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Soybean

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Meet the editor

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Contents

Preface

Soybean is an agricultural crop of tremendous economic importance. Soybean and food items derived from it form dietary components of numerous people, especially those living in the Orient. The health benefits of soybean have attracted the attention of nutritionists as well as common people.

The literature on soybean research is voluminous. This prompted InTech Open Access publisher to embark on this meaningful project of inviting eminent scientists in different arenas of soybean research to contribute articles in their areas of specialization.

Due to the explosion of knowledge, few people in one discipline of soybean research are conversant with every other aspect of soybean research. Hence a compilation of the soybean literature and sorting it under different categories and distribution in separate volumes would facilitate investigators and students to familiarize themselves with the diverse areas of soybean research.

The volume focuses on soybean biochemistry, chemistry and physiology, with the great majority of articles classified under biochemistry.

In the biochemistry section, the health promoting effects of soybean constituents, regulation of soybean isoflavonoid biosynthesis, safety aspects of soybean food, hypersensitivity to soybean oil, and effect of fungal infection on soybean cultivation are discussed.

In the chemistry section, soybean fiber, soybean fatty acids, heavy metal uptake by soybean, and analysis of glyposate and aminorrethylphosphoric acid residues in soybean are covered.

In the physiology section, the relationship between soybean consumption and obesity. The effect of salinity stress on soybean, and teh effect of a lepidopteran insect on soybean are described.

Readers will find this book illuminating and enjoyable and investigators will gain inspirations for further research.

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Part 1

Biochemistry

1

Hypersensitivity Reaction to Generic Drug-Containing Soybean Oil

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1. Introduction

Over the last years the use of generic drugs has increased in the European Union due to their minor economic cost. The main regulatory request to market these products is their equivalence to the original compounds.
We describe two women that presented anaphylaxis after intake of generic omeprazole. The two patients had previously tolerated original non-generic omeprazole. Dot-blot assay revealed that the serum of the two patients was positive to generic omeprazole and soy.
The diagnosis of soy allergy should not be excluded in cases of drug hypersensitivity. We suggest to tests soy in all hypersensitivity reaction to generic drugs.

2. Brief report

Over the last years the use of generic drugs has increased in Spain and in the European Union due to their minor economic cost. The main regulatory request to market these products is their equivalence to the original compounds.
Soy bean is an additive of some drugs. Hypersensitivity reactions to soy bean oil as a result of drug intake have been scarcely reported[1-3]. Recent studies on analytic investigation on protein content in refined seed oils suggest that fully refined seed oils should be taken into account in the context of allergic reactions and would benefit of further toxicological studies[4].
We describe two women (58 and 81 years old) previously diagnosed of allergic asthma due to pollen, that presented a severe drug reaction (asthma, angioedema, hypotension), few minutes after intake of a capsule of a generic omeprazole. The two patiens have been previously tolerated non-generic omeprazole.
After informed consent the following tests were carried out:
Patient 1: Skin prick tests (SPT) and ImmunoCAP assay (Phadia, Uppsala, Sweden) were positive with *Lolium perenne* and soy bean in the first woman (wheal mean diameter

with soy extract 20 mm, specific IgE to soy 9.01 KU/L) and with a dilution 1/10 in saline 0.9% of the powder contained in a capsule of the generic omeprazole (wheal mean diameter 14 mm).

Patient 2: This woman, previously diagnosed as asthmatic asthma due to grass pollen and oral syndrome after eating lentils, presented a wheal mean diameter with soy extract of 16 mm and a specific IgE to soy of 23 KU/L. SPT with omeprazole extract gave a wheal mean diameter of 12 mm. A positive control (histamine, 10 mg/mL) and a negative control (saline 0.9%) were also used.

SPT performed with non-generic omeprazole were negative. SPT performed with generic omeprazole extract were positive in other five patients sensitized to soy bean (wheal mean diameter 10 mm).

IgE-Dot Blot (Bio Rad, California, USA) assay was performed with the powder contained in a capsule of the generic omeprazole A and a capsule of generic omeprazole B reconstituted in 600 µL of 20% ethanol/water (v/v), and extract of soybean (4 mg/mL of protein). The assay revealed as the serum of the two patients, was positive to generic omeprazole (A and B) and soybean extract but negative to diluents' wells, and the same samples was negative against the serum of a non-atopic patient. (Figure 1)

Although incidences of anaphylactic reactions induced by proton pump inhibitors of H2 are rare, they can life threatening. Skin prick tests and oral challenge tests may be useful for the diagnosis, but immunological test never confirm the presence of specific IgE antibodies to active principles of these drugs[5,6].

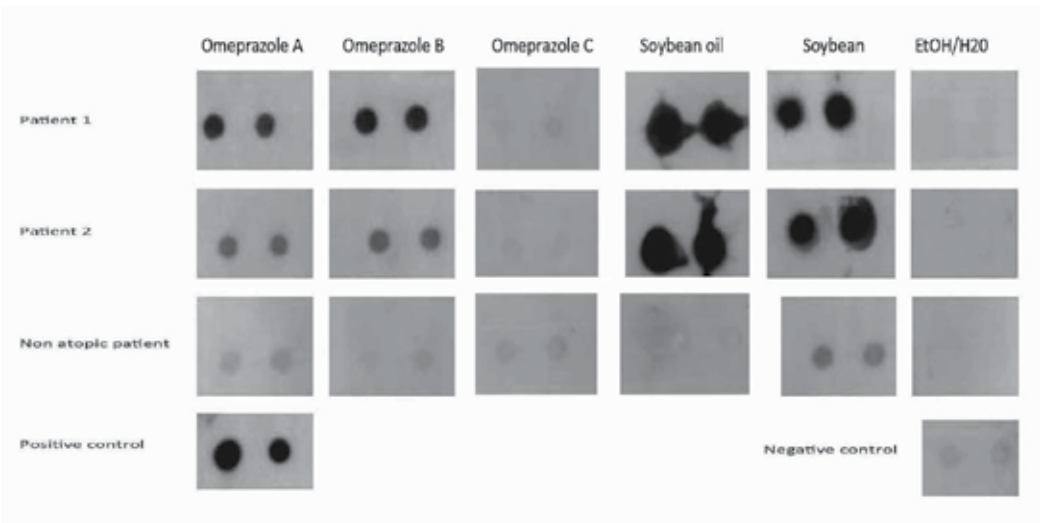

Fig. 1. Responses of serum from Patients 1 and 2 and from a nonatopic patient to generic omeprazole from two different manufacturers, to nongeneric omeprazole, to soybean oil, to soybean extract, and to diluents are shown. The response to *Dermatophagoides pteronyssinus* of serum from a patient who was sensitized to *D. pteronyssinus* extract, which was used as a positive control of the assay, is also shown.

Our observation suggests that an immediate hypersensitivity to drug-containing soy oil may cause anaphylaxis reaction in patients previously sensitized to this legume. The first report of possible anaphylaxis after a drug containing soybean oil implicated the drug propofol[2]. Since propofol contains both egg lecithin and soybean oil, its use is contraindicated in patients with hypersensitivities to these component. Several other drugs may have a food component, resulting in contraindications and warnings in product labelling.

Several commercially important refined vegetable oils are derived from plants which are recognized as potent food allergens (peanut, soy). Full refining of oils results in the almost complete removal from oils of protein, which is responsible for allergic reactions. However, it is uncertain whether the minute amounts remaining could provoke allergic reactions in highly susceptible individuals. This has led to a vigorous debate about the safety of refined oils and specifically whether to label each oil individually because of the potential risk of allergenicity [7].

Until active principles are clearly indicated in drug labellings, excipients and other minor additives are not included and only defined as excip. c.s. Since the introduction of generic drugs to the pharmaceutical market a debate exists whether they are well-investigated and of high quality. There is some uncertainly about whether evidence of bioequivalence is enough to guarantee efficacy and safety of generic drugs[8]. Food allergy consumers depend on ingredient labels for allergen avoidance[9]. However the drug labelling may not indicate the form or source of the allergen, and individuals who currently avoids foods may presented severe allergic symptoms after intake of drugs with minimal protein content such as soy oil, soy lecithin or lysozyme[10]. There are now reliable assays for the determination of soybean proteins in processed foods that may be applicable in drugs[11].

Soy is a clinical relevant allergenic source. The diagnosis of soy allergy should not be excluded in cases of drug hypersensitivity. We suggest to tests soy in all hypersensitivity reaction to generic drugs.

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2

Safety Aspect in Soybean Food and Feed Chains: Fungal and Mycotoxins Contamination

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1. Introduction

Soybean (*Glycine max* L. Merr.) is an Asiatic leguminous plant cultivated in many parts of the world for its oil and proteins, which are extensively used in the manufacture of animal and human foodstuffs (FAO, 2004a; Hepperly, 1985). The production reached 47.5 million tons during the 2006/2007 harvest season ranking Argentina third as soybean producer in the world. In Argentina, during the last quarter of the century, soybean production has increased at an unprecedented rate from a cultivated area of 38.000 hectares in 1970 to 16 million hectares today. Around 70% of the soybean harvested is processed, providing 81% and 36% of the world's exported soybean oil and meal, respectively (SAGPyA, 2010). Soybean is often attacked by fungal infections during cultivation, or post-harvest (in transit or in storage), significantly affecting its productivity. Seeds and infected harvest debris are the main sources of primary infections, and the level of seed damage depends on environmental conditions such as high relative humidity, dew, and temperatures above 25 ^{o}C. These species can be potential mycotoxin producers. Mycotoxins (from "myco" fungus and toxin) are relatively low-molecular weight, fungal secondary metabolic products that may affect exposed vertebrates such as animals in a variety of ways. Mycotoxins are considered secondary metabolites because they are not necessary for fungal growth and are simply a product of primary metabolic processes. The functions of mycotoxins have not been clearly established, but they are believed to play a role in eliminating other microorganisms competing in the same environment. They are also believed to help parasitic fungi invade host tissues. The amount of toxins needed to produce adverse health effects varies widely among toxins, as well as within each person's immune system (Brase et al., 2009).

Some mycotoxins are carcinogenic, some are vasoactive, and some cause central nervous system damage. The mycotoxins can be acutely or chronically toxic, or both, depending on the kind of toxin, the dose, the health, the age and nutritional status of the exposed individual or animal, and the possible synergistic effects between mycotoxins. The most frequently studied mycotoxins are produced by species of *Aspergillus*, *Penicillium, Fusarium* and *Alternaria*.

There is an increasing world consumer demand for high quality and inocuous food and drink products with the lowest possible level of contaminants such as mycotoxins. As a result, the food industry in the developed world demands raw ingredients of the best

quality and that conform to statutory limits where these have been set for mycotoxins. Because the mycotoxins are unavoidable, it is important to know how the concentrations of mycotoxins present in raw materials change through the food and feed chains. The development of prevention strategies today has been predominantly based on using the HACCP approach and to identify the critical control points in the pre- and post-harvest food chain. This approach enables strategies for minimizing consumer exposure to be developed through appropriated management of the products (Sanchis & Magan, 2004; Scudamore 2004).

2. Fungal and mycotoxin contamination in soybean at preharvest stages

2.1 *Fusarium* species dynamic and their mycotoxins at different soybean reproductive growth stages

The occurrence of fungi in soybean seeds has received far more attention than the occurrence of fungi in pods and flowers. This is understandable from a practical standpoint: infected seeds and infected seedlings developing from them represent greater economic risks in soybean production, and seed contamination with mycotoxins represents a health risk to human and animals (Roy et al., 2001).

The mycoflora of soybean seed may be affected by environmental factors, geographic location, cultural practice and degree of host susceptibility to pathogens (Villaroel et al., 2004) Previous studies carried out in Argentina evaluated the fungal contamination on freshly harvested soybean or soybean seeds under storage (Boca et al., 2003; Broggi et al., 2007). However, other studies have demonstrated that the isolation frequency of fungi from living plant tissues differs from those of senescing or dead tissues of soybean plants. For this reason, three reproductive stages of soybean development and samples of flowers, pods and seeds were examined. The flowers were obtained during R2 growth stage (full bloom), while pods and seeds were recovered during R6 (full seed) and R8 (full maturity) growth stages. The mycoflora isolated from flowers, pods and seeds were dominated by two genera: *Alternaria* and *Fusarium*, at similar levels across all the stages. Among *Fusarium* isolates, 45% were isolated from pods, 38% from seeds and 17% from flowers. *Fusarium* contamination across different stages showed that the high isolation frequency was found in pods and seeds at stage R6 (full seed), being the a_w of immature seeds 0.992. At stage R8 (full maturity), the water content of the seeds dropped dramatically to 0.70 and the percentage of *Fusarium* spp. also diminished compared to stage R6. Villarroel et al. (2004) showed that *Fusarium* spp. isolated at R6 and R8 growth stages from pods and seed tissues were significantly greater on conventional than on transgenic cultivars. In our study, a transgenic cultivar with tolerance to glyphosate was used since more than 90% of the planted area in Argentina belonged to this category (Lopez et al., 2008). The *Fusarium* species identified are showed in the Fig. 1, being *F. equiseti* the most frequently recovered from flowers, pods and seeds (40% of isolates), followed by *F. semitectum* (27%) and *F. graminearum* (11%). The distribution of *Fusarium* species was similar in the three parts of the plants and the reproductive stages evaluated.

Members of *Fusarium* genus are known to produce a broad spectrum of toxins including trichothecenes of A- and B-types. Among B-type trichothecenes, deoxynivalenol (DON) and nivalenol (NIV) are important mycotoxins produced by members of the *F. graminearum* species complex (*Fg* complex). DON is the most distributed *Fusarium* mycotoxin and occurs world-wide in crops from temperate regions. NIV also occurs in cereals and has been

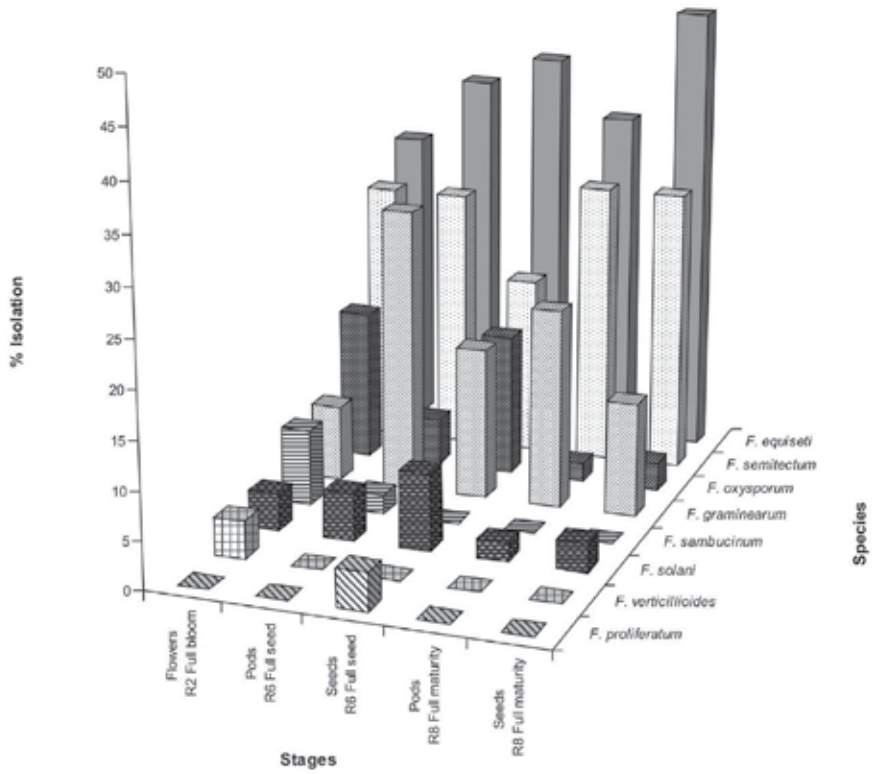

Fig. 1. *Fusarium* species distribution at different soybean reproductive stages of development.

extensively found in Japan and Korea, and at relatively low levels in samples from Europe, Southern Africa and South America (Placinta et al., 1999). DON is associated with feed refusal, vomiting and suppressed immune functions (WHO, 2001), and NIV is more toxic to humans and domestic animals than DON (Ryu et al., 1988). Due to their toxicity, international regulations limit the content of DON in the food chains (FAO, 2004a; Verstraete, 2008). Trichothecenes also are potent phytotoxins (Eudes et al., 2000), with DON being more phytotoxic than NIV (Desjardins et al., 2006).

In our study, soybean seed samples from each reproductive stage (R6 and R8) were evaluated for DON and NIV contamination. Out of 40 samples, two were contaminated with DON at levels of 1.6 μg/g (R6 stage) and 0.9 μg/g (R8 stage). Only one sample at stage R8 showed T-2 toxin contamination at level of 280 μg/kg. Neither NIV nor HT-2 were detected (Barros et al, 2008b). These results demonstrate that the soybean could be contaminated with mycotoxins at low levels and the presence of *Fusarium* species potentially toxigenic might be considered for further studies on natural contamination. Generally, grains with a moisture content equivalent to less than 0.70 a_w (<14.5% moisture by weight) will not be subject to fungal spoilage and mycotoxin production (Aldred and Magan, 2004). In the present study, similar a_w was observed in seeds at stage R8, this fact suggest that the mycotoxin contamination occurred between stages R6 and R7 were the level of a_w was around 0.99. Other studies have analyzed the importance of soybean pathogens such as *Diaporthe/Phomopsis* complex and *Cercospora spp.* in tissues prior to harvest maturity (Baird et al., 2001; Ploper et al., 1992; Roy et al., 2001; Villarroel et al., 2004). However, this is the first

report to analyze the *Fusarium* species diversity and densities and mycotoxin contamination during the soybean grain ripening.

2.2 Chemotype and genotype within *Fusarium graminearum* species complex

Based on the DON contamination on soybean seeds at stages R6 and R8, we considered interesting to investigate the toxigenic ability of isolates belonging to *F. graminearum* species complex. Strains of *F. graminearum* usually express one of three sets of trichothecene metabolites either: (i) nivalenol and acetylated derivatives (NIV chemotype), (ii) deoxynivalenol and 3-acetyldeoxynivalenol (3-ADON chemotype), or deoxynivalenol and 15- acetyldeoxynivalenol (15-ADON chemotype) (Ward et al. 2002). Surprisingly, *Fusarium* isolates that produce both DON and NIV (NIV/DON chemotype) have been reported and described as "unknown" chemotypes (Quarta et al., 2006; Ward et al., 2002).

The 15-ADON chemotype is predominant in North America and the 3-ADON chemotype is predominant in same areas in Asia, including China, Australia, and New Zealand (Guo et al., 2008). Due to the toxicological differences between NIV and DON (Desjardins and Proctor, 2007), it is important to determine the chemotypes of strains present in a given region on different crops. With the identification of the genes responsible for trichothecene biosynthesis, PCR assays have been developed to distinguish the toxin producing genotypes (Chandler et al., 2003; Jennings et al., 2004; Lee et al., 2001; Waalwijk et al., 2003). Primers based on the sequences of alleles at *Tri3*, *Tri5* and *Tri7* have been designed to differentiate the three toxin genotypes (Quarta et al., 2005, 2006).

Examination of trichothecene profile among the strains isolated from the soybean agroecosystem revealed that the 15-ADON was the dominant chemotype identified by chemical and PCR analysis (Barros, personal communication). Similar results were obtained in previous studies that evaluated the toxigenic potential of *F. graminearum* isolates from wheat in Argentina (Alvarez et al., 2009; Reynoso et al., 2011) and South of Brazil (Scoz et al., 2009). However, a 12% of the isolates showed an unusual pattern of trichothecenes with a simultaneous DON and NIV production. The finding of isolates with an unusual pattern of trichothecenes production (DON and NIV producers) in this study agree with previous reports by Reynoso et al. (2011) and Fernandez Pinto et al. (2008) who identified this type of strains from wheat in Argentina. This fact is not surprising since e regular crop rotation is soybean/wheat and the pool of strains could move through one to another agroecosystem.

Several reports examined the trichothecene production by strains of *F. graminearum* isolated from cereals in Argentina based on chemical analyses (Alvarez et al., 2009; Faifer et al., 1990; Fernandez Pinto et al., 2008; Lori et al., 1992; Molto et al., 1997). However, this is the first report on determine trichothecene chemotype and genotype among the *Fg* complex isolated from soybean in Argentina. Although a good relation between multiplex PCR (genotype) and chemical analysis (chemotype) was observed, more studies are necessary to evaluate variations within the field populations on soybean. For example, in North America the 15-ADON chemotype is predominant, but recent molecular surveillance has shown that 3-ADON chemotype is replacing 15-ADON from eastern to western Canada (Ward et al., 2008).

The *Fg* complex is composed of at least twelve lineages (O'Donnell et al., 2008). *F. graminearum* populations from wheat in Argentina are genotypically diverse, and belong to *F. graminearum* lineage 7 (Ramirez et al., 2006, 2007) also termed *F. graminearum sensu stricto* (O'Donnell et al., 2000, 2004). Further studies using molecular markers and sequence analysis on the *F. graminearum* species complex population isolated from soybean

agroecosystem are in progress, these results will allow us to know the isolates characteristics such as genotype, genetic diversity, lineage and pathogenicity.

3. *Alternaria* species and their mycotoxins on soybean at harvest time

A diverse group of saprophytic and parasitic fungi can colonize and infect soybean pods and seeds prior harvesting (Villarroel et al., 2004). *Alternaria* and *Fusarium* species are the most commonly isolated fungi from soybean in Argentina and in others regions of the world (Boca et al., 2003; Broggi et al., 2007; Gally et al., 2006; Roy et al., 2001). The most common *Alternaria* specie found on soybean seeds is *A. alternata*.

From a survey done on *Alternaria* species contamination on soybean samples (n=50) harvested in two provinces of Argentina during two harvests seasons (2006-2007 and 2007-2008) years. We observed that around 80% of the samples presented *Alternaria* spp. contamination at levels ranging from 2 to 84%. One hundred and forty strains were morphologically indentified at species level according to Simmons (1992, 2007) based mostly on the three-dimensional sporulation patterns. Seventy five strains were identified as *A. alternata*, 65 as *A. infectoria*, 8 as *A. oregonensis*, 5 as *A. graminicola* and 1 as *A. tritimaculans*, respectively. These results were noticeable, considering that except *A. alternaria*, all the other strains were members of the *A. infectoria* (morphological) group and also all belonged to the infectoria species-group, which is genetically distinct and phylogenetically distant from the other species-group (brassicola species-group and the alternata species-group). The *A. infectoria* group comprises at least 30 known species (Andersen et al., 2009). Morphologically the *A. infectoria* group differs from others *Alternaria* species-groups in the three dimensional sporulation pattern (Simmons and Roberts, 1993). The mains characteristic for the *A. infectoria* group is the production of small conidia in branched chains with long, geniculate multilocus secondary conidiophores between conidia (Simmons, 2007). This data were the first report on the presence of member of species belonging to the *A. infectoria* group on soybean. Recently, *A. infectoria* have also been isolated in high frequency on wheat probably due to changes in cropping systems in most of the different agroclimatic zones in Argentina (Ramirez et al., 2005). Also, Perello et al. (2008) have associated *A. infectoria* as the ethiological agent of black point in wheat grains in Argentina. We explain the presence of *A. infectoria* and *A. infectoria* group on soybean as a consequence of the practice of double cropped soybean cultivation widely used in our country, which consist of sowing the soybean immediately after harvesting a wheat crop.

Most *Alternaria* species are saprophytes that are commonly found in soil or on decaying plant tissues. Some species are opportunistic plant pathogens that, collectively, cause a range of diseases with economic impact on a large variety of important agronomic host plants including cereals, ornamentals, oil crops and vegetables. *Alternaria* species are also well known as post-harvest pathogens. Some *Alternaria* species are well known for the production of toxic secondary metabolites, some of which are powerful mycotoxins that have been implicated in the development of cancer in mammals (Thomma, 2003). Among these metabolites with mammalian toxicity are alternariol (AOH), alternariol monomethyl ether (AME) (Logrieco et al., 2003; Ostry, 2008). Recently have been reported that AOH and AME posses cytotoxic, genotoxic and mutagenic properties *in vitro* (Brugger et al., 2006; Fehr et al., 2009; Lehmann et al., 2006; Wollenhaupt et al., 2008), and there is also some evidence of carcinogenic properties (Yekeler et al, 2001). Tenuazonic acid (TA) is a mycotoxin and phytotoxin, produced primarily by *A. alternata*, but also by other phytopathogenic *Alternaria* species (Logrieco et al., 2003). This toxin is considered as a

possible causal factor of Onyalai, a human hematological disorder (Ostry, 2008). TA also exhibits phytotoxic, insecticidal, zootoxic, cytotoxic, antibacterial and antiviral activity. TA has been shown to be more toxic than other mycotoxins produced by *Alternaria* species such as altenuene (AE), alternariol (AOH) and alternariol monomethyl ether (AME) (Ostry, 2008). These mycotoxins have been demonstrated to be produced by *Alternaria* species on wheat, tomato, sorghum, pecans, sunflower and on cotton (Scott, 2001; Ostry, 2008).

3.1 Ecophysiology of *Alternaria alternata* on soybean based media

Fungal growth and mycotoxin production result from the complex interaction of several factors and, therefore, an understanding of each factor involved is essential to understanding the overall process and to predict and prevent mycotoxin development (Chamley et al., 1994). Temperature and water activity (a_W) are the primary environmental factors that influence growth and mycotoxin production by several *Alternaria* species in cereals and oil seeds (Etcheverry et al., 1994; Magan & Baxter, 1994; Magan & Lacey 1984; Torres et al., 1992; Young et al., 1980).

Prevention of mycotoxin contamination of food raw materials is now considered more effective than subsequent control. Thus, hazard analysis critical control point (HACCP) approaches are being developed to examine the critical control points (CCPs) at which mycotoxigenic fungi and mycotoxins may enter a range of food and feed chains (Aldred & Magan, 2004). Therefore, accurate information is needed on the impact of key environmental factors such as a_W, temperature and their interactions, and on identifying marginal and optimum conditions for growth and toxin production. Few studies have attempted to build two-dimensional profiles for growth and mycotoxin production by *Alternaria* species (Sanchis & Magan, 2004). Due to the lack of information on *Alternaria* growth and toxin production on soybean or culture medium based on soybean, we decided to evaluate the effect of water activity (a_W; 0.995, 0.98, 0.96, 0.94, 0.92 and 0.90), temperature (5, 18, 25 and 30°C), incubation time (7-35 days) and their interactions on mycelial growth and AOH, AME and TA on soybean extract agar by two *A. alternata* strains isolated from soybeans in Argentina. Maximum growth rates were obtained at the highest a_W (0.995) and 25°C with grow decreasing as the water availability of the medium was reduced. Maximum amount of AOH was produced at 0.98 a_W and 25 °C for both strains. Maximum AME production was obtained for both strains at 30 ºC, but a different a_W 0.92 and 0.94 for the strains RC 21 and RC 39 respectively. Maximum TA production was obtained for both strains at 0.98 a_W, but at 30 and 25 ºC for the strains for RC 21 and RC 39 respectively. The concentrations range of three toxins varied considerably depending on a_W and temperature interactions assayed. Further, the three metabolites were produced over the temperature range from 5 to 30 ºC and a_W range from 0.92 to 0.995. Although at 5 and 18 ºC little of any mycotoxin was produced at a_W lower than 0.94. Two-dimensional profiles of a_W x temperature were developed from these data to identify areas where conditions indicate a significant risk from *A. alternata* growth and mycotoxin accumulation on soybean (Fig. 2) (Oviedo et al., 2009a, 2010). All the conditions of a_W and temperature were maximum production of the three toxins are those found during soybean development in the field. Thus, field conditions are likely to be conducive to optimum grow and toxin production of this specie.

Taking in to account these results, it appears that different combinations of a_W and temperature are necessary for optimal production of these 3 toxins by *A. alternata* and that the limiting a_W for detectable mycotoxin production is slightly greater than that for growth.

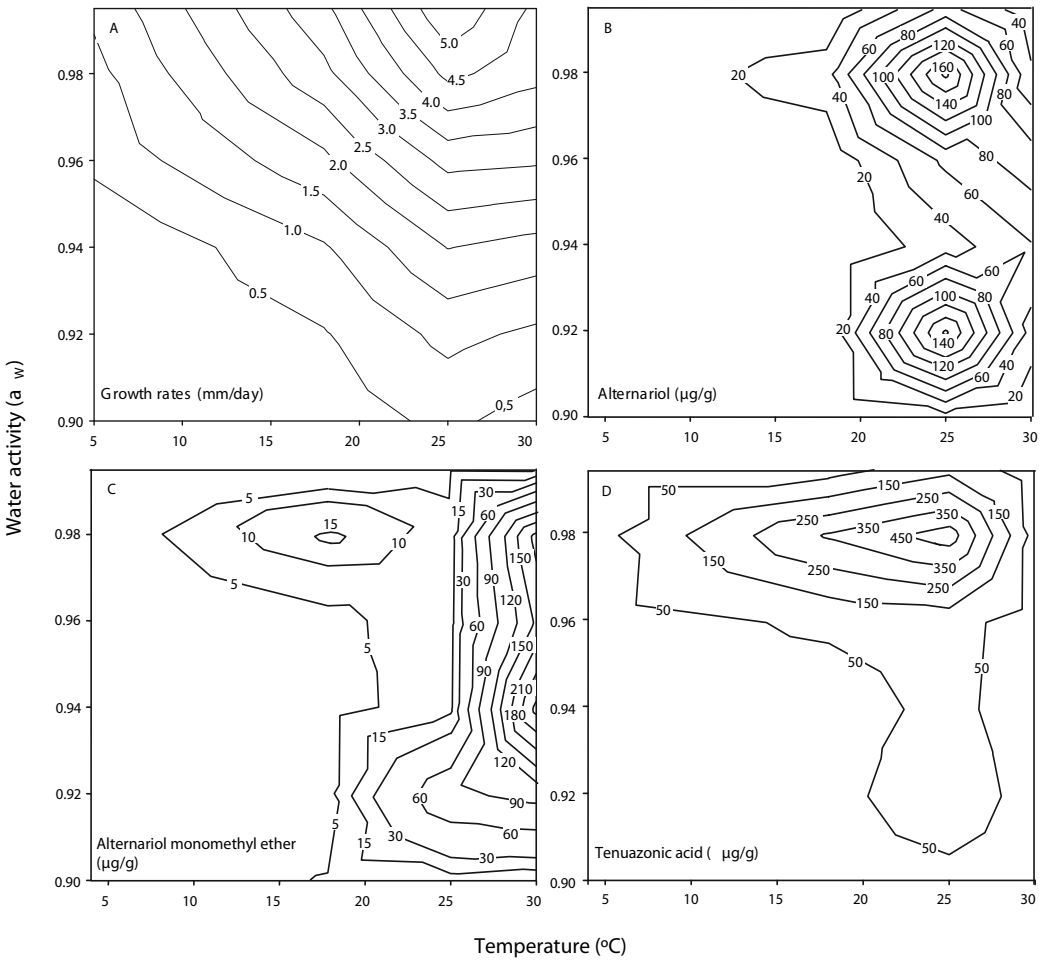

Fig. 2. Comparison of profiles for growth and different mycotoxins by *Alternaria alternata* on soybean based media.

In the present study, the knowledge of interacting environmental conditions provides very useful information for predicting the possible risk factors for AOH, AME and TA contamination of soybean. The a_W and temperature range used in this study simulate those occurring during grain ripening. Also, the data demonstrated the contrasting impact of a_W, temperature and incubation time on growth and AOH and AME production by the two strains examined. The knowledge of AOH, AME and TA production under marginal or sub-optimal temperature and a_W conditions for growth can be important since improper storage conditions accompanied by elevated temperature and moisture content in the grain can favour further mycotoxin production and lead to reduction in grain quality.

3.2 Natural contamination of *Alternaria* mycotoxins on soybean seeds

Alternaria toxins have recently received much attention, both in research programs and in risk assessment studies. At present, no statutory or guideline limits set for *Alternaria* mycotoxins have been set by regulatory authorities (FAO, 2004b). Current data on the

natural occurrence of *Alternaria* toxins point to low human dietary exposure. Further studies are necessary to develop strategies for safe food and feed supplies by developing detection methods, identifying *Alternaria* mycotoxins risk in the production chain, determining the critical control points, and developing preventive measures.

Numerous methods have been developed for AOH and AME determination in different agricultural commodities (Logrieco et al., 2009; Ostry, 2008; Scott, 2001); however there was no an available technique for determining these mycotoxins in soybean. Solid-phase extraction columns have been used for extraction and clean-up of AOH and AME in apple juice and wheat. The natural occurrence AOH and AME on soybean seeds harvested in Argentina was evaluated. Both toxins were simultaneous detected by using HPLC analysis coupled with a solid phase extraction column clean-up. Characteristics of this in-house method such as accuracy, precision and detection and quantification limits were defined by means of recovery test with spiked soybean samples. From a survey of 50 soybean seed samples evaluated for AOH and AME contamination, it was found that 44% of them were contaminated with AME. AME was found in levels ranging from 62 to 1,153 ng/g. Although a limited number of samples were evaluated, this data were the first report on the natural occurrence of *Alternaria* toxins in soybean seeds and is relevant from the point of view of animal public health (Oviedo et al., 2009b). Also the results showed that AOH and AME are produced on soybean seeds at harvest time. This data agree with previous studies (Oviedo et al., 2009a, 2010) in which we have demonstrated that the environmental conditions (a_W and temperature) optimum for growth and mycotoxin production by *A. alternata* on soybean-base media were similar to those occurring during soybean development in the field until harvest.

During the last five years numerous studies dealing with AOH and AME toxicity have been published. Both mycotoxins have been reported to have genotoxic, mutagenic and carcinogenic effects (Ostry, 2008; Logrieco et al., 2009). Also, have been suggested that, the mutagenicity of AOH may have bearing on the carcinogenicity of this mycotoxin. Recently Tiemann et al. (2009) have demonstrated that AOH and AME, at similar concentration levels found in the present study, negatively affected progesterone synthesis in porcine granulosa cells *in vitro*. In view of the fact that granulosa cells directly influence the metabolic and structural growth of the oocyte (Albertini et al., 2001), exposure to AOH or AME may eventually affect reproductive performance by interfering with follicular development in swine and possibly other mammalian species. Feedstuff should therefore be carefully controlled for *Alternaria* toxins content.

4. Fate of fungal and mycotoxin contamination during soybean meal production process

Soybean production and its by-products (oil and meal) form one of the most important economic activities in Argentina. At present, Argentina is the world´s first exporter of soybean meal and oil and the third producer of soybean, behind USA and Brazil (Lopez et al., 2008). Hygienic safety of soybean and by-products depends on fungal contamination among other microorganisms. However, with European Union legislation imminent, the consideration of mycotoxins is becoming increasingly important.

The fungal and mycotoxin contamination on soybean used in the soy meal production was examined, in order to identify critical control points (CCP_s) in the process. Respect to fungal contamination, the levels of fungal propagules in all points of the process were no higher

than 10^4 cfu/g, value considered safe by GMP14 normative. However several potential toxigenic fungi were detected, especially species belonging to the genera *Fusarium* and *Aspergillus*. Among *Fusarium* species, *F. verticillioides* was most frequently recovered (60% of isolates), followed by *F. oxysporum, F. subglutinans, F. proliferatum, F. semitectum* and *F. graminearum*. The genus *Aspergillus* was the second most frequent genus isolated and the dominant *Aspergillus* species identified belong to the section *Flavi* (*A. flavus*) and section *Nigri* (*A. niger* aggregate). According to the species identified, the natural occurrence of aflatoxins (produced mainly by *A. flavus*), fumonisins (produced by *F. verticillioides* and *F. proliferatum*) and deoxynivalenol (DON) and zearalenone (produced mainly by *F. graminearum*) were analyzed at six points in soybean meal production process. Previously, in order to evaluate the natural occurrence of these mycotoxins, adequate methodologies for their determination on soybean and soy meal by using HPLC analysis were optimized (Barros et al., 2008a, Barros personal communication).

Aflatoxin B_1 and fumonisin B_1 were detected in a few samples at low levels and no zearalenone contamination was observed. DON showed higher incidence than aflatoxins and fumonisins and was detected in different points of the process at ppm levels. However, *F. graminearum*, the main responsible for cereal contamination with DON in Argentina, was recovered at very low frequency. This result showed that DON contamination of soybean seed occurred at field stage previous to harvest. For this reason, the dynamics of *Fusarium* populations at field stage and specifically in the reproductive growth stages where the soybean seed is developed was evaluated.

5. Conclusion

This chapter summarizes new information on toxigenic fungi and mycotoxin contamination on soybean at preharvest, harvest and processing stages. Also data on ecophysiology of the most important genus and species isolated are provided. Although, *Alternaria* mycotoxins are not yet regulated, their toxicity is at present under revision. *Fusarium* mycotoxins are regulated both in food and feedstuff. All the presented information are relevant from the point of view of food safety, since mycotoxins are natural contaminants and their presence is unavoidable. It is important to reduce their presence and optimized prevention strategies at all stages of food and feed chains.

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3

Properties of Angiotensin I - Converting Enzyme(ACE)

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1. Introduction

The life-style diseases, such as obesity, diabetes and hypertension, and cardiovascular disease have been increasing in Japan recently. The mortality rate due to heart disease or vascular disease has been 2nd and 3rd of total death toll. The hypertension is a most important disease because it may induce cerebral apoplexy and cardiovascular disease.
Recently, new aspects of food functions have been drawing considerable attention.
Among them are the physiological functions of some food components in relation to certain ailments. One of the representatives is the epidemiological relationship between foods and hypertension (1), (2). Some foods are known to be effective in suppressing the development of hypertension (3), which suggests the existence of some components having medicine-like action on the regulation systems of blood pressure.
The renin-angiotensin system is considered to be a blood pressure regulation system which is apt to be affected by food components (4). The angiotensin I-converting enzyme (ACE) is the key enzyme in the rennin angiotensin system. Therefore, food components which inhibit ACE have the possibility to lowering of blood pressure.
Based on this point of view, the effects of ACE inhibitor from various food materials, and fermented foods were surveyed.

2. Angiotensin I - Converting Enzyme(ACE) in the renin-angiotensin system

ACE (peptide dipeptidase, E.C.3.4.15.1) plays an important physiological role in rennin angiotensin system to regulate both the arterial blood pressure, and salt and water balance(5). The system starts by the conversion of angiotensinogen to a pre-hypertensive hormone angiotensin I by the action of rennin which is secreted by the kidney(Fig.1). The angiotensin I is further converted to angiotensin II, the active form of the hormone, by the action of ACE. Angiotensin II raises blood pressure by acting directly to blood vessels, sympathetic nerves and adrenal glands. ACE is an important target for inhibitor since it performs the last step in the biosynthesis of the octapeptide angiotesin II. ACE also destroys the vasodilating peptide bradykinin.
Therefore, food components which inhibit ACE have the possibility to suppress hypertension by decreasing the formation of angiotensin II.
In fact, oral dose of synthetic ACE inhibitor like captopril to hypertensive patients becomes the first choice for the medical treatment.

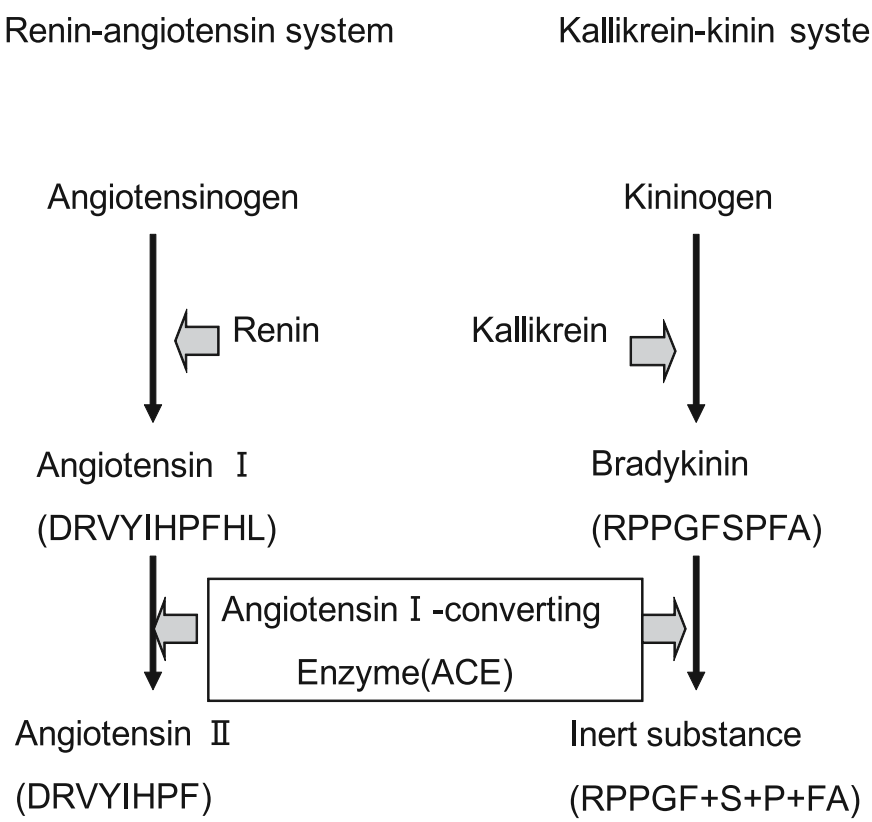


Fig. 1. Enzymatic action of ACE

The enzyme seems to be similar in rabbit, dog, rat, pig, and man and to be present in a large number of tissues including lung, kidney, plasma, and brain and in the endothelial lining of the vasculature. The ACE is a glycoprotein.

The most abundant forms have molecular weights in the range 130,000-140,000kDa, of which about 25% is carbohydrate by weight. It has a broad tolerance for substrates, although it will not hydrolyze peptide bonds containing a secondary amino group nor are peptide bonds containing acidic residues in the S'_2 subsite good substrates.

3. ACE inhibitor

Since snake venom peptide inhibitors played a role in establishing the clinical value of ACE inhibitors (6), many peptide derivatives of inhibitor have been developed(7).

Many natural products have also been examined for ACE inhibitory activity, and teprotide from *Brothrops Jararaca* (8), aconvenin from *Streptomyces* (9), and others (10,11) have been identified. ACE inhibitors such as captopril and enalapril have been as antihypertensive drugs. Therefore, the inhibiton of ACE can reduce blood pressure.

In recent years, several ACE inhibitory peptides from food protein sources have been isolated and reported, such as casein (12), tuna (13) and sardine (14).

However, most of them were peptides of enzymatic hydrolyzates from food protein source. There have been a few reports about non-peptidyl ACE inhibitors, polyphenols (15), and nicotianamine (16).

3.1 ACE inhibitor of various fermented foods and protease hydrolyzates from various food protein

ACE inhibitory activity was measured with 11 kinds (31 items) of fermented foods (17) (Table 1). The degrees of ACE of the various fermented food inhibition was expressed as IC_{50}, defined as the sample concentration that inhibits 50% of ACE activity. The degrees of ACE inhibition by the food samples were compared based dry weight.

The IC_{50} of three kinds of cheese (blue, camembert, and red cheddar), dried bonito, and natto were particularly very low (0.16-0.2mg/ml). Nyufu and temphe caused moderated inhibition (IC_{50} of 0.66mg/ml). ACE inhibition by miso paste is relatively weak.

Among the tree kinds of miso, interestingly, the miso made from rice did not inhibit ACE. These results suggested that the material for fermentation or fermentation process may affect ACE inhibitory activity of the final products. Similary, a vinegar from apple and a mirin produced by an ordinary method did not inhibit ACE.

This might suggest that these foods contains inhibitory substances produced during fermentation. Considering the IC_{50} values based on dry weight, cheese (expect for cottage cheese), fish sauce, say sauce, natto, and nyufu had high ACE inhibiting power.

Protease hydrolyzates of various food proteins which indicate ACE inhibitory activity have been reported to have hypotensive activity (12, 13, 14). Recent studies found several ACE inhibitory peptides isolated from the hydrolyzates of proteins by various kinds of protease (18-19) (Table 2).

3.2 ACE inhibitor in various beans

The two reports about ACE inhibitory activity of soy sauce have recently been reported. Kajimoto reported that say sauce decreases blood pressure in dog (20). It suggested that soy sauce contains a substance that promotes histamine absorption as the causative agent of blood pressure decreased effect.

Kinoshita *et al.* found an inhibitory compound of the ACE in say sauce (16).

ACE inhibitor in the high molecular weight (Hw) fraction from soy sauce by gel filtration chromatography decreased blood pressure in hypertensive rats (SHR) and two-kidney Goldblantt hypertensive rats (2HGH rats). The main ACE inhibitor in the Hw fraction was identified as nicotianamine (*N*-[*N*-(3-amino-3-carboxypropyl) -3-amino-3-carboxypropyl] -azetidine-2-carboxylic acid) (Fig.2). Nicotianamine is abbreviated as NA.

They found that soybeans contain a large quantity of nicotianamine.

They suggested that the origin of the nicotianamine in soy sauce is soybeans.

The inhibition of ACE and NA content of 41 different beans were reported (21) (Table 3). Among these beans, particularly high ACE inhibitory activity was found in pea, broad bean and soybean. The NA content was the highest in pea, followed by soybean and Lenties.

The strength of ACE inhibition of beans was correlated to the amount of NA content.

This shows that ACE inhibition of beans is attributable to NA content.

Fermented foods	Raw Materials/Kinds	IC_{50}(mg/ml) [m]
Liquid products		
Fish sauce	Samon	1.69
	Sardine [a]	1.43
	Sardine [b]	3.15
	Anchovy	1.35
Mirin	General	ND
	Old style	85.62
Sake	General	5.27
	Junmai [c]	3.77
	Ginjo [d]	6.95
	Genmai [e]	4.61
Soy sauce	Koikuchi [f]	1.72
	Koikuchi [g]	1.35
	Usukuti [h]	2.75
	Saishikomi [i]	1.25
	Shiro [j]	17.8
	Tamari [k]	0.96
	Tamari [l]	0.71
Vinegar	Several corps	10.05
	Rice	8.94
	Apple	ND
Solid products		
Cheese	Camembert	0.16
	Blue	0.27
	Red cheddar	0.16
	Cottage	ND
Dried bonito	Bonito	0.24
Miso	Rice	ND
	Barley	2.38
	Soybean	5.35
Natto	Soybean	0.19
Nyufu	Soybean	0.66
Temphe	Soybean	0.51

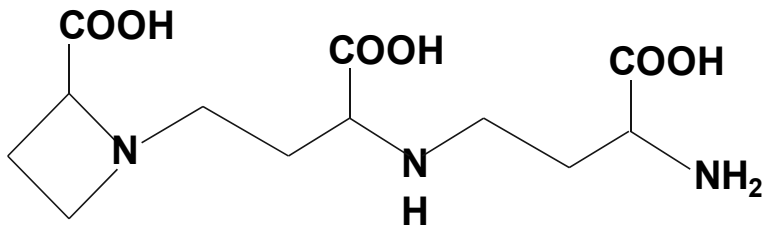
ND:no detected
a Made by the traditional method.
b Made by the enzymatic method.
c No ethanol is added throughout the manufacturing procedure.
d Made from intensively polished rice and fermented at low temperature with no or little ethanol added, and then carefully refined.
e Made from unpolished rice and aged for 3 year.
f The ratio of defatted soybean: wheat is about 1:1 in the starting material.
g The ratio of raw soybean: wheat is about 1:1.
h Soy sauce with weak color. The starting material is the same as that of koikuchi.
i Refermented soy sauce using soy sauce instead of salt water for fermentation.
j Almost all of the material is wheat. Its color is weaker than that of usukuchi.
k Almost all of the material is defatted soybean.
l Almost all of the material is raw soybean.
m The values of solid products indicate those of the extracts by 10 times volume of hot distilled water.

Table 1. ACE Inhibitory Activities of Various Fermented Foods(17)

Peptides	Origin
GPAGAHyp	Gelatin
GPHypGTDGAHyp	Gelatin
FFVAPFPEVFGK	Bovine α_{s1}-casein
AVPYPOQ	Bovine β-casein
TTMPLW	Bovine α_{s1}-casein
YIPIQYVLSR	Bovine β-casein
VHLPPP	Corn-zein
VHLPP	Corn-zein
LPP	Corn-zein
LRP	Corn-zein
LSP	Corn-zein
LQP	Corn-zein
AVN	Fig sap
PIP	Fig sap
LYPVK	Fig sap
LKY	Euphausia protein
PTHIKWGD	Tuna muscle
YKSFIKGYPVM	Sardine muscle
KVLAGM	Sardine muscle
IVGRPRHQG	Dry bonito
IKP	Dry bonito
SVAKEL	Bonito internal organ
FCF	White egg albumin
IIY	Wheat glyadine
YRILEF	Soy protein
FVIPAGY	Soy protein
IAPNY	Rice protein
IYPRY	Refined sake

Amino acids were shown by one chracter mark.
Hyp:hydroxyl proline

Table 2. Peptidyl ACE inhibitor from foods and food proteins



Nicotianamine

Fig. 2. Structure of nicotianamine.

Family (Nomenclature)	IC_{50} (mg/ml・raction mixture)	NA (mg/dry 100g)
Common bean *(phaseolus vulgaris)*	0.1	37(n=10)
Pea *(pisum sativum)*	0.08	50.7(n=5)
Cowpeas *(vigna unguiculata)*	0.39	18.7(n=5)
Broad bean *(Vicia faba)*	0.09	25.5(n=3)
Soybean *(Glycine max)*	0.11	39.5(n=6)
Sword bean *(Canavalic gladiata)*	0.4	11.9(n=1)
Chickpea *(Cicer qrietinum)*	79.6	31.5(n=1)
Lentis *(Lens culinarics)*	88.7	36(n=1)

NA:Nicotianamine

Table 3. ACE inhibitory activities of various beans(21)

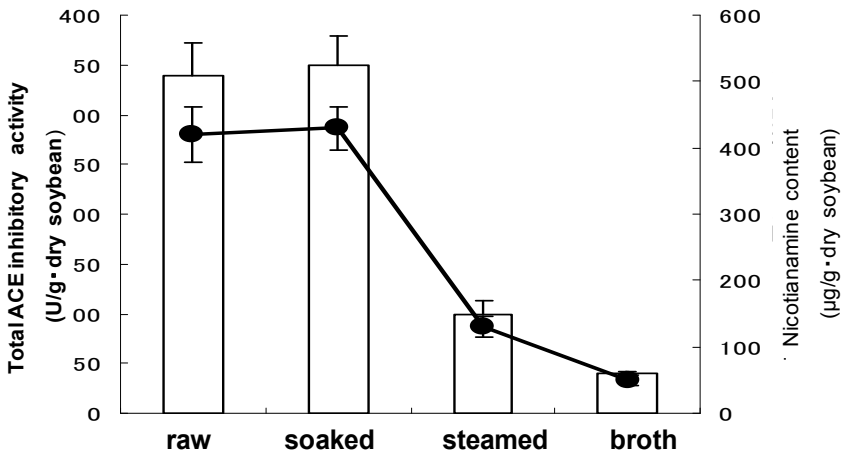
3.3 ACE inhibitor in various vegetables and mushrooms.

NA content in various vegetables and mushrooms have been reported .
NA content of Hayatouri(*Sechium edule*), Seri(*Oenanthe javanica*) ,Ashitaba(*Angelia keiskei*), Moroheiya (*Corchorus olitorius*), Kureson (*Nastrutium officinale*), Nigauri (*Nomordica charantia*) were 1.0 , 1.2, 1.7, 2.2, 3.7, 1.5 mg/100g/fresh matter, respectively(22). Of the 23 mushrooms, marked ACE inhibitory activity was observed in *Rhodophyllus clypeatus, Lyophyllum semitable,* and *Pholiota adipose*. The NA content of *Rhodophyllus clypeatus* was 13.2mg / dry 100g (23).

4. Isolation of Nicotianamine (NA) from soybean broth and changes in its content in the soybean steaming process.

Soybean broth (SB), a by-product of miso, soy sauce and natto as tradtional japanese foods manufacturing, is discarded as an industrial waste (24). The change in the ACE inhibitory

activity in the soybean steaming process was investigated to evaluate the use of SB as a functional food material. An ACE inhibitor was isolated from soybean broth by ion-exchange and gel chromatography. The results of the chemical identification by TOF-mass, IR, and NMR spectra analysis corresponded to those of Kristensen et al for NA (25). The yield of pure NA from SB was 0.037% (dry matter).
In the soybean steaming process at 121ºC for 30 min, the ACE inhibitory activity was the same in both raw and soaked soybeans, but decreased to 30% of raw soybeans in steamed soybeans and 10 % in SB (Fig.3).

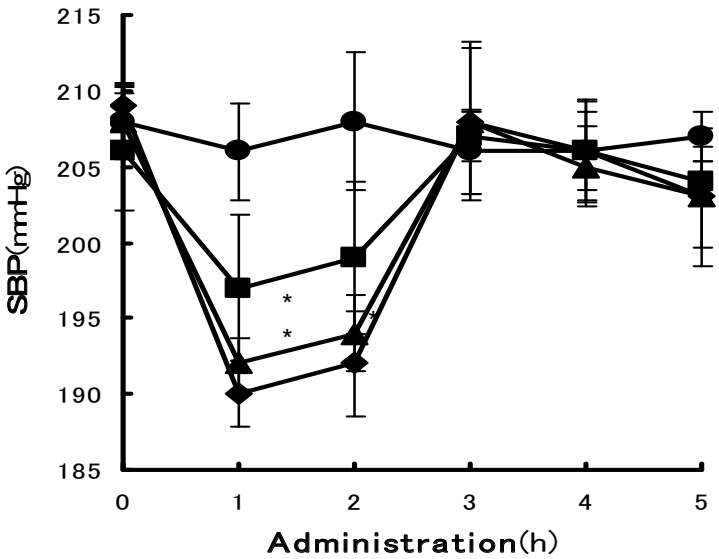


One unit of ACE inhibitory activity is defined as the amount of inhibitor needed to inhibit 50% of the ACE activity(IC_{50} value).
Means ±SD(n=3), □: total ACE inhibitory activity (U/g・dry) ● :nicotianamine content (μg/g・dry)

Fig. 3. The changes of ACE Inhibitory activity and Nicotianamine(NA) content of beans and broth in the steaming process

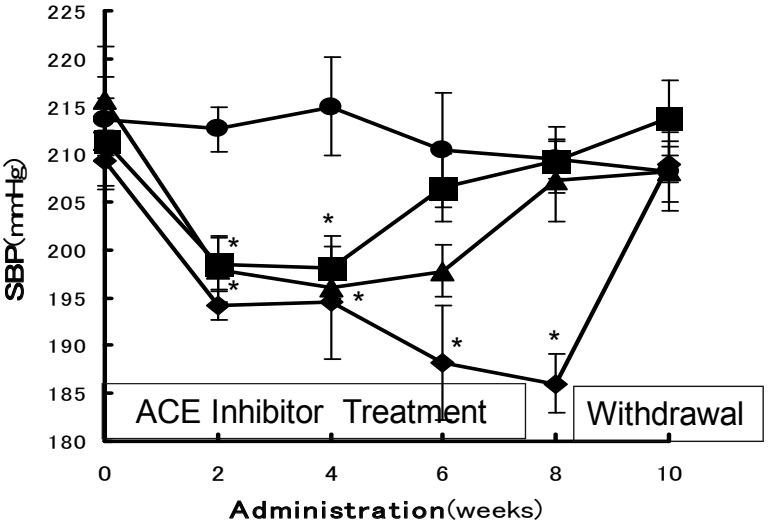
5. Antihypertensive effects of nicotianamine (NA) in spontaneously hypertensive rats

The effect of the ACE inhibitor NA form soybean broth on blood pressure was investigated in spontaneously hypertensive rats (SHR) upon single and long-term administration (26).
Single oral dose of NA (0.9, 4.5, 9.0mg/kg body weight) decreased blood pressure 1h after administration, and to the control level 3h after administration (Fig.4).
Long-term oral dose of NA(0.9mg and 4.5mg/kg body weight) decreased blood pressure for 4 weeks after administration, while that of NA(9.0mg/kg body weight)was decreased for the full 8-weeks feeding period(Fig.5).



Symbols: ●: control: saline at a dose of 5.0ml/kg body weight; ■: NA(0.9mg/kg・b. w.); ▲: NA(4.5mg/kg・b. w.); ◆: NA(9.0mg/kg・b. w.)
Each value is expressed as mean ±S.E.M. (n=6).
Significant difference from the control group:*p<0.05

Fig. 4. Effects of single administration of nicotianamine(NA) on systolic blood pressure (SBP) in SHR



Symbols: ●: control: NA (0.0mg/kg body weight); ■: NA (0.9mg/kg・b. w.); ▲: NA (4.5mg/kg・b. w.); ◆: NA (9.0mg/kg・b. w.)

Fig. 5. Effects of long-term administration of nicotianamine(NA) on systolic blood pressure (SBP) in SHR
Each value is expressed as mean ±S.E.M. (n=6).
Significant difference from the control group:*p<0.05

At 8 weeks after administration, serum NA content in SHR was determined by amino acid analyzer and revealed that NA was not detected in the blood of SHR (0.9mg and 4.5mg/kg body weight group), while 32.6±7.3 μg/ dL NA was detected in the 9.0mg/kg body weight group.

It was suggested that NA absorbed from the intestine decreased the systolic blood pressure (SBP) in SHR, and an appropriate NA level (9.0mg/kg body weight group) may provide long-term antihypertensive effects upon administration.

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4

Chemical Ecology Studies in Soybean Crop in Brazil and Their Application to Pest Management

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1. Introduction

Considering the current state of soybean production and markets around the world, it is readily apparent that it is possible to divide the countries in the world in two halves: producer and consumers'. Consumers' countries are mainly those belonging to the European Union that have their need for proteins used for animal feeding supplied in their majority by soybean seed or meal imports (Dros, 2004). The majority of soybean production is shared (80%) between four countries: the United States, Brazil, Argentina and China (Dros, 2004). Therefore, if we consider only those countries that may supply their internal needs and exporting either seeds, meals or oils, only the USA, Brazil and Argentina remain as exporting countries (Daydé et al., 2009). In this context, Brazil is currently the world second largest soybean producer (18%) and exporter (19%), with a cultivated area for soybean around 23 million ha and production around 3 ton/ha, reaching yearly a total production of approximately 68 million ton (CONAB, 2010).

Worldwide, scientists in different countries are trying to increase both the productivity and profitability of the agricultural sector of their economies, to feed growing populations and to increase the quality of life for millions of people. In recent years there has been a growing concern about environmental changes, and about how we are using the resources available in natural habitats. These concerns have triggered a search for natural products as a source of medicines, cosmetics, fuel, nutrients for humans and animals, and, especially, as alternatives to synthetic pesticides. These have led to an awareness of the importance of studying natural products, and of conserving the natural habitats that remain largely unexplored in the tropics and subtropics. Within this context, and bearing in mind that research with semiochemicals has been producing more efficient tools for insect pest management, this review opens opportunities for discussions of new technologies to reduce the use of toxicant chemical insecticides against insect pest species. The development of new monitoring methods based on semiochemical interactions, sexual pheromone baits, species-specific attractants (including natural enemies), molecular biology studies and vibrational communication offers great prospects for its application in Pest Management and Area Wide Pest Management Programs of insect populations.

Despite Brazil being one of the largest producers and exporters of soybean in the world (CONAB 2010), millions of tonnes of grains and products are lost every year due to damage by pests, mainly stink bugs (Hemiptera: Pentatomidae) (Panizzi, 1997, 2007).

Phytophagous stink bugs feed on a wide variety of plants, including numerous important crop plants such as grains, forage and fiber crops, vegetables, and perennial crops such as tree fruits and nuts (Panizzi, 2000). Damage is caused by both immatures and adults, but only adults are winged and capable of long-distance movement. Injury to young seeds, fruits, or nuts produces necrotic lesions and often results in premature abortion, while attacked leaves may wilt and die. Stink bugs are also known or implicated as vectors of plant pathogens such as yeast, fungi, and bacteria (Pannizzi 1997).

Stink bugs are reported attacking soybean crops during reproductive stages (Panizzi, 1997), and throughout this period they suck pods and grains causing direct damage and indirect injury. In Brazil, several species of stink bugs have been associated with soybean, and the more frequently observed are: *Euschistus heros* (Fabricius), *Nezara viridula* (Linnaeus), *Piezodorus guildinii* (Westwood), *Chinavia impicticornis* (Stål), *Chinavia ubica* (Rolston), *Dichelops melacanthus* (Dallas), *Dichelops furcatus* (Fabricius), *Thyanta perditor* (Fabricius) and *Edessa meditabunda* (Fabricius) (Panizzi and Slansky, 1985; Panizzi and Corrêa Ferreira, 1997; Medeiros et al., 1997; Laumann et al., 2008b; Vieira et al., 2008) (Figure 1). In this species complex the brown stink bug, *Euschistus heros* is the key pest, principally in central Brazil, due to its high abundance and heavy damage to the crop.

The stink bugs produce a wide variety of chemical compounds that show potential to be used as a toll for managing these insects. Among these compounds are the pheromones, which can be classified as either sexual, alarm or aggregation pheromones (Aldrich, 1988). Stink bug management by means of the use of semiochemicals is an alternative that has shown potential for application in soybean and other crops (Aldrich, 1988; Borges et al., 1998a; b; 2010; Cullen & Zallom, 2000; 2005; 2006). Effective bug management hinges on the rapid detection of these invasions so that appropriate control measures can be implemented before serious crop damage occurs.

Associated with this more ecologically based approach, egg parasitoids and soybean resistant varieties also can be manipulated to manage stink bugs. Egg parasitoids (Hymenoptera: Scelionidae) are the principal natural enemies of stink bugs. These egg parasitoids have biological attributes and behavioral responses to host density that could lead to density-dependent parasitism and efficient local regulation of stink bug populations (Laumann et al., 2008; 2010). Some species of this parasitoid family are being considered and used in many countries for biological control of stink bugs, by mass or inoculative liberations, especially the cosmopolitan *Trissolcus basalis* (Wollaston) (Caltagirone, 1981; Clarke, 1990) and the neotropical *Telenomus podisi* Ashmead (Côrrea-Fereira, 2002) (Figure 1).

The egg parasitoids might be used through mass release and their behavior might be managed using semiochemicals from its host or plants. The use of natural enemies as biological control tool of pests has been confirmed as very positive to the economy and environment. Studies have reported that around $ 20.92 billions of dollars are lost to native pests in the USA, and when the natural control is acting these losses decrease to $ 7.32 billion (Losey & Vaughan, 2006). Within natural control are included parasitoids, predators, pathogens, climatic conditions and host-plant resistance. Soybean resistant varieties have been recommended for pest control because the pest populations can be reduced to levels below the economic threshold reducing the disturbance and pesticide inputs to the agroecosystem without extra costs for the growers. Additionally, the resistant cultivars

could be compatible with chemical, microbial, and cultural control methods, including synergistic effects with natural enemies. Therefore, it is important that host-plant resistance combine positively with the action of natural enemies.
In this review we will give an overview of the main semiochemicals related to the Brazilian soybean stink bug complex, illustrating the diversity of the compounds and the possibilities of using these compounds in pest control. Other topics related to chemical ecology and behavioral ecology of stink bugs, their host plants and their natural enemies will be considered.

2. Pheromones and other stink bugs semiochemicals

Semiochemicals mediate interactions of insects with other organisms and they can be used in different ways for population monitoring, mass trapping, sexual confusion and in indirect applications, such as behavioral manipulation of natural enemies with parasitic wasps, that use both sex pheromones and defensive compounds during their foraging behavior (Vinson, 1985; Borges et al., 1998a; Bruni et al., 2000; Fiaboe et al., 2003). Defensive compounds also are used as defence against predators, since many are repellent, irritant or toxic (Eisner, 2003).

2.1 Defensive compounds

The stink bugs from the soybean complex, *Chinavia impicticornis*, *Chinavia ubica*, *Dichelops melacanthus*, *Euschistus heros* and *Piezodorus guildinii* have had their blends of defensive compounds evaluated both qualitatively and quantitatively. All these studies showed that the blends of defensive compounds are species-specific. Thus, different species may have exactly the same compounds in the blend, but these compounds are present in different ratios.
This specificity can potentially play an important role in intra-specific communication and may help to understand the phylogeny of some pentatomid groups, through chemotaxonomy, and in understanding the evolution of insect defence. For example the sister species, *C. impicticornis* and *C. ubica* are more similar to each other than the species *E. heros*, *P. guildinii* and *D. melacanthus* (Fig. 2A) (Pareja et al., 2007).
The main compounds identified from the glands of Brazilian stink bugs are: 2-alkenals (aldehydes with an insaturation on the second carbon), mainly the *trans* isomer with very small quantities of the *cis* isomer; saturated aliphatic hydrocarbons; and 4 oxo-(*trans*)-2-alkenals (C_6, C_8 and C_{10}). Esters, alcohols and unsaturated hydrocarbons are present as well, but in lower quantities and in some species, such as for the males of *P. guildinii*, many sesquiterpenes were identified (Borges et al., 2007). The 4-oxo-alkenals are present in higher quantities in nymphs of the pentatomids when compared with MTG of adult insects (Aldrich, 1988). These compounds were responsible for separating nymphal blends from adult blends of Neotropical pentatomids (Fig. 2B) (Pareja et al., 2007).
The biological role of most of these defensive compounds still needs to be studied. Three of the compounds that were identified in higher concentrations in the Brazilian species, namely (*E*)-2-octenal, (*E*)-2-decenal and (*E*)-2-hexenal, were reported initially as defensive compounds (Gilby & Waterhouse, 1967) and later as alarm pheromones (Pavis et al., 1994, Lockwood, 1987). The esters, although in lower concentrations when compared to the aldehydes have also been shown to have biological importance, mainly as an alarm pheromone in *N. viridula* (Lockwood & Story, 1987).

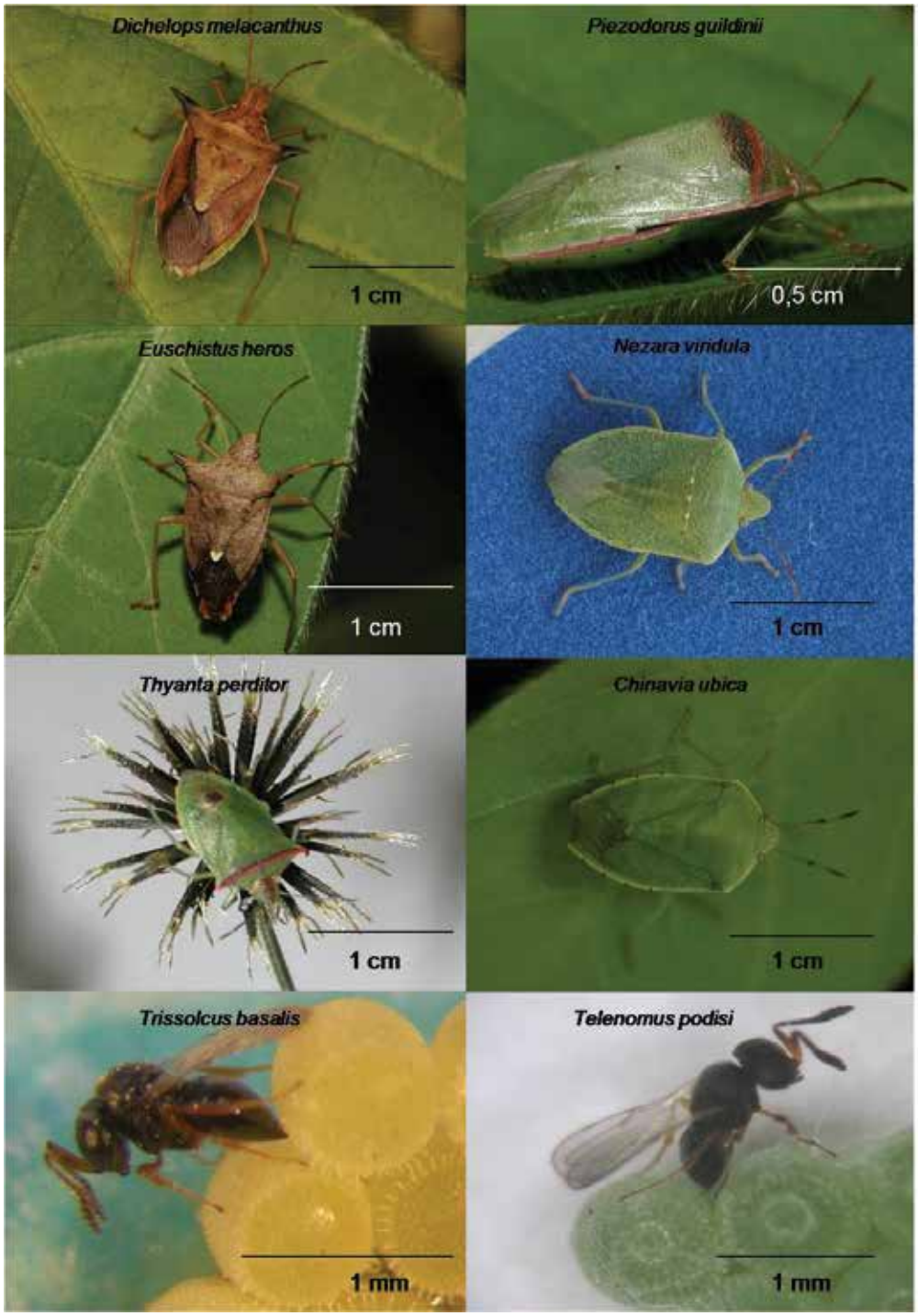


Fig. 1. The main stink bugs and their natural enemies commonly find on soybean crops in Brazil. Stink-bugs pictures by Cecília Vieira, parasitoids photographs by Raúl Laumann.

These aldehydes have also been shown to have important anti-microbial action, in particular against the entomopathogenic fungus *Metarhizium anisopliae* (Metschnikoff) Sorokin (Borges et al., 1993). These blends could therefore be important in determining susceptibility of these insects to pathogens, a consideration of likely importance in biological control efforts.
The aliphatic hydrocarbons are present in large quantities, and in most species tridecane is one of the principal compounds found in gland or aeration extracts (Moraes et al. 2005a, Aldrich et al., 1989, 1994, Borges et al., 2007, Pareja et al., 2007). In *Edessa rufomarginata* (De Geer) (Howard & Wiemer, 1983) and *Edessa meditabunda* (Moraes et al., unpublished data) undecane is the most abundant aliphatic hydrocarbon. Lockwood & Story (1985) found that tridecane has an action as both an aggregation and as an alarm pheromone for *N. viridula*, in a dose-dependent manner, though recently, Fucarino et al. (2004) did not find any biological activity for tridecane in *N. viridula*. Lockwood & Story (1985) only found effects at very high (alarm) and very low (aggregation) concentrations. It is difficult to determine whether these concentrations are biologically relevant, or those used by Fucarino et al. (2004) are more realistic, since very high amounts (up to 1 mg) of tridecane can be present in the MTG at any given time. However the aggregation effect at very low concentrations showed that these insects can detect the compound in small amounts and this is likely to be a true aggregation effect. Aldrich (1988) proposed that one of the functions of the aliphatic hydrocarbons is to serve as solvents that modulate the evaporation of the other compounds, and this can explain the high quantities of these compounds found in extracts of the stink bugs.
The 4-oxo-(*E*)-2-alkenals are compounds that have only been reported in the Hemiptera. Fucarino et al. (2004) showed that 4-oxo-(*E*)-2-decenal elicits aggregation behavior in first instar nymphs of *N. viridula*, as had been proposed by Borges & Aldrich (1992). However there is no further information on the biological activity of these compounds, since they are unique to these species, and therefore difficult to obtain. Compounds with very similar mass spectra to, and eluting just after 4-oxo-(*E*)-2-hexenal have been detected in Pentatomidae (Pareja et al., 2007) and other Hemiptera (Drijfhout et al., 2002). However, it is not yet clear whether these are produced by the insects or are breakdown or rearrangements of 4-oxo-(*E*)-2-hexenal.

2.2 Sex pheromones

The Hemiptera show a fantastic variety in the chemical structure of the few sex pheromones identified so far. In contrast to the Lepidoptera, the Hemiptera do not show a structural pattern of the compounds associated to families or subfamilies (Moraes et al., 2008a). However, for some species of Pentatomidae it is possible to observe a structural pattern inside the same genus.
So far all the studies with stink bugs have detected the male as the producer of the sex pheromone (McBrien & Millar, 1999, Moraes et al., 2005a, Borges et al. 2006, Borges et al., 2007). In addition males are responsible for emitting sounds that attract females during courtship and mating (Čokl et al., 2000, Moraes et al., 2005b).

Species of *Nezara* group

Nezara group include more than 100 species with 8 genera and they are cosmopolitan with higher diversity in Afro- and Neotropical regions. Schwertner & Grazia (2006) conducted a cladistic study of this group considering *Chinavia* a valid genus that include Nearctic and Neotropical species formerly classified as *Acrosternum*. Following this new classification all *Acrosternum* from the regions cited above will be refereed as *Chinavia* in this text.

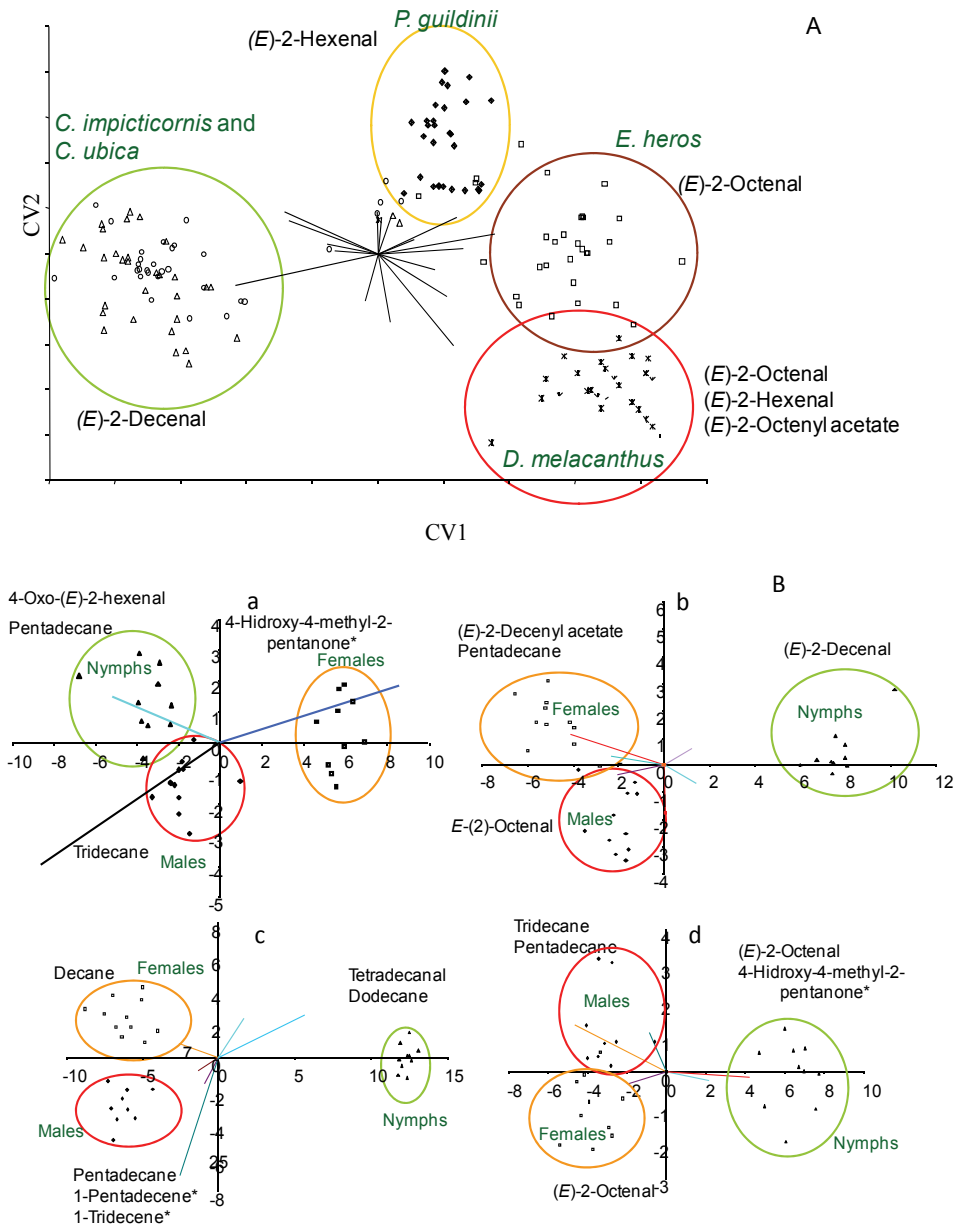


Fig. 2. Canonical variates analysis (CVA) biplot for (A) the analysis differentiating between species, and (B) the stage analysis independent of different species a) *Chinavia impicticornis*, b) *Chinavia ubica*, c) *Euschistus heros* and d) *Dichelops melacanthus*. The points are the individual scores for each replicate, calculated from the CVA equation that maximises differences between treatments along the two dimensions defined by the two canonical variates (CV1 and CV2). The lines are the loadings for each of the variates (compounds), only the main compounds that separate the species and different stages insects are shown. Asterisks indicate tentative identification.

Of the species within the genus *Nezara* and *Chinavia* studied from different geographic areas in the world the major components identified were: *trans*-(*Z*)-bisabolene epoxide (*trans*-*Z*-EBA) ((*Z*)-(1′*S*,3′*R*,4′*S*)(-)-2-(3′,4′-epoxy-4′-methylcyclohexyl)-6-methyhepta-2,5-diene) and the corresponding *cis*-isomer (*cis*-*Z*-EBA) (Baker et al., 1987; Aldrich et al., 1987; 1993; McBrien et al., 2001) (Table 1). These studies showed that the specificity is guaranteed by the different ratios of the two components produced by the different species (Aldrich et al., 1993; McBrien et al., 2001; MCB Moraes, RA Laumann & M Borges, unpublished data). Miklas et al. (2000) using solid phase micro-extraction (SPME) methodology with *N. viridula* from different parts of the world reported that there is a great variability in the quantities produced of the sex pheromones between males of the same species, but the ratio of *cis* to *trans*-*Z*-EBA is constant.

Only two species of genus *Nezara, N. antennata* and *N. viridula*, have had their sex pheromone identified, but nine *N. viridula* populations were studied from different geographical areas. All of these populations showed the *trans* isomer in higher quantities than the *cis* isomer. In contrast to the *Nezara* species, the *Chinavia* species presented higher quantities of the isomer *cis*-*Z*-EBA. The exceptions are *C. pennsylvanica* (Say), which has a ratio close to one between the isomers, and *C. impicticornis*, which only produces the *trans* isomer (Moraes et al., 2008a). It is worthwhile to notice that C. *impicticornis* has the ratio between the components more similar to the blend identified on *Nezara spp.* than the others *Chinavia* species.

The function of the ratio of *trans* to *cis*-Z-EBA is still unclear. Aldrich et al. (1989) reported that in laboratory experiments males of *N. viridula* from Japan mated with females of *N. viridula* from the USA (Mississippi), and the offspring were fertile and produced a ratio of *trans* to *cis*-Z-EBA of 1.53, which is intermediate between the two parental blends. Electroantenography experiments with female antenna of *Chinavia ubica* and *C. impicticornis* showed that the females responded to both isomers *cis* and *trans* (*Z*)-EBA, but in bioassays they did not responded for heterospecific males, what indicate that they have receptors for different isomers, and the ratio between the compounds is essential to locate the partners of the same species (M.C.B, Moraes; R.A. Laumann & M. Borges data not published).

Bioassays in the laboratory carried out with *N. viridula* from Brasilia, Brazil, showed that females were attracted only to synthetic EBA when both isomers are present in the correct ratio (Borges, 1995). The absolute configuration was identified as being (1´*S*,3´*R*,4´*S*) (1) (Baker et al., 1987), and later Borges (1995) reported that females did not show the same level of response (attraction) to the racemic mixture than to the (1´*S*,3´*R*,4´*S*) enantiomer. A similar result was obtained by McBrien et al. (2001) working with *Chinavia hilaris* (Say). Females of C. *hilaris* responded only to the pheromonal mixture containing both *cis* and *trans*-*Z*-EBA in the same ratio released by the males, additionally the absolute configuration identified for this species was the same as found by Baker et al. (1987).

Euschistus heros

The pheromone components of *E. heros* were identified by (Borges et al., 1994, and Aldrich et al., 1994) and consists of three components: methyl 2,6,10-trimethyldecanoate, methyl 2,6,10 trimethyldodecanoate, and methyl 2*E*,4*Z* decadienoate (Table 1). Zhang et al. (2003) confirmed the ratio among the three components by previously proposed (Borges & Aldrich, 1994; Aldrich et al., 1994; Borges et al., 1998a) as being: 53 % methyl 2*E*,4*Z*-decadienoate, 3% methyl -2,6,10- trimethyldodecanoate and 44 % methyl- 2,6, 10-trimethyltridecanoate. Recently, Moraes et al. (2008b) carried out a study with E. *heros* that

revealed the importance of understanding the physiology and the behaviour of stink bugs in order to identify the correct sex pheromone blend. When the insects were aerated with food (*Phaseolus vulgaris* (L.) pods) males of *E. heros* released all three components in the ratio reported by Zhang et al. (2003) during seven consecutive days. On the other hand, when the insects were kept in aeration without food, after 48 h they stopped releasing the main component of the sex pheromone (methyl-2*S*,6*R*,10*S*-trimethyltridecanoate), and they released in higher quantities the first component of the blend, methyl-(2*E*,4*Z*)-decadienoate.
The biological activity of the three components of *E. heros* species was confirmed in a laboratory bioassay and showed that methyl 2,6,10-trimethyltridecanoate was the main component in attracting females (Borges et al., 1998a). Costa et al. (2000) carried out a set of experiments that showed that the racemic mixture of methyl 2,6,10 trimethyltridecanoate was efficiently attractive to females in laboratory bioassays. Methyl-2,6,10-trimethyltridecanoate has 8 possible stereoisomers. The absolute configuration of methyl 2,6,10 trimethyltridecanoate was determined by means of bioassays using the 8 stereoisomers separately, which were synthesized by Mori & Murata (1994). Costa et al. (2000) reported that females respond better to isomer 2*S*,6*R*,10*S* than the other isomers when compared with a solvent. Borges et al. (1998b) using the racemic mixture of methyl 2,6,10 trimethyltridecanoate captured *E. heros* in field traps. The experiments were carried out placing rubber septum with 10 µg of racemic mixture of methyl 2,6,10 trimethyltridecanoate placed in a soybean crop (Borges et al., 1998b). Traps containing the racemic mixture caught a higher number of *E. heros*, along with another pentatomid, *P. guildinii*, when compared with a trap containing only the septum with solvent (Borges et al., 1998b). Additionally, the racemic mixture in a lure formulation showed great power to attract *E. heros* in field conditions and a potential to be use for population monitoring of this species (Borges et al., 2010).

Thyanta perditor

Males of *T. perditor* produce the ester, methyl-2*E*,4*Z*,6*Z*-decatrienoate ((*E*2,*Z*4,*Z*6)-10:COOMe) as the main sex pheromone component (Moraes et al. 2005a) (Table 1). The same component was also identified in the pheromone blend of the two nearctic species *Thyanta pallidovirens* and *Thyanta custator acerra* (Millar et al., 1997; McBrien et al., 2002). As in *Nezara* and *Chinavia* species, *Thyanta* spp have a very similar blend, and one of the compounds is the same, which could suggest that closely related species of stink bugs share the same or similar blends of compounds as sex pheromone and specificity is due to different ratios of these compounds. Bioassays in the laboratory showed that 4 µg of the (2*E*,4*Z*,6*Z*) methyl decatrienoate was attractive to females of *T. perditor* (Moraes et al., 2005a). In addition to bioassays in the laboratory, the sex pheromone of *Thyanta perditor* was the subject of field trials. Traps baited with 1 mg (*E*2,*Z*4,*Z*6)-10:COOMe, were more efficient in capturing *T. perditor* than control traps. Additionally, traps baited with the sex pheromone captured a significantly higher number of insects than the sampling cloth technique, as well as, some tachinid parasitoids of stink bugs (R.A., Laumann; M.C.B., Moraes; A., Krimian & M., Borges not published). Results from field tests showed that the (*E*2,*Z*4,*Z*6)-10:COOMe has potential for monitoring populations of *T. perditor*, and this technique is more easily performed, than the traditional cloth sampling.

Piezodorus guildinii

The Neotropical redbanded stink bug, *P. guildinii* is one species of the complex of stink bugs that are serious pests of soybean in Central and South America, especially in Brazil (Panizzi & Rossi, 1991). Borges et al. (1999a) reported the identification of two components of *E. heros*

sex pheromone in the sex pheromone blend of *P. guildini,* methyl-2*S*,6*R*,10*S*-trimethyltridecanoate and methyl-2,4,6- trimethyldodecanoate. After this, Borges et al. (2007) identified a new compound specific to males when gas chromatograms of extracts of volatiles collected from sexually mature virgin males and females were compared. This compound was not found in aerations of sexually immature females or males, and its retention time and mass spectra matched the sesquiterpene β-sesquiphellandrene (Borges et al., 2007) (Table 1). The quantitative analysis of 10 extracts obtained from volatile collection of 20 males resulted in a mean liberation of sexual pheromone of approximately 40 ng/24 h.
For *Piezodorus guildinii* the bioassays showed that females of the species responded preferentially to (7*R*) - β-sesquiphellandrene and the females also showed response to the isomer (7*S*) - β-sesquiphellandrene (Borges et al., 2007). Furthermore, the role and biological activity of *P. guildinii* compounds as a mediator of reproductive behavior under natural conditions still remains to be determined.
It is likely that the pheromone will act in concert with the substrate-borne vibrational signals that this insect uses for communication over shorter ranges (Moraes et al., 2005b). Thus, developing pheromone-based monitoring systems for this insect may be, in the near future, considered an integration of both the pheromonal (chemical) and vibrational (physical) signals to be incorporated into trap design.

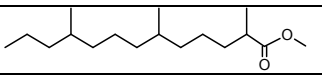
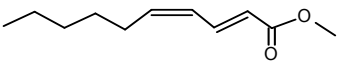
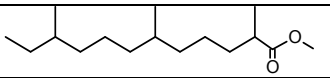
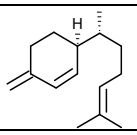
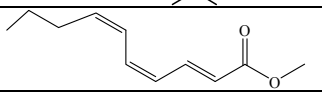
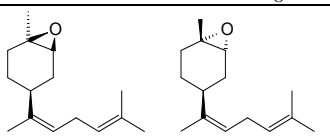
Compounds	Structure	Species
Methyl 2,6,10-trimethyltridecanoate		*Euschistus heros*
Methyl *E* 2,*Z*4- decadienoate		*Euschistus heros*
Methyl 2,6,10-trimethyldodecanoate		*Euschistus heros*
(7*R*)-(+)-β-Sesquiphellandrene		*Piezodorus guildinii*
Methyl *E*2, *Z*4, *Z*6-decatrienoate		*Thyanta perditor*
cis-*Z*-Epoxybisabolene *trans*-*Z*-Epoxy-bisabolene		*Chinavia* sp. *Nezara* sp.

Table 1. Sex pheromone structures from the complex of the Brazilian stink bugs (Hemiptera: Pentatomidae) soybean pests.

2.3 Stink bug semiochemicals and behavior of natural enemies'

The potential for utilising semiochemicals to manage parasitic Hymenoptera to improve biocontrol programs has been discussed by several authors (Smiths 1982; Lewis & Nordlund, 1985, Lewis & Martin 1990; Powell & Pickett, 2003). The identification of factors leading to host recognition, acceptance and oviposition, and *in vitro* rearing and mass

production may offer possibilities for making previously artificial and unacceptable hosts, acceptable after treatment with kairomones, for example (Vinson, 1985).

The selection process of a host by a parasitoid involves both location and recognition behavior that precedes oviposition (Vinson, 1984, 1985). In each phase of the selection process, physical and chemical stimuli acting as cues, play an important role and may alter parasitoid behavior (Weseloh, 1981; Nelson & Roitberg, 1995). Therefore, the cues may have different origins. These can be derived from the host plant, the host itself or from a host product. In this case, they are called kairomones (Noldus & van Lenteren, 1983; Blum, 1996). Some of these cues are concerned with host location and involve volatile semiochemicals that act at long-distances (Sales et al., 1979; Aldrich et al., 1984; Bin et al., 1987; Borges et al., 1998b; Dicke, 1994). Other cues act at short range and are involved in host recognition (Buleza & Mikheev, 1978; Leonard et al., 1987; Nordlund et al., 1987; Borges et al., 1998b). Various stimuli have a certain range over which they can provide information to the parasitoid. Once the parasitoid is within a potential host community, shape and semi-volatile factors increase in importance and when the host is contacted, contact chemicals, shape, size and texture then become important (Vinson, 1984).

Scelionidae that parasitize eggs from stink bugs can use several types of semiochemicals for long-range localization of habitat, microhabitat, and hosts: volatiles from plants damaged by stink bug oviposition or feeding (Colazza et al., 2004; Moraes et al., 2005c, 2008c); sex pheromones (Aldrich, 1995; Borges et al., 1998a,b; 2003, Bruni et al., 2000; Silva et al., 2006); volatile defensive secretions from the metathoracic (adults) or dorsal abdominal (nymphs) glands of stink bugs (Aldrich 1995, Mattiacci et al., 1993; Borges & Aldrich 1992); or crude whole body extracts of stink bugs (Colazza et al. 1999; Salerno et al. 2006). Volatiles from non-host stages of stink bugs, such as pheromones or defensive compounds, as well as contact chemicals (traces left by walking insects) can also be used for host location, recognition, and acceptance leading to successful oviposition (Bin et al. 1993; Borges et al., 1999b, 2003; Colazza et al., 1999; Conti et al. 2003). Physical stimuli such as visual and resonance cues may also be involved in successful host search (Borges et al., 1999b).

The foraging behavior of *Telenomus podisi* in the presence of selected stimuli from its host, *Euschistus heros* were recorded in a closed arena bioassay. The parasitoids were given the choice between single and combined stimuli presented to them simultaneously (egg mass; virgin males and females volatile extracts; volatile extracts of sexually mature males and females; synthetic standards of male sex pheromone; (*E*)-2-hexenal, a component of the alarm pheromone, hexane and air). To find the host egg, *T. podisi* primarily uses the sensory cues released from the male insects, and the results suggest that the eggs did not release any volatile that could enhance or decrease the attraction of the egg parasitoid. *T podisi* responded to the male extract, sex pheromone components and to (*E*)-2-hexenal (Silva et al., 2006). In brief, in a multisensory condition, *T. podisi* use olfactory signals to find its host at long distance. This confirms previous studies that host location is oriented primarily by olfactory cues released directly out from the non target host egg, which is more detectable and reliable (Borges et al., 1998a).

The long range host searching behavior of the egg parasitoids *Telenomus podisi* and *Trissolcus basalis* is differentially influenced by the blends of volatiles released from the metathoracic glands of different stink bug species. We have studied whether such variable response is due to different individual components of these glands and whether these responses reflect host preferences. Y-tube olfactometer bioassays were carried out with crude extracts of metathoracic glands of five different host species of neotropical stink bugs. Furthermore, we

tested the parasitoids´ response to synthetic standards of individual compounds identified in these stink bug glands. Results showed that females of *T. basalis* and *T. podisi* responded differentially to crude gland extracts of the different species of host stink bugs and to the compounds tested. The parasitoid *T. basalis* showed a positive taxic behavior to *Nezara viridula* methathoracic gland extracts (Laumann et al., 2009) its preferred host species (Sujii et al., 2002). Furthermore, *T. basalis* responded positively to 4-oxo-(*E*)-2-hexenal and (*E*)-2-decenal, two components that are in higher quantities present in *N. viridula* glandular secretion. The compound 4-oxo-(*E*)-2-hexenal modified the kinetics traits of *T. basalis* walking pattern and suggests that this compound might stimulates the searching behavior of this parasitoid (Laumann et al., 2009).

The parasitoid *T. podisi* was attracted to crude gland extracts of the preferred host (*Euschistus heros*) and had its walking pattern altered when stimulated with 4-oxo-(*E*)-2-hexenal. In addition, *T. podisi* also responded positively to (*E*)-2-hexenal and to the hydrocarbon tridecane, both of which are defensive compounds released from the metathoracic glands by several stink bugs. These results indicate some degree of specialization in the response of two generalist parasitoid species towards defensive secretions of stink bugs (Laumann et al., 2009).

The use of semiochemicals for behavioral manipulation of parasitoids have been proposed and discussed extensively. In recent years, semiochemicals have also been discussed as a tool to improve biological control (Vet & Dicke, 1992; Lewis & Martin, 1990; Powell & Pickett, 2003). Specific knowledge about host - parasitoid relationships mediated by semiochemicals are important for improving the effectiveness of applications of semiochemicals in integrated pest control.

2.4 Vibrational communication in stink bugs

Aside from chemical communication stink bugs use vibratory signals during mating behavior. Vibratory signals provide information about species, sex, position on a plant and receptivity to mating (Čokl et al., 2000; Čokl & Virant-Doberlet 2003; Miklas et al., 2003a; b; Gogala, 2006). Vibratory signals emitted by pentatomids are efficiently transmitted through the plant (Čokl et al, 2005). Species- and sex-specific songs have been described in several species, and different vibratory songs have been described and correlated with reproductive behaviour in the Neotropical species *Chinavia impicticornis*, *Chinavia ubica*, *Euschistus heros*, *Piezodorus guildinii* and *Thyanta perditor* (Fig. 3) (Moraes et al., 2005b; Lopes et al., 2006).

Neotropical stink bug species feed and mate on the same host plants and in Brazil constitute major components of the soybean pest complex. During mating they communicate with species and sex specific vibratory signals. Songs differ between species in the time structure and amplitude modulation of their units. The repertoire of *C. impicticornis*, *E. heros* and *T. perditor* fits into the scheme described for most investigated stink bugs: females call with a sequence of pulses which differ among species in their duration and repetition rate, and males respond with courtship songs of species specific temporal structure and amplitude modulation of complex pulse trains (Čokl & Virant-Doberlet, 2003; Gogala, 2006). Female calling and male courtship songs are the main constituents of vibratory communication between sexes in the mating period. The other vibratory emissions represent either transitional song, support recognition during close-range courtship or are involved in male rivalry. First recorded vibratory emissions of *P. guildinii* confirm that genus *Piezodorus* represents an exception between Pentatominae (Moraes et al., 2005b). Irregularly repeated female vibratory signals do not trigger typical male courtship responses like in small stink

bugs *Holcostethus strictus* (Fabricius) and *Murgantia histrionica* (Hahn). On the other hand, complex rivalry with extensive frequency modulation of pulses as described also in *P. lituratus* opens a new insight into the role of vibratory communication in stink bugs (Moraes et al., 2005b).

Except for *P. guildinii,* songs of investigated species from Brazil fit well into the general pattern of vibrational communication in Pentatominae, though several questions remain unanswered. Studies on the functional value of statistically determined differences between songs are lacking and there are no data about the role of different host plants on the quality of vibratory signals exchanged during communication at different distances. Bimodal communication with olfactory and vibratory signals needs detailed studies on the nature of chemical signals involved and finally signals of other modalities like touch, vision and even low frequency near-field sound emitted by mates immediately before copulation (Moraes et al., 2005b). This knowledge could be applied in stink bug management if, in the future, the technology development can design micro-devices emitting vibrational signals that could be a powerful tool to be applied in the field to enhance the efficiency of the capture of stink bugs, and also to attract and keep the natural enemies in the field.

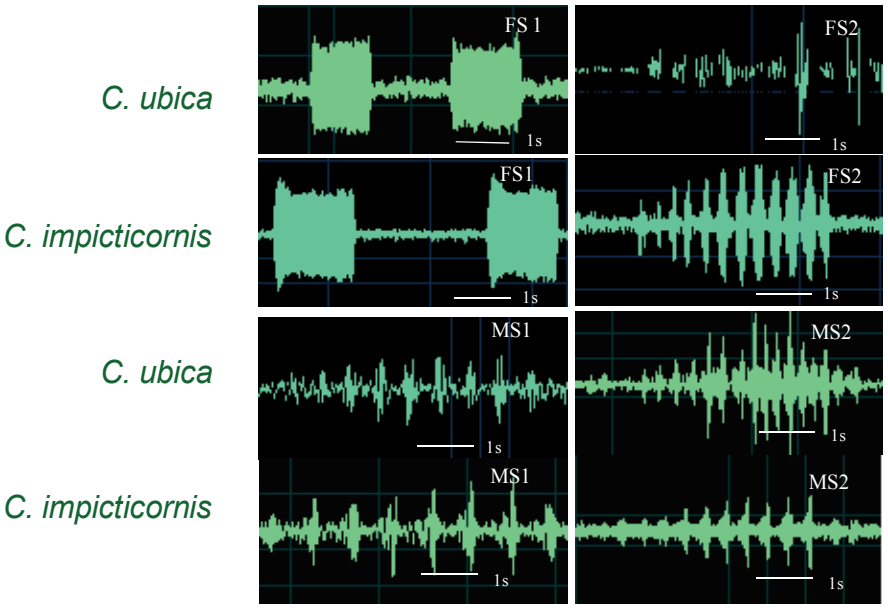


Fig. 3. Example of songs emitted by different *Chinavia* species stink bugs. FS1 = Female song 1; FS2 = Female song 2; MS1 = Male song 1; MS2 = Male song2.

Vibratory signals emitted by pentatomids are efficiently transmitted through the plant (Čokl et al., 2005) and can be used by natural enemies to obtain information about host presence and position.

Males and females of the Neotropical brown stink bug, *Euschistus heros*, communicate with vibratory songs transmitted through plants. The egg parasitoid *Telenomus podisi*, a natural enemy of these bugs, shows taxis to the vibratory signals of female songs on a plant and on an artificial substrate. This reaction is specific to the signal type since directionality is elicited by the songs of *E. heros* females, but not males or duet songs or by continuous pure tone vibratory signals. Vibratory signals do not influence other components of *T. podisi*

foraging behavior, such as linear velocity, turning rate and tortuosity. These results are the first register of a parasitoid eavesdropping sexual vibratory signal of insects. When *T. podisi* females were stimulated with songs of *E. heros* females they showed an oriented response (taxis) toward the emission points, with significant differences in the first choice and residence time in areas (leaves of plants or arms of the arena) vibrated with the songs. Additionally, a higher turning rate of *T. podisi* females when stimulated with this song indicates some effect on their kinetic locomotion behavior (Laumann et al. 2007). In addition to selectively responses to females' songs *T. podisi* also can discriminate between songs of different stink bugs species showing selective responses toward song for their preferred host *E. heros* (R. Laumann; A. P. S. Lopes; M.C.B. Moraes; A. Čokl & M. Borges, not published).
The use of vibratory signals of *E. heros* females over intermediate distances (on the same plant) can be favoured because these cues can be more reliable than chemical or visual cues from eggs. *Telenomus podisi* probably uses the vibratory signals emitted by females to locate sites where there is a high probability of finding eggs. This hypothesis can be valid in the case that stink bug females copulate and oviposit on the same plant; field experiments are needed to confirm or reject this hypothesis (Laumann et al., 2007).

2.5 Odor chemoreception

Insects communicate through visual, vibrational, chemical and tactile signals, the last two being perceived by sensory receptors known as sensilla located mainly in the antennae, legs and bodies labial (Silva et al., 2010). There are several studies reporting the importance of morphology and ultrastructure of the antennal sensilla (chemosensory hairs) to understand the mechanisms of olfaction (Hallberg et al., 1994; Isidoro et al., 2001; Gómez & Carasco, 2008); however, for pentatomids, few studies have been conducted in this area. In this group of insects the representative studies have focused on the morphology and ultrastructure of antennal sensilla of *Lygaeus kalmii* (Slifer & Sekhon, 1963), *Lygus lineolaris* (Dickens et al., 1995; Chinta et al., 1997), *Nezara viridula* (Brézot et al., 1997) *Odontopus nigricornis*, *Cyclopelta siccifolia* and *Chrysocoris purpurea* (Rani & Madhavendra, 2005).
To characterize chemical communication in stink bugs, it is necessary to know the structure of the olfactory system of these insects. Therefore, the morphology, abundance, and distribution of antennal sensilla in males, females and 5th instar nymphs of *E. heros, P. guildinii* and *E. meditabunda* were studied.
A transitional difference in sensilla density between nymphs and adults was recorded and this factor seems to be a common phenomenon reported for the stink bug *N. viridula* by Brézot et al. (1997). There is a general tendency for insects to increase the number of receptors at the adult stage, particularly chemoreceptors, such as basiconic and trichoid sensilla (Catalá, 1997). As reported by Chapman (1982), this may be related to different chemical communication. The increase in the number of chemoreceptors at the adult stage would reflect additional sensorial requirements for reproduction and dispersal in the host community.
Studies of ultrastructure and function of sensilla and labial parts were initiated through scanning electron microscopy (SEM) with the aim of identifying the olfactory sensilla on the antennae of *E. heros* and other stink bugs species. These studies may help in the interpretation of how the signal processing occurs in the insect and, indirectly, to develop more efficient tools to control these pests (Silva et al., 2010).
In addition to the main chemosensory receptors, the trichoid and basiconic sensilla, two additional types were identified: the knob-shaped and the slit-tipped basiconic sensilla. The sexual dimorphism in numbers of sensilla trichodea (ST1), sensilla basiconica (SB1) and

(SB2) constitutes an important feature in the chemical communication of these stink bugs. The results presented are important for ongoing studies on mating and host plant seeking behaviors in these species, and more detailed studies regarding the odor perception in the pentatomid group (Silva et al., 2010) (Fig. 4).

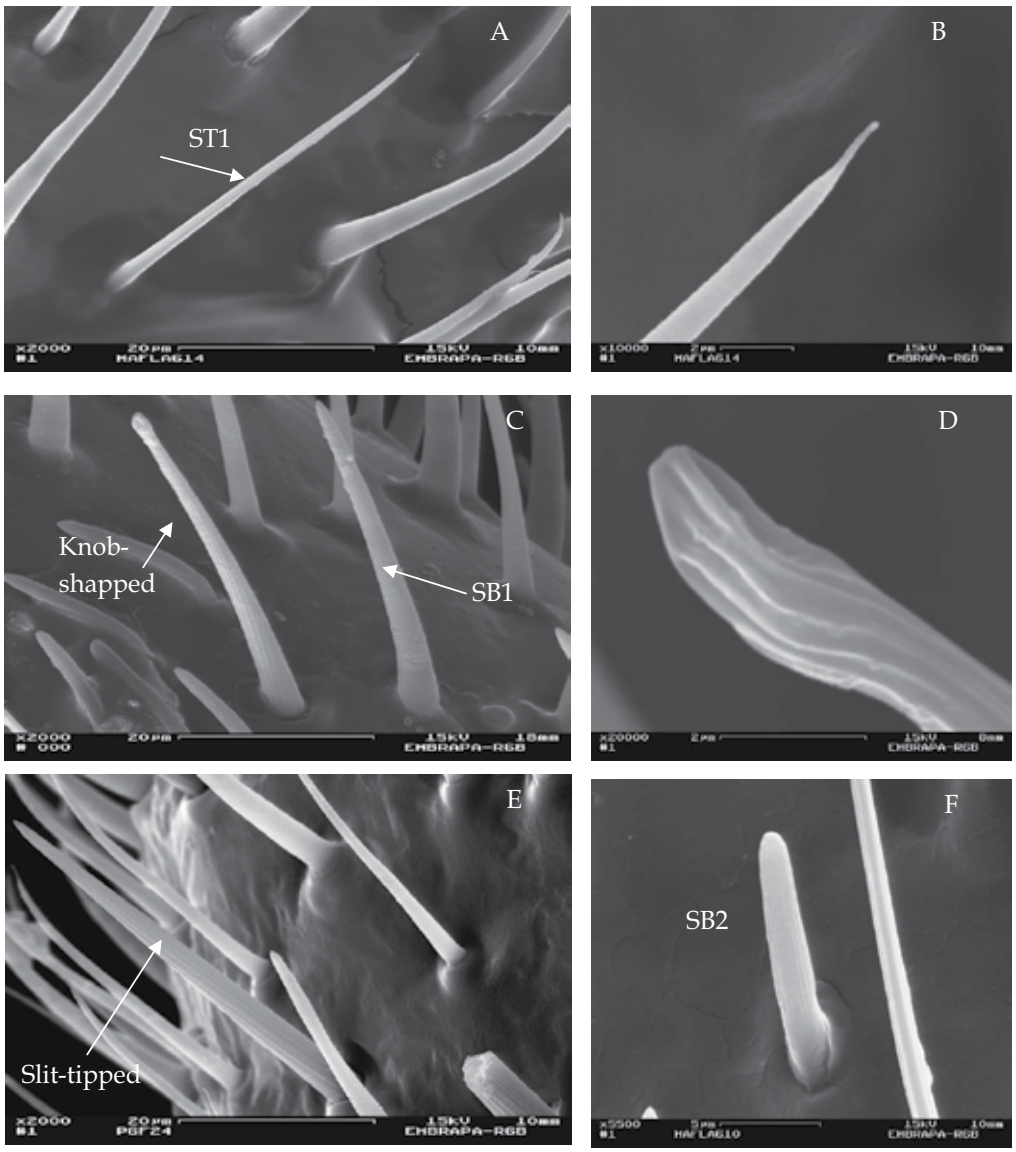


Fig. 4. SEM micrographs of the olfactoty sensilla on the antennae *of Euschistus heros* and others stink bugs. (A) The long straight, sharp-tipped sensillum trichoid 1(ST1) with smooth wall on the flagellum of *E. heros*. (B) Tip of the sensillum at higher magnification showing its hooked point. (C) knob-shaped and basiconic sensillum type 1 (SB1). (D) Tip of the sensillum at higher magnification showing deep grooves. (E). Long slit-tipped sensillum with longitudinal grooves. (F) Basiconic sensillum type 2 (SB2).

Going deeply into the sensilla structure, chemical signals cross the pores and are transported through the sensillum lymph to interact with the chemoreceptors, which in turn activate the cascade of events leading to spike activity in sensory neurons (Pelosi, 1996; Stengl et al., 1999). The most important proteins involved in the recognition of chemical cues comprise odorant-binding proteins (OBPs), and odor degrading enzymes (ODEs), which are involved in peripheral olfactory processing, and the chemoreceptor proteins family formed by the olfactory receptors (ORs). Insect OBPs are small globular proteins (about 135–220 amino acids long) that bind and solubilize hydrophobic odorants such as pheromones or allelochemicals, and transport and deliver them to chemoreceptors located on the sensory neurons (Matsuo et al., 2007; Laughlin et al., 2008). In the first case they are called pheromone binding proteins (PBPs) and in the second general odorant binding proteins (GOBPs) (Leal et al., 1999; Scaloni et al., 1999). In the antennae the OBPs are compartmentalized in specific sensilla, the PBPs are related to the sensilla trichodea (Steinbrecht et al., 1992) and GOBPs in sensilla basiconica (Laue et al., 1994). The CSPs (also known as olfactory specific-D, OS-D, or sensory appendage protein, SAP) comprise another class of small binding proteins (about 130 amino acids long), which are expressed in antennal and non-antennal tissues and are capable of binding odorants, however showing less binding specificity than the OBPs (Jacquin-Joly et al., 2001). The ODEs are selectively evolved to degrade pheromones and allelochemicals (Rybczynski et. al, 1989). Such enzymes attack specific functional groups, such as acetate esters, aldehydes, alcohols, ketones and epoxides. A few semiochemical degrading enzymes have been identified and characterized in detail in contrast with current efforts on OBPs and ORs. The ORs are expressed by olfactory receptor neurons (ORNs) and localized in the membranes of the ciliated dendrites as seven-transmembrane domain receptors of about 400 amino acids that bind environmental compounds, thereby transforming the chemical signal into the activation of neurons in the higher processing centres in the brain, which in turn mediate the appropriate behavior (Clyne et al., 1999; Engsontia et al., 2008).

All these protein components (OBP, ODE, and OR, sometimes CSP) of the olfactory system are extensively prospected as candidates for the development of biotechnological tools for pest control by different research groups around the world. Since the identification of the first PBP in the silk moth *Antheraea polyphemus* (Cramer) (Vogt & Riddiford, 1981), many other labs became interested in molecular components of the olfactory system mainly for insects of the Lepidoptera order. These became faster in insects from different orders with the advance of the genome assessment, greatly expanding knowledge of the range of species possessing these proteins and knowledge of the size and diversity of the olfactory gene families. Until now the genomes of nine insect species from different orders have been sequenced, allowing us to compare the organization richness of the olfactory system and helping to clarify the evolution of the olfaction machinery (Table 2). Nevertheless, the majority of the olfactory proteins identified are putative, based on their sequence similarity with sequences stored in a database, therefore the functional validation has not been provided in most of the cases.

In the Hemiptera order 21 OBPs have been identified and only for two hemipterans the genome sequencing information was provided, *Acyrthosiphon pisum* (Harris) (Aphidoidea) and *Rhodnius prolixus* Stål (Reduviidae). The group of scientists at Embrapa Genetic Resources and Biotechnology in Brazil is putting effort into sequencing the genome of at least one stink bug species, *Euschistus heros*, from the soybean complex. Using cDNA library construction from antennae and sequencing of 1000 clones two putative OBPs (called

Insect	OBP/CSP	OR
Acyrthosiphon pisum (Hemiptera: Aphidoidea)	49.6/5.4	86.9
Anopheles gambiae (Diptera: Culicidae)	60	-
Apis mellifera (Hymenoptera: Apidae)	58	16
Nasonia sp (Hymenoptera: Pteromalidae)	1	15
Tribolium castaneum (Coleoptera: Tenebrionidae)	3	13

Table 2. Genes representation of the olfactory system in insect genomes available in the NCBI site (National Center for Biotechnology Information).

*Eher*OBP1 and *Eher*OBP2, Genbank HM347779) and three putative CSPs (called *Eher*CSP1, *Eher*CSP2 and *Eher*CSP3) (Genbank in submission) have been identified. The *Eher*OBP1 presented the highest similarity to *Lygus lineolaris* (E-value 2e-20) and the *Eher*OBP2 to *Cimex lectularius* L. (E-value 7e-07) (Fig 5a). By BLAST similarity search, both *Eher*OBPs are hypothetically related to PBP family, and they are expressed in antennae in the gender producer of the sexual pheromone (males) and in the gender attracted (females), as evidenced by quantitative PCR analyses. The *Eher*OBP1 and *Eher*OBP2 are also expressed in legs, wings, abdomen and heads (without antennae), although in smaller amounts than in the antennae. The *Eher*CSPs identified are also expressed in the antennae of males and females and are more similar to each other than to the others CSP of other hemipterans (Fig. 5b) (L.R. Farias, M. Borges, M.C.B. Moraes, R.A. Laumann, D.P. Paula, unpublished data).

Fig. 5. Partial alignments of some putative OBPs identified in Hemiptera order with: a. Deduced amino acid sequences of the putative *Eher*OBP1 and *Eher*OBP2; b. Nucleotide sequences of the putative *Eher*CSP1, EherCSP2 and *Eher*CSP3 (ClustalX 1.81 program and edition using BoxShade 3.21). The gray color indicates the similarity and the black color the identity. The sequences are indicated by the GenBank code followed by the abbreviation of the genus and species. The species are: *Sitobion avenae* (Sav); *Acyrthosiphon pisum* (Apis); *Myzus persicae* (Mper); *Nasonovia ribis-nigri* (Nrib); *Lygus lineolaris* (LAP); *Aphis fabae* (Afa); *Aphis gossypii* (Agos); *Nilaparvata lugens* (Nlug); *Rhodnius prolixus* (Rpr); *Apolygus lucorum* (Aluc); *Cimex lectularius* (Clec); *Adelphocoris lineolatus* (Ali).

Considering the number of OBPs identified in other insect species and also by the proteomic results of the antennae (Fig. 6), it can be inferred that there is the potential to identify more olfactory proteins from *E. heros*. The differences in the polypeptide composition in the proteomic profile of the antennae of nymphs, males and females *E. heros* confirm the sexual dimorphism in numbers of sensilla trichodea and basiconica showed by the antenna morphology studies.

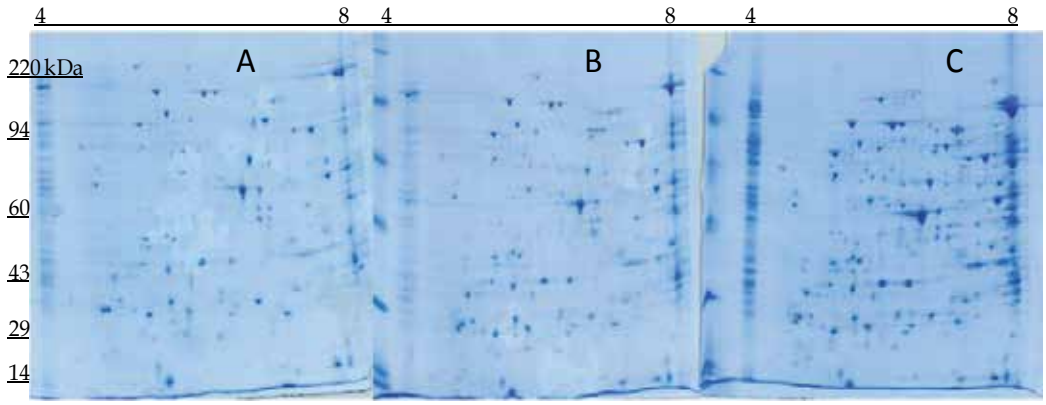


Fig. 6. Proteomic profile of total *E. heros* antennae proteins by two-dimensional SDS-PAGE. The pH linear range of the first dimension gel (isoelectric focusing = IEF, 13 cm) is indicated above the gels. The positions of protein standards (kDa) used in the second dimension gel (SDS-PAGE 15%) are indicated at the left of first gel. A. Fifth instar nymphs, B. Males and C. Females (600 antennae each, ~500 µg of total protein), both virgins 12 days-old.

The efforts concerning the identification of more chemoreception proteins are being continually conducted in order to know the complete genetic charge of the pheromone olfaction and in this way systemically knowing how the recognition of pheromones works, as well as the potential to recognize different categories of pheromones at the same time, and the developing of the olfaction according to the stink bug life cycle and the biotic and abiotic factors that can influence chemoreception. Furthermore the studies will be expanded to the other stink bugs species in order to richly characterize the olfaction mechanisms in stink bugs and by this way contribute to the development of biotechnological tools to aid in pest management schemes.

3. Tri-trophic interactions soybean - stink bugs - natural enemies

Plants commonly suffer continuous damage by insects in their natural habitat. There are two main strategies of defence against herbivore feeding damage. In one strategy, the plants directly affect the insect; for example, the plant reduces the supply of essential metabolites to the herbivore or synthesizes compounds that minimize herbivore feeding, such as antinutritive or antidigestive compounds, which is known as direct defence (Dicke, 1994a; b; Kessler & Baldwin, 2002). In the other strategy, the plant affects the herbivore indirectly by attracting their natural enemies, such as parasitoids, which is known as indirect defence (Dicke, 1994a; b; Kessler & Baldwin, 2002).

Several studies have demonstrated that plants injured by herbivore feeding produce specific blends of odours, which can be attractive to parasitoids and predators. The profile of the volatiles emitted is markedly different from those of undamaged or mechanically damaged plants (Dicke & van Loon, 2000; De Moraes et al., 1998; Kessler & Baldwin, 2001; Turlings et al., 1990; 1998; Hoballah & Turlings, 2001). The volatile organic compounds (VOCs) emitted by plants are of fundamental importance in the association involving plants, phytophagous insects and their natural enemies.

The possibility of plant volatiles to play important role in the attraction and retention of the natural enemies is researched by scientists the world over. Chemical ecology studies using soybean in Brazil are concerned with the possibility of utilizing plant volatiles to manipulate the behavior of the egg parasitoids of the stink bugs, for their application in pest management programs.

3.1 Stink bugs in soybean (interaction with plants, bioecology and damage)

Most phytophagous pentatomids are polyphagous, feeding on cultivated and uncultivated (wild) plants. They are major pests of economically important crops throughout the world, including legumes such as soybean and beans; cereals such as rice and wheat; and tree crops such as citrus, oil palms and coconut, cocoa, and coffee (Panizzi, 1997 and references therein).

Phytophagous pentatomids feed sucking up nutrients from host plants using their stylets. They can feed in different structures of the plants but seed and immature fruits are the preferred feeding sites (Panizzi et al., 2000). In soybean and other legumes stink bugs feed mainly on pods and grains causing direct damage such as seed abortion, reduction of oil contain and mechanical damage or indirect injury like fungal transmission and physiological alterations (Villas-Bôas et al., 1990; Sosa-Gómez & Moscardi, 1995; Boethel et al., 2000).

The preferred feeding sites are ephemeral resources, therefore a succession of host plants are necessary to develop successive generations along crop seasons or continuous breeding (Panizzi, 1997). Specific host plant relationships may be found in different crops and geographical sites, additionally some species show feeding preferences by certain plant taxa (Panizzi, 1997).

The host plant/stink bug interaction is also related to the bio-ecology of the insect. In temperate climates adult stink bugs enter a reproductive diapause after the crop season (fall) and spend the winter protected in different places (under the bark of tress, litter or culture debris) (Ehler, 2000, Mourao & Panizzi, 2000). In spring they develop a first generation in wild hosts and then migrate to cultivated crops where the numbers of generations that develop depend on climatic conditions (Ehler, 2000).

In tropical or sub-tropical conditions the phenology can be different. For example, in southern Brazil (Parana State) Panizzi (1997) describe continuous generations for *N. viridula* and *P. guildinii* and a diapause period in the winter for *E. heros*. On the other hand, in central Brazil (Federal District) *E. heros* and others pentatomids, due to high temperatures throughout the year, could be found during dry season (winter for temperate and sub-tropical regions of the southern hemisphere) on alternative hosts, wild or cover crops plants that grow in more humid situations, with reproductive activity and developing successive generations. Additionally the host plant spectra used by pentatomids in central Brazil is different than that observed in southern region of the country (Laumann et al., 2008b; Vieira et al., 2008).

The specific knowledge of cultivated and wild hosts may be relevant to stink bug management (Panizzi, 1997). Information about invasion time in the crop fields could help in monitoring and control, knowledge of alternative host plants could help to develop trap crops and the dynamic of the use of crop/wild hosts and their influence in natural enemy guilds could be useful to biological control strategies. Additionally, the semiochemical complex related to crop/wild host plants could be useful to identify chemicals to repel or attract stink bugs or attract and retain natural enemies in specific areas.

3.2 Interaction soybean stink bugs - Direct and Indirect defenses

Working on signalling through volatiles induced by *E. heros* on two legumes has demonstrated that these volatiles influence the attraction and retention of the egg parasitoid, *T. podisi*. Air-borne extracts obtained from two host plants of *E. heros*, soybean, *Glycine max* L., and pigeon pea, *Cajanus cajan* (L.)(Leguminosae), produce a different blend of emitted volatiles when attacked by adult males or females and nymphs of the pest species compared with the undamaged plants. The same results were obtained when the plants were treated with extracted saliva of *E. heros*, mechanically introduced into the plants. This indicates that some substance in the saliva contributed to the release of the volatiles (Moraes et al., 2005c).

Olfactometer studies with female *T. podisi* and treated plants confirmed the significant preference of the egg parasitoid for herbivory damaged plants by *E. heros* when compared to undamaged plants On the other hand, volatile extracts obtained from soybean subjected to the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, a non-host species for *T. podisi*, showed a different blend of volatiles compared to those obtained from plants damaged by *E. heros* feeding, and the extract obtained from *A. gemmatalis* herbivory damaged plants did not attract the egg parasitoid. These results indicate that *E. heros* causes the induction of indirect defense of soybean and it is possibly caused by an elicitor present in the pest saliva. This work has demonstrated the potential of these volatiles to play an important role in the attraction and retention of the egg parasitoid, *T. podisi* (Moraes et al., 2005c).

Phytohormones have been used as inductors of defense of plants to improve the resistance of the plants to pests (Birkett et al., 2000; Moraes et al., 2008d; Pickett et al., 2007). *cis*-Jasmone is a volatile compound derived from fatty acid pathway, which has been used to induce the chemical defense of plants (Birkett et al., 2000; Moraes et al., 2008d). Soybean plants treated with *cis*-jasmone also had their chemical defense induced provoking changes in the volatile organic compounds (VOCs) profile when compared to untreated soybean (L.) (var. BR16) and the volatiles released by the *cis*-jasmone treated soybean attracted the egg parasitoid *T. podisi*. The main components induced by *cis*-jasmone were camphene, myrcene, (*E*)-ocimene, methyl salicylate and (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT). Thus, *cis*-jasmone appears to induce defence pathways in soybean similar to those induced by stink bug damage, and this phenomenon appears to be a promising tool for the manipulation of beneficial natural enemy in future sustainable stink bug control strategies (Moraes et al., 2009).

These results suggest that *cis*-jasmone has great potential for the development of new strategies for stink bug control using the natural defense of plants to attract natural enemies in soybean crops. Further laboratory and field studies are underway to evaluate how *cis*-jasmone can be used in a productive agricultural context, not only with soybean but with other economically important Brazilian crops (Moraes et al., 2009).

3.3 Soybean varieties and response to stink bugs

Several studies have shown that herbivore induced plant volatiles act directly on herbivores and indirectly on their natural enemies (Moraes et al., 2005c and references therein). However, little has been reported about the effect of herbivore damage on resistant and susceptible plant cultivars and its effect on natural enemies. Thus, to evaluate the attraction of *Telenomus podisi* and *Euschistus heros* to different types of damage (herbivory, herbivory+oviposition and oviposition) in two stink bug resistant soybean cultivars, Dowling and IAC100, and one susceptible cultivar, Silvânia were carried out. In olfactometer studies, the parasitoids were attracted to herbivory and herbivory+oviposition damaged soybean plants when compared to undamaged soybean plants for the resistant cultivars, but did not show preference for the susceptible cultivar Silvânia in any of the damage treatments. In olfactometer studies, *E. heros* females did not show preference for odors of damaged or undamaged soybean plants of the three cultivars studied. The susceptible cultivar Silvânia produced a very low level of herbivory induced plant volatiles (HIPVs) whereas resistant cultivars (IAC100 and Dowling) produce a higher quantity of HIPVs (Michereff et al., 2009). The compounds that most contributed to the divergence between damaged soybean plants compared to undamaged plants were (*E,E*)-α-farnesene, methyl salicylate, (*Z*)-3-hexenyl acetate and (*E*)-2-octen-1-ol. In addition, it is apparent that each cultivar has a different response to herbivore damage, eliciting different responses by the egg parasitoid. Inasmuch, the level of indirect defense in response to herbivory or oviposition damage in resistant cultivars is greater than that in susceptible cultivars and that there is a synergistic effect between the different types of damage in soybean in attraction of the egg parasitoid *T. podisi* (Michereff et al., 2009; Moraes et al., 2008c).

In brief, *E. heros* induces the indirect defense of soybean resistant cultivars Dowling and IAC100 and *T. podisi* is attracted to the blend of herbivory and herbivory+oviposition damaged plants. *E. heros* did not distinguish in an olfactometer bioassays between damaged and undamaged soybean nor between resistant and susceptible cultivars. Further laboratory studies are necessary to investigate the importance of these compounds in the attraction of *T. podisi*. Once understood, these volatiles could help to develop new strategies for control of *E. heros* using natural enemies in soybean crops (Michereff et al., 2009).

In addition, the effects of direct defences of different soybean genotypes on nymphal development of the southern green stink bug *Nezara viridula*, showed that in resistant genotypes the mortality of nymphs was around 66.2% and in susceptible cultivar it was around 27.5% (Piubelli et al., 2003). The quantities of flavonoids in soybean appear to be involved in the direct defence and resistance against herbivorous (Piubelli et al., 2005).

These studies may be used to develop new tools for pest management such as attracting naturally the natural enemies and influencing, in this way, the level of parasitism that may occur or to control herbivorous insects increasing natural pest mortality. Selection of semiochemicals, from plants, with the power to increase the beneficial action of parasitoids will enable the use of an efficient methodology for management of stink-bugs with minimal impact on the environment.

3.4 Metabolic pathways induced

The composition of herbivore induced volatiles can change among plant and herbivore species, even when comparing different plant varieties, different developmental stages of the plant and under abiotic stress (Viswanathan et al., 2007; Moraes et al., 2005c; 2008c; Turlings et al., 1998).

The herbivore induced volatiles come from several different metabolic pathways, the most important are the isoprene-derived terpenoids and fatty acid derived the green leaf volatiles, mainly when the damage is provoked by chewing insects (Arimura et al., 2009). Pathogens and sucking insects, like aphids and white flies, has a unique aspects that they induce a third and important pathway, the salicylic acid pathway, in a higher expression compared to when plants are attacked by chewing insects (Waling, 2000; Kaloshian & Walling, 2005).

Terpenoids are produced from the assembly of the C_5 building block called isoprene units (2-methyl-1,3-butadiene), which can originate from the mevalonic acid pathway or the pyruvate pathway (Gerhenzon and Kreis, 1999, Arimura et al., 2009). The monoterpenes (C_{10}) and diterpenes (C_{20}) are synthesized in plastids and the sesquiterpenes in the cytosol. Other important class of terpenes, the homoterpenes, such as TMTT, can be produced from sesquiterpenes and diterpenes, therefore they come from cytosol or plastid route. The fatty acid derived compounds come from the octacanoid signaling pathway, which breaks down fatty acids (linolenic and linoleic acid) to provide the green leaf volatiles (GLV), which includes, C_6 aldehydes, alcohols, and their esters, and also jasmonic acid and its derivatives, such as *cis*-jasmone and methyl jasmonate (Hatanaka, 1993; Dudareva et al., 2006; Pickett et al., 2007). Salicylic acid dependent responses are very important upon attack by plant pathogens (Walling, 2000; Kaloshian & Walling, 2005), the compounds derived from this pathway are aromatic compounds, for example, methyl salicylate and indole, that have important functions in insect-plant communication (De Boer & Dicke, 2005).

Stink bugs in general feed preferentially on soybean seeds, but some species, such as *Edessa meditabunda* prefer the vegetative parts of the plants (Panizzi, 2007). The stink bugs use a stylet to pierce the tissue, destroying few cells, resulting in a minor mechanical damage, and consuming great quantities of fluid during long periods (Panizzi, 2007).

Soybean plants damaged by *E. heros* feeding induced the three main pathways described above, both those involved with chewing herbivore damage (terpenoids and fatty-acids) and also the salicylic acid pathway. The main herbivore induced volatiles in soybean were methyl salicylate, the GLV, (*Z*)-3-hexenyl acetate and the sesquiterpene, (*E*,*E*)-α-farnesene. The soybean response to stink bug feeding resembles that to pathogens in many ways as described for aphids and white flies by Kaloshian and Walling (2005). Another interesting difference in the plant´s response when damaged by stink bug feeding when compared to chewing insects is that for chewing insects the green leaf volatiles are produced and released immediately after the attack and the terpenoids only few hours later or when the plants reach the next photosynthetic phase, (next day). In soybean plants damaged by stink bugs the green leaf volatiles and terpenoids were induced only after 72-96 hours of damage (Moraes et al., 2005c; 2008c; 2009). The interactions between pathways and the timing of the compounds released are raising many interesting questions that will need further of work to be responded.

3.5 Herbivory induced volatiles and natural enemy behavior

Egg parasitoids have a short time frame in which their host eggs are suitable for parasitism, and in several systems these parasitoids respond to plant volatiles induced by feeding damage of the herbivore as a means of finding suitable hosts. By reviewing all the work carried out regarding indirect defence induced by pentatomids, it has been demonstrated that these volatiles play an important role in the attraction and retention of the egg

parasitoids, *T. basalis* and *T. podisi* (Colazza et al., 2004; Moraes et al., 2005c; 2008c; 2009). As discussed above, the volatiles derived from the host plants appear to be a powerful tool for the manipulation of egg parasitoid behaviour and their exploitation for pest control. The potential use for that should be attracting beneficial insects in selected environments and influencing the levels of parasitism that may occur by the use of indirect defence of plants may enhance integrated pest management.

We conclude that indirect defences are produced by these plants, when attacked by *E. heros*, as an induced response indirectly favouring the egg parasitoid as a biological control agent of this harmful soybean pest species (Moraes et al., 2005c; 2008c; 2009).

The results obtained and recorded from all studies regarding the tritrophic interactions in the Brazilian soybean crop; indicate that plant volatiles maybe one of the main strategy used by this parasitoid for location of habitats where stink bugs eggs are more likely to be found (Moraes et al., 2005c; 2008c; 2009).

4. Application to stink bugs management

The economic importance of stink bug damage to soybeans, combined with the necessity of developing more integrated management of stink bug populations, are motivating researchers worldwide looking for methods to reduce pesticide use for stink bug control (Corrêa-Ferreira & Moscardi, 1996; Panizzi & Corrêa-Ferreira, 1997; Venzon et al., 1999; Corrêa-Ferreira & Panizzi, 1999; Knight & Gurr, 2007). The concept of integrated pest management (IPM) is evolving toward a more sustainable management system in which external chemical interventions are a last therapeutic resort. Sustainable agriculture requires management of the ecosystem so as to conserve the natural enemies that are instrumental in suppressing pest populations (Knight & Gurr, 2007; Weiss et al., 2009; Moraes et al., 2009).

While the sustainable agriculture movement has been gaining momentum, the genetically engineered crop revolution has begun in earnest with releases of *Bt*-cotton, -corn and -potato, with genetically engineered strains of soybean, sorghum, canola, alfalfa and wheat soon to follow. In *Bt*-crops, the primary pests are suppressed by the expressed *Bt* toxins, alleviating the need for insecticides for control of these insects (Naranjo, 2009 and references therein). However, *Bt* toxins are not effective against sucking insects (Hemiptera) (P. Roberts, University of Georgia College of Agricultural and Environmental Sciences, Athens, GA, USA, personal communication; Sharma & Pampapathy, 2006; Torres & Ruberson, 2008) and, as a consequence, have surfaced as the new primary pests in these transgenic crops. The complex of stink bugs around the world has always been difficult to control, and the advent of reduced tillage practices has intensified this problem (Fidelis et al., 2003; Chocorosqui & Panizzi, 2004; Seffrin et al., 2006).

The use of sex pheromone baited traps for monitoring stink bugs catches mainly the adult sexually mature females individuals (Borges et al., 1998a) but damage is caused by both adult and immature stink bugs (Millar et al., 2002). Therefore, for efficient population monitoring, which might counts also the immatures insects, the pheromone baited traps technology could be able to establish an accurate relation between insects trapped with the population density in the field, to indicate precisely the critical changes in population dynamics and behaviour of stink bugs in the field, that could be used for timing control measures for key pest as *E. heros*.

The effectiveness of the synthetic sex pheromone of the Neotropical brown stink bug, *E. heros*, was evaluated both in laboratory and field assays. The pheromone-baited traps were

effective in field tests even at low bug population densities, as compared with the usual monitoring technique, shake cloth sampling. Traps around borders or in the centre of soybean fields caught similar numbers of bugs. Trap captures showed a positive relation with field populations, as monitored with the shake cloth technique, during the reproductive phase of the soybean crop from the R1 to R5 developmental stage, i.e., from pod formation to pod fill. Some cross-attraction was also observed, with *Piezodorus guildinii* and *Edessa meditabunda* also being caught in pheromone-baited traps, suggesting that these insects respond to the sex pheromone or to the defensive compounds released by *E. heros* captured in traps. In brief, the results showed that traps baited with 1 mg of the sex pheromone efficiently caught bugs and that the lures lasted one month under field conditions, and that traps could be used only in the borders of the crop area at a density of one trap every 200 meters (Borges et al., 2010).

In conclusion, the synthetic *E. heros* sex pheromone can be used to monitor the seasonal fluctuations in stink bug populations infesting soybean. The results gave a more precise indication of the critical changes in population dynamics and behavior of these bugs in the field, that can be used for timing control measures for the key pest, *E. heros,* with more precision in soybean in the Central Region of Brazil. Because environmental conditions vary in different regions of Brazil, ranging to wet tropical in the north to temperate in the south, a more complete evaluation of this technology in other soybean producing regions is needed to validate or adapt the sex pheromone baited trap technology to different environments and stink bug guilds (Borges et al., 2010). Additionally, in recent year stink bugs have adapted to new crops such as cotton (Willrich et al., 2004 a; b; c; 2005), maize (Townsend & Sedlacek 1986, Avila & Panizzi, 1995) and sunflower (Panizzi & Machado-Neto, 1992; Malaguido & Panizzi 1999), so that the monitoring technology of pheromone baited traps for stink bugs could lead to establish effective biocontrol strategies that contribute to solving current and future stink bug pest problems.

Another case of study in Brazil was concerned with the species *Thyanta perditor* that is one of the stink bugs of the guild attacking soybean and may be found on others crops such as wheat, sunflower and sorghum. The field attractiveness of synthetic *T. perditor* pheromone baited traps were tested using the two-liter transparent plastic soft drink bottles traps baited with rubber septa impregnated with the treatments: 1mg methyl-(*E*2,*Z*4,*Z*6)-decatrienoate ((*E*2,*Z*4,*Z*6)-10:COOMe), the male sex pheromone of *T. perditor*, and traps with rubber septa impregnated with hexane (control). The experiment was performed in a soybean field during the soybean reproductive stages. Traps were monitored every week and the captures were compared to the population density estimated by the sampling cloth and visual inspection monitoring techniques. Traps baited with (2*E*,4*Z*,6*Z*)-10:COOMe were more effective in capturing *T. perditor* than control traps. The *T. perditor* pheromone traps also showed cross-attraction to other species of stink bugs such as *E. he*ros, *E. meditabunda*, *P. guildinii* and *N. viridula*. In addition, results of this case of study showed that, the synthetic sex pheromone of the Neotropical stink bug *T. perditor* is a powerful female attractant and is highly efficient for population monitoring (Laumann, Moraes, Krimian & Borges not published).

The sampling cloth technique is a reference method for stink bug monitoring and is considered to be a non biased method to estimate the stink bug species population (Kogan & Pitre, 1980). The similar performance of the pheromone traps and the sampling cloth to estimate the species occurrence and their relative abundance could indicate that the pheromone baited traps may be a tool not only for population density estimations but also

to identify the relative composition of the stink bug guilds, at least in guilds where the pheromone baited traps used are that of the dominant species.

Future pest management strategies should accommodate the semiochemical relationships including crop plants and herbivore enemies to take full advantage of stabilizing trophic webs. In the past decades, scientists have worked on the chemical ecology of different stink bug species in an attempt to identify semiochemicals that might be useful for monitoring pest populations (Borges et al., 1998a; Millar et al., 2002) or for manipulation of the behaviour of beneficial insects (Borges & Aldrich, 1994a; Borges et al., 1998a; 1999).

Furthermore, it is likely that the pheromone will act in concert with the substrate-borne vibrational signals that this insect uses for communication over shorter ranges (Moraes et al., 2005c). It may be possible to develop pheromone-based monitoring systems for this insect, particularly if vibrational signals also could be incorporated into the trap design. Such monitoring systems, when used in conjunction with strategies such as trap cropping, may provide an integrated pest management solution for control of these pest bug species.

Regarding the application of semiochemicals for the manipulation of the behavior of the egg parasitoids to stink bugs management, Peres (2004) showed that application of (*E*)-2-hexenal in field conditions, in southern Brazil, may result in increased parasitism of eggs naturally deposited by caged females, in a week-long experiment. On the other hand, in a full crop season time experiment applications of (*E)*-2-hexenal in soybean plots (400 m^2) showed that the treated areas with 4 mg of (*E*)-2-hexenal applied in rubber septa increased the number of scelionid wasps in relation to non treated areas, however the increase in parasitoid abundance was not associated with the occurrence and intensity of parasitism (Vieira 2010). In another study where *cis*-jasmone was sprayed in a soybean field, the structure of the community of Scelionidae in terms of species richness, was similar in general between treatment and control plots and equity was higher in treated plots. The total number of Scelionidae, mainly *Telenomus* spp. and *Trissolcus* spp. was significantly higher in treated plots. However, no significant difference in rates of parasitism and number of stink bugs were found between plots (Vieira, 2010).

These results suggest that semiochemicals have the potential to be used as a behavior manipulator of egg parasitoids. Notwithstanding, prior to an efficient field use in pest management specific concentrations, dosages, formulations and application times need to be established to obtain a total comprehension of their influence in scelionid wasp field behavior.

5. References

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Natural Antimicrobials for Biopreservation of Sprouts

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Spain

1. Introduction

In recent years there has been an increase in consumers demands for mungbean, alfalfa, soybean, radish and other seed sprouts (Rosas and Escartin, 2000) that are usually eaten raw in salads or in sandwiches. Seed sprouts have been part of the human diet since old times in countries such as Japan where they are widely consumed. Tthe interest in consuming fresh green sprouts has extended all over the world because they are considered to provide health benefits (Rosas and Escartin, 2000). A great variety of seed sprouts can be found at present in the market, such as adzuki bean (*Phaseolus angularis*), alfalfa (*Medicago sativa*), broccoli (*Brassica oleracea* convar. *botrytis*), cress (*Lepidium sativum*), lentil (*Lens culinaris*), mung bean (*Phaseolus aureus*), soybean (*Glycine max*), white mustard (*Sinapis alba*), green and yellow pea (*Pisum sativum*), onion (*Allium cepa*), radish (*Raphanus sativus*), rice (*Oryza sativa* L.), rye (*Secale cereale*), sesame (*Sesamum indicum*), sunflower (*Helianthus annuus*) and wheat (*Triticum aestivum*), although the most popular are alfalfa, soybeans, mung beans and raddish (Taormina et al., 1999). Seed sprouts are usually eaten raw in salads or in sandwiches, and concerns for the safety of these raw foods have increased lately. Sprouts are grown from seeds placed in environmentally controlled, hydroponic conditions and incubated in warm, moist, nutrient-rich conditions, which are ideal environments for microbial growth. The seeds usually carry microbial loads comprised between 3 and 6 log CFU/g, including pseudomonads and enterobacteriaceae as main components (Andrews et al., 1982; Prokopowich and Blank, 1991; Robertson et al., 2002; Splittstoesser et al., 1983). The bacterial load increases rapidly during the sprouting process, reaching from 6 to 8 log CFU/g after two days in one study (Fu et al., 2001) and between 7.8 and 8.8 in another (Weiss et al., 2007). Other reports have indicated final counts of up to 8-9 log CFU/g in commercial sprouts (Patterson and Woodburn, 1980; Prokopowich and Blank, 1991). In addition, the pathogenic bacteria can survive on sprouts through the typical refrigerated shelf life of the products (Harris et al., 2003). Recent studies indicate that pathogenic bacteria can survive both on and in plant tissues (Lynch et al., 2009). For example, when alfalfa seeds contaminated with *Escherichia coli* O157 or with *Salmonella* are sprouted, the bacteria enter the growing sprout, and appear throughout the deep tissues of the young plants (Itoh et al.,

1998; Charkowski et al., 2002). The bacterial cells located inside plant tissue will be refractile to inactivation by common disinfection methods.

A wide variety of pathogens have been isolated from sprouted seeds (including alfalfa, mung bean, cress, soybean, and mustard): *Aeromonas hydrophila, Salmonella* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus, Yersinia enterocolitica* and *Vibrio cholerae* (Beuchat, 1996; Harris et al., 2003). Outbreaks have been associated primarily with *Salmonella* serotypes but have also been attributed to *B. cereus*, *E. coli* O157:H7, and *Y. enterocolitica* (Harris et al., 2003). Alfalfa sprouts rank in the first place of associated outbreaks, followed by far by clover, radish, and mung bean sprouts. This is due to the fact that alfalfa sprouts are the most popular type of seed sprouts that are commonly eaten raw, while others such as mung beans are often cooked before consumption. Therefore, it is necessary to plan intervention strategies to decrease the risks for transmission of human pathogenic bacteria through sprouted seeds. In this chapter we discuss how different biocontrol strategies can be applied either alone or in combination with other intervention strategies to improve the control of pathogenic bacteria most frequently found in sprouts (Fig. 1).

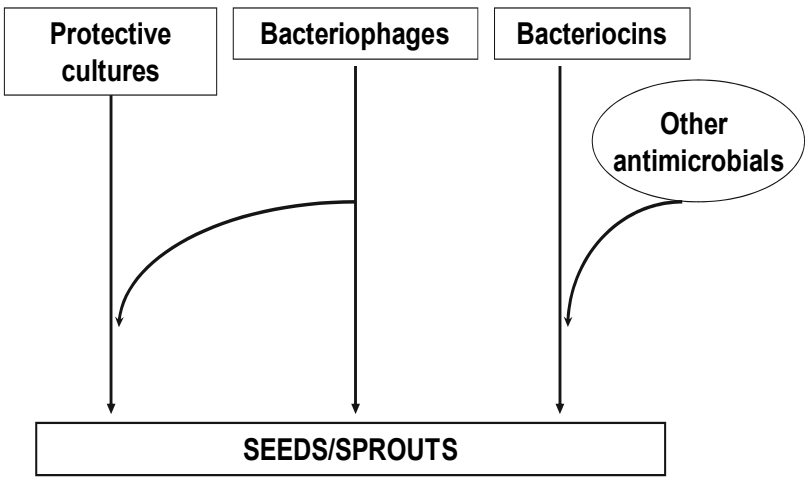

Fig. 1. Intervention strategies based on biopreservation to decrease the risk of transmission of human pathogenic bacteria in sprouts.

2. Inoculation with protective cultures

One of the oldest approaches for biopreservation of foods is based on the natural interactions that microbes play in Nature. Such interactions can be redirected and exploited to selectively inhibit microbial populations of concern, such as pathogenic or spoilage bacteria in food ecosystems. Microbial antagonism is usually based on a combination of mechanisms which include competition for space and nutrients, and selectively changing the environmental conditions, eg. by acidification, or production of an array of antimicrobial substances that include organic acids, oxygen radicals, siderophores, antibiotics, lytic enzymes, and bacteriocins. The bacteriocins were defined by Jack *et al*. (1995) as ribosomally synthesized antimicrobial peptides or proteins. When using live microbial antagonists as protective cultures in food biopreservation, there is a number of limitations and rules that need to be taken into consideration (Galvez et al., 2007): i) the protective cultures must not

be pathogenic or produce toxins against humans or animals, and must not modify the organoleptic properties of the food product; ii) they must be able to multiply in the food and produce antagonistic activity at the food storage temperature; ii) they must be efficient in controlling the bacterial pathogen of concern under the common food storage and processing conditions.

There has been a great interest in the application of bacteriocins and bacteriocin-producing strains (especially those produced by the LAB) on the preservation of foods of animal origin, but to a much less extent on vegetable foods such as sprouted seeds (Gálvez et al., 2008; Abriouel et al., 2010). Cai et al. (1997) isolated a collection of bacteria from mung- and soy bean-sprouts from retail stores and tested them for anti-listerial activity. Ten strains showed highest inhibitory activity, among which one strain was identified as a nisin-producing *Lactococcus lactis* subsp. *lactis*. This strain was able to reduce the levels of *L. monocytogenes* by 1.0-1.4 logs in Caesar salad after storage for 10 days at 7 or 10ºC. Similar results were reported when the bacteriocin-producing *Enterococcus faecium* ATCC 19434 strain was tested on the salad for comparison (Cai et al., 1997). However, none of the strains were tested on sprouts. The enterococcal strain *Enterococcus faecalis* A-48-32 (which produces the cyclic peptide bacteriocin AS-48) was inoculated as a protective culture against *B. cereus* on soybean sprouts stored at 15 and 22 ºC simulating temperature-abuse events (Cobo Molinos et al., 2008a). Enterococci multiplied rapidly on sprouts at the two temperatures tested. In both cases, bacteriocin activity could be recovered from the sprouts during days 1 to 3 of storage, but not after more prolonged periods. In cocultures at 15 ºC, growth of *B. cereus* was completely inhibited for the whole storage period, with viable cell counts being significantly lower than control cultures for the first 5 days of storage. By contrast, cocultivation with the A-48-32 strain at 22 ºC only produced some growth inhibition of *B. cereus* during the first 3 days of storage.

Bennik et al. (1999) tested two bacteriocin-producing *Pediococcus parvulus* strains (isolated from minimally-processed vegetables and one *Enterococococcus mundtii* ATO6 strain (isolated from chicory endive) that produced the pediocin-like mundticin ATO6 (Bennik et al., 1998). Only *E. mundtii* was capable of bacteriocin production at 4-8ºC, a reason why this strain was selected for experiments to control *L. monocytogenes* on mungbean sprouts, which were stored under modified atmosphere (MA) of 1.5%O_2/20%CO_2/78.5%N_2 at 8ºC. However, in spite of its capacity for growth and bacteriocin production under MA, this strain failed to inhibit the growth of *L. monocytogenes* on sprouts and for this reason was not likely to be feasible for application as a protective culture (Bennik et al., 1999). More recently, the bacteriocin-producing strain *E. mundtii* CUGF08 isolated from alfalfa sprouts was shown to produce an anti-listerial bacteriocin called mundticin L (Feng et al., 2009). This bacteriocin is highly similar to mundticin ATO6, differing in only by one amino acid residue in the YYGNGV (YYGNGL) motif and the last two amino acid residues, which are reversed as in the almost identical mundticin SK (Kawamoto et al., 2002). The antagonistic capacity of this strain on sprouts has not been reported so far.

In one study, approximately 120 strains of indigenous microbiota from raw vegetables (including alfalfa and clover sprouting seeds, Romaine lettuce, and pre-peeled baby carrots) were tested for their ability to inhibit the growth of *Salmonella* Chester, *L. monocytogenes, E. coli,* or *Erwinia carotovora* subsp. *carotovora* (Liao and Fett, 2001). Six isolates capable of inhibiting the growth of at least one pathogen were isolated and identified: *Bacillus* spp. (three strains), *Pseudomonas aeruginosa* (one strain), *Pseudomonas fluorescens* (strain A3), and yeast (strain D1). When strains A3 and D1 were tested on green pepper, the growth of *Salmonella* Chester and *L. monocytogenes* was reduced by 1 and 2 logs, respectively, over a

period of 3 days. The authors stressed the potential of these two strains as biocontrol agents for reducing the growth of these two pathogens on fresh produce products, although their inhibitory effects on sprouts were not determined.

Matos and Garland (2004) tested different potential biological control inoculants against *Salmonella* in alfalfa. Inocula included raw bacterial suspensions derived from market sprouts or laboratory-grown alfalfa sprouts (obtained by sonication of sprouts in a buffered peptone solution) and the *P. fluorescens* 2-79 strain. This strain was originally isolated from the rhizosphere of wheat and shown to suppress take-all, a major fungal root and crown disease of wheat and barley. A cocktail of four different serovars of *Salmonella* were used, all of them isolated from sprout-related outbreaks (*Salmonella* Newport H1275, *Salmonella* Anatum F4317, *Salmonella* Stanley H0558, and *Salmonella* Infantis F4319). According to results of the competitive exclusion bioassays, *P. fluorescens* 2-79 was able to reduce the growth of *Salmonella* on alfalfa sprouts by 4.22, 4.24, and 1.81 logs on days 1, 3, and 7, respectively. The laboratory-grown sprout inoculum also had its highest inhibitory effect on *Salmonella* at the beginning of the growth period with log reductions of 2.56 on day 1; however, viable cell reductions then decreased to 0.21 on day 3 and 0.71 logs on day 7. The best results were observed for the market sprout inoculum, which reduced the number of *Salmonella* cells by 1.61, 2.80, and 5.48 log on days 1, 3 and 7, respectively. The market sprouts inoculum achieved the highest log reduction of *Salmonella* on day 7 than any of the other experimental treatments. Apparently, the natural populations of bacteria found in commercial sprouts were better adapted to utilization of an array of nutrient substrates, suggesting competition with the *Salmonella* for available resources as a potential mode of action. Unfortunately, the authors did not characterize the microbial composition of this inoculum or how it could be propagated and standardized as a commercial inoculant.

The potential of *P. fluorescens* 2-79 as a biocontrol agent on sprouts was further investigated by Fett (2006). The addition of a *P. fluorescens* 2-79 inoculum to the seed soak water prior to the germination of alfalfa seeds previously inoculated with a cocktail of *Salmonella enterica* strains led to outgrowth inhibition of the salmonellae through the complete 6-days sprouting period, without an adverse effect on sprout yield or appearance. At day 6, the counts of *S. enterica* were between 4 and 5 log CFU/g lower compared to seeds not treated with the antagonist. Final cell numbers of *S. enterica* on the sprouted seeds were shown to be dependant on the cell density of the inoculum and on the application or not of a sanitation treatment (Liao, 2008). Remarkably, the population of salmonellae on sanitized seeds was approx. 1 log higher than that on nonsanitized seeds which contained 2 logs higher number of native microflora. In another study, a *Pseudomonas jessenii* strain isolated from raddish sprouts showed high inhibitory activity against a variety of target bacteria (*L. innocua, S. aureus, Bacillus subtilis, S*. Typhimurium, *E. cloacae* and other *Pseudomonas* strains). When tested as a protective culture in challenge experiments against *S.* Senftenberg on hydroponically grown mung bean seeds inoculated simultaneously with the two bacteria, *Salmonella* showed a reduced growth on the sprouts, which resulted in cell counts exceeding 3 log and 2 log CFU/g below the control after 24 and 48 h incubation, respectively (Weiss et al., 2007). The best results were obtained when the seeds were preinoculated with *P. jenssenii* on day 0 and challenged with *Salmonella* Senftenberg on day 1 of sprouting. Pre-inoculation with *P. jenssenii* completely suppressed growth of *S.* Senftenberg during germination of sprouts. After 7 days, while the salmonellae reached 8 log CFU/g in the control samples, counts on sprout samples with the pre-treated seeds were below 1 log CFU/g.

Enterobacter asburiae is commonly associated with plants and has been used as a biocontrol strain for inhibiting the growth of enteric pathogens such as *Salmonella* and *E. coli* O157:H7 (Cooley et al., 2003). *E. asburiae* JX1 isolated from mungbean sprouts exhibited stable antagonistic activity against a broad range of *Salmonella* serovars (Agona, Berta, Enteritidis, Hadar, Heidelberg, Javiana, Montevideo, Muenchen, Newport, Saint Paul, and Typhimurium DT104) (Ye et al., 2010). Mung beans inoculated with a cocktail of the *Salmonella* serovars in combination with *E. asburiae* JX1 attained much lower levels of salmonellae (1.16 log CFU/g) than the single cocktail inoculated samples (6.72 log CFU/g) after 4 days of sprouting. The inhibitory activity of *E. asburiae* JX1 was attributed partially to nutrient competition, but also to the production of inhibitory substance(s) released in solid medium but not in liquid cultures.

3. Application of lytic bacteriophages

Lytic bacteriophages are gaining interest as biocontrol agents because they are highly species-specific, and therefore can target the pathogenic bacteria of concern without affecting other bacteria that may be of interest in the particular food system where they are being applied. The self-propagating capacity of lytic bacteriophages is also an advantage concerning the amount of inoculant to be added, decreasing in principle the processing costs. Phages have been shown to control the growth of pathogens such as *L. monocytogenes*, *Salmonella* and *Campylobacter jejuni*, as well as spoilage organisms in fruit, dairy products, poultry and red meats (Greer, 2005; Hudson et al., 2005; Rees and Dodd, 2006). Nevertheless, due to their high strain specificity, it is often necessary to apply complex phage mixtures, making the phage selection procedures rather complicated. Factors such as strain sensitivity, transient resistance, and host range specificity of the bacteriophages clearly seem to limit their application as biocontrol agents on sprouts.

Pao et al. (2004) tested the potential of lytic phages (the ***Myoviridae*** Phage-A, targeting ***Salmonella*** Typhimurium and ***Salmonella*** Enteritidis, and the ***Siphoviridae*** Phage-B, specific for ***Salmonella*** Montevideo) in experimentally contaminated broccoli and mustard seeds. *Salmonella* counts increased to a greater extent in mustard seeds than in broccoli seeds during soaking. Application of Phage-A achieved a 1.37 log suppression of ***Salmonella*** growth on mustard seeds. The mixture of Phage-A and Phage-B caused a 1.50 log suppression of ***Salmonella*** growth in the soaking water of broccoli seeds. Because of the observed host specificity of phages, the authors stressed the importance of developing phage mixtures that can control a broad range of potential contaminants. However, experiments with two *Salmonella* bacteriophages isolated from enrichment of sewage samples (SSP5 and SSP6, belonging to the *Myoviridae* and *Siphoviridae* families, respectively) against a *Salmonella* Oranienburg strain (isolated from an outbreak of salmonellosis associated with alfalfa sprouts in Australia) provided unsatisfactory results. In liquid cultures, maximum reductions of *Salmonella* counts of ca. 1 log unit were achieved after 3 h of incubation. However, the remaining viable population was refractile to a second bacteriophage addition, entering a temporary non-specific phage resistance stage (Kocharunchitt et al., 2009). The temporary character of resistance was further demonstrated because bacterial colonies isolated from agar plates after the phage challenge showed the same degree of phage sensitivity as the original strains. On alfalfa seeds experimentally contaminated with *S.* Oranienburg, phage treatment (SSP6) caused a still lower reduction of *Salmonella* levels after 3 h of presoaking (as compared to liquid cultures) and did not inhibit

growth of the *Salmonella* population thereafter. The phage survived on both treated and control seeds as well as in their treatment solutions, indicating that the phage could survive throughout the sprouting process.

In a more recent study, a cocktail of six bacteriophages (F01, P01, P102, P700, P800, and FL 41) isolated from manure effluent sampled from pig or feedlot farms was prepared and evaluated for controlling a cocktail of *Salmonella* serovars in sprouting seeds (Ye et al., 2010). Mung beans inoculated with *Salmonella* and sprouted over a 4-day period attained levels of 6.72 log CFU/g. Levels of *Salmonella* were reduced to 3.31 log CFU/g when the pathogen was coinoculated with bacteriophages. However, by using a combination of *E. asburiae* JX1 and bacteriophages, the levels of *Salmonella* associated with mung bean sprouts were only detected by enrichment. With alfalfa sprouts derived from seeds inoculated with *Salmonella* alone, the counts of the enteric pathogen attained levels in the order of 7.62 log CFU/g. However, in the presence of *E. asburiae* JX1 and bacteriophages, no *Salmonella* was recovered even when samples (25-g batches) were enriched. The biocontrol preparation was effective at controlling the growth of *Salmonella* under a range of sprouting temperatures (20 to 30 ^{o}C). The combination of *E. asburiae* JX1 and bacteriophages represents a promising, chemical-free approach for controlling the growth of *Salmonella* on sprouting seeds.

4. Treatment with bacteriocin preparations

Bacteriocins can be concentrated from cultured broths of their producer strains and used as partially-purified preparations in food systems. Nisin and pediocin PA-1/Ach are available on the market for application in foods. These convenient, natural preservatives, can be added to foods in a more flexible way than protective cultures or phage preparations, and the bacteriocin dose can be adjusted to maximize its bactericidal effects. Very often, bacteriocins are used in food systems as part of hurdle technology, whereby their bactericidal effects are enhanced by other antimicrobial factors with which they may act synergistically.

Compared to meat and dairy products, where the application of bacteriocins has been studied extensively, much less work has been done on vegetable foods. In mungbean sprouts dipped in a solution of purified mundticin ATO6 (200 U/ml) or coated with an alginate film containing the bacteriocin (200 U/ml), a 2 log-decline of listerial counts was observed after treatments (Bennik et al., 1999). Although outgrowth of the listeria was not inhibited during storage of samples, counts obtained for the mundticin-treated samples were always lower compared to the untreated control. The mundticin-alginate coating provided the best results, especially within days 5 and 10 of incubation. Application of bacteriocins immobilized in edible coatings has been investigated in greater details for other types of foods. Bacteriocin immobilization has several advantages like a more regular delivery of bacteriocin molecules to the food substrate, lower inactivation of bacteriocin by interaction with food components or enzymes, and protection from cross contamination.

Nisin and pediocin solutions were tested as possible sanitizer treatments on cabbage, broccoli, and mung bean sprouts against a bacterial cocktail of five *L. monocytogenes* strains (Bari et al., 2005). Two hours after inoculation of the listeria, the samples were washed vigorously with agitation for 1 min with different antimicrobial solutions (0.02 M EDTA, 2% sodium lactate, 0.02% potassium sorbate, 0.02% phytic acid, 10 mM citric acid, and 50 mg/l of nisin, 48 mg/l of pediocin, or combinations of bacteriocins and chemical preservatives). Viable cell count determinations after treatments indicated an overall lower efficacy for all

treatments on mung bean sprouts compared to the other vegetable substrates, resulting in reductions of 0.5 log CFU/g or lower compared to samples washed simply with distilled water. Antilisterial activity of pediocin in sprouts was potentiated by citric acid, while the activity of nisin was by phytic acid. The most effective antimicrobial treatment combination on mung bean sprouts was nisin plus pediocin plus phytic acid, which caused a 1.2-log CFU/g reduction compared to washing treatment with distilled water. This combined treatment also reduced the native microflora by ca 1.4 log CFU/g. However, the fate of survivors after treatment was not investigated. It would be interesting to know whether the surviving bacterial populations were able to multiply or decreased during further storage of the treated samples.

Enterocin AS-48 is a broad-spectrum cyclic antimicrobial peptide produced by *E. faecalis* and *E. faecium* strains (reviewed by Maqueda et al., 2004). This bacteriocin has been widely investigated in food systems against foodborne pathogenic bacteria such as *L. monocytogenes, S. aureus, B. cereus, E. coli* or *S. enterica* (Abriouel et al., 2010; Ananou et al., 2005; Cobo Molinos et al., 2005, 2009a; Grande et al., 2006, 2007a, b; Martínez Viedma et al., 2008, 2009a, 2009b; Muñoz et al., 2007). Partially-purified preparations of enterocin AS-48 can be produced easy on semi-synthetic media (Abriouel et al., 2003) and on whey-based substrates (Ananou et al., 2010), which makes this bacteriocin an amenable antimicrobial for application in foods. Because of its broad spectrum of inhibition and increased stability due to its cyclic structure, enterocin AS-48 can be a sound candidate for decontamination of vegetable foods containing *L. monocytogenes* and other foodborne bacteria sensitive to this bacteriocin.

In experiments carried out for enterocin AS-48 on sprouts, *L. monocytogenes* was able to grow without bacteriocin on alfalfa and soybean sprouts and in green asparagus at temperatures of 6 to 22ºC and to reach high cell numbers (up to 6 log CFU/g, depending on temperature) during storage (Cobo Molinos et al., 2005). Sprouts inoculated with *L. monocytogenes* were treated by immersion for 5 min in distilled water or in bacteriocin solutions of 5, 12.5, and 25 µg/ml. The effect of bacteriocin treatment was directly proportional to bacteriocin concentration and inversely proportional to storage temperature of samples after treatment. In alfalfa and soybean sprouts, the concentration of viable listeria was reduced by approx. 2 log units by the 25 µg/ml bacteriocin immersion treatment. Washing treatments with 12.5 µg/ml bacteriocin caused apparently much lower reductions of listeria populations in soybean sprouts compared to alfalfa sprouts (1-1.5 log cycles). Nevertheless, for both alfalfa and soybean sprouts, the bacteriocin concentrations of 12.5 as well as 25 µg/ml reduced the concentrations of viable listeria below the detection levels from day 1 to day 7 of storage at temperatures of 6ºC as well as 15ºC. These results indicated that residual bacteriocin adsorbed to the treated sprouts was able to provide a protective effect after treatment for samples stored under a broad interval of refrigeration temperatures (Cobo Molinos et al., 2005). Nevertheless, incubation of the treated samples at a higher temperature of 22ºC seriously compromised the protective effect of the bacteriocin. In soybean sprouts, the concentration of viable cells was reduced much more slowly, and in alfalfa sprouts the listeria were able to multiply even in the samples treated with 25 µg/ml bacteriocin. For green asparagus, no viable listeria were detected during storage at 15ºC of samples treated with bacteriocin concentrations of 12.5 and 25 µg/ml. The bacteriocin apparently had a lower effect at 6ºC (reducing the viable concentration of listeria below detection levels only for 25 µg/ml bacteriocin after day 3 of storage) and did not avoid growth of the listeria in the samples stored at 22ºC.

In order to improve the bactericidal effects against listeria, the bacteriocin (25 µg/ml) was applied on green asparagus in washing treatments in combinations with a variety of antimicrobials: acetic acid, citric acid, lactic acid, potassium lactate, sodium propionate, potassium sorbate, sodium nitrite and nitrate, trisodium-tri-metaphosphate, potassium thiosulfate, potassium permanganate, n-propyl *p*-hydroxybenzoate, *p*-hydoxybenzoic acid methyl esther, hexadecylpyridinium chloride, peracetic acid, and sodium hypochlorite (Cobo Molinos et al., 2005; Table 1). The combinations of AS-48, acetic acid, citric acid, sodium propionate, potassium sorbate or sodium nitrite had limited or no effect on viability of listeria compared to the effect of AS-48 alone. Remarkably, solutions containing AS-48 plus lactic acid (0.1% and 0.5%), sodium lactate (0.1% and 0.5%), n-propyl *p*-hydroxybenzoate (0.1% and 0.5%), *p*-hydroxybenzoic acid methyl esther (0.5%), peracetic acid (80 ppm), sodium hypochlorite (100 ppm), potassium nitrate (100 ppm), or tri-sodium tri-metaphosphate (0.5%) reduced viable counts of listeria below detection limits upon application of the immersion treatment and/or further storage for 24 h. A lower increase of AS-48 activity was noticed for hexadecylpyridinium chloride (0.5%), sodium thiosulphate (0.01 N), tri-sodium phosphate (1.5%) and potassium permanganate (25 ppm). This study provided a variety of combinations of enterocin AS-48 and sanitizers that improved the efficacy for decontamination of *Listeria* while at the same time decreasing the effective concentration of sanitizers to be added.

Since most foods (including sprouts) are complex ecosystems where mixed microbial populations coexist, the impact of bacteriocin treatment was investigated on soybean sprouts during storage at 10ºC. Changes in microbial populations during storage of bacteriocin-treated foods may provide insights on the global impact of bacteriocin treatment in addition to the selective inhibition of the bacterial pathogen being studied by selective counting methods. For doing these studies, culture-independent methods based on the total DNA of the bacterial community are frequently used, and the results of amplification of species-specific DNA regions are analysed by denaturing gel electrophoresis (DGGE). Results from sprout samples treated with enterocin AS-48 revealed modifications of the microbial populations during storage, apparently increasing the proportion of *Enterococcus* and *Leuconostoc* bacteria and decreasing the levels of Gram-negative bacteria (such as *Pantoea, Escherichia* and *Enterobacter*) on the sprouts during storage (Cobo Molinos et al., 2009b). These results indicate that bacteriocin treatment with enterocin AS-48 has additional effects besides inhibition of *L. monocytogenes* on sprouts, disturbing the microbial balance. The consequences of these changes for the biopreservation of sprouts and for the survival of pathogenic and spoilage bacteria need to be investigated in deeper details.

Bacillus cereus is a toxin-producing common soil inhabitant that is often present in a variety of foods, including those of vegetable origin (Granum, 2001). In soybean sprouts, endospore-forming bacteria were found in the order of 2 log CFU/g, and 53 out of 55 *B. cereus* isolates were found to produce diarrheic enterotoxins (Kim et al., 2004). A study was carried out on application of a washing treatment with enterocin AS-48 (25 µg/ml) for decontamination of sprouts and green asparagus challenged with *B. cereus* and *Bacillus weihenstephanensis* and stored at temperatures of 6, 15 or 22ºC (Cobo Molinos et al., 2008a). The best results were obtained for samples refrigerated at 6 ºC, in which the washing treatments reduced the population of *B. cereus* by 1 to 1.6 log cycles, and the remaining viable population was reduced below detection levels after days 1 to 3 of storage. By contrast, reductions of viable cell counts obtained after treatments at 15 or 22 ºC were much lower in the three types of food tested, and the remaining viable cells multiplied during

storage of the treated samples. A similar trend was observed for samples challenged with *B. weihenstephanensis*, with reductions of viable counts up to 3.4 log cycles after treatment, complete inhibition of outgrowth during storage at 6ºC for up to 7 days, and proliferation of survivors during storage at 15 and 22ºC. In order to improve the efficacy of treatments, the bacteriocin (25 µg/ml) was tested in combination with a variety of chemicals (lactic acid, sodium lactate, sodium hypochlorite, tri-sodium tri-metaphosphate, hexadecylpyridinium chloride, peracetic acid, polyphosphoric acid, carvacrol, hydrocinnamic acid, n-propyl *p*-hydroxybenzoate and *p*-hydroxybenzoic acid methyl esther) against *B. cereus* in alfalfa sprouts stored at 15ºC (Cobo Molinos et al., 2008a; Table 1). The bactericidal effect of treatments was enhanced significantly by addition of AS-48 for carvacrol (0.3%), cinnamic acid (0.3%), hydrocinnamic acid (0.5%), polyphosphoric acid (at 0.1 and 0.5%), peracetic acid (40 ppm), hexadecylpyridinium chloride (0.5%), sodium hypochlorite (100 ppm), and tri-sodium tri-metaphosphate (0.5%). Interestingly, for some of the combined treatments with AS-49 (with sodium hypochlorite, peracetic acid, polyphosphoric acid, and hydrocinnamic acid) the levels of *B. cereus* remained below detection limit after 24 h of treatment, indicating that treatments were effective in preventing outgrowth of possible survivors or that no surviving cells were left by the treatments. The degree of protection afforded by these combined treatments was tested in alfalfa sprouts challenged with *B. cereus* and with *B. weihenstephanensis,* and stored at 15ºC for one week. In samples treated with carvacrol, cinnamic and hydrocinnamic acids in combination with AS-48, viable cell counts of *B. cereus* remained significantly lower compared to each individual treatment during most part or the whole storage period. The best results were obtained for the combinations of AS-48 and sodium hypochlorite, hexadecylpyridinium chloride, peracetic acid and polyphosphoric acid, which reduced the population of *B. cereus* below detection limits for the whole or at least most of the storage period. The combinations of 25 µg/ml AS-48 and hydrocinnamic acid, peracetic acid, sodium hypochlorite, polyphosphoric acid and hexadecylpyridinium chloride also reduced the population of *B. weihenstephanensis* below detection limits for one week when tested on alfalfa sprouts (Cobo Molinos et al., 2008a).

Bacteriocins from Gram-positive bacteria are usually not active against Gram-negative bacteria, with some exceptions. However, Gram-negative cells can be rendered sensitive to these bacteriocins when exposed to treatments that damage the bacterial outer membrane, which acts as a barrier against diffusion of bacteriocin molecules to the cytoplasmic membrane where they exert their lethal action. Exposure to chelating agents, acids, or sublethal heat, are often used to sensitize Gram-negative bacteria to bacteriocins. Since most of the Gram-negative bacteria are resistant to enterocin AS-48, the bacteriocin was tested on sprouts in combination with several sensitizing treatments. Once the synergistic effects were demonstrated in washing treatments against *S. enterica* as test organism, they were corroborated on other Gram-negative species. Inactivation of *S. enterica* cells inoculated on soybean sprouts increased greatly when sprouts were heated for 5 min at 65ºC in an alkaline solution (25 µg/ml, pH 9.0) of enterocin AS-48 (Cobo Molinos et al., 2008b). Washing treatments containing AS-48 (25 µg/ml) and 1.5% lactic acid, 1.5% trisodium phosphate, 0.5% trisodium tri-metaphosphate, 0.1% polyphosphoric acid, 80 ppm peracetic acid, 0.5% hexadecylpyridinium chloride, 100 ppm sodium hypochlorite, 0.5% n-propyl *p*-hydroxybenzoate, 0.5% p-hydroxybenzoic acid methyl esther and 2% hydrocinnamic acid significantly reduced the population of *S. enterica* to a much greater extent that the single treatments (Table 1). The best results were obtained for combinations of AS-48 and lactic acid, peracetic acid, as well as polyphosphoric acid.

	L. monocytogenes	*B. cereus*	*S. enterica*
Acetic acid	X		
Citric acid	X		
Propionate	X		
Sorbate	X		
Lactic acid	X	X	X
Lactate	X	X	X
Nitrite	X		
Nitrate	X		
EDTA			X
Tri-sodium phosphate	X		X
Tri-sodium tri-metaphosphate	X	X	X
Thiosulphate	X		
Permanganate	X		
Propyl-*p*-hydroxybenzoate	X	X	X
p-Hydoxybenzoic acid methyl esther	X	X	X
Peracetic acid	X	X	X
Hexadecylpyridinium chloride	X	X	X
Sodium hypochlorite		X	X
Polyphosphoric acid		X	X
Carvacrol		X	
Cinnamic acid		X	
Hydrocinnamic acid		X	X

Table 1. Antimicrobials that potentiate the efficacy of enterocin AS-48 on sprouts (X). Unmarked combinations were not tested or did not potentiate bacteriocin activity.

The efficacy of enterocin AS-48-polyphosphoric acid treatment for decontamination of sprouts was tested on other Gram-negative bacteria, including species involved in human disease and in food spoilage *E. coli* O157:H7, *Shigella flexneri, Shigella sonnei, Enterobacter aerogenes, Y. enterocolitica, A. hydrophila* and *P. fluorescens*). Enterocin addition increased the bactericidal effects of treatments in all cases, although there were large differences in the concentrations of polyphosphoric acid required for a complete inactivation and prolonged protection from one bacterial species to another. For example, *E. coli* required at least 0.4% polyphosphoric acid for complete inactivation while *Y. enterocolitica* was very sensitive to low polyphosphoric acid treatment (0.1-0.2%). *A. hydrophila* and *E. aerogenes* were very sensitive to combined treatments with enterocin AS-48 plus 0.5% and 1% polyphosphoric acid, respectively. *P. fluorescens* was also very sensitive to 1% polyphosphoric acid (with an average decrease of 5 log CFU/g after the combined treatment), but it required a higher concentration of at least 2% to avoid overgrowth during storage. The shigellae were the most resistant of all bacteria to the combined treatment. *S. sonnei* was not completely eliminated by treatments containing 2% polyphosphoric acid and *S. flexneri* was even more resistant, although the combined treatment with 2% polyphosphoric acid always reduced the surviving fraction and avoided overgrwth during storage of the samples. These results suggest that combined treatments with polyphosphoric acid at 1% are sufficient to suppress the most common Gram-negative pathogens found in sprouts (that is, *S. enterica* and *E. coli*)

as well as others such as *Y. enterocolitica* and the spoilage *Pseudomonas*. Other bacteria which are much less common, such as the shigella, would require a polyphosphoric acid concentration of at least 2% for inactivation on sprouts. Therefore, the disinfection capacity of the combined washing treatments could be adjusted depending on the estimated risks.
The bacteriocins from Gram-negative bacteria have seldom been exploited in food preservation, although they could be useful in the control of enteric pathogens. Many Gram-negative species have shown to produce bacteriocins, but those produced by *E. coli* strains (or colicins) have been studied in greater details (Riley and Gordon, 1996). A recently isolated *E. coli* strain Hu194 (from a human fecal sample) was capable of inhibiting 22 *E. coli* strains of serotype O157:H7 and it also exhibited some degree of antimicrobial activity against *Salmonella.* This inhibition was mediated by the production of a colicin named Hu194 (Nandiwada et al., 2004). Semi-crude colicin Hu194 was applied by mixing and drying on alfalfa seeds challenged with three different *E. coli* O157:H7 strains. The bacteriocin treatment caused variable effects depending on the strain. One of the strains was successfully inactivated (5 log CFU/g reduction) from inoculated alfalfa seeds, without any bacterial overgrowth being observed for up to 5 days of further incubation. By contrast, the other two strains required 20-fold higher colicin concentrations to achieve a reduction of 3 log cycles (Nandiwada et al., 2004). This reduction was slightly enhanced after soaking the inoculated seeds for a longer period of time, and the maximum bacterial reduction was typically observed during the first 2 days after soaking the seeds in the colicin extract suspension. The variations observed in strain sensitivity need to be further investigated by testing a larger number of strains in order to establish the minimum bacteriocin dose that is needed to achieve an acceptable reduction of viable counts for the target bacteria.

5. Conclusions and perspectives

Biocontrol strategies aimed at reducing the transmission of human pathogenic bacteria through the consumption of sprouts seem a promising alternative strategy to the use of chemical preservatives or at least to reduce the concentrations of preservatives to be used. Application of live bacterial cultures requires a careful selection of strains that are well adapted to the food substrate and storage temperature of sprouts, and is somehow limited by their capacity for an effective in situ production of antimicrobial substances and/or competitive exclusion of the pathogens. Strains of Gram-negative bacteria belonging to *Pseudomonas* and *Enterobacter* genera seem to be promising for the control of *Salmonella* on sprouts. Intervention strategies based on lytic bacteriophages require a careful selection of complex phage preparations in order to overcome efficacy limitations due to strain specificity. Bacteriophages may act synergistically with antagonistic bacteria against selected human pathogenic bacteria. This is an interesting approach that has very seldom been exploited and where further studies are required. The lytic enzymes produced by bacteriophages could also be exploited as antimicrobial agents on sprouts (either alone or in combination with other treatments), minimizing the impact of phage specificity. However, this approach has not been investigated yet. Some bacteriocins (especially those produced by the lactic acid bacteria) have shown interesting results when tested on sprouts. The efficacy of these bacteriocins can be enhanced in combination with other antimicrobials, decreasing the concentrations of chemical preservatives required for disinfection of sprouts and protection from bacterial overgrowth during shelf life storage. The application of bacteriocins from Gram-negative bacteria for biopreservation of sprouts has been seldom

investigated. This is an interesting field for new research as well. In addition, the application of cocktails based on protective cultures, bacteriophages and bacteriocins should also be investigated as broad-spectrum intervention strategies capable of simultaneous inhibition of mixed bacterial populations of human pathogenic bacteria on sprouts.

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6

Asian Soybean Rust – Meet a Prominent Challenge in Soybean Cultivation

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1. Introduction

The people inhabiting our planet are expected to touch the nine million mark within the next 40 years. Feeding the world population adequately is a problem which has been faced for a long time, and although considerable efforts have been made in tackling global food security, further solutions are needed to increase the supply of basic staples in the coming decades. Generally, there are two major strategies to approach this goal for food production: firstly, to grow more crop plants on newly reclaimed land or to increase yields on existing farm land by using improved cultivars; and secondly, to diminish the actual losses in crop yield arising due to pests, diseases or from the competition for nutrients with weeds. In view of the size of the challenge it is reasonable to assume that only a combination of different approaches can achieve global food security. However, implementation of the first strategy may have negative ecological impacts on existing ecosystems, and a higher input of fertilizer or water, both of which are expensive or limited, will be a consequence. The second strategy, by contrast, displays an attractive alternative because it deals with the conservation of already produced biomass. Thus, in principle, would not need any further input of resources for crop growth. It was estimated in 1994 that the actual losses due to insects, microbes and weeds reduced the theoretically achievable global crop yield by 42% (Oerke et al., 1994). Reducing this depletion would be a valuable contribution to ensure food security.
This chapter will deal with soybean as one of the world's major staple crops and strategies will be presented to combat yield losses caused by a prominent foliar pathogen known as Asian soybean rust caused by the fungus *Phakopsora pachyrhizi* (Fig. 1). Generally, all parts of soybean plants are targeted by different pathogens and pests during the growing season, e.g. roots by cyst nematodes, young seedling by different *Pythium*, *Rhizoctonia* or *Phytophthora* species and leaves e.g. by *Phakopsora pachyrhizi*. Asian soybean rust (ASR) affects yield because of premature leaf drop and reduced green leaf area which affects the photosynthetic capacity of the plant. Generally, agronomists try to keep plant diseases in check by breeding resistant cultivars or, more recently, by using biotechnological tools. However, it must be noted that the latter approach which includes the generation and release of genetically modified plants into nature are discussed controversially in public debates. Unlike for some other important plant diseases, neither breeding for resistant cultivars nor the biotechnological approach have so far led to novel soybean genotypes that could withstand all isolates of the ASR fungus.

Fig. 1. Disease symptoms caused by the Asian soybean rust fungus on soybean plants. Brownish spots become visible on the leaf surface of susceptible soybean plants eight to ten days after inoculation with the pathogen *Phakopsora pachyrhizi* (left picture). Disease symptoms, which are reminiscent of oxidised metal (rust), are referred to as 'rust pustules' and consist of an aggregation of individual fungal uredospores (right picture). These uredospores are dispersed by wind or water and each of them is able to generate a novel infection site on healthy plant tissue of the same plant or plants on distant field plots.

Here, we report on our research to get a deeper insight into the interaction of a soybean plant with the ASR fungus. In the short term, we aimed at targeting novel traits which might confer resistance to soybean against this disease and identifying crucial elements required by the pathogen to be virulent on soybean; and at the end of the day both approaches might channel into the development of a novel cultivar that effectively withstands *P. pachyrhizi*.

1.1 What is the problem?

The phenomenon of global warming has let to considerable changes in environmental conditions of ecosystems worldwide. Many plant pathogens and pests are extending their ranges and emerging in habitats where they had not been present before. While sporadic invasion by these species had been terminated in the past mostly due to freak, unfavorable weather conditions, nowadays they may survive more frequently and become endemic. The problem of pathogen dispersal is compounded by increased human mobility and long-distance travel. A recent example is the invasion of the Asian soybean rust fungus, *Phakopsora pachyrhizi*, into South and North America. Initially, a less aggressive relative of *P. pachyrhizi*, known as *Phakopsora meibomiae* and referred to as the Latin-American isolate, was indigenous in South-America where it was not considered a major problem in soybean cultivation. The situation changed dramatically with the appearance of the Asian-Australian isolate, *P. pachyrhizi*, which by now has reached top ranking among soybean diseases in South-America, e.g. in 2003 the disease spread through Brazil and caused losses estimated at US$ 2 billion (Yorinori et al., 2005). In 2004 ASR was reported for the first time in the

continental United States and since then it has attracted a lot of attention because of its potential to cause high yield losses within the corn belt in the Midwestern US (Schneider et al., 2005). In contrast to other soybean diseases no cultivars are available which can withstand all races of this pathogen, thus farmers rely on the costly use of fungicides to manage the threat (Miles et al., 2007). Because of its economical and ecological impact fungicidal treatments are seen critically. Furthermore, emerging resistance of the pathogen against certain fungicide classes has to be expected. Accordingly, new strategies are needed to combat this growing threat and below we present approaches currently pursued in our lab.

1.2 Our approach

Generally, research into fungal plant diseases at the academic level could be done by concentrating on the plant or fungal partner in the interaction. The plants can be investigated for traits conditioning susceptibility and trying to fortify the natural defense arsenal and the pathogen by analyzing the infection process, aiming at identifying an Achilles heel which could be used in subsequent approaches to avert infection of plants.

In our lab we follow a holoistic strategy and investigate both partners. As a source for traits for the potential improvement of plant defenses, we exploited a phenomenon known as nonhost resistance. The term "nonhost resistance" describes the resistance of plants against pathogens for which the plant species in question is not considered a host (Heath, 2001). For example, a pathogen of grasses would not necessarily be expected to infect other plants, e.g. soybean, to which it has not had the opportunity to adapt in the course of evolution. In natural ecosystems, as well as in agricultural habitats, nonhost resistance mediates a robust protection against pathogenic invaders and it was therefore often suggested that the mechanisms of nonhost resistance could be exploited to improve the resistance of crop plants (reviewed by Thordal-Christensen, 2003). The model plant *Arabidopsis thaliana* displays nonhost resistance against the Asian soybean rust and this enables us to take advantage of the extensive tool-box for genetic and molecular investigations which already exists. Thus, it has been shown that Arabidopsis mutants with defects in one or several of the so-called PEN (penetration) genes are compromised in nonhost resistance against another important pathogen, namely *Blumeria graminis* f. sp. *hordei*, which is the powdery mildew pathogen of barley (Stein et al., 2006; Schweizer, 2007). We tested the impact of mutations in these genes on infections with *P. pachyrhizi* on Arabidopsis in growth chamber experiments. Alterations in the infection process were monitored by macroscopic and microscopic evaluation. Testing of several other Arabidopsis mutants and analyses of defense gene activation were used to investigate the possible involvement of other resistance mechanisms in nonhost resistance of Arabidopsis against *P. pachyrhizi*.

In a second line of experiments we worked towards a better understanding of the infection process by *P. pachyrhizi* on soybean. By now it is widely accepted that phytopathogens actively create an accomodating environment in their cognate host by interfering with the plant's defense machinery. Such a scenario is well documented for bacterial pathogens which possess a dedicated secretory pathway to deliver so-called effector molecules into the host cells which then sabotage essential resistance mechanisms (Romer et al., 2007). It could be speculated that rust fungi, such as *P. pachyrhizi*, also recruit such molecules to avoid recognition or the initiation of defense, although no experimental proof for this hypothesis exists so far. Nevertheless, if this assumption were to be correct, and if identification of the

effector molecules were possible, these molecules or their targets inside the plant cell could be valuable targets in future plant protection programs. Testing this hypothesis in the soybean/*P. pachyrhizi* interaction is difficult because the pathogen has an obligate biothrophic life-style which means that it grows and multiplies solely on living plant tissue. This adds to the difficulty of purifying fungal-derived molecules because contamination with plant material must be expected. To minimize this drawback, we followed an approach of firstly isolating fungal infection structures from infected plant material and then trying to identify fungal genes expressed solely at this stage of infection. Purification of the desired infection structures was achieved using affinity chromatography. After sampling of sufficient material a snap-shot of active genes was taken by isolating RNA and sequencing the entire transcriptome. In this way, we obtained a large data set representing sequences of actively expressed genes. The challenging task is now the informed selection of interesting *P. pachyrhizi* genes which are crucial for successful infection amongst a group of genes which are not involved in pathogenicity.

2. The pathogen

The development of novel plant protection strategies is intrinsically tied to an in-depth understanding of the prerequisites of both interaction partners such that disease becomes established. This includes detailed knowledge of the life-style and the infection mechanism of a given pathogen as well as the defense reactions displayed by the host. The next paragraphs document the current status of knowledge in these areas.

2.1 Pathogen life-cycle and host range

Rust fungi belong to a group of fungi called basidiomycetes. They differ from other groups of fungi because the cells in most stages of the life-cycle contain two separate haploid nuclei, and the mycelium is referred to as dikaryotic. Rust fungi of different species cause disease on several host plants but all of them have a so-called obliagtely biotrophic life-style in common; which means that these pathogens can grow and multiply solely on living host tissue. However, it has to be mentioned that after intensive efforts it is now possible to grow some rust fungi in axenic culture apart from the host plant. However, this has so far not been achieved successfully for the Asian soybean rust fungus *P. pachyrhizi*.

Infection of soybean plants by *P. pachyrhizi* starts with the germination of a uredospore on the leaf surface (Fig. 2). Growth of the germ tube is terminated by the formation of a specialized, globose infection structure called an appressorium. The appressorium is separated from the germ tube by a septum. Since germination and appressorium formation also occur on water agar plates and artificial membranes it seems that both processes do not involve plant-derived signals (Koch and Hoppe, 1988). In these experiments a direct correlation was found between the frequency of appressorium formation and the pore size of the artificial membranes, which suggested the involvement of thigmo- rather than chemodifferentiation (Staples and Macko, 1994; Koch and Hoppe, 1988).

Penetration of *P. pachyrhizi* into host epidermal cells is initiated within the appressorium by building of an internal structure called the appressorial cone which then elongates into the penetration peg (Bromfield, 1984). Direct penetration of the host cuticle is a special feature of the Asian soybean rust, as opposed to stomatal penetration which is usual for the majority of other rusts. Epidermal cells of host plants undergo cell death after penetration by

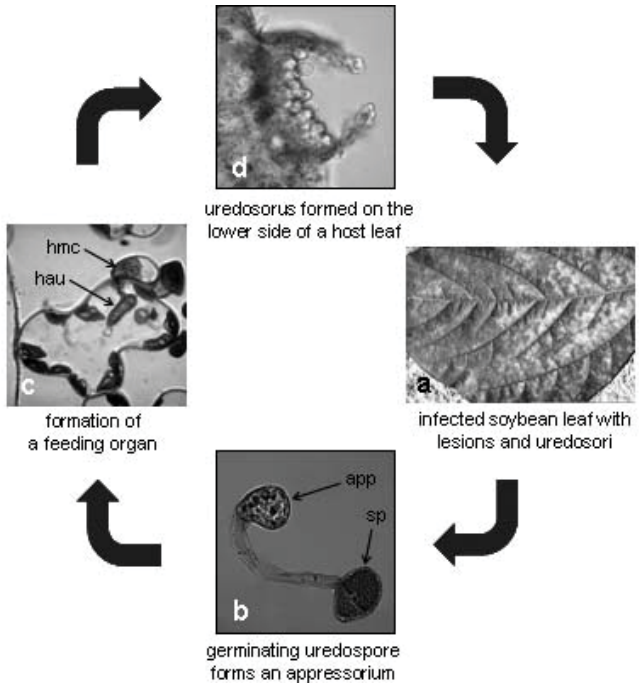

Fig. 2. Life-cycle of *P. pachyrhizi* on soybean. a) Uredospores of *P. pachyrhizi* are released from infected leaves and dispersed by wind. b) Upon contact with suitable surfaces, a uredospore germinates and forms an appressorium. c) Tissue of infected leaves is heavily colonized by fungal hyphae which give rise to haustorial mother cells (hmc) from which haustoria (hau) are inserted into host mesophyll cells. d) Asexual reproduction of *P. pachyrhizi* is completed by generating novel uredospores in uredosori. sp, uredospore; app, appressorium; hau, haustorium; hmc, haustorial mother cell; modified from (Goellner et al., 2010).

P. pachyrhizi; a feature which is generally uncommon among biotrophic plant pathogens in general and particularly in rust diseases (Keogh et al., 1980). However, penetration hyphae do not appear to be affected by this cell death event and grow on, branching after they reach the mesophyll and differentiating haustorial mother cells (Koch et al., 1983). The latter give rise to the formation of specialized infection structures called haustoria, which are the essential feeding organs of the pathogen. Haustoria are common among all obligate biotrophic fungal plant pathogens and failure to produce them is a knock-out criterion for infection by these pathogens. Once haustoria are successfully established the fungal mycelium intensively colonizes the host tissue and the production of new uredospores completes the pathogens life-cycle (Koch et al., 1983).

Worthy of special note is the broad host range of the Asian soybean rust fungus, a feature which is uncommon among the majority of other rusts. Actually, it has been documented that *P. pachyrhizi* is able to infect and sporulate on 31 different species in 17 different genera of leguminous plants (Ono et al., 1992). Using artificial inoculation methods under laboratory conditions this range of host plants can even be expanded. However the increased time span needed to sporulate on the latter plants in comparison to *P. pachyrhizi's* intrinsic soybean host indicates a lower degree of compatibility.

2.2 *P. pachyrhizi*'s journey around the world

The degree of attention which was directed towards the Asian soybean rust after invasion of the continental US has been nearly unprecedented for an emerging plant healt problem (Stokstad, 2004). Cultivation of soybean is proposed to have started in the eastern half of northern China in the 11th century B.C. and soybean-associated diseases spread together with its propagation range throughout the eastern hemisphere. Hence, Asian soybean rust appeared in Australia in 1934 and in South America in 1976 (Vakili and Bromfield, 1976; Bromfield, 1984). It has to be noted that this first outbreak of the disease in the New World was due to the less aggressive Asian soybean rust isolate *Phakopsora meibomiae*, which is referred to as the Latin-American isolate (Bonde et al., 2006). Not until 2001 was the more aggressive Asian-Australian pathogen species *P. pachyrhizi* found on the American continent (Freire et al., 2008). After a first report from Paraguay, the pathogen spread to Bolivia, Brazil and Argentina. Possibly with tropical storms the pathogen spread north to the continental US but, due to its inability to overwinter, it was not able to cause great yield losses so far (Isard et al., 2005; Christiano and Scherm, 2007). Because uredospores are not frost-tolerant and the more robust teliospores have never been found for this pathogen, inoculum has to overwinter in the more temperate South during the winter. At this soybean-free time of the year alternative host plants such as the immigrant weed kudzu (*Pueraria montana*) can serve as a potential host. This alternation between domesticated and wild host species creates the source for initial infection at the beginning of the each soybean growing season (Fabiszewski et al., 2010).

3. The plant

Although plants are continuously exposed to pathogenic microorganisms disease seems to be an exception. It must be concluded, therefore, that plants have evolved efficient strategies to avoid disease. Since a constant activation of metabolic responses such as defense when they are not needed has high fitness-costs (Heil and Baldwin, 2002), most of the defense reactions rely on an inducible system (Jones and Dangl, 2006). On the other hand, pathogens have evolved mechanisms to circumvent recognition in order to successfully establish disease or, to actively suppress the execution of downstream signal networks involved in plant defense (Dodds and Rathjen, 2010). The following paragraphs will give a short introductory overview on the defense reactions displayed by soybean upon attack by the Asian soybean rust and on actual strategies pursued to combat the threat.

3.1 Breeding for disease resistance and other disease management strategies

Firstly, it has to be noted that none of today's soybean cultivars express resistance against all known races of *P. pachyrhizi*. Thus, it has to be kept in mind that at present, protection of planted soybean against the Asian soybean rust is incomplete and yield losses have to be expected in cases where virulent races of the pathogen encounter soybean communities.

So far five resistance genes, referred to as *Rpp1* to *Rpp5*, have been identified, each conferring resistance against a particular race of *P. pachyrhizi* (Bromfield and Hartwig, 1980; Hartwig and Bromfield, 1983; Bromfield, 1984; Morceli et al., 2008). These resistance genes have been identified genetically but not yet cloned. Screening for resistance against *P. pachyrhizi* is generally done using three different infection phenotypes: the highest degree of protection was observed in the so-called "immune" response where no disease symptoms were found on soybean leaves upon inoculation with the pathogen (Bromfield, 1984; Pham

et al., 2009). By contrast, susceptible interactions are characterized by tan-colored lesions, referred to as "TAN"-type, in which 2-5 uredia (syn. uredosori) are produced on average. An intermediate resistance phenotype occurred on plants showing reddish-brown lesion ("RB"-type) with little or no sporulation (Bromfield, 1984; Pham et al., 2009). Break-down of resistance against *P. pachyrhizi* conferred by single dominantly-inherited genes is well known due to the capacity of the fungus to form new races (Bromfield, 1984). A strategy to circumvent this problem could be the pyramiding of different resistance genes in a single cultivar, making it much more complicated for a pathogen to overcome resistance, or by incorporating novel sources of resistance. Thus, close relatives of *Glycine max*, e.g. *G. sojae* and other *Glycine spp.*, have been screened for resistance against *P. pachyrhizi* (Hartman, 1992; Hartman et al., 2005). Another approach has aimed at characterizing the spectrum of resistance in *P. pachyrizi's* alternate host kudzu (Jordan et al., 2010). So far, none of these approaches has led to the generation and field release of novel soybean cultivars.

The introgression of known resistance genes into the genetic background of other cultivars can be assisted by the availability of associated molecular markers (Babu et al., 2004). Efforts towards this goal have been made in soybean by mapping the genomic location of the *P. pachyrhizi* resistance genes (*Rpp1-Rpp5*) to molecular linkage groups (Hyten et al., 2007; Monteros et al., 2007; Garcia et al., 2008; Silva et al., 2008). These markers could e.g. be used to determine whether resistance genes of newly identified soybean accessions with resistance against *P. pachyrhizi* map to already known loci (Ray et al., 2009).

Asian soybean rust could also be kept in check using fungicide treatments. Yield losses could be avoided by prophylactic fungicide treatments between the early flowering (R1) and seed filling (R5) stages if the pathogen is around (Doerge and Trybom, 2008). The current practice for controlling the disease with chemicals in the U.S. includes a combination of timely observation and application of appropriate fungicides. This had kept soybean yield losses to a minimum even in 2007 which was a year of great expansion of Asian soybean rust occurrence in the U.S. (Doerge and Trybom, 2008). Past experience has told us that resistance against fungicides may develop (Steffens et al., 1996; van den Bosch and Gilligan, 2008) and the risk is minimized by using mixtures of chemicals belonging to different classes or chemicals with different modes of action. Presently, strobilurins and triazoles are used effectively to manage the disease. However, additional fungicides are needed since some had only emergency authorization for use in the U.S. (section 18 labels).

Besides chemical control of Asian soybean rust, it might also be possible to use biological control agents. Thus, it was reported that uredospores of *P. pachyrhizi* are colonized by fungal hyperparasites such as *Verticillium psalliote* (Saksirirat and Hoppe, 1990, 1991). However these data have been generated under laboratory conditions and field test have not been performed yet. It seems questionable, therefore, whether biological control could substitute chemical disease control or substantially contribute to its effectiveness (Goellner et al., 2010).

Since the source for the initial *P. pachyrhizi* inoculum at the start of the growing season comes from the immigrant weed kudzu, reducing the density of this weed host may help. However, due to the wide range of kudzu this seems not to be feasible. On the contrary, by using modelling of pathogen spillover between soybean and kudzu it turned out that applying efficient disease management for the soybean host will reduce infections on the wild host species (Fabiszewski et al., 2010). Currently, none of the discussed strategies are successful on their own in controlling Asian soybean rust and it seems that a combination of different approaches is most promising.

4. Results

In this section some results from our lab showing novel insights into plant defense mechanisms which are efficient in restriction of the Asian soybean rust disease are presented. Firstly, we employed the model plant species Arabidopsis *thaliana* which did not show any disease symptoms after inoculation with *P. pachyrhizi* and secondly, we isolated fungal haustoria from *P. pachyrhizi* to learn more about the cross-talk between pathogen and host.

4.1 Exploiting a model plant species reveals novel defense traits against *P. pachyrhizi*

Without doubt Arabidopsis is currently the best-understood plant species and consequently large tool boxes are available for forward and reverse genetic approaches. Therefore, it was not astonishing that plant pathologists also started to investigate Arabidopsis-pathogen interactions, including ASR. As described above, nonhost resistance is displayed by a plant species against pathogens from other host plants and it is believed that this type of resistance is conditioned multifactorially and emerged in the course of evolution.

We have tested a range of different Arabidopsis ecotypes collected all around the world for their response to inoculation with *P. pachyrhizi* and none of them showed disease symptoms or became infected (Loehrer et al., 2008). This result was taken as an indication that Arabidopsis and the Asian soybean rust indeed present a nonhost type of interaction. Obviously, Arabidopsis must have defense strategies which protect it effectively against this pathogen. To get further insights into the underlying mechanisms, we made use of a collection of Arabidopsis mutant plants which previously had been shown to be compromised against another nonhost pathogen of Arabidopsis, i.e. barley powdery mildew. These mutants are collectively called *pen*-mutants (penetration) and, so far, encompass three different genetic loci. While inoculation of *pen1-1* and *pen 2-1* mutants with *P. pachyrhizi* did not result in disease symptoms, pronounced necrotic spots were found on leaves of *pen3-1* mutant plants (Loehrer et al., 2008).

We further analysed the interaction of the *pen*-mutants with *P. pachyrhizi* by microscopy. Interestingly, we observed that *P. pachyrhizi* was able to invade epidermal cells on both Arabidopsis wild-type and mutant plants. But the striking difference was that the fungus was able to invade the mesophyll solely on Arabidopsis *pen* mutants (Fig. 3).

To further underpin this observation a quantitative assessment of *P. pachyrhizi* infection sites was done for each genotype. Therefore, cellular interaction phenotypes were grouped into five different categories each representing a typical fungal developmental stage and the absence or presence of a corresponding plant defence reaction (Fig. 4). At least 100 infection sites were inspected and categorized per infected leaf and for statistical reasons three leaves from different plants were analysed for each plant genotype. Importantly, we observed that in plants without a *pen*-mutation the fungus was not able to develop substantially behind the border between epidermal and mesophyll tissue. However, penetration of the epidermis was found regularly. In contrast, in *pen*-mutants the pathogen always invaded the mesophyll and the strongest impact on mesophyll cell collapse was found on Arabidopsis plants carrying the *pen3-1* mutation (Fig. 4).

We further investigated the requirements of nonhost resistance in Arabidopsis against *P. pachyrhizi* by analyzing Arabidopsis mutants carrying mutations not only in genes associated with penetration defense but also in post-penetration resistance mechanisms. Among other mutants, we identified *pen2-1pad4-1* which showed a previously unseen degree of compatibility towards *P. pachyrhizi* as indicated by the formation of haustoria-like

structures inside mesophyll cells. Hence, it must be concluded that the pathogen had overcome a further barrier of nonhost resistance, i.e. penetration resistance of mesophyll cells, in this mutant (Fig. 5). The haustoria of biotrophic fungi are essential feeding organs which are indispensable for continuation of their life-cycle beyond exhaustion of conidial resources. Successful formation of the haustorial stage is therefore thought to play a key function in establishment of biotrophy and is a prerequisite for the completion of the life-cycle by release of a new spore generation. However, it must be stated that in Arabidopsis *pen2-1pad4-1* mutants, irrespective of the initiation of haustorium formation, sporulation of *P. pachyrhizi* was not observed.

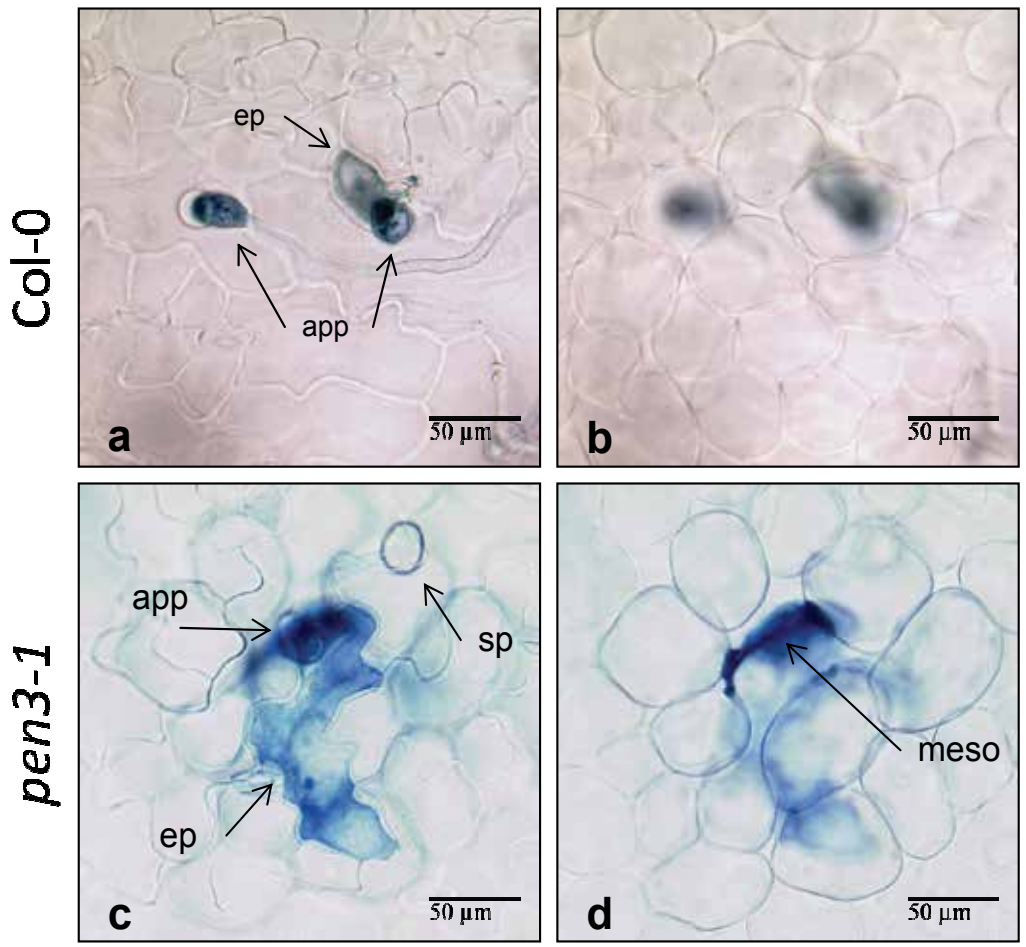

Fig. 3. Interaction sites between *P. pachyrhizi* and *Arabidopsis thaliana* at the cellular level. Leaves of Arabidopsis wild-type (Col-0) and mutant (*pen3-1*) plants were harvested 1 day after inoculation with uredospores of *P. pachyrhizi* and stained with trypan blue. Bright-field microscopy was done using a Leica DMR microscope. For each genotype, optical sections were recorded focussing on the epidermal cell layer (a and c) or on the mesophyll tissue (c and d). app, appressorium; ep, trypan blue-stained epidermal cell; meso, collapsed mesophyll cell stained with trypan blue; sp, uredospore

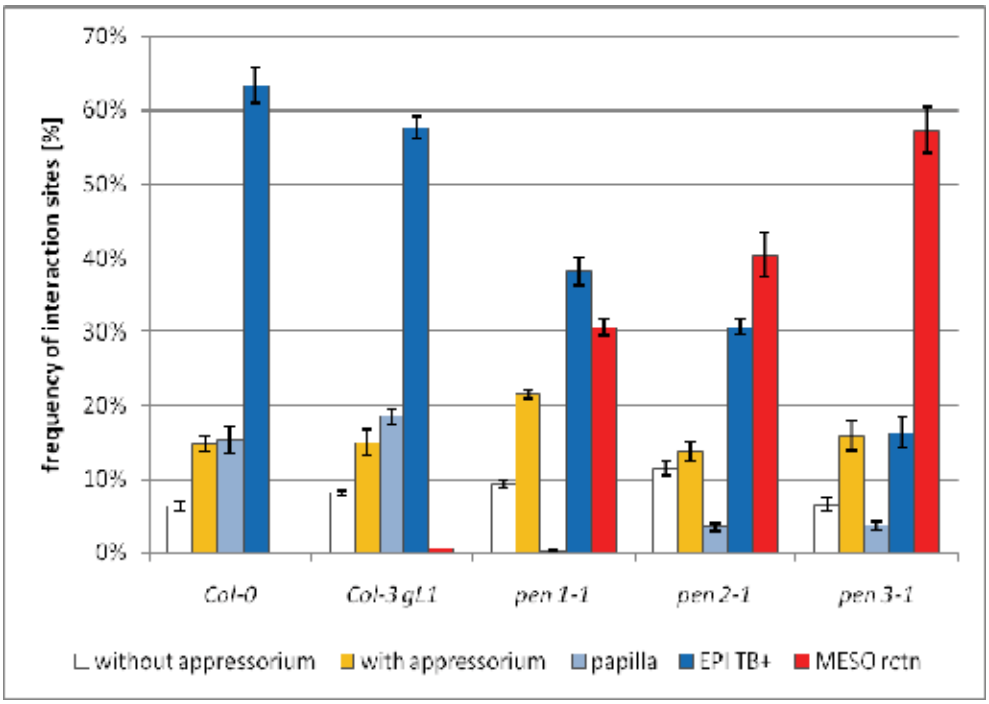

Fig. 4. Quantitative microscopic evaluation of infection sites of *P. pachyrhizi* on leaves of *Arabidopsis thaliana*. Different leaves of Arabidopsis wild-type and mutant plants were harvested 4 days post inoculation with uredospores of *P. pachyrhizi* and stained with trypan blue. Each interaction site was grouped into a particular category: the first comprises spores that had germinated but did not develop appressoria by the time of analysis (without appressorium); the second class encompasses germinated spores with an appressorium (with appressorium); the third category describes infection sites where fungal penetration is countered by the formation of a papilla (papilla); the fourth class represents infection sites with penetrated epidermal cells that retain the trypan blue stain (EPI TB+) and the fifth category comprises infection sites at which the fungus had proceed into the mesophyll tissue and caused cell collapse (MESO rctn). At least 4 leaves have been analysed per genotype and approximately 100 interaction sites were inspected per leaf. Frequencies are given as mean and standard error. Modified after (Loehrer et al., 2008).

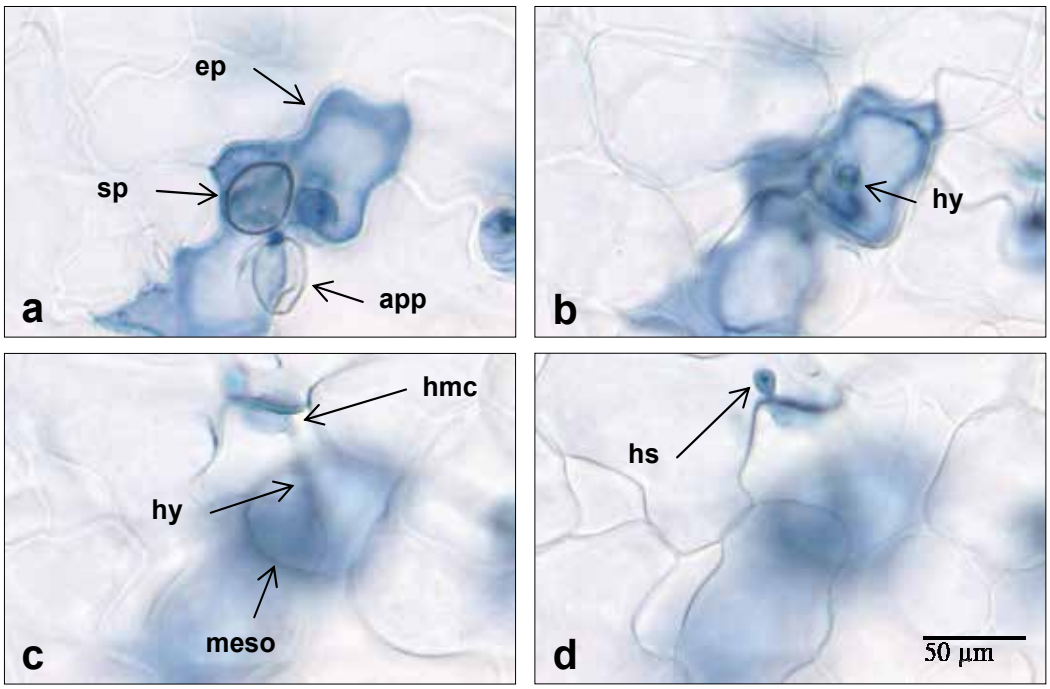

Fig. 5. Impaired penetration and post-penetration resistance mechanisms gave rise to the formation of a haustorium-like structure. Pictures a-d display optical sections at a particular infection site of *P. pachyrhizi* on an Arabidopsis *pen2-1pad4-1* mutant plant. Bright-field micrographs were taken by step-wise focusing on: (a) a fungal uredospore (sp) and the respective appressorium (app), (b) an epidermal cell (ep) penetrated by a hypha (hy), (c) a fungal infection hypha growing in the intercellular space between mesophyll cells and form a haustorium mother cell (hmc) and (d) a haustorium-like structure (hs) build inside a mesophyll cell.

4.2 Analysis of the fungal-plant interactome to unravel *P. pachyrhizi's* Achilles' heel

As discussed in the previous chapters, the successful initiation of the haustorial stage is a crucial event in the life-cycle of biotrophic fungal pathogens. During this process *P. pachyrhizi* penetrates cell walls of host mesophyll cells and invaginates their plasmamembrane without rupture. This gives rise to a double-layered membrane-bound compartment representing a venue of intimate contact between host and pathogen. Crosstalk, that is interchange of signal molecules across this compartment, is likely and knowledge of the interacting molecules might help to understand the basis of the disease.

Aiming at the identification of signal molecules which are released from *P. pachyrhizi's* haustoria, we first started to prepare intact haustoria from infected soybean leaves using a protocol established by Hahn and Mendgen (1992). This procedure exploits surface properties of rust haustoria which permits them to bind to the lectin Concanavalin A (Con A). The protocol depicted in Fig. 6 was established for *P. pachyrhizi* at the University of Konstanz in close cooperation with the group of Prof. R. Vögele. Each round of extraction ended with a small fraction containing the desired haustoria. These final fractions were rarely contaminated with chloroplasts whereas amyloplasts were found more frequently (Fig. 7).

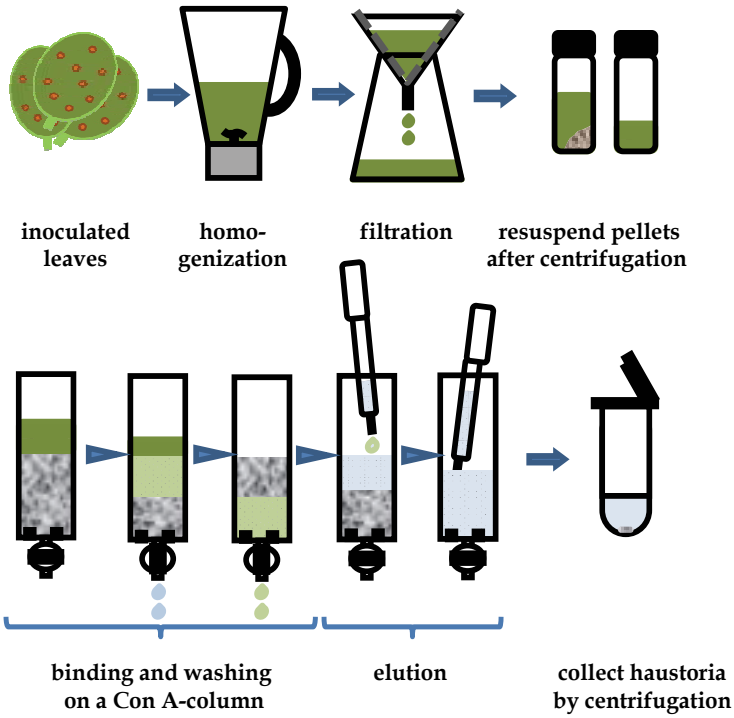

Fig. 6. Flow-chart of haustoria isolation using Con A-affinity chromatography. Soybean leaves showing severe disease symptoms 8 to 9 days after inoculation were homogenized and filtrated through nylon gauze. After centrifugation the pellet containing the haustoria was resuspended in binding buffer and transferred onto an equilibrated Con A-sepharose column. After binding of the haustoria to Con A, the column was washed several times with fresh buffer to elute remaining cell debris. Afterwards, haustoria were mechanically eluted from the column by strongly agitating the matrix using a pipette. The supernatant containing the haustorial fraction was then transferred to a microcentrifuge tube for further processing.

Several rounds of chromatography were needed to collect sufficient haustorial tissue for down-stream processing. Predicting that genes encoding proteins necessary for the establishment of haustoria are under transcriptional control, we sequenced the whole haustorial transcriptome (Loehrer and Schaffrath, unpublished). Finally, we ended up with 111,440 *de-novo* assembled contigs from which 50 candidate genes were chosen based on structural features commonly found within known or predicted fungal effector genes (Ellis et al., 2007; Ellis et al., 2009). The functional testing of our candidate genes for an involvement in haustorium initiation requires the transformation of the pathogen. However, transformation of biotrophic fungi in general, and rust fungi in particular, is still a challenging task (Gregory et al., 2010). Efforts towards the implementation of a test system for gene function in *P. pachyrhizi* using different experimental set-up are in progress in our lab.

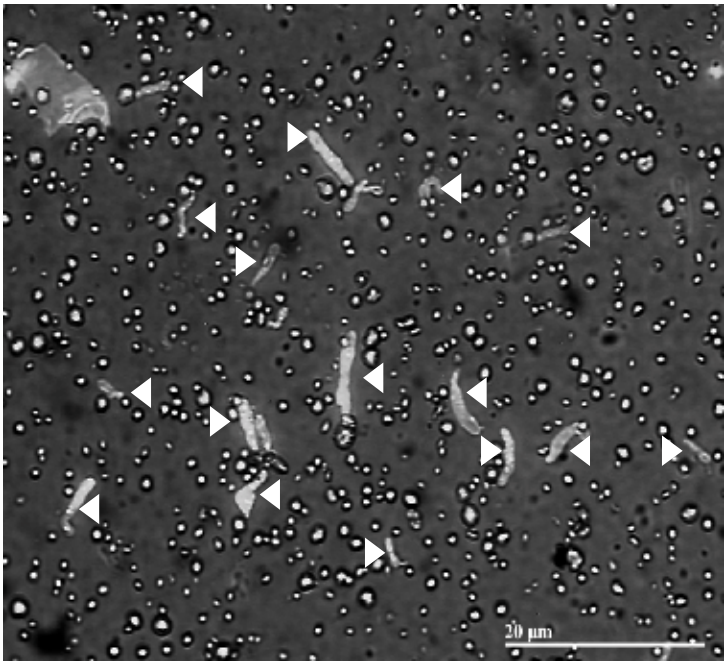

Fig. 7. Fraction enriched with haustoria of *P. pachyrhizi*
Haustoria were isolated form soybean leaves heavily infected with the Asian soybean rust pathogen *P. pachyrhizi* using Con A-affinity chromatography. Haustoria are labeled with white triangles. For details of purification please see text and Fig. 6.

4.3 Discussion

Undoubtedly, there is a constantly growing need for staple crops worldwide. Breeding for varieties with higher yield has contributed to solve this problem (Evenson and Gollin, 2003). Another promising perspective to meet this challenging task is the improved protection of plants against yield losses due to pests and diseases. Although considerable progress had been made towards this goal, the emergence of pathogens with novel virulence spectra or the invasion by previously inconspicuous pathogen species into new habitats bear novel problems which undermine these successes. A recent example for this scenario is the occurrence of a novel pathogen species causing Asian soybean rust (*Phakopsora pachyrhizi*, Asian-Australian type) across the American continent which is more aggressive than the endogenous Latin-American species *Phakopsora meibomiae*.

In an effort to identify novel sources for resistance against this pathogen we switched over to the plant model species *Arabidopsis thaliana* which shows complete resistance against the Asian soybean rust (Loehrer et al., 2008). This type of resistance is referred to as nonhost resistance and Arabidopsis is specifically suited to study this phenomenon because a whole range of different mutants is available which differ in their nonhost response to various pathogens (Thordal-Christensen, 2003). We investigated for the first time the interaction between Arabidopsis and *P. pachyrhizi* in our lab and we started, therefore, with a detailed cytological analysis of the fungal infection process on wild-type Arabidopsis plants. Doing so, we observed that the fungus was not only able to penetrate epidermal cell walls but also grew further to the border of the mesopyll tissue where the infection process came to an end

(Loehrer et al., 2008). Penetration of epidermal cells of nonhost plants was a known phenomenon for *P. pachyrhizi* (Hoppe and Koch, 1989). We further verified this observation using barley plants and, most importantly, we could show that the inhibition of cell death in epidermal tissue of barley limits penetration success of the pathogen (Hoefle et al., 2009). It must be concluded, therefore, that *P. pachyrhizi* did not attack plants like a blind battering ram but rather utilizing subtle methods to cause cell death which might contribute to successful infection by avoiding active defense reactions.

Looking at Arabidopsis mutant plants with impaired penetration resistance, we observed that after the breakdown of this first line of defense *P. pachyrhizi* was able to grow intercellularly into the mesophyll tissue and build haustorium mother cells (Loehrer et al., 2008). However, the pathogen was not able to invade mesophyll cells and establish haustorium-like structures until diminishing of a second line of defense in Arabidopsis *pen2-1pad4-1* double mutants. Conversely, it could be concluded that PEN2 and PAD4 proteins are important for keeping *P. pachyrhizi* in check, at least in Arabidopsis. Further experiments will reveal whether the same is true for homologous proteins in soybean. Our investigations with Arabidopsis and *P. pachyrhizi* led to a novel, rather unexpected, theory, namely that the pathogen 'hides' its bitrophic nature to force the ostensible host to invest in an inappropriate defense scenario. It appears as if *P. pachyrhizi* comes along as a 'wolf in sheep's clothing'. This hypothesis came from gene expression studies which showed that the biotrophic pathogen *P. pachyrhizi* triggers transcription of genes typically activated in Arabidopsis in response to necrotrophic pathogens (Loehrer et al., 2008). This activation, in turn, is associated with a shut-down in transcription of genes normally involved in defense against biotrophic pathogens due to a negative cross-talk of related signaling molecules (Spoel et al., 2003). Taken together, our experiments with Arabidopsis and *P. pachyrhizi* reveal the cell survival machinery and the artificial induction of genes associated with defense against biotropic fungi as potential targets to engineer durable resistance against this pathogen in soybean.

Having a closer look at the pathogen, the sequencing of *P. pachyrhizi's* haustorial transcriptom had provided us with a lot of necessary information to identify crucial steps for its pathogenicity. However a major drawback to further progresses is the current lack of whole genome information for *P. pachyrhizi* in public databases. This makes functional testing of candidate genes, despite the general problem with the set-up of transformation systems for biotrophic fungal plant pathogen, a very challenging task.

5. Conclusions and outlook

Our investigations have brought novel insights into our understanding of the interaction between soybean plants and the causal agent of Asian soybean rust, *P. pachyrhizi*. Using novel approaches, we have identified so far unnoticed plant traits which seemed to be crucial to resist the pathogen. The next critical and challenging task will be the implementation and incorporation of these features into elite soybean cultivars. It must be mentioned, however, that plant pathogen interactions are dynamic systems where modifications in one partner selects for a compensating reaction in the other partner, a scenario best documented in the evolutionary arms race between pathogen virulence factors and plant resistance genes. Therefore, we must be aware that the introduction of novel resistance traits into soybean using classical breeding programs or genetically modified plants might lead to counteraction by *P. pachyrhizi* to overcome these novel barriers. Thus, it

is also indispensable for a sustainable plant protection strategy to increase our knowledge on the requirements of *P. pachyrhizi* to act as a successful pathogen. The in depth understanding of its pathogenesis might help in anticipating potential future activities of the pathogen in response to the introduction of soybean varieties with novel traits. In sum, it is obvious that Asian soybean rust can only be conquered using a holistic approach including researchinto both the plant and the pathogen.

6. Acknowledgements

In this section some results are presented that had been obtained in collaboration with the University of Konstanz. Hence, we greatly acknowledge help of Prof. Kurt Mendgen with scanning electron microscopy and Prof. Ralf Vögele for his help with the isolation of haustoria from rust fungi. Work at the Department of Plant Physiology is supported by BASF Plant Science Company GmbH. ML is supported by a grant of RWTH Aachen University. Prof. Alan J. Slusarenko is greatly acknowledged for his very helpful comments on the manuscript.

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7

Soybean Oil and Meal as Substrates for Lipase Production by *Botryosphaeria ribis*, and Soybean Oil to Enhance the Production of Botryosphaeran by *Botryosphaeria rhodina*

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1. Introduction

Soybean oil, a common vegetable (plant seed) oil, has traditionally been used as a food in cooking and salad dressings, and more recently, as a feedstock for the production of biodiesel fuels (Bajaj et al., 2010; Ghaly et al., 2010). After extracting the oil from soybean seeds, the residue constitutes an important by-product called soybean meal, or oil-seed cake. This edible by-product, a rich source of proteins and amino acids, especially tryptophan, threonine and lysine (Ramachandran et al., 2007), has been used as a livestock feed, as well as a nutrient source and fermentable substrate for producing microbial lipases (Ul-Haq et al., 2002), and other enzymes (Singhania et al., 2009).

The fatty acid composition of soybean oil is typically: 53.8 % linoleic ($C_{18:2}$), 20.8 % oleic ($C_{18:1}$), 11.4 % palmitic ($C_{16:1}$), 9.3 % linolenic ($C_{18:3}$), 4.4 % stearic ($C_{18:0}$), and 0.3 % arachidic acids ($C_{20:0}$) (Ghaly et al., 2010). Besides the applications cited above, soybean oil was demonstrated an effective inducer for the production of fungal enzymes (lipases) that degrade plant seed oils (Messias et al., 2009). It has also been reported to enhance the synthesis of pleuromutilin by *Pleurotus mutilis*, an antibiotic effective against gram-positive bacterial pathogens (Hu et al., 2009), and promoted the production of laccases by *Botryosphaeria rhodina* MAMB-05 when added to nutrient medium (Dekker et al., 2007).

Lipases (EC 3.1.1.3; triacylglycerol acylhydrolases) are hydrolytic enzymes, which catalyse the hydrolysis of the ester linkages of long-chain acylglycerols to glycerol and free fatty acids. These enzymes also conduct interesterification, transesterification and ammonolysis reactions. Lipases are ubiquitous among microorganisms being produced by bacteria, actinomycetes, filamentous fungi and yeasts, and have found applications in various sectors of commerce (Li & Zong, 2010). The main industrial application of lipase is still restricted to their use in laundry detergents to remove fats and oil stains (Hasan et al., 2010). They are also used in various food and agro-chemical industries, e.g., processing foods, treatment of fatty effluents, synthesis of biosurfactants, removal of resins (pitch) in processing paper from wood cellulose pulps, and as

biocatalysts in biotransformation reactions in the semi-synthesis of drugs (Jaeger & Eggert, 2002; Gotor-Fernández et al., 2006). More recently, their applications have been extended to catalysis of transesterification of plant seed oils (triacylglycerols) for biodiesel production (Hasan et al., 2006; Bajaj et al., 2010; Ghaly et al., 2010).
Several genera of fungi, and mainly *Aspergillus, Colletotrichum, Penicillium*, and *Rhizopus*, have been studied for lipase production (Treichel et al., 2010), and more recently, the genus *Botryosphaeria* was described as a good producer of lipases by our research group. Among the nine isolates of *Botryosphaeria* studied, a strain of *Botryosphaeria ribis* EC-01 was found to produce high lipase titres (Messias et al., 2009).
The genus *Botryosphaeria* has been studied by our research group since 1995 (Barbosa et al., 1995) when an isolate of *Botryosphaeria* sp. MAMB-05 was found to be ligninolytic producing a polyphenol oxidase (laccase), and concomitantly secreted an exopolysaccharide (EPS) into the culture fluid during growth. The EPS was described as a β-glucan (Dekker & Barbosa, 2001), and characterized at the structure level as a β-(1→3),(1→6)-D-glucan, and named botryosphaeran (Barbosa et al., 2003). The production of botryosphaeran could be increased by the combined addition of soybean oil and the surfactant, Tween 80, to the nutrient media during submerged liquid fermentation (Silva et al., 2007).
In this chapter, we report on the comparison of different vegetable oils, including soybean oil, as substrates to produce lipases by *Botryosphaeria ribis* EC-01 by submerged fermentation, as well as the influence of nitrogen and phosphate sources on lipase production. A comparison of soybean meal with other oil-seed cakes (castor bean and corn kernel) as fermentative substrates for lipase production by *Botryosphaeria ribis* EC-01, as well as the effect of adding mineral nutrients to soybean meal to enhance lipase production by this fungal strain, is also discussed. The effect of adding soybean oil and Tween-80 to nutrient media to increase botryosphaeran production by *Botryosphaeria rhodina* MAMB-05 is also presented.

2. Experimental procedures

2.1 Materials

All plant seed oils (canola, coconut, corn, olive, soybean, and sunflower) were of food grade. Castor bean oil and meal were purchased from Remy Comércio e Beneficiamento de Mamona (Londrina-PR, Brazil); and hempseed oils (raw and refined) from Prairie Emerald (Hemp Oil, Canada). Corn steep liquor (Milhocina®) and corn meal were kindly donated by CornProducts Brazil (Mogi Guaçu-SP, Brazil), and soybean meal by IMCOPA (Cambé-PR, Brazil).

2.2 Methods

2.2.1 Microorganisms

Botryosphaeria rhodina MAMB-05 (Barbosa et al., 1995; 1996) and *Botryosphaeria ribis* EC-01 (Silveira et al., 1996) were isolated from the stem of eucalypt trees.

2.2.2 Preparation of inoculum and growth conditions

Fungal isolates were maintained on potato-dextrose agar (PDA) slants at 4 °C, and sub-cultured at three-monthly intervals. From PDA the fungi were transferred to agar plates containing glucose (10 g/L), minimum salts medium (VMSM; Vogel, 1956), and agar (20 g/L), and left at 28 °C for 5 d. Following growth, four plugs (0.7 cm-diameter) were taken from the mycelial-colonized agar with the aid of a sterile cork borer and used to inoculate Erlenmeyer flasks containing nutrient medium.

2.2.3 Enzyme production by submerged fermentation

Fungal cultures were developed in submerged fermentation on nutrient medium (VMSM) containing plant seed oils (1 %, v/v) as described by Messias et al., (2009) for lipase production. To evaluate the effects of nitrogen (N) and phosphate (P) on lipase production, *Botryosphaeria ribis* EC-01 was cultured on VMSM containing soybean oil (1 %, v/v) as the carbon source, and each of the different N and P sources (see below) for 5 days at 28 °C. N (i.e., NH_4NO_3, 0.2 % (w/v) and P (i.e., KH_2PO_4, 0.5% (w/v) in the VMSM medium were replaced separately by each of the inorganic salts: $NaNO_3$ (0.4 % (w/v), NH_4Cl (0.4 % (w/v), $(NH_4)_2SO_4$, (0.2 % (w/v), and Na_2HPO_4, NaH_2PO_4, K_2HPO_4, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$; each at 0.5 % (w/v). Organic N sources included: urea, peptone, yeast extract and corn steep liquor (each of 0.2 %, w/v, concentration). In experiments evaluating the effect of P source on lipase production when *Botryosphaeria ribis* EC-01 was cultured on soybean oil (1 %, v/v), three phosphates (KH_2PO_4, K_2HPO_4, Na_2HPO_4 each at 0.5 %, w/v) were added to VMSM, but the initial pH of the medium was not adjusted. In a separate experiment, the initial pH value of the nutrient media was adjusted from 3.5 to 9.5 with 1 M HCl or 1 M NaOH, and lipase production evaluated. In experiments examining the effect of nutrient supplementation of oil-seed meals on lipase production, water or VMSM were added to the oil-seed meals (castor bean, corn kernel and soybean at 1 %, w/v, final concentrations), and *Botryosphaeria ribis* EC-01 grown in submerged fermentation for 5 days at 28 °C. In separate experiments, the effect of soybean meal concentration was evaluated using final concentrations of 0.5, 1.0, 2.0, 4.0 and 6.0 % (w/v) made up in only distilled water. All experiments were conducted in replicates of three, and the results represent the means ± SD.

2.2.4 Enzyme assays

The extracellular fluids (ECF) arising from submerged fermentation were used as the source of lipases, and were obtained after removal of the fungal mycelium by centrifugation at 1,509 x *g* at room temperature for 15 min.

Lipase activity was assayed against *p*-nitrophenyl palmitate (*p*NPP, Sigma) as substrate according to Winkler & Stuckmann (1979). The reaction was carried out in 50 mM sodium phosphate buffer (pH 8.0) at 55 °C (Messias et al., 2009). Absorbance was measured spectrophotometrically (410 nm, molar extinction coefficient of *p*-nitrophenol (*p*NP) was 15,000 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the release of 1 μmol of *p*NP per min under the assay conditions.

2.2.5 Harvesting and determination of mycelium biomass

Fungal cultures grown in submerged fermentation were harvested and mycelium removed by centrifugation (2,240 x *g*/15 min at 4 °C). The supernatant recovered was then filtered through glasswool, and collected for analysis. The fungal biomass (mycelium) was washed once with distilled water and measured gravimetrically after drying (70 °C) to constant weight in an oven. In experiments where fungal isolates were cultivated on soybean meal, fungal biomass was not quantified.

2.2.6 Analytical determinations

Extracellular protein was measured by a modified Lowry's method (1951) as described by Hartree (1972) when the fungus was grown on soybean oil, and by the Bradford method

(Bradford, 1976) where soybean meals were the fermentable substrate. Bovine serum albumin was used as the standard. Total sugars were determined by the phenol-sulfuric acid method (DuBois et al., 1956), and reducing sugars by the method of Nelson (1944) and Somogyi (1945); glucose was used as standard for both methods.

2.2.7 Determination of botryosphaeran and fungal biomass

Botryosphaeran production by *Botryosphaeria rhodina* MAMB-05 and its isolation from the ECF through precipitation with ethanol was developed as described by Steluti et al., (2004), and Silva et al., (2007).

3. Soybean oil as carbon source for lipase production by submerged fermentation

The endophytic, ascomyceteous fungus, *Botryosphaeria ribis* EC-01, is a constitutive and inductive lipase producer. A constitutive producer of lipase, because this enzyme was always expressed independent of the carbon source used in the culture medium including glucose (normally considered a catabolite repressor); and inductive, because lipase titres (U/mL) could be enhanced when carbon sources such as fatty acids (e.g., oleic acid), vegetable oils (soybean oil), surfactants (Tween-80), and emulsified lipids (stearic acid plus Triton X-100) were incorporated in nutrient medium (Messias et al., 2009). Several complex substrates have been reported to enhance lipase synthesis. Examples include wheat bran, rice bran, sugarcane bagasse, and oil-seed cakes derived from coconut, olive, sesame (Ul-Haq et al., 2002). However, vegetable oils, free fatty acids, hydrolysable esters, Tween surfactants, bile salts and glycerol appear to be essential supplements to enhance lipase yields (Gupta et al., 2004; Treichel et al., 2010).

Several vegetable oils were used as sole carbon source and compared to induce lipase production by *Botryosphaeria ribis* EC-01.

3.1 Effect of different vegetable oils as sole carbon source for the production of lipases by *Botryosphaeria ribis* EC-01

Among the vegetable oils tested as sole carbon source for *Botryosphaeria ribis* EC-01, raw hempseed oil and sunflower oil produced highest lipase titres, followed by canola oil, soybean oil, and olive oil (Table 1). Lowest activity was observed for coconut oil. In terms of specific lipase activity (U/mg), refined hempseed oil induced highest activities, followed by coconut oil and soybean oil, while olive oil was least. These results indicate that the composition of vegetable seed oils (nature of the fatty acids constituting the acylglycerols) affected *Botryosphaeria ribis* EC-01 metabolism (Table 1). Pogori et al., (2008) investigated lipase production by *Rhizopus chinensis* CCTCC M201021 and showed that soybean oil enhanced lipase production and was highest among other oils studied. Comparatively, *Botryosphaeria ribis* EC-01 was previously shown to produce highest lipase titres on soybean oil and glycerol, while eight isolates of *Botryosphaeria rhodina* produced significantly lower enzyme titres (Messias, 2008). Despite the source of the plant seed oils used as carbon source and the lipase yields produced by *Botryosphaeria ribis* EC-01, the fungal biomass observed did not have high variation (Table 1).

Plant seed oil	Lipase activity (U/mL)	Specific activity (U/mg*)	Fungal biomass (g/L)
Soybean	23.0 ± 0.82	138.4 ± 2.47	14.8 ± 0.42
Sunflower	27.7 ± 1.16	112.8 ± 1.79	12.0 ± 0.36
Olive	22.7 ± 2.41	40.1 ± 0.63	11.9 ± 0.20
Hempseed (refined)**	20.1 ± 1.35	227.6 ± 2.75	15.5 ± 0.24
Hempseed (raw)	28.0 ± 0.79	97.2 ± 3.52	14.8 ± 0.27
Canola	26.8 ± 2.45	44.7 ± 3.61	12.6 ± 0.58
Coconut	10.0 ± 0.50	170.0 ± 0.28	14.1 ± 0.35

* mg protein; ** degummed oil

Table 1. Comparison of lipase production by *Botryosphaeria ribis* EC-01 grown on different vegetable oils as sole carbon source for 5 days by submerged fermentation.

3.2 Effect of nitrogen source on lipase production by *Botryosphaeria ribis* EC-01 on soybean oil as sole carbon source

Nitrogen constitutes essential micronutrients for microbial growth, and can play an important role in enzyme production and their optimization. These nutritional requirements are present in defined nutrient media (synthetic medium), as well as complex components such as peptones, yeast extract, malt extract, and also agro-industrial residues containing all the components necessary for microorganism development (Treichel et al., 2010).

Both organic (urea, corn steep liquor, yeast extract, peptone) and inorganic (NH_4NO_3 or $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$) N sources were evaluated for the production of lipase by *Botryosphaeria ribis* EC-01. In all grown cultures, soybean oil and KH_2PO_4 were used as the carbon and P sources, respectively. The highest specific activities (32.2 U/mL and 89.3 U/mg) were observed in cultures containing NH_4NO_3 (Table 2). Microorganisms generally produce higher lipase levels on organic N sources (Sharma et al., 2001). *Aspergillus* sp., for instance, produced twice as much lipase in culture medium containing peptone than in the presence of NH_4NO_3 (Cihangir & Sarikaya, 2004), while the production of intracellular lipases by *Rhizopus oryzae* was higher on corn steep liquor (Essamri et al., 1998). For the genus *Botryosphaeria*, inorganic N sources, such as $(NH_4)_2SO_4$ and NH_4NO_3 were reported as having the best effect on production of laccases by *Botryosphaeria rhodina* MAMB-05 (Dekker et al., 2007), whereas for lipases, the highest specific activity produced by *Botryosphaeria ribis* EC-01 was observed for media containing NH_4NO_3 (Table 2).

The final pH values of the culture medium after 5 days growth ranged from 4.7 to 8.8, except that for NH_4Cl, where the final pH was 1.78. NH_4^+ is the most readily assimilated cation amongst inorganic N sources, as the N atom is at the same oxidation level (-3) as the N atom in biological molecules (amino acids, purines and pyrimidines). Considering that NH_4^+ is a weak acid, NH_4^+ can dissociate in fermentation medium to NH_3 and H^+. Ammonia enters cells by means of passive diffusion, whereas nitrate requires nitrate and nitrite reductase enzymes (both of which are NADPH-dependent) to be converted to NH_3. This may be a likely reason for the low final pH values observed with NH_4Cl, i.e., NH_3 was probably rapidly removed by the fungus from the culture medium leaving only H^+ and Cl^- ions, hence the acidity (Miranda et al., 1999; Galvagno & Forchiassin, 2004).

Nitrogen Source	Lipase activity (U/mL)	Specific activity (U/mg)	Final pH of culture medium	Fungal biomass (g/L)
None	1.29 ± 0.05	3.59 ± 0.46	6.48 ± 0.04	8.67 ± 1.09
Urea	18.26 ± 0.87	12.75 ± 0.71	8.80 ± 0.07	7.84 ± 0.28
Corn Steep Liquor	8.93 ± 0.35	25.51 ± 2.27	6.41 ± 0.03	10.37 ± 0.36
Yeast extract	1.89 ± 0.45	5.71 ± 1.40	6.88 ± 0.10	11.51 ± 0.66
Peptone	15.41 ± 2.20	32.46 ± 5.90	7.36 ± 0.02	13.08 ± 0.85
$(NH_4)_2SO_4$	7.00 ± 1.69	27.46 ± 4.37	4.73 ± 0.30	10.00 ± 0.85
NH_4Cl	25.24 ± 4.51	37.40 ± 5.71	1.78 ± 0.13	11.19 ± 0.50
NH_4NO_3 *	32.19 ± 1.97	89.34 ± 2.56	6.87 ± 0.12	12.27 ± 0.14
$NaNO_3$	4.52 ± 0.73	15.69 ± 2.99	7.17 ± 0.05	12.19 ± 0.27

* N source normally used in minimum salts medium (VMSM)

Table 2. Effect of nitrogen sources on lipase production by *Botryosphaeria ribis* EC-01 grown on soybean oil for 5 days by submerged fermentation.

Low lipase yields were observed in the presence of yeast extract, as well as the inorganic salt, $NaNO_3$. Despite the low lipase yields, *Botryosphaeria ribis* EC-01 growth was generally higher when the nutrient media was supplemented with N rather than without added N (Table 2).

After 5 days growth on nutrient medium containing soybean oil and supplemented with corn steep liquor, yeast extract or peptone as N sources, the total sugars (represented by polysaccharides and glyco-conjugates in these N sources) consumed by the fungus were low (2, 18 and 32 %, respectively). The sugar content (measured as reducing sugars), however, presented by these N sources increased 10-fold at the end of fermentation. The results indicated that *Botryosphaeria ribis* EC-01 preferentially used soybean oil as the primary carbon source for growth and lipase production, as evidenced by the sugar content remaining at the end of fermentation.

3.3 Effect of phosphate source and initial pH on the production of lipase by *Botryosphaeria ribis* EC-01 using soybean oil as sole carbon source

K_2HPO_4, KH_2PO_4 or Na_2HPO_4 are typical phosphates used in nutrient media for lipase production by fungi (Macris et al., 1996; Gulati et al., 1999; Fadiloglu & Erkmen, 1999; Lima et al., 2003; Lin & Ko, 2005; Wang et al., 2008). Several P sources were compared for lipase production by *Botryosphaeria ribis* EC-01, and are shown in Table 3. The highest specific activities of lipase produced were observed in media containing K_2HPO_4. Fungal biomass was highest on KH_2PO_4, followed by K_2HPO_4.

The final pH values in fungal cultures grown on media containing Na_2HPO_4 and K_2HPO_4 were alkaline (8.0 and 8.2, respectively), and acidic (pH 3.8) for $NH_4H_2PO_4$, probably due to the rapid uptake of NH_4^+ by the fungus as explained above.

A comparison of the effect of three different P sources (K_2HPO_4, KH_2PO_4, Na_2HPO_4) on lipase production by *Botryosphaeria ribis* EC-01 grown on soybean oil and NH_4NO_3 as N source is presented in Figure 1. The profiles of the specific lipase activities differed for each of the P sources evaluated, with highest activity (285 U/mg) being produced on K_2HPO_4

Sources of phosphate	**Lipase activity** (U/mL)	**Specific activity** (U/mg)	**Final pH of culture medium**	**Fungal biomass** (g/L)
Na_2HPO_4	40.83 ± 1.95	104.23 ± 3.20	8.05 ± 0.06	9.40 ± 1.00
NaH_2PO_4	29.07 ± 1.10	94.50 ± 9.19	6.45 ± 0.06	7.25 ± 0.30
KH_2PO_4 *	32.19 ± 1.98	89.34 ± 2.56	6.87 ± 0.12	12.27 ± 0.14
K_2HPO_4	38.23 ± 3.71	201.91 ± 2.51	8.15 ± 0.09	11.76 ± 0.41
$NH_4H_2PO_4$	39.93 ± 6.47	68.50 ± 4.62	3.81 ± 0.57	10.92 ± 0.33
$(NH_4)_2HPO_4$	10.43 ± 2.09	30.89 ± 5.80	5.48 ± 0.18	10.40 ± 0.25

* P source normally used in the minimum salts medium (VMSM)

Table 3. Effect of phosphate sources on the growth and production of lipases by *Botryosphaeria ribis* EC-01 on soybean oil for 5 days by submerged fermentation.

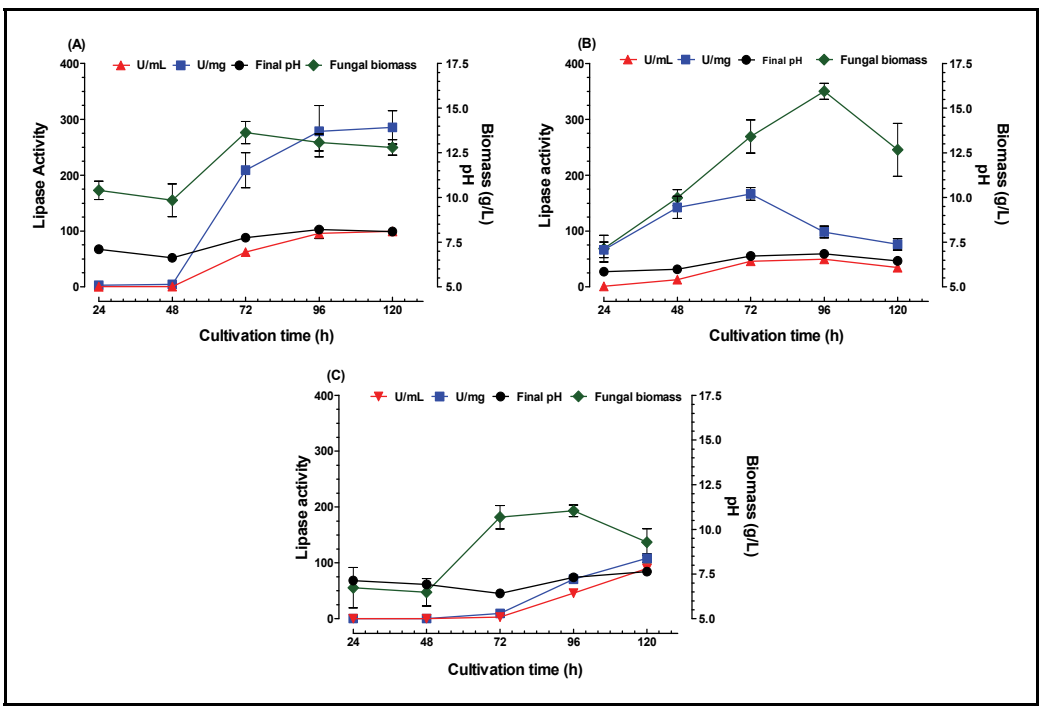

Fig. 1. Growth profiles comparing lipase production by *Botryosphaeria ribis* EC-01 on soybean oil in media containing NH_4NO_3 as N source, and three different phosphate sources: (**A**) K_2HPO_4, (**B**) KH_2PO_4 and (**C**) Na_2HPO_4 in submerged fermentation. The initial pH of the culture medium was not adjusted.

and the least on Na_2HPO_4 (108 U/mg). In the case of the di-cation phosphate salts, lipase production occurred during late log phase of growth (after 72 h), whereas for KH_2PO_4, lipase commenced after 24 h and decreased after 72 h.

According to Jaeger and coworkers (1994), the initial pH of the nutrient medium may be related to an increase of lipase production, and values ranging from 7.0 to 8.0 had a positive

effect in lipase synthesis. The same observation was described for a strain of *Aspergillus oryzae* which produced highest lipase titres at initial pH 6.0, whereas the peak of lipase production was observed when the final pH reached 8.0 (Ohnishi et al., 1994).
The influence of initial pH within the range 3.5 to 9.5 on lipase production by *Botryosphaeria ribis* EC-01 grown on soybean oil in media containing the 3 different P sources (KH_2PO_4, K_2HPO_4 and Na_2HPO_4) is presented in Figure 2.

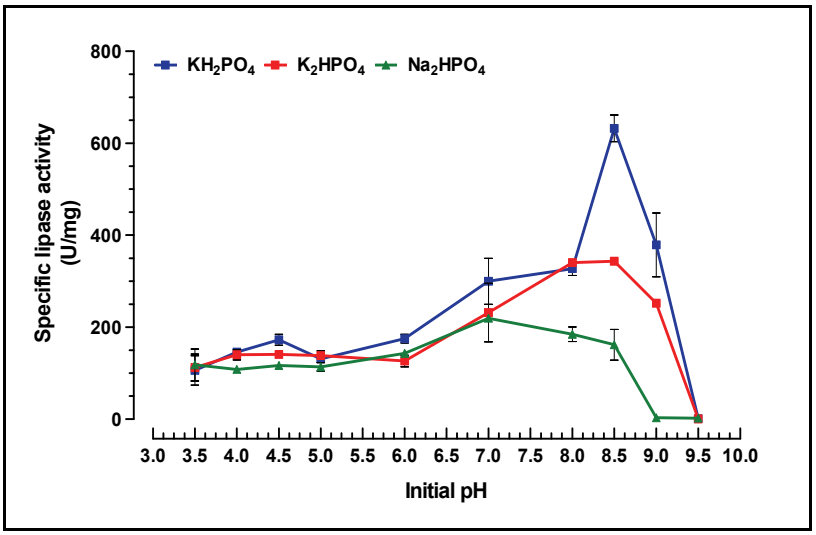

Fig. 2. Influence of initial pH on the production of lipases by *Botryosphaeria ribis* EC-01 cultured for 5 days on soybean oil, and media containing NH_4NO_3 as N source and either KH_2PO_4, K_2HPO_4 or Na_2HPO_4 as phosphate sources.

Highest specific lipase activity (632.6 U/mg) was achieved on KH_2PO_4 and an initial pH of 8.5. The enzyme profile for K_2HPO_4 was similar, but the specific activity was lower (350 U/mg), while in the case of Na_2HPO_4, the best initial pH was 7.0 producing a specific activity of 200 U/mg. It is clear from these results that not only the initial pH value of the nutrient media affected lipase activity, but also the type of P source was important in enhancing lipase specific activities. Similar observations of the effect of initial pH on lipase production have been reported for *Fusarium globulosum* (Gulati et al., 2005), and *Aspergillus terreus* (Gulati et al., 1999) when grown on maize oil, and reported the optimal initial pH being 7.0 and 9.0, respectively. In another example, *Cryptococcus* sp. S-2, produced highest lipase activity when grown on triolein and yeast extract at an initial pH of 5.6 (Kamini et al., 2000). From these observations, it is concluded that initial pH has a significant effect on the production of fungal lipase. The composition of the culture medium such as carbon, N and P sources, inoculum size, as well as culture conditions (shaking rate, temperature, air/medium ratio) will also have interaction effects on microbial lipase production (Lin et al., 2006). Response surface methodology was employed to optimize medium nutrients to produce lipase by *Geotrichum* sp, in order to study the effect of carbon sources (soybean oil, olive oil and glucose) and concentrations of N sources (corn steep liquor and NH_4NO_3). The optimized condition was obtained using NH_4NO_3 (2.1 - 2.5 %), corn steep liquor (13 - 15 %) and soybean oil (0.6 %), and resulted in lipase titres of 20 U/mL (Burket et al., 2004).

4. Soybean meal as substrate for lipase production by submerged fermentation

As is common with most commercial enzymes, lipase is associated with high production costs, generally because the enzymes are produced by submerged fermentation (Castilho et al., 2000). *Botryosphaeria ribis* EC-01 is able to produce lipases both in submerged and solid-state fermentations (Messias et al., 2009; Costa et al., 2009).

Oil-seed cakes are rich in protein and are recognized as being good food supplements, and some have been used for feed applications in poultry, fish and pig production. They also add value to various biotechnological processes such as the production of enzymes, antibiotics and mushrooms by fermentation (Ramachandran et al., 2007). Oil-seed cakes, being rich in protein, can serve as a source of nitrogen for enzyme production including lipases.

Three oil-seed meals (soybean, castor bean, corn kernel) were compared as substrates for their effect on lipase production by *Botryosphaeria ribis* EC-01 when cultivated at a concentration of 1 % (w/v) in the absence (distilled water only) and presence of minimum salts medium (VMSM). The results are presented in Table 4 and showed that the addition of VMSM to the nutrient medium containing soybean and castor meals strongly decreased lipase production by *Botryosphaeria ribis* EC-01. However, the addition of VMSM increased lipase production when corn kernel meal was the substrate. These results indicate that soybean and castor bean meals contain sufficient nutrients to support the growth of *Botryosphaeria ribis* EC-01 and subsequent production of lipases. In this case, the use of these meals could lower the costs of lipase production.

The results presented in Table 5 shows that soybean and castor bean meals have higher protein concentrations than corn kernel meal, which may also explain the capacity by *Botryosphaeria ribis* EC-01 to produce higher lipase activity.

Minimal salts medium (VMSM)	**Oil-seed meal**	**Lipase activity**		
		(U/mL)	(U/mg*)	(U/g ds**)
None ***	Soybean	13.4 ± 0.63	142.5 ± 5.31	47.5 ± 2.26
	Castor bean	12.3 ± 0.38	88.0 ± 1.30	43.8 ± 1.37
	Corn kernel	0.8 ± 0.20	17.3 ± 3.26	3.0 ± 0.75
Presence	Soybean	0.1 ± 0.03	1.7 ± 0.66	1.1 ± 0.67
	Castor bean	0.8 ± 0.07	7.2 ± 0.41	2.8 ± 0.23
	Corn kernel	4.5 ± 0.09	28.9 ± 0.49	16.7 ± 0.33

* mg protein; ** dry substrate; *** replaced by distilled water

Table 4. Comparison of lipase production by *Botryosphaeria ribis* EC-01 grown on three different oil-seed meals in the absence and presence of minimal salts medium for 5 days by submerged fermentation.

Different soybean meal concentrations (from 0.5 to 6 %, w/v) were evaluated on lipase production by *Botryosphaeria ribis* EC-01 in submerged fermentation in order to enhance enzyme activities. The results are presented in Table 6, which shows a comparison of lipase production [titres (U/mL), specific activity (U/mg of protein), and yield (U/g of dry meal substrate, ds)].

Oil-seed meal	Carbohydrate	Lipid	Protein	Ash	Moisture
Soybean	30.7	0.7	49.4	6.2	13.0
Castor bean	47.2	1.4	31.8	7.1	12.5
Corn kernel	66.9	1.2	23.1	1.8	7.1

Table 5. The chemical composition of oil-seed meals (g/100 g; Costa, 2008).

One percent soybean meal was the concentration that promoted highest lipase activity when expressed as specific activity (182.5 U/mg) and enzyme yield (67.6 U/ds). Considering that the oil-seed meals are rich in proteins, highest lipase titres (79.4 U/mL) were obtained on 6 % soybean meal (Table 6), and do not really correspond as an expression of high lipase activity compared to the specific activity (182.5 U/mg). Hence, when using oil-seed meals as fermentable substrates for lipase production, one should consider all 3 measures of enzyme activity (U/mL, U/mg, and U/g ds).

Tan et al., (2004) compared soybean bean meal, defatted soybean meal and soybean protein (all at 4 %) as substrates for lipase production by *Penicillium camembertii* Thom PG-3. The lipase activities obtained were 75.2, 128.4 and 78.8 U/mL, respectively. Also present in the nutrient medium were cyclodextrin (0.5 %), olive oil (0.75 %), K_2HPO_4 (0.5 %) and $(NH_4)_2SO_4$ (0.1 %), and these supplements would surely affect lipase production.

Soybean meal (%, w/v)	Lipase activity		
	(U/mL)	(U/mg*)	(U/g ds**)
0.5	5.6 ± 0.50	95.1 ± 7.73	39.7 ± 2.73
1.0	19.0 ± 1.37	182.5 ± 2.64	67.6 ± 5.14
2.0	34.0 ± 0.03	129.7 ± 0.11	59.1 ± 0.29
4.0	35.9 ± 0.92	38.5 ± 0.90	32.3 ± 2.94
6.0	79.4 ± 6.56	76.3 ± 6.58	46.0 ± 3.82

* mg protein; ** dried substrate

Table 6. Comparison of lipase production by *Botryosphaeria ribis* EC-01 grown on increasing concentrations of soybean meal under submerged fermentation for 5 days.

Some comparisons on lipase production by submerged and solid-state fermentations have been discussed in terms of U/mL and U/g ds for several microorganisms cultivated on different substrates, and no conclusion could be achieved in terms of highest enzyme titres because the processes are dependable on the microorganism, the type of substrate fermented, and the conditions used for cultivation (Treichel et al., 2010). *Botryosphaeria ribis* EC-01, for example, produced lower yields of lipase activity when grown by solid-state fermentation (Costa, 2008) than compared to the results obtained herein by submerged fermentation.

Phosphate was also added to the oil-seed meal in order to supplement and enhance lipase production. A comparison of adding KH_2PO_4 and K_2HPO_4 is shown in Figure 3. The addition of KH_2PO_4 significantly enhanced lipase production in terms of specific activity.

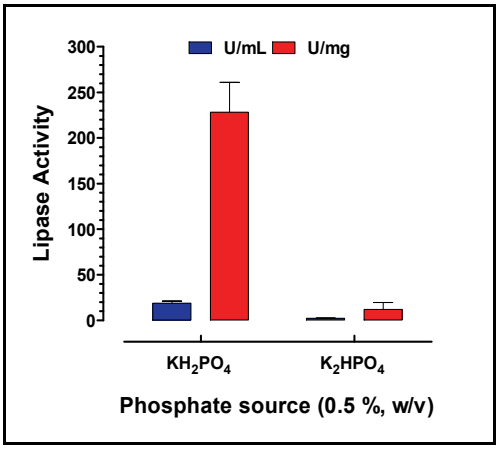

Fig. 3. Comparison of phosphate source on the production of lipases by *Botryosphaeria ribis* EC-01 grown on soybean meal (1% w/v) for 5 days by submerged fermentation.

5. Soybean oil emulsification to enhance the production of the exopolysaccharide botryosphaeran produced by *Botryosphaeria rhodina* MAMB-05

Exopolysaccharides (EPS) are biopolymers secreted extracellularly by several microorganisms including fungi. These carbohydrate macromolecules possess important industrial applications and interesting biological activities. Because of their physical properties, they have been used in foods as emulsifying, stabilizing and thickening agents, as well as in pharmaceutical formulations, and as drug delivery agents (Sutherland, 1998).

Botryosphaeria rhodina isolate MAMB-05 produces an EPS named botryosphaeran, a β-1,3;1,6-D-glucan comprising 22 % ramification. The side branches consist of glucosyl and gentiobiosyl residues linked to the β-1,3-D-glucan backbone chain by β-1,6-bonds (Barbosa et al., 2003). When grown on different carbohydrate substrates, *Botryosphaeria rhodina* MAMB-05 produced a family of botryosphaerans that differed only in the degree of branching (Steluti et al., 2004). *Botryosphaeria rhodina* MAMB-05 grown on fructose presented a higher degree of branching (31 %) compared to that when grown on sucrose and glucose (21-22 %) as carbon sources. In each case, the degree of branching affected the physical properties (viz., rheology) of the botryosphaerans produced (Corradi da Silva et al., 2005). Botryosphaeran exists in solution in a triple helical conformation (Giese et al., 2008); an important property manifesting biological response modifying activity.

Vegetable seed oils, fatty acids and surfactants such as Tween-80 (polyoxyethylene sorbitan mono-oleate) when added to nutrient media, are known to enhance the production of fungal β-glucans (West & Reed-Hamer, 1995). The enhanced production is possibly due to the oils providing an additional energy source to the fungus, thus shunting glucose for synthesis into β-glucan.

As some β-1,3-glucans have important industrial and pharmaceutical applications, and botryosphaeran is no exception, their inclusion as new materials in commercial applications is dependent upon their scale of production. Effective strategies to increase the yields of exopolysaccharides in fermentation processes are therefore important. Vegetable seed oils are readily available commercially and are considered low-cost. Their use in nutrient media

to enhance fungal β-glucan production can therefore be a promising means of increasing their yields for commercial purposes.

Botryosphaeria rhodina MAMB-05 when grown on basal media (glucose (10 g/L) plus VMSM) in which soybean oil (1 %, v/v) was incorporated demonstrated that botryosphaeran production could be enhanced during the course of submerged fermentation (Figure 4).

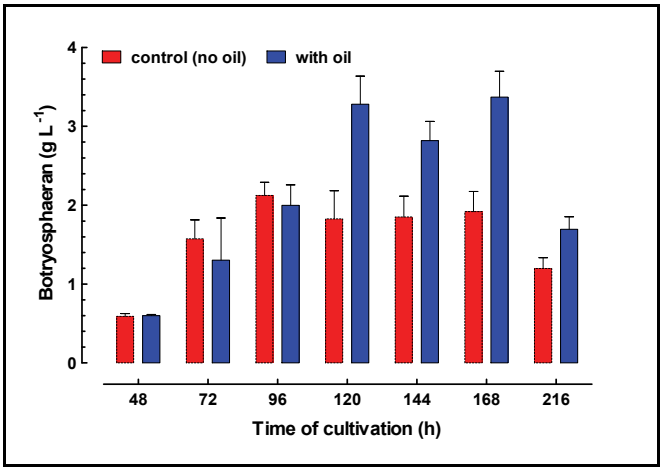

Fig. 4. Profile for the production of botryosphaeran by *Botryosphaeria rhodina* MAMB-05 grown in the presence and absence of soybean oil (Silva, 2007).

The addition of the surfactant Tween 80 to basal medium was also effective in promoting botryosphaeran production, and the combined presence of soybean oil and Tween-80 could further increase botryosphaeran yields (Figure 5). Structural characterization of the derived products revealed no structural abnormalities compared to botryosphaeran produced in the absence of oil and Tween-80 (Silva et al., 2007).

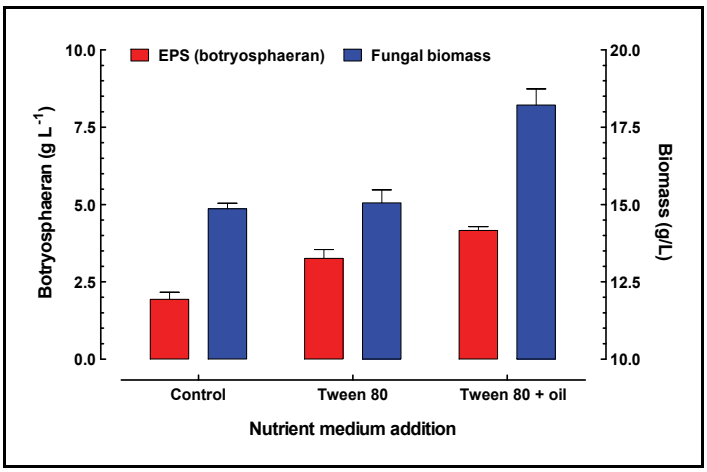

Fig. 5. Comparison of botryosphaeran and biomass production by *Botryosphaeria rhodina* MAMB-05 grown on glucose in the absence (control) and combined presence of Tween-80 and soybean oil, when added to the basal medium (Silva, 2007).

There was no significant difference in botryosphaeran production when using different commercial brands of soybean oil (Figure 6). The presence of *tert*-butylhydroquinone (preservative for unsaturated vegetable oils) in one commercial soybean oil brand did not affect botryosphaeran production by *Botryosphaeria rhodina* MAMB-05.

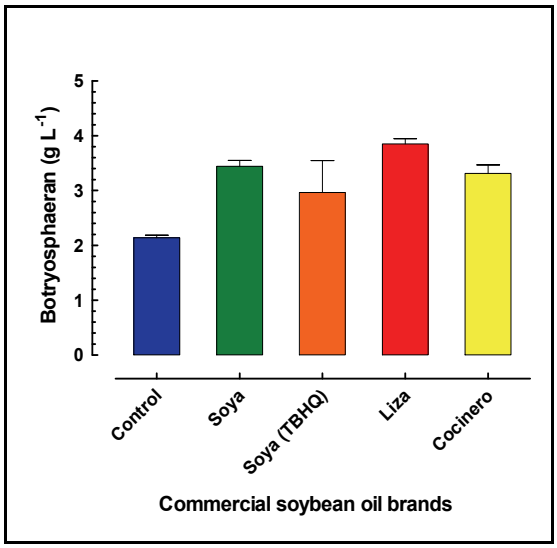

Fig. 6. Influence of different Brazilian commercial soybean oil brands on the production of botryosphaeran by *Botryosphaeria rhodina* MAMB-05 (n = 3; $p < 0{,}001$); TBHQ, *tert*-butylhydroquinone (Silva, 2007).

6. Conclusions

Soybean oil was an effective carbon source for *Botryosphaeria ribis* EC-01 to produce lipases by submerged fermentation, and NH_4NO_3 was the best nitrogen source. The source of phosphate also influenced lipase production, and the initial pH of the nutrient medium had a significant effect on promoting lipase activity.

Soybean meal was also an excellent substrate for lipase production by *Botryosphaeria ribis* EC-01, and did not require supplementation with nutrients to increase lipase activity when grown by submerged fermentation. KH_2PO_4 was an exception, and when added to soybean meal significantly increased the specific lipase activity.

Soybean oil in the presence of Tween 80 enhanced the production of botryosphaeran for *Botryosphaeria rhodina* MAMB-05, and there was no significant difference using various commercial brands of soybean oil.

7. Acknowledgment

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8

Non-denatured Soybean Extracts in Skin Care: Multiple Anti-Aging Effects

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1. Introduction

Non-denatured soybean extracts contain all the ingredients of the soybean in their natural conformations, which is different from soy foods that must be denatured, or from soy isoflavone-based cosmetic products. The multiple benefits of non-denatured soybean extracts for skin care range from skin lightening, extracellular matrix enhancement and delayed hair growth, to a protective effect from photodamage and possibly from skin cancer.

The discovery of the role of the keratinocyte receptor Protease-Activated Receptor-2 (PAR-2) in keratinocyte-melanocyte interactions and pigment transfer resulted in the identification of novel agents that affect skin color. The inhibition of serine proteases was found to reduce PAR-2 activation and therefore to alter skin color. A search for naturally occurring serine protease inhibitors identified non-denatured soybean extracts. These extracts inhibit PAR-2 activation and induce human skin lightening. Non-denatured soybean extracts also enhance collagen and elastin synthesis, and protect existing elastin fibers from degradation, therefore providing anti-aging benefits such as reducing skin sagging and wrinkling. Additionally, non-denatured soybean extracts delay hair growth, resulting in thinner and smaller hair shafts and reduced visibility of undesired hair growth. Topical pretreatment with non-denatured soybean extracts reduces UV-induced edema and erythema, and additionally reduces UVB-induced DNA and cellular damage, which could lessen the risk of skin cancer. Thus non-denatured soybean extracts could serve as inexpensive, safe and efficacious natural agents, which provide multiple skin care benefits, ranging from protection to restoration.

2. Non-denatured soybean extracts

Denaturation, a process induced by external agents or stressors, leads to changes in the tertiary structure of proteins, resulting in the loss of their activity. Conventional soybean extracts used in the cosmetic and food industries are typically denatured. The cosmetic industry uses mainly organic extracts of soybeans, which are enriched with isoflavones (reviewed in Thornfeldt 2005). The organic solvent extraction process removes or inactivates most of the soybean proteins while concentrating the isoflavones. The food industry takes major steps to inactivate the soy proteins, particularly Soybean Trypsin

Inhibitor (STI), which interferes with the process of food digestion. Industry standards include heating under pressure and alkaline soaking, which denature most of the soybean proteins (Liu, 1999). Interestingly, soybeans have been used in Asia for centuries as a dietary source of protein, and they have always been fermented to inactivate STI, thereby preventing the inhibition of trypsin activity which is needed for digestion of dietary proteins.

The non-denatured soybean extracts discussed in this chapter are different from soy foods and from cosmetic soy preparations in two major aspects. First, the non-denatured soybean extracts contain all the ingredients at the same proportions as in the native bean. No soybean constituent is removed or is specifically enriched during the processing of the beans, and all components of the beans remain in a "soy powder" preparation that serves as raw material. Second, the soy powder is kept in its natural, non-denatured state, with all the soybean proteins, and in particular STI, present and active. Non-denatured soybean extracts, therefore, cannot be used for dietary consumption, as they contain active STI (see e.g. Paine et al, 2001; Seiberg et al, 2001).

3. The Soybean proteins STI and BBI are serine protease inhibitors

The proteins STI, a Kunitz-type trypsin inhibitor, and BBI, the Bowman-Birk protease inhibitor (reviewed in (Birk, 1985; Kennedy, 1998), were first isolated from soybeans in the early 1940's. STI (reviewed in Birk, 1985) is a protein of 181 amino acid residues and a tertiary structure which is dependent on two disulfide bridges (reviewed in Song et al., 1998). BBI is an 8 kDa protein with 81 amino acid residues and seven disulfide bonds that is a weak trypsin inhibitor and a strong chymotrypsin inhibitor (Billings et al., 1992). STI is heat labile (Song et al., 1998), while BBI has a stable conformation even after its disulfide bonds are broken by heating or treatment with acid and pepsin (Birk, 1985). STI and BBI are found only in the bean, and not in any other part of the soy plant (Birk, 1985). BBI is known for its chemo-preventive effects in different types of cancer (reviewed in Kennedy, 1998) and a soybean extract enriched in BBI has been under evaluation for chemo-prevention in clinical studies. STI and BBI have not been studied in the past for their topical effect on skin pigmentation, skin aging or skin cancer. This chapter summarizes the biological activities of STI, BBI, and non-denatured soybean extracts (containing STI and BBI), and describes molecular and cellular mechanisms affected by these agents, which lead to superior skin care and "anti-aging" benefits.

4. Skin aging

Skin aging is a complex phenomenon induced by endogenous (chronological) and exogenous (extrinsic) factors. Chronological aging is a genetically programmed, unavoidable process, while extrinsic influences (e.g., sunlight, wind, heat, cigarette smoke, or exposure to chemicals) could be at least partially controlled by the individual (reviewed in Uitto, 1986; Baumann, 2007; Uitto, 2008; Leyden,1990). Among the extrinsic factors, ultraviolet (UV) irradiation (sunlight, tanning beds) is the single most important cause of skin aging (reviewed in Uitto, 1986; Baumann, 2007; Uitto, 2008; Leyden, 1990). UV exposure leads to numerous changes in the skin, including epidermal and dermal thinning, a decline in collagen production and in the functionality of the dermal elastic network, and an uneven distribution of pigment deposition, all resulting in an aged-skin phenotype. Additionally, UV exposure is a major factor in the development of skin cancer. Most skin cancers are

induced by the cumulative exposure to UV irradiation, and therefore appear mainly on aged skins (see e.g. Strom and Yamamura, 1997).

5. Non-denatured soybean extracts reduce pigment production and deposition

The irregular pigmentation displayed in photo-damaged skin includes numerous types of hyperpigmented lesions, commonly referred to as "age spots". These UV-induced lesions are the result of uneven distribution of pigment cells, local loss of melanocytes, hyperactivity of melanocytes, and modified keratinocyte-melanocyte interactions (reviewed in Ortonne 1990). Therapies for hyperpigmented lesions range from cryotherapy and energy-based devices (e.g. laser, pulsed light), to chemical peels, and to topical pharmaceutical depigmenting agents (e.g. hydroquinone, tretinoin, adapalene), (Ortonne 2006). However, many of these treatments are expensive, or are accompanied by undesired effects (reviewed in Jimbow and Jimbow, 1998). The consumer needs safe, efficacious and cost effective depigmenting agents, and additionally desires "natural" treatments, with the expectations of safety, effectiveness and mildness at reasonable cost.

Early searches for depigmenting agents used cultured melanocytes (pigment cells) and assayed for either inhibition of tyrosinase activity (the rate-limiting enzyme in pigment production) or for selective melanocyte cytotoxicity. To better represent the complexity of intact skin, we used keratinocyte-melanocyte co-cultures in our studies. The co-culture system enabled us to identify keratinocyte-melanocyte interactions that are involved in pigment transfer, and later to modulate these interactions in a way that could alter skin color (Seiberg et al., 2000a, 2000b). A keratinocyte receptor, PAR-2, was identified as a phagocytic receptor that regulates the keratinocyte ability to ingest melanosomes (Sharow et al, 2000). Since PAR-2 is activated by serine proteases like trypsin (reviewed in (Déry et al., 1998, 1999)), its activation is inhibited by serine protease inhibitors. The modulation of the PAR-2 pathway was indeed shown to affect skin color. Activators of PAR-2 induced a visible skin darkening, and the inhibition of PAR-2 activation by serine protease inhibitors led to visible skin lightening (Seiberg et al 2000a, 2000b).

A search for botanical agents with a serine protease-inhibitory activity had identified non-denatured soybean extracts, and using the keratinocyte-melanocyte co-culture system documented that these extracts reduce pigment deposition (Paine et al, 2001). Non-denatured soybean extracts were later shown to reduce pigment deposition in human skin and to decrease the pigmentary levels of human age spots (Hermanns et al, 2000, Wallo et al, 2007). Non-denatured soybeans extracts contain the active trypsin inhibitors STI and BBI. Therefore, these purified proteins were tested, in parallel with the complete non-denatured soybean extract, for their ability to modulate pigment production and distribution. Non-denatured soybean extracts, or STI or BBI alone, were found to inhibit PAR-2 activation, resulting in cytoskeletal and cell surface reorganization and reduced keratinocyte phagocytosis (Paine et al, 2001). The depigmenting activity of these agents, as well as their ability to prevent UV-induced pigmentation, was demonstrated both in vitro and in vivo (Paine et al, 2001). Importantly, the non-denatured soybean extracts were superior to either STI or BBI alone in their depigmenting effect, even though the concentrations of STI and BBI within these extracts were much lower than when tested individually (Paine et al, 2001). These studies suggest that the non-denatured soybean extracts contain additional active agents besides STI and BBI, which either contribute directly to the depigmenting effect, or

enhance the delivery or the activity of STI and/or BBI in the skin. Examples of the depigmenting activity of non-denatured soybean extracts are shown in figure 1 (Images courtesy of Dr. C.B. Lin and D. Rossetti).

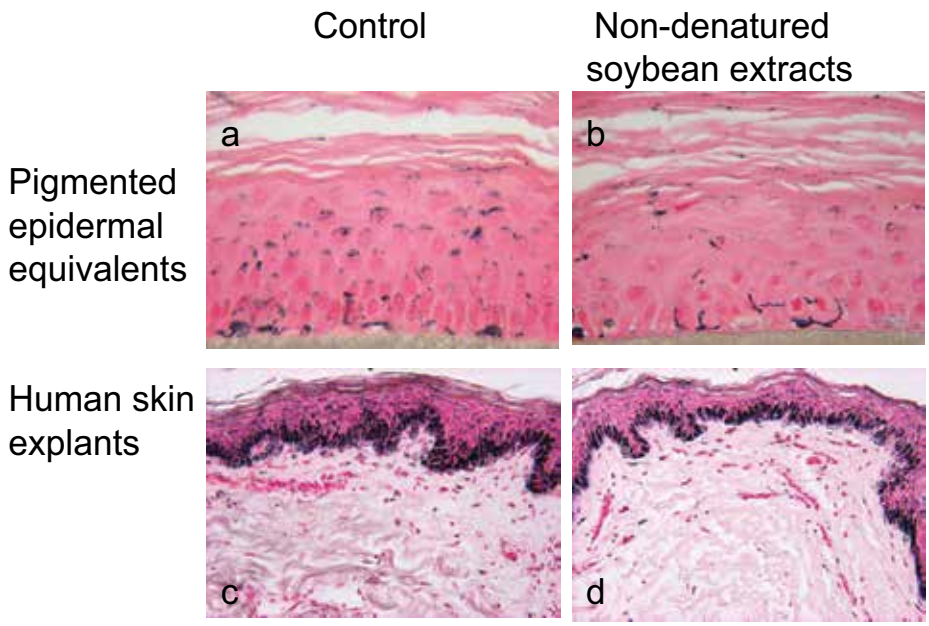

Fig. 1. **Non-denatured soybean extracts reduce pigment production and deposition.** (a-b) Pigmented epidermal equivalents were topically treated with 2% non-denatured soybean extracts in PBS, or PBS alone, once daily, 5 days/week. Following nine days in culture the tissues were stained with Fontana-Masson (F&M) staining, to document pigment deposition. A marked reduction was observed in the treated equivalents as compared to control. (c-d) Human skin explants were obtained from surgical procedures with informed consent and institutional board approval. Explants were maintained in culture for eight days, with or without topical daily treatment, 5 days/week, with a formulation containing 2.5% non-denatured soybean extract. F&M staining documented a strong reduction in pigment production and deposition in the human skin following the non-denatured soy extract treatment. Images are courtesy of Dr. CB Lin and D. Rossetti.

6. Non-denatured soybean extracts enhance the dermal extracellular matrix and the elastic fiber network

Elastin fiber production is reduced with aging (reviewed in Uitto, 1986, 2008; Pasquali-Ronchetti and Baccarani-Contri, 1997). The age-induced decline in skin elasticity results from slower tissue regeneration, from lower elastin synthesis levels, and from the increased production and secretion of elastases. UV exposure further decreases the functionality of the elastic fiber network, as excessive elastin production and abnormal cross-linking create elastotic material ("solar elastosis") with reduced elastic capacity (reviewed in Uitto 1986, 2008). Preventing or reversing skin ageing includes the use of sunscreens and sun avoidance behavior, the use of anti-oxidants, and the use of agents like retinoids, which inhibit

collagenases and promote collagen production (Baily et al, 1990; reviewed in Fisher and Voorhees, 1998). Only a few agents are available to directly enhance the balanced synthesis and assembly of the elastic fiber network. The inhibition of fibroblast-derived elastases following chronic UVB irradiation was found to protect the dermal elastic network of the skin from fragmentation, and to reduce wrinkle formation in rodents (Tsukahara et al, 2001a, b). Searching for botanical extracts for anti-aging skin care use, the non-denatured soybean

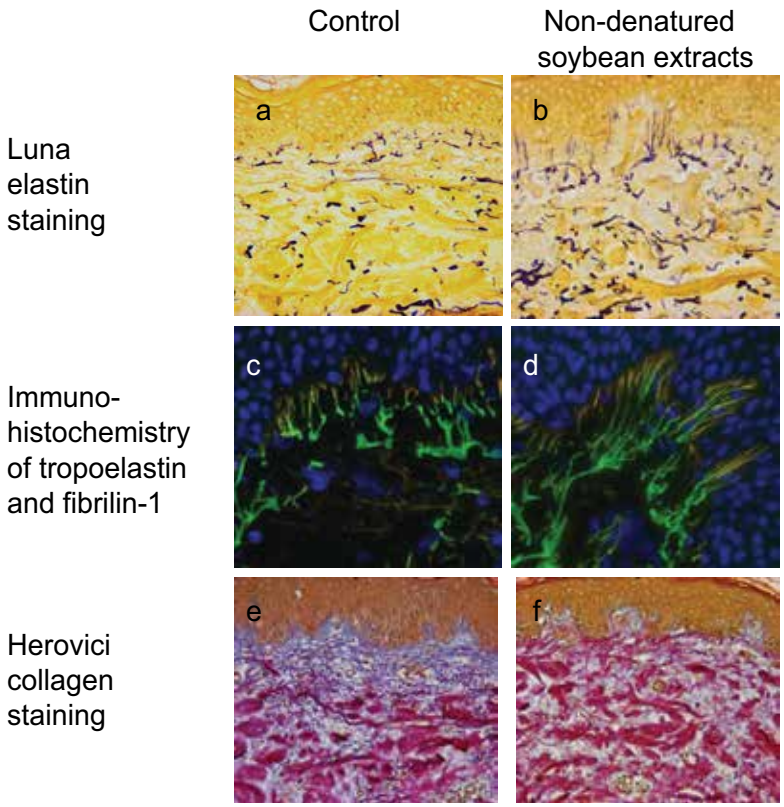

Fig. 2. **Non-denatured soybean extracts enhance elastin and collagen synthesis.** Human skin explants were obtained from surgical procedures with informed consent and institutional board approval. Explants were maintained in culture for eight days, with or without topical daily treatment, 5 days/week, with a formulation containing 2.5% non-denatured soybean extract. Histological and histochemical stainings were performed to document elastin and collagen fibers. (a-b) Luna elastin staining. Mature elastin fibers are stained purple-brown. Topical treatment with non-denatured soybean extracts results in enhanced elastic fiber network. (c-d) Immuno-histochemical staining of tropoelastin (the elastin fiber monomer, green) and fibrillin-1 (an elastin-accessory protein, red). Co-localization is viewed as yellow. Non-denatured soybean extracts induce the synthesis of tropoelastin and fibrillin-1, and their co-localization documents the site of elastin fiber formation. (e-f) Herovici staining documents mature collagen fibers in magenta-red. Topical treatment with non-denatured soybean extracts induces the production of collagen fibers. Images are courtesy of Dr. CB Lin and D. Rossetti.

extracts were found to have elastase-inhibitory activities (Zhao et al, 2009). In addition, non-denatured soybean extracts were found to induce the synthesis of collagen and elastin, and to promote the correct assembly of new elastin fibers, providing a complete protection and restoration to the dermal extracellular matrix (see Figure 2, images courtesy of Dr. C.B. Lin and D. Rossetti).

In vitro studies using both purified elastases and cultured fibroblasts demonstrated that non-denatured soybean extracts could affect the extracellular matrix. The enzymatic activity of several elastases was inhibited by these extracts, and, to a lesser extent, by STI or BBI, while soy isoflavones did not show any elastase-inhibitory activity (Zhao et al, 2009).. The non-denatured soybean extracts also protected elastic fibers produced by cultured fibroblasts from degradation by exogenously-added elastases (Zhao et al, 2009). Additionally, these extracts exhibited elastin-enhancement activities. A dose-dependent induction of expression of the elastin gene was documented using an elastin promoter-luciferase reporter gene, and was confirmed by mRNA analysis of treated fibroblasts. The synthesis of new tropoelastin monomers, as well as of elastin-accessory proteins, and the assembly of new elastin fibers, was documented by histological staining (Zhao et al, 2009).

The elastin-enhancing activity of the non-denatured soybean extracts was confirmed in vivo. Histological analysis of mice and swine skins topically treated with non-denatured soybean extracts showed a significant increase in the elastic fibers network, with an accompanied increase in elastin mRNA and in desmosine content (Zhao et al, 2009). When human skins, transplanted onto immuno-deficient mice, were topically treated with these extracts, an increase was documented in the expression of elastin, elastin-accessory proteins, and collagen (Zhao et al, 2009). Human skin explants treated ex-vivo with non-denatured soybean extracts and analyzed by histological staining and immunohistochemistry showed an increase in elastin, fibrilin-1 and collagen production (see Figure 2; Images courtesy of Dr. C.B. Lin and D. Rossetti). These data suggest that non-denatured soybean extracts not only protect extracellular matrix from degradation, they also induce collagen and elastin synthesis, and increase the total amount of stable, cross-linked elastin fibers.

7. Non-denatured soybean extracts reduce hair growth

Unlike other mammals, humans no longer use their hair for environmental protection, but keep or remove their hair for social and cosmetic purposes. Many procedures are used to remove unwanted hair, from simple home treatments like shaving, to laser and light therapies. These methods differ in the duration of hair elimination after removal, their price range, their pain and discomfort levels, and their possible undesired effects (reviewed in Olsen, 1999). Shaving, the most popular hair-removal method, requires daily treatments, may result in nicks and cuts, may increase the risk of infection, may leave a perception of an increased rate of hair growth, and may leave undesirable stubble. An alternative to at-home hair removal is hair dying or bleaching, used to reduce hair visibility in particular body areas. However, such methods are less preferred as the emerging portions of the hair shafts are always darker than the already-treated parts. An alternative to these methods is desired; particularly a method that would reduce undesired hair growth, with a safe and simple at-home procedure. As the biological activities of the non-denatured soybean extracts were further explored, they were found to delay hair growth, resulting in smaller and thinner hair shafts (Seiberg et al, 2001). The use of a skin care product containing these extracts, therefore, would reduce the visibility of undesired hair growth.

Topical daily treatments with STI or BBI visually delayed hair growth and reduced the length of the hair shafts of treated mice, with reduced hair follicles size observed histologically. Non-denatured soybean extracts (containing STI, BBI and isoflavones) were superior to either STI or BBI alone in this inhibitory activity. Non-denatured soybean extracts led to delayed and reduced hair growth, and hairs were visibly thinner, more "directionally organized", and smoother to touch, relative to untreated controls (Seiberg et al, 2001). Histological analysis confirmed these observations, and documented smaller size and less developed hair follicles (see Figure 3). A statistically significant effect on hair follicle dimensions was observed, with hair shaft diameter reduced by an average of 42%, and hair bulb diameter reduced by an average of 23.8% (Seiberg et al, 2001). Additionally, the depigmenting effect of STI and BBI led to lighter colored hair shafts, which could potentially contribute to the reduced visibility of undesired hair growth. Heat-denatured soybean extract, or commercially available pasteurized soymilk, had no effect on hair growth or hair appearance (Seiberg et al, 2001), further supporting the involvement of intact STI and BBI in the hair growth inhibitory effect.

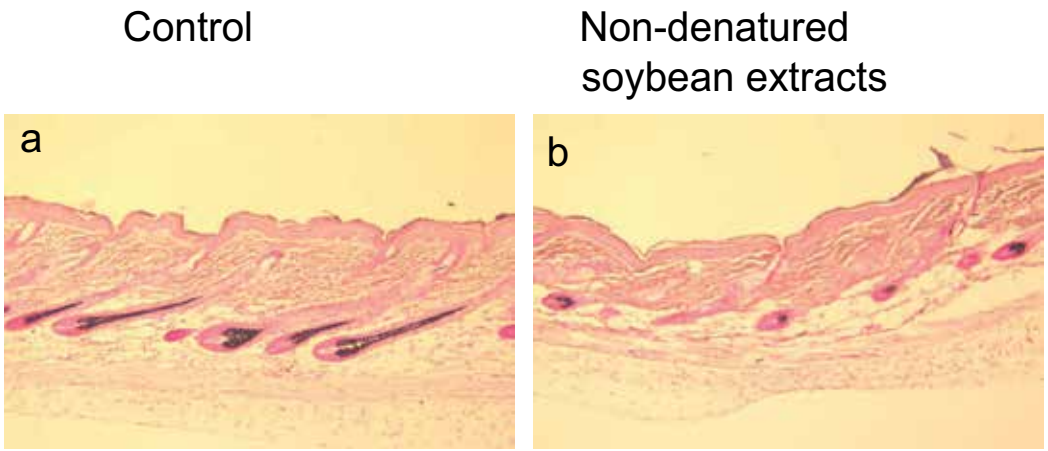

Fig. 3. **Non-denatured soybean extracts delay hair growth.** C57Bl/6 mice were wax depilated to induce a new hair cycle, and were treated daily with non-denatured soymilk (b) or remain untreated (a) as in (Seiberg et. al., 2001). F&M staining of skin biopsies taken at day 7 of the hair cycle demonstrate delayed follicular development, and reduced hair follicle dimensions and pigment deposition in the soy-treated mice.

The non-denatured soybean extracts contain about 0.001% isoflavones. Phytoestrogens were shown to reduce hair growth *in vitro* (Hoffman et al, 1997), but to promote hair growth in mice and in volunteers with alopecia (Okajima and Harada, 2008). Therefore, the effect of the soybean-derived phytoestrogens on hair growth was studied, alone (0.001%), and in combination with the whole non-denatured soybean extract (total 0.002% isoflavones). Isoflavones alone reduced mouse hair growth, but not as effectively as non-denatured soybean extracts alone. The combination of non-denatured soybean extracts, supplemented with 0.001% soybean-derived isoflavones, reduced hair growth to a higher degree than each preparation alone (Seiberg et al, 2001), suggesting complementary roles for STI, BBI and isoflavones in the hair growth inhibitory effect. These data suggest that non-denatured soybean extracts may serve as a natural alternative treatment for reducing the visibility of undesired grown hair..

8. Non-denatured soybean extracts inhibit the COX-2 pathway, contributing to anti-aging and chemo-preventive effects

Chronic inflammation has been linked to numerous skin diseases and conditions, including skin aging (Reviewed in Thornfeldt, 2008), and many skin care products, therefore, contain anti-inflammatory agents. Additionally, inflammation has been linked to epithelial skin tumors, and anti-inflammatory drugs are being studied for the prevention and treatment of non-melanoma skin cancers (Reviewed in Mueller, 2006). Cyclooxygenase-2 (COX-2), the main UV-responsive COX isoform in human skin, is involved in UV-induced skin inflammation and carcinogenesis (reviewed in Zhan and Zheng (2007); Rundhaug et al, 2007). UV-induced COX-2 expression plays a major role in UV-induced inflammation, edema, keratinocyte proliferation and epidermal hyperplasia, as well as in the generation of oxidative DNA damage. Repeated exposure to UV leads to chronic upregulation of COX-2 expression and chronic inflammation, contributing both to accelerated skin aging and to an increased risk of skin cancer. The induction of COX-2 expression by UV is higher in aged human skin as compared to young human skin, and aged human skin produces higher amounts of prostaglandin E_2 (PGE_2, the product of the COX-2 pathway) relative to young skin (Seo et al, 2003). These data suggest that the aging of the skin may increase the susceptibility for developing both photoaging and carcinogenic processes. Oral and topical COX-2 inhibitors have chemopreventive activity against chemically- and UV-induced skin cancer in numerous animal models (reviewed in Zhan and Zheng (2007); Rundhaug et al, 2007). The topical applications of soy isoflavones to mouse skins before UVB exposure reduced the expression of COX-2 (Chiu et al, 2009).

Non-denatured soybean extracts were found to inhibit the COX-2 pathway both in vitro and in vivo (Chen et al, 2008). Cultured keratinocytes or epidermal equivalents were pretreated with non-denatured soybean extracts and then thoroughly washed prior to UV exposure. In the absence of treatment, UV irradiation induced COX-2 expression and PGE_2 secretion, and induced the activation of p38 MAP kinase. Non-denatured soybean extracts reduced UVB-induced COX-2 expression and PGE_2 secretion, and inhibited p38 MAP kinase activation in vitro (Chen et al, 2008). Mice pre-treated topically with non-denatured soybean extracts, then extensively washed prior to UV-exposure, had reduced levels of COX-2 expression in their skins at 24 hr post UVB exposure, as compared to mice exposed to UVB alone, confirming the COX-2 inhibitory activity of the non-denatured soybean extracts in vivo (Chen et al, 2008). Examples of the COX-2 inhibitory activity of the non-denatured soybean extracts, and of the reduction in UV-induced PGE_2 secretion are shown in Figure 4. The COX-2 inhibitory activity of the non-denatured soybean extracts suggests the possible delay of photoaging and skin cancer processes in human skin.

9. Non-denatured soybean extracts reduce skin cancer progression in high-risk mice

The photoprotective effects of polyphenols, including green tea polyphenols, resveratrol, and soy isoflavones like genistein, on UV-induced skin inflammation, oxidative stress and DNA damage, have been extensively documented (Reviewed in Nichols and Katiyar, 2010). Additionally, the chemopreventive effects of the soy protein BBI have been demonstrated, and an extract of soybeans enriched in BBI has been under evaluation in numerous human clinical studies for more than ten years (Kennedy, 1998). Oral feeding of mice with a non-denatured

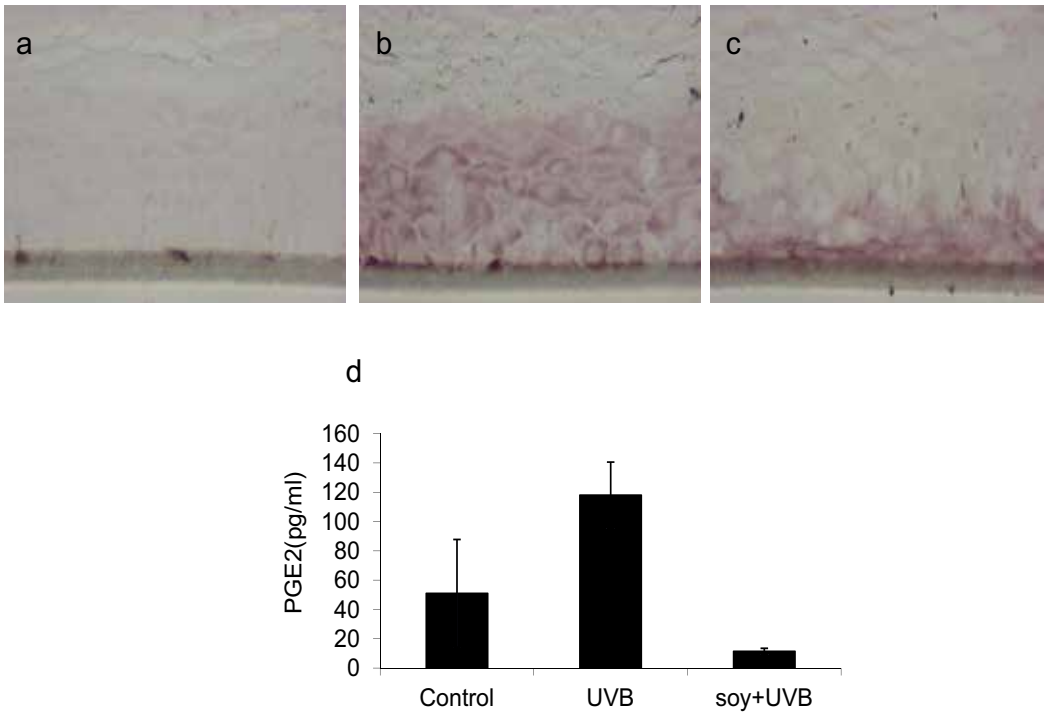

Fig. 4. **Non-denatured soybean extracts inhibit the COX-2 pathway**. Epidermal equivalents were treated with 2% non-denatured soybean extracts in PBS or with PBS alone for 24 hours, then washed extensively, followed by UVB exposure (100 mJ/cm^2) as in (Chen et al., 2008). Samples were collected at 24 hours post UVB exposure for COX-2 immuno-histochemical staining. (a) PBS. (b) PBS followed by UVB. (c) 2% non-denatured soybean extracts followed by UVB. COX-2 staining of the treated equivalents demonstrates the inhibitory effect of non-denatured soybean extracts on UVB-induced COX-2 protein levels. (d) Culture media were analyzed for PGE_2 secretion. UV induced, and non-denatured soybean extracts inhibited the secretion of the inflammatory mediator PGE_2.

soymilk protein supplement led to reductions in skin tumor numbers and volume when tumors were chemically induced (Limtrakul et al, 1993), however undesired gastro-intestinal effects were observed during this study. Since non-denatured soybean extracts contain isoflavones and BBI, and since they possess COX-2 inhibitory activity, the use of these extracts as topical chemo-preventive agents was evaluated.

Hairless mice exposed for 20 weeks to chronic low levels of UV light become "high risk mice", which are tumor-free, but with a high risk of developing skin tumors during the next several months in the absence of additional UV exposure. The high-risk, UV-irradiated mice (with no visible skin lesions) were topically treated with non-denatured soybean extract, heat-denatured soybean extract, STI, or BBI, or with water or BSA as controls (Huang et al, 2004). Topical applications of non-denatured soybean extract but not of heat-denatured soybean extract to these high risk mice reduced the incidence and slowed the growth and progression of skin tumors (see Fig. 5a). Similar topical applications of STI or BBI also reduced the incidence, and slowed or inhibited the formation and growth of skin tumors,

but to a lesser extent than the whole non-denatured soybean extract. The number of tumor-bearing mice, the number of tumors per mouse, and the volume of the visible part of the tumors, were all reduced the most upon the topical treatment of non-denatured soybean extracts (Huang et al, 2004). Histopathological examination of skin sections from the untreated mice and from mice treated with heat-denatured soybean extracts all documented numerous squamous cell carcinoma (SCC) lesions. The non-denatured soybean extracts-treated skins, in contrast, had only very small lesions, with no dysplasia or carcinoma (Figure 5b-d, images courtesy of C. Paine). These data might suggest that non-denatured soybean extracts could be topically applied to sun-exposed human skin to prevent sunlight-induced skin damage and to reduce the risk of skin tumor formation and progression.

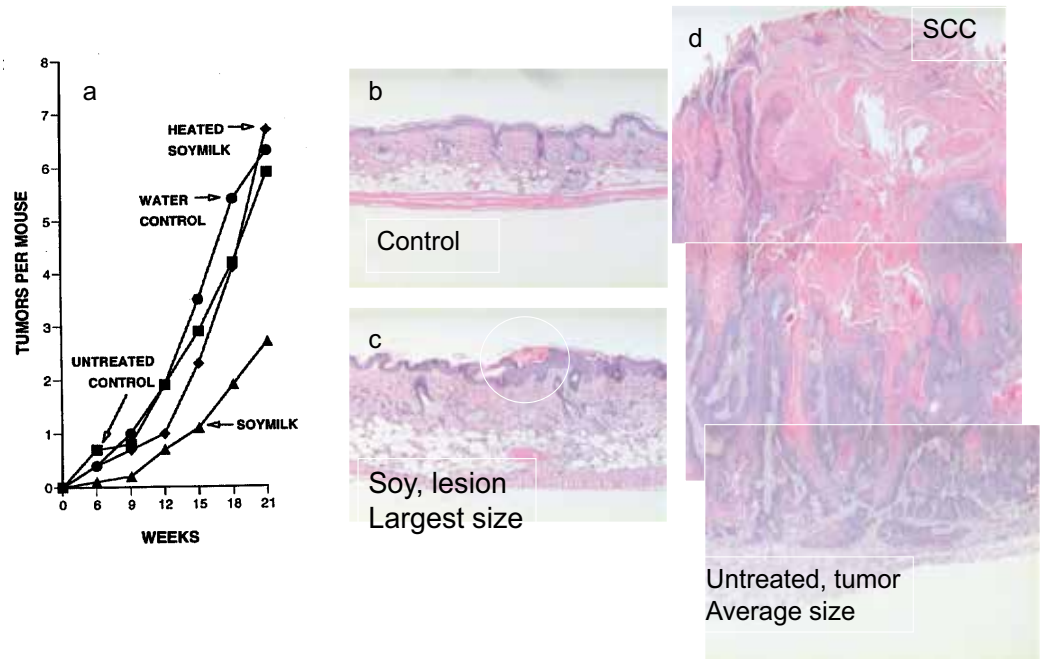

Fig. 5. **Non-denatured soybean extracts delay the progression of skin cancer in high-risk mice.** SKH-1 mice were irradiated with UVB (30 mJ/cm^2) twice weekly for 20 weeks and UVB irradiation was then stopped, as in (Huang et al., 2004). Three weeks later, the mice were topically treated with non-denatured soybean extract ("soymilk"), heat-denatured soybean extract ("heated soymilk"), or water control, once a day, 5 days/week, for 21 weeks. (a) The number of visible tumors per mouse was determined every 3 weeks. Topical treatment with non-denatured soymilk, but not with heat-denatured soymilk, led to a marked reduction in the number of tumors per mouse. (b-d) H&E staining of visible skin lesions and visually unaffected skins was performed at the end of the study. (b) Control skin (not exposed to UV and untreated). (c) Largest visible lesion of a non-denatured soymilk-treated skin. (d) An average lesion in the high-risk, untreated group shows a well-differentiated SCC. All images are of same magnification. Topical treatment with non-denatured soymilk significantly reduced the severity of the developing skin lesions. Images are courtesy of C. Paine.

10. Non-denatured soybean extracts reduce skin cancer risk by multiple mechanisms

Non-denatured soybean extracts, as all botanical extracts, contain multiple agents, which are only partially characterized. It is expected, therefore, that such extracts would act by multiple mechanisms. Studies of the mechanisms of action of the chemopreventive activity of non-denatured soybean extracts identified effects both at the initiation and the progression of skin tumors, with multiple mechanisms affecting the tumor and the microenvironment (Chen et al, 2008). Topical pretreatment of hairless mice or Yucatan swine with non-denatured soybean extracts for several days prior to UVB exposure, prevented or reduced UVB-induced DNA damage (T-T dimers) and apoptosis. This reduction in the cumulative DNA damage in skin is, at least partially, mediated via the activation of the cell-cycle regulatory protein Chk1 (Chen et al, 2008). Non-denatured soybean extracts inhibit metallo-proteinases (MMPs) expression in vivo, suggesting the inhibition of dermal ECM remodeling, which is required for tumor progression. Vascular endothelial growth factor (VEGF)-induced endothelial tube formation in Matrigel was inhibited in vitro by non-denatured soybean extracts, suggesting a possible inhibitory effect on angiogenesis and tumor progression. Taken together with their ability to inhibit multiple proteases and to enhance ECM production (Zhao et al, 2009), their ability to inhibit the COX-2 pathway (Chen et al, 2008), and their content of intact STI and BBI, non-denatured soybean extracts could affect the tumor and the microenvironment at multiple stages of skin cancer initiation and development. Topical treatment with non-denatured soybean extracts, therefore, could reduce the risk of skin cancer development, by reducing UVB-induced DNA and cellular damage, and reducing microenvironment processes.

11. Clinical studies with non-denatured soybean extracts

An initial human clinical study using non-denatured soybean extracts for skin care was performed on hyperpigmentary lesions (Hermanns et al, 2000). Non-invasive image analysis was performed with high magnification pictures obtained with a UV-emitting unit and a sensitive fluorescence video recording, which could detect subclinical mottled skin pigmentation. Non-denatured soybean extracts led to significant skin lightening after a 3-week treatment (Hermanns et al, 2000), suggesting, for the first time, that the inhibition of the PAR-2 pathway might present a novel approach for the treatment of pigmentary skin lesions.

The effect of non-denatured soybean extracts on hair growth was first evaluated in three Caucasian males with dark facial hair. Panelists treated one side of their face with the extracts, daily, immediately after shaving. By the forth week, the hair of the treated side was visually lighter and shorter, and felt smoother to touch. Image analysis of the treated areas four hours after shaving documented reduced length and thickness of the hair stubble at the treated side, with a statistical significant difference in all measured parameters (Seiberg et al, 2001). Examples of images from this study are shown in figure 6a-b. For the next proof of concept study, hair was wax-depilated of two symmetrical areas of the legs, below the knee, in two females, and one leg was treated daily, for four weeks, with non-denatured soybean extracts. Hair growth on the treated site was visually reduced, with the hair shafts visibly shorter and thinner than those of the control sites (Seiberg et al, 2001).

Examples of images from this study are shown in Figure 6c-d. Later studies documented similar effects on women's facial hair. These observations confirmed the effect of non-denatured soybean extracts on delaying human hair growth, and suggested that this effect is not related to the method of hair removal or to the body part being treated.

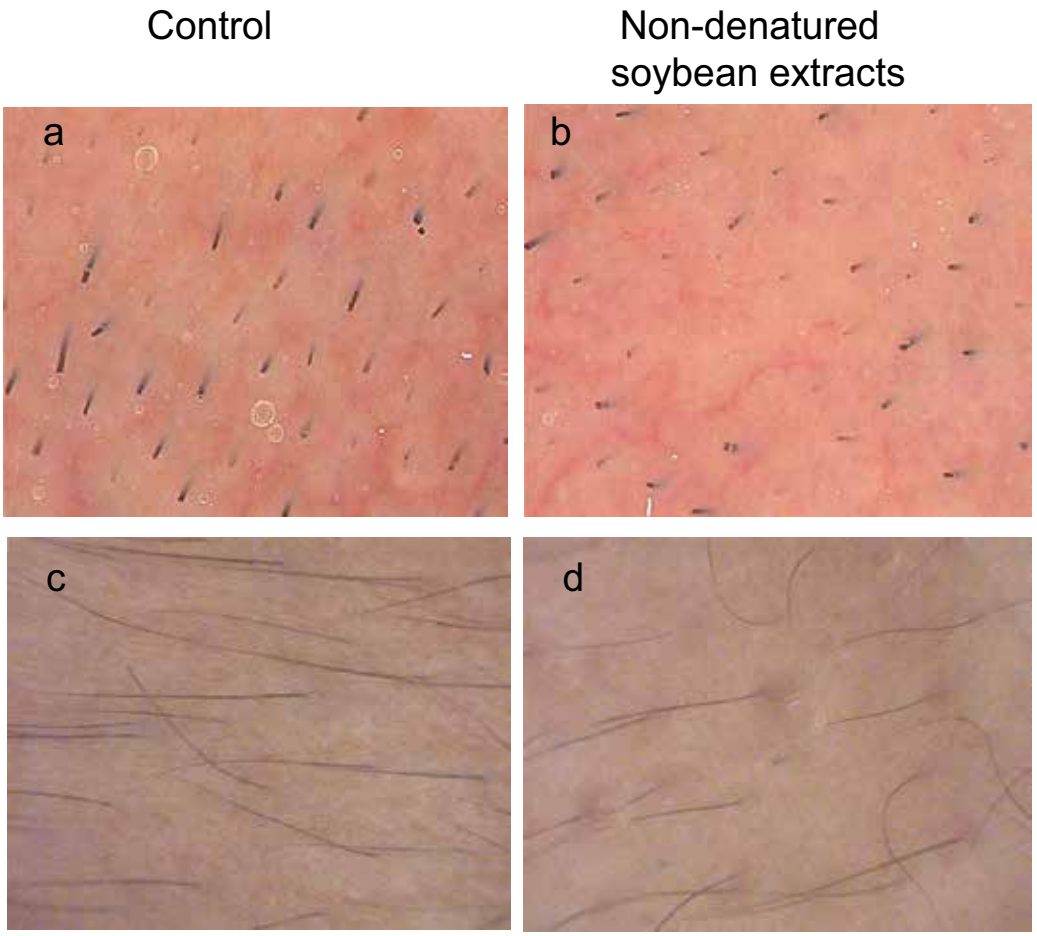

Fig. 6. **Non-denatured soybean extracts reduce human hair length and thickness.** (a-b) Caucasian males treated the right side of their face with non-denatured soybean extract, immediately after shaving. Digital pictures taken after four weeks of treatment, four hours after shaving, demonstrate reduced growth rate and dimensions of the hair stubble at the treated side (b), relative to the untreated control (a). (c-d) Hair was wax-depilated of two symmetrical areas of female legs, below the knee, and one leg was treated daily, for four weeks, with non-denatured soybean extract . Images taken at four weeks of treatment, six days after shaving, document reduced density and dimensions of the hair shafts at the treated side (d), relative to the untreated control (c).

The non-denatured soybean extracts were next evaluated in a placebo-controlled clinical study for their effect on UV-induced erythema, hyperpigmentation and peeling (Wu et al, 2001). Subjects were exposed to UV irradiation of about 1.5-2 MED, following by daily

applications of the non-denatured soybean extract. Diffused reflectance spectroscopy measurements and independent dermatologist assessment concluded that UV-induced erythema was reduced at 24 hrs after the application of non-denatured soybean extracts. At 7 days post irradiation, peeling was observed on some placebo or untreated sites, but not on the non-denatured soy-treated sites (Wu et al, 2001). This clinical, placebo-controlled study clearly demonstrates that non-denatured soybean preparations are beneficial for daily defense against solar irradiation. An example of the effect of pretreatment with non-denatured soybeans extracts on UV-induced erythema and peeling is shown in figure 7.

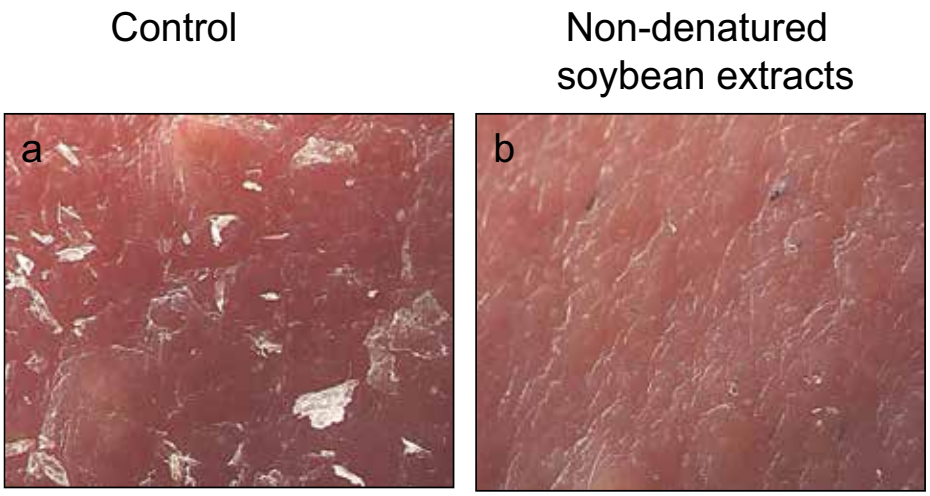

Fig. 7. **Non-denatured soybean extracts reduce UV-induced erythema and peeling of human skin.** A Caucasian male treated the right side of his face with non-denatured soybean extract, immediately after shaving, for six weeks, and was then exposed to solar irradiation during recreational activity. Digital Hi-Scope pictures taken 24 hrs post UV exposure, demonstrate reduced redness and peeling at the treated side (b), relative to the untreated control (a).

A double blind, placebo-controlled study of the efficacy of a moisturizer containing non-denatured soybean extracts was performed to evaluate the effects of this moistuizer on skin tone, skin pigmentation, and other photoaging-induced effects, in 63 women with moderate photo-damage (Wallo et al, 2007). Efficacy was monitored by clinical observations and self-assessments on weeks 0, 2, 4, 8, and 12, and by non-invasive measurements (such as colorimetric evaluations and digital photography) on weeks 0, 4, 8, and 12. The non-denatured soybean extract-containing moisturizer was well tolerated, and was more effective than the placebo in improving numerous photoaging parameters of skin color and texture, including overall skin tone, mottled pigmentation, blotchiness, dullness, fine lines, overall texture, and overall appearance (Wallo et al, 2007). Examples of the effect of the non-denatured soy extract moisturizer on skin pigmentation and on facial wrinkles are shown in

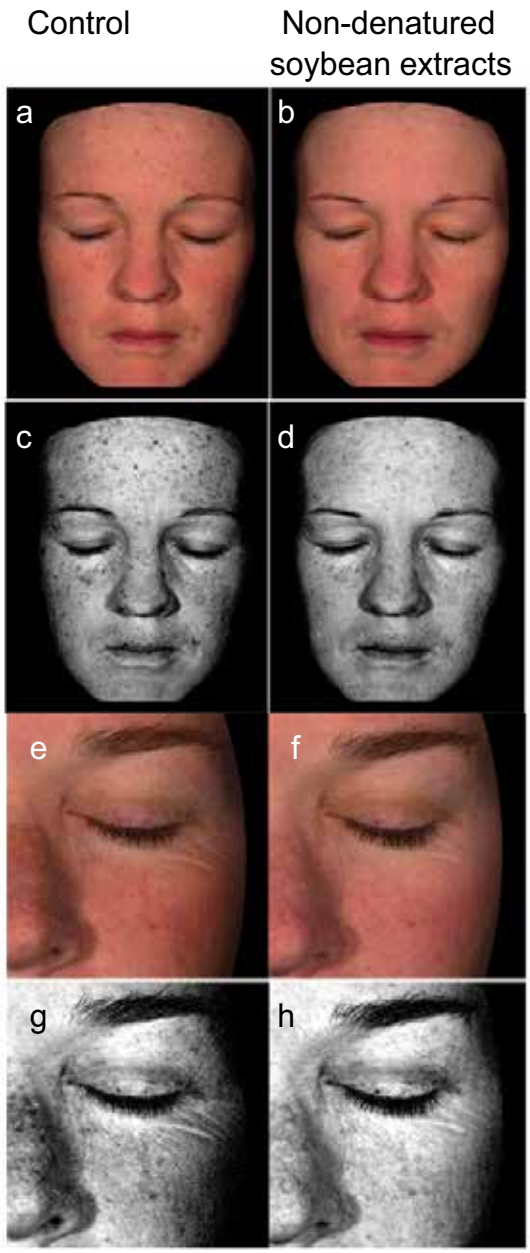

Fig. 8. **Non-denatured soybean extracts reduce signs of photoaging.** Women's faces were treated for 12 weeks, twice daily, with a moisturizer containing 2% non-denatured soybean extracts. A cross-polarized (a-b, e-f) and a black-and-white (blue-channel) of cross-polarized (c-d, g-h, enable visual enhancement of pigmentation changes) photographs were taken at baseline and at the end of the treatment period. (a-d) The images demonstrate improvement in skin tone, texture and radiance parameters following the treatment with non-denatured soybean extracts. (e-h) Images demonstrate improvement in wrinkling ("crow's feet") and in pigmentation parameters. Images are courtesy of W. Wallo.

figure 8 (Images courtesy of W. Wallo). The differences in photoaging parameters were significant from week 2 to week 12 for all above parameters (except for dullness, which started at week 4), suggesting that a moisturizer containing non-denatured soybean extracts is very effective in ameliorating photoaging-induced skin effects.

12. Conclusion

Preclinical and clinical studies, as well as mechanistic understanding, confirm that non-denatured soybean extracts, their formulations and their products, are safe and effective in improving numerous skin care parameters. Thus, the use of non-denatured soybean extracts provides multiple skin care benefits, ranging from protection and restoration, to aesthetic benefits.

13. Acknowledgement

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9

Soybean: Africa's Potential Cinderella Food Crop

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1. Introduction

Soybean (*Glycine max* [L.] Merrill) is an important oriental crop whose agronomic characteristics were apparently well known in China before 2200 BC. It is a legume that grows in tropical, sub tropical and temperate climates. Originally domesticated in China around 1700-1000 BC, soybean is now cultivated throughout East and South East Asia where people depend on it for food, animal feed and medicine. Soybean reached North America in 1765 via London, and Africa through missionaries in the early 19th century (CGIAR, 2005).

The fact that soybean is fastly becoming the 'toast' of many countries beside China is not in doubt. However, this cannot be rightly said of African countries as a whole. According to FAO data of 2005, 95.2 million ha of land were used for soybean cultivation worldwide and total production was 212.6 million tones. The three major soybean-producing countries were USA (29 million ha), Brazil (23 million ha) and Argentina (14 million ha). Seed production from the cultivated hectares of land were 83, 51 and 38 million tones for USA, Brazil and Argentina respectively. In a strong contrast, the same source revealed that in the whole of Africa, soybean was grown on an average of 1.16 million ha with an average production of 1.26 million tones.

Malnutrition and food insecurity are not being eradicated fast enough; in Africa, they are on the increase. The resources to significantly reduce this tragedy are not that large (Hadad & Martorell, 2002). Already, it is projected that Sub Saharan Africa, with its combination of high population growth and lagging economic performance, the number of malnourished children is expected to increase by six million compared with 1997 (Rosegrant & Meijer, 2002). As it has been earlier opined that resources to tackle the menace of malnutrition is not all that large in Africa, there is a need for critical analysis of what Africa has, and how best it could be utilized, in surmounting the hydra-headed problems of hunger and malnutrition which incidentally constitute the major African challenges.

Considering both Africa's climatic and edaphic factors, tropical soybean varieties can be cultivated in about half of Africa's land. In addition, there is a greater possibility with the introduction of newly developed IITA genetically modified drought tolerant soybean. On the understanding of the well established soybean's medicinal and food values, incorporation of soybean into Africans' diet is a sure way of stemming the tide of malnutrition in Africa. The present review article is focusing on the role of soybean in tackling the menace of malnutrition in the past, present and future Africa.

2. Soybean improvement programme in Africa

In 1908, soybean seeds (which were sourced from Manchuria) were found to be suitable replacement for linseed and cottonseed in the city of Hull, Great Britain. However, subsequent to Sino-Japanese war (which Japan won), Britain, which owned many colonies in Africa, decided to test systematically, the ability of soybeans to produce good yields in British colonies. Sir Alfred Jones and Mr. Grenville Turner were the leader of the British expendition. Incidentally, in the year 2007, the five leading soybean producers in Africa namely Nigeria, South Africa, Uganda, Zimbabwe and Egypt all have strong historical ties to Great Britain (Shurtleff & Aoyagi, 2009). However, it is worthy of note that the introduction of soybeans into Africa was characterized with many pitfalls such as no or poor germination, crop failure after germination and low yields. For instance, in 1908, soybeans planted on 1.35 acres at Moor Plantation, Ibadan. Nigeria failed. While still recounting on Nigeria's soybean experience, Ezendinma (1964), reported that in 1937, ten varieties were obtained from USA and one each from Malaya and British Guiana. Of these, only one US variety, Otootan, the Malayan and Creole from British Guiana survived; the rest either failed to germinate in the first planting. Furthermore, in 1940 more introductions were made from Philiphines and Trinidad. It was also reported that eight varieties were introduced from Southern Rhodesia in 1942, and in 1949 a total of eleven varieties were brought into Nigeria from India, Ceylon and the Philiphines. The sudden change in ambient temperature in transit was thought to be responsible for the rapid deterioration of the embryos and the subsequent failure of the seeds to germinate (Ezendinma, 1964). However, later advances in scientific knowledge pointed to other directions as *Bradyrhizobium japonicum* populations required for effective nodulation of soybeans are found to be non-endemic to African soils (Hadley & Hymowitz, 1973). Also, in the 1970s, there was a little interest and effort in Africa to grow and improve soybean because of extremely low yields and seed viability, high shattering rate and limited postharvest use.

Consultative Group on International Agricultural Research (CGIAR) soybean research is based in Sub Saharan Africa and has progressed very well in both crop improvement and postharvest processing and utilization. To avoid the need to inoculate soybean with *B. japonicum*, soybean breeders at the International Institute of Tropical Agriculture (IITA) (which was founded by CGIAR), Nigeria, developed new soybean genotypes for Africa, known as *Glycine* cross (TGX), which nodulate with *Bradyrhizobium* spp populations indigenous to African soils (Pulver et. al., 1985). Also, since the 1970s soybean breeders at the CGIAR's IITA in Ibadan, Nigeria have been successfully working on developing improved varieties of soybean that fix more nitrogen from the atmosphere without *Rhizobium* inoculation, are high yielding, store well, and are resistant to pod shattering. Furthermore, soybean improvement activities in IITA include the development of dual-purpose varieties that are tolerant to phosphorus-deficient soils and have enhanced capacity to kill seeds of the parasitic weed *Striga hermonthica* which attack cereals. Such varieties were found to have high grain yield and produce large amounts of biomass, making them useful as a soil-building rotation crop and as fodder for livestock. So far, seventeen IITA-bred tropical soybean varieties have been released by national agricultural research and extension system (NARES) of several West and Central African countries including Nigeria, Benin, Ghana, Democratic Republic of Congo, Togo and Uganda. Unfortunately, support for soybean research among NARES has declined in the recent time.

The great landmark of IITA's breeding effort on soybean is far from being obscured. It was reported that when IITA started soybean improvement research in 1974, the average yield per hectare in Africa was 660 kg/ha and total production was only 0.2 million tones. Thirty years later, using IITA-developed varieties, the average yield in West African countries increased by more than 50% and 67% in the whole of Africa. Currently, twenty-one African countries now produce soybean with Nigeria, South Africa and Uganda being the highest three producers (IITA, 2008)

3. Soybean production and utilization in Africa

Africa is the most tropical of the continents. Most of Africa has high temperatures throughout the year. The most important factor differentiating its climates is the amount, duration and seasonal distribution of rainfall. The different climatic zones found in Africa include tropical wet, tropical summer rainfall, semiarid, arid, highland and Mediterranean (Newman et al., 2007). Due to the different climatic zones found in Africa and other biotic and abiotic constraints, soybean cannot readily grow in all parts of Africa.

The extent to which soybean can be cultivated in different regions of Africa is shown in Figure 1. With the influx of IITA genetically modified drought tolerant soybean variety, there is a possibility of growing soybean in semiarid and arid regions which have earlier being dubbed unsuitable for soybean cultivation. Going by the 2008-2009 records, Nigeria is still the highest Africa's soybean producer (39%), closely followed by South Africa (35%) while Uganda is the third highest African producer (14%). Figure 2 shows the significant soybean producers in Africa. A critical comparison of Figures 1and 2 would reveal that maximizing Africa's soybean production potential is far from being realized.

According to Newman et al. (2007), since at least the mid-19th century African economies were increasingly reworked to meet the needs of industrial Europe. Soybean, one of the Agricultural raw materials from the turn of the 20th century was not an exception. The British effort of systematically testing soybean in her colonies was targeted at ensuring a sustainable raw materials supplies for the Great Britain. A comparison of Nigeria's contribution to the Commonwealth and World's soybean production between 1938 and 1960 is presented in Table 1. The fact that soybean has not been largely incorporated into African diet up to the first eighty years of the 20th century also contributed to its being majorly an export crop then. However, sequel to the soybean awareness in some African countries in the 1980s, the situation has greatly changed as many soy based African meals were developed. Presently, Africa's soybean production cannot match up with her demand. According to FAO, Africa spent US$ 1 billion in 2004 to import soybean and soy oil. Of this, US$752 million was for soybean oil and US$254 million was for soybean grain/meal.

Year	Nigeria	Commonwealth	World
1938/39	Nil	4	12,562
1954/55	9	144	19,661
1955/56	10	162	20,666
1956/57	16	159	23,780
1957/58	15	190	24,581
1958/59	14	182	27,570
1959/60	4	188	27,501

Table 1. Commonwealth and World's soybean production (000 tonnes) between 1938 and 1960

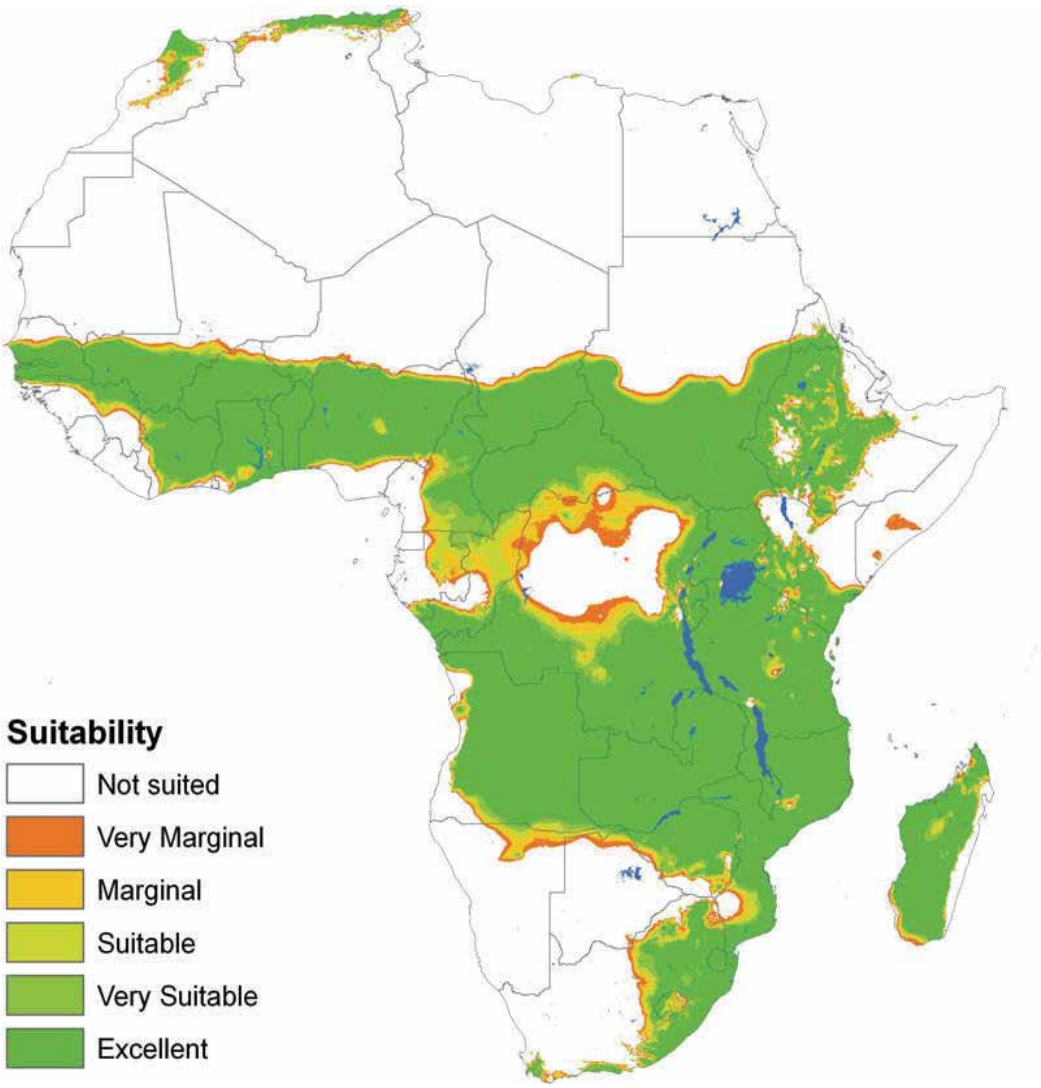

Fig. 1. Soybean suitability map for Africa (IITA, 2008)

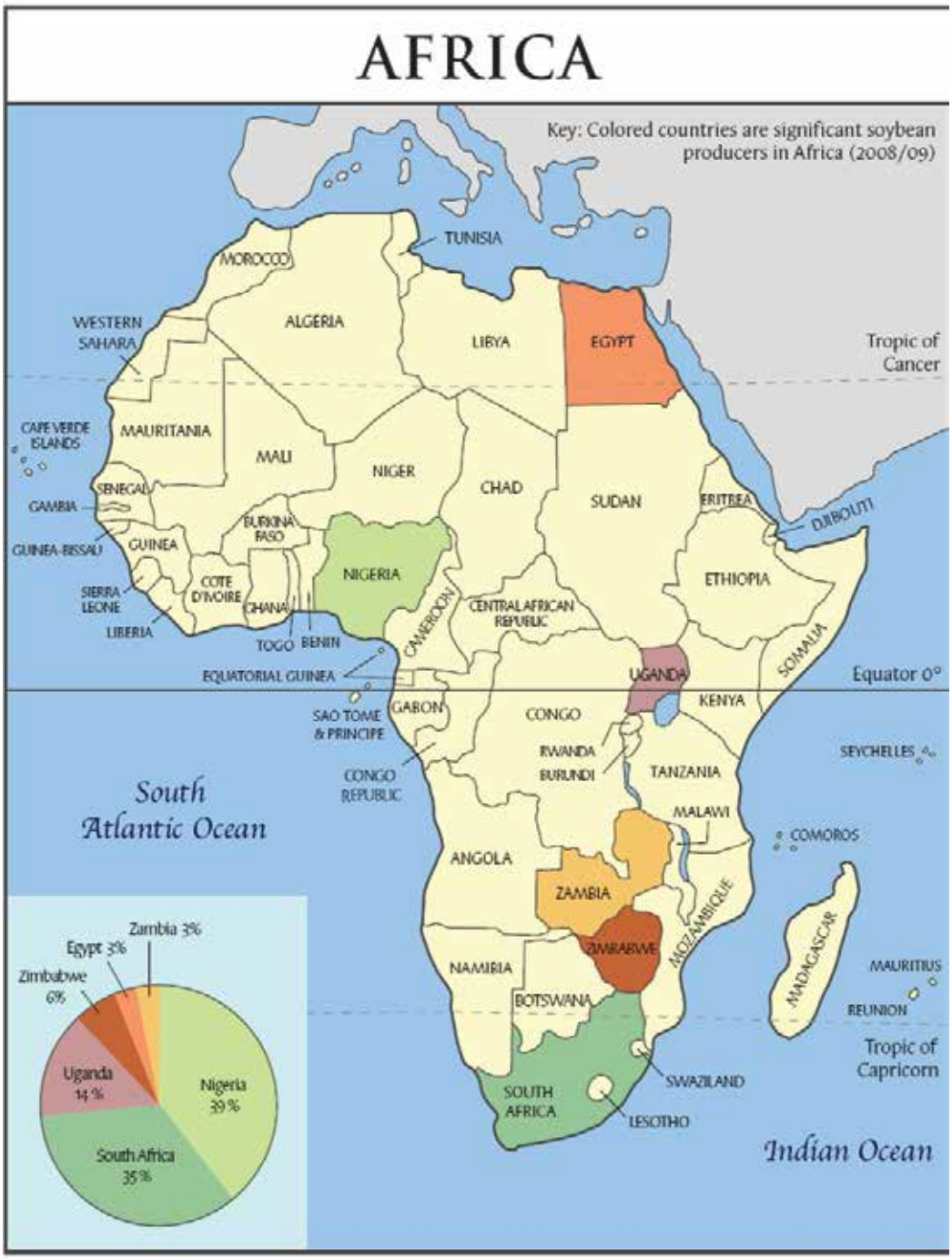

Fig. 2. Significant soybean producers in Africa (Shurtleff & Aoyagi, 2009)

4. Soy foods in Africa

Although the value of soybean as high protein source has been recognized, its use as human food was not immediately developed in Africa. Difficulties of soaking, preparation, cooking and a certain lack of palatability apparently precluded its use by many African people in the past era (Onichie, 1965). However, it should be stressed that as far back as 1938 commercial soy food products began to find their way into Africa, even where soybean was not cultivated. For instance, ProNutro, the earliest known commercial soy food product in Africa, was introduced into South Africa by Hind Brothers and Co Ltd (Odendal, 1965). Soy foods appeared in Ethiopia in 1945 (Shurtleff & Aoyagi, 2009), while Cape Verde, Eritrea and Liberia had their first contact with soy foods in 1960. Furthermore, in 1965, a black American and Seventh-day Adventist medical missionary, Dr D.W. Harrison started black Africa's first soy foods Company- Africa Basic Foods in Uganda. In a bid to incorporate soybean into Africa diet and improve nutrition, in 1965, a west African gruel derived from maize was fortified with soybean to produce Soy-Ogi by Akinrele in Nigeria. Much more later, the United State Development Agency (USDA) Food for Peace Programme employed soy food in her program in Mauritania and Djibouti in 1973 and 1978 respectively to solve problems of Malnutrition and Protein deficiency.

The recent expansion of soybean farming in Nigeria is attributable to both the development of improved varieties and soy-based food products. It is in this regards, that CGIAR's IITA also focused on soybean post harvest research. At IITA, post harvest researchers, have developed appropriate technologies that reduce labor and time requirements for processing, and improve the product quality and quantity. Various soybean-processing machines, such as a flour mill (dry mill or grinder, an extruder/expeller), a screw press and thresher, have been adapted for use in Sub- Saharan Africa and have been widely adopted by both small and medium scale processors (CGIAR, 2005). Altogether, about one hundred and fifty food products with good nutritive value and consumer acceptability have been developed by IITA food technologists. Many of these new products have been tried out in villages across the region, modified by local people to suit local culinary tastes, and ultimately adopted with increasing popularity.

In addition to IITA research efforts on incorporation of soybean into African diet, various independent researchers have reported on the possible use of soybean in addressing the major Africa's challenges- Hunger and Malnutrition. Soybean seeds have been exploited for the production of food products such as Soybean daddawa (Popoola & Akueshi, 1986), Soybean fortified gari and tapioca (Sanni & Sobamiwa 1994; Kolapo & Sanni 2005; Kolapo & Sanni 2009), cereal-based traditional weaning food (Osundahunsi & Awor 2003), Soy-Coconut milk based yoghurt (Olubamiwa & Kolapo 2008a), Soy-Cow milk based yoghurt ((Olubamiwa & Kolapo, 2008b) and Soy-corn milk (Kolapo & Oladimeji, 2008).

The utilization of soybean food product in Africa is rapidly gaining momentum. In Nigeria, for example, the private sector is becoming a major player in the market for soybean and soy products. Nigeria now has more than 65 soybean processing plants ranging in size from small village-level mills to plants established by food processing giants such as Nestle Plc and Cadbury Plc. These big processors use soybean to boost the protein contents of baked goods, breakfast cereals, weaning foods and dairy products. In a related development, an alliance between Sun Opta Inc and Specialized Protein Products (SPP) of South Africa is targeted at manufacturing and selling liquid and powdered soy ingredients and soy milk beverages in Africa and other International markets. Sales of liquid and powdered products from this alliance are expected to grow over time to over $15 million.

5. Soybean in nutrition and health

Soybean is often described as the miracle golden bean, the pearl of the Orient, the Cinderella crop of the century, the meat that grows on vines, the protein hope of the future and the salvation crop among others. These attributions are mainly due to the relatively high protein content (about 40%) contained in soybean seeds. It also contains approximately 20% fat. The fatty acids in soybean are majorly unsaturated types e.g. oleic and linoleic acid. Unlike the saturated fat in animal protein, it is suitable in reducing heart ailment which may be caused or aggravated by excessive intake of cholesterol from animal fat. Soybean also provide adequate amount of carbohydrate, digestible fibres, minerals, vitamins etc. In addition to its high food value, it is one of the least expensive sources of protein when compared to eggs, milk, beef and cowpea (IAR&T, 1988).

Soybean is a major source of vegetable protein and oil for human and animal consumption and also for industrial use. Soybean oil is widely used throughout the world for human consumption as margarine, salad, cooking oils and shortenings (Lapades, 1977). Nutritionally, soy proteins are superior among vegetable proteins since they contain good supplies of essential amino acids, though they are slightly deficient in some sulphur containing amino acids such as cysteine and methionine. In a report by IAR&T (1987), it was shown that soybean ranks the highest among leguminous crops in terms of protein utilization and efficient ratio compared with other plant sources. For instance, soybean has a higher total digestible nutrient percentage of 91.99% compared to cowpea with 79.52%. Thus soybean consumption is more relatively helpful in solving nutrition protein-intake problem among the poor people. Though it lack starches, soybean contain such other carbohydrates as cellulose, pectin and phytic acid. Not only does cellulose promote good elimination together with other indigestible fibre, it helps in maintaining good physical condition and preventing rectal cancer. (Tokuji & Asako, 1984).

Studies by Lassitar (1981) revealed that soybean oil is highly digestible, high in polyunsaturated fatty acids, about 85.0% with no cholestrol. The studies also showed that mature soybean seed contains vitamins such as thiamine, niacin, riboflavin, cholin, vitamins E and K. These vitamins are necessary for normal body growth and development. Whole soybean contains 1.6% potassium, 0.3% calcium, 0.3% magnesium and minute quantity of iron, zinc and copper. The mineral present in soy products can contribute to the overall requirement especially for children and pregnant women. (NAERLS/FDA, 1994)

Lecithin is also present in soybean. Davis (1979) reported that adequate diet including soya lecithin will free the cholestrol deposits in the blood vessels, suspend the particles in the blood, carry them away and metabolise them. In this way, the blood vessels are free and blood pressure returns to normal. It is on this premise that Sanni (2000) recommends that people who cannot afford lecithin should include soyabeans in their diet.

There is no doubt, a strong nexus between nutrition and health. The treatise by Scrimshaw et al. (1968): Interaction of Nutrition and Infection, set out a paradigm which has endured for almost a half century. Similarly, Solomon (2007) stated that demographics, food supply and epidemiological transitions determine the specific relevance of the malnutrition-infection interaction in individual circumstances. The strong link between a good nutrition and a good health must have been responsible for the invaluable role which soybean has been playing in maintaining a better health in its consumers for many centuries. Data from different regions of the world have shown that consumption of food containing soybean and soybean products has been associated with improved heart disease risk factor, reduced osteoporosis, alleviation of menopausal symptoms, reduced cancer risk, diabetes and serum

cholesterol. Soybean consumption also help in reducing obesity; this is consequent upon soybean isoflavones especially which cause production of fewer and smaller fat cells (Naaz et al., 2003). Report has shown that soybean could help minimize coronary heart disease through controlling cholesterol, blood pressure, vascular function and direct effects on the cells of the artery wall (AHA, 2000). Men which were at risk of developing coronary heart disease consuming soybean diets have been found to have significant reductions in both diastolic and systolic blood pressure (Sagara et al., 2004).
Studies have established the beneficial effect of soybean to diabetic patients particularly Non-Insulin Dependent Diabetes Mellitus (NIDDM). The protein and fibre in soybeans can prevent high blood sugar level and help in keeping blood sugar levels under control. In addition, the proportion of potassium to sodium (3/1- 11/1) makes soybean an ideal food for diabetes mellitus patients (Lijuan et al., 2000). Furthermore, Texeira et al. (2000) submitted that soy protein help diabetic patients prevent kidney diseases and improve the cholesterol profile. There are evidences that soy foods may help reduce bone loss that typically occurs after menopause. Soya isoflavone can help women with low bone mineral content prevent hip fractures in post menopausal years (Chen et al. 2003; Anderson 2003; Koh et al. 2009). In areas of the world where soybeans are eaten regularly, rates of colon cancer, as well as some other cancers including breast cancer tend to be low. Soybean contains relatively high amounts of glucosycermide, which may be the reason for the cancer –preventive effect of eating soy foods (Symolon et al., 2004). In more recent studies, it was established that soy food consumption was significantly associated with decreased cancer recurrence and death (Shu et al., 2009; Guha et al., 2009).

6. Africa's nutrition situation

Many of the developing world's poorest producers and most undernourished households depend on Root and Tubers (R&T) as a contributing, if not principal, source of food and nutrition (Alexandratos, 1995). In part, these farm households value R&T because R&T produce large quantities of dietary energy and have stable yields under conditions in which other crops may fail (Alexandratos, 1995). Among these culprit developing countries; African countries are of major concern. More than 30 edible and edible species of R&T are grown today. Foremost among them in terms of aggregate output and estimated value of production are cassava, potato, sweet-potato and yam. Other prominent R&T include cocoyam, ginger, taro, yam bean as well as Andean R&T such as arracacha, mashua,oca and ulluco (Scott et al., 1992).
The diets of people in many developing countries (of which substantial part are in Africa) comprise mainly starchy materials. Unfortunately, animal sources of proteins, which are used to compliment the starchy diets are expensive and out of reach for low-income families (Obatolu et al., 2007). In Nigeria, statistics on cassava production and consumption indicated cassava as major national food and cash crop. According to Ouraga-Djoussou & Bokanga (1998), annual consumption of cassava in Nigeria doubled to 250 kg per capita between 1983 and 1994. Cassava production increased from 14.4 milliom mt to 31.1 million mt during 1982-97 (FAO 1999). The increase in consumption and output of cassava can be attributed to several factors. Given Nigeria's low per capita income and rapid population growth, cassava has served as both a basic staple and food security crop; the ban on cereal imports between 1987 and 1990 provided an added stimulus to its production (Adeniji et al., 1997). The crop's multiple uses have also facilitated greater consumption. Roots are

consumed in fresh, boiled form; as toasted granules widely known as *gari*; as chips/flour (or *lafun*); and as unsteamed wet paste (or *fufu*) (Nweke, 1994).

Because the demand for cassava as a food commodity has remained strong, commercial sales of both processed products and fresh roots as raw material for food processing have become a highly profitable undertaking, due in part to technical improvements in processing and the introduction of high yielding varieties (Nweke et al., 1988). According to estimated expenditure elasticities for processed cassava (*gari*), urban households treat it as normal good (Nweke et al., 1994). In another estimates, based on the six-country Collaborative Study of Cassava in Africa (COSCA), expenditure elasticities for rural households hover around 1.0 for fresh and processed cassava (Ezemenari et al., 1998), which is similar to the elasticities for high-value foods. From these studies it was concluded that continued urbanization and improvements in income are likely to translate into continued strong demand for cassava in Nigeria and other Africa countries covered by the study. However, this trend signals a great danger for a populace which depends majorly on cassava on the understanding that cassava roots are an excellent source of carbohydrates but are deficient in protein, vitamins and some minerals. It is therefore not surprising that both Protein Calorie Malnutrition (PCM) and Macro and Micro Nutrients Malnutrition (MMNM) are common characteristics of many African populations. Consequently, it is equally not suprprising that the 2009 Global Acute Malnutrition (GAM) prevalence values in many African countries were either poor, serious or critical; with a very few being acceptable (Figure 3).

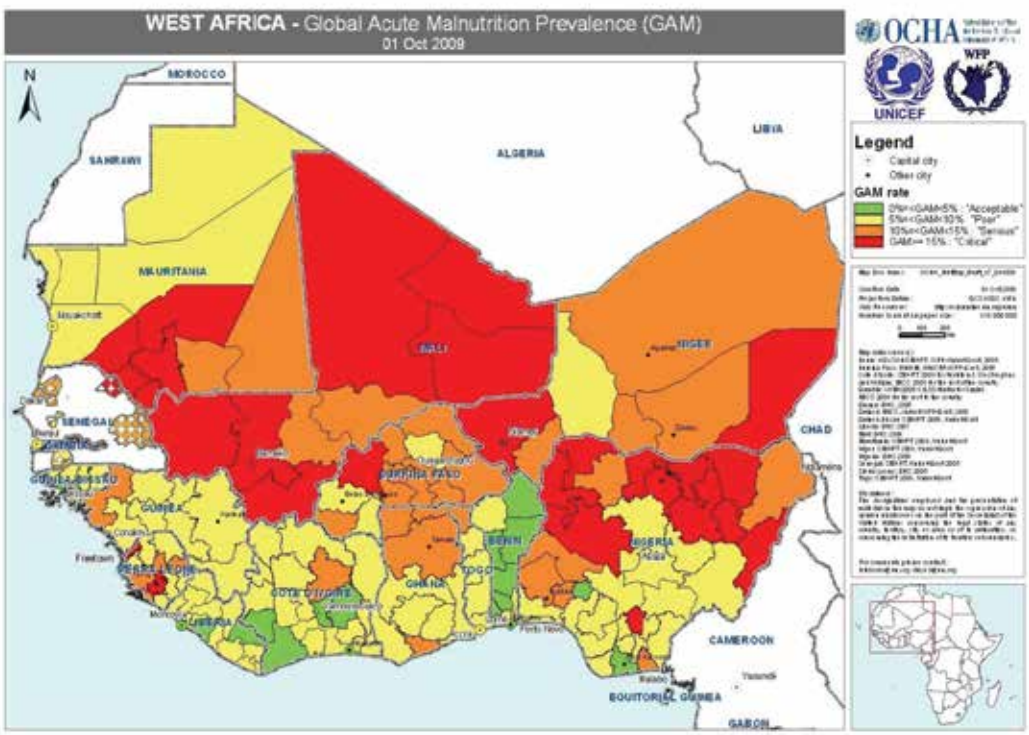

Fig. 3. Global Acute Malnutrition Prevalence (OCHA, 2009)

While addressing the state of nutrition in Africa, another important factor which deserves serious attention is the changing diet and the accompanied disease of lifestyle. Previously in African countries, most populations were very poor and they ate frugally. Their intakes of energy and fat were relatively low, but those of fiber-containing foods were high, as is usual with high consumption of plant foods (Lube, 1971). These are still the dietary features of those living traditional lifestyles and, to a large extent, current rural Africans (Vorster et al., 1994). In the past, prevalences of chronic diseases due to lifestyle were low (Trowell, 1960). However, within the past generation, diets have changed with increases in energy and fat intake but decreases in fiber intake (Bourne et al., 1993). Such diets, with ongoing changes have been associated with the variably rising prevalences of the nutrition-related diseases (Walker 1996; Walker & Segal 1997). In South Africa and some other countries in Sub-Saharan Africa, among Africans, changes in diet and other environmental factors particularly among urban dwellers have evoked large rises in the occurrences of some chronic disorders and disease of lifestyle but hardly any rises in others. Thus, major increases have occurred in obesity in women, hypertension and diabetes in both sexes and cerebrovascular disease (Walker 1996; Walker & Segal 1997). Judging from the experiences of the various populations described, it seems likely that, within the next generation, a marked rise in the occurrence of Coronary Heart Disease in urban African population would be inevitable (Walker et al., 2002).

7. Soybean and the future Africa

There is no doubt that Malnutrition is one of the major Africa's health challenges. As stated earlier, this hydra-headed problem manifests in form of Protein Calorie Malnutrition (PCM) and Macro and Micro Nutrient Malnutrition (MMNM). The resources to tackle this menace is not all that large in Africa; and with the worsening economic performance in many African countries, the end of malnutrition in Africa, is not in sight if some critical and decisive steps are not urgently taken.

In 2000, the world pledged through the Millenium Development Goals (MDGs 1 and 2) to halve hunger and ensure Universal Primary Education by the year 2015. It has since been recognised that school feeding programmes could be a key to achieving these goals, because they bring many synergies that support each other. In response, some national governments in Africa launched School Feeding and Health Programmes. In a particular school feeding attempts by World Food Programme (WFP) in areas of high food insecurity(which included many African countries), fortified biscuits and take home rations have been used (Gelli et al., 2009). However, a study by Kolapo & Sanni (2009) suggested that using a West African foods such as gari and tapioca which are fortified with whole soybean flour rather than `piecemeal` fortifications will help meet Recommended Daily Allowance (RDA) of many macro and micro nutrients

Reports from Nigeria, Zimbabwe and Kenya have shown that families growing and utilising soybean are more healthier than those families that do not use soybean as part of their diets. Owing to the greatly invaluable nutritive and health benefits furnished by soybased foods, there is a need for a renewed, concerted and sustainable incorporation of soybean into African diet. The IITA soybean success story recorded particularly in Nigeria, Zimbabwe, Uganda and South Africa must be replicated in other African countries.

As stated earlier, previous reports have shown that the changing diets of African is excercibating disease of life style such as diabetes, obesity and the likes. Incidentally,

adoption of soybean has been shown to reduce the incidence of such disease. As the old adage goes, a wealthy nation is a product of a healthy nation. In this regards, various African national governments should consider soybean as being pivotal to building a healthy population. The attributes of soybean as a cinderella food crop can be utilised by African leaders in solving the challenges of food insecurity in the continent as soybean production technolgy matches the resource of small-scale farmers with the principal inputs being good seed of an adapted variety and useful information. The report of Rukuni (2002) shows how investment, technology and national institutions must interact to bring about food security in Sub-Saharan Africa, the region of the world where all indicators of hunger are worsening. There is no other time other than now which African leaders are to rise to this challenge; and in doing so, soybean should be considered as the cinderella food crop.

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10

Protease Inhibitors, Lectins, Antifungal Protein and Saponins in Soybean

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1. Introduction

Many investigations have been conducted on soybean during the past decade since it is a unique dietary source of isoflavones which display a diversity of biological activities and reduce the risk of some chronic diseases. Soybean is distinctive in that it has a high content of isoflavones which allegedly diminish the risk of cancer, cardiovascular disease, and osteoporosis, and also alleviate menopausal symptoms. Isoflavones are weakly estrogenic. The isoflavone genistein also affects signal transduction. Soyfoods and isoflavones have arrested the attention of many researchers on account of their potential role in preventing and treating cancer and osteoporosis. The low breast cancer mortality rates in Asia and the putative antiestrogenic infer that soyfood intake reduces breast cancer risk. Soy or isoflavones may decrease the risk of prostate cancer. The low estrogenic activity of soybean isoflavones and their structural resemblance to the synthetic isoflavone ipriflavone, which elevates bone mineral density in postmenopausal women, suggest that soy or isoflavones may decrease the risk of osteoporosis.
Besides isoflavones, soybean also produces protease inhibitors, lectins, and antifungal proteins, which have important biological activities. This review encompasses a discussion of isoflavones, protease inhibitors, lectins and antifungal proteins.

2. Protease inhibitors

Protease inhibitors in soybeans can reduce protein digestion, and induce pancreatomegaly and enhance chemically induced pancreatic tumors in some animals (Grant, 1989). The quantity of protease inhibitors ingested would not have any adverse consequences in humans (Liener, 1994). The trypsin / chymotrypsin inhibitor (Bowman-Birk inhibitor) found in soybeans has been studied as an anticancer agent (Kennedy, 1995).
A large number of protease inhibitors belonging to various types have been purified from different kinds of legumes. In the study of Fang et al. (2010b), by using liquid chromatography techniques, a Kunitz type trypsin inhibitor (KBTI) was isolated from Korean large black soybeans. It exhibited a molecular mass of 20107.645 Da and inhibited the proteases trypsin and alpha-chymotrypsin with an activity of 8520 BAEE units/mg and

24 BTEE units/mg, respectively. Its trypsin inhibitory activity demonstrated pronounced thermal stability (0-100 °C) and stability over a wide range of pH values (pH 3-11). KBTI reduced the activity of HIV-1 reverse transcriptase activity with an IC_{50} value of 0.71 µM. It evoked the release of pro-inflammatory cytokines including TNF-alpha, IL-1beta, IL-2 and interferon-gamma at the mRNA level. KBTI exerted low antiproliferative activity toward CNE-2 and HNE-2 nasopharyngeal cancer cells, MCF-7 breast cancer cells, and Hep G2 hepatoma cells. It was devoid of mitogenic, ribonuclease and antifungal activities.

A 19-kDa trypsin inhibitor with an N-terminal amino acid sequence highly homologous to Kunitz-type trypsin inhibitors was purified from seeds of Chinese black soybean *Glycine max* cv. "Small Glossy Black" using a procedure that involved anion exchange chromatography on Q-Sepharose, cation exchange chromatography on SP-Sepharose and anion exchanger chromatography on DEAE-cellulose. It was bound on all three ion exchangers. It inhibited trypsin with an IC_{50} of 19 µM and chymotrypsin with an IC_{50} of 14.3 µM. Its trypsin inhibitory activity was relatively pH stable and thermostable. It was preserved in the pH range pH 3-pH 13 and in the temperature range 0 °C-60 °C. The trypsin inhibitory activity was reduced in the presence of dithiothreitol (from 5 to 25 mM) in a dose-dependent manner indicating the paramount importance of disulphide bonds to the activity. It inhibited HIV-1 reverse transcriptase with an IC_{50} of 0.16 µM, and exerted antiproliferative activity toward MCF-7 breast cancer cells with an IC_{50} of 4.3 µM and HepG2 hepatoma cells with an IC_{50} higher than 25 µM. The trypsin inhibitor was devoid of antifungal activity and mitogenic activity towards mouse splenocytes (Ye and Ng, 2009a).

A trypsin inhibitor from the seeds of Hokkaido large black soybeans possessed an N-terminal amino acid sequence that closely resembled those of 8-kDa Bowman-Birk trypsin inhibitors. The trypsin inhibitor was unbound on SP-Sepharose but bound on the anion exchangers DEAE-cellulose and Mono Q. It exerted antiproliferative activity toward breast cancer (MCF-7) cells and hepatoma (HepG2) cells with an IC_{50} of 35 and 140 µM, respectively. The trypsin inhibitory activity of the inhibitor was retained following thermal treatment up to 100 °C for 30 min and after exposure to the pH range 2-13 for the same duration. The trypsin inhibitor inhibited HIV-1 reverse transcriptase with an IC_{50} of 38 µM. Moreover, there was no antifungal activity toward *Fusarium oxysporum* and *Mycosphaerella arachidicola* (Ho and Ng, 2008).

Chinese 'Large Black Soybeans' produce a 60-kDa lectin and a 20 kDa trypsin inhibitor. Both proteins were absorbed on Q-Sepharose, but could be separated from one another on Mono Q. Further purification was achieved by gel filtration on Superdex 75. Both trypsin inhibitor and lectin were stable from pH 3 to 13 and from 0 °C to 65 °C. The trypsin inhibitor was stable at pH as low as 2, and it inhibited trypsin and chrymotrypsin with an IC_{50} of 5.7 µM and 5 µM, respectively. Its trypsin inhibitory activity was reduced in the presence of dithiothreitol indicating the importance of the disulphide bond to the activity. Both trypsin inhibitor and lectin inhibited HIV-1 reverse transcriptase (IC_{50} = 44 and 26 µM) and proliferation of HepG2 hepatoma cells (IC_{50} =9 6 and 17 µM) and MCF7 breast cancer cells (IC_{50} = 42 and 13.5 µM) (Ye and Ng, 2009b).

Studies utilizing different types of protease inhibitors as anticarcinogenic agents in vivo and in vitro systems have recently been reviewed. These studies suggest that the protease inhibitors which prevent carcinogenesis affect processes in the early stages of carcinogenesis, although they can be effective at long time periods after carcinogen exposure in both in vitro and in vivo systems. While there is strong evidence that these protease inhibitors can affect both the initiation and promotion stages of carcinogenesis, they have no

effect on already transformed cells. The results have suggested that the first event in carcinogenesis is a high frequency epigenetic event and that a later event, presumably genetic, leads to the malignant state. Protease inhibitors appear capable of reversing the initiating event, presumably by stopping an ongoing cellular process begun by carcinogen exposure. The major lines of investigation on the mechanism of the protease inhibitor suppression of carcinogenesis relate to the ability of anticarcinogenic protease inhibitors to affect the expression of certain oncogenes, and the levels of certain types of proteolytic activities. The anticarcinogenic protease inhibitors have no observable effects on normal cells, but can reverse carcinogen-induced cellular changes for several different end-points studied. The most direct method of determining the mechanism of action of the anticarcinogenic protease inhibitors is to identify and characterize the proteases with which they interact. In the cells of the in vivo and in vitro systems in which protease inhibitors can prevent carcinogenesis, only a few proteases have been observed to interact with the anticarcinogenic protease inhibitors. Proteases have been identified by both substrate hydrolysis and affinity chromatography (Kennedy, 1995).

3. Lectins

Lectins, a class of proteins that reversibly and non-enzymatically bind specific sugars, have been purified from different kinds of legumes. In the study of Fang et al. (2010a), a lectin (KBL) with a molecular mass of 48 kDa was isolated from Korean large black soybeans. The specific hemagglutinating activity of the lectin was 4096 titer/mg. The metal chelator EDTA brought about a decline of hemagglutinating activity. The activity could be reinstated by addition of Fe^{3+} ions and divalent cations such as Ca^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, and Pb^{2+}. The lectin was specific to sugars including D-(+)-galactose, D-(+)-raffinose, L-(+)-arabinose, alpha-D-(+)-melibiose, and alpha-lactose, the hemagglutinating activity of the lectin could be inhibited by these sugars. The lectin manifested remarkable thermal stability and stability over a wide range of pH values. The lectin demonstrated HIV-1 reverse transcriptase inhibitory activity with an IC_{50} of 1.38 µM. However, it lacked antifungal, cytokine inducing, mitogenic and ribonuclease activities. In addition, it did not inhibit proliferation of nasopharyngeal cell lines at concentrations up to 20 µM.

A lectin was purified from the seeds of the cultivar of soybean (*Glycine max*), called the small glossy black soybean. The purification protocol involved anion exchange chromatography on Q Sepharose, cation exchange chromatography on SP Sepharose and fast protein liquid chromatography anion exchange chromatography on Mono Q followed by gel filtration on Superdex 75. The dimeric 50 kDa melibiose-binding lectin with an N-terminal sequence identical to that of soybean lectin was bound on all three ion exchangers. Of all the sugars tested, melibiose most potently inhibited the hemagglutinating activity of the lectin. The lectin was stable between pH 3-12 and 0-70 ^{o}C. The lectin elicited maximal mitogenic response from murine splenocytes at about the same molar concentration as Con A. Although the magnitude of the maximal response was smaller, the soybean lectin suppressed the activity of HIV-1 reverse transcriptase and exerted antiproliferative activity toward breast cancer MCF7 cells and hepatoma HepG2 cells with an IC_{50} of 2.82 µM, 2.6 µM and 4.1 µM, respectively. The lectin lacked antifungal activity. Another lectin isolated from a different cultivar of soybean called little black soybean was grossly similar to small glossy black soybean lectin but the former had a larger subunit molecular mass (31 kDa), a more potent mitogenic activity and lower thermostability. The data suggest that different soybean cultivars produce similar but not identical lectins (Lin et al., 2008).

4. Antifungal proteins

A 25 kDa monomeric antifungal protein with an N-terminal amino acid sequence exhibiting homology to a segment of chitin synthase, was purified from the seeds of the black soybean *Glycine soja*. The protein was designated as glysojanin. It potently inhibited mycelial growth of the fungi *Fusarium oxysporum* and *Mycosphaerella arachidicola*. It inhibited HIV-1 reverse transcriptase with an IC_{50} of 47 μM, [methyl-^{3}H]thymidine incorporation by mouse splenocytes with an IC_{50} of 175 μM, and ^{3}H-leucine incorporation into proteins in the cell-free rabbit reticulocyte lysate with an IC_{50} of 20 μM. The isolation protocol involved anion-exchange chromatography on DEAE-cellulose, affinity chromatography on Affi-gel blue gel, cation-exchange chromatography by fast protein liquid chromatography on Mono S, and gel filtration by fast protein liquid chromatography on Superdex 75 (Ngai and Ng, 2008).

5. Saponins

Saponins are glycosides composed of a lipid-soluble aglycone consisting of either a sterol or, more often, a triterpenoid structure linked to water-soluble sugar residues that vary in their type and quantity. Legumes are the major sources of dietary saponins. The same bean can have different types of saponins. Saponins are very poorly absorbed. Most saponins form insoluble complexes with 3-β-hydroxysteroids and interact with and form large, mixed micelles with bile acids and cholesterol. Although saponins were shown to lower cholesterol in some animal species, the hypocholesterolemic effects of saponins in humans are more speculative (Milgate and Roberts, 1995). Saponins may have anticancer properties, as suggested by a recent rodent study that found that a saponin-containing diet (3% by wt) inhibited by about two-thirds the development of azoxymethane-induced preneoplastic lesions in the colon (Koratkar and Rao, 1997). However, given that human intake of saponins is generally 200–300 mg/d whereas total food intake is <500 g (dry weight), it is not clear to what extent these results in rodents are relevant to humans (Ridout et al., 1988).
The effect of saponins isolated from soya bean flour on the incidence of aberrant crypt foci (ACF) induced by azoxymethane (AOM) in the colonic wall of CF1 mice was investigated. Four weekly injections of AOM, a known colon carcinogen, were administered to mice. One week after the last injection, mice were placed on an AIN-76 diet supplemented with 3% soya bean saponins or continued on the basal AIN-76 diet. Another group of mice was placed on the saponin diet without AOM initiation to observe the effect of saponins on the growth characteristics of mice. Dietary intake of soya saponins significantly reduced the incidence of ACF at the end of 14 weeks (postinitiation). Noninitiated mice maintained on a similar soya bean saponin-supplemented diet did not show any adverse effects on the growth and overall health of the animals. These findings suggest that soya bean saponins can play an important role in inhibiting the incidence of ACF in the colon of mice (Koratkar and Rao, 1997).
Daidzein, an isoflavone in soybeans, suppresses the growth of HL-60 cells implanted in mouse subrenal capsules (Jing et al., 1993). Genistein inhibits the proliferation of a diversity of both hormone-dependent and hormone-independent cancer cells *in vitro* with an IC_{50} between 5 and 40 mM (2–10 mg/mL), including breast (Peterson and Barnes, 1991; 1996; Pagliacci et al., 1994; Peterson et al., 1996; So et al., 1996; Clark et al., 1996; Zava and Duwe, 1997), prostate (Peterson and Barnes, 1993; Naik et al., 1994; Kyle et al., 1997), colon (Kuo et

al., 1997; Kuo, 1996), and skin (Rauth et al., 1997) cancer cells (Adlercreutz and Mazur, 1997; Akiyama and Ogawara, 1991; Constantinou and Huberman 1995). Genistein suppresses the metastatic activity of breast (Scholar and Toewa, 1994) and prostate (Santibáñez et al., 1997) cancer cells *in vitro* independent of the effects on cell growth. The anticancer effects of genistein *in vitro* (Wei et al., 1993) are attributed to its inhibitory effects on enzymes that play a role in signal transduction, including ribosomal S6 kinase (Linassier et al., 1990), MAP kinase (Thorburn and Thorburn, 1994), and tyrosine protein kinases (Akiyama et al., 1987). Genistein also inhibits the activity of DNA topoisomerase II (Constantinou et al., 1990) and raises the *in vitro* concentrations of transforming growth factor β (TGF β) (Peterson et al., 1998) which may suppress growth of cancer cells (Benson and Colletta, 1995; Benson et al., 1996; Markowitz and Roberts, 1997). Genistein has an important role as a potent inhibitor of angiogenesis *in vitro* (Messina, 1999).

Genistein exerts an inhibitory action on the growth of both estrogen-dependent and estrogen-independent breast cancer cells *in vitro*, but it is not certain if cellular concentrations of genistein *in vivo* can reach *in vitro* concentration capable of suppressing growth of breast cancer cells. Genistein inhibits proliferation of induced serum and epidermal growth factor–stimulated normal human mammary epithelial cells with IC_{50} values substantially lower than those for transformed human breast epithelial cells (Peterson and Barnes, 1994) suggesting that soy intake may inhibit the initiation of cancer cells, rather than impeding the proliferation of existing cancer cells. Because synergistic effects between genistein and daidzein have been observed *in vitro*, it would be of interest to examine their concerted action *in vivo* (Evans et al., 1995; Franke et al., 1995). The possibility remains that other components of soybeans, individually or in conjunction with isoflavones, account for the hypothesized anticancer effects of soyfoods. Wrensch et al (1991), McMichael-Phillips et al (1998), and Cassidy et al (1994; 1995) demonstrated that soy or isoflavones are potentially capable of producing physiological actions related to breast cancer risk. Cassidy et al (1994) noticed that the intake of soy, in particular isoflavone-rich soy (Cassidy et al., 1995), prolongs the follicular phase and circulation levels of gonadotropins. Brown and Lamartiniere (1995), Lamartiniere et al (1995), and Murrill et al (1996) were of the opinion that early consumption of soyfoods by young girls may prevent breast cancer development later in life. Neonatal or prepubertal exposure to genistein inhibits the development of dimethylbenz(a) anthracene-induced mammary tumors in rodents and prolongs the latency period (Brown and Lamartiniere, 1995; Lamartiniere et al., 1995; Murrill et al 1996).

Genistein exerts and antiproliferative action on both androgen-dependent and androgen-independent prostate cancer cells *in vitro* (Peterson and Barnes, 1993; Naik et al., 1994). It also diminishes the potential of prostate cancer cells to metastasise independent of cell growth inhibition. Concomitantly tyrosine phosphorylation of an unidentified molecular species declines (Santibáñez et al., 1997). Though the role played by estrogen in prostate cancer has not been clarified, estrogens are effective in the therapy of metastatic prostate cancer (Pienta and Esper, 1993). Hence the potential estrogenic effects of isoflavones may be protective. Genistein inhibits 5-α-reductase in genital skin fibroblasts and benign hyperplastic prostate tissue (Evans et al., 1995). This enzyme converts testosterone into a more potent form dihydrotestosterone, which stimulates the growth of prostatic tissue. Ross et al (1992) showed that biomarkers of 5-α-reductase activity are higher in white and black men compared with Japanese men. The *in vitro* results of Evans et al (1995) agree with data from Lu et al (1996), showing that following 1 month of soymilk consumption (36 oz/d), the

serum concentration of 3α, 17β-androstanediol glucuronide, a dihydrotestosterone metabolite, was significantly lowered. In mice fed a diet containing soy for 9 months, the incidence of prostatic dysplasia, considered to be a preneoplastic prostate lesion, was significantly diminished. At 12 months, however, difference between the 2 groups was much attenuated. These results are in line with the epidemiologic data noted above and also with the results of a study of MNU-induced prostate tumors in Lobund-Wistar rats (Pollard and Luckert, 1997).

Rats receiving a diet containing soy had a shorter latency period than those fed a diet containing soy high in isoflavones with a small quantity of isoflavones (Pollard and Luckert, 1997). A diet containing soy flour (33% by weight) for 4 months inhibited tumor growth in rats with Dunning R3327 PAP tumors implanted (Zhang et al., 1997). Schleicher et al (1998) Genistein (50 mg/kg body weight) given to rats subcutaneously in the dorsal scapular area every 12 h stranting at the time of tumor cell transplantation inhibited development of prostate tumor implanted with prostate cancer cells and totally inhibited development of lung metastases. Dalu et al (1998) reported that genistein (1 mg genistein/g diet) caused a decline in weight of the dorsolateral and ventral prostates and down-regulated the expression of tyrosine-phosphorylated proteins in rats. genistein dose-dependently inhibited ^{3}H-thymidine incorporation in cultured benign prostatic hypentrophy tissue and prostate cancer tissue (Geller et al., 1998) Although genistein suppressed growth of prostatic cancer cells in vitro, when the metastatic MAT-Lylu prostate cancer cells were injected into the right flank of rats, oral treatment with genistein did not suppress the prostate tumors development (Naik et al., 1994). The doses utilized more closely resembled human dietary intake than those employed in the investigations of Schleicher et al (1998) and Dalu et al (1998). Higher doses of genistein injected intraperitoneally had little effect on tumor growth (Naik et al., 1994). There are limited human data available for use in addressing the soy–prostate cancer hypothesis, although Severson et al (1989) noted that tofu consumption might produce a lower risk of prostate cancer. Isoflavones are detected in the prostatic fluid, at the highest concentrations in men from soyfood-consuming countries (Morton et al., 1997). Isoflavones are concentrated several-fold in the prostatic fluid compared to plasma concentrations,.

Genistein inhibits DNA synthesis in human prostate cells *in vitro* and reduces effect of testosterone in prostate cancer development in rats (Jenkins et al., 2003; Adlercreutz et al., 2000). However, a daily soybean intake sufficient to bring about a decline LDL-cholesterol, does not effect serum concentration of prostate specific antigen (Adlercreutz, 2002). Antifungal proteins (Ma et al., 2009) lectins/hemagglutinins (Lin et al., 2008) and protease inhibitors (Fang et al., 2010) demonstrate antiproliferative activity toward tumor cells *in vitro*. Some of these show pH stability and thermostability and thus their aforementioned activities may be retained *in vivo*.

Fotsis et al (1993) observed that genistein at high concentrations interfered with the action of bovine microvascular cells to invade collagen gels and form capillary-like structures when treated with basic fibroblast growth factor. Antiangiogenic agents by preventing tumor-stimulated angiogenesis, inhibit tumor growth beyond a size of 1–2 mm and thus from becoming clinically insignificant (Folkman and Klagsbrun, (1987). A low genistein concentration is required to inhibit angiogenesis *in vitro* (Adlercreutz and Mazur, 1997).

The antimetastatic activity of soybean saponin has been examined by assessing matrix production of the metalloproteinases MMP-2 and MMP-9 in HT-1080 cells (Kang et al., 2008). MMP-2 and MMP-9 mRNA expression levels were determined by RT-PCR analysis and the levels of secreted MMP-2, MMP-9 and tissue inhibitor of metalloproteinase-2 (TIMP-

2) were assessed by gelatin zymography and/or ELISA. The invasion of a Matrigel-coated membrane by human fibrosarcoma HT-1080 and HT-29 colon cancer cells was determined by counting the migrated cells. Exposure of HT-1080 cells to soybean saponin diminished the mRNA expression of and attenuated the secretion of MMP-2 and MMP-9. However, the secretion of TIMP-2 was enhanced in a dose-dependent manner. The invasion of HT-1080 cells through a Matrigel-coated membrane was suppressed. The antimetastatic activity of soybean saponin was further confirmed in an *in vivo* mouse experiment in which CT-26 colon cancer cells were injected via the caudal vein after administering soybean saponin in the diet. The incidence of metastatic tumor colonization of lungs in mice underwent a mild decline 14 days after injection of CT-26 cells via the caudal vein. Thus, soybean saponin reduces tumor cell metastasis by inhibiting production of MMP-2 and MM-9 production and enhancing TIMP-2 secretion.

The structural resemblance between estrogen, isoflavones and the synthetic isoflavone, 7-isopropoxyisoflavone (ipriflavone), which increases bone mass in postmenopausal women (Valente et al., 1994; Brandi, 1992), inhibits osteoclast activity in vitro (Tsude et al., 1986) raises speculation about the benfits of isoflavones bone health. The ipriflavone has to be metabolized in order to be maximally effective. Daidzein, a soybean isoflavone is one of the metabolites.

That genistein in particular affects bone density in rats (Blair et al., 1996; Fanti et al., 1998). Blair et al (1996) observed that the dry femoral mass of ovariectomized rats fed 30 mmol genistein in particular /day for 4 weeks was 12% higher ($P < 0.05$) than that of the controls. In a study by Fanti et al (1998), after 21 days of subcutaneous injection of 5 and 25 mg genistein/g body wt, tibial bone mineral loss in ovariectomized rats was significantly reduced. Potter et al (1998) reported that following 6 months of treatment, lumbar spine bone mineral density was significantly increased in postmenopausal women who had a daily intake of 40 g soy protein containing 2.25 mg isoflavones/g protein, while bone density remained unchanged in women who ingested the same amount of soy protein but containing only 60 % isoflavones (1.39 mg isoflavones/g protein). Some insight has been gained into the possible mechanism(s) regulating the effect of isoflavones on bone health in rats. Isoflavones may both stimulate and inhibit bone formation. Fanti et al (1998) reported that genistein augmented the number of osteoblasts and the serum osteocalcin level, but had no effect on number of osteoclasts.On the other hand, Blair et al (1996) noted that genistein inhibited avian osteoclast protein synthesis *in vitro* probably due to its inhibitory effects on tyrosine phosphorylation. Estrogen and tamoxifen, which inhibit bone resorption, induce apoptosis in osteoclasts, an effect which is antagonized *in vitro* by antibodies to TGF-β (Hughes et al., 1996). Since genistein enhanced TGF-β *in vitro*, the effect of the isoflavone bone resorption may involve TGF-β.

The decrease in hepatic cholesterol synthesis is caused by a decrease in serum insulin concentration because insulin activates an enzyme involved in cholesterol synthesis and, on the other hand, it might be attributed to a change in the hepatic bile acid profile (Mälkki, 2001).

The isoflavones in diet containing soybean may retard atherosclerotic progression by virtue of their inhibitory activity toward LDL oxidation, which evokes a series of events forming atherosclerotic plaques. Isoflavones may elicit a cholesterol-lowering action due to interaction with estrogenic receptors, and structural similarity between isoflavones in a diet containing and their metabolites and estrogens. (Anderson et al., 1999). Various clinical studies have disclosed the importance of consumption of soybean protein with its isoflavones to achieve a hypocholesterolemic effect (Lichtenstein, 1998; Farriol et al., 2006).

The Asian diet is rich in soybean foods compared with the western diet (Craig, 1997). Inclusion of isolated soybean protein with isoflavones in the diet of postmenopausal women causes a decline in the incidence of hot flashes (Albertazzi et al., 1998; Setchell and Cassidy, 1999).

In postmenopausal women, isoflavones exert a weak estrogenic action and hence could be exploited as a dietary alternative or supplement to hormone replacement therapy (Setchell and Cassidy, 1999; Duffy et al., 2003). Improved cognitive ability was observed in postmenopausal women after intake of soybean extract containing isoflavones. Two types of estrogenic receptors (ER-α and ER-β) are found in the brain. Isoflavones bind preferentially to ER-β receptors, which play a role in cognitive function and are found in abundance in brain regions involved in cognition (Duffy et al., 2003).

From the foregoing account it can be seen that isoflavones represent important bioactive components of soybean. In addition, protease inhibitors, lectins and antifungal proteins in soybean display a multiplicity of health promoting activities such as antitumor, mitogenic and antimicrobial activities (Table 1). Hence the intake of soybean is beneficial to health.

6. Conclusion

The above review of literature has revealed that protease inhibitors in some cultivars of soybean have marked thermostability and pH stability and may account for the reduced incidence of cancer in populations favouring a diet rich in soybeans. Soybean saponins also may account for part of the antitumor activity in soybeans. Soybean saponins also display other health promoting effects such as anti-osteoporotic activity. Lectins and antifungal proteins also exhibit antitumor and other activities. Thus a regular dietary intake of soybeans is beneficial to health and should be encouraged.

Bioactive component s of soybean	Biological activities
Protease inhibitors (Bowman – Birk and Kunitz types)	Antitumor, HIV-1 reverse transcriptase inhibitory
Lectin	Antitumor, HIV-1 reverse transcriptase inhibitory
Antifungal protein	Antifungal, HIV-1 reverse transcriptase inhibitory, anti-mitogenic
Saponins	Hypocholesterolemic , anti-atherosclerotic , increase bone mineral density

Table 1. Bioactive components of soybean and their biological activities

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11

SOYBEAN: A Multifaceted Legume with Enormous Economic Capabilities

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1. Introduction

Soybean is the broad bean classified under plant legume with its scientific name as *Glycine max*. It is known for more than 3000 years in Southeastern Asia. Due to its versatility and exceptional health benefits it is being cultivated across the whole world. Different varieties of this truly amazing legume are available throughout the year. It is found in varied of sizes as well as seed coat colors, right from black, brown, blue, yellow or mottled. Like other beans, soybeans grow in pods and come under category of edible seeds.

About 85% of the world's soybeans are processed annually into soybean meal and oil. Approximately 98% of the soybean meal is crushed and further processed into animal feed with the balance used to make soy flour and proteins. Of the oil fraction, 95% is consumed as edible oil; the rest is used for industrial products such as fatty acids, soaps and biodiesel. It is one the most widely researched and health-promoting cheapest food. The key benefits are related to their excellent protein content (contains all 8 essential amino acids), high levels of essential fatty acids, numerous vitamins and minerals, isoflavones, and fiber. Most of the soy products act as perfect replacements for meat and dairy products specifically required for vegetarians. For children, soybeans are effective in their growth and development. Soybeans have considerable amounts of *a*-linolenic acid, omega-6 fatty acid and isoflavones (genistein and daidzein) (Messina, 1995). While the isoflavones present reduces the risk of developing cancer of breast, cervical, ovarian, lung and colon, protein helps in lowering cholesterol levels, thus helpful in reduction heart and blood pressure associated diseases.

Soybean is one of the important crops taken into consideration as candidature of genetically modified (GM) foods due to its great demand worldwide. GM organisms have had specific changes introduced into their DNA by genetic engineering techniques. These techniques are much more precise than mutagenesis and breeding where an organism is exposed to radiation or chemicals to create a non-specific but stable change. The herbicide resistant gene has been taken from bacteria and inserted into soybean that has led to resistance to glyphosate or glufosinate herbicides. United States (US) stands first in using GM soybean and also it is one of the major exporters across the world. There are various issues associated with genetic modification, particularly its long term affects on environment. The technique seems to be analogous to nuclear power, nobody loves it, but climate change has made genetic modification, a most important requisite. It offers both faster crop adaptation and a biological, rather than chemical approach leading to tremendous increase in crop yield.

Soybean contains about 30% of soluble and insoluble carbohydrates. The primary soluble carbohydrates in the soybean are: stachyose, raffinose and sucrose. These sugars play significant role in determining viability and germinating ability of soybean seeds. The amounts of these sugars vary according to the variety of soybean and its growing conditions (Rotundo & Westgate, 2009). The oligosaccharides raffinose and stachyose are significantly important for human health by promoting growth of bifido bacteria present in the lower intestine. Bifido bacteria has significant role in reducing the incidence of many diseases of lower tract including colon cancer (Rada *et al*., 2002).

Soybean is a nutritious meal with tremendous food value. It has been explored worldwide for its inclusion in variety of food and is also an important constituent for animal feed. Soybean has been explored for variety purpose to have its better economic capabilities. It is the source of various enzymes, particularly important in seed imbibition and germination. Many of these enzymes have industrial significance and due to their plant origin are more acceptable, particularly by food industry. The present chapter is based on economic capabilities of soybean in variety of fields and its emerging significance in enzyme based industries.

2. History

Linguistic, geographical and historical evidence suggest that the soybean emerged around 11th century B.C. (during Shang dynasty) in the eastern half of north China. The plant's wild precursor was a recumbent vine, *Glycine max* var. *ussuriensis*. During the early centuries of domestication, the soybean was nothing like as important in the Chinese diet as it is today. In fact it may well have been far more useful as a fertilizer than as a food-ploughed back into the soil to enriching it for other crops such as wheat or millet. The soy plant belongs to member of a family of plants that has the ability to draw nitrogen from the air, impart it into the soil through its roots thus enriching poor soils.

By the first century A.D. it reached central and south China, as well as peninsular Korea. The movement of the soybean within the primary gene center is associated with the development, consolidation of territories, and degeneration of Chinese dynasties. From about the first century A.D. to the Age of Discovery (15-16th century), soybeans were introduced into several countries and land races developed in Japan, Indonesia, Philippines, Vietnam, Thailand, Malaysia, Burma, Nepal and north India. These regions comprise the secondary gene center. The movement of the soybean throughout this period was due to the establishment of sea and land trade routes and the rapid acceptance of the plant as a staple food by other cultures like Indonesians. For centuries, the soybean has been the cornerstone of East Asian nutrition. In the late 16th century and throughout the 17th century European visitors to China and Japan had noted in their diaries the use of a peculiar bean from which various food products were produced. They noted that the Japanese flavor fish dishes with a certain sauce called misol, what Europeans call gravy is made from a bean grown in various localities of Japan. In 1665, Friar Domingo Navarrete described tofu as a common and cheapest food of China. In the 17th century soy sauce was a common item of trade from the East to the West. In 1679, John Locke noted that mango and soy are two sauces brought to England from the East Indies. It was not until 1712, when Engelbert Kaempfer, who lived in Japan during 1691 and 1692 as a medical officer of the Dutch East India Company, published in his book *Amoenitatum Eroficum*, that the Western world fully understood the connection between the cultivation of soybeans and its utilization as a food plant. It reached

the Netherlands before 1737 as Linnaeus described the soybean in the *Hortus Cliffortianus* which was based on plants cultivated in the garden at Hartecamp. In 1739, soybean seeds sent by missionaries in China were planted in the Jardin des Plantes, Paris, France. In 1790, soybeans were planted at the Royal Botanic Garden at Kew, England and in 1804 they were planted near Dubrovnik, Yugoslavia. The soybeans were grown for taxonomic or display purposes in Netherlands, France and England. However, the soybeans grown in Yugoslavia were harvested, cooked, mixed with cereal grain and then fed to chickens for increased egg production.

Soybeans made their first appearance in the US in the 18th century, planted by an American who brought them back from China. American farmers began planting soybeans in the 19th century; yet, it was not until the early 20th century, when nutrition pioneers such as George Washington Carver and John Harvey Kellogg began discovering and promoting the health benefits of soybeans to receive greater public attention. Interest in the nutritional benefits of soybeans has steadily increased only in the past few years. This has come about in response to the recent scientific research that is supporting the numerous ways that soybeans can promote health. Today, the US is the world's leading commercial producer of soybeans (www.soyinfocenter.com, www.fas.usda.gov).

3. Soybean in meals

Soybeans have been a part of traditional food for human population in Asian and Eastern part of the world for several decades. Recently it has become popular in other parts of the world. They are high in protein, fiber and unsaturated fat, and rich in vitamins and minerals. They also show many anticarcinogenic properties related to the unique benefits of soy isoflavones, phytochemicals which exert biological effects in humans and other animals (de Mejia *et al.*, 2003). In recent years, breakthroughs in food science and processing have made it possible to use soybean ingredients in new ways, creating foods that are familiar to consumers incorporating parts of the soybean for functional or nutritional purposes. This has greatly expanded the food processing industries to use soybeans and soy-based ingredients.

3.1 Traditional Soy-Foods (Liu, 2004)

3.1.1 Tofu

It is a regular part of the diet in many Asian nations and is available across the US and in most Western nations. It is soft, white and almost cheese-like food favored for its versatility, mild flavor and high nutritional value. Since it is naturally processed it retains the soybean's important nutrients such as the isoflavones. It is made from soymilk by adding a coagulant (calcium sulfate) to the milk to form curds that are shaped and pressed into cakes. Depending on the coagulant used, tofu is rich in minerals and is an excellent source of high-quality protein (10-15%), polyunsaturated fats *viz*. linoleic and linolenic acids (5-9%) and B vitamins. Tofu is versatile and nutritious as it can be used in soups, salads, pastries, sandwiches, and spreads. It is relatively low in carbohydrates and in fiber (as the pulp was removed), making it easy to digest.

3.1.2 Soy-milk

Liquid extract of soybean used in the preparation of tofu, or as a nutritious beverage. It can be consumed by people who are dairy sensitive (lactose intolerant) or by strict vegetarians who eat no animal proteins. Soymilk is an excellent source of protein, B-vitamins and iron.

There are also a number of soymilks available that are fortified with vitamins and minerals, such as β-carotene, calcium, docosahexaenoic acid and omega-3 fatty acid. It has low levels of saturated fat and no cholesterol. The popularity of soymilk has grown in US and Europe since 1980s when refrigerated soymilks came onto the market.

3.1.3 Green vegetable soybeans

This simple and nutritious soyfood is really just the whole soybean picked at its peak of green maturity, at a time when it is high in sucrose and chlorophyll, and has a firm texture. It is harvested in the pod, and sold either in the pod or shucked, after being blanched and frozen. Because they are picked when their sugar levels are high, green vegetable soybeans are very sweet. Green vegetable soybeans contain ~13% protein same as in tofu, and are naturally high in calcium.

3.1.4 Tempeh

Traditional fermented soyfood from Indonesia and is quite unique in its texture, flavor and versatility. It is made from the whole soybean, which has been de-hulled, cracked and cooked in water with added vinegar to reduce the pH. Once cooked, the soybeans are mixed with the spores of the *Rhizopus oligosporus* fungus and left to incubate for 24 h at around 88°F. Various grains or seeds may be mixed in during processing to vary the taste and texture of the final product. It contains ~19% protein, is higher in fiber than tofu, and is a significant source of vitamins and minerals.

3.1.5 Miso

It is a rich and flavorful paste made from fermented and aged whole soybeans, or from soybeans in combination with wheat, barley or rice using fungal starter *Aspergillus oryzae* under specific conditions. This salty paste is a treasured soup-base and flavoring ingredient used throughout Japan, Korea, Taiwan, Indonesia and China. It has unique medicinal properties and is believed to help reduce the effects of environmental poisons on the body, and contains enzymes and bacteria that can aid in digestion. It is high in protein but contains large amount of sodium therefore should be consumed sparingly.

3.1.6 Soy sauce

It is the most well-known and popular traditional soyfoods, used extensively as a flavoring ingredient in most Asian cooking. There is variety of soy sauce in the market: tamari (naturally processed), shoyu (processed with a fermented wheat starter) etc. Much of the soy sauce sold today is made with hydrolyzed vegetable protein (HVP) with added sugar, color and preservatives. All soy sauces are high in sodium due to which it has been precluded to use as nutritional food. From a nutritional prospective, tamari has highest protein level followed by shoyu and then the HVP-based soy sauce.

3.1.7 Natto

It is a whole soybean food (very popular in Japan) that is produced by fermenting small, cooked soybeans with *Bacillus natto* until they develop a sticky, viscous coating. It has a strong taste and aroma and is definitely for the adventurous eater. It can be found frozen, or fresh, and will last about a week refrigerated.

3.1.8 Okara

It is the fibrous remains of the soybean after it has been processed to make soymilk. It is very high in moisture content and contains the insoluble carbohydrates and dietary fiber of the soybean, as well some remaining protein and fat. If fully cooked, it is blend in flavour and is an excellent ingredient to add to breads and other baked goods. It is not usually sold in stores, as it is very wet, heavy and highly perishable.

3.1.9 Soy Sprouts

Fresh, crisp sprouts of germinated soybeans sold after having grown for 5 to 7 days. They are a traditional food of Korea and eaten either raw or in prepared food dishes. They are high in protein and fiber, and contain vitamin C.

3.1.10 Soybean oil

It is produced from soybeans by mechanical or solvent extraction. Crude soybean oil is further filtered to produce salad and cooking oils. In the US, most of what is sold as "vegetable oil" in the stores is really pure soybean oil. In other countries, it is often labeled as soybean oil, or soya oil. It is the primary oil used in the food processing industry and is found in vegetable oil shortenings and margarine. Soybean oil is high in polyunsaturated fats, low in saturated fats, and a good source of linoleic and linolenic acids, the omega-3 and omega-6 fatty acids also found in fish oils.

3.2 Second generation Soy-foods

Soy-foods manufacturers have been very responsive to consumer demands for convenient, healthful foods. As a result, a wide array of "second generation" soy-foods is available in the marketplace today, catering to different tastes and use preferences. Here are some second generation soy-foods that are currently available:

3.2.1 Soy-nuts

Crunchy nuts have been prepared by dry roasting or oil roasting of whole or split soybeans that have first been soaked in water. They can be sold with salt or other flavoring ingredients added as a coating. Soy-nut butter is a paste of ground soy-nuts that have been prepared in a similar manner to peanut butter, and may have salt, sweeteners and additional oil added.

3.2.2 Meat alternatives

This is the category made from tofu, tempeh, textured soy flour, textured soy concentrate, isolated soy protein and wheat gluten. Products may take the shape of burgers, hot dogs, sausages, luncheon meats, ground meat and meatballs. Most products are made with a combination of vegetable protein ingredients to achieve the best texture and are flavored for a particular use.

3.2.3 Cheese alternatives

Block, sliced, spreadable and grated cheese alternatives may be made from soymilk, tofu or other vegetable protein ingredients. They can be flavored with cheese like American cheese, mozzarella, cheddar, Monterey jack, Parmesan etc. Most of these products are made with some amount of casein (from cow's milk) which is responsible for melting action in cheese when heated.

3.2.4 Soymilk yogurt

Soymilk yogurt is made in the same manner as cow's milk yogurt by inoculating pasteurized soymilk with cultures like *Acidophilus*, *Bifidos* etc. It tastes very similar to cow's milk yogurt and is available in a variety of styles and flavors. It is very high in protein, a great source of isoflavones and can be used as it is or in recipes calling for yogurt.

3.2.5 Nondairy frozen desserts

Nondairy frozen desserts are produced in the same manner as their dairy counterparts. They may be prepared from a base of soymilk, soymilk yogurt, tofu or isolated soy protein. Some brands, such as the pioneering Tofutti brand, have created a loyal niche for themselves, but the category as a whole still struggles to reach a broad market.

4. Soybean as source of nutrition (www.soyinfocenter.com, www.fas.usda.gov, www.whfoods.com)

4.1 A Health-promoting meat replacer

Soybeans are regarded as equal in protein quality to animal foods. Just one cup of soybeans provides 57.2% of the Daily Value (DV) for protein for less than 300 calories and only 2.2 g of saturated fat. **Table 1** shows the nutritional values of 100 g of soybean. Soy protein tends to lower cholesterol levels, while consuming protein from animal sources tends to raise them, since they also include saturated fat and cholesterol. In addition to healthy protein, some of soybeans' nutritional high points include a good deal of well-absorbed iron: 49.1% of the DV for iron in that same cup of soybeans; plus 37.0% of the DV for Nature's relaxant, magnesium; and 41.2% of the DV for essential omega-3 fatty acids.

4.2 Stay lean with soy

Active isoflavone, genistein found in soy helps in staying lean as it produces fewer and smaller fat cells. It was found that when laboratory animals have eaten diets containing 500-1500 ppm of genistein led to decrease in fat content by ~40% than the animals eating regular chow. Comparable amount of genistein in humans could easily be consumed by simply including traditional soy foods, such as tofu, soy milk, tempeh, and miso as part of their diet which provides about 30-40 mg per serving.

4.3 Eat whole soy foods, not purified soy products for optimal health

A cancer-preventive ability of soy foods markedly reduced in highly purified soy products and supplements. Further, processed soy foods can stimulate the growth of pre-existing estrogen-dependent breast tumors. Soy foods contain complex mixtures of bioactive compounds that interact with one another to promote health, while the partially purified isoflavone-containing products consumed lost many of the biologically active components compare to whole soy foods. In one of the study, laboratory animals were divided into four groups, one of which received no soy and served as the control group, while the others were given whole soy flour, soy extract, a mixture of isoflavones, or genistein in pure form. In the animals given minimally processed soy food *viz*. soy flour, tumors neither grew nor regressed. But in the animals given dietary soy products containing isoflavones in more purified forms, the tumors grew. It is most important for postmenopausal women with estrogen-responsive breast cancers. Avoid processed soy products and supplements that contain isoflavones in more purified forms (Cassidy *et al*., 1994).

4.4 Soybean oil: Nutritional analysis

Soybean oil is 61% polyunsaturated fat and 24% monounsaturated fat, which is comparable to the total unsaturated fat content of other vegetable oils (~ 85%). Like other vegetable oils, soybean oil contains no cholesterol.

4.4.1 Polyunsaturated vs Saturated fats

Nutrition experts recommend limiting total fat consumption to 30% or less of the total daily calories and limiting saturated fats to 10% or less. Saturated fatty acids raise blood cholesterol which can thicken arterial walls and increase the risk of heart disease. Populations with diets low in saturated fats have the lowest death rates. As a result, the replacement of saturated fats with reasonable amounts of polyunsaturated fats, such as those found in soybean oil, is recommended.

4.4.2 Essential fatty acids

Soybean oil is rich in polyunsaturated fatty acids, including the two essential fatty acids: linoleic (50%) and linolenic (8%). They aid the body's absorption of vital nutrients, precursors to hormones that regulate smooth muscle contraction, blood pressure, and the growth of healthy cells in human.

4.5 Soy protein: Nutritional benefits

Almost 40% of the calories in soybeans are derived from protein causing soybeans to be higher in protein than other legumes and many animal products. The quality of soy protein is highly notable and approaches the quality of meat and milk. Unlike many other good sources of protein, soybeans are low in saturated fat, low-density lipoproteins, triglycerides and are cholesterol-free. Soybean is the best and cheapest source for protein supplementation with respect to other plant and animal sources. It is a healthy adjunct to a varied diet high in fiber, fruits and vegetables and moderate in fat helpful in losing weight and improves sports performance. There is a huge debate regarding importance of soy-protein over other sources, especially whey protein is the most important matter of concern. Both of them are high-quality proteins and have a Protein Digestibility Corrected Amino Acid Score (PDCAAS) of 1.0. Soy protein isolate is 90% protein on a dry-weight basis, is highly digestible (97%) and allows the essential amino acids *viz*. valine, isoleucine and leucine absorbed across the gastrointestinal tract. Processing of soy protein requires a water-washed process allowing isoflavones to remain intact and removal of all gas-producing carbohydrates and fat. In contrast to whey protein, which suffers two major disadvantages; primarily its high lactose content and expensive processing. It is estimated that 75% of adults worldwide show some decrease in lactase activity during adulthood. The frequency of decreased lactase activity ranges from nearly 5% in Northern Europe, up to 71% for Southern Europe, to more than 90% in some African and Asian countries. Further, processing of whey protein requires two processes: micro-filtration and ion exchange. Both of these processes are very expensive, due to which whey protein is the most costly protein source in the market.

According to comparative analyses of whey and soy protein, later has almost double content of glutamine and arginine. Glutamine is the primary carrier of nitrogen to skeletal muscle and other tissues in the body, helps in buffering lactic acid buildup in the blood and muscles, boosts muscle protein activity, increases growth hormone levels and strengthens immune capacity. Arginine plays a key role in stimulating the release of anabolic hormones

that promote muscle formation, reducing physiological stress, and maintaining a strong and healthy immune system. Soy protein has 18% of branched chain amino acids (BCAA) while whey concentrate has 20%. BCAA are used as an energy source during exercise. During endurance activity, nitrogen is removed from the BCAA and converted to alanine, which is transported *via* the bloodstream from the muscle to the liver where it is converted to glucose. Glucose from the liver returns to muscle to supply energy for fueling exercise (www.soybean.org, www.bodybuildingforyou.com).

5. Physiological significance of carbohydrates

During seed germination of soybean, the total soluble carbohydrate content of the cotyledons and embryo axis declines rapidly during the first 3 days of germination. Depletion began earlier in the embryo axis with respect to the cotyledon. The total carbohydrate content of the cotyledons of plants grown in light and dark was approximately the same for the first 7 days of germination. Between day 9 and 13 the total carbohydrate content of the cotyledons of soybean seedlings grown in dark was higher than that of plants grown in light. The reducing sugar content of light-grown soybean cotyledons increased by ~5-fold during the first 9 days of germination, whereas that of dark-grown soybean cotyledons increased more slowly during this interval. Reducing sugars in the embryo increased during the early stages of germination until they approximately equaled the total carbohydrate. Between day 4 and 13, oil was depleted more rapidly in the cotyledons of seedlings grown in light than those grown in the dark. The reserve carbohydrates of soybean embryos and cotyledons consisted primarily of low molecular weight oligosaccharides, particularly sucrose, stachyose, and raffinose. These compounds decreased rapidly during germination. Stachyose and raffinose declined rapidly in the cotyledons by day 3 and disappeared by day 9 of germination. Sucrose increased slightly during the first 3 days but decreased steadily after the 3rd day. Fructose and glucose appeared in day-1 cotyledons, with the former reaching a maximum value on the 5th day and the latter on the 9th. Stachyose, raffinose, and sucrose were also the major sugars found in the 0-day embryo axes. Stachyose and raffinose were depleted completely by around day 3 in the embryo axes while sucrose decreased between day 5 and day 13. Fructose and glucose appeared in day-1 embryo axes, continued to increase through day-5 of germination, and decreased thereafter (Tsung Min Kuo *et al*., 1990; Bernal-Lugo & Leopold, 1992; Sitthiwong *et al*., 2005; Dierking & Bilyeu, 2009). Raffinose, stachyose and verbascose were thought to be involved in seed protection during the desiccation process by stabilization of the membrane. Raffinose, stachyose in combination with phytin plays significant role towards protection against imbibitional chilling. Sucrose is exceptionally effective in protecting membrane integrity in dry systems, as well as being one of the best vitrifying sugars. Raffinose is known to enhance the protective effects of sucrose by limiting crystallization. In quiescent seeds, the main soluble embryonic carbohydrate reserves are sucrose, usually associated with lesser amounts of the oligosaccharides, raffinose, stachyose, and/or verbascose. The decline of seed quality during storage leading to decrease in the growth rate of the germinating axis termed as "vigor" and subsequently loss of germinating ability (germinability) (Sharma *et al*., 2007). The major factors leading to decline of vigor and germinability of the seed are:

- Decline in soluble carbohydrate contents with seed aging lead to limited availability of respiratory substrates for germination.

- Depletions of disaccharides lessen the protective effects of sugars on structural integrity of membranes as well as limit the ability of the seeds to maintain the vitrified state, a non-crystalline liquid state of high viscosity.
- The presence of reducing sugars also leads to deterioration of protein components through Amadori and Maillard reactions.

6. Source of various enzymes having industrial significance

Soybean has enormous economical significance due to its high food value, role in oil industries as well in bio-diesel production. Leguminous plants are source of various industrial enzymes, particularly found during seed imbibitions and germination as well as those involved in nitrogen metabolism (Dey, 1984). Recently, the approach is emphasizing to use soybean which is a legume for extraction of enzymes at industrial scale helpful for its economic significance. Most importantly, extract prepared from soybean used for enzyme isolation while leaving behind the residues is useful for various purposes *viz*. animal fodder, oil extraction, bio-diesel production etc. For various industrial applications, enzymes so extracted from leguminous seeds are immobilized onto suitable matrices leading to improvement in physico-chemical properties (Das *et al*., 1997; Kayastha & Srivastava, 2001; Tripathi *et al*., 2007; Dwevedi *et al*., 2009; Kumar *et al*., 2009). **Figure 1 (A, B)** show immobilized *β*-galactosidase from *Pisum sativum* onto Amberlite MB150 beads and gold nanoparticles (Dwevedi and Kayastha, 2009) being used for lactose hydrolysis at industrial scale. Implementation of soybean at industrial scale as source of various enzymes will make it a multifaceted crop with enormous economic capabilities. Following are few enzymes known from soybean having specific industrial applications.

6.1 Cellulases (Kemmerer & Tucker, 1994)

It is used commercially for food processing in coffee, textile industry, in laundary detergents, paper and pulp industries. They are even used for pharmaceutical applications, fermentation of biomass into biofuels, used as treatment for phytobezoars, a form of cellulose bezoar found in the human stomach.

6.2 *α*- and *β*-Amylases (Tripathi *et al*., 2004; Kumari *et al*., 2010; Kumari & Kayastha, 2011)

Amylases find application in bread making and in clothing and dishwasher detergents to dissolve starches from fabrics and dishes, production of sugars from starch, such as in making high-fructose corn syrup.

6.3 Proteases (Oh *et al*., 2004)

They are used to dissolve gelatin off scrap film, allowing recovery of its silver content, remove cloudiness produced during storage of beers. They are used in manufacturing biscuits in lowering the protein level of flour.

6.4 Phytases (Hamada, 1996; Hegeman *et al*., 2001)

They have been used as animal feed additive in diets largely for swine and poultry, and to some extent for fish. They help in improving the utilization of phosphate from phytate.

Therefore, including adequate amounts of phytase in the diets for simple-stomached animals reduces the need for orthophosphate supplementation of the feed. As a result, the environment is protected from pollution by phosphorus containing manure because of the reduced (~50%) faecal phosphate excretion by the animals. There is also a great potential for the use of phytases in processing and manufacturing of food for human consumption, but up to now, no phytase product for a relevant food application has found its way to the market. Technical improvements by adding phytases during food processing have been reported for bread-making, production of plant protein isolates, corn wet milling and the fractionation of cereal bran.

6.5 Transglutaminases (Kang & Cho, 1996; Lilley *et al*., 1998)

They have significant role in food industry, used in cold bond meat pieces**,** attach bacon to the surface of meat**,** improves texture of cheese and yogurt by reducing water loss, etc.

6.6 Ureases (Kumar *et al*., 2009)

The enzyme is used in the field of diagnosis to determine urea in the blood serum, to decompose urea in the artificial kidneys. In food industry, urea has been marked as an undesirable substance, particularly in biologically fermented food products such as sake, beer, wine, soy sauce etc. Presence of urea in food products gives bitter taste, cause coloring or deterioration of the flavor and cause lowering of food safety. Urease is being used in treatment of waste water plant particularly from agriculture, rich in urea, in secondary and tertiary oil recovery etc.

6.7 Peroxidases (Chatfield & Dalton, 1993)

They are used for treatment of industrial waste waters, *viz*. phenols (important pollutants) are oxidized to phenoxy radicals, which participate in reactions leading to production of polymers and oligomers that are less toxic than phenols by the enzyme. Furthermore, peroxidases can be an alternative option of a number of harsh chemicals, eliminating harsh reaction conditions. There are many investigations about the use of peroxidase in many manufacturing processes like adhesives, computer chips, car parts, and linings of drums and cans.

6.8 *α*-Galactosidases (Herman & Shannon, 1985)

The enzyme works in the digestive tract to break down the complex or branching sugars (polysaccharides and oligosaccharides) in foods such as legumes (beans and peanuts) and cruciferous vegetables (cauliflower, broccoli, cabbage, brussels sprouts, among others). The enzyme breaks those complex sugars into simple sugars, making these foods somewhat more digestible, and reducing intestinal gas. The polysaccharides and oligosaccharides found in these foods otherwise pass through the small intestine unaffected. Once in the large intestine, those sugars may be metabolized by intestinal flora, fermenting to produce the gases that cause discomfort. The approach is moving to use oral *a*-galactosidase for solving gastric problems.

7. Agricultural status and economic importance of soybean

Soybean stands first in the world as edible oil and occupies important place in the economy. Soybean de-oiled cake is being exported and earning foreign exchange. Soybean

cake is edible and is used for preparation of different bye-products such as Nutri Nuggets. Soybean de-oiled meal is recommended as animal feed. From soybean whole seed, soy-milk obtained and considered to be the best health drink for infants and soya cheese is also considered as health food. Recent advancements in food processing have transformed the bitter, gray, beany-tasting Asian beverage into a product that Western consumers will accept.

The world soybean price has increased tremendously by ~50% in the year 2007-2008 (remarkable year in the soybean history) with respect to previous years due to strong world demand (**Figure 2**). Area expansion and yield improvement have greatly stabilized its price for the last few years. Brazil's soybean area is expanded annually by about 3% for the last few years due to increment in its price. It has been estimated that by 2017, Brazil will surpass the US to become the leading producer in the world. Falling real domestic prices and expansion of urban area have led to very limited growth of soybean production in China. On the other end, robust economic growth has encouraged domestic consumption of soybean by 40% over the baseline period. Import of oilseeds and domestic crush make China, the world's leading soybean importer for the last 10 years (**Figure 3**). This strong demand for soybeans becomes a focus of attention for major exporting countries (Brazil and the US dominate the soybean net exports market). Paraguay has emerged as an important soybean net exporter and holds a 7.6% share of the world market. Argentina, the leading soybean meal net exporter, ships 98% of its production to the world market because of its differential export tax.

World soybean meal production grows by 2.5% per year in response to rising feed demand. China increases its consumption by 3.4% annually because of strong expansion in its livestock sector whereas that of US increases by 19% (**Figure 4**). The volume of net exports in the soybean meal market increases by 32% in the whole world. Significance of soybean oil in the production of biodiesel has led to increment industrial use of soybean oil in countries like Argentina, Brazil, and the US by 187%, 208%, and 101%, respectively over the year 2007-2008. Despite the domestic biodiesel mandate, Argentina still dominates world soybean oil exports, satisfying 72% of the world market. Brazil and the US together account for 21% of world soybean oil net exports by 2017-2018. China and India's combined share of net imports holds 48% of the world market.

All soybean producers pay a mandatory assessment of 0.5% to 1% of the net market price of soybeans. About $80 million annually has been invested in United Nations soybean's program to strengthen the position of soybeans in the marketplace and expand domestic as well as foreign markets for uses for soybeans and its products. State soybean councils from Maryland, Nebraska, Delaware, Arkansas, Virginia, North Dakota and Michigan provide another $2.5 million for the purpose of research. Private companies like Archer Daniels Midland (ADM) also contribute their share. ADM spent $4.7 million for advertisement. Soy milk has posted the biggest gains, soaring from $2 million in 1980 to $300 million in the US in 2001 (www.soygrowers.com, www.agmrc.org, www.soyconnection.com, www.agbioforum.org).

8. Soybean in biotechnology

The GM foods are most commonly used to refer to crop plants created for human or animal consumption using the latest molecular biology techniques. These plants have been modified in the laboratory to enhance desired traits such as increased resistance to

herbicides and pest, cold, drought and salinity tolerance, improving nutritional content, in pharmaceuticals and phytoremediation. The enhancement of desired traits can be traditionally undertaken through breeding but it is very time consuming and are often not very accurate. US is the largest commercial producer of GM crops (~99 million acres are devoted to GM crops) with respect to 13 other countries (Argentina, Canada, China, Australia, Bulgaria, France, Germany, Mexico, Romania, South Africa, Spain, and Uruguay). Soybeans and corn are the top two most widely grown GM crops followed by cotton, rapeseed (or canola) and potatoes.

Approximately 70% of the GM soybeans are produced in the world. Currently, in the United States GM soybeans have reached the marketplace to be use in animal feed. The major question aroused about the effect of GM soybeans on the animals. As reviewed from the results of 23 research experiments conducted over the past few years have revealed that there was negligible effects. In one of the study, separate groups of chickens, dairy cows, beef cattle and sheep were fed with GM as well as traditional soybean as a portion of their diet. Each experiment independently confirmed that there is no significant difference in the ability of animals to digest the GM crops and no significant difference in the weight gain, milk production, milk composition, and overall health of the animals when compared to animals fed with traditional crops. It was also concluded that human consumption of milk, meat and eggs produced from animals fed GM crops would be as safe as products derived from animals fed conventional crops (www.biotechknowledge.monsanto.com). The soybeans used in the studies were produced by inserting a gene that causes the plant to be tolerant to the environmentally friendly herbicide glyphosate. This tolerance to glyphosate allows farmers to spray and kill weeds without killing the soybeans. In other studies the nutritional value of GM soybeans were compared with traditional ones. These studies showed significantly negligible difference in the nutritional composition of the beans (Herdt, 2006).

Biotechnologists feel that GM crops hold the answer of feeding the growing population of the world. It has been estimated that the supply of food required to adequately meet human nutritional needs over the next 40 years. It is quantitatively equal to the amount of food previously produced throughout the entire history of humankind. With the current world population at about six billion, and the estimated 10 billion people expected by the year 2040, modern methods of biotechnology must be used to produce enough feed for livestock and food for humans. GM foods have the potential to solve many of the world's hunger and malnutrition problems, and to help protect and preserve the environment by increasing yield and reducing reliance upon chemical pesticides and herbicides. Yet there are many challenges ahead for governments, especially in the areas of safety testing, regulation, international policy and food labeling. Many people feel that genetic engineering is the inevitable wave of the future and that we cannot afford to ignore a technology that has such enormous potential benefits. However, we must proceed with caution to avoid causing unintended harm to human health and the environment as a result of our enthusiasm for this powerful technology (Bonny *et al*., 2008).

9. Soybean in treatment of various diseases

9.1 Lowers blood pressure and cholesterol levels, helpful for treatment of heart related diseases

Effect of soy protein and soy isoflavones on blood pressure and cholesterol levels were studied in 61 middle-aged men, at high risk of developing coronary heart disease. For five

weeks, half the men consumed diets containing at least 20 g of soy protein and 80 mg of soy isoflavones per day. The effects on their blood pressure, cholesterol levels, and urinary excretion of isoflavones were measured, and then compared to those of the other half of the men who were given a placebo diet containing olive oil. The men consuming soy in their diet were found to have significant reductions in both diastolic and systolic blood pressure. Not only was their total blood cholesterol significantly lower, but their HDL cholesterol level significantly increased (Candy, 1996).

Soy protein has been shown in some studies to be able to lower total cholesterol levels by 30% and lower LDL levels by as much as 35-40%. This is important because high levels of cholesterol, especially LDL cholesterol, tend to become deposited into the walls of blood vessels, forming hard plaques. If these plaques grow too large or break, they can cause a heart attack or stroke. Some studies have even shown that soy protein may be able to raise HDL cholesterol levels. HDL cholesterol travels through the body collecting the cholesterol that has been deposited in the arteries, so it can be taken away and removed by the liver. One of the main goals of atherosclerosis treatment and prevention, therefore, is to lower LDL cholesterol levels while raising HDL levels. And soy is one food that may be able to do both at once. In addition, soybeans also contain very good amounts of fiber. When eaten, the fiber in soybeans binds to fats and cholesterol in food, thus less is absorbed. In addition, soybeans' fiber binds to bile salts and removes them from the body. Since the liver gets rid of cholesterol by transforming it into bile salts, their removal by fiber forces the liver to use more cholesterol to form more bile salts, leads to lowering of overall cholesterol levels (Beretz *et al*., 1988).

A recently discovered bioactive peptide found in soybeans, *lunasin* is likely to be a key factor in soy's cholesterol lowering actions. A study in which *lunasin* was added to human-liver cells demonstrated the compound's potential to significantly lower cholesterol by inhibiting the expression of the gene responsible for body's internal production of cholesterol and increasing the expression of a gene that reduces levels of LDL cholesterol in the blood. When added to a cell culture of HepG2 liver cells, lunasin slashed HMG-CoA reducstase expression by 50%. HMG-CoA is the gene that directs production of HMG-CoA reductase, the enzyme responsible for cholesterol biosynthesis. At the same time, *lunasin* increased by 60% the expression of the gene which produces LDL cholesterol receptors that help clear plasma cholesterol (Anthony, 2000).

9.2 Increased nitric oxide production

Soy protein protects against atherosclerosis by increasing blood levels of nitric oxide which helps in blood vessel dilation, inhibit oxidative damage of cholesterol and the adhesion of white cells to the vascular wall. It was found that when laboratory animal which are apoliprotein-E deficient (a condition leading to development of atherosclerosis) are fed with soy protein have significant increase in blood level of nitric oxide. Intensive study has concluded that soy protein has led to increased blood levels of L-arginine (the amino acid that the body uses to produce nitric oxide) and nitric oxide metabolites. Thus, soy protein rich diet helps in protection against atherosclerosis (Henrotin *et al*., 2003).

9.3 Stabilize blood sugar at healthy levels, lowers diabetes risk and protect against diabetes-related kidney and heart disease

Soybeans can be very beneficial for diabetics, particularly type 2 diabetes. The protein in soybeans, and also in other legumes, is excellent for diabetic patients. The protein and fiber

in soybeans prevent high blood sugar levels and help in keeping blood sugar levels under control. Diabetes patients are especially susceptible to atherosclerosis and heart disease, which is the number one killer of persons with diabetes. Keeping cholesterol levels low with soybeans may be useful for preventing these heart problems. In addition, soybeans have been shown to lower high triglyceride levels. Triglyceride levels tend to be high in diabetic patients, and high triglyceride levels are another factor of diabetics' increased risk for heart disease. A study was conducted on 64,227 middle-aged Chinese women with no previous history of diabetes, cardiovascular disease or cancer for 5 years. It was revealed an inverse association between eating legumes and incidence of type-2 diabetes. A high intake of all legumes resulted in a 38% reduction in risk, while a high intake of soybeans, specifically, was associated with a 47% risk reduction. Soy yogurt plays an important role in controlling high blood pressure in type-2 diabetic patients. High blood pressure is one of the problem for which diabetics are at increased risk. Soy yogurt contains angiotensin-I converting enzyme (ACE-I), which plays important role in the constriction of blood vessels and is a target of blood pressure-lowering medications (ACE-inhibitors) (Villegas *et al*., 2008).
A small clinical trial was conducted on type 2 diabetes patients with nephropathy (diabetes-related kidney damage) suggested that soy protein can help diabetics hearts and kidneys from damage caused by the disease. The study was conducted on 14 type 2 diabetes male and female patients receiving medical care at an educational university hospital and private kidney disease clinic. For the first seven weeks, patients followed a diet (0.8 g/kg of protein, based on 70% animal and 30% vegetable protein) typically recommended to control nephropathy. After a washout period they were re-admitted for another 7-week cycle consuming a diet containing 35% soy protein and 30% vegetable protein. Following the soy diet, all patients experienced significant reductions in total cholesterol, triglyceride and LDL-cholesterol, while levels of beneficial HDL cholesterol remained stable and renal function improved. Specifically, the patients' urinary urea nitrogen (a protein component that is not normally leaked into the urine) and proteinuria (protein in the urine, another indicator that the kidneys are beginning to fail) were both much lower on the soy protein diet (Teixeira *et al*., 2004).

9.4 Minifies chronic inflammation

Egg yolks are the richest source of choline, followed by soybeans. Intake of diet rich in choline (~300 mg per day) containing food leads to reduction of inflammatory markers like C-reactive protein**,** interleukin-6, tumor necrosis factor alpha by ~20%. Each of these markers of chronic inflammation has been linked to a wide range of conditions including heart disease, osteoporosis, cognitive decline and Alzheimer's, and type-2 diabetes. It has also been found that choline in association with betaine (found naturally in vegetables such as beets and spinach) work together in cellular process of methylation, which is not only responsible for the removal of homocysteine, but is involved in turning off the promoter regions of genes involved in inflammation. Recommended daily intakes of choline is 550 mg per day for men and 425 mg a day for women (www.whfoods.com).

9.5 Promote gastrointestinal health

A sphingolipid called soy glucosylceramide found in soybeans reduces colon cancer risk. When laboratory animals exposed to carcinogen were given a diet containing 1% soy glucosylceramide, the proliferation of colon cancer cells were dropped by 56%. When the

same diet was given to a strain of animals bred to spontaneously develop colon cancer, the rate at which tumors formed was dropped by 37%. Soy sphingolipids provided this protection by affecting the expression of 96 different genes in the cells that form the lining of the intestines. Soy's effects on these genes resulted in a decrease in the production of two factors associated with cancer initiation and promotion: hypoxia-induced factor 1 alpha and transcription factor 4. Other plants also contain sphingolipids but soy contains relatively high amounts of glucosylceramide due to which it is much effective in cancer prevention. The fiber in soybeans also provides preventative therapy for colon cancer. Fiber is able to bind to cancer causing toxins and remove them from the body, so they can't damage colon cells (www.florahealth.com).

9.6 Protection against cancer of prostate, breast and endometrium

A 9-year Japanese study involving 43,509 men ranging in age from 45 to 74 years found that eating soy food lead to significant lowering of localized prostate cancer risk. Among the men who were older than 60, the protective effect was strongest. It has been suggested that isoflavone found in soybeans protectively alter men's metabolism of estrogen (men do produce some estrogen! the prostate is the primary locus of estrogen production.). Soy's effect of increasing the amount of 2-hydroxy estrogen produced in relation to the amount of 16-hydroxyestrone made in prostate helps in prevention of prostate cancer. Earlier research linking soy to protection against prostate cancer has suggested that soy's isoflavones reduce testosterone levels and inhibit 5-alpha-reductase (converts testosterone to its most potent form, DHT) which are linked to prostate growth and male baldness. A recent study of human prostate cancer cells demonstrated some of the mechanisms behind genistein's anti-prostate cancer effects. Genistein blocks cell cycling and prevent the proliferation of cancerous cells in the prostate by inducing *apoptosis* of abnormal cells. Another study found that genistein protected cells in healthy men from an increase in free radical production by inhibiting the activation of an important inflammatory agent called NF-kappa B and by decreasing levels of DNA adducts (a marker of DNA damage). In addition to genistein, another isoflavone found in soybeans called *daidzen* has also demonstrated protective action against prostate cancer.

Soy's isoflavones also contribute to protective effect against breast cancer in women. The women, who ranged in age from 40 to 59 years, filled out a dietary questionnaire that included questions about soy consumption and were followed for 10 years. Whether prior postmenopausal, women who reported eating three or more cups of miso soup per day had a 40% lower risk of developing breast cancer compared to women who reported consuming less than one cup per day. Women with the highest intakes of isoflavones compounds in soyfoods that can bind to estrogen receptors in the body and block out human estrogen, thus lessening its effects had a 54% lower risk of developing breast cancer compared to those whose intake of isoflavones was lowest.

Soy foods may also reduce risk of endometrial cancer (cancer affecting the lining of the uterus). Eating soy foods is one of the reason due to which Asian women have the lowest incidence of endometrial cancer in the world. The study included over 800 women (aged 30-69 years) with endometrial cancer, and a disease-free, matched control group of over 800 women. When the data was evaluated, a significant inverse association was found between frequency of eating soy foods and endometrial cancer risk. Soybeans contain isoflavones genistein and *daidzein* (1000 times less potent than human estrogen) that can bind to

estrogen receptors in the body, blocking out human estrogens and providing a much more gentle estrogenic effect. Moreover, minimally processed soy foods are rich in dietary fiber, which has been shown to lower estrogen levels (Arjmandi *et al*., 1998; Tong *et al*., 1999; Miyazawa *et al*., 1999; Zhou *et al*., 1999).

9.7 Leads to healthy transition through menopause and reduction in various symptoms during post-menopause in women

It has been found that consuming isoflavone-containing soy foods significantly inhibits bone loss and stimulates bone formation in menopausal women. Women whose daily diets provided soy isoflavones had much lower amounts of deoxypyridinoline (Dpyr, a bone resorption marker) in their urine, and much higher amounts of bone-specific alkaline phosphatase (BAP, a bone formation marker) in their blood (Welty *et al*., 2007).

Women's risk for cardiovascular disease (CVD) increases after menopause, in part because levels of risk factors including homocysteine and excess body iron tend to rise. In this study, researchers looked at the effect of soy protein, specifically soy's isoflavones and phytate, on CVD risk factors in 55 postmenopausal women. To do so, they randomly assigned the women to 1 of 4 soy protein (40 g per day) groups: soy containing both its native phytate and native isoflavones, native phytate but low isoflavone, low phytate and native isoflavone, or low pytate and low isoflavone. After just 6 weeks, women in the groups given soy protein with its native phytate had significant reductions in homocysteine and excess iron concentrations. Soy protein with native isoflavones had no effect. Most interesting about this study is the fact that the phytate in soy, a compound which, because of its mineral-binding effects, has sometimes been considered problematic, that is responsible for some of the CVD-protective effects soy offers postmenopausal women (Kritz-Silverstein & Goodman-Gruen, 2002).

Eating soy nuts also resulted in significant improvements in the women's scores on the menopausal symptom quality of life questionnaire: a 19% average decrease in vasomotor symptoms score, 12.9% reduction in psychosocial symptoms score, 9.7% decrease in physical symptoms score, and a 17.7% reduction in sexual symptoms score (Welty *et al*., 2007).

Despite the fact that the isoflavones found in soy have only 1/1,000th the potency of human estrogens, and epidemiological studies indicate that populations consuming diets high in soy have lower rates of breast cancer, the safety of consuming soy has been questioned because hormone replacement therapy has been found to increase breast cancer risk. Now, an animal study conducted at Wake Forest University Baptist Medical Center, has found that consuming the amount of soy phytoestrogens that would be ingested when soyfoods are included in the diet (in women, about 129 mg/day of isoflavones) does not increase risk of breast or uterine cancer, and appears to be protective. The researchers measured a number of markers of cancer risk: breast density, numbers of dividing breast and uterine cells, sex steroid receptor expression, and blood levels of estrogen. In the monkeys receiving Premarin, levels of all cancer markers increased significantly. In contrast, the monkeys given soy with or without its isoflavones had no increase in any of the cancer markers. And animals receiving soy with isoflavones actually had significantly lower levels of estrogen than the animals given soy from which the isoflavones had been removed. The researchers concluded that the high dietary levels of soy isoflavones did not increase markers related to uterine and breast cancer risk in the laboratory animals studied. Wood's research team has now begun investigating whether soy isoflavones can be used to block breast cell proliferation triggered by estrogen replacement therapy. The theory is that since soy

Nutrients	Nutritional Value
Water	8.5 g
Energy	416 kcal
Protein	36.5 g
Fat (total lipid)	19.9 g
Fatty acids, saturated	2.9 g
Fatty acids, mono-unsaturated	4.4 g
Fatty acids, poly-unsaturated	11.3 g
Carbohydrates	30.2 g
Fiber	9.3 g
Ash	4.9 g
Isoflavones	200 mg
Calcium	277 mg
Iron	15.7 mg
Magnesium	280 mg
Phosphorus	704 mg
Potassium	1797 mg
Sodium	2.0 mg
Zinc	4.9 mg
Copper	1.7 mg
Manganese	2.52 mg
Selenium	17.8 µg
Vitamin C (ascorbic acid)	6.0 mg
Thiamin (vitamin B_1)	0.874 mg
Riboflavin (vitamin B_2)	0.87 mg
Niacin (vitamin B_3)	1.62 mg
Panthotenic acid (vitamin B_5)	0.79 mg
Vitamin B_6	0.38 mg
Folic acid	375 µg
Vitamin B_{12}	0.0µg
Vitamin A	2.0µg
Vitamin E	1.95 mg

Table 1. Nutritional Values of Soybeans (per 100g)

isoflavones, but not estrogens, are similar enough in structure that they can bind to estrogen receptors, they can prevent the much more powerful human estrogen from doing so, thus reducing its effects in the body. Until this research provides more answers, it looks like soyfoods may, at the very least, reduce cancer risk in postmenopausal women with healthy breast tissue.

One of the more popular uses of soybeans lately has been in the treatment of menopausal symptoms. Soybeans contain active compounds called *isoflavones* that act like very weak estrogens in the body. These phytoestrogens bind to estrogen receptors and may provide enough stimulation to help eliminate some of the uncomfortable symptoms that occur when natural estrogen levels decline. Studies have shown that women who consume soy foods report a significant reduction in the amount of hot flashes that they experience. There is also some evidence that soy foods may even be able to help reduce the bone loss that typically occurs after menopause. And as women's risk for heart disease significantly increases at menopause, soybeans numerous beneficial cardiovascular effects make it a particularly excellent food to consume frequently as menopause approaches (Wood *et al*., 2004).

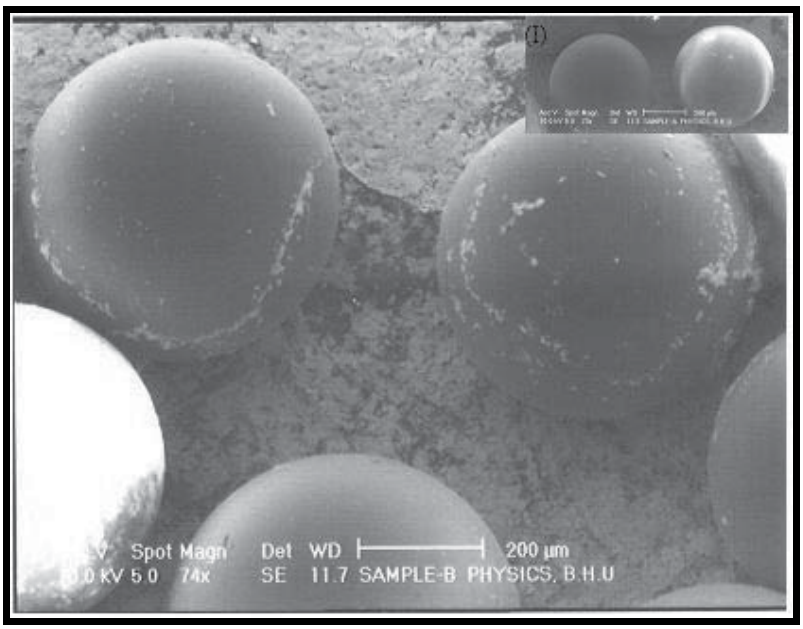

Fig. 1A. Scanning electron micrograph of *Ps*BGAL (*β*-galactosidase from *Pisum sativum*) immobilized on Amberlite MB-150 beads and its control (inset) with scale bar of 200 μm (adapted from Dwevedi & Kayastha, 2009).

10. Future perspectives

Soybean has been the most studied legume due to its enormous economical significance as well as health benefits. Intensive research has been going on to understand soybean at its genetic level, particularly understanding of the organization, complexity, and distribution of the gene and topography of its repetitive sequences. Although, the distribution of genic and repetitive sequences in soybean is known but a detailed analysis is lacking. This gap could be filled by a combination of cytogenetic analyses, targeted sequencing and functional genomic analyses. Phenotypical functional genomics systems, particularly gene knockout systems and improvements in transformation efficiencies are needed.

Soybean is the number one oilseed crop in the world and provides a multi-billion-dollar source of high-quality protein. The rich genomic resources available for soybean make it a model crop legume. The gene discovery stemming from structural and functional genomics research in soybean will certainly lead to new products and to varieties with improved nutritional and agronomic characters.

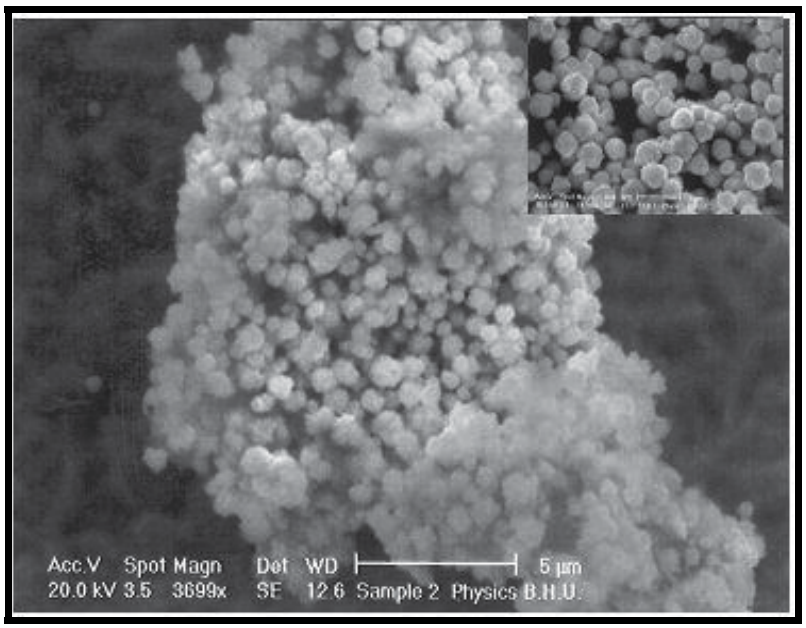

Fig. 1B. Scanning electron micrograph of immobilized *Ps*BGAL onto gold nanoparticles and its control (inset) without enzyme with scale bar of 5 μm, respectively (adapted from Dwevedi *et al*., 2009).

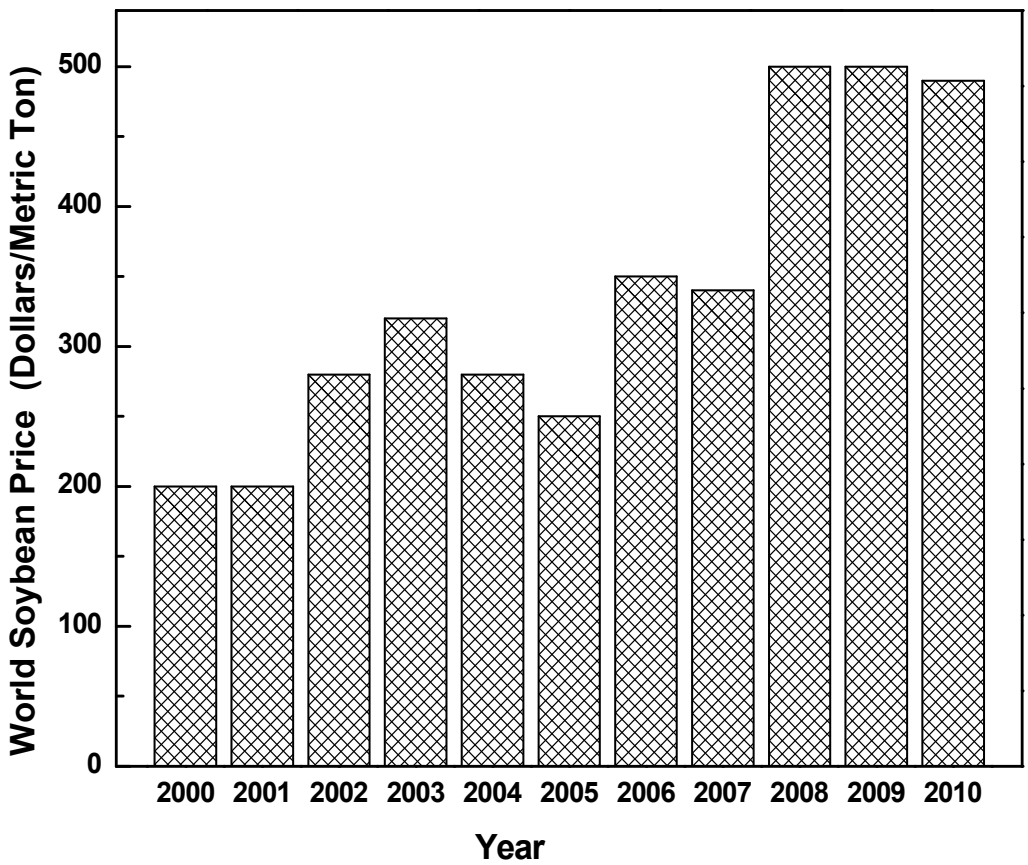

Fig. 2. Increase in the price of Soybean across the world for the last 10 years

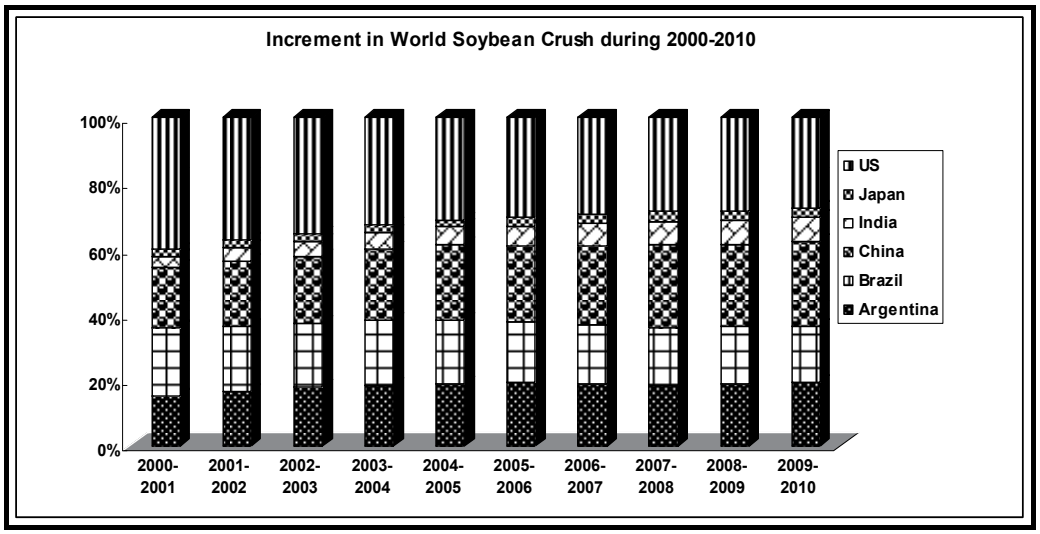

Fig. 3. Increase in world Soybean crush for variety of purpose for the last 10 years

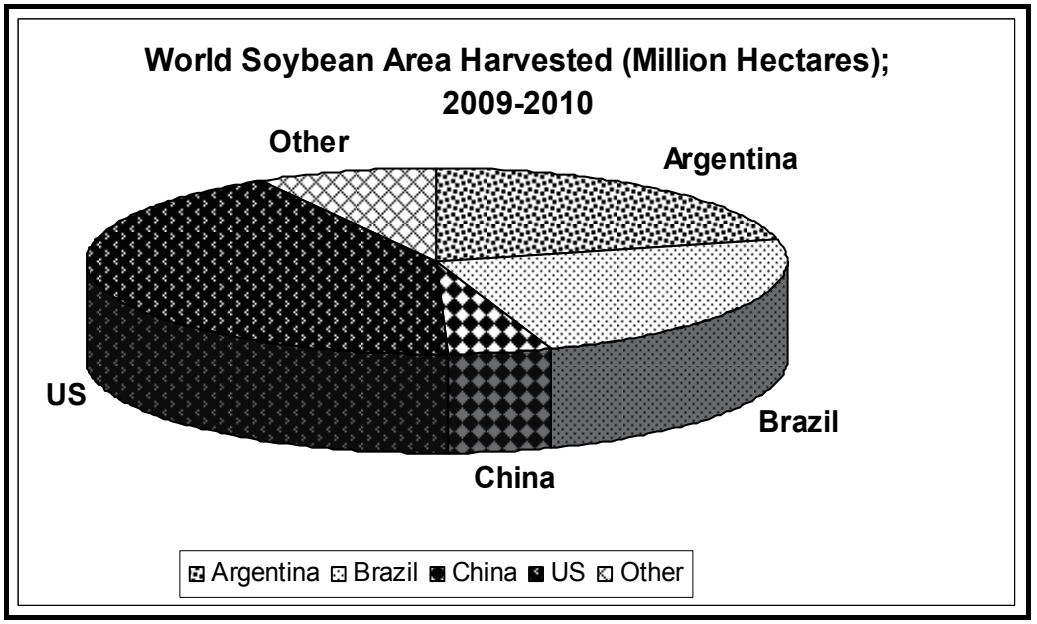

Fig. 4. Area which is used for Soybean cultivation for the last year (2009-2010)

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12

Tempe and Mineral Availability

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1. Introduction

Calcium plays crucial roles in the formation of bones and teeth, cell growth, and metabolism. However, the rate of absorption of calcium in the small intestine and its bioavailability is low, because it can easily crystallize, insolubilize, and precipitate at the physiological pH. Therefore, despite that calcium plays many crucial roles in development and other process, the bioavailability of this essential mineral tends to be scarce.
Certain food ingredients, such as casein phosphopeptides (CPPs) and nondigestible oligosaccharides, are known to improve the absorption of calcium by preventing its crystallization in the small intestine.
Tempe, which is produced by the fermentation of boiled soybean by means of *Rhizopus* culture, is a traditional soyfood in Indonesia. Soyfoods are generally considered an unfavorable source of calcium because soybean is rich in phytate that chelates calcium and retards its absorption. However, the phytate content in tempe is established to be less than half of that in soybean. Accordingly, it is expected that the consumption of tempe may in contrast improve the absorption of calcium from the small intestine.
In this chapter, I first emphasize the importance of dietary calcium and the problems associated with calcium deficiency. Second, I discuss an animal study that, which our group conducted to assess the effect of soybean fermentation on calcium absorption in growing male rats. It was found that the calcium absorption ratio of the rats fed tempe was significantly higher than that of the rats fed unfermented soybean. The high calcium absorption ratio of the tempe group may have been caused by both low phytate content and peptides that were produced during the fermentation of soybean by *Rhizopus*. This section also describes other components such as CPPs, which improve the availability of calcium. Third, I describe the results of my *in vitro* study; in particular, I discuss the ability of minerals, such as calcium and nonheme iron, to solublilize in the presence of phytate. It was determined that the solubilization of nonheme iron was affected by the concentration of phytate, whereas that of calcium was not. Finally, I present the potential challenges and future possibilities in this area.

2. The roles of calcium and absorption

Dietary calcium plays crucial roles in the formation of bones and teeth, contraction of muscles, activation of enzymes, secretion of hormones, and various functions of the cell, including excitation of nerves and aggregation of blood. Dietary calcium is absorbed by, not only the upper gastrointestinal tract including the duodenum, but also by the jejunum and

ileum. However, generally the rate of absorption of calcium from the small intestine and the bioavailability of calcium have been reported to be low because calcium easily crystallizes, insolubilizes, and precipitates at the physiological pH (Saito and Lee, 1998; Heuvel et al., 1999) Accoringly, at present, calcium is the sole mineral that is deficient in the Japanese diet according to the latest nutrition survey.

3. The ingredients in food that affect calcium absorption

The ingredients in food that affect calcium absorption are shown in Table 1 (Uenishi, 2001). In this section, I describe CPPs and phytate in more detail.

CPPs, the components made by digestions of milk casein, are suggested to increase the passive transport of calcium in the small intestine by preventing its crystallization due to the phosphorylated serine residues in the CPP molecule (Saito and Lee, 1998). However, it has also been reported that the preferable effect of CPP is affected by its concentration and the existence of dietary fiber (Hansen et al., 1997; Bennett et al., 2000).

Phytate (inositol-6-phosphate) interacts with calcium strongly and forms the phytate salt of calcium at a molar ratio from 1:1 to 1:6. Because the salt is water-insoluble at a high molar ratio and our gastrointestinal tract lacks phytase, which is the enzyme that digests phytate, we cannot utilize the insoluble calcium salt (Zhou & Erdman, 1995). Soyfoods are generally considered an unfavorable source of calcium supply because soybean is rich in phytate that retards the absorption of calcium (Anderson and Wolf, 1995), although, soybean has a relatively high contents of calcium.

Accelerate	Suppress
Casein phosphopeptide (CPP)	Phytate
Lactate	Oxalate
Oligosaccharide	Dietary fiber
Vitamin D	Excessive phosphorus
Lysin	Saturated fatty acid
Arginin	
n-3 Polyunsaturated fatty acids	
Materials that accelate gastric acid	

Table 1. Food ingredients that affect calcium absorption.

4. Tempe

Tempe, the traditional soy-fermented soyfood prepared by salt-free fermentation with *Rhizopus microsporus*, was originally developed in Central Java, Indonesia. Because there are some kinds of tempe made from ingredients other than whole soybean, both traditional tempe and a couple other types are shown in Fig. 1. Tempe made from soybeans is rich in not only soy protein, glycosidized isoflavones, and vitamin B, all of which are originally present in soybean, but also in free amino acids and isoflavone-aglycones that are produced during the fermentation process (Karyadi and Lukito, 1996; Ikeda et. al., 1999). Therefore, tempe intake has been reported to decrease the effects of chronic degenerative diseases,

including cancer, coronary disease, osteoporosis, and menopausal symptoms, due to its high antioxidative activity (Watanabe et. al., 2007) and the decreasing effects of plasma low-density lipoprotein (LDL) levels (Nout and Kiers, 2004).
Moreover, it has also been reported that the phytate content in tempe decreases to less than half of that present in soybeans, due to phytase during fermentation (Mugula, 1992). Moreover, in an *in vitro* experiment, it has been reported that calcium precipitation was drastically decreased in tempe compared with that in unfermented soybean, mainly due to the presence of peptides with molecular weight of 10,000 or more in tempe (Japan patent, JP 2004-57204 A.). Therefore, it was expected that an improvement in calcium absorption would occur by fermentation of soybean during tempe processing.

Fig. 1. Photos of different kinds of tempe made from (a) soybean, (b) okara, and (c) kapok. Okara is soybean fiber that remain after making tofu. Kapok is a deciduous tree.

5. Dietary tempe and calcium absorption.

In this section, I describe the results of an animal study that examined the effect of dietary tempe on calcium absorption (Watanabe et. al., 2008).

5.1 Experimental procedure

Three-week-old male Sprague-Dawley rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed individually in stainless steel cages at room temperature (20-25 °C) with a 12h light/dark cycle (light from 8:00 to 20:00). They were fed a commercial, nonpurified chow diet (F-2; Funabashi Farms, Chiba, Japan) for a week. The animals were subsequently grouped into 3 dietary groups, consisting of 5 rats per group, and they were fed the indicated experimental diets for 4 weeks.
The experimental diets were formulated based on the AIN-93G specifications. Soybean, conventional tempe, and anaerobic-tempe were used as the protein sources of the experimental diets. Conventional tempe and anaerobic-tempe were prepared as previously described (Aoki et al., 2003). Briefly, steamed soybeans were incubated aerobically with *R. microsporus* IFO 32002 for 20h (to obtain conventional tempe), and then anaerobically incubated for 5h (to obtain anaerobic-tempe).
The calcium and phosphorus levels were adjusted by addition of $CaCO_3$ and K_2HPO_4, respectively. The magnesium level of the diet was not adjusted. The calcium contents of soybean, tempe, and anaerobic-tempe were 6.1, 5.9, and 6.1 g/kg, respectively. The magnesium contents were 1.4, 1.1, and 0.9 g/kg, respectively. Furthermore, the phosphorus contents were 4.5, 4.1, and 4.3 g/kg, respectively.

All animals received daily-prepared fresh diets *ad libitum* for 4 weeks. The rats were housed in metabolic cages for 3 days before the end of this test period, and the feces and urine were collected from each rat. An HCl solution (1 mol/L) was added to each flask for collecting urine. The apparent mineral absorption ratio was calculated using the following formula: apparent absorption ratio = (intake - fecal excretion - urinary excretion) / intake × 100.
At the end of the 4-weeks test period, the rats were sacrificed by decapitation under diethyl ether anesthesia. Their blood was collected and centrifuged at 1700 × g for 10 min to separate the plasma.

5.2 Results and discussion

The results of the calcium balance tests for 3 consecutive days are shown in Fig. 2. The group order based on the calcium absorption ratio for the 3 days tested was as follows: tempe group > anaerobic-tempe group > soybean group. In particular, the absorption ratio of the tempe group was higher than those of the other 2 groups, although no significant difference was observed between the groups on the third day. Furthermore, no significant differences were found in the calcium contents in the femur and plasma samples among the dietary groups (data not shown).

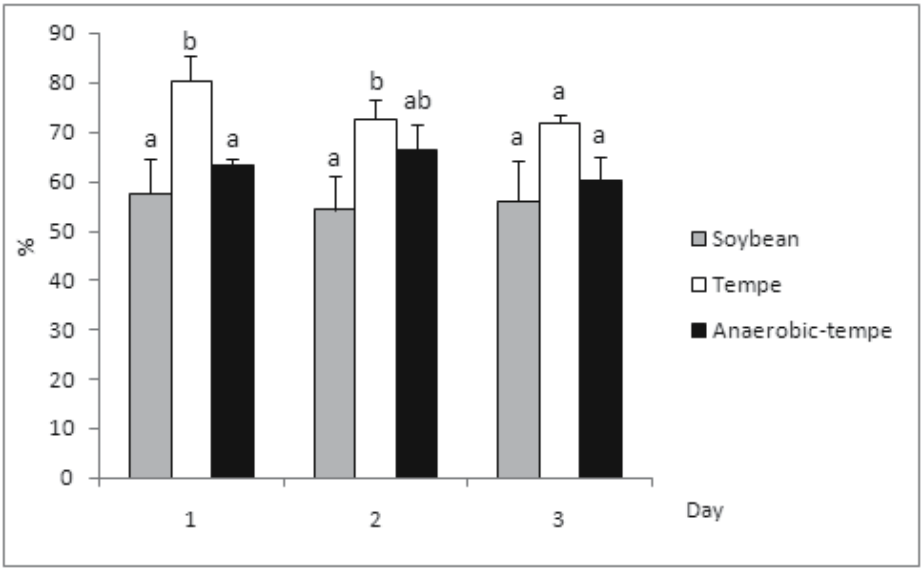

Fig. 2. Calcium absorption ratio of 5 rats (means ± SE.) from each of the soybean, tempe, and anaerobic-tempe groups through the 3 days before the end of the test period. The different letters on top of the bars indicate significant differences among groups on each day ($p < 0.05$).

Despite that the occurrence of CPP-like peptides are not expected in fermented soy products, some peptides that prevent the crystallization of calcium and promote its absorption, such as peptides rich in acidic amino acids (Muramoto et al., 1994), may be

produced during fermentation. The calcium absorption ratio of the anaerobic-tempe group was remarkably less than that of the tempe group. Therefore, the effective peptides in the tempe may be hydrolyzed during anaerobic fermentation because, as previously reported, the contents of free amino acids and oligopeptides increases considerably during anaerobic fermentation carried out to produce anaerobic-tempe (Watanabe et al., 2006).

The phytate contents in soybean, tempe, and anaerobic-tempe were 18.4, 13.4, and 1.60 g/kg, respectively. The particularly low level of phytate in the anaerobic-tempe may be due to the hydrolysis of phytate during anaerobic fermentation (Sutardi and Buckle, 1988). However, since a reverse correlation was not observed between calcium absorption and phytate content, the effect of phytate on calcium absorption was determined to be restricted.

Isoflavones in soyfoods were also reported to enhance calcium absorption in rats (Zafer & Weaver, 2004). The isoflavone content of unfermented soybean was the highest among the 3 experimental soyfoods in this study; both the tempe and anaerobic-tempe showed identical isoflavone content (data not shown). Therefore, isoflavones may not be the major factor responsible for the high calcium absorption ratio of the tempe group observed in this study.

The absorption ratios of magnesium in each of the diet groups are shown in Fig. 3. In contrast with the calcium absorption results, the magnesium absorption ratio of the tempe group was lower than those of the other 2 groups, although no significant difference was observed on the second day.

The absorption ratios of phosphorus for each of the diet groups are shown in Fig. 4. Similar to the magnesium absorption results, the phosphorus absorption ratio of the tempe group was lower than those of the other 2 groups, although a significant difference was observed only on the third day.

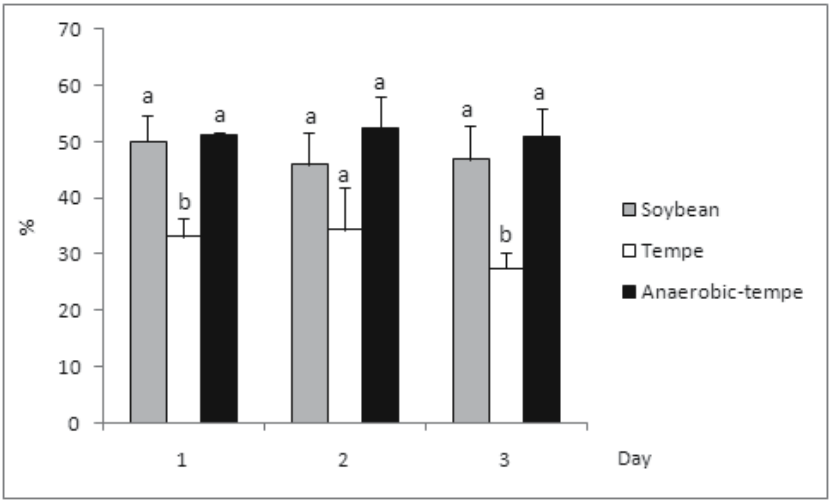

Fig. 3. Magnesium absorption ratio of 5 rats (means ± SE) from the soybean, tempe, and anaerobic tempe groups during the 3 days before the end of the test period. The different letters above the bars indicate significant differences among the groups on each day ($p < 0.05$).

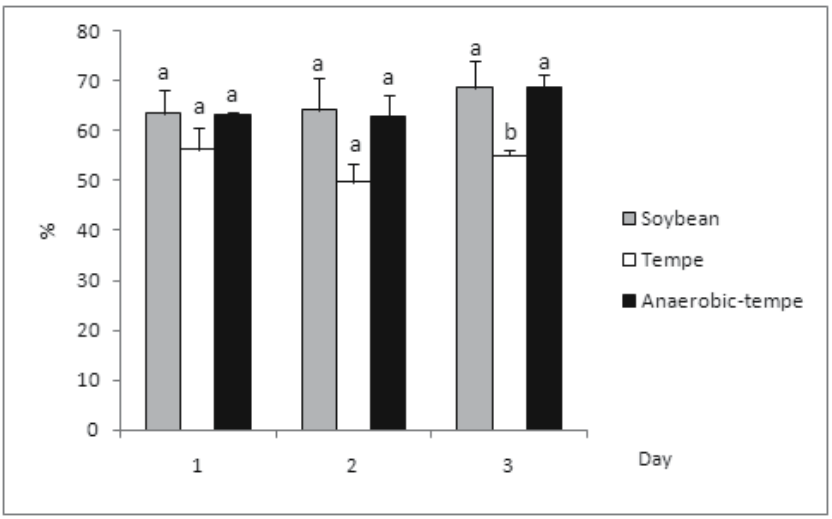

Fig. 4. Phosphorus absorption ratio of 5 rats (means ± SE) from the soybean, tempe, and anaerobic tempe groups during the 3 days before the end of the test period. The different letters above the bars indicate significant differences among the groups on each day ($p < 0.05$).

6. Phytate contents and calcium solubility

Because the effect of phytate on calcium absorption was found to be restricted in our animal study, as described in section 5, I also describe the results of an *in vitro* calcium solubility assay using some kinds of soyfoods, including tempe, in this section.

6.1 Experimental procedure

Two kinds of unfermented soybean, 5 kinds of Japanese tempe, and 5 kinds of Indonesian tempe were used. All of these soyfoods were freeze-dried and then powdered. The powders were subsequently used as the experimental samples in the calcium solubility assay.

Fifty milligrams of each powdered experimental sample, 100 µL of 20 mM calcium chloride solution, and 20 mM phosphate buffer (pH 7.0) were mixed and then incubated for 30 min at 37 °C. Each mixture was centrifuged for 10 min at 9100 × g, and then the calcium concentration of the supernatants was measured using Calcium E-test (Wako Pure Chemical Industries, Ltd., Osaka, Japan). A mixture using distilled water instead of 20 mM calcium chloride solution was used as the blank.

6.2 Results and discussion

The correlation between calcium solubility and the phytate contents in some kinds of soyfoods is shown in Fig. 5. The longitudinal axis shows the ABS of the experimental solution. A low ABS indicates low solubility of calcium. Because the calculated correlation coefficient was -0.445, which indicates a weak negative correlation, high phytate contents may induce low calcium solubility in this condition. However, because the correlation coefficient was not particular high, other factors other than phytate are likely also important for solubilizing calcium.

Furthermore, there are a number of inconsistent studies about the relationship between calcium absorption and phytate content in diet.
One study reported that a diet that consisted of 20% phytate-free soybean protein increased the calcium absorption ratio by only 1% in growing male rats, although the magnesium and zinc absorption ratios were increased by 5-10% compared with a diet of soybean protein and casein (Fukui et al., 1997). In another study that used mature and immature rats, the phytate level did not affect the absorption of calcium (Mason et al., 1993). Furthermore, another study that used infant formulas determined that the phytate level in a diet that contained soybean protein did not affect mineral bioavailability, such as calcium and phosphorus, in rats (Churella, 1989).
In contrast, another report indicated that a diet that consist of 20% phytate-free soybean protein increased both the absorption and retention ratios of calcium, magnesium, phosphorus, iron, and zinc, compared with a diet of soy protein isolate and casein in rats (Kamao et al., 2000). With the exception of the aforementioned study, other investigatiors have reported an unfavorable effect of phytate on calcium availability (Anderson & Wolf, 1995; David & Wolf, 1987).

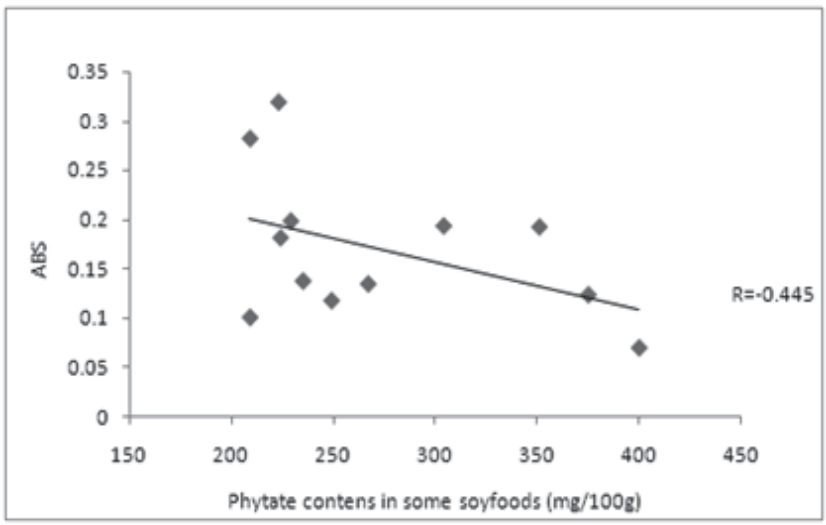

Fig. 5. Correlation between calcium solubility and the phytate content in different kinds of soyfoods. The longitudinal axis shows the ABS of the experimental solutions; A low ABS indicates a low solubility of calcium.

Moreover, the addition of phytate to diets has been demonstrated to decrease the absorption ratios of magnesium and phosphorus, but not calcium (Miyazawa et al., 1996).

7. Phytate contents and nonheme iron solubility

In this section, I describe the results of an *in vitro* nonheme iron solubility assay using some kinds of soyfoods, such as tempe. Nonheme iron reportedly reduced in the stomach and absorbed by the duodenum and proximal jejunum. The absorption ratio of nonheme iron is also low, because the nonheme iron is likely to be crystallized, insolubilized, and precipitated similarly to calcium. The low availability of nonheme iron, which occupies a

large proportion of a typical diet, is one of the factors that lead to a lack of iron as a nutrient (Clydesdale, 1983).

7.1 Experimental procedure

Samples for the nonheme iron solubility assay were prepared by the method described in section 6.1. Furthermore, 50 mg of each powdered experimental sample, 30 µL of $FeCl_3$ solution (1.68 mg/mL HCl), and 500 µL of MOPS buffer (pH 7.5) were mixed, and subsequently incubated for 30 min at 37 °C. The mixtures were centrifuged at 9100 × g, and the concentration of the supernatants was measured using Fe C-test (Wako Pure Chemical Industries, Ltd.). A mixture using distilled water instead of $FeCl_3$ solution was used as the blank.

7.2 Results and discussion

The correlation between nonheme iron solubility and phytate content in some kinds of soyfoods is shown in Fig. 6. The longitudinal axis shows the ABS of the experimental solutions. A low ABS indicates low solubility of nonheme iron. Because the calculated correlation coefficient was 0.807, which indicates a strong positive correlation, a high phytate content may induce high nonheme iron solubility in this condition, whereas higher phytate content suggested to induce lower mineral solubility.

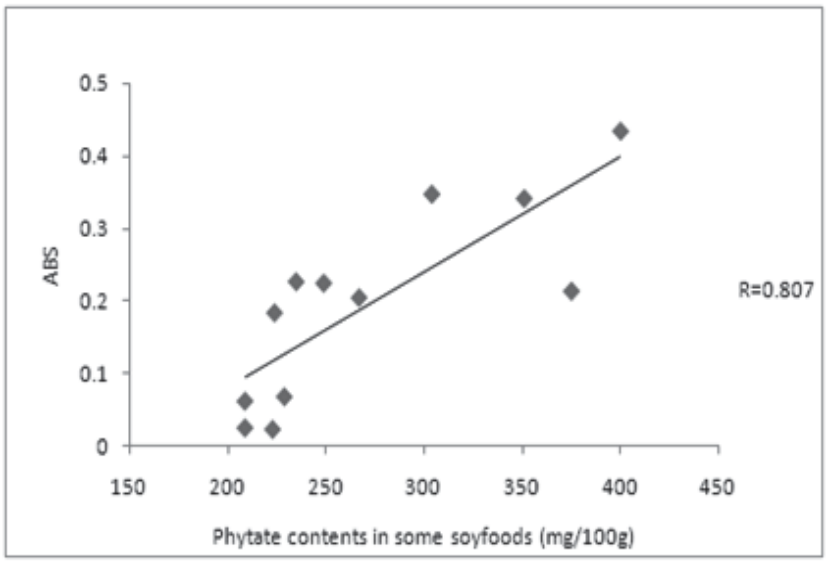

Fig. 6. Correlation between nonheme iron solubility and phytate content in some kinds of soyfoods. The longitudinal axis shows the ABS of the experimental solution; A low ABS indicates low solubility of nonheme iron.

Similar to the existing literature on calcium absorption, there are also some inconsistent studies about the relationship between phytate contents in diets and nonheme iron absorption.
Dietary phytate has been suggested to decrease the bioavailability of iron due to the formation of iron and phytate in the small intestine (Minihane & Rimbach, 2002). The absorption ratio of iron from bread containing bran was remarkably lower than that from

white bread. Moreover, the absorption ratio of iron from the bread with bran with removed phytate from an HCl treatment increased to the same level as from white bread (Hallberg, 1984).
In contrast, although excessive administration of phytate created complexes of phytate with iron and calcium, the absorption ratios of iron and calcium were unaffected (Graf & Eaton, 1984).
The formation of phytate-iron complexes are suggested to involve either monoferric phytate that complexes with 1 iron, or ferric phytate that complexes with 2 or more irons. While monoferric phytate is contained in wheat, ferric phytate is contained in soybean. Furthermore, the bioavailability of monoferric phytate is high relatively and that of ferric phytate is low (House & Welch, 1987; Morris & Ellis, 1976; Ambe et al., 1987).

8. Conclusion

As evidence by the findings presented in this chapter, the relationship between phytate concentrations and mineral availability has remained unclear. The relationship between the solubility and both calcium and iron, as determined by the methods in section 5.1 and 6.1, are shown in Figs. 7 and 8, respectively.

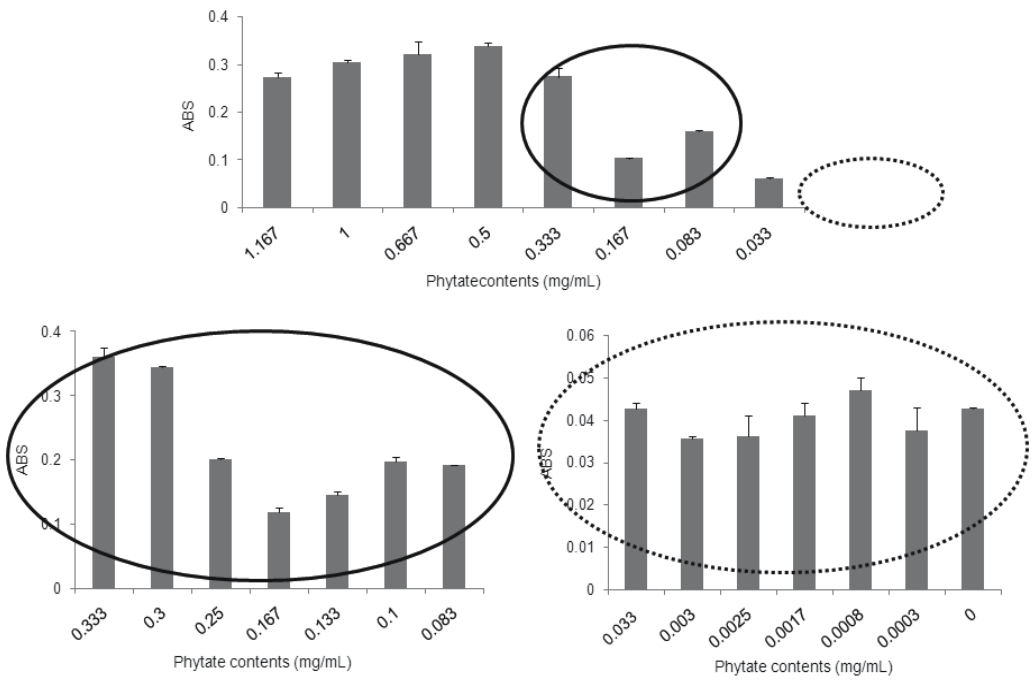

Fig. 7. The relationship between phytate contents and solubility of calcium based on an *in vitro* assay. The longitudinal axis shows the ABS of the experimental solutions. A low ABS indicates low solubility of calcium. The upper graph panel indicates calcium solubility in a range of phytate contents from 1.167 mg/mL to 0.033 mg/mL. Furthermore, the left lower panel indicates calcium solubility in the phytate contents of the concentration range shown in the solid line circle on the upper panel. Similarly, the right lower panel indicates the solubility at concentrations in the area shown in the dotted line circle on the upper panel.

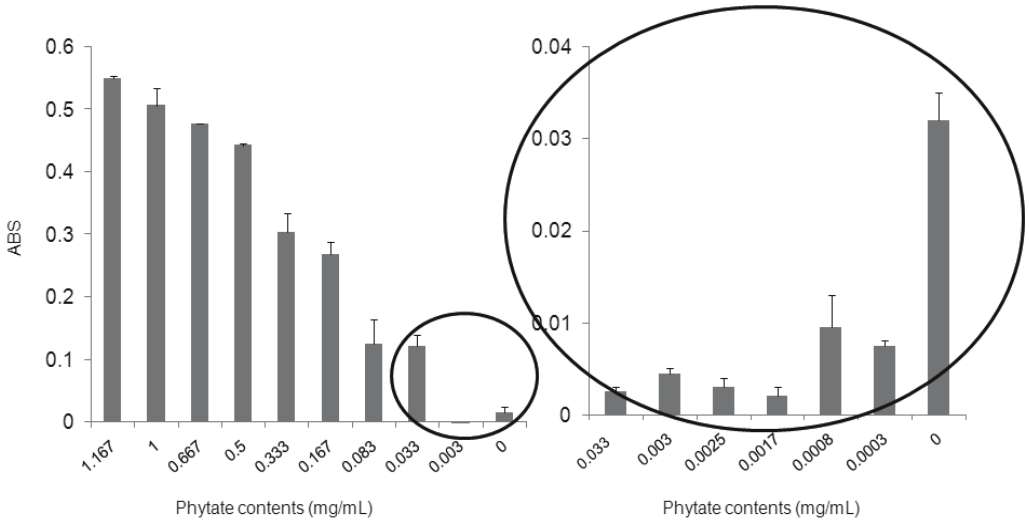

Fig. 8. The relationship between phytate content and solubility of iron based on an *in vitro* assay. The longitudinal axis shows the ABS of the experimental solutions. A Low ABS indicates low solubility of nonheme iron. The left panel show the iron solubility in the phytate contents ranging from 1.167 mg/mL to 0 mg/mL. The right panel indicates the iron solubility at concentration shown in the circle on the left panel.

As shown in Fig. 7, calcium solubility is maximized an optimal concentration of phytate. In this study, the optimal concentration of phytate was determined to be 0.167 mg/mL. At the higher and lower phytate concentrations, the solubility was not lower than at the optimal concentration.

As shown in Fig. 8, nonheme iron solubility decreased gradually in the phytate concentration from 1.167 mg/mL to 0.033 mg/mL. On the other hand, in the concentration lower than 0.033 mg/mL, the solubility in the 0 mg/mL was the highest.

Despite that I indicated a relationship between phytate concentration and both calcium and nonheme iron solubility, the physiological concentrations of calcium and nonheme iron in the small intestine remain unclear. Therefore, further detailed investigations were needed to elucidate the role of phytate in the solubility, absorption, and bioavailability of minerals such as calcium and nonheme iron.

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Soluble Carbohydrates in Soybean

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1. Introduction

Soybean seeds accumulate soluble carbohydrates throughout development and maturation. Soluble carbohydrates may have important roles in seed germination, seed desiccation tolerance, and cold stress tolerance. The analysis of soluble carbohydrates in soybean seeds and other plant parts at various growth stages (Obendorf et al., 1998b & 2009; Kosina et al., 2009), under environmental stresses (Blackman *et al*., 1992; Buitink et al., 2004; Caffrey et al., 1988; Koster & Leopold, 1988; Obendorf et al., 1997; Rosnoblet et al., 2007; Obendorf et al., 2008b) and in mutant lines (Sebastain et al., 2000; Hitz et al., 2002; Obendorf et al., 2008b & 2009) has provided valuable information about the potential roles of these compounds. The methods for analysis of soluble carbohydrates involve extraction of compounds and quantification by HPLC or high resolution gas chromatography. A special method for measuring transport unloading from the seed coat to the embryo has been developed (Thorne & Rainbird, 1983; Rainbird et al., 1984; Ellis & Spanswick, 1987; Gomes et al., 2005; Kosina et al., 2009 & 2010). In the leaves of soybean plants, glucose is converted to glucose-6-phosphate and then to *myo*-inositol. Maternally, *myo*-inositol is converted to D-pinitol (1D-3-*O*-methyl-*chiro*-inositol) through D-ononitol (1D-4-*O*-methyl-*myo*-inositol) as an intermediate; *myo*-inositol also is converted to D-*chiro*-inositol. Three cyclitols (*myo*-inositol, D-pinitol, and D-*chiro*-inositol) along with sucrose are transported to developing seeds via the phloem where they are unloaded from the seed coat into the apoplastic space surrounding the embryo. During seed maturation the transported free cyclitols accumulate as galactosyl cyclitols, digalactosyl cyclitols, or trigalactosyl cyclitols in the axis and cotyledons of the maturing embryos. Sucrose accumulates as sucrose and as raffinose family oligosaccharides (RFO; raffinose, stachyose and verbascose), *myo*-inositol accumulates as galactinol series oligosaccharides (galactinol, digalactosyl *myo*-inositol and trigalactosyl *myo*-inositol), D-*chiro*-inositol accumulates as fagopyritols (fagopyritol B1, fagopyritol B2, and fagopyritol B3), and D-pinitol accumulates as galactopinitols (galactopinitol A, galactopinitol B, ciceritol, and trigalactopinitol A). In the seed, *myo*-inositol also is converted to phytic acid (*myo*-inositol hexa*kis*phosphate) which is stored in seed protein bodies as phytin (the potassium, sodium, and magnesium salts of phytic acid), a major source of phosphorus and cation chelation. Raffinose family oligosaccharides and phytin can result in reduction of the digestibility and the economic, environmental and dietary value of soybean seed. Consumption of RFO from mature seed products results in flatulence in humans and non-ruminants in addition to reduced digestibility in chickens and pigs (Sebastian et al., 2000). Reducing raffinose and stachyose accumulation in soybean seeds results in increases in metabolizable energy in soybean feed (Sebastian et al., 2000) and reduces flatulence in

humans (Suarez et al., 1999). When consumed, phytic acid is a major inhibitor of both calcium (Heaney et al., 1991) and iron (Lynch et al., 1994) absorption in humans and also results in high phytate concentrations in the manure of chickens and pigs (Sebastian et al., 2000; Hitz et al., 2002). Phosphorus runoff from manure accumulates in lakes and streams resulting in their subsequent eutrophication (Sharpley et al., 2003). In comparison, consumption of products with low phytic acid improves mineral absorption (Heaney et al., 1991; Lynch et al., 1994) and reduces livestock fecal and urinary total phosphorus by 40% with an increase of less damaging and essential nutrient, absorbable (digestible) inorganic phosphorus (Htoo et al., 2007). Mutants have been identified which reduce the accumulation of these undesirable compounds in soybean seeds.

Several useful reviews on soluble carbohydrates in seeds have been published (Dey, 1990; Horbowicz & Obendorf, 1994; Avigad & Dey, 1997; Obendorf, 1997; Loewus & Murthy, 2000; Górecki et al., 2001; Kadlec et al., 2001; Peterbauer & Richter, 2001; Raboy, 2009). This chapter describes the soluble carbohydrate composition of soybean seeds, the structures and biosynthetic pathways, accumulation of soluble carbohydrates during seed development and maturation and their degradation during hydration and germination, a description of changes in soluble carbohydrates in soybean seeds expressing mutant *stc1* and *mips* phenotypes, and the trade-off between improved nutritional quality and agronomic performance of seeds with modified soluble carbohydrate composition.

2. Soluble carbohydrate extraction and analysis

2.1 Greenhouse growth of soybean plants

Locally (42° north latitude) adapted genotypes and cultivars of soybean (maturity groups I and II with indeterminate growth habit) are grown throughout the year in a greenhouse at 27°C day (14 hours) and 21°C night (10 hours) with natural sunlight supplemented 14 hours daily with 740 μmole cm^{-2} $hour^{-1}$ incandescent light from 1000 watt BU Sylvania metal-halide lamps positioned above the plants (Fig. 1). After inoculation of soybean seeds with

Fig. 1. Soybean plant growth in greenhouse.

Bradyrhizobium japonicum, three seeds are placed at 1-cm depth in moist greenhouse soil mix in 4-L pots. The soil mix is composed of equal volumes of silty clay loam soil and artificial medium. The artificial medium contains 0.2 m^3 coarse vermiculite, 0.2 m^3 peat moss, 0.5 kg ferrous sulfate, and 1 kg commercial fertilizer (10-10-10, % as N, P_2O_5, and K_2O equivalents). At 1 week after emergence, seedlings are thinned to 1 plant per pot. Plants are thoroughly watered and fertilized with 2 g pot^{-1} of commercial fertilizer (20-20-20, % as N, P_2O_5, and K_2O equivalents) in water at weekly intervals. Plants are debranched to promote pod set on the main stem, and plants are rotated on the greenhouse bench weekly. This method provides a continuous supply of soybean plants, pods, and seeds at all stages of seed development and maturation for experimentation throughout the year. Pods and seeds may be selected at specific growth stages to synchronize samples for experimentation.

2.2 Soybean stem-leaf-pod explants

Soybean stem-leaf-pod explants which include one internode, one leaf, and one pod with three immature seeds (280–300 mg fresh weight each; about 35 days after pollination; at mid-seed fill before accumulation of RFO, fagopyritols, and galactopinitols) are prepared for feeding exogenous substrates (Gomes et al., 2005; Obendorf et al., 2008a; Kosina et al., 2010) and analysis of seed coat unloading (Kosina et al., 2010). The cut, basal end of the internode (stem) of each explant is placed in a 125-mL Erlenmeyer flask (one explant per flask) containing 100 mL of a feeding solution (Fig. 2). Each solution is loaded into an explant through the cut stem and transported to the leaf by the transpiration stream and to the seed coat through the phloem (Fig. 2). The effect of feeding specific substrates on the composition of mature dry seeds can be determined after feeding the explants for 1-2 weeks followed by slow drying of the explants to facilitate maturation of the seeds (Gomes et al., 2005; Obendorf et al., 2008a).

Fig. 2. Soybean stem-leaf-pod explants in cyclitol solutions or a control solution.

2.3 Soybean seed coat cup

The use of seed coat cups (Fig. 3), formed by surgically removing the immature embryo from the immature soybean seed forming an empty seed coat, is a useful technique to study compounds unloaded from the seed coat into the apoplastic space surrounding the embryo

(Thorne & Rainbird, 1983; Rainbird et al., 1984; Ellis & Spanswick, 1987; Gomes et al., 2005; Kosina et al., 2009 & 2010). Seed coat cup unloading analysis is performed on the middle seed using the surgical method of removing the distal half of the seed coat and the entire embryo from the intact seed coat cup (Fig. 3; Thorne & Rainbird, 1983; Ellis & Spanswick, 1987; Gomes et al., 2005; Kosina et al., 2009 & 2010). Because buffer, salts and mannitol (Thorne & Rainbird, 1983) interfere with derivatization of soluble carbohydrates for analysis by gas chromatography, unloaded compounds are collected in water (Gomes et al., 2005; Kosina et al., 2009 & 2010). Freshly prepared, empty seed coat cups are rinsed two times with distilled water to remove residues and fragments left over from the excision process (Ellis & Spanswick, 1987). The seed coat cup is filled with 200 µL ddH_20 and four 200-µL samples are collected at 30-min intervals for 2 hours (cups refilled after each sampling). An equal volume of ethanol and a known amount of internal standard are added to the sample which is dried and derivatized for analysis by gas chromatography.
Sucrose unloading rates into surgically prepared seed coat cups are comparable to the sucrose unloading rates in plants that are not surgically altered (0.5-1.0 µmole h^{-1}; Thorne & Rainbird, 1983). We have successfully used the seed coat cup method to identify the soluble carbohydrates unloaded by seed coats on intact soybean plants (Gomes et al., 2005; Kosina et al., 2009) and also by seed coats on soybean stem-leaf-pod explants after feeding specific substrates to explants (Kosina et al., 2010).

Fig. 3. Soybean seed coat cup on plant. Seed coat cups are filled with distilled water. Samples are taken at 30-minute intervals for 2 hours and analyzed for soluble carbohydrates unloaded from seed coats.

2.4 Extraction of water-soluble carbohydrates

Soybean, other oil seeds or seed parts (one axis, one or two cotyledons, one seed coat) may be finely pulverized by placing seeds or seed parts in liquid nitrogen and grinding the frozen tissues to a fine powder with a mortar and pestle that is pre-chilled with liquid nitrogen. Tissue (3 to 300 mg) pulverization is easily performed with frozen immature seeds or frozen mature dry seeds or seed parts. A single seed, axis, cotyledon, or seed coat may be prepared for extraction and analysis. Soluble carbohydrates may be extracted in water or hot water. Unfortunately, water extracts may also include contaminating proteins, hydrolytic enzymes, and sometimes cell wall or membrane components. Extraction with aqueous alcohol (water:ethanol, 1:1, v/v) minimizes contamination and activity of hydrolytic enzymes. Passing the aqueous ethanol extract through a 10,000 molecular weight cut-off filter can remove many of the contaminating protein components. Heating the aqueous

ethanol extract to 80°C may inactivate hydrolytic enzymes and minimize degradation of oligosaccharides and galactosyl cyclitols. Heating acidic plant tissue extracts may result in specific artifacts. For example, glutamine readily cyclizes to pyrrolidone carboxylic acid at 100°C (Chibnall & Westall, 1932). *myo*-Inositol may undergo chemical isomerization after heating under specific conditions (Sasaki et al., 1988; Taguchi et al., 1997). Therefore, seed culture media are sterilized by ultafiltration (Saab & Obendorf, 1989; Obendorf et al., 1990; 1998a; 1998b; Wettlaufer & Obendorf, 1991). The filtrate of the aqueous ethanolic extracts of seeds or seed parts may be evaporated under a stream of nitrogen gas at room temperature leaving a dry residue for liquid chromatography or for derivatization in preparation for analysis by gas chromatography. Larger volumes may be freeze-dried when extracting, concentrating, and purifying standards from plant materials.

Typically, the frozen powder from one soybean axis is homogenized with 0.6 mL of ethanol:water (1:1, v/v) containing 100 µg of phenyl α-D-glucoside as internal standard in a ground-glass tissue homogenizer, one soybean cotyledon is extracted in 2.0 mL of ethanol:water (1:1, v/v) containing 300 µg of phenyl α-D-glucoside as internal standard in a ground-glass tissue homogenizer, and one soybean seed coat is extracted in 1.0 mL of ethanol:water (1:1, v/v) containing 100 µg of phenyl α-D-glucoside as internal standard in a ground-glass tissue homogenizer. The extracts are centrifuged at 15,000 x g in a microfuge. Aliquots (500 µL) of the cleared supernatants are passed through a 10,000 molecular weight cut-off filter (Nanosep 10K Omega, Pall Life Sciences, Ann Arbor, Michigan, USA), 200 µL of each filtrate is dried under a stream of nitrogen gas and stored over P_2O_5 overnight to remove traces of water. The dried residues are derivatized with trimethylsilyl-

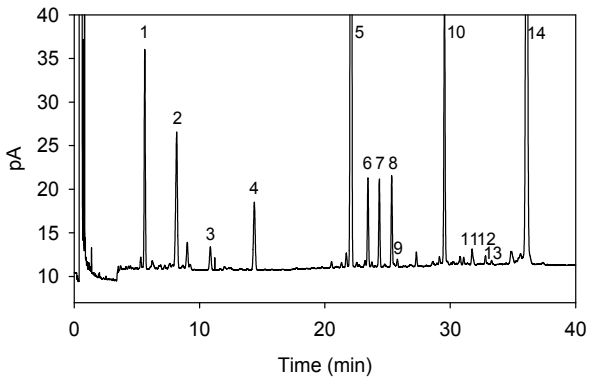

Fig. 4. Gas chromatogram of mature soybean seed cotyledon extract. Dried residues of extracts including the internal standard phenyl α-D-glucoside were derivatized with trimethylsilylimidazole (TMSI):pyridine and analyzed by gas chromatography (Horbowicz & Obendorf, 1994) with minor changes (Gomes et al., 2005) on an HP-1MS capillary column (15 m length, 0.25 mm internal diameter, 0.25 µm film thickness). Identification of peaks: D-pinitol (**1**), D-*chiro*-inositol (**2**), *myo*-inositol (**3**), phenyl α-D-glucoside (internal standard) (**4**), sucrose (**5**), galactopinitol A (**6**), galactopintiol B (**7**), fagopyritol B1 (**8**), galactinol (**9**), raffinose (**10**), ciceritol (**11**), fagopyritol B2 (**12**), DGMI (digalactosyl *myo*-inositol) (**13**), and stachyose (**14**). Soybean seeds expressing the mutant *stc1* phenotype may accumulate small amounts of trigalactosyl cyclitols (not shown in illustration) including trigalactosyl pinitol A (TGPA, 37.8 min), fagopyritol B3 (39.9 min), and trigalactosyl *myo*-inositol (TGMI, 40.4 min).

imidazole:pyridine (1:1, v/v) for analysis by high resolution gas chromatography (Fig. 4). We use silanized glass inserts for drying and derivatization in preparation for analysis by gas chromatography to reduce the potential for chemical isomerization.

2.5 Analysis of soluble carbohydrates

High resolution gas chromatography is the preferred method of analysis of soybean soluble carbohydrates which are relatively small oligomers (monomers to tetramers). Fifteen to thirty different soluble carbohydrates may be identified with good resolution on a single chromatogram (Fig. 4). We use long-cup laminar cup splitter liners (Catalog #20802, Restek International, intltechsupp@restek.com) in the split injection port to facilitate volatilization of the high molecular weight trimethylsilylated carbohydrates (di- and trigalactosides). Some researchers prefer to use direct on-column injection (Traitler et al., 1984).

High pressure liquid chromatography (HPLC) may be preferable for separation of larger oligosaccharides (larger than verbascose, a pentamer) but resolution of monosaccharides and separation of different cyclitols and different galactosyl cyclitols are sometimes problematic when using HPLC.

Analysis of soluble carbohydrates by gas chromatography (Horbowicz & Obendorf, 1994) requires that pure authentic compounds be used as reference standards. Fortunately, many soluble carbohydrates found in soybean seeds are commercially available (see Kadlec et al., 2001, for a listing of sources). Some of the galactosyl cyclitols are not available commercially and must be extracted from plant sources. Kadlec et al. (2001) itemize several plant sources from which standard cyclitols and galactosyl cyclitols may be isolated and provide detailed comparisons of commonly used methods of analysis. Some useful references for the preparation of cyclitols, galactopinitols, fagopyritols, and galactosyl *myo*-inositols include: Ford, 1985; Schweizer et al., 1978; Quemener & Brillouet, 1983; Schweizer & Horman, 1981; Nicolas et al., 1984; Gantner et al., 1991; Horbowicz & Obendorf, 1994; Horbowicz et al., 1998; Szczecinski et al., 1998 & 2000; Obendorf et al., 2000; Steadman et al., 2001; Streeter, 2001; Frank et al., 2009). Recently, the structures of fagopyritol B3, digalactosyl *myo*-inositol (DGMI) and trigalactosyl *myo*-inositol from buckwheat seeds have been confirmed by NMR (Gui, W., Lemley, B.A., Keresztes, I., Condo, A., Steadman, K.J. & Obendorf, R.L., unpublished).

3. Soluble carbohydrate composition of mature seeds

Mature dry soybean seeds may contain 15 to 20 different soluble carbohydrates amounting to approximately 15 to 25% of dry weight (Table 1). Raffinose family oligosaccharides, predominantly stachyose in mature dry soybean seeds, are α-galactosyl derivatives of sucrose (Fig. 5). Sucrose and RFO are the major soluble carbohydrates in soybean seeds (Amuti & Pollard, 1977; Kuo et al., 1988; Horbowicz & Obendorf, 1994; Obendorf et al., 1998b). Other soluble carbohydrates include α-galactosyl derivatives of the cyclitols *myo*- inositol (galactinol and sometimes digalactosyl *myo*-inositol and trigalactosyl *myo*-inositol) (Fig. 6), D-pinitol (galactopinitol A and sometimes digalactosyl pinitol A (ciceritol) and trigalactosyl pinitol A (Fig. 7), and galactopinitol B (Fig. 8), but the digalactosyl pinitol B and trigalactosyl pinitol B oligomers have not been detected in soybean seeds), and D-*chiro*-inositol (fagopyritol B1 and sometimes the di- and tri- galactosyl oligomers fagopyritol B2 and fagopyritol B3) (Fig. 9). Small amounts of *myo*-inositol, D-pinitol, and D-*chiro*-inositol may also be detected in mature

dry seeds. Other than small amounts of maltose (Table 1), reducing sugars are in low concentrations, or not detected, in mature dry seeds. Stachyose and other RFO are not digested by humans, chickens, pigs, and other non-ruminant animals but are microbially fermented in the lower gut resulting in flatulence and reduced feed efficiency (Gitzelmann & Auricchio, 1965; Rutloff et al., 1967; Price et al., 1988; Sebastian et al., 2000).

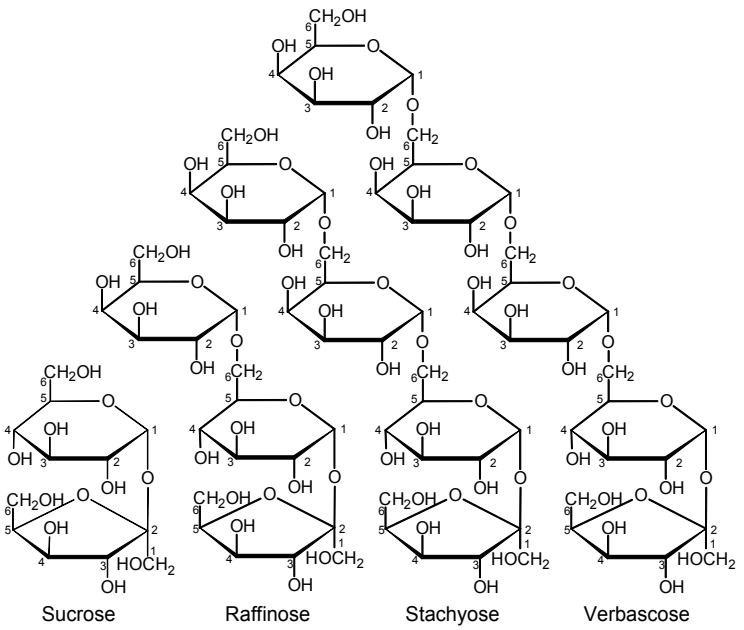

Fig. 5. Raffinose family oligosaccharides (RFO; raffinose, stachyose, and verbascose) are mono-, di- and tri-galactosyl derivatives of sucrose. Sucrose and stachyose are the major soluble carbohydrates in mature soybean seeds.

4. Synthesis of soluble carbohydrates

The enzyme hexokinase (EC 2.7.1.1) converts glucose to glucose-6-phosphate. The enzymes *myo*-inositol-phosphate synthase (MIPS; EC 5.5.1.4) and *myo*-inositol-phosphate monophosphatase (IMP; EC 3.1.3.25) convert glucose-6-phosphate to *myo*-inositol (Fig. 10). 1D-*myo*-Inositol-3-phosphate, the name preferred by biochemists, and 1L-*myo*-inositol-1-phosphate, the name preferred by chemists, are the same structure. Of the four *Mips* genes (*Mips1*, *Mips2*, *Mips3*, *Mips4*) identified in soybean, *Mips1* is highly expressed in immature seeds (Hegeman et al., 2001; Hitz et al., 2002; Nunes et al., 2006; Chiera & Grabau, 2007), especially in cotyledons (Hitz et al., 2002; Chappell et al., 2006). *Mips2*, *Mips3* and *Mips4* are poorly expressed in immature seeds; by contrast *Mips4* is highly expressed in leaves (Chappell et al., 2006).

The free cyclitols *myo*-inositol, D-ononitol, D-pinitol, and D-chiro-inositol (Fig. 11) are present in soybean leaves (Streeter, 2001; Streeter *et al*., 2001). D-Ononitol is an intermediate in the conversion of *myo*-inositol to D-pinitol in leaves of legumes (Dittrich & Brandl, 1987). The

enzyme *myo*-inositol *O*-methyl transferase (IMT; EC 2.1.1.129) converts *myo*-inositol to D-ononitol (Vernon & Bohnert, 1992; Vernon et al., 1993; Wanek et al., 1995). The enzyme(s) for conversion of D-ononitol to D-pinitol is unknown but is believed to be a two-step oxidoreductase with an inosose as an intermediate (Fig. 11; Obendorf, 1997). Likewise, the

Soluble carbohydrate	Soybean Line			
	CHECK	LRS	LRSP1	LRSP2
	mg (g dry weight)$^{-1}$ ± SE			
Sucrose	57.22 b	84.84 b	212.12 a	91.96 b
Raffinose	16.70 a	0.82 c	5.58 b	6.65 b
Stachyose	133.60 a	6.44 b	3.27 b	3.28 b
Verbascose	6.90 a	1.60 b	0 c	0 c
myo-Inositol	0.60 ab	1.77 a	1.20 a	0.03 b
Galactinol	0.55 b	8.93 a	0 b	0.01 b
Digalactosyl *myo*-inositol	0.34 b	8.38 a	0 b	0.03 b
Trigalactosyl *myo*-inositol	0 b	0.43 a	0 b	0 b
D-Pinitol	6.67 b	0.60 c	28.63 a	10.36 b
Galactopinitol A	7.39 a	9.34 a	1.20 b	1.29 b
Galactopinitol B	5.47 a	3.15 b	1.14 c	0.91 c
Ciceritol	2.00 b	9.17 a	0.03 c	0.10 c
Trigalactosyl pinitol A	0 b	1.22 a	0 b	0 b
D-*chiro*-Inositol	0.40 b	0.06 b	1.47 a	0.16 b
Fagopyritol B1	4.57 a	2.59 b	2.10 b	2.14 b
Fagopyritol B2	0.69 a	1.33 a	0.05 b	0.09 b
Fagopyritol B3	0 b	0.27 a	0 b	0 b
Fructose	0.62 b	0.56 b	5.76 a	0.27 b
Glucose	0.33 b	0.41 b	1.75 a	0.25 b
Maltose	2.75 a	5.27 a	5.36 a	5.48 a
Total soluble carbohydrates	246.87 ab	147.18 bc	269.63 a	123.98 c
Total RFO	157.21 a	8.86 b	8.85 b	9.92 b
Total α-galactosides	178.28 a	53.66 b	13.36 c	14.49 c
Ratio (sucrose:RFO)	0.51 c	11.24 b	36.46 a	10.36 b
Ratio (sucrose:α-galactosides)	0.43 c	1.72 c	20.07 a	6.83 b

Table 1. Soluble carbohydrates in cotyledon tissues from seeds of four soybean lines (from Obendorf et al., 2008b). For comparisons between soybean lines, means not connected by the same letter are significantly different ($P < 0.05$) after a Tukey correction for multiple comparisons. RFO = raffinose family oligosaccharides (raffinose + stachyose + verbascose).

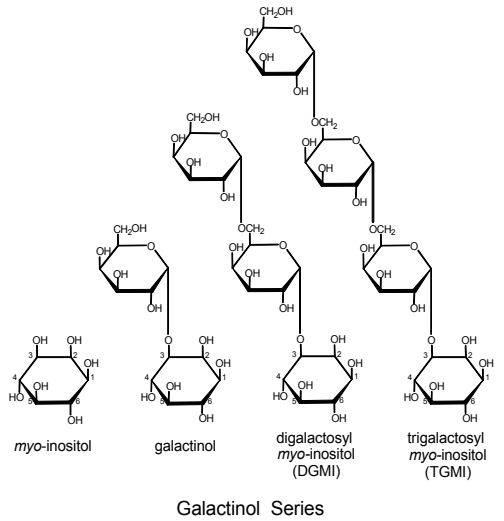

Fig. 6. Galactinol series. Small amounts of galactinol (α-D-galactopyranosyl-(1→3)-1D-*myo*-inositol or α-D-galactopyranosyl-(1→1)-1L-*myo*-inositol) and sometimes digalactosyl *myo*-inositol are detected in soybean seeds. Seeds with low RFO accumulation or with elevated galactinol also may have increased amounts of digalactosyl *myo*-inositol and detectable amounts of trigalactosyl *myo*-inositol.

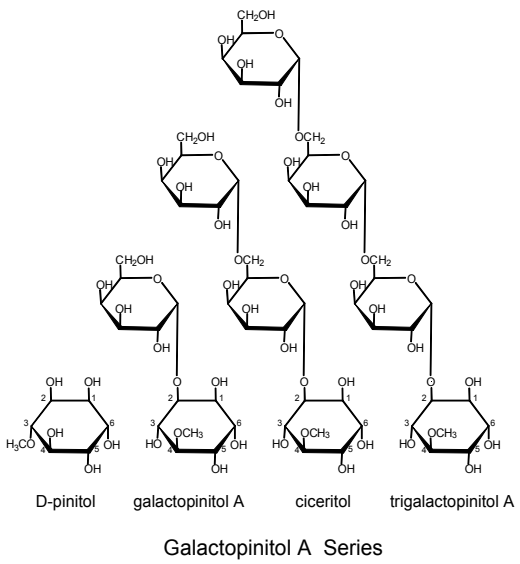

Fig. 7. Galactopinitol A series. Normal soybean seeds accumulate mostly galactopinitol A (α-D-galactopyranosyl-(1→2)-1D-4-*O*-methyl-*chiro*-inositol) and small amounts of ciceritol (digalactosyl pinitol A). Soybean seeds expressing the *stc1* mutant, with low raffinose synthase activity and low accumulation of stachyose, may accumulate small amounts of trigalactosyl pinitol A. In the galactopinitol A series, the galactosyl residues attach to the 5-position of D-pinitol; upon attachment, the carbons are renumbered: 6 becomes 1, 5 becomes 2, and 3 becomes 4.

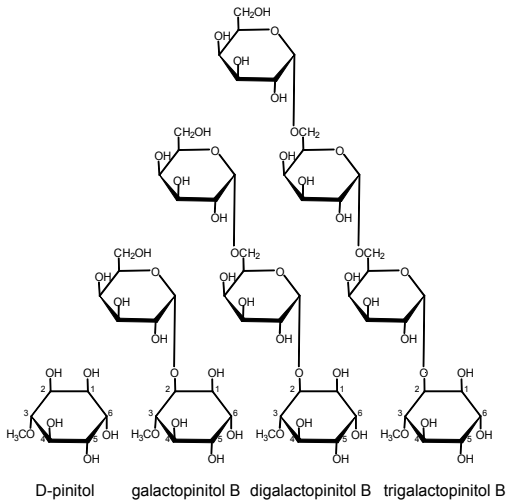

Fig. 8. Galactopinitol B series. Only galactopinitol B (α-D-galactopyranosyl-(1→2)-1D-3-*O*-methyl-*chiro*-inositol) accumulates in soybean seeds. Digalactosyl pinitol B and trigalactosyl pinitol B have not been detected in soybean. In the galactopinitol B series, the galactosyl residues attach to the 2-position of D-pinitol.

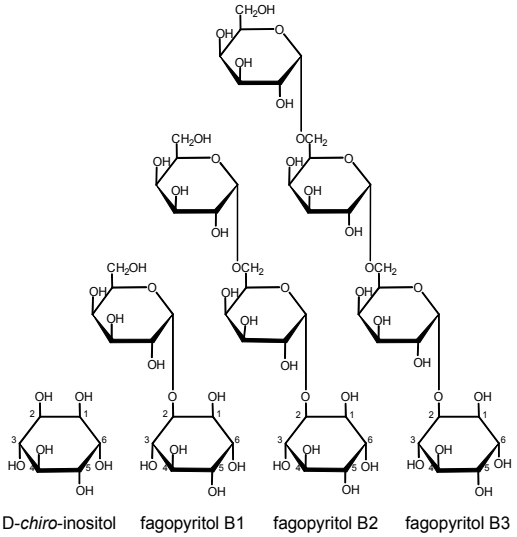

Fig. 9. Fagopyritol B series. Fagopyritol B1 (α-D-galactopyranosyl-(1→2)-1D-*chiro*-inositol) is the dominant fagopyritol in normal mature soybean seeds. Seeds fed free D-*chiro*-inositol or seeds expressing the mutant *stc1* phenotype with low RFO accumulation also may have fagopyritol B2 and small amounts of fagopyritol B3. Only the fagopyritol B series compounds are detected in mature soybean seeds. Fagopyritol A series compounds, (fagopyritol A1, α-D-galactopyranosyl-(1→3)-1D-*chiro*-inositol) have not been detected in soybean.

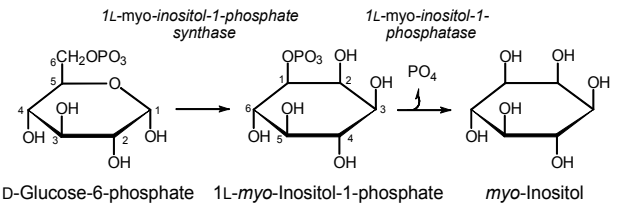

Fig. 10. Synthesis of *myo*-inositol. The structures 1D-*myo*-inositol-3-phosphate and 1L-*myo*-inositol-1-phosphate are the same structure.

enzyme(s) for conversion of *myo*-inositol to D-*chiro*-inositol is unknown but is believed to be a two-step oxidoreductase with an inosose as an intermediate (Fig. 11). There is no evidence to support the proposed synthesis of D-*chiro*-inositol from D-pinitol in soybean (Obendorf, 1997; Obendorf et al., 2004). Surgically removing the immature embryo from immature soybean seed to form an empty seed coat cup has been used to study compounds unloaded from the seed coat into the apoplastic space surrounding the embryo (Thorne & Rainbird, 1983; Rainbird et al., 1984; Ellis & Spanswick, 1987; Gomes et al., 2005; Kosina et al., 2009 & 2010). Sucrose (90% of C), amides (glutamine, 52% of N; asparagine, 19% of N) and amino acids are the most abundant compounds unloaded by soybean seed coats (Rainbird et al., 1984; Ellis & Spanswick, 1987).

Additionally, *myo*-inositol, D-pinitol and D-*chiro*-inositol are transported to the seed and unloaded from the seed coat into the apoplastic space surrounding the developing embryo (Gomes et al., 2005; Kosina et al., 2009 & 2010). The reducing sugars glucose, fructose, and maltose are detected in variable but small amounts.

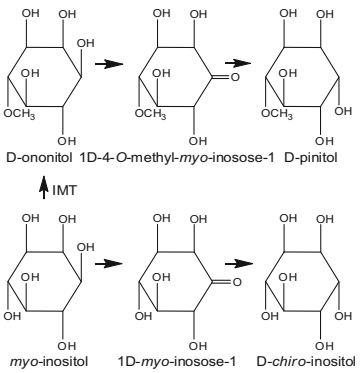

Fig. 11. Soybean cyclitols. Soybean leaves have four cyclitols: *myo*-inositol, D-ononitol (1D-4-*O*-methyl-*myo*-inositol), D-pinitol (1D-3-*O*-methyl-*chiro*-inositol) and D-*chiro*-inositol. *myo*-Inositol is converted to D-pinitol through D-ononitol as an intermediate in legume leaves (Dittrich and Brandl, 1987). The enzyme *myo*-inositol *O*-methyl transferase (IMT; EC 2.1.1.129) converts *myo*-inositol to D-ononitol. The enzyme(s) for conversion of D-ononitol to D-pinitol is unknown but is believed to be a two-step oxidoreductase with an inosose as an intermediate. The enzyme(s) for conversion of *myo*-inositol to D-*chiro*-inositol is unknown but is believed to be a two-step oxidoreductase with an inosose as an intermediate. There is no evidence to support the proposed synthesis of D-*chiro*-inositol from D-pinitol in soybean. *myo*-Inositol, D-pinitol and D-*chiro*-inositol are transported to the seed and unloaded from the seed coat into the apoplastic space surrounding the developing embryo. *myo*-Inositol also may be synthesized in the embryo during seed development.

D-Ononitol, galactinol, galactopinitols, fagopyritols, raffinose, stachyose, and verbascose are not detected in seed coat cup exudates (Gomes et al., 2005; Kosina et al., 2009 & 2010). Seed coat cup unloading rates for D-*chiro*-inositol, *myo*-inositol, D-pinitol, and sucrose average 5.1, 3.6, 32.9, and 147.7 μg hour^{-1}, respectively, on soybean plants (Kosina et al., 2009). *myo*-Inositol also may be synthesized in the embryo during soybean seed development because *Mips* (wild-type *Mips* sequence designation GM mI 1-PS-1A, GenBank accession number AY038802) is expressed in immature cotyledons (Hitz et al., 2002; Chappell et al., 2006). Synthesis of D-pinitol and D-*chiro*-inositol have not been reported in normal soybean embryos. D-Ononitol, the intermediate in the conversion of *myo*-inositol to D-pinitol (Fig. 11) is not detected in soybean seed coat exudates (Gomes et al., 2005; Kosina et al., 2009 & 2010), in soybean embryos (Horbowicz & Obendorf, 1994), or in non-transgenic somatic embryos (Chiera et al., 2006). Transgenic somatic embryos of soybean containing the myo-*inositol* O-*methyl transferase* (*IMT*) gene from *Mesembryanthmum crystallinum* led to an increase in D-ononitol in embryos, compared to non-transgenic embryos, and an increase in D-pinitol in maturing embryos (Chiera et al., 2006).

Sucrose is transported from leaves to seeds, unloaded by seed coats to the apoplastic space surrounding the embryo, and taken up by immature soybean embryos as the major carbon source for seed growth. Raffinose synthase (RFS, EC 2.4.1.82) transfers a galactosyl residue from galactinol (the galactosyl donor) to sucrose (the galactosyl receptor) to form raffinose (Fig. 12). Stachyose synthase (STS, EC 2.4.1.67) transfers a galactosyl residue from galactinol to raffinose to form stachyose (Fig. 12), the most abundant RFO in normal soybean seeds (Table 1) (Avigad & Dey, 1997; Peterbauer & Richter, 2001). Verbascose synthase (VBS) transfers a galactosyl residue from galactinol to stachyose to form verbascose (Fig. 12), usually in small amounts in soybean seeds.

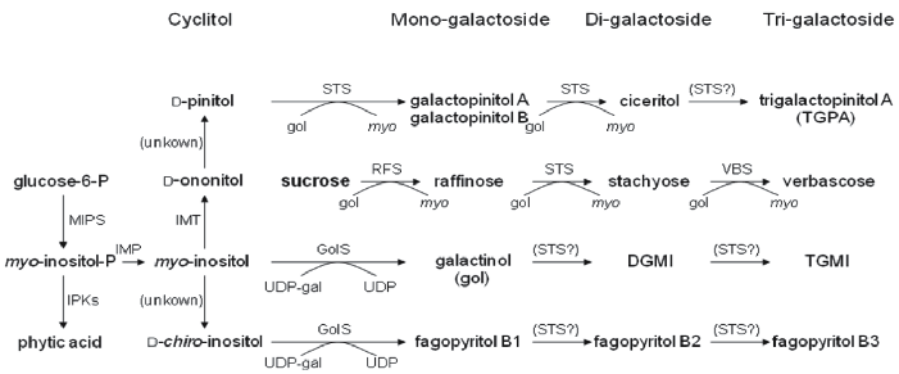

Fig. 12. Proposed pathways for synthesis of cyclitols, cyclitol galactosides, and raffinose family oligosaccharides (from Obendorf et al., 2009). Parentheses (unknown) by an arrow indicates that an enzyme catalyzing the reaction has not been identified. Some reactions may be reversible. DGMI, digalactosyl *myo*-inositol; TGMI, trigalactosyl *myo*-inositol; gol, galactinol; GolS, galactinol synthase (EC 2.4.1.123); IMP, *myo*-inositol-phosphate monophosphatase (EC 3.1.3.25); IPKs, *myo*-inositol-phosphate kinases; MIPS, *myo*-inositol-phosphate synthase (EC 5.5.1.4); *myo*, *myo*-inositol; RFS, raffinose synthase (EC 2.4.1.82); STS, stachyose synthase (EC 2.4.1.67), (STS?) indicates STS, or a similar enzyme, is proposed by extrapolation but has not been demonstrated experimentally; UDP, uridine diphosphate; UDP-gal, uridine diphosphate galactoside; VBS, verbascose synthase.

Maternally synthesized *myo*-inositol may be transported to the soybean seed and unloaded by the seed coat into the apoplastic space surrounding the immature embryo (Gomes et al., 2005; Kosina et al., 2009 & 2010). *myo*-Inositol also may be synthesized in the embryo tissues since *Mips* is expressed in embryos (Hitz et al., 2002; Chappell et al., 2006). Galactinol synthase (GolS, EC 2.4.1.123) transfers a galactosyl residue from UDP-galactose (the galactose donor) to *myo*-inositol (the galactosyl acceptor) to form galactinol (α-D-galactopyranosyl-(1→3)-1D-*myo*-inositol or α-D-galactopyranosyl-(1→1)-1L-*myo*-inositol) (Fig. 12; Obendorf et al., 2004). High availability of *myo*-inositol in embryos leads to elevated galactinol which in turn leads to elevated synthesis of RFO (Fig. 12; Karner et al., 2004). Elevated galactinol may also lead to accumulation of its higher oligomers, digalactosyl *myo*-inositol (DGMI) and trigalactosyl *myo*-inositol (TGMI) (Fig. 12). Enzymes responsible for the accumulation of these higher oligomers of galactinol have not been characterized experimentally, but are predicted to be a stachyose synthase or an enzyme similar to stachyose synthase (STS?; Fig. 12). Metabolism of *myo*-inositol is difficult to follow because *myo*-inositol has multiple roles and forms multiple products including galactinol (Fig. 6), other cyclitols (Fig. 11), cell wall components, membrane components and phytic acid (Loewus & Murthy, 2000; Raboy, 2009).

D-Pinitol is synthesised in leaves (Dittrich & Brandl, 1987; Streeter, 2001; Streeter et al., 2001), transported to seeds, and unloaded by soybean seed coats into the apoplastic space surrounding immature embryos (Figs. 11 & 12; Gomes et al., 2005; Kosina et al., 2009 & 2010). In the embryo, stachyose synthase (STS, EC 2.4.1.67) transfers a galactosyl residue from galactinol to D-pinitol to form galactopintol A plus galactopinitol B (Peterbauer & Richter, 2001; Fig. 12), isomeric compounds due to the presence of the *O*-methyl group of pinitol (Figs. 7 & 8). Only galactopintol A forms higher oligomers in soybean seeds (Fig. 12). Stachyose synthase transfers a galactosyl residue from galactinol (or from galactopinitol A) to glactopinitol A to form ciceritol (Fig. 12; Hoch et al., 1999; Peterbauer and Richter, 2001). It is proposed that stachyose synthase or a similar enzyme also transfers a galactosyl residue from galactinol to ciceritol to form trigalactosyl pinitol A (Fig. 12). Digalactosyl pinitol B and trigalactosyl pinitol B have not been detected in soybean.

Like D-pinitol, D-*chiro*-inositol also is transported to seeds and is unloaded by the seed coat into the apoplastic space surrounding the embryo (Gomes et al., 2005; Kosina et al., 2009 & 2010). Galactinol synthase transfers a galactosyl residue from UDP-galactose (the galactosyl donor) to D-*chiro*-inositol (the galactosyl acceptor) to form fagopyritol B1 (α-D-galactopyranosyl-(1→2)-1D-*chiro*-inositol) (Fig. 12; Obendorf et al., 2004). Soybean galactinol synthase does not form fagopyritol A1 (α-D-galactopyranosyl-(1→3)-1D-*chiro*-inositol) and cannot utilize D-pinitol as a galactosyl receptor to form galactopintols (Obendorf et al., 2004).

5. Modification of soluble carbohydrates in soybean seeds

Seeds of four proprietary soybean [*Glycine max* (L.) Merrill] lines (Table 2) with normal raffinose, stachyose and phytin (CHECK) seeds expressing the normal *Stc1* and *Mips* phenotype; low raffinose and stachyose (LRS) seeds expressing the mutant *stc1* phenotype; low raffinose, stachyose, and phytin (LRSP1, LRSP2) seeds expressing the mutant *mips* phenotype (wild-type *Mips* sequence designation GM mI 1-PS-1A, AY038802; Hitz *et al*., 2002) were provided by Steve Schnebly, Pioneer Hi-Bred, A DuPont Business. All were advanced breeding lines in related, but not isogenic, Group II maturity agronomic backgrounds developed by traditional breeding. The *stc1* and *mips* alleles in these breeding lines are described by Sebastian *et al*. (2000), Hitz *et al*. (2002), and Meis *et al*. (2003).

	CHECK	LRS	LRSP1	LRSP2	References
Raffinose	normal	low	low	low	Sebastian et al., 2000 Hitz et al., 2002
Stachyose	normal	low	low	low	Sebastian et al., 2000 Hitz et al., 2002
Phytic acid	normal	normal	low	low	Sebastian et al., 2000 Hitz et al., 2002
Mutant	*Stc1* normal *Mips* normal	*stc1* mutant *Mips* normal	*Stc1* normal *mips* mutant	*Stc1* normal *mips* mutant	Sebastian et al., 2000 Hitz et al., 2002 Meis et al., 2003
Imbibitional chilling	tolerant	tolerant	sensitive	sensitive	Obendorf et al., 2008b
Field emergence	normal	normal	reduced	reduced	Meis et al., 2003
myo-Inositol-phosphate synthase activity in seeds	normal	normal	low	low	Hitz et al., 2002
Raffinose synthase activity in seeds	normal	low	normal	normal	Hitz et al., 2002
Stachyose synthase activity in seeds	normal	normal	normal	normal	Hitz et al., 2002
Galactinol synthase activity in seeds	normal	normal	normal	normal	Hitz et al., 2002
Galactinol	normal	high	low	low	Sebastian et al., 2000 Hitz et al., 2002
RFO	normal	low	low	low	Sebastian et al., 2000 Hitz et al., 2002
Galactopinitols	normal	higher	lower	lower	Obendorf et al., 2008b
Fagopryitol B1	normal	normal	normal	normal	Obendorf et al., 2008b
Fagopyritols B2 + B3	low	increased	low	low	Obendorf et al., 2008b
Trigalactosyl cyclitols	very low	increased	very low	very low	Obendorf et al., 2009
Feeding D-*chiro*-inositol increased fagopyritol B1	yes	yes	yes	yes	Obendorf et al., 2008a
Feeding *myo*-inositol increased RFO	no	no	yes	yes	Obendorf et al., unpublished Hitz et al., 2002

Table 2. Soybean seed phenotypes of four breeding lines.

Soybean seeds with low raffinose and low stachyose (LRS phenotype) expressing a mutant *stc1* gene conferring reduced raffinose synthase (RFS) activity but normal stachyose synthase (STS) and galactinol synthase (GolS) activities (Sebastian et al., 2000; Hitz et al., 2002) have field emergence and yield comparable to seeds with normal raffinose and stachyose (Neus *et al*., 2005) (Table 2). The low raffinose and stachyose (LRS) phenotype is associated with a novel raffinose synthase allele, RS2 (Dierking & Bilyeu, 2008). LRS seeds expressing the mutant *stc1* phenotype have increased accumulation of galactosyl cyclitols (fagopyritols and galactopintitols) (Obendorf *et al*., 2008b, 2009) and are tolerant to imbibitional chilling (Obendorf *et al*., 2008b) (Table 2). Seeds with low raffinose, stachyose and phytin (LRSP phenotype with 50% less phytin than the normal *Mips* phenotype) expressing a mutant *mips* gene conferring reduced *myo*-inositol-phosphate synthase (MIPS) activity (Sebastian *et al*., 2000; Hitz *et al*., 2002) have decreased field emergence, especially when seeds are produced in subtropical environments (Meis *et al*., 2003), and also are sensitive to imbibitional chilling (Obendorf *et al*., 2008b) (Table 2). Seeds expressing the mutant *mips* phenotype (wild-type *Mips* sequence designation GM mI 1-PS-1A, AY038802; Hitz et al., 2002) with low stachyose and phytin (LRSP1, LRSP2) accumulate very small amounts of galactosyl cyclitols (galactinol, galactopinitols, fagopyritol B2, fagopyritol B3) (Obendorf *et al*., 2008b, 2009), but these seeds can accumulate galactinol, raffinose and stachyose after incubation with *myo*-inositol (Hitz *et al*., 2002) (Table 2). Seeds and isolated embryos of all four lines accumulate fagopyritol B1 after incubation with D-*chiro*-inositol followed by slow drying (Obendorf et al., 2008a; Obendorf, R.L., Sensenig, E.M., Byrt, E.M., Owczarczyk, A.B., Ohashi, M., & Schnebly, S.R., unpublished).

6. Loss of soluble carbohydrates during seed germination

Normal, mature, dry soybean seeds have a high concentration of stachyose (Fig. 13A; Hsu et al., 1973; Amuti & Pollard, 1977; Obendorf et al., 1998b). Upon hydration, stachyose and raffinose concentrations in axis tissues decline to low concentrations before germination (radicle emergence) at 18 hours, followed by an increase in reducing sugars (monosaccharides) (Hsu et al., 1973; Koster & Leopold, 1988). The loss of stachyose in axis tissues (Fig. 13A) correlates with the loss of desiccation tolerance when measured as the rate of leakage from axes after imbibition, desiccation, and rehydration, and emergence of radicles (germination) and shoots following various durations of pre-imbibition (Fig. 13B) (Koster & Leopold, 1988). Loss of non-reducing sugars (raffinose, stachyose, sucrose) is associated with a transient accumulation of starch in axis and cotyledon tissues of soybean seeds (Von Ohlen, 1931; Adams et al., 1980) and with the onset of isocitrate lyase (ICL, EC 4.1.3.1) synthesis and subsequent lipid mobilization after germination (Polanowski & Obendorf, 1991). Conversion of sugars to starch during seed hydration facilitates the increase in solute potential (smaller negative values). Solute potential of axis tissues of hydrating seeds increases to about -1.4 MPa before germination (Egli & TeKrony, 1993).

7. Accumulation of soluble carbohydrates during seed development

During the first 21 days after pollination, the soybean pod increases to about 35 mm in length. After 21 days, the seeds increase in size and expand within the pod (Fig. 14, left). Soybean seeds increase in dry weight in a linear response to maximum fresh weight (Fig. 15A), color changes from green to yellow at maximum seed dry weight (physiological

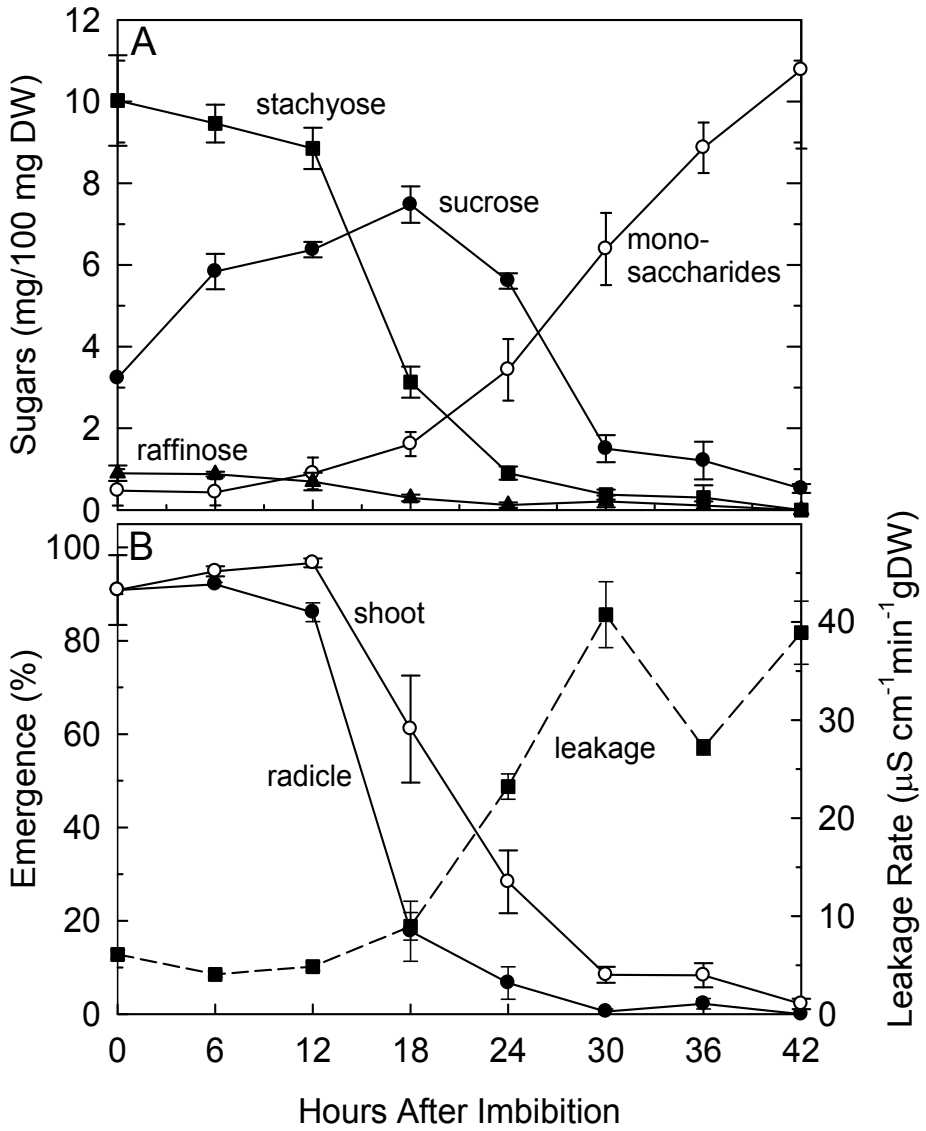

Fig. 13. Changes in soluble sugars and loss of desiccation tolerance during imbibition and post-germination in soybean. A, Changes in sucrose, raffinose, stachyose, and mono-saccharides in axes during imbibition and post-germination of soybean seeds. Radicle emergence (germination) at 18 hours. B, Rate of leakage (squares) from axes after imbibition, desiccation, and rehydration, and emergence of radicles (closed circles) and shoots (open circles) after desiccation following various durations of pre-imbibition (adapted from Koster & Leopold, 1998; this material is copyrighted by the American Society of Plant Biologists and is reprinted with permission).

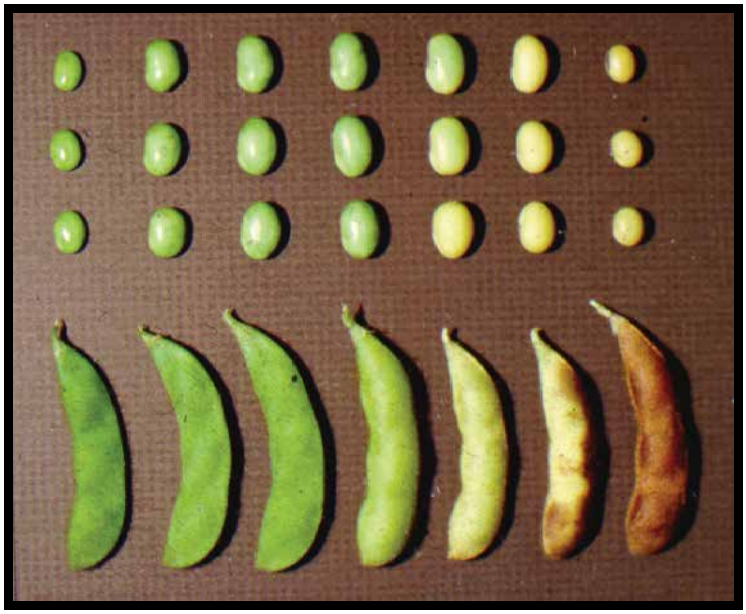

Fig. 14. Soybean seed development and maturation. The number of days after flowering is approximately 24 (left), 34, 38, 48, 54, 58, and 70 (right, mature dry seed).

maturity or mass maturity) (Fig. 15E), and seeds begin to shrink from the pod wall and decrease in size as they begin to lose water during seed desiccation to their mature, dry size (Fig. 14, right). During the linear phase, the soybean seed dry weight growth rate is typically about 5 mg seed^{-1} day^{-1} in the field (Rubel et al., 1972; Obendorf et al., 1980), 8 mg seed^{-1} day^{-1} in the greenhouse (Obendorf et al., 1980), 3-4 mg seed^{-1} day^{-1} in pod culture (Obendorf et al., 1983), and 5-25 mg seed^{-1} day^{-1} in isolated seed culture depending on starting size (Obendorf et al., 1984). Physiological maturity or mass maturity is defined as time of maximum seed dry weight accumulation and represents the cessation of seed dry matter growth. Physiological maturity of individual soybean seeds typically occurs at about 50 days after pollination when seed coat color changes from green to yellow (Fig. 14) and seed moisture is about 60% on a fresh weight basis (Obendorf et al., 1980 & 1998b; Fig. 15). Translocation of sucrose from photosynthate into the embryo ceases when the seed coat changes from green to yellow and seed respiration declines rapidly (TeKrony et al., 1979). Likewise, cotyledons cease to take up sucrose when the cotyledon color changes from green to yellow (Vernooy et al., 1986). At this time, transport of water and nutrients into the seed ceases and the seed begins to shrink as it loses water. Shrinkage of the seed from the pod wall is the most reliable indicator of the cessation of soybean seed growth on plants (Crookston & Hill, 1978). At the time seed growth ceases, the osmotic potential of embryo tissues is about -1.8 MPa (Saab & Obendorf, 1989; Egli, 1990; Slawinska & Obendorf, 1991). Axis tissues turn yellow and cease growth before cotyledons (Obendorf et al., 1984 & 1998b). The pattern of yellowing begins at the radicle tip and progresses up the hypocotyl to the cotyledonary node, whereas cotyledons turn yellow from the edge to the center of each cotyledon (Obendorf et al., 1984 & 1998b). Raffinose and stachyose accumulate late during seed maturation (the yellowing and drying phases; Fig. 15) (Amuti & Pollard, 1977; Yazdi-Samadi et al., 1977; Dornbos & McDonald, 1986; Lowell & Kuo, 1989; Obendorf et al., 1998b

& 2009). About 70% of the RFO, and likewise the galactosyl cyclitols, accumulate after maximum seed dry weight (physiological maturity) during the phase of seed drying (Obendorf et al., 2009). The monogalactosides (raffinose, galactopinitol A, galactopinitol B, fagopyritol B1) start to accumulate when the embryo tissues begin to yellow, followed by the digalactosides (stachyose, ciceritol, fagopyritol B2, digalactosyl *myo*-inositol) and finally the trigalactosides (verbascose, trigalactosyl pinitol A, fagopyritol B3, trigalactosyl *myo*-inositol) during the desiccation phase of seed maturation (Obendorf et al., 2009).
Immature soybean seeds can be precociously matured by slow drying (Adams et al., 1983; Blackman et al., 1992). Germination of immature soybean seeds is similar for seeds undergoing slow drying or for seeds held at high relative humidity to prevent drying (Fig. 16A). Seeds undergoing slow drying develop desiccation tolerance whereas seeds held at high relative humidity do not develop desiccation tolerance (Fig. 16B). Seeds undergoing slow drying accumulate stachyose (Fig. 16D) and are desiccation tolerant (Fig. 16B). In contrast, seeds held at high relative humidity do not accumulate stachyose (Fig. 16D) and do not develop desiccation tolerance (Fig. 16B). Stachyose is not required for germination per se as accumulation of stachyose was not required for germination of seeds held at high relative humidity (Fig. 16A; Blackman et al., 1992). Inhibition of RFO degradation in hydrated soybean seeds did not decrease germination under laboratory conditions with minimal environmental stress, suggesting that RFO metabolism is not an obligatory requirement for soybean germination per se (Dierking & Bilyeu, 2009). These observations do not negate a role of RFO as seed storage reserves. The results merely mean that other readily mobilized reserves are in sufficient supply to meet the needs for germination. Galactinol is the galactosyl donor for the formation of stachyose (Fig. 12). Seeds which are held at high relative humidity do not accumulate stachyose (Fig. 16D) and these seeds accumulate more galactinol (Fig. 16C) than seeds held at high relative humidity. Similarly, LRS seeds expressing the *stc1* mutant have low raffinose synthase activity (Table 2) resulting in lower raffinose, stachyose, and verbascose accumulation but more galactinol than CHECK seeds expressing normal *Stc1* with normal raffinose synthase activity and larger accumulations of raffinose, stachyose, and verbascose (Table 1, Table 2). LRS seeds also accumulate more di- and tri-galactosyl cyclitols than CHECK seeds. LRS seeds are desiccation tolerant, tolerant to imbibitional chilling (Obendorf et al., 2008b), and have normal field emergence (Meis et al., 2003) (Table 2), perhaps because these seeds, that are low in RFO, accumulate more galactosyl cyclitols (Tables 1 & 2). Buckwheat seeds normally accumulate only very small amounts of raffinose and stachyose, but buckwheat seeds do accumulate fagopyritols and are desiccation tolerant with a high germination percentage (Horbowicz & Obendorf, 1994; Horbowicz et al., 1998). It is proposed that fagopyritols and other galactosyl cylitols can function in the same way as stachyose in conveying seed desiccation tolerance and seed performance (Horbowicz & Obendorf, 1994; Horbowicz et al., 1998) in LRS soybean seeds (Obendorf et al., 2008b).

8. Phytic acid

Phytic acid (*myo*-inositolhexa*kis*phosphate) can account for about 75% of the total seed phosphorus and accumulates mostly in seed cotyledon protein bodies as potassium, magnesium, and manganese salts of phytic acid (phytin) (Raboy, 2009). Ingested phytic acid is not efficiently hydrolyzed by humans, chickens, pigs, or other monogastric animals and may contribute to reduced uptake of iron, zinc, and calcium (Heaney et al., 1991; Lynch et

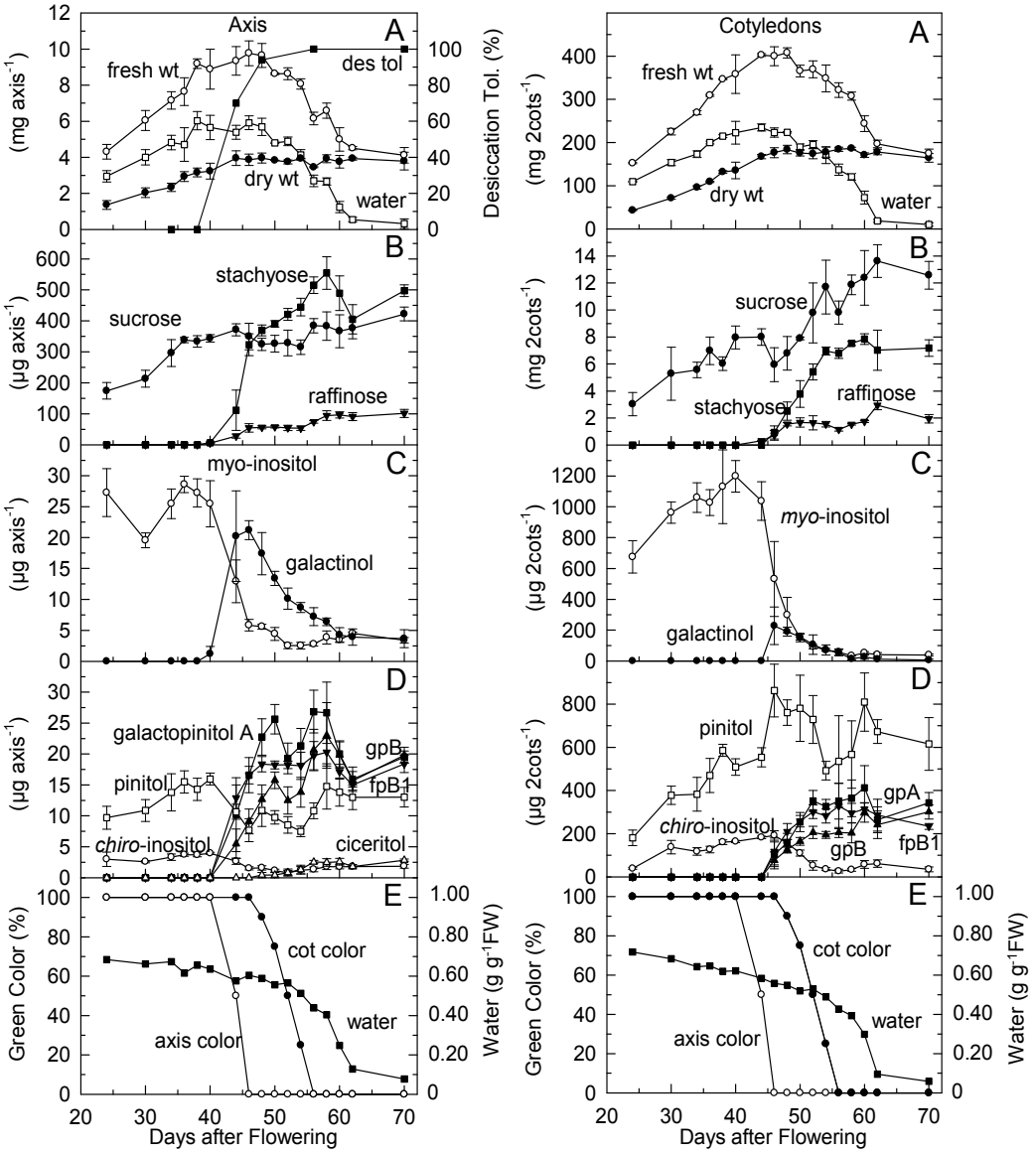

Fig. 15. Soluble carbohydrate accumulation in soybean embryos (axis on left; cotyledons on right) in normal soybean seeds during development and maturation in the greenhouse (from Obendorf et al., 1998b). A, Fresh weight, dry weight, water content, and desiccation tolerance. B, Sucrose, raffinose, and stachyose. C, *myo*-Inositol and galactinol. D, D-Pinitol, galactopinitol A (gpA), galactopinitol B (gpB), ciceritol, D-*chiro*-inositol and fagopyritol B1 (fpB1). E, loss of axis green color, cotyledon green color, and water concentration.

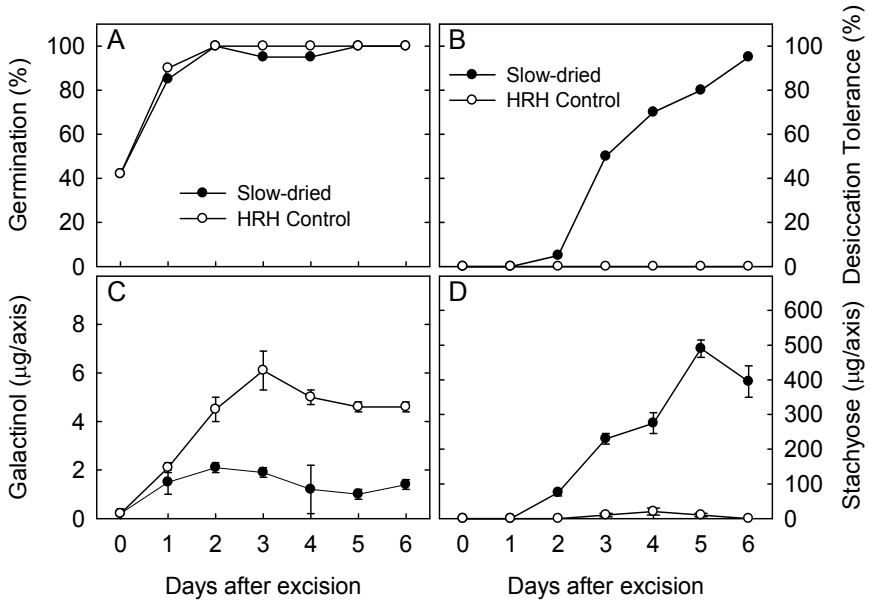

Fig. 16. Stachyose accumulation correlates with seed desiccation tolerance. A) Germination percentage for excised developing soybean seeds undergoing slow drying (filled circles) or high relative humidity (HRH; open circles) for 0 to 6 days after excision. Samples of 20-25 seeds were removed on successive days of each treatment and placed on moist germination paper. Percentage of germination (i.e., radicle emergence) was determined after 7 days on moist germination paper. B) Development of desiccation tolerance during slow drying (filled circles) or high RH control (open circles) treatments. Samples of 20-25 seeds were desiccated rapidly at 13% RH and then tested for germination. C) Content of galactinol in axes of excised developing soybean seeds undergoing slow drying (filled circles) or high RH control (open circles) for 0 to 6 days after excision. D) Content of stachyose in axes of excised developing soybean seeds undergoing slow drying (filled circles) or high RH control (open circles) for 0 to 6 days after excision. For C and D, values are mean ± SE of the mean of five samples of five axes each. Note the different scales on the y axis (adapted from Blackman et al., 1992; this material is copyrighted by the American Society of Plant Biologists and is reprinted with permission).

al., 1994) and contribute to phosphorus pollution through manure from animals fed phytic acid in seed and grain concentrates (Sebastian et al., 2000; Hitz et al., 2002). Seeds with reduced phytic acid can germinate in laboratory studies under minimal environmental stress. Therefore, there is considerable interest in lowering the phytic acid in soybean seed products commonly used in animal feeds. Soybean seeds expressing the mutant *mips* phenotype have reduced phytic acid (Sebastian et al., 2000; Hitz et al., 2002) but also have reduced field emergence compared to seeds expressing the normal *Mips* phenotype that accumulate normal amounts of phytic acid (Meis et al., 2003). Another approach using mutants homozygous for *lpa1* and *lpa2* (low phytic acid genes 1 and 2) also produce a low phytic acid phenotype, but these soybean seeds have reduced field emergence compared to seeds expressing the normal phytic acid phenotype (Oltmans et al., 2005). Additional research is needed to obtain low phytic acid phenotypes that result in field emergence

comparable to normal phytic acid phenotypes. Soluble carbohydrates metabolite profiling of low phytic acid (*lpa*) mutant soybean seeds detected reduced *myo*-inositol, galactinol, raffinose, stachyose, galactopinitol A, galactopinitol B, and fagopyritol B1 compared to the wild type (Frank et al., 2009). These results are similar to those observed for LRSP1 and LRSP2 seeds expressing the mutant *mips* phenotype (Tables 1 & 2).

9. Conclusions

Using soybean stem-leaf-pod explants, we fed free cyclitols to the cut stems of soybean explants and followed the changes in soluble carbohydrates downloaded from explant seed coats and also in mature dry seeds from explants. The results demonstrate that increasing the supply of D-*chiro*-inositol in maternal tissues can result in increased accumulation of fagopyritols in seeds expressing the mutant *stc1* phenotype with low RFO, in seeds expressing the mutant *mips* phenotype with reduced raffinose, stachyose and phytin, and in seeds expressing the normal *Stc1* and *Mips* phenotype with normal levels of raffinose, stachyose and phytin. Increasing *myo*-inositol may increase accumulation of phytic acid and/or RFO in seeds. Therefore, it is proposed that increasing the conversion of *myo*-inositol to D-*chiro*-inositol in soybean leaves and subsequent transport of D-*chiro*-inositol to the seeds for accumulation as fagopyritol B1 in maturing seeds may improve the field performance of mature soybean seeds expressing the mutant *mips* phenotype (Kosina et al., 2010).

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14

Endoplasmic Reticulum Stress Response

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1. Introduction

The endoplasmic reticulum (ER) is a key organelle that serves as the gateway for newly synthesized proteins into the secretory pathway. Following synthesis, secretory proteins are exported from the ER to various cellular compartments where they fulfill their inherent biological roles. The correct trafficking of proteins through the secretory pathway is accomplished by sorting-specific motifs in the secretory proteins that are recognized by transporters that translocate the proteins to their cellular destination. As a sorting and protein-processing organelle, the ER contains protein-folding and protein-processing mechanisms for the maturation of newly-synthesized secretory proteins and a quality control mechanism to prevent abnormal proteins from reaching their final destination. Under normal conditions, the ER's processing capacity is dynamically balanced with the protein synthesis rate. Disruption of the equilibrium between the cell's secretory activity and the ER's processing and folding capacities promotes a condition that is known as ER stress. In general, perturbation of ER homeostasis by ER stress leads to the accumulation of unfolded proteins in the lumen of the organelle, which triggers a cytoprotective signaling pathway designated as the unfolded protein response (UPR). To restore ER homeostasis, activation of the UPR culminates in transient and general down-regulation of protein translation, up-regulation of ER folding functions to enhance the ER's processing capacity and induction of the ER-associated protein degradation-related quality control mechanism that ensures the disposal of unfolded and abnormal proteins. However, if the stress condition persists and UPR fails to restore ER homeostasis, a cell death signal is activated as the ultimate attempt for survival. In soybean, the ER is a prime organelle partly due to the high secretory activity of developing seeds, which accumulate high levels of storage proteins in protein bodies originating in the secretory pathway. As in any other eukaryotic organism, ER stress triggers the evolutionarily conserved UPR in soybean, but it also participates in crosstalk with several other adaptive signaling responses, such as osmotic stress-induced cell death and ER stress-induced programmed cell death.

2. Structure and constitutive function of the endoplasmic reticulum

The endoplasmic reticulum (ER) is a large, versatile and flexible organelle consisting of a high-surface-area endomembrane system into which various groups of peripheral and integral proteins are incorporated. The constitutive function of the ER is not restricted to protein synthesis and the modification and control of cellular Ca^{2+} concentration. Due to the presence of signaling proteins, the ER also acts as a regulator of signaling pathways.

There are numerous functional domains in the ER of plant cells that extend into areas in connection with other organelles in which specific products accumulate (Sparkes et al., 2009). For instance, the junction between the outer nuclear envelope and the ER forms a gated domain that controls the exchange of proteins between the two organelles. Likewise, the exportation sites to the Golgi, designated ERESs (ER exit sites), are important junctions in the secretory pathway that mediate the transport of soluble and membrane proteins from ER to Golgi.

The ER possesses three distinct sub-compartments: the rough ER, the smooth ER and the nuclear envelope, and these are interlinked by a membrane network that extends as tubules (smooth) and cisternae (rough) throughout the cytosol (Sparkes et al., 2009). The rough ER represents the entry of proteins into the secretory pathway. Soluble and membrane proteins are translocated from this pathway to their final destination: the ER, Golgi, vacuole or plasma membrane.

The rough endoplasmic reticulum (ER) is a major site of protein biosynthesis, and translation occurs on the cytosolic face of the ER via polysomes associated with the ER membrane (Vitale and Denecke, 1999). Nascent proteins are co-translationally translocated across the ER membrane through aqueous channels designated translocons. Protein synthesis initiates in the cytosol, leading to the exposure of an N-terminal peptide signal that is recognized by the ribonucleoprotein SRP (signal recognition particle). Upon binding to the signal peptide, the SRP mediates the association of the nascent ribosome-peptide-SRP complex with the ER membrane through interactions with the SR receptor and ER membrane proteins. After association with the ER membrane, the signal peptide is transferred from the SRP to the translocon, where it is positioned to drive the synthesis of the protein into the lumen of the ER. The Sec61 complex is part of the translocon and plays a key role in the translocation of newly-synthesized proteins into the lumen of the ER. In plants, homologs of Sec61 subunits have been shown to share similar functions with their yeast and mammalian counterparts (Hartmann et al., 1994). The hydrolysis of the signal peptide is carried out by specific proteases called signal peptidases, which are located on the luminal side of ER. The removal of the signal peptide from newly-synthesized proteins is crucial for the maintenance of ER homeostasis (Coleman et al., 1995). Mutants that are defective in this process, such as the maize floury2 mutant, display altered phenotypes and enhanced accumulation of molecular chaperones involved in protein folding (Fontes et al., 1991; Gillikin et al., 1997). In the case of ER integral membrane proteins that have a transmembrane segment, this hydrophobic segment functions as a stop transfer signal that interrupts the translocation process of the nascent peptide. Then, the hydrophobic residues migrate laterally to the interior of the membrane, creating transmembrane domains that anchor the protein in the ER membrane.

Once inside the ER, the polypeptide chain may be modified by branched N-linked glycans if they have glucosylation sites for attachment of the oligosaccharide core. This process leads to the initiation of folding. The endoplasmic reticulum (ER) provides a folding environment that facilitates the proper folding and assembly of secretory proteins, which are prerequisites for their movement through the secretory apparatus. A set of ER-resident proteins that includes molecular chaperones and folding enzymes associates with newly synthesized polypeptides to promote the proper folding and assembly of oligomeric secretory proteins (see below). The folding of glycoproteins is facilitated by the calnexin/calreticulin system of molecular chaperones, whereas the folding of many other proteins is adenosine triphosphate (ATP)-dependent in a process mediated by ER-resident

members of the heat shock protein family. The tertiary structure of secretory proteins is stabilized by intra- and inter-chain disulfide (S) bonds, which are catalyzed by the protein disulfide isomerase (PDI).

Proteins that have not acquired the correct conformation must be identified and transported back to the cytosol or to the vacuoles for degradation in a process that is mediated by the ER-associated protein degradation (ERAD) machinery. This process of protein folding as well as unfolded protein identification and targeting for degradation underlies the mechanism of quality control in the organelle that ensures that abnormal proteins do not reach their final destination.

One of the most dynamic domains of ER is the exportation site to the Golgi that is designated the ERES (ER exit site). This important junction in the secretory pathway mediates the transport of soluble and membrane proteins from the rough ER to the Golgi apparatus through the COPII complex, which is a type of vesicle coat protein that transports proteins in anterograde transport (Lee & Miller, 2007). COPII refers to the specific coat protein complex that initiates the budding process on the ER membrane. The coat consists of large protein subcomplexes that are made of four different protein subunits. The transport may be bidirectional, and retrograde movement from Golgi to ER is mediated by COPI. This retrograde transport promotes the endocytosis of extracellular molecules and the recycling of proteins and membranes to maintain the integrity of different compartments.

During the process of protein export from ER to Golgi, the exported proteins are recruited on the cytosolic side of the ER membrane by the COPII complex. The assembly of the complex is activated by interactions of GTPases with GEFs (guanine-nucleotide exchange factor) to control the process of budding vesicles off from the ER membrane. The exit of this complex is balanced by the entry of the COPI complex into the ER. COPI is a protein that coats vesicles transporting proteins from the *cis* end of the Golgi complex back to the rough ER, where they were originally synthesized. The coat consists of large protein subcomplexes that are made of seven different protein subunits.

The vacuole is an organelle that may also be a direct target of proteins from the ER, which are transported to the vacuoles via a Golgi apparatus-independent pathway. For instance, maize endosperm protein bodies emerge directly from the ER by the accumulation of zeins (the maize storage proteins) as an insoluble matrix in the organelle (Herman and Larkins, 1999). Zeins belong to the class of seed proteins known as prolamins. In many cereals, prolamins are sequestered in the ER and form protein bodies that may remain in the ER or be delivered directly to the vacuole for storage (Chrispeels & Herman, 2000). In soybean, the biogenesis of seed protein bodies is predominantly via Golgi-mediated transport of storage proteins to vacuoles. Nevertheless, there is also compelling evidence for a Golgi-independent form of protein body biogenesis in soybean seeds (Mori et al. 2004).

The ER is also the storage site for the calcium that is used in many signal transduction cascades. Finally, if the cell encounters adverse physiological conditions that ultimately affect the environment of the ER, protein folding will be hampered and unfolded proteins will accumulate. A signal transduction response called the unfolded protein response (UPR) is initiated in the ER to protect the cell until normal conditions are restored.

2.1 Endoplasmic reticulum-resident molecular chaperones

The protein concentration in the lumen of the ER is estimated to be 100 mg/mL. Such a high concentration is believed to promote protein aggregation, which is particularly facilitated by the exposure of hydrophobic regions of newly synthesized proteins that otherwise would

not be exposed in their native conformation. As a consequence, the ER contains a set of resident proteins called reticuplasmins, including enzymes and chaperones, that function in the synthesis, folding and assembly of newly synthesized proteins in the ER.

2.1.1 ER members of the HSP family: Binding Protein (BiP) and Hsp94.

The molecular chaperone BiP (GRP78) is a 78-KDa protein that belongs to the HSP70 (Heat shock protein 70 kDa) family and represents one of the best characterized molecular chaperones of the ER. BiP has been shown to play a dynamic role in the regulation of various ER-supported processes, including regulation of molecular chaperone levels, translocation of secretory proteins into the ER lumen, catalysis of protein folding, targeting of incorrectly folded proteins for degradation and perception of ER stress (Hendershot, 2004). BiP binds and protects newly synthesized proteins when they are in an unfolded state, but it is released from them before maturation to allow folding of the substrate protein.

BiP harbors an N-terminal ATPase domain and a domain for substrate binding at the carboxyl-terminus. Therefore, like all Hsp70 family members, BiP binds and hydrolyzes adenosine triphosphate (ATP), which controls its chaperone function (Gething, 1999). Upon binding to ATP, Hsp70 undergoes a conformational change ("open" configuration), which allows it to associate with unfolded substrates. The presence of a DnaJ co-factor in the complex catalyzes the rapid hydrolysis of ATP to adenosine diphosphate (ADP), stabilizing the complex formed between Hsp70 and the unfolded protein substrate. The next step in the Hsp70 adenosine triphosphatase (ATPase) cycle is the exchange of ADP by ATP in the nucleotide binding cleft, which "reopens" the Hsp70 protein and releases the unfolded protein.

Soybean has four BiP genes: soyBiPA, soyBiPB, soyBiPC and soyBiPD, which are differentially expressed in different organs (Kalinski et al, 1995; Figueiredo et al., 1997; Cascardo et al., 2001). *soyBiPD* is expressed in all organs, whereas the expression of *soyBiPB* is restricted to leaves. *soyBiPA* transcripts are detected in leaves, roots and seeds, and *soyBiPC* RNA is confined to leaves, seeds and pods. Like all plant BiPs, conditions that promote the accumulation of unfolded proteins in the ER, such as treatment with tunicamycin or AZC (L-azetidine-2-carboxylic acid), strongly induce soybean BiPs. Soybean *BiP* expression has also been shown to respond to a variety of abiotic and biotic stress conditions, including water stress, fungus infestation, insect attack, nutritional stress, and elicitors of the plant-pathogenesis response (Carolino et al., 2003).

Soybean BiP exists in interconvertible phosphorylated and non-phosphorylated forms, and the equilibrium can be shifted to either direction in response to different stimuli (Cascardo et al., 2000). In contrast to tunicamycin treatment, water stress stimulates phosphorylation of BiP species in cultured soybean cells and stressed soybean leaves. Although the tunicamycin-induced BiP forms are unmodified and osmotic stress-induced BiP forms are phosphorylated, both treatments cause the conversion of oligomeric BiP to the monomeric forms (Carolino et al., 2003). In mammalian cells, modification of BiP is associated with its oligomerization, and it is generally accepted that the non-phosphorylated form is the biologically active BiP species in the folding pathway because tunicamycin-induced BiP or BiP bound to nascent proteins is unmodified (Freiden et al., 1992). Thus, the modification of plant BiP protein in response to water stress differs from the usual pattern of post-translational modifications of eukaryotic BiPs. The simplest explanation for these results is

that phosphorylation of soybean BiP by osmotic stress may serve as a distinct regulatory function, and because it is not restricted to the oligomeric form of BiP, it may occur at different sites. This argument is supported by the observation that despite its phosphorylation state, the soybean BiP isoform from water-stressed leaves exhibits protein-binding activity and associates with a water stress-induced 28 kDa polypeptide (Cascardo et al., 2000).

Soybean BiP has also been shown to associate detectably with normal storage proteins *in vitro* (Gillikin et al., 1995). However, direct evidence for a role of plant BiP in storage protein folding and maturation includes the demonstration that BiP associates co-translationally with rice prolamin storage proteins (Li et al., 1993) and binds to exposed sites on monomers of phaseolin but not to the trimeric form of the bean protein (Foresti et al., 2003). Thus, the BiP binding site is buried in the mature bean protein, supporting the notion that BiP action precedes and contributes to the maturation of legume storage proteins.

Glucose-regulated protein 94, also designated endoplasmin or Hsp94, is also a member of the heat shock family, and it is an abundant ER molecular chaperone. It seems to be encoded by a single gene in the Arabidopsis genome, the SHEPHERD (SHD) gene. Arabidopsis SHD is induced by ER stressors and exhibits chaperone activity with very specific substrate binding activity (Martınez & Chrispeels, 2003; Klein et al., 2006). At-SHD is implicated in the synthesis of clavata proteins; thus, null alleles of SHD display defects in meristem size control similar to those of clavata mutants (Ishiguro et al., 2002). Glycine max Hsp94 is also represented by a single gene in the soybean genome, but there is no information with respect to the function of soybean GRP94.

2.1.2 Calnexin/Calreticulin system

Calnexin and calreticulin are lectins that function as components of the major protein folding machinery of the ER and specifically bind glycoproteins that carry monoglucosylated N-linked glycans (Parodi, 2000). Calreticulin is a soluble protein in the ER lumen, whereas calnexin is a type 1 membrane protein. In eukaryotes, a large fraction of secretory proteins are glycosylated in the ER by attachment of a Glc3Man9GlcNac2 oligosaccharide core that has 14 monosaccharide moieties. The three glucoses in the most external portion of the oligosaccharide are numbered G12, G13 and G14. As the protein enters into the folding/unfolding cycle, the removal of G14 and G13 is initiated by the action of glycosidases I and II. Glycosidase I is a membrane glycoprotein tightly associated with the translocon, and it hydrolyses α-1,2 glycosidic bonds to remove the G14 of oligosaccharide chains on secretory proteins. In plants, the relevance of this hydrolysis for protein folding has been demonstrated by the incapacity of phaseolin to assemble into the mature trimeric protein if the hydrolysis of G14 is blocked (Lupattelli et al., 1997). Glycosidase II hydrolyses α-1,3 glycosidic bonds to release G13 from the oligosaccharide core on the protein. After removal of G13 and G14, the glycoproteins associate with calnexin and calreticulin for proper folding and protection against misaggregation. The recognition of folding glycoproteins by calnexin and calreticulin is mediated by protein-linked monoglucosylated oligosaccharides. The glycoproteins are released from their calreticulin/calnexin anchor through the action of glycosidase II, which releases G12 to leave an unglycosylated oligosaccharide on the protein. Monoglucosylated glycans are then recreated by UDP-Glc:glycoprotein glucosyltransferase (GT), a soluble luminal enzyme, and they are thus recognized again by the lectins if they are linked to improperly folded protein

moieties. The deglucosylation-reglucosylation cycle continues until proper folding is achieved. In this system, GT behaves as a sensor of glycoprotein conformation by specifically glucosylating N-linked glycans in misfolded glycoproteins and thus retaining them in the calnexin/calreticulin chaperone cycle.
As ER-resident molecular chaperones, both calreticulin and calnexin from soybean are induced by ER stressors, such as tunicamycin and AZC (Irsigler et al., 2007). Under hyperosmotic conditions, soybean calnexin is up-regulated and accumulates in the plasma membrane (Nouri & Komatsu, 2010).

2.1.3 PDI family

Protein disulfide isomerases (PDIs) also play important roles in the folding of nascent polypeptides. More specifically, they are involved in the formation of disulfide bonds on secretory proteins in the ER. PDIs are represented by a small family in soybean with at least two copies: GmPDIS-1and GMPDIS-2 (Wadahama et al., 2007). Their domain structures contain two thioredoxin-like domains, a and a', and an ERp29c domain. In cotyledon cells, both proteins are distributed to the endoplasmic reticulum and protein storage vacuoles. Data from coimmunoprecipitation and crosslinking experiments indicate that GmPDIS-1 associates with proglycinin, a precursor of the seed storage protein glycinin, in the cotyledon. Levels of GmPDIS-1, but not of GmPDIS-2, increase in cotyledons with the accumulation of glycinin during seed development and under ER-stress conditions. In contrast, GmPDIS-2, but not GmPDIS-1, is induced by osmotic stress (Irsigler et al., 2007).

2.2 The endoplasmic reticulum-associated protein degradation system (ERAD)

Quality control in the ER is associated with two fundamental aspects of protein metabolism: the retention of incompletely or incorrectly folded proteins in the ER and the targeting of irreversibly misfolded proteins for degradation. This quality control mechanism increases the efficiency of correctly folded proteins, prevents the export of defective proteins, restores the amino acid pool and maintains the homeostasis of the endomenbrane system. Defective proteins are eliminated by the ERAD (ER-associated degradation) system, which recognizes terminally unfolded proteins, retrotranslocates them to the cytosol, promotes their ubiquitinization and degrades them.
Several homologs of ERAD-associated genes have been identified in soybean, such as genes encoding polyubiquitin, a ubiquitin conjugating enzyme, the alpha subunit of the proteasome, CDC48 and Derlin (Irsigler et al., 2007). However, the functions of these soybean homologs have not been demonstrated experimentally. Further evidence that a quality control mechanism operates in legumes is the demonstration that bean seed storage glycoproteins (*Phaseolus lunatus*) associate irreversibly with BiP and accumulate in the ER if glucosylation is blocked by treatment with tunicamycin (Sparvoli et al., 2000).

3. ER stress and the unfolded protein response

The ER is the gateway for secretory proteins, which are synthesized by ER-associated polyribosomes and interact with molecular chaperones as they enter the lumen of the organelle. The ER-resident molecular chaperones facilitate the folding and assembly of newly synthesized proteins into a competent conformation that allows their translocation further in the secretory pathway (Liu & Howell, 2010). However, if the secretory proteins

fail to acquire a native conformation, they are targeted for degradation in a process mediated by the ER-associated degradation system (ERAD). Therefore, under normal conditions, the rate of protein synthesis is balanced with the rate of protein processing and folding within the lumen of the ER. Any condition that disrupts the homeostasis of the organelle and the proper folding and maturation of secretory proteins promotes the accumulation of unfolded proteins in the lumen of the organelle, which results in ER stress. Cells respond to ER stress by activating a protective signaling cascade designated the unfolded protein response (UPR), which allows information about the folding status of proteins in the lumen of the ER to be transmitted to the nucleus and cytosol. In mammals, ER stress is sensed and the UPR is transduced by three distinct classes of ER transmembrane proteins: PERK, ATF6 and Ire1p. Upon activation, these receptors act in concert to transiently attenuate protein synthesis, up-regulate ER folding capacity and degrade misfolded proteins. However, if the stress persists and the UPR is unable to restore ER homeostasis, a cell death signal is activated as an ultimate attempt for survival.

Components of the plant UPR that are the proximal sensors include Ire1p homologs and ATF6-related proteins, designated AtbZIP28 and AtbZIP60 (Urade, 2009; Liu & Howell, 2010). Two Ire1p homologs were found in Arabidopsis (AtIre1-1 e AtIre1-2), and one was found in rice (OsIre1). Plant Ire1p homologs contain an Ire1p-like receptor configuration with a stress sensor luminal domain at the N-terminus, a transmembrane segment, and a kinase domain and a ribonuclease domain at the C-terminus. AtIre1-1 and AtIre1-2 are type I transmembrane proteins with a one-pass transmembrane segment in their central portion. AtIre1 is located in the nuclear envelope and in regions of the ER contiguous with the nucleus.

Three lines of evidence indicate that the C-terminal domains of plant Ire1p homologs are functional (Urade, 2009; Liu & Howell, 2010). First, the C-terminal domains of plant Ire1p homologs display high sequence conservation with the kinase/endonuclease C-terminal portion of yeast Ire1 and contain the DLKPQN motif, which resembles motives found in Ser/Thr kinases. In addition, AtIRE1 and OsIre1 exhibit autophosphorylation activity in vitro. Finally, mutation of the lysine residue in the AtIre1-1 DLKPQN motif totally abolishes the autophosphorylation activity of the receptor. In spite of having a ribonuclease domain at the C-terminus, its putative ribonuclease activity has not been demonstrated in plants.

The N-terminal domains of AtIre1-1, AtIre1-2 and OsIre1 function as ER stress sensors (Urade, 2009). The N-terminal domains of plant Ire1p homologs have been functionally characterized by complementation assays in yeast. In these experiments, the N-terminal domain of plant Ire1p, when fused to the C-terminal domain of yeast Ire1, was able to perceive ER stress in tunicamycin-treated yeast cells. Although the N-terminal domains of plant IRE1 homologs can functionally replace yeast IRE1, downstream components of the IRE1 signaling pathway have yet to be identified in plants.

AtbZIP60 and AtbZIP28 are ER stress-induced leucine zipper (bZIP) transcription factors that are anchored to the ER membrane under normal conditions and may serve as ER stress sensors and transducers, such as the mammalian ATF6 transducer (Liu & Howell, 2010). ATF6 is a transcription factor that is anchored to the ER membrane under normal conditions, and it has a C-terminal ER stress-sensing domain oriented to the ER lumen. In response to ER stress, ATF6 is translocated to the Golgi, where it is specifically cleaved by S1P and S2P proteases to release its N-terminal transcription factor domain. The released ATF6 domain is targeted to the nucleus, where it activates the coordinated up-regulation of a set of genes encoding ER chaperones, folding enzymes and ERAD components. Likewise,

the plant ATF6 homolog AtbZIP28 is located in the ER membrane under normal conditions. However, under stress conditions, AtbZIP28 undergoes regulated intermembrane proteolysis by S1P and S2P proteases. The released bZIP domain is translocated to the nucleus, where it acts in concert with the heterotrimeric NF-Y complex to activate UPR genes. The NF-Y complex is composed by the transcriptional factors NF-YA4, NF-YB3 and NF-YC2.
AtbZIP60 is a type II transmembrane transcriptional factor. It also undergoes regulated proteolysis by an unknown mechanism that is not dependent on the S1P and S2P proteases (Urade, 2009). Expression of a truncated form of AtbZIP60 harboring the bZIP domain up-regulates UPR target genes under normal conditions, indicating that the regulated release of AtbZIP60 from the membrane may underlie the transduction mechanism of an ER stress signal.
Comprehensive genome-wide evaluations of ER stress-induced changes in gene expression have provided evidence that the UPR operates in a similar fashion in both soybean and Arabidopsis (Irsigler et al., 2007). Inducers of ER stress, such as tunicamycin and AZC, promote the up-regulation of a class of genes that functions in (i) protein folding, (ii) ERAD and (iii) translational regulation. In the protein folding category, the up-regulated genes include ER-resident molecular chaperones such as BiP, calreticulin, calnexin, and the folding catalyst protein disulfide isomerase (PDI). ERAD-associated genes that are up-regulated by ER stress in soybean include those encoding polyubiquitin, ubiquitin conjugating enzyme, the alpha subunit of the proteasome, CDC48 and Derlin. Examples of ER stress-induced genes that are potential regulators of protein translation are a translational inhibitor protein (AW508686), eukaryotic translation initiation factor 3 subunit 10 (AW317679), translation elongation factor 1-gamma (AI960794) and a series of up-regulated ribosomal protein genes. These genomic analyses suggested that soybeans, like Arabidopsis and mammals, have evolved at least three different mechanisms that mediate UPR: (1) transcriptional induction of genes encoding chaperones and vesicle trafficking proteins, (2) attenuation of genes that encode secretory proteins, and (3) upregulation of the ER-associated protein degradation (ERAD) system for rapid disposal of unfolded proteins in the ER.
As further evidence that the UPR operates in soybean, the promoters of soybean BiP genes contain functional ER stress cis-acting elements (ERSEs), and soybean BiP functions as a regulator of the UPR as it does in mammals and yeast (Buzeli et al., 2002; Costa et al., 2008). Nevertheless, the roles of plant BiP in stress perception and in the mechanism of signal propagation remain to be elucidated. Overexpression of soybean BiP inhibits activation of the UPR and attenuates ER stress in response to tunicamycin, a potent inducer of ER stress (Alvim et al., 2001; Costa et al., 2008). Soybean BiP has also been demonstrated to confer tolerance to drought in transgenic soybean and tobacco plants (Alvim et al., 2001; Valente et al., 2009). The apparent increase in drought tolerance mediated by BiP has not been associated with typical short-term and long-term avoidance responses or with typical tolerance mechanisms. BiP overexpression confers resistance to drought through an unknown mechanism that may be related to ER function and cross-talk with the osmotic signaling response.

4. Integration of ER stress and the osmotic stress response: the NRP-mediated cell death response.

The potential of the endoplasmic reticulum (ER) stress response to accommodate adaptive pathways and its integration with other environmentally induced responses has been the

subject of studies in recent years (Liu & Howell, 2010). The most well-characterized ER-based integrative pathway in plants is the ER stress and osmotic stress integrated response that is mediated by N-rich proteins and transduces a cell death signal (Figure 1; Costa et al., 2008). This integrative pathway was first identified by genome-wide approaches and expression profiling studies, which revealed the existence of a modest overlap of the ER stress- and osmotic stress-induced transcriptomes in soybean seedlings treated with PEG (an inducer of osmotic stress) or tunicamycin and AZC, potent inducers of ER stress (Irsigler et al., 2007). In fact, these global expression-profiling analyses revealed that only a small

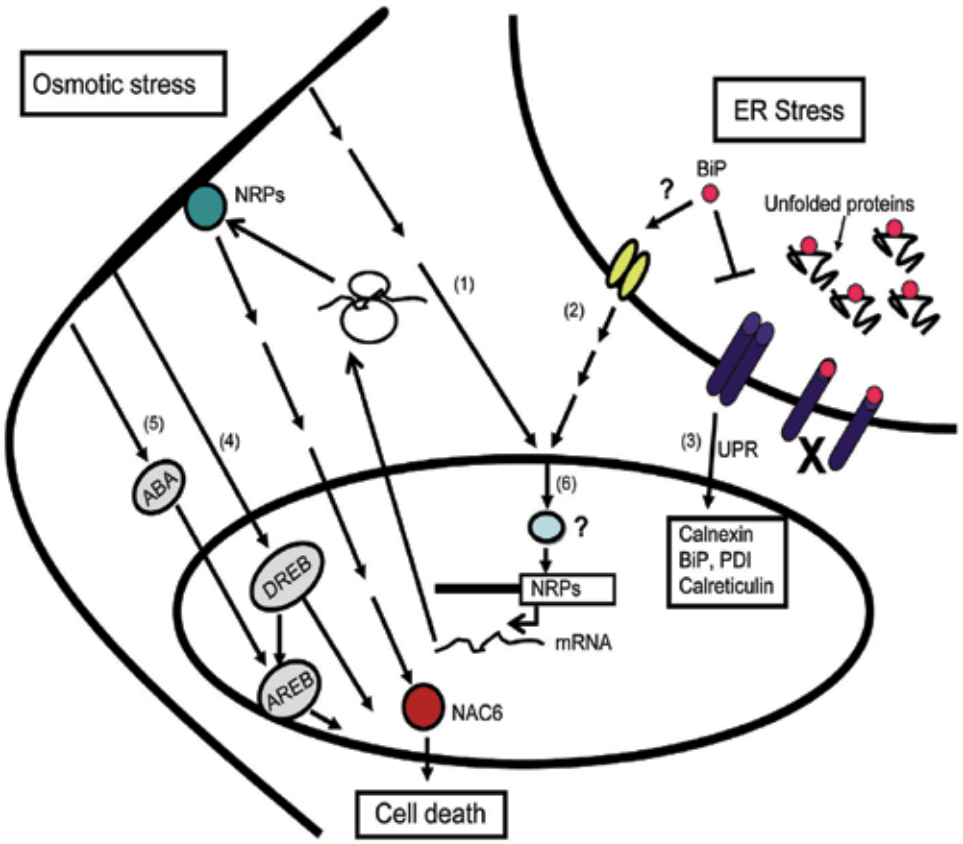

Fig. 1. Osmotic- and ER-stress integrating pathway.
ER stress and osmotic stress activate two independent signaling pathways (1 e 2), which converge upon *NRP-A* and *NRP-B* expression to activate an osmotic- and ER-stress integrating pathway, also called the integrated pathway (6). The ER-stress signaling branch (2) of the integrated pathway is distinct from the UPR (3). However, whether the osmotic-stress signaling branch (1) of this integrated pathway shares components with the ABA-dependent (4) and ABA-independent response (5) to drought is unknown at present. Enhanced accumulation of the membrane-associated NRPs activates a cascade to induce the expression of the nuclear transactivator *NAC6* that in turn promotes cell death. BiP, an ER-resident molecular chaperone, regulates the UPR. Whether BiP also modulates the integrated pathway remains to be determined.

proportion of the up-regulated genes (only 5.5%) represented a shared response that integrates the two signaling pathways. These co-regulated genes have been considered to be downstream targets of the integrated pathway based on similar induction kinetics and a synergistic response to the combination of osmotic and ER stress-inducing treatments. Genes in this integrated pathway with the strongest synergistic induction encode proteins with diverse roles, such as plant-specific development and cell death (DCD) domain-containing proteins (NRP-A and NRP-B), a ubiquitin-associated (UBA) protein homolog and NAC domain-containing proteins (GmNAC6). This integrated pathway diverges from the molecular chaperone-inducing branch of the unfolded protein response (UPR) in several ways. While UPR-specific targets are inversely regulated by ER and osmotic stresses, the co-regulated target genes require both signals for full activation. Furthermore, BiP (binding protein) overexpression in soybean prevents activation of the UPR by ER stress inducers but does not affect the induction of the integrated target genes.

NRP-A and NRP-B share a highly conserved C-terminal DCD (development and cell death) domain in addition to a high number of asparagine residues at their more divergent N-termini. This structural organization places NRP-A and NRP-B in subgroup I of the plant-specific DCD-containing proteins. NRPs are critical mediators of ER and osmotic stress-induced cell death in soybean (Costa el al., 2008). The cell death response mediated by NRPs resembles a programmed cell death event. Overexpression of NRPs in soybean protoplasts induces caspase-3-like activity and promotes extensive DNA fragmentation. Furthermore, transient expression of NRPs in plants causes leaf yellowing, chlorophyll loss, malondialdehyde production, ethylene evolution, and induction of the senescence marker gene CP1.

Like NRPs, GmNAC6, another target of the integrated pathway, is strongly induced by cycloheximide, a potent inducer of cell death in soybean suspension cells, and it promotes cell death when ectopically expressed in tobacco leaves (Pinheiro et al., 2009). GmNAC6 belongs to the class of NAC (NAM, ATAF1, ATAF2 and CUC2) domain-containing proteins, which comprise a large family of plant-specific transcription factor genes represented by at least 101 sequences in the soybean genome that are clustered into 15 different sub-families. Members of this family are involved in development and stress response.

The NAC transfactors are organized into a general structure that consists of a highly conserved N-terminal domain involved in DNA binding (called the NAC domain) and a C-terminal region highly divergent in sequence and length that functions as the activation domain (Hegedus et al., 2003). The NAC domain contains nearly 160 amino acid residues divided into five subdomains (A–E) of conserved blocks intercalated with heterogeneous blocks or gaps. In the ER and osmotic stress-induced cell death response, GmNAC6 acts downstream of NRPs as it is strongly induced by ectopic expression of NRPs in soybean protoplasts (unpublished), and it promotes cell death when expressed *in planta* (Pinheiro et al., 2009).

In mammalian cells, ER stress has been shown to trigger both ER stress-specific apoptotic pathways and shared PCD signaling pathways elicited by other death stimuli (Malhotra & Kaufman, 2007). The NRP-mediated PCD signaling fits the concept of a shared pathway induced by ER stress and other stimuli, which is consistent with the localization of NRPs either as cytosolic or membrane-associated proteins. This localization of NRPs, their rapid response to programmed cell death inducers along with their capacity to induce caspase-3-like activity in soybean protoplasts are all consistent with a model in which NRPs act as adaptor proteins that activate upstream initiator caspases. Several cell surface death

receptors have been identified in mammalian cells as the mediators of the extrinsic cell death-inducing pathway (Ashkenazi & Dixit, 1998). Upon activation by ligand binding, the cytoplasmic domain of these death receptors recruits adaptor proteins to activate caspase cascades that result in morphological changes associated with apoptosis. Identification of the downstream targets of NRPs and the regulators of their induction will be crucial to elucidating the underlying mechanism of this ER- and osmotic stress-induced cell death signaling pathway that transduces a PCD signal into multiple hallmarks of leaf senescence.

5. Acknowledgements

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Regulation of Isoflavonoid Biosynthesis in Soybean Seeds

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1. Introduction

Isoflavonoids are biologically active plant natural products synthesized via general phenylpropanoid pathway. They accumulate predominantly in plant species belonging to family *Leguminosae*. Isoflavonoids play numerous roles in the interaction between plants and environment, where they act as inducers of nodulation genes during symbiosis between legumes and *Rhizobium* bacteria (Ferguson & Mathesius 2003, Phillip 1992) and also function as precursor molecules for the production of phytoalexins during plant-microbe or plant-insect interactions (Aoki et al. 2000, Dixon 1999, Dixon & Ferreria 2002). Many epidemiological and clinical trials have suggested a positive role for isoflavonoids in human health and nutrition (Aerenhouts et al. 2010, Cederroth & Nef 2009). The core isoflavones have structural similarity to beta-estradiol (Fig. 1) and possess affinity for oestrogen receptors (Molteni et al. 1995). Due to their structural resemblance with beta-estradiol, isoflavonoids have been associated with chemo-preventive activities against hormone dependent cancers such as breast cancer and post-menopausal ailments (Dixon 2004, Limer & Speirs 2004). Research data over the past decade suggests that the dietary intake of isoflavonoids may also be associated with many additional health benefits such as reduction of risk of cardiovascular diseases, osteoporosis, loss of bone mass intensity (reviewed in Messina 1999, Rochfort & Panozzo 2007, Zhang & Yu 2009). Due to these pharmaceutical and nutraceutical properties associated with isoflavonoids and their use in functional foods, there is a growing interest in these compounds and the plants that produce them.

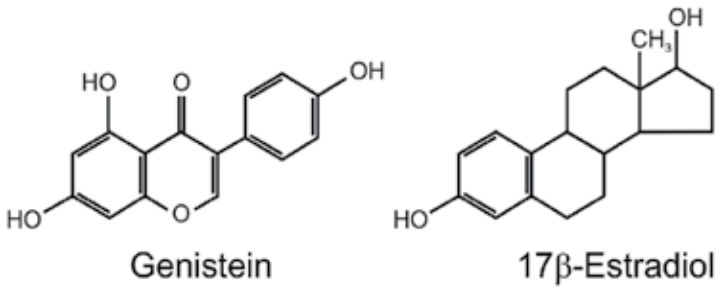

Fig. 1. Structure of isoflavone genistein and 17β-Estradiol.

Soybean (*Glycine max* [L.] Merr.) seeds contain large amount of isoflavonoids and are primary source of these compounds in human diet. Since isoflavonoids are phytoestrogens, the presence of these compounds in some foods such as soy-based formula for infants is controversial (Chen & Rogan 2004, Setchell 2001). Despite its continuous use for more than four decades, no clear correlation between soy isoflavones and negative effects in infant health has been reported. Nevertheless, seed isoflavonoid content has gained considerable attention in soybean breeding programmes for developing soybean cultivars with both high and low isoflavonoid levels that satisfies consumer's needs.

The amount of isoflavonoids present in soybean seed is a complex trait that is highly variable and is determined by multiple genetic and environmental factors that are largely unknown (Gutierrez-Gonzalez et al. 2010, Hoeck et al. 2000). This complexity of mechanism of isoflavonoid accumulation in soybean seeds poses a challenge to breeders for cultivar development. Thus understanding the mechanism of regulation of isoflavonoid synthesis and their accumulation in soybean, identifying the genetic factors involved in the regulation, dissecting the mechanism of how those factors interact with each other and with the environmental components are critical.

2. Biosynthesis of isoflavonoids in soybean

Isoflavonoids are synthesized by a legume specific branch of general phenylpropanoid pathway. Other branches of phenylpropanoid pathway are common to all plant species and produce lignin, proanthocyanidins, anthocyanin, phlobaphenes (Fig. 2). Chalcone is the first step in the production of flavonoids and isoflavonoids (Hahlbrock & Scheel 1989) which requires an enzymatic reaction catalyzed by the enzyme chalcone synthase (CHS), a plant specific polyketide synthase (Schröder 2000). Soybean contains nine *CHS* genes (*CHS1*-*CHS9*) among which *CHS7* and *CHS8* are critical for isoflavonoid biosynthesis (Dhaubhadel et al. 2007). Legume plants produce two kinds of chalcones, tetrahydroxy chalcones (naringenin chalcone) and trihydroxy chalcone (isoliquiritigenin chalcone) whereas non-legume plants only produce tetrahydroxy chalcones (naringenin chalcone). The production of isoliquiritigenin in legumes is a result of additional enzymatic reaction catalyzed by a legume specific enzyme chalcone reductase (CHR). Both naringenin and isoliquiritigenin chalcones then get converted into their corresponding flavanones by chalcone isomerase (CHI). Soybean *CHI* gene family consists of seven members that are grouped into four subfamilies: *CHI1, CHI2, CHI3,* and *CHI4* (Ralston et al. 2005). These genes are further categorized into different types based on their catalytic abilities where type I *CHI*s (such as *CHI2*) is common to all higher plants, convert naringenin chalcone to naringenin, and type II *CHI*s (such as *CHI1*) are almost entirely exclusive to legumes, possess additional capacity to catalyze the conversion of isoliquiritigenin to liquiritigenin (Ralston et al. 2005, Shimada et al. 2003).

The branch point enzyme that introduces isoflavonoid specific branch in the phenylpropanod pathway is 2-hydroxyisoflavanone synthase (isoflavone synthase, IFS). IFS is a microsomal cytochrome P450 monooxygenase enzyme that catalyzes a 2, 3 aryl ring migration of flavanones to their corresponding flavones and subsequent hydroxylation of the resulting C-2 radical. This reaction also requires reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen molecule and yields 2-hydroxyisoflavanone. The reaction product of IFS, 2-hydroxyisoflavanone, is extremely unstable and undergoes dehydration by forming a double bond between C-2 and C-3 by a dehydratase to form

genestein or daidzein (Hakamatsuka et al. 1998). Two *IFS* genes, *IFS1* and *IFS2*, have been identified in soybean (Jung et al. 2000, Steele et al. 1999) about a decade ago by two research groups independently that brought a major break through in the attempts to metabolically engineer isoflavonoids in nonlegumes. These two IFSs differ from one another by 14 amino acid residues; however, both IFS1 and IFS2 convert the naringenin and liquiritigenin flavanones to their corresponding isoflavones (Dhaubhadel et al. 2003, Jung et al. 2000).

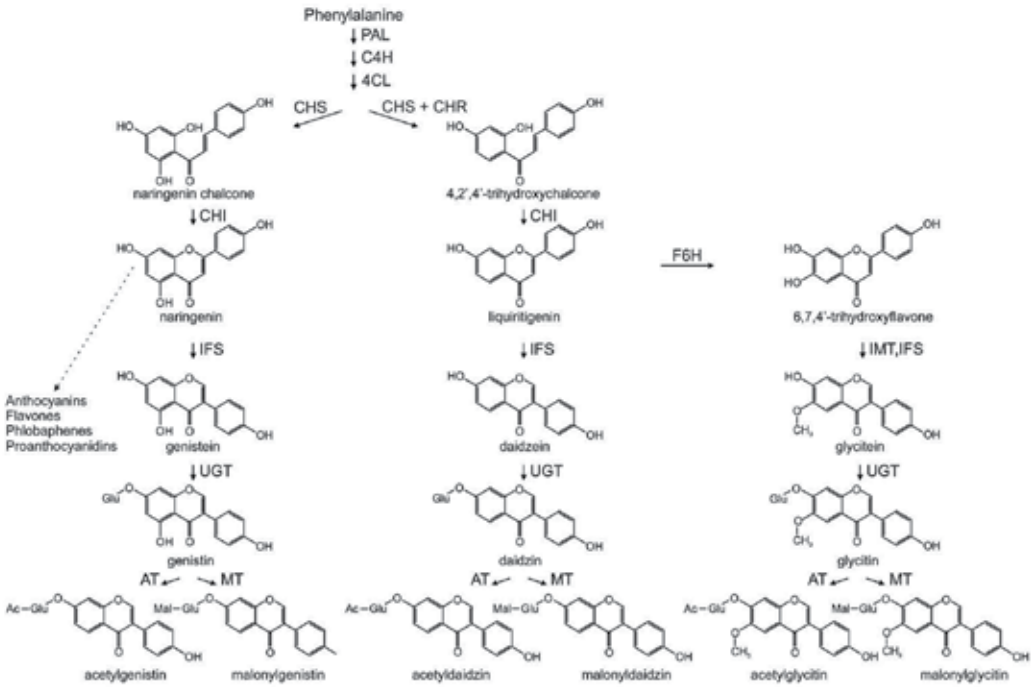

Fig. 2. Soybean seed isoflavonoid biosynthesis pathway. Dotted arrow indicates multiple steps involved in the synthesis of other phenylpropanoids. PAL, phenylalanine ammonia lyase; 4CL, 4-coumarate-CoA-ligase; C4H, cinnamate-4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; F6H, flavonone-6-hydroxylase; UGT, glycosyl-transferase; IFS, 2-hydroxyisoflavanone synthase; IMT, isoflavone methyl-transferase; MT, malonyl-transferase; AT, acetyl-transferase; (modified from Dhaubhadel *et al*., 2003).

Soybean seeds contain nine different isoflavonoids with three core isoflavone aglycones (daidzein, genistein and glycitein). The aglycones are the most bioactive forms of isoflavonoids. Each of these aglycones may also be present as their corresponding 7-*O*-glycosides (daidzin, genistin and glycitin) and malonyl glycosides (6″-*O*-malonyldaidzin, 6″-*O*-malonylgenistin and 6″-*O*-malonylglycitin) (Kudou et al. 1991) (Fig. 2). The malonyl glycoside form of isoflavonoids are thermally unstable and get converted into acetylglycosides (6″-*O*-acetyldaidzin, 6″-*O*-acetylgenistin and 6″-*O*-acetylglycitin) forms during processing of soybeans and soy foods (Griffith & Collison 2001). The conjugation of glycosyl- and malonyl-groups to the isoflavone aglycones is catalyzed by uridine diphosphate glycosyltransferase (UGT) and malonyltransferase (MT), respectively. The

addition of glycosyl- and malonyl groups to the aglycones confers the metabolite with the increased water solubility and reduced chemical reactivity. The conjugation process also increases the *in vivo* stability of the metabolite and alters their biological activity (Jones & Vogt 2001). In soybean, the UGT73F2 and GmMT7/GmIF7MaT are isoflavonoid specific UGT and MT that are involved in glycosylation and malonylation of soybean isoflavones, respectively (Dhaubhadel et al. 2008, Suzuki et al. 2007) providing stability and solubility to aglycone molecules and possibly helping in their compartmentalization to central vacuole of the cell or transport to the site of accumulation. Even though it has been found that malonyl derivatives of isoflavonoids are highly unstable, the majority of soybean seed isoflavonoids accumulate in the form of malonyl- conjugates followed by glycosyl- conjugates while the amount of daidzien, genistien and glycitein is negligible (Fig. 3). It is not known what stabilizes the malonyl derivatives of isoflavonoids *in planta*.

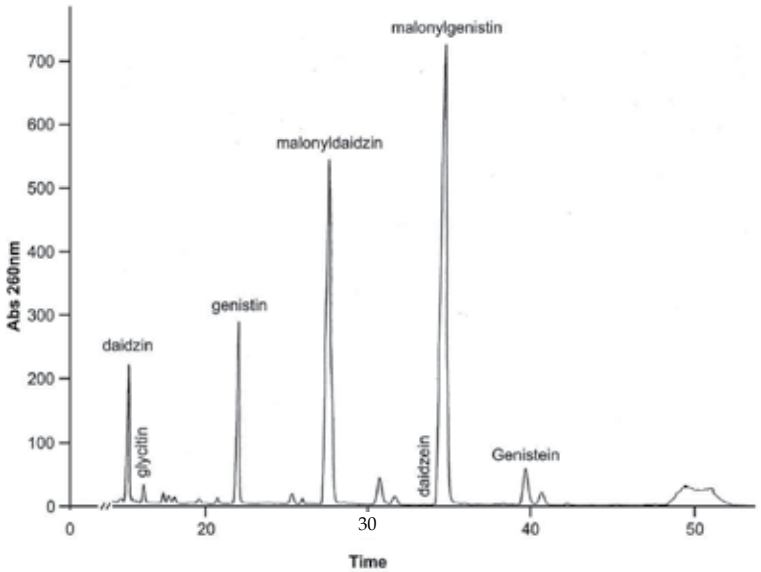

Fig. 3. Chromatogram showing seed isoflavonoid accumulation profiles analysed by HPLC. Isoflavonoids were extracted from mature soybean seeds in 50% acetonitrile and separated using HPLC.

3. Metabolic engineering of isoflavonoids

Due to health related benefits associated with the dietary intake of isoflavonoids such as prevention against various hormone-dependent cancers, (Dixon & Ferreria 2002), there is considerable interest in engineering non-legume plants such as tomato, broccoli or lettuce to introduce isoflavonoid phytoestrogen or to generate soybean plants with altered isoflavonoid content. Metabolic engineering of isoflavones in non-legume plants possessing active flavonoid branch of phenylpropanoid pathway has been conducted successfully (Yu et al. 2000). However, attempts in these lines have produced very low concentration of isoflavonoids (Jung et al. 2000, Yu et al. 2000). Since IFS is the entry point enzyme into isoflavonoid biosynthesis and is not present in nonlegume plants, these earlier efforts for metabolic engineering of isoflavonoids were focussed on introducing soybean IFS into corn,

tobacco and *Arabidopsis thaliana* (Jung et al. 2000, Liu et al. 2002, Yu et al. 2000). Introduction of soybean *IFS* gene into *Arabidopsis* could yield low levels of genistein glycosides (Liu et al. 2002). However, this constitutive production of isoflavonoids in *Arabidopsis* resulted into competition for flavanone between endogenous flavonol synthesis and IFS, affecting the flavonol pathway disproportionally. Since naringenin is the substrate for both IFS and flavonoid 3'-hydroxylase (F3'H), the limitation for isoflavonoid synthesis in *Arabidopsis* is at the level of naringenin where partitioning of flux between isoflavonoid and flavonoid biosynthesis pathway occurs. This conclusion was supported by the results where high levels of genistein conjugates were obtained by expressing soybean *IFS* in *Arabidopsis* mutant line *tt3/tt6* that lacks F3'H and flavonol production (Liu et al. 2002). Interestingly, as in soybean, the genistein produced in *Arabidopsis* were conjugated. The conjugated sugars were same as that are conjugated to kaempferol and quercetin but with different position specificity suggesting that conjugation is highly critical for isoflavonoid accumulation. It is possible that engineered isoflavone aglycones may perform as poor substrates for endogenous UGTs and MTs of the host plant, and ultimately get turned over resulting into its reduced accumulation (Liu et al. 2002). Therefore, it is crucially important to confirm the presence of UGTs and MTs that are functionally similar to isoflavonoid specific UGT and MT before plants are selected for metabolic engineering of these metabolites.

The recent efforts on genetic engineering of isoflavonoids have been geared towards introducing and/ or modifying the expression levels of IFS together with other structural genes involved in isoflavonoid biosynthetic pathway (Deavours & Dixon 2005, Lozovaya et al. 2007, Sreevidya et al. 2006, Subramanian et al. 2005) or introducing foreign transcription factors to up-regulate the synthesis of the metabolites (Yu & McGonigle 2005, Yu et al. 2003). One of the *in planta* functions of isoflavones is to serve as signal molecules for rhizobia to establish symbiotic association with legumes for the formation of nitrogen-fixing root nodules (Pueppke 1996, Spaink 2000). There is also a great deal of interest in exploiting this property of isoflavones and transferring the symbiotic nitrogen fixing ability to other crops of economical importance such as rice (Ladha & Reddy 2003, Reddy et al. 2002). Introduction of isoflavonoids in high value crops that normally do not produce these metabolites will reduce the cost of chemical nitrogen fertiliser input as well as may enhance their nutritional value. Introduction of soybean *IFS* gene was sufficient to produce genistein conjugates in rice (Sreevidya et al. 2006). As observed in *Arabidopsis*, tobacco and maize BMS cell line (Liu et al. 2002, Yu et al. 2000), the endogenous UGTs were able to glycosylate genistein in transgenic rice expressing soybean *IFS* gene (*35S-IFS*). Analysis of *35S-IFS* transgenic rice plants was performed for its ability to enhance *nod* gene expression in rhizobia. The results showed that both leaf and root extracts from transgenic *35S-IFS* lines stimulated *nod* gene expression in *Bradyrhizobium japonicum* USDA110 (Sreevidya et al. 2006). It will be of utmost interest if the transgenic *35S-IFS* lines could form nodules that participate in nitrogen fixation. Isoflavonoids also function as phytoalexins in plants. These are defence related compounds produced by the plant upon pathogen infection. Engineering the isoflavonoid pathway in non-legume plants or boosting the production of phytoalexins in legumes may make plants more resistant to pathogens and also reduce the use of chemical pesticide/fungicides. A direct role of isoflavonoids in disease resistance was demonstrated by RNAi silencing of *IFS* expression in soybean which resulted into reduction of the level of isoflavonoid accumulation leading to drastic increase in disease symptoms upon *Phytophthora sojae* infection (Subramanian et al. 2005). The results suggested that increased levels of isoflavonoids may enhance plant innate defense system.

Several metabolic engineering attempts are made to modify the level of isoflavonoids in soybean seeds for the enhancement of the nutritional value of the seed. Expression of several phenylpropanid genes such as *PAL*, *CHS*, *CHI* and *IFS* was performed in different combinations that resulted in change in the isoflavonoid levels in soybean seeds (Yu et al. 2003, Zernova et al. 2009). However, the most significant level of increase in isoflavonoid in seeds was achieved by introducing the maize C1 and R transcription factors in soybean (Yu et al. 2003). Isolated from maize, C1 is an R2R3 MYB transcription factor that requires an R MYC co-factor for its function in anthocyanin production (Grotewold et al. 1998). A chimeric protein CRC was produced, in which R was inserted between the DNA binding and activation domains of C1, and introduced into soybean. The transgenic soybean lines overexpressing chimeric CRC produced significantly higher levels (up to four times) of isoflavonoids than that of wild-type soybean seeds. The increase in isoflavonoid levels in transgenic soybean CRC lines was achieved when the complete flow of substrate was diverted to the isoflavonoid pathway by blocking the expression of flavonoid biosynthetic genes (Yu et al. 2003). Realising the complexity of phenylpropanoid pathway, a better understanding of complete biosynthetic pathway and the associated branch pathways, their regulation and cross connection with branch pathways is crucial for metabolic engineering of isoflavonoids.

4. Regulation of isoflavonoid biosynthesis in soybean seeds

Since soybean seed is the main source of isoflavonoids for human consumption, much research is focussed on understanding the biosynthesis and accumulation of these compounds in seeds. As shown in Fig. 4, the accumulation of isoflavonoids in soybean embryos increases as seeds approach towards maturity [50-70 days after pollination (DAP)]. After 70 days of pollination, soybean seed slowly starts losing water, shrinks in size and goes into dormant condition. The level of seed isoflavonoids reaches to maximum level in mature dry seeds (Dhaubhadel et al. 2003). The measurements of accumulation of isoflavonoids in soybean seeds (Fig. 4) were performed by harvesting seeds from field

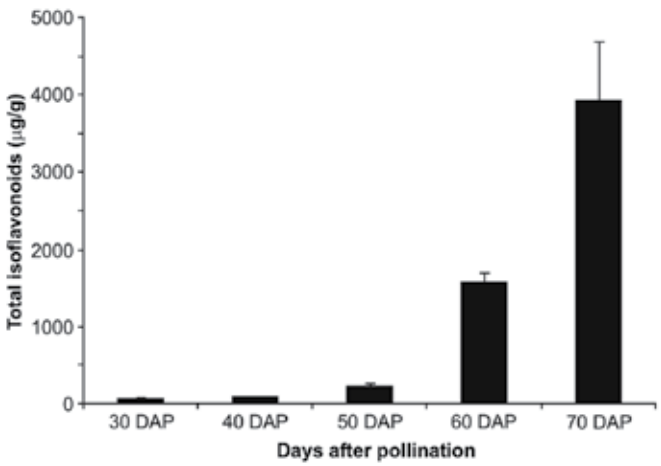

Fig. 4. Total isoflavonoid content in developing soybean embryos measured by HPLC. Data are mean values from three independent experiments. DAP- days after pollination

grown soybean plants at various stages of seed development and separating the embryos from seed coat for isoflavonoids extraction. The embryo extracts were hydrolysed to convert malonyl-derivatives to their corresponding glycosides and levels of six different isoflavonoids (daidzein, glycitein, genistein, daidzin, glycitin and genistin) were measured by high-performance liquid chromatography (HPLC).

For the detail analysis of seed isoflavonoid content, soybean cv Harosoy63 was grown under controlled condition. Flowers were tagged on the first day of pollination and seeds harvested every day starting from 40 DAP to 75 DAP for isoflavonoid measurement by HPLC. The results suggested that glycitein and its derivative level remained constant from early to late seed maturity in soybean. However, both daidzein and genistein and their conjugates accumulation increased during seed development suggesting that it is the synthesis and accumulation of daidzein and genistein and their conjugates that contribute to the increased accumulation of isoflavonoids in seeds during the later stages of seed development (Fig 5).

It has been demonstrated in the past that many plant natural products are often transported from the site of synthesis to the site of accumulation. Examples include nicotine in tobacco which is biosynthesized in roots, then translocated to leaves and finally accumulated in central vacuole of leaves by a novel multidrug and toxic compound extrusion (MATE)-type transporter, Nt-JAT1 (Hashimoto & Yamada 2003, Shitan et al. 2009, Shoji et al. 2000). Similarly glucosinolates are synthesized in leaves and translocated to seeds during development in *Tropaeolus majus* (Lykkesfeldt & Moller 1993). A carrier mediated system has been proposed for accumulation of glucosinolates in developing rapeseed embryos (Chen & Halkier 2000, Gijzen et al. 1989). To investigate if similar pattern of transport and accumulation occurs in soybean seed isoflavonoids, the ability of soybean embryos to synthesize isoflavonoids were assessed and maternal effect on seed isoflavonoids was determined (Dhaubhadel et al. 2003). The precursor feeding [^{14}C Phe] experiment demonstrated that together with leaf and pod tissues, soybean embryos were also able to incorporate radiolabelled Phe into isoflavonoids demonstrating the ability of embryos to synthesize these compounds within the tissue. In addition, the *in vitro* uptake study and determination of maternal effect on seed isoflavonoid content revealed that there may be involvement of transport mechanism in the accumulation of isoflavonoids in soybean seeds. A significantly large amount of isoflavonoids were also found in leaf and stem tissues which may ultimately get channelled into the sink tissues such as seeds. The study demonstrated that soybean embryos have an ability to synthesize isoflavonoids, however, isoflavonoids synthesized in maternal tissues may contribute to the total seed isoflavonoid accumulation at maturity (Dhaubhadel et al. 2003).

As discussed previously, the majority of seed isoflavonoids are accumulated in conjugated form (Fig. 3). The soybean seed isoflavone aglycones are predominantly present in their malonylglycoside form followed by glycoside derivatives. These conjugation processes are catalyzed by MT and UGT, respectively. Involvement of UGT and MT in isoflavonoid biosynthesis was demonstrated previously, however, the genes encoding these enzymes were identified only recently. Two independent studies, using functional genomics approach and homology based strategy, identified *GmMT7/GmIF7MaT* as isoflavonoid specific malonyltransferase (Dhaubhadel et al. 2008, Suzuki et al. 2007). Heterologously produced GmMT7/GmIF7MaT was able to transfer malonyl group to isoflavone glycosides.

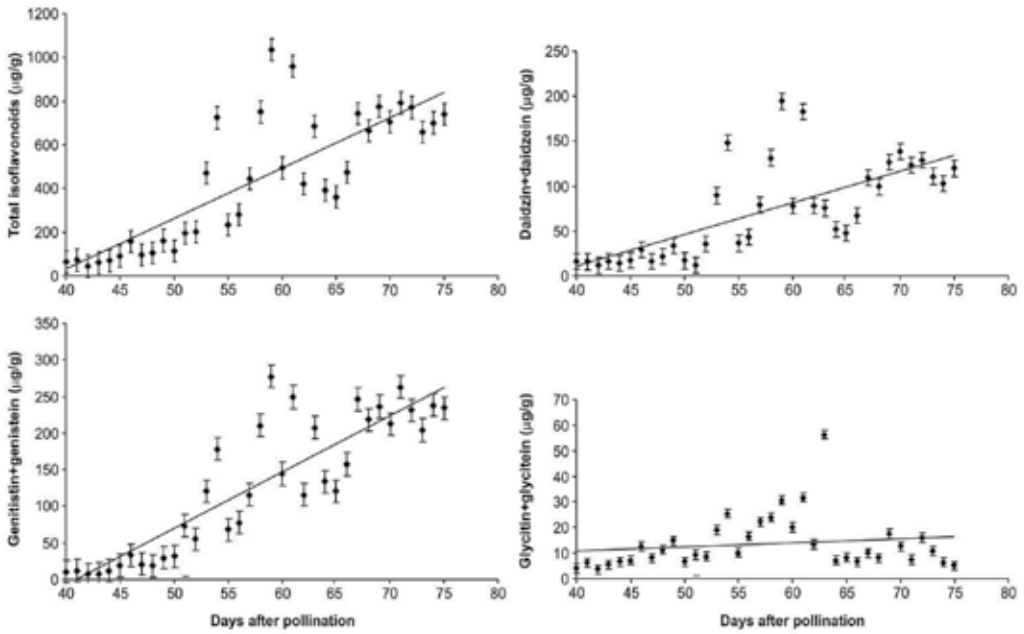

Fig. 5. Detail accumulation pattern of isoflavonoids in soybean seeds measured by HPLC. Data are mean values from two independent experiments. Numbers indicate days after pollination.

The recombinant GmMT7 was used to produce radiolabelled [^{14}C] malonylgenistin and malonyldaidzin which were subsequently used in the feeding experiment to investigate if externally supplied isoflavonoids can accumulate in embryos or not. The results obtained were similar to when [^{14}C] labelled Phe, a precursor for isoflavone synthesis, was used. As shown in Fig. 6, a soybean branch containing developing pods and leaves were taken and [^{14}C] labelled Phe was fed through the stem for 24 h at 24^{0}C followed by autoradiography. Incorporation of precursors to isoflavonoids was induced by cold treatment of the soybean plant at 4^{0}C for 3 days before feeding radiolabelled isoflavonoids or precursors. The results suggested that both [^{14}C] labelled Phe and [^{14}C] labelled isoflavonoids were transported to mature leaves and embryos. The HPLC analysis was performed to confirm the incorporation of [^{14}C] Phe into isoflavonoids (data not shown). The ^{14}C signal in embryos was only detected in opened pods (Fig. 6A) but not in closed pods. Furthermore, a high amount of transport was observed in mature embryos compared to young embryos (Fig. 6B). These data support the long distance transport of isoflavonoids to the site of accumulation as observed in case of many other secondary metabolites. Identification of the specific transporter involved in accumulation of isoflavonoids in soybean seed will help in understanding how these metabolites are accumulated in the sink tissues as well as may aid in metabolic engineering of these compounds in seeds.

A global gene expression analysis during embryo development was performed in soybean where gene expression profiles of two soybean cultivars (RCAT Angora-high isoflavonoid cultivar and Harovinton-low isoflavonoid cultivar) that contrasted in seed isoflavonoid content were compared (Dhaubhadel et al. 2007). Since isoflavonoid level is influenced by environmental factors (Eldridge & Kwolek 1983, Tsukamoto et al. 1995, Vyn et al. 2002), the

analysis consisted of the samples collected from two different locations grown at two different years. Gene expression analysis was performed using soybean cDNA microarray (Vodkin et al. 2004). In deed, environmental effects on the gene expression of the developing seeds were large and surpassed cultivar specific differences. Among 5910 genes that are differentially expressed in one of the five stages of embryos included in the study,

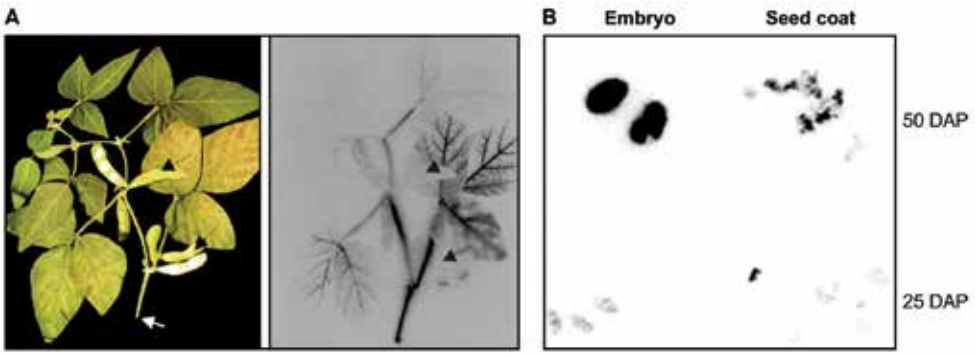

Fig. 6. Transport of [^{14}C] isoflavonoids in the sink tissues. A. [^{14}C] Phe was fed to a detached soybean branch containing leaf, developing pod and flowers for 24 h followed by autoradiography. The white arrow indicates stem part where feeding was performed. B. Autoradiography of dissected embryos (25 and 50 DAP) harvested from A. DAP-days after pollination.

CHS7 and *CHS8* expression were higher in RCAT-Angora compared to Harovinton. The expression profiles of these two genes correlated well with the isoflavonoid accumulation in seed during development suggesting a critical role for *CHS7* and *CHS8* genes in seed isoflavonoid synthesis. Both coding sequences and 5' upstream regions of *CHS7* and *CHS8* genes are closely related compared to their similarities with other members of the *CHS* gene family (Yi et al. 2010). RNAi gene silencing of *CHS8* gene in soybean hairy roots resulted in reduced transcript levels of both *CHS7* and *CHS8* genes which led to reduced isoflavonoids in the roots. *In silico* analysis of promoter regions of *CHS7* and *CHS8* identified several *cis*-regulatory factors that are common to both the genes, however, some unique motifs were also identified for each these gene. From the promoter analysis it has been speculated that *CHS7* may be involved in induced isoflavonoid synthesis whereas the role for *CHS8* possibly in isoflavonoid synthesis under normal condition. However, these speculations have to be verified experimentally (Yi et al. 2010).

The transcriptional regulation of phenylpropanoid pathway is one of the most extensively studied regulatory pathways in plants (Grotewold 2005, 2008, Ni et al. 1996, Weisshaar & Jenkins 1998). Even though several different groups of transcription factors such as MYBs, bHLH, WRKY, MADS box and WD40 have been shown to regulate the pathway in a coordinated manner (Gonzalez et al. 2008, Hartmann et al. 2005, Ramsay & Glover 2005), MYB transcription factors are largely studied (Jin & Martin 1999) and have been involved in majority of steps in flavonoid biosynthetic pathway. In *Arabidopsis*, an R2R3 MYB transcription factor regulates *CHS* gene expression thereby affecting the flavonoid composition (Mehrtens et al. 2005, Stracke et al. 2007). The maize *C1* gene and *Arabidopsis PAP1* gene belong to MYB transcription factor family and both of them regulate anthocyanin biosynthesis (Cone et al. 1986, Martin & Paz-Ares 1997). C1 is an R2R3 MYB

that requires a bHLH containing MYC co-factor R for its function (Grotewold et al. 1998). In the presence of R, C1 recognizes "CAACCACC" motif present in the *cis*-acting regions of the phenylpropanoid genes and activates the entire pathway (Grotewold et al. 2000). For metabolic engineering of isoflavonoids, a chimeric protein CRC was introduced in soybean under a seed-specific phaseolin promoter. Transgenic soybean plants overexpressing CRC gene produced four times more isoflavonoids in seeds compared to wild type soybean (Yu et al. 2003). This effect was only observed when transcription of flavanone 3-hydroxylase was suppressed to block anthocyanin branch of the pathway and total flux of substrates was diverted to isoflavonoid biosynthesis which suggested a strong competition for the precursor substrates by different branches of phenylpropanoid pathway. The expression levels of many phenylpropanoid genes such as *PAL, C4H, CHR, DFR* including *CHS* was increased in CRC transgenic soybean seeds (Yu et al. 2003). These results along with others in *Arabidopsis*, maize and several other plants have shown the regulation of *CHS* gene by MYB transcription factors (Sablowski et al. 1994, Solano et al. 1995, Stracke et al. 2007). Recently, a functional genomic approach was used to search for transcription factor that regulates expression of *CHS8* gene and isoflavonoid synthesis. The study identified GmMYB176, an R1 MYB transcription factor, as a regulator for *CHS8* expression (Yi et al. 2010). Most of the plant MYBs and those that are involved in phenylpropanoid pathway, consist of R2 and R3 repeats (Romero et al. 1998) and belong to the R2R3 MYB group. Some MYB-like proteins with R1 repeats have also been identified from several plant species (Baranowskij et al. 1994, Feldbrugge et al. 1994, Rubio-Somoza et al. 2006, Wang et al. 1997). However, GmMYB176 is the only R1 MYB transcription factor identified so far which is involved in plant secondary metabolism. GmMYB176 recognizes a 23 bp motif containing TAGT(T/A)(A/T) sequence within the *CHS8* promoter and binds with it for *CHS8* gene regulation. The RNAi silencing of *GmMYB176* in soybean hairy roots reduced the level of isoflavonoids in the transgenic roots demonstrating the functional role of GmMYB176 in isoflavonoid biosynthesis. The GmMYB176 silenced transgenic hairy roots showed reduced transcript levels of *GmMYB176*, *CHS8* and *IFS* genes. However, overexpression of GmMYB176 in soybean hairy roots neither increased expression levels of isoflavonoid biosynthetic genes nor the metabolite level. The results suggested that the regulation of *CHS8* gene by GmMYB176 may involve requirement for additional co-factors for its function in root pointing towards a cooperative and combinatory mechanism of gene regulation. Eukaryotic gene expression is generally regulated by multi-protein complex which involves combinatorial action of several transcription factors. Such interactome include protein-protein interactions, as well as protein-DNA interactions (Libault et al. 2009). An example of combinatorial regulation in plants is the direct interaction between two distinct classes of transcription factors, C1/P1 and R/B, required for the biosynthesis of anthocyanin in maize (Goff et al. 1992, Goff et al. 1990). Identification of GmMYB176 interactome will highlight the complexity of isoflavonoid regulation and regulation of other metabolic processes associated with it.

Even though GmMYB176 is localized in nucleus, it does not possess a nuclear localization signal. It was suspected that a protein-protein interaction may be involved in the localization of GmMYB176 in nucleus (Yi et al. 2010). Sequence analysis predicted presence of pST binding sites within GmMYB176, to which 14-3-3 protein may bind thereby assisting in its nuclear localization. Deletion of one of the predicted 14-3-3 protein site within GmMYB176 changed its subcellular localization suggesting that 14-3-3 may regulate the nuclear localization of GmMYB176. In plants, 14-3-3s are thought to be involved in a variety of signaling processes (Bai et al. 2007, Paul et al. 2008, Schoonheim et al. 2009, Sehnke et al.

2002, Xu & Shi 2006). They regulate activities of a wide array of target proteins via protein-protein interactions which involves binding with phosphoserine/phosphothreonine residues in the target proteins (Muslin et al. 1996, Yaffe et al. 1997) or by some unknown mechanism (Aitken 2006, Fu et al. 2000). Using Bimolecular Florescent Complementation and targeted yeast two-hybrid assays, it has been shown that GmMYB176 interacts with soybean 14-3-3 protein, SGF14d (Dhaubhadel & Li 2010). Soybean genome contains 18 14-3-3 genes among which 16 are transcribed (Li & Dhaubhadel 2010). Identification of 14-3-3 specific for GmMYB176 regulation/localization will provide a deeper understanding of isoflavonoid synthesis and regulation in soybean.

5. Future research

Although the therapeutic medicinal properties associated with consumption of soy isoflavonoids and their role in plant-microbe interaction including nitrogen fixation and defence mechanism is well established and substantial amount of efforts already focussed towards the metabolic engineering of isoflavonoids in legumes and nonlegumes plants, the task of manipulating the levels of isoflavonoids has been an enormous challenge. The major problems in this endeavour are the complexity of trait (Gutierrez-Gonzalez et al. 2010) and its inter-connection with other major branches of the phenylpropanoid pathway. The individual genes involved in isoflavonoid biosynthetic pathway are well known for several decades and regulation of these genes are starting to be understood. The availability of soybean genome sequence makes it possible to identify the members of each gene family that are involved in isoflavonoid specific pathway. The future research should gear towards identifying the regulators for isoflavonoid specific genes and their regulons in the pathway, determining how different branches of the phenylpropnoid pathway are interconnected, and how the metabolon is formed. The knowledge generated will allow us to modify the approach necessary for efficient metabolic engineering of these compounds for human health or for plant protection and nitrogen fixation in crops of agronomic importance.

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16

Nondestructive Estimation of the Contents of the Functional Elements in Soybean by Near Infrared Reflectance Spectroscopy

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1. Introduction

In recent days, the consumers' demands for the agricultural products are highly upgraded and widely diversified. Their attentions are focused not only on the major constituents, the nutritional constituents, or the palatability, but also on the physiologically functional activities. Soybean (*Glycine max* L.) is a major (oilseed) crop, and a good source of nutrition such as protein and oil. In Japan, soybean is used for producing the excellent traditional foods such as tofu, miso, soy-sauce, and boiled beans. It is also used for inventing the new industrial foods such as snack foods. In order to make the consumption of the soybeans increase, it is indispensable to make the high-values added to them. For example, the proper contents of physiologically functional elements give highly added values to soybeans. Soybean contains some functional elements such as isoflavones, thiamine (Vitamin B_1), riboflavin (Vitamin B_2) and tocopherols (Vitamin E). Isoflavones have function for preventing osteoporosis (Yamori, 2001). Thiamine (Vitamin B_1), riboflavin (Vitamin B_2) and tocopherol have relations to carbohydrate metabolism and anti-oxidant function, respectively (Tsujimura, 2004). However, the conventional methods for the determination of these physiologically functional elements are much labor intensive. A simple and rapid method for the estimation of them is necessary for screening soybean varieties for the plant breeding. Further, a nondestructive analytical method is needed so that the sample seeds can be used for breeding and sowing after the analyses and selections.

Near infrared spectroscopy (NIRS) has been understood as one of the most powerful analytical tools in the agro-food sector (Shenk et al., 2007; Williams, 2006). Hymowitz et al. (1974), Choung et al. (2001), and Tajuddin et al. (2002) reported the oil and protein analysis of soybean using NIRS. Now, NIRS is one of the official methods for determining major constituents of soybean in the trade based on the quality (Osoborne & Fearn, 1986; USDA FIGS, 1996). Furter, Pazderniket al. (1997), and Kovalenko et al. (2006) tried the determination of amino acid composition of soybeans by NIRS. Hollung et al. (2005) reported the evaluation of nonstarch polysaccharides and oligosaccharide content by NIRS. Li et al. (2009) analyzed lecithin and by-products in the soybean oil processing by using NIRS as a quality control tool. Also, Sato et al. (1998, 2002) reported the estimation of the contents of major constituents, the level of the deterioration indices, and the fatty acid

composition in soybean by NIRS. Then, if other criteria can be estimated by NIRS method, this method will gain greater position in the soybean analysis. In this chapter, the feasibility of NIRS for the estimation of the contents of some functional elements, i.e., isoflavones, thiamine, riboflavin and tocopherol in soybean seeds was examined.

2. Materials and methods

2.1 Soybean samples

Forty-eight samples were cultivated in various areas from northern to southern part of Japan in 2003. The varieties of samples used were as follows: Toyokomachi, and Toyomusume (produced in Hokkaido Prefectural Tokachi Agricultural Experiment Station); Ohsuzu, Suzuyutaka, and Ryuhou (produced in National Agricultural Research Center for Tohoku Region (Akita)); Ayakogane, Enrei, Koganedaizu, Sakukei-4-gou, Suzuyutaka, Tachinagaha, Tachiyutaka, Tamaurara, Tamahomare, Harosoy, Fukuyataka, Houen, Miyagi'oojiro, Yumeminori, Ohsodenomai, Kiyomidori, Akikogane, Enrei, Sachiyutaka, Suzuotome, NattoShouryuu, and Miyagi'ohshiro, (produced in National Institute of Crop Science (Tsukuba)); Enrei, Sachiyutaka, Tamahomare, Fukuyutaka, and Shintanbaguro (produced in National Agricultural Research Center for Western Region (Kagawa)); Fukuyutaka, Kurodamaru, Kiyomidori, and Shinanoguro (produced in our National Agricultural Research Center for Kyushu Okinawa Region (Kumamoto)). One Chinese (Baimei_Baishan) and two USA varieties (from Harrowvinton and from Ohio) were also included. These samples were collected and sent to our research center and were milled by a ultra-centrifugal mill ZM1000 (Retsch Co., Germany) through a screen (φ=1.0mm). All the powdered samples and all the whole grain samples were packed in sealed polystyrene containers (LABORAN Pack, AS ONE Co., Osaka) and were stored at 5°C until being analyzed.

2.2 Chemical measurements

Table 1 described the contents of the isoflavones, thiamine, riboflavin and tocopherols determined by HPLC method (Nishiba et al., 2007). The respective components such as glycosides (daidzin, glycitin, and genistin), malonyl glycosides, acetyl glycosides and aglycons (daidzein, glycitein, and genistein) were determined in this process, and the total isoflavone content was calculated as the summation. Also, the respective tocopherol content was determined, and the total content was calculated.

2.3 Near infrared spectroscopic measurements and statistical analysis.

2.3.1 NIR analysis with an InfraAlyzer 500 (IA500)

An InfraAlyzer 500 (Bran + Luebbe (B+L) GmbH, Norderstedt, Germany) (Photo 1) was used to measure the NIR reflectance spectra in the wavelength range from 1100 to 2500 nm at 2-nm intervals. Samples were packed in a standard cell on a standard drawer for soybean powder (about 3 g), or packed in a whole grain cell on a moving drawer for intact plural soybean seeds (about 60 g). Also, NIR spectra were measured for a single seed in a single grain cup on a standard drawer. See Chapter 28 for the sample presentation method. The samples were divided into two sets: a calibration set (n=36) and a prediction set (n=12) as in Table 1, where their fundamental statistics were described. The unit is mg (100 g DW)$^{-1}$. By the way, the amounts of acetyl glycitin and glycitein were almost none as described in Table 1, and then, their statistical analyses were not carried out in the following. In the tocopherol

analysis case, samples were divided into two sets: a calibration set (n=16) and a prediction set (n=7) as in Table 1.

		calibration set (n = 36 or 16)				prediction set (n = 12 or 7)			
		range		mean	std	range		mean	std
glycoside	daidzin	8.41 -	56.71	24.55	11.68	11.57 -	43.59	21.34	10.63
	glycitin	2.31 -	11.81	6.37	2.50	2.27 -	12.81	6.10	3.36
	genistin	14.40 -	87.49	36.24	16.26	17.72 -	57.65	32.70	13.56
	total	26.28 -	154.01	67.16	28.77	34.31 -	109.93	60.14	25.25
malonyl glycoside	malonyl daidzin	31.82 -	192.27	95.77	40.54	39.36 -	162.15	84.90	39.08
	malonyl glycitin	4.77 -	22.22	12.60	4.49	4.70 -	25.98	11.81	6.02
	malonyl genistin	51.79 -	264.06	133.72	50.15	65.12 -	200.18	125.77	42.95
	total	102.96 -	473.30	242.08	89.84	119.91 -	368.42	222.48	82.47
acetyl glycoside	acetyl daidzin	0.04 -	1.64	0.69	0.35	0.06 -	1.53	0.71	0.47
	acetyl glycitin	0.00 -	0.00	0.00	0.00	0.00 -	0.00	0.00	0.00
	acetyl genistin	0.21 -	1.82	0.88	0.40	0.00 -	1.76	0.87	0.48
	total	0.26 -	3.46	1.56	0.73	0.10 -	3.25	1.59	0.92
aglycone	daidzein	0.25 -	2.66	1.07	0.59	0.26 -	1.72	0.78	0.45
	glycitein	0.00 -	0.23	0.01	0.04	0.00 -	0.00	0.00	0.00
	genistein	0.35 -	2.82	1.37	0.65	0.32 -	1.87	1.08	0.50
	total	0.60 -	5.48	2.45	1.20	0.58 -	3.58	1.86	0.93
total isoflavone	total	133.44 -	633.42	313.26	116.83	156.96 -	482.24	286.07	107.79
Vitamin B	thiamine	0.57 -	0.90	0.70	0.09	0.56 -	0.81	0.69	0.08
	riboflavin	0.20 -	0.28	0.23	0.02	0.21 -	0.26	0.23	0.01
tocopherol (toc)	α-toc	0.77 -	10.83	3.23	2.44	2.26 -	7.08	3.49	1.60
	β-toc	0.41 -	4.21	1.33	1.00	0.58 -	1.70	1.07	0.36
	γ-toc	11.19 -	21.69	17.09	2.80	9.10 -	19.16	16.08	3.13
	δ-toc	5.59 -	15.46	9.85	3.02	5.32 -	12.45	7.90	2.33
	total	25.27 -	37.59	31.50	3.69	20.67 -	35.93	28.53	4.60
	α-toc equivalence	2.76 -	13.29	5.57	2.60	4.09 -	9.41	5.60	1.72

The unit is [mg (100 g DW)$^{-1}$]. std: standard deviation

Table 1. The fundamental statistics of the samples to be analyzed.

Photo 1. An instrument : An InfraAlyzer 500

Multiple linear regression (MLR) analysis of the NIRS data with the HPLC data was carried out using IDAS software (B+L), an accessory software of IA500, on the calibration set. When the first- and second-derivative NIR spectra were calculated, the default parameters were used. The validations of the calibration equations obtained, or the prediction process, were

carried out using the prediction set. The Unscrambler (version 9.6; Camo Co., Norway), which was a software for the data-analysis and is sold separately, was also used on the IA500 data for partial least square regression (PLSR) or principal component regression (PCR) analysis. The authors analyzed the data not only on the original spectra, but also on the derivative spectral data, i.e., pretreated spectral data. In this case, the conditions to calculate the derivatives were as follows: gap 11, segment 10 for the first derivative (abbreviated as d1); and gap 10, segment 11 for the second derivative (d2). The gap and segment are the parameters in the Gap-Segment derivatives. Gap is the length of the interval that separates the two segments that are being averaged, and segment is an interval over which data values are averaged.

2.3.2 NIR analysis with a SpectraStar 2400 (SS2400)

A SpectraStar 2400 (Unity Scientific, USA) (Photo 2) was also used to measure the NIR reflectance spectra in the wavelength range from 1200 to 2400 nm at 1-nm interval. The sample presentation methods for powder and plural whole seeds were in the same manner as IA500 case.

Photo 2. An instrument: A SpectraStar 2400

The SensoLogic (Sensologic GmbH, Germany) was used on SpectraStar 2400 data with chemical data for MLR analysis, and PLSR/PCR analysis. The conditions to obtain the derivatives for the pretreatment of NIR spectra were as follows: gap 10, segment 10 for the first derivative (abbreviated as d1), gap 10, segment 10 for the second derivative (d2), and gap 5, segment 5 for the other second derivative (d22). First, the data analyses were carried out on the calibration set. Then, the validations of the calibration equations obtained were checked using the prediction set.

2.3.3 NIR analysis with an MPA, Multi Purpose FT-NIR Analyzer

An MPA (Multi Purpose FT-NIR Analyzer, Bruker Optics, Germany) was also used for the NIR measurements: wavenumber = 4000-12000cm^{-1}, resolution = 16cm^{-1} (Photo 3). The sample types analyzed were powdered soybeans, plural whole soybean seeds, and a single soybean seed. They were measured using the specified cells on the specified modules depending upon their types (Photo 4). The Opus (Bruker Optics, Germany) was used for the statistical analysis of PLSR and PCR with an automatic analysis. By the way, the calibration and prediction sample sets were different from above because of the automatic analysis.

Photo 3. An instrument: The FT-NIR spectrometry MPA

Photo 4. The sample presentation methods for an FT-NIR spectrometry MPA
(powder, plural seeds, and a single seed)

2.3.4 NIR analysis with an NIRFlex N-500

An NIRFlex N-500 (Buchi, Swiss) was also used to measure the FT-NIR reflectance spectra of the powder samples and whole kernel soybean seeds in the wavenumber range from 4000 to 12000 cm^{-1} with resolution = 8 cm^{-1} (Photo 5). Each sample was packed in a test tube or a petri dish for the measurement with using the specified module (Photo 6). NIRCal5 (Buchi, Swiss) was used for the automatic statistical analysis.

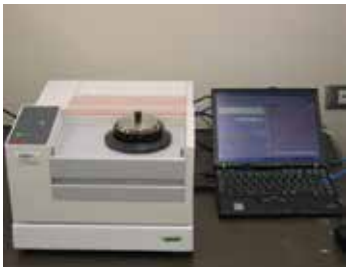

Photo 5. An instrument: An NIRFlex N-500

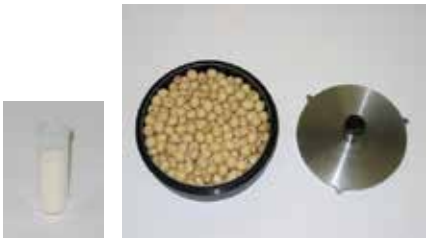

Photo 6. The sample presentation methods for an NIRFlex N-500.
(a test tube for a powder, and a dish and a lid for plural seeds)

3. Results and discussion

3.1 An InfraAlyzer 500

3.1.1 MLR analysis

Table 2 describes the calibration process (left side) and the prediction results (right side) developed for powdered soybean analysis with IDAS: the selected wavelengths in the calibration equations, the correlation coefficient (r), the standard error of calibration (SEC), the standard error of prediction (SEP), mean-corrected SEP (MC-SEP), and bias. These calibrations provided the best prediction in the validation process. In the table, when the contribution ratio, r^2, exceeded 0.5 in the prediction, the results are described in bold letters. For the total isoflavone content described at the last column of the isoflavone section, SEP was adequate for the estimation. The selected wavelengths were mainly due to C-H bonds (Osborne et al. 1993). The counts of wavelengths selected were also adequate, i.e., not so many wavelengths. Furthermore, especially as for powdered soybean, the respective components, such as glycosides and malonyl glycosides, also could be estimated separately, as described in Table 2, where the contribution ratios of the respective components, glycosides and malonyl glycosides, exceeded 0.5. On the other hand, the contents of acetyl glycosides and aglycons were poorly estimated because of their small range fluctuations.

		Calibration				Validation			
		treatment	selectred wavelengths	r	SEC	r	SEP	MC-SEP	Bias
glycoside	**daidzin**	**raw**	**1680, 2236**	**0.74**	**8.17**	**0.83**	**6.00**	**6.26**	**-0.24**
	glycitin	**d1**	**1747, 1943, 2147, 2383, 2391, 2471**	**0.90**	**1.22**	**0.80**	**2.22**	**2.10**	**0.94**
	genistin	**raw**	**1188, 1692, 2184, 2236**	**0.89**	**8.10**	**0.93**	**6.63**	**6.92**	**-0.09**
	total	**raw**	**1188, 1692, 2184, 2236**	**0.88**	**14.68**	**0.90**	**12.99**	**13.56**	**0.10**
malonyl glycoside	**malonyl daidzin**	**d1**	**1115, 1131, 2195, 2275, 2311,2347, 2383**	**0.96**	**12.30**	**0.95**	**15.43**	**14.98**	**5.69**
	malonyl glycitin	**d1**	**1851, 1939, 2307, 2419, 2463, 2475**	**0.91**	**2.03**	**0.74**	**4.30**	**4.33**	**1.12**
	malonyl genistin	**raw**	**1700, 1724, 2220**	**0.85**	**27.84**	**0.89**	**21.03**	**20.61**	**-7.26**
	total	**raw**	**1680, 2244**	**0.79**	**57.01**	**0.84**	**159.45**	**51.81**	**151.53**
acetyl glycoside	**acetyl daidzin**	**d1**	**1115, 1651, 1743, 2171, 2235**	**0.83**	**0.22**	**0.79**	**0.33**	**0.32**	**-0.11**
	acetyl genistin	**raw**	**1912, 1936, 2396, 2400**	**0.75**	**0.29**	**0.75**	**0.34**	**0.35**	**-0.04**
	total	d1	1271, 1751, 1755, 2107, 2191, 2347, 2363	0.93	0.30	0.66	0.69	0.71	0.05
aglycone	daidzein	d2	1354, 1746	0.59	0.50	0.57	0.53	0.39	0.38
	genistein	raw	2188, 2192	0.54	0.57	0.39	0.53	0.51	0.20
	total	raw	1556, 1668, 2296, 2364, 2376, 2380	0.82	0.76	0.02	1.36	1.26	0.63
total isoflavone	**total**	**raw**	**1188, 1688, 2184, 2236**	**0.92**	**48.88**	**0.95**	**38.51**	**39.94**	**-4.54**
Vitamin B	thiamine	raw	1852 2296 2320	0.57	0.08	0.38	0.08	0.08	0.02
	riboflavin	d2	1386 1482 1926 2338	0.71	0.01	0.62	0.01	0.01	0.00
tocopherol (toc)	α-toc	raw	1596	0.28	2.50	0.55	1.38	1.45	-0.32
	β-toc	**raw**	**1100**	**0.19**	**1.04**	**0.78**	**0.45**	**0.26**	**0.38**
	γ-toc	**d2**	**2142 2442**	**0.85**	**1.62**	**0.75**	**2.19**	**2.26**	**-0.63**
	δ-toc	raw	1176 1244 1260 1264 1412	0.98	0.80	0.49	3.06	2.61	1.87
	total	d2	1190 1322 1694 2282 2370 2434	0.90	2.11	0.40	6.04	4.55	4.33
	α-toc equivalence	raw	1596	0.26	2.69	0.57	1.42	1.53	-0.08

r : Correlation coefficient between chemical method and NIR method.
SEC: Standard error of calibration.; SEP: Standard error of prediction.; MC-SEP: Mean-corrected SEP.
(The bold letters mean that the contribution ratio, r^2, exceeded 0.5 in the validation.)

Table 2. The calibration and the prediction results (powdered soybean) for IA500 data.

Table 3 describes the calibration process and the prediction results developed for intact plural seeds analysis. SEP for the total isoflavone content described at the last column of the isoflavone section, was also small enough for the estimation. The selected wavelengths were mainly due to C-H bonds. Some of the respective components in intact plural soybean seeds still could be estimated. However, the contents of acetyl glycosides and aglycons were poorly estimated.

		Calibration				Validation			
		treatment	selected wavelengths	r	SEC	r	SEP	MC-SEP	Bias
glycoside	**daidzin**	**d2**	**1630, 1746, 2294**	**0.79**	**7.62**	**0.80**	**6.50**	**6.73**	**0.88**
	glycitin	d1	1203, 1947, 2335	0.81	1.55	0.49	2.92	3.05	-0.08
	genistin	raw	2236, 2280, 2336, 2360	0.80	10.50	0.50	12.84	13.41	0.18
	total	raw	1712, 1732, 2236, 2296, 2320, 2368	0.89	14.39	0.59	22.37	22.42	-6.30
malonyl glycoside	**malonyl daidzin**	**d2**	**1186, 1230, 1322, 1370, 1742, 2290, 2378**	**0.95**	**14.03**	**0.82**	**25.68**	**26.68**	**-2.65**
	malonyl glycitin	d1	1199, 1443, 1739, 1959, 2335, 2415	0.89	2.25	0.58	5.14	5.30	0.84
	malonyl genistin	**d1**	**1395, 1723, 2099, 2255, 2291, 2327, 2351**	**0.94**	**19.54**	**0.87**	**21.75**	**22.62**	**-6.30**
	total	**raw**	2192, 2208, 2232, 2236, 2272, 2336, 2356	0.92	38.97	0.44	151.29	73.54	133.91
acetyl glycoside	acetyl daidzin	raw	1112, 1124, 1132, 1136, 1144, 1304	0.79	0.24	0.66	0.35	0.36	-0.07
	acetyl genistin	raw	1648, 1660, 2268, 2280, 2288	0.83	0.24	0.57	0.39	0.41	-0.03
	total	d1	1395, 1655, 2255	0.68	0.56	0.69	0.70	0.73	-0.08
aglycone	**daidzein**	**raw**	**1256, 1260, 1268**	**0.66**	**0.47**	**0.85**	**0.39**	**0.24**	**0.31**
	genistein	d1	1263, 1643, 1927, 2287	0.66	0.52	0.40	0.49	0.49	0.16
	total	raw	2140, 2148, 2184, 2212, 2236, 2256, 2264	0.76	0.88	0.56	1.00	0.84	0.60
total isoflavone	**total**	**d2**	**1630, 1746, 2294**	**0.85**	**65.89**	**0.82**	**63.43**	**65.63**	**8.67**
Vitamin B	thiamine	d1	1527 1931 2095 2371	0.63	0.08	0.33	0.08	0.08	-0.02
	riboflavin	**raw**	**1120 1132 1256 1268 1296**	**0.77**	**0.01**	**0.80**	**0.01**	**0.01**	**0.00**
tocopherol (toc)	α-toc	d2	1554	0.41	2.37	0.05	1.78	1.92	0.09
	β-toc	raw	1160 1256 1456 2040	0.93	0.44	0.46	0.48	0.50	0.12
	γ-toc	d2	2314	0.55	2.50	0.57	2.58	2.79	0.03
	δ-toc	d2	1202 1246	0.88	1.57	0.14	2.96	2.94	1.14
	total	**raw**	**1176 1228 2428 2432**	**0.91**	**1.88**	**0.78**	**5.03**	**3.57**	**3.79**
	α-toc equivalence	d2	1442 1550	0.57	2.38	0.30	1.69	1.82	-0.15

see footnotes in Table2.

Table 3. The calibration and the prediction results (intact plural soybean seeds) for IA500 data.

The counts of wavelengths selected were proper, i.e., not too many. Generally, one wavelength can be selected for each 5 to 15 samples in MLR analysis (Hruschka, 2001), i.e., three to seven wavelengths can be selected in this case, because 36 samples were used for developing the calibration equations. Further, in the prediction process, different samples from the calibration set were used to check the overfitting. Both calibration equations for the estimation of the total isoflavone content obeyed this rule. Further, as for the powder analysis, the calibrations for some of the respective components of isoflavone in the powder were also adequate. On the other hand, for intact plural soybean seeds, the contribution ratio (r^2) was low, even when many wavelengths were selected for developing calibration equations for the respective components.

Figure 1 shows the prediction results of the total isoflavone analyses for the powder (Fig.1-a)), and for the intact plural seeds (Fig.1-b)). The correlation coefficient (r) between the chemical method and NIR method, and the standard error of prediction (SEP) were also described. The SEP value was one third to one half of SD described in Table 1. Comparing from the SEPs in Fig.1 with the standard deviation of the samples (about 110 mg(100 gDW)$^{-1}$ as described in Table 1), the author consider that the total isoflavone content could be estimated.

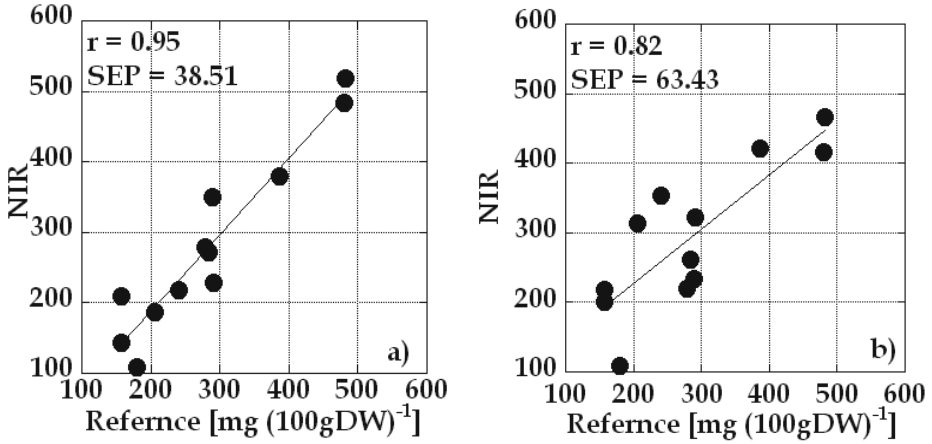

Fig. 1. The results of NIR analysis developed for total isoflavone: a) powdered soybean; b) intact plural soybean seeds with MLR analysis on IA 500 data.

3.1.2 PLSR/PCR analysis with the Unscrambler on IA 500 data.

Table 4 describes the results of PLSR/PCR analysis obtained using the Unscrambler, the calibration process (left side) and the prediction results (right side) developed for powdered soybean. The treatment on the original spectra, the number of factors, the correlation coefficient (r), the standard error of calibration (SEC), root mean squared error of prediction (RMSEP), the standard error of prediction (SEP), and bias were described. The better cases were described among PLSR and PCR. For the total isoflavone content, SEP was adequate for the estimation. Further, especially as for powdered soybean, the respective component, glycoside and malonyl glycoside, also could be successfully estimated separately, as described in Table 4, where the contribution ratios of the respective components, glycosides and malonyl glycosides, exceeded 0.5. The respective analysis of the total of the malonyl

glycosides was drastically improved from the results of MLR analysis (Table 2). On the other hand, acetyl glycoside and aglycon contents were poorly estimated as in MLR analysis.

		Calibration				Validation			
		Treatment	Factors	r	SEC	r	RMSEP	SEP	Bias
glycoside	**daidzin**	**d1**	**pls-6**	**0.78**	**7.45**	**0.80**	**6.88**	**6.79**	**2.27**
	glycitin	**d1**	**pls-9**	**0.89**	**1.16**	**0.77**	**2.40**	**2.32**	**0.92**
	genistin	**raw**	**pls-8**	**0.85**	**8.64**	**0.90**	**6.63**	**6.49**	**2.31**
	total	**raw**	**pls-8**	**0.85**	**15.22**	**0.90**	**12.21**	**11.99**	**4.14**
malonyl glycoside	**malonyl daidzin**	**d1**	**pls-7**	**0.91**	**17.25**	**0.91**	**17.68**	**17.95**	**4.16**
	malonyl glycitin	**d2**	**pls-12**	**0.93**	**1.68**	**0.74**	**4.25**	**4.37**	**0.76**
	malonyl genistin	**d2**	**pls-7**	**0.90**	**22.31**	**0.90**	**21.78**	**22.71**	**-1.25**
	total	**d1**	**pls-7**	**0.92**	**36.59**	**0.93**	**34.31**	**35.68**	**3.13**
acetyl glycoside	acetyl daidzin	raw	pls-6	0.71	0.25	0.51	0.40	0.41	-0.08
	acetyl genistin	d1	pls-1	0.42	0.37	0.69	0.39	0.40	0.02
	total	d1	pls-1	0.42	0.67	0.61	0.77	0.80	0.01
aglycone	daidzein	d2	pls-3	0.60	0.48	0.37	0.54	0.47	0.30
	genistein	d1	pls-8	0.74	0.44	0.45	0.61	0.55	0.31
	total	d1	pls-9	0.77	0.77	0.50	1.23	1.02	0.74
total isoflavone	**total**	**raw**	**pls-8**	**0.91**	**48.83**	**0.95**	**40.01**	**38.88**	**14.66**
Vitamin B	thiamine	d2	pls-1	0.22	0.09	0.58	0.07	0.08	0.01
	riboflavin	raw	pls-2	0.50	0.01	0.61	0.01	0.01	0.00
tocopherol (toc)	α-toc	raw	pls-1	0.19	2.47	0.45	1.49	1.55	-0.39
	β-toc	**d2**	**pls-3**	**0.87**	**0.50**	**0.70**	**1.53**	**1.14**	**1.11**
	γ-toc	d2	pls-2	0.57	2.38	0.25	3.45	3.71	-0.28
	δ-toc	d2	pcr-3	0.71	2.18	0.51	2.22	2.20	0.90
	total	raw	pls-1	0.13	3.78	-0.42	5.95	5.44	3.17
	α-toc equivalence	raw	pla6-1	0.18	2.64	0.48	1.51	1.62	-0.15

see footnotes in Table 2.

Table 4. The calibration and the prediction results (powdered soybean) with the Unscrambler on IA500 data.

Table 5 describes the calibration process and the prediction results developed for intact plural seeds. As for the total isoflavone content, SEP was fair enough for the estimation. As for intact plural soybean seeds, the results were improved: some of the respective component also could be estimated. The respective analysis of the total of the malonyl glycosides was also drastically improved. However, acetyl glycoside and aglycon contents were still poorly estimated.

The total isoflavone content could be estimated not only with powdered soybean but also with intact plural soybean seeds. It is the similar level as shown in Fig. 1. However, the content of the respective isoflavone component could be estimated for powdered soybean as described in Table 2-5. The present findings suggest that the total isoflavone content of the soybean seeds could be estimated for simple, rapid, and nondestructive breeding selection by the NIRS method. The respective elements in the powder could be estimated. PLSR and PCR analyses were also tried, and the results were similar to those obtained by MLR analysis. Further, for total malonyl glycoside, the bias was drastically improved by PLSR analysis.

The estimations for some of the contents of Vitamin B, and tocopherol were fair for rough estimation despite of their small range fluctuations. As for Vitamin B, considering from the

SEPs in Table 2-5 with comparing the standard deviation in Table 1: 0.08 for thiamin, and 0.01 for riboflavin. These contents might be fairly estimated.

		Calibration				Validation			
		Treatment	Factors	r	SEC	r	RMSEP	SEP	Bias
glycoside	**daidzin**	**d1**	**pls-11**	**0.96**	**3.34**	**0.81**	**6.98**	**7.28**	**0.33**
	glycitin	d2	pls-20	0.99	0.26	0.65	2.56	2.67	-0.13
	genistin	**d2**	**pls-12**	**0.97**	**4.10**	**0.84**	**8.33**	**7.84**	**-3.63**
	total	**d2**	**pls-12**	**0.97**	**6.96**	**0.84**	**16.19**	**15.43**	**-6.62**
malonyl glycoside	**malonyl daidzin**	**d2**	**pls-13**	**0.98**	**7.44**	**0.94**	**14.83**	**14.87**	**-4.15**
	malonyl glycitin	**raw**	**pls-12**	**0.99**	**0.75**	**0.73**	**4.16**	**4.33**	**-0.40**
	malonyl genistin	**d2**	**pls-11**	**0.96**	**13.43**	**0.93**	**15.91**	**16.36**	**-2.81**
	total	**d2**	**pls-12**	**0.98**	**19.86**	**0.95**	**26.64**	**26.52**	**-8.07**
acetyl glycoside	acetyl daidzin	raw	pls-6	0.69	0.26	0.63	0.36	0.38	-0.05
	acetyl genistin	raw	pcr-6	0.68	0.30	0.69	0.36	0.37	-0.02
	total	raw	pcr-6	0.67	0.55	0.69	0.68	0.71	-0.07
aglycone	daidzein	d2	pls-7	0.81	0.35	0.36	0.55	0.49	0.29
	genistein	raw	pls-8	0.83	0.36	0.40	0.63	0.52	0.39
	total	d2	plst-6	0.75	0.80	0.36	1.14	0.98	0.66
total isoflavone	**total**	**d2**	**pls-12**	**0.97**	**26.57**	**0.96**	**32.80**	**30.28**	**-15.34**
Vitamin B	thiamine	d2	pcr-1	0.14	0.09	0.24	0.08	0.08	0.01
	riboflavin	d1	pls-3	0.63	0.01	0.54	0.01	0.01	0.00
tocopherol (toc)	α-toc	d2	pcr-1	0.02	2.52	0.03	1.62	1.73	-0.24
	β-toc	**d2**	**pls-1**	**0.46**	**0.91**	**0.74**	**0.46**	**0.28**	**0.38**
	γ-toc	raw	pls-1	0.13	2.87	0.35	3.17	3.27	0.95
	δ-toc	raw	pls-7	0.95	0.95	0.40	2.52	2.47	1.04
	total	raw	pcr-1	0.25	3.70	0.16	5.35	4.90	2.82
	α-toc equivalence	d1	pls-1	0.28	2.18	0.01	1.85	1.97	0.31

see footnotes in Table 2.

Table 5. The calibration and the prediction results (intact plural soybean seeds) with the Unscrambler on IA500 data.

3.1.3 Statistical analysis for total isoflavone content on a single seed analysis.

As for a single seed analysis, considering from SEP, NIRS may be available for nondestructively estimating the total isoflavone content in both MLR- and PLSR-analysis cases (Table 6, upper columns). The scattering graphs of these results were shown in Fig.2.

		Calibration		**Prediction**			
		r	**SEC**	**r**	**SEP**	**bias**	**MC-SEP**
single seed analysis	**MLR Analysis by IDAS**	**0.77**	**79.62**	**0.80**	**69.92**	**23.25**	**68.87**
	PLS Analysis by Unscrambler	**0.89**	**53.70**	**0.79**	**69.01**	**19.33**	**69.20**
plural seeds analysis adapted on a single seed spectrum	**MLR Analysis by IDAS**	**------**	**------**	**0.83**	**62.99**	**18.21**	**62.98**
	PLS Analysis by Unscrambler	**------**	**------**	**0.96**	**158.84**	**155.33**	**34.70**

r : Correlation coefficient between chemical method and NIR method.
SEC: Standard error of calibration; SEP: Standard error of prediction; MC-SEP: Mean-corrected SEP
The unit is mg $(100gDW)^{-1}$

Table 6. Results of statistical analysis for total isoflavone content by NIR on a single seed analysis.

The same level of SEP was obtained in this single seed analysis case as plural soybean seeds analysis case (3-1-2 section). Kudou et al. (1991) reported that isoflavone distributed mainly in hypocotyls of soybean seeds, i.e., on the surface of a seed, and this might be why the SEP was not so bad for an intact seed. (Sato et al., 2009b)

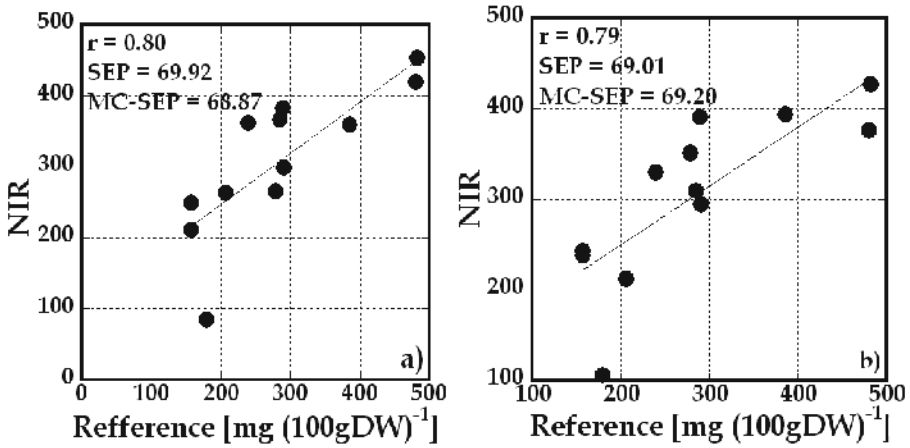

Fig. 2. The scattering graphs of the prediction results on a single seed analysis estimating the total isoflavone content by a) MLR- and b) PLSR-analysis.

3.1.4 Adaptation of calibrations obtained by plural seed analysis to single seed spectra

On the other hand, when the calibrations developed for plural seeds were adapted on a single seed spectrum case, the results of both MLR- and PLSR-analysis cases were described in the lower columns of Table 6. The scattering graphs of these results were shown in Fig.3. In this case, the bias and skew emerged as shown in Fig.3 (right). The reason is that its reflectance spectrum is a similar one as plural one, but its level is lower in a single seed case

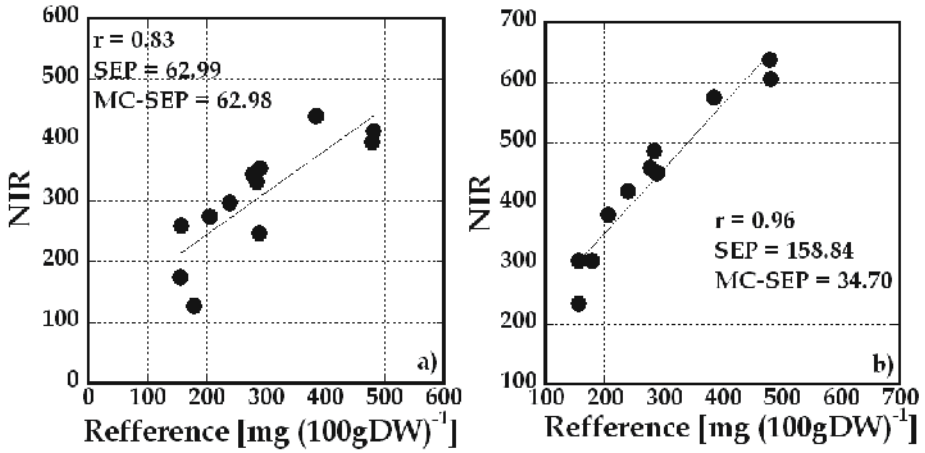

Fig. 3. The scattering graphs of the results adaptation of plural seeds analysis on a single seed for estimating the total isoflavone content by a) MLR- and b) PLSR-analysis.

than in plural seeds case. However, considering from MC-SEP, the level of the total isoflavone content could be estimated. The same level of MC-SEP was obtained in this single seed analysis case as plural soybean seeds analysis case. NIRS may be available for the nondestructive estimation of the total isoflavone content by both MLR- and PLSR-analysis cases on a single seed spectrum (Sato et al., 2009a).

3.2 Analysis on SpectraStar 2400 data(Sato et al., 2008)

3.2.1 MLR analysis

Table 7 describes the calibration and the prediction results developed for powdered soybean by MLR analysis with the SensoLogic: spectral treatment, selected wavelengths for calibration equations, the correlation coefficient (r), the standard error of estimate (SEE), root mean square error of prediction (RMSEP), bias, and the standard error of prediction (SEP). These calibrations provided the best prediction among the prediction results. The figures in bold letters mean the contribution ratio, r^2, exceeds 0.5. As for the total isoflavone content described in the last column of the isoflavone section, SEP was good enough for the estimation. The selected wavelengths were mainly due to C-H bonds (Osborne et al., 1993). The counts of the selected wavelengths in the calibration were a few and proper. Further, especially as for powdered soybean, the respective components, such as glycosides and malonyl glycosides, also could be estimated separately as described in Table 7. The estimations for acetyl glycoside,, and aglycon contents were not good enough because of their small range fluctuations. The estimation for riboflavin content was fair.

Table 8 describes the calibration and the prediction results developed for intact plural soybean seeds analysis. As for the total isoflavone content, SEP was also good enough for the estimation. The selected wavelengths were mainly due to C-H bonds, and the counts of wavelengths were a few and proper. As for intact plural soybean seeds, some of the respective components, such as malonyl daidzin and malonyl genistin, still could be estimated. On the other hand, the estimations for acetyl glycosides, aglycons contents were also not good enough because of their small range fluctuations.

Figure 4 shows the scattering graphs, which are the prediction results of the total isoflavone content between chemical method and NIR method: a) as for powdered soybean, and b) as for intact plural soybean seeds. Considering from the SEPs in Fig.4 with comparing its standard deviation (107.79 [mg $(100g\ DW)^{-1}$] described in Table 1, the isoflavone content might be fairly estimated in both cases.

3.2.2 PLSR/PCR analysis on SpectraStar 2400 data

Table 9 describes the calibration and the prediction results developed for powdered soybean by PLSR/PCR analysis. The number of factors was described instead of wavelengths. The better case among PLSR and PCR was described. As for the total isoflavone content at the last column of the isoflavone section, SEP was good enough for the estimation. Further, especially as for powdered soybean, the respective components, such as glycosides, malonyl gylucosides, and acetyl genistin, also could be estimated separately. On the other hand, the estimations for other acetyl glycosides and aglycons contents were not good enough because of their small range fluctuations. The estimation for thiamine or total tocophrol content was fair.

Table 10 describes the calibration and the prediction results developed for intact plural soybean seeds analysis. As for the total isoflavone content, SEP was fair enough for the

		Calibration				Prediction			
		treatment	calibration equations or wavelengths	r	SEE	r	RMSEP	bias	SEP
glycoside	**daidzin**	**d2**	**1684, 1766, 2223**	**0.79**	**7.62**	**0.85**	**5.97**	**-0.10**	**6.24**
	glycitin	**raw**	**1710, 2284, 2320**	**0.76**	**1.72**	**0.77**	**2.33**	**0.30**	**2.41**
	genistin	**d22**	**1820, 1887, 2002, 2264**	**0.86**	**8.80**	**0.87**	**7.97**	**3.58**	**7.44**
	total	**d22**	**1488, 1798, 2260**	**0.82**	**17.53**	**0.90**	**12.90**	**6.11**	**11.86**
malonyl glycoside	**malonyl daidzin**	**d2**	**1355, 1488, 1756, 1780, 2176, 2262**	**0.93**	**17.10**	**0.93**	**15.56**	**1.78**	**16.15**
	malonyl glycitin	**d1**	**1458, 1807, 2361**	**0.81**	**2.78**	**0.74**	**4.41**	**0.59**	**4.56**
	malonyl genistin	**d2**	**1314, 1329, 1737, 2063**	**0.88**	**25.43**	**0.92**	**17.66**	**-1.56**	**18.37**
	total	**d2**	**1315, 1336, 1738, 1757, 2065**	**0.90**	**42.88**	**0.94**	**28.70**	**-1.78**	**29.91**
acetyl glycoside	acetyl daidzin	raw	1705, 2328, 2336	0.63	0.29	0.62	0.36	0.03	0.38
	acetyl genistin	d1	1431, 1655, 1664, 1828	0.71	0.30	0.56	0.40	-0.04	0.42
	total	**d22**	**1542, 1809, 1886, 2000**	**0.80**	**0.47**	**0.76**	**0.61**	**-0.12**	**0.63**
aglycon	daidzein	raw	1344, 1368, 1376, 1396, 1790, 1813, 1824, 1835, 1873	0.86	0.36	0.64	0.50	0.14	0.50
	genistein	d1	1330, 1389, 1437, 1617, 1824, 1836, 2005, 2308	0.90	0.33	0.52	0.55	0.22	0.52
	total	d2	1299, 1612, 1829, 2037, 2112	0.87	0.65	0.49	1.08	0.40	1.05
total isoflavone	**total**	**d2**	**1685, 1767, 2022**	**0.87**	**61.11**	**0.94**	**43.63**	**-8.38**	**44.72**
Vitamin B	thiamine	d22	1230, 2142	0.59	0.08	0.26	0.09	0.02	0.09
	riboflavin	d2	1410, 1496, 1636, 1818, 2030, 2320	0.79	0.01	0.63	0.01	0.00	0.01
tocopherol (toc)	**α-toc**	**d2**	**1888, 2153, 2343**	**0.92**	**1.12**	**0.87**	**1.23**	**0.15**	**1.31**
	β-toc	d1	1272, 1822, 2196, 2314, 2359	0.95	0.41	0.54	0.45	-0.09	0.48
	γ-toc	raw	2287, 2330, 2364, 2372, 2380, 2400	0.98	0.65	0.60	2.81	-0.98	2.84
	δ-toc	d1	1655, 2339	0.91	1.42	0.44	2.24	0.81	2.25
	total	d2	1415, 1499, 1820, 1889, 2069, 2282	1.00	0.38	0.64	4.31	1.90	4.18
	α-toc equivalence	raw	2369, 2377, 2395	0.90	1.30	0.69	1.66	0.29	1.77

r : Multiple correlation coefficient between chemical method and NIR method.
SEE : Standard error of estimate
RMSEP : Root mean square error of prediction
SEP : Standard error of prediction.

Table 7. The calibration process and the prediction results for powdered soybean with MLR analysis on SpectraStar 2400 data.

		Calibration				Prediction			
		treatment	wavelength	r	SEE	r	RMSEP	bias	SEP
glycoside	**daidzin**	**d2**	**1623, 1741, 2293**	**0.80**	**7.51**	**0.73**	**7.44**	**1.62**	**7.59**
	glycitin	d22	1568, 1745, 2204	0.82	1.52	0.44	3.02	0.08	3.16
	genistin	d2	1621, 1743, 2293	0.80	10.32	0.65	10.45	1.73	10.77
	total	**d22**	**1548, 1713, 2187**	**0.87**	**15.24**	**0.79**	**16.51**	**4.24**	**16.66**
malonyl glycoside	**malonyl daidzin**	**d2**	**1301, 1554, 2170**	**0.84**	**23.39**	**0.88**	**18.62**	**-0.86**	**19.42**
	malonyl glycitin	d2	1256, 1365, 1473, 1527, 1629, 1841, 1974, 2115, 2158, 2216, 2350	0.95	1.68	0.63	5.03	1.76	4.92
	malonyl genistin	**d22**	**1350, 1369, 1671, 1706, 1893, 1982**	**0.94**	**18.34**	**0.86**	**22.06**	**-3.52**	**22.75**
	total	**d2**	**1260, 1316, 1662, 1745, 2291**	**0.89**	**44.12**	**0.88**	**40.23**	**0.80**	**42.01**
acetyl glycoside	acetyl daidzin	d22	1566, 1600, 1751, 1830, 1846, 2185	0.91	0.16	0.68	0.35	0.02	0.36
	acetyl genistin	d1	1937, 2102, 2347	0.72	0.30	0.55	0.41	0.02	0.42
	total	d2	2051, 2255	0.63	0.59	0.56	0.76	0.02	0.79
aglycon	daidzein	d22	1464, 1682, 2256, 2375	0.80	0.37	0.41	0.51	0.24	0.47
	genistein	d22	1542, 1682,2376	0.78	0.43	0.21	0.58	0.19	0.58
	total	d2	1746, 2166	0.48	1.09	0.31	1.10	0.45	1.05
total isoflavone	**total**	**d22**	**122 1 , 1550, 1708, 2187**	**0.91**	**51.88**	**0.88**	**57.26**	**2.66**	**59.75**
Vitamin B	thiamine	d1	1636, 1824	0.52	0.08	0.63	0.07	0.00	0.07
	riboflavin	d22	1352, 1643, 2060, 2371	0.62	0.01	0.29	0.01	0.00	0.01
tocopherol (toc)	**α-toc**	**d22**	**1226, 1733, 2355**	**0.86**	**1.45**	**0.72**	**1.15**	**-0.26**	**1.21**
	β-toc	raw	1716, 2350, 2363	0.84	0.63	0.35	0.54	0.17	0.56
	γ-toc	raw	1259, 1272	0.75	2.06	0.46	3.03	0.31	3.25
	δ-toc	raw	2268, 2295, 2323	0.91	1.46	0.04	3.65	1.61	3.55
	total	d22	1537, 2186, 2187, 2400	0.89	2.01	0.53	4.11	1.30	4.21
	α-toc equivalence	**d22**	**1226, 1733, 2355**	**0.86**	**1.53**	**0.80**	**1.09**	**0.00**	**1.18**

see footnotes in Table 7.

Table 8. The calibration and the prediction results for intact plural soybean seeds with MLR analysis on SpectraStar 2400 data.

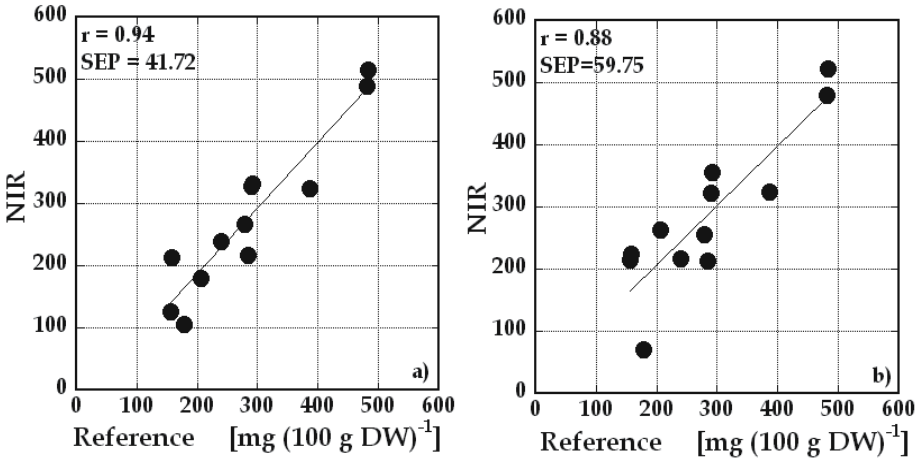

Fig. 4. The results of NIR analysis developed for total isoflavone: a) powdered soybean; b) intact plural soybean seeds with MLR analysis on SpectraStar 2400 data.

		Calibration				Prediction			
		treatment	factors	r	SEE	r	RMSEP	bias	SEP
glycoside	**daidzin**	**d22**	**pls-10**	**0.94**	**4.82**	**0.90**	**4.82**	**-1.18**	**4.88**
	glycitin	**raw**	**pls-4**	**0.72**	**1.87**	**0.82**	**2.41**	**0.41**	**2.48**
	genistin	**d22**	**pcr-18**	**0.83**	**13.72**	**0.93**	**5.53**	**0.25**	**5.78**
	total	**d1**	**pcr-11**	**0.84**	**18.93**	**0.82**	**16.12**	**4.16**	**16.26**
malonyl glycoside	**malonyl daidzin**	**d1**	**pls-6**	**0.90**	**19.32**	**0.94**	**14.28**	**5.90**	**13.58**
	malonyl glycitin	**raw**	**pls-4**	**0.72**	**3.36**	**0.71**	**4.77**	**1.12**	**4.85**
	malonyl genistin	**d2**	**pls-7**	**0.90**	**25.36**	**0.95**	**15.18**	**0.60**	**15.84**
	total	**d2**	**pls-9**	**0.94**	**35.96**	**0.95**	**30.17**	**-0.25**	**31.52**
acetyl glycoside	acetyl daidzin	raw	pls-2	0.49	0.32	0.60	0.38	-0.02	0.40
	acetyl genistin	**d1**	**pcr-1**	**0.38**	**0.38**	**0.75**	**0.37**	**0.01**	**0.39**
	total	d1	pls-1	0.47	0.66	0.64	0.75	-0.01	0.78
aglycon	daidzein	raw	pcr-1	0.49	0.53	0.27	0.55	0.28	0.49
	genistein	d1	pcr-16	0.85	0.47	0.47	0.65	0.26	0.62
	total	raw	pcr-1	0.45	1.09	0.13	1.17	0.55	1.08
total isoflavone	**total**	**d22**	**pls-10**	**0.97**	**36.36**	**0.92**	**42.50**	**-6.06**	**43.94**
Vitamin B	**thiamine**	**raw**	**pls-1**	**0.31**	**0.09**	**0.83**	**0.06**	**0.01**	**0.06**
	riboflavin	d2	pls-1	0.52	0.01	0.34	0.01	0.00	0.01
tocopherol (toc)	α-toc	d2	pcr-4	0.65	2.23	0.69	1.42	0.73	1.31
	β-toc	**d2**	**pcr-1**	**0.07**	**1.06**	**0.73**	**0.40**	**0.28**	**0.30**
	γ-toc	d22	pcr-1	0.14	2.96	0.48	3.32	1.22	3.34
	δ-toc	d22	pcr-7	0.99	0.64	0.71	2.18	1.24	1.93
	total	**d22**	**pls-5**	**0.93**	**1.70**	**0.79**	**4.35**	**3.25**	**3.13**
	α-toc equivalence	**d2**	**pcr-4**	**0.66**	**2.38**	**0.73**	**1.59**	**1.07**	**1.28**

see footnotes in Table 7

Table 9. The calibration and the prediction results for powdered soybean with PLSR/PCR analysis on SS2400 data. [mg (100g DW)-1]

estimation. As for intact plural soybean seeds, some of the respective component such as malonyl daidzin, and malonyl genistin, still could be estimated. On the other hand, the estimation for glycosides, acetyl glycosides, and aglycons were not good enough because of their small range fluctuations.

As for Vitamin B, considering from the SEPs in Table 7-10 with comparing the standard deviation: 0.08 [mg (100g DW)$^{-1}$] for thiamin, and 0.01 for riboflavin. These contents might be fairly estimated.

The feasibility of NIRS for the estimation of the contents of isoflavone in soybean seeds was examined. As for SpectraStar 2400 case, considering from SEP, NIRS may also be available for estimating the total isoflavone content. Even if the whole seeds analysis case, that of SEP was fair enough for their estimations. Further, especially as for powdered soybean, the respective components of the isoflavones, such as glucosides and malonyl glucosides, could be estimated separately. However, the estimations of acetyl glucosides and aglycons contents were not good because of their small range fluctuations. PLSR/PCR analysis were also tried, and the similar results were obtained and some were improved.

		Calibration				Prediction			
		treatment	factors	r	SEE	r	RMSEP	bias	SEP
glycoside	daidzin	d22	pls-11	0.95	4.33	0.63	9.06	0.80	9.43
	glycitin	d22	pls-15	0.99	0.57	0.53	3.20	1.43	2.99
	genistin	d2	pcr-10	0.78	12.34	0.60	12.06	2.96	12.21
	total	d22	pls-11	0.96	10.27	0.60	22.57	2.85	23.38
malonyl glycoside	**malonyl daidzin**	**d22**	**pls-11**	**0.98**	**10.64**	**0.92**	**15.68**	**1.39**	**16.31**
	malonyl glycitin	d22	pcr-7	0.56	4.24	0.41	5.78	1.50	5.83
	malonyl genistin	**d22**	**pls-12**	**0.97**	**14.46**	**0.87**	**21.21**	**-0.99**	**22.13**
	total	**d22**	**pls-11**	**0.97**	**24.95**	**0.91**	**33.75**	**2.48**	**35.15**
acetyl glycoside	acetyl daidzin	d1	pls-3	0.65	0.28	0.52	0.40	0.01	0.42
	acetyl genistin	d1	pls-3	0.57	0.35	0.47	0.43	0.04	0.45
	total	d1	pcr-4	0.58	0.64	0.52	0.80	0.07	0.83
aglycon	daidzein	d1	pcr-1	0.04	0.61	-0.40	0.54	0.30	0.47
	genistein	raw	pcr-1	0.10	0.66	-0.44	0.60	0.27	0.56
	total	raw	pcr-1	0.15	1.21	-0.36	1.15	0.59	1.03
total isoflavone	**total**	**d22**	**pls-11**	**0.97**	**34.70**	**0.88**	**51.34**	**5.73**	**53.28**
Vitamin B	thiamine	d22	pls-1	0.28	0.09	0.29	0.08	0.01	0.08
	riboflavin	d1	pls-1	0.29	0.02	0.07	0.01	0.00	0.01
tocopherol (toc)	α-toc	d1	pcr-1	0.02	2.60	0.11	1.62	-0.24	1.73
	β-toc	d22	pls-1	0.62	0.84	0.68	0.50	0.39	0.34
	γ-toc	d1	pls-1	0.37	2.77	0.61	2.63	0.70	2.74
	δ-toc	raw	pcr-1	0.66	2.42	0.23	3.28	1.75	2.99
	total	raw	pcr-1	0.41	3.59	0.31	5.19	2.80	4.73
	α-toc equivalence	d1	pcr-1	0.08	2.77	0.10	1.70	0.02	1.84

see footnotes in Table 7

Table 10. The calibration and the prediction results for intact plural soybean seeds with PLSR/PCR analysis on SS2400 data.

3.2.3 Data transfer (Sato et al., 2009a)

Table 11 described the calibration and prediction results, that are analyzed using SensoLogic with these NIRS data converted from InfraAlyzer 500 data into those of SpectraStar2400 data. The similar SEP results were obtained as in Table 7. Then, the obtained equations were adopted on those of prediction set of original data of SpectraStar2400. The SEP of the total isoflavone content was 70.82 [mg (100g DW)$^{-1}$] in the MLR analysis of the powder, and it shows that total isoflavone level was able to be estimated in stead of bias and skew. Further, using the original calibration set of SpectraStar2400, the bias and skew correction were carried out and the SEP was drastically improved to 45.72 [mg (100g DW)$^{-1}$]. The similar results were obtained in PLSR analyses, and also in the analysis of plural seeds.

			Calibration		Prediction			
			r	SEC	r	RMSEP	bias	SEP
powder	MLR Analysis	analysis on converted data	0.90	56.91	0.94	43.37	-1.47	45.27
		adopted on SpectraStar2400 data	before bias and skew correction		0.91	68.87	12.05	70.82
			after bias and skew correction		0.91	44.18	5.96	45.72
powder	PLSR Analysis	analysis on converted data	0.86	66.54	0.90	51.75	0.54	54.05
		adopted on SpectraStar2400 data	before bias and skew correction		0.91	83.94	-57.13	64.23
			after bias and skew correction		0.91	46.17	9.67	47.16
plural seeds	MLR Analysis	analysis on converted data	0.90	56.31	0.78	71.37	-2.03	74.52
		adopted on SpectraStar2400 data	before bias and skew correction		0.72	109.95	-68.76	89.61
			after bias and skew correction		0.72	74.70	2.81	77.97
plural seeds	PLSR Analysis	analysis on converted data	0.92	56.17	0.82	73.84	32.11	69.45
		adopted on SpectraStar2400 data	before bias and skew correction		0.88	91.62	66.80	65.50
			after bias and skew correction		0.88	56.23	18.22	55.57

see footnotes in Table 7

Table 11. The results of data transfer trial. The unit is mg (100g DW)$^{-1}$.

3.3 Analysis on Bruker Data

Table 12 described the calibration and the prediction results of the estimation of total isoflavone content with automatic analysis. Figure 5 shows the scattering plots of the prediction results of total isoflavone analysis. As for soybean powder analysis, judging from RMSEP, NIRS may be available for estimating the total isoflavone content. Even if the plural seeds analysis case, RMSEP was also good enough for the nondestructive estimation. Further, the respective components of the isoflavones, such as glycosides and malonyl glycosides, could be estimated separately as for powdered soybean and a part of plural soybean seeds cases (data abbreviated). As for a single seed analysis case, the precision was fair. Table 13 described the calibration and the prediction results of the estimation of Vitamin B and total tocopherol content with automatic analysis. The estimations for the contents of Vitamin B, and tocopherol were fair for rough estimation despite of their small range fluctuations.

	Calibration (n=36)			Prediction (n=12)			
sample type analyzed	rank	r	RMSEE	r	RMSEP	bias	RPD
soybean powder	9	0.974	31.9	0.971	24.9	6.71	3.99
plural soybean seeds	8	0.996	11.8	0.935	36.3	-5.48	2.81
a single seed soybean	8	0.814	77.1	0.886	53.1	1.91	2.16

rank: the number of PLSR/PCR vectors
r: Correlation coefficient between chemical method and NIR method.
RMSEE: Root mean square error of estimation. [mg/100gDW]
RMSEP: Root mean square error of prediction. [mg/100gDW]
RPD: ratio of standard error of prediction to standard deviation.

Table 12. The calibration and the prediction results of the estimation of total isoflavone content with automatic analysis on Bruker data.

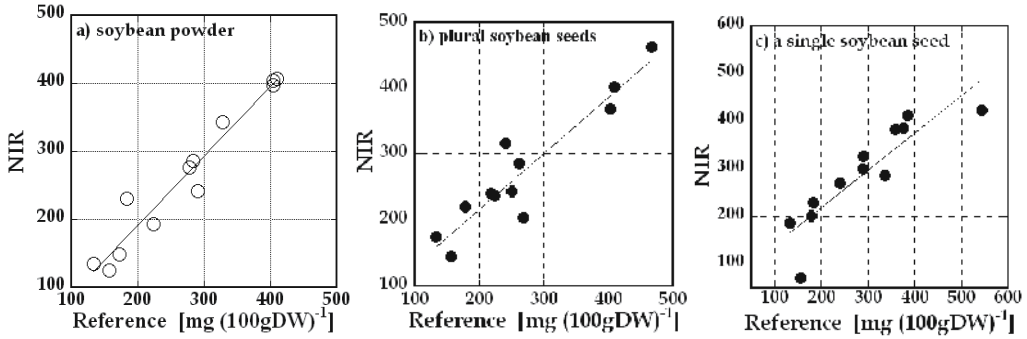

Fig. 5. Estimation of total isoflavone content. a) soybean powder; b) intact plural soybean seeds; c) a single soybean seed on Bruker data.

3.4 Analysis on Buchi Data

Table 14 described the calibration and the prediction results of the estimation of total isoflavone, Vitamin B and total tocopherol content with automatic analysis. As for soybean powder analysis, judging from SEP, NIRS may be available for estimating the total isoflavone content. Even if the plural seeds analysis case, SEP was also good enough for the nondestructive estimation. Further, the respective components of the isoflavones, such as glucosides and malonyl glucosides, could be estimated separately as for powdered soybean and a part of plural soybean seeds cases (data abbreviated). As for a single seed analysis case, the precision was fair. The estimations for the contents of Vitamin B, and tocopherol were fair for rough estimation despite their small range fluctuations.

4. Conclusions

The feasibility of some types of the near infrared spectroscopy (NIRS) for the estimations of the contents of isoflavones, thiamine (Vitamin B_1), riboflavin (Vitamin B_2), and tocopherol (Vitamin E) in soybean seeds was examined with MLR and PLSR/PCR analysis. Considering from the standard error of prediction, NIRS may be available for estimating the total isoflavone content not only as for powdered soybean but also as for intact plural soybean seeds. Vitamin B and tocopherol contents were fair enough for rough estimation

despite of their small range fluctuations. Trials of a single seed analysis, and the data transfer were also carried out.

The authors already reported the NIRS analysis of major constituents and the deterioration indices in the soybean (Sato et al. 1994), and the fatty acid composition in soybean (Sato et al. 2002). There were some trials to estimate amino acids composition in soy by NIRS method (Kovalenko et al., 2006; Pazdernik et al., 1997). In this study, some of physiologically functional elements can be estimated by NIRS method. The present findings showed that the isoflavone content could be estimated by the NIRS method, and the NIR method increase the value in soybean analysis. The NIRS method will gain greater position in the soybean analysis by these results.

sample type analyzed	constituents	Calibration (n=36 or 16)			Prediction (n=12 or 7)			
		rank	r	RMSEC	r	RMSEP	bias	RPD
soybean powder	thiamine(VB_1)	5	0.457	0.1	0.865	0.03	-0.01	1.94
	riboflavin(VB_2)	9	0.874	0.01	0.762	0.01	0.01	1.43
	total tocopherol	6	0.987	0.95	0.954	1.1	-0.02	3.07
plural soybean seeds	thiamine(VB_1)	9	0.853	0.05	0.826	0.07	-0.01	1.69
	riboflavin(VB_2)	7	0.812	0.01	0.86	0.01	0	1.85
	total tocopherol	7	0.972	1.15	0.8	3.12	-0.43	1.64
a single seed soybean	thiamine(VB_1)	4	0.371	0.09	0.741	0.06	-0.04	1.49
	riboflavin(VB_2)	1	0.228	0.01	0.537	0.02	0.01	1.11
	total tocopherol	5	0.727	3.83	0.978	1.03	-0.61	4.45

see footnotes in Table 12.

n: counts of samples (n = 36 for isoflavone, and Vitamin B, or 16 for tocopherol) and so on .

Table 13. The calibration and the prediction results of the estimation of Vitamin B and tocopherol with automatic analysis on Bruker data.

sample type analyzed	constituents	Calibration (n = 36 or 16)		Prediction (n = 12 or 7)		
		r	SEC	r	SEP	bias
soybean powder	total isoflavone	0.913	48.35	0.912	48.35	4.54
	thiamine(VB_1)	0.751	0.06	0.747	0.08	0.02
	riboflavin(VB_2)	0.738	0.01	0.758	0.02	0.00
	total tocopherol	0.803	2.67	0.804	2.82	1.02
plural soybean seeds	total isoflavone	0.941	40.13	0.934	40.61	20.83
	thiamine(VB_1)	0.860	0.04	0.873	0.06	0.02
	riboflavin(VB_2)	0.777	0.01	0.813	0.02	0.01
	total tocopherol	0.610	3.87	0.759	3.31	2.40

Table 14. The calibration and the prediction results of the estimation of total isoflavone, Vitamin B and tocopherol content with automatic analysis on Buchi data.

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17

Isoflavone Content and Composition in Soybean

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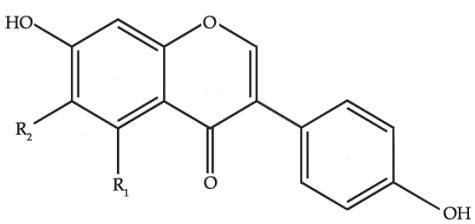
1. Introduction

Soybean seed has been used in a diet of East-Asian population for centuries. Western population has raised interest in this item after numerous epidemiological and clinical studies have showed that, due to the large consumption of soybean, there is less incidence of cardio-vascular disease, osteoporosis, and certain types of cancer in Japan and China, in comparison to western countries (Scheiber et al., 2001; Chiechi et al., 2002; Potter et al., 1998; Barnes et al, 1994; Adlercreutz & Mazur 1997; Sarkar & Li, 2003; Lee et al., 2003b; Moriguchi et al., 2004; Ikeda et al., 2006; Wu et al., 2008).
Several classes of biologically active compounds are found in soybean, but it is considered that isoflavones are most responsible for its favourable influence on health (Crouse et al., 1999). Isoflavones are natural occurring substances, present in some plants, which are structurally similar to estrogens and can exibit weak estrogen-like effects. For this reason, they are classified as phytoestrogens: plant-derived compounds with activity similar to estrogens. Isoflavones are not widespread in nature and can be found almost exclusively in the plants of the *Leguminosae* family (Anderson & Wolf, 1995; Philips et al., 2002; Romani et al., 2003). In that sence soybean is one of few isoflavone sources in human nutrition, which explains its wide use through different food products and isoflavone-rich dietary supplements.

1.1 Chemical structures of isoflavones

Isoflavones are polyphenolic compounds which exist in twelve differents chemical forms (Lee et al., 2004). Daidzein, glycitein and genistein are the aglycone forms of isoflavones (Fig.1). In conjuction with sugars, they build the β-glucosides (daidzin, glycitin



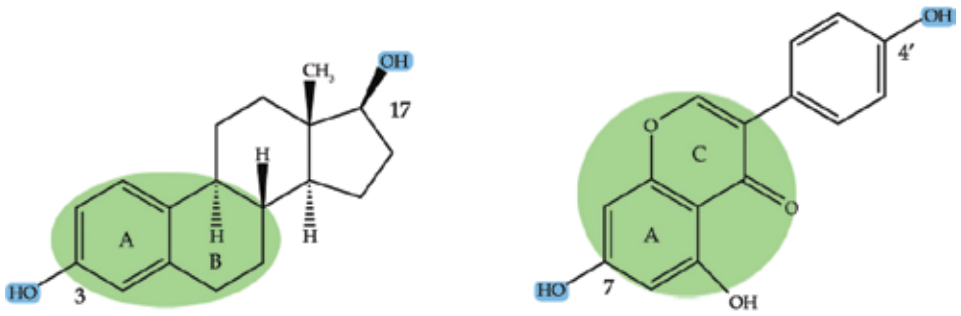
Name	R1	R2
Daidzein	-H	-H
Glycitein	-H	$-OCH_3$
Genistein	-OH	-H

Fig. 1. Chemical structures of aglycone forms of isoflavones

and genistin), the 6''-O-malonyl glucosides (malonyl daidzin, malonyl glycitin, and malonyl genistin) and the 6''-O-acetyl glucosides (acetyl daidzin, acetyl glycitin, and acetyl genistin). The aglycone structures can be found in very small amounts in soybean, while the glycoside forms are dominant. However, isoflavones in glycoside forms are inactive, because hydrolysis and the release of the aglycone component are essential for the absorption of isoflavones in the digestive tract (Day et al., 1998). For this reason aglycones are considered to be biologically active forms of isoflavones.

1.2 Biological activity of isoflavones

Isoflavones perform most of their biological effects through modulation of estrogenic receptors (ER), as a result of their structural similarity with human estrogens (Cederroth & Nef, 2009). By comparing the chemical structures of genistein and estradiol (Fig.2) it can be noticed that the genistein rings A and C are similar to the estradiol rings A and B and the distance between hydroxyl groups is almost identical in both molecules.



Estradiol Genistein

Fig. 2. Structures of estradiol and genistein

The isoflavone affinity towards estrogenic receptors results in numerous effects on estrogen-regulated systems, including cardiovascular, metabolic, reproductive, skeleton, and central nervous system. A significant characteristic of isoflavones is their capacity to bond to both subtypes of estrogenic receptors (ERα and ERβ), but mainly to β receptors. Such specific affinity towards estrogenic receptors allows them to perform estrogenic and anti-estrogenic effects, depending on the type of the tissue and the endogenous estrogen levels (Kupier et al., 1997; Kupier et al., 1998). Tissue-selective activity of isoflavones is important because anti-estrogenic effects in reproductive tissue can decrease the risk of hormone-dependent cancers (breast, uterus, and prostate cancer), while estrogenic effects in other tissues can impact preventively towards osteoporosis and hypercholesterolemia. In comparison with physiological estrogens, isoflavones are very weak estrogens which, on the molar basis, have from 10^{-2} to 10^{-4} activities of 17β-estradiol (Biggers & Curnow, 1954; Bickoff, 1962). Genistein has ten times higher estrogenic activity comparing to daidzein (Branham et al., 2002; Diel et al., 2000; Diel et al., 2004), whereas glycitein has the highest estrogenic potential in vivo (Song et al., 1999). Genistein and glycitein can be biodegraded into metabolites with no estrogenic activity (Cassidy et al., 2000; Simons et al., 2005), while daidzein can be metabolised into equol, which has higher estrogenic potential than daidzein (Setchell et al., 2002).

Soy isoflavones and their metabolites can also exhibit the biological activity independent from their interactions with estrogenic receptors (Barnes et al., 2000). They inhibit the synthesis and activity of the particular enzymes included in estrogenic metabolism, as well as the activity of tyrosine kinase (Whitehead et al., 2002; Akiyama et al., 1987). Besides, isoflavones can act as antioxidants (Ruiz-Larrea et al., 1997; Wiseman et al., 2000; Djuric et al., 2001, Malencic et al., 2008).

1.3 Dietary supplements on the basis of soyben isoflavones

Soy isoflavones, among other phytoestrogens, are used as the alternative to estrogen hormone replacement therapy in menopause. Concerns about potential side effects of hormone therapy have resulted in the increased interest for the usage of soy-based dietary supplements (Nelson et al., 2006). However, the efficiency of these supplements can vary significantly as a result of uneven quality of the preparations, differences in populations and individual differences of patients. For example, the isoflavone content in soy-based dietary supplements can vary among producers, and even among series of the same producer (Cesar et al., 2006).

Labels of soy-based dietary supplements mainly inform about the total isoflavone content, while the information on the individual isoflavone contents is not provided. Nevertheless, biological potential of preparations with the same total isoflavone content can be different due to the variations of isoflavone composition in primary raw material (soybean seed) (Ceran et al., 2007; Tepavcevic et al., 2009). Thus, it is exceptionally important to determine individual isoflavone contents in the soybean seed. Exploring of the factors which can influence the isoflavone content and composition in soybean enables the selection of the raw material which potentially has the most beneficial effect on health.

1.4 Factors that influence the isoflavone content and composition in soybean seed

The total isoflavone content in soybean seeds ranges from 0.05% to 0.50% of dry material. Distribution of individual isoflavones within these values can vary significantly, although it is known that daidzein with its conjugates and genistein with its conjugates are present in nearly equal amounts in soybeans, whereas glycitein and its conjugates are present in lesser amounts (Lee et al., 2004). Variations in content and composition of the soybean isoflavones occur as a consequence of different factors, among which the most examined are the genotype of the seed, as well as the year and location of seeding.

1.4.1 Genotype

According to the previous reports, genotype significantly influences the content and composition of isoflavones in soybean seed (Wang & Murphy, 1994; Hoeck et al., 2000; Lee et al., 2003c). Thus, the determination of isoflavone profiles in different soybean varieties has become the subject of numerous studies, with the aim of selecting genotypes that have better healthpromoting characteristics. Majority of these reports come from the countries which are the largest exporters of soybean at the world market, such as the USA, Brazil, Argentina, China, and India.

American soybean varieties have high total isoflavone levels, with genistein and its conjugates as predominant isoflavone forms. Xu and Chang (2008) reported that the total isoflavone content in thirty soybean genotypes (from the North Dakota – Minnesota region) ranged between 1.18 and 2.86 mg/g of the seed, out of which 69% were genistein and its

conjugates. Another study, conducted on eight American soybean genotypes, showed that the content of total isoflavones varied from 2.05 to 4.22 mg/g of the seed, with malonyl genistin as the most abundant component, comprising 42% of the total isoflavone content, in the average (Wang & Murphy, 1994). Nine American genotypes form Virginia (Chung et al., 2008) had the total isoflavone content ranging from 2.50 to 3.20 mg/g of the seed, out of witch 75-84% was malonyl genistin. The study on fifteen Korean soybean varietes showed, however, that the total isoflavone contents in these genotypes were significantly higher than in the seeds from other countries, ranging from 1.88 to 9.49 mg/g of the seed (Lee et al., 2003a). Analysis of individual isoflavones in soybeans from China and Korea demonstrated that Chinese genotype contained higher isoflavone amounts than Korean genotype (Lee et al., 2007). Brazilian soybean genotypes had considerably lower isoflavone concentrations in comparison with American and Korean genotypes, according to the study by Genovese and collaborators (2005). They reported that total isoflavone content in thirteen Brazilian genotypes ranged between 0.57 and 1.88 mg/g of the seed. Low content of total isoflavones in Brazilian genotypes were found by Ribeiro and collaborators (2007), as well. In eighteen different soybean genotypes, they found the total isoflavone levels ranging from 0.9 to 1.21 mg/g of the seed. Genotype from Ecuador had 0.68 mg of total isoflavones per g of the seed, which is in the range of Brazilian genotypes, but far below isoflavone content in American and Korean genotypes (Genovese et al., 2006). The average total isoflavone content in Indian seeds, according to the study by Devi and collaborators (2009), was 0.76 mg/g of the seed.
The wide range of total isoflavone values, from 0.68 mg/g in Ecuador seed up to 9.49 mg/g in Korean seed are due to the significant differences in explored soybean genotypes, but also may reflect the diverse conditions of seeding, as well as the variations in methods of isoflavone determination between the studies.

1.4.2 The year and location of seeding

The year and location of soybean seeding can considerably influence the isoflavone content in the seed, while their influence on the isoflavone composition is less distinctive. According to Wang and Murphy (1994), the year of seeding substantially influenced the total and individual isoflavone contents, but not the isoflavone distribution in soybeans. In the same study, the seeding location did not have a significant influence either on the total and individual isoflavone contents, or their distribution. The study by Hoeck and collaborators (2000), however, showed that the location of seeding, beside the genotype of seed and the year of seeding, can impact the isoflavone contents in soybean. Lee and collaborators (2003a) concluded that the seeding year affects isoflavone contents more than the location or genotype. They reported that the range of total isoflavone contents in fifteen soybean varieties varied from 2.20 to 6.45 mg/g in 1998, from 3.19 to 9.50 mg/g in 1999 and from 2.93 to 4.83 mg/g in 2000. Tsukamoto and collaborators (1995) suggested that part of the effects attributed to years and locations in research studies may reflect differences in the temperatures that occur during seed development, as a result of the date of planting. Hence, it can be concluded that the diversity of isoflavone contents depend on unknown climatic and environmental factors and genetic variation.

2. Our works

Throughout our research, we have analysed the content and composition of isoflavone in domestic and introduced soybean genotypes grown in Vojvodina, the northern region of

Serbia. Previous reports about isoflavone content in soybean seeds grown in Vojvodina have not been found, as this region (belonging to Central Europe) is not generally accepted for ecological surroundings of soy. Our research (Cvejic et al., 2009; Tepavcevic et al., 2010) involved sixty different genotypes of soybean grown on the experimental fields of the Institute of Field and Vegetable Crops in Rimski Šančevi, Novi Sad, during 2004, 2005, and 2006 (Table 1).

2004	2005	2006
1. LN92-7369	21. Alisa x Lori	41. Černovitska 9
2. 1581/99	22. Alisa x Linda	42. Veselovska
3. 1511	23. Alisa x Meli	43. Amurska
4. 1499/99	24. Alisa x Tara	44. Čerivnica stepu
5. Lori	25. Alisa x 1499	45. Mandzurska
6. Linda	26. Alisa x BL-8	46. Venera
7. Balkan	27. Alisa x Sava	47. Valjevka
8. BL-8	28. Alisa x Venera	48. Ana
9. Alisa	29. Alisa x Morava	49. NS-L-410015
10. Tara	30. Alisa x Balkan	50. Galina
11. Meli	31. Balkan x Sava	51. Sargent
12. Sava	32. Balkan x Venera	52. MN 1801
13. Venera	33. 1499 x Sava	53. Ne1900
14. Morava	34. Sava x Venera	54. Barnes
15. LN92-1581 x 1581/99	35. Venera x Morava	55. MN 0901
16. 1499/99 x 1581/99	36. (LN92 x 1581) x (1499 x 1518)	56. Chang Nong 5
17. 1499/99 x 151	37. (1499 x 1511) x (Lori x LN92)	57. JJ 96021
18. Lori x LN92-158	38. (Linda x LN92) x (Balkan x BL-8)	58. Ba 28
19. Linda x LN92-158	39. Balkan x (Balkan x BL-8)	59. NM 97002/1
20. Balkan x BL-8	40. BL-8 x (Balkan x BL-8)	60. Jilin Provinca

Table 1. Examined soybean genotypes

The sample from 2004 included twenty genotypes, among which fourteen were the already cultivated varieties, and the remaining six were their F1 hybrids. Seeds from 2005 (twenty different genotypes) represented F1 hybrids of the genotypes from 2004. Within the sample from 2006, twenty genotypes of different origin (Russia, the USA, China and Serbia) were analysed. The presented sample enabled analysis of the influence of particular factors on isoflavone content in soybean, such as hybridisation, seeding year and the origin of seed. The obtained results were also important for comparison with the isoflavone content and composition of soybeans grown in other regions.

The analysis of soybeans grown in 2004 suggested that the total isoflavone values of all the twenty genotypes were considerably higher than some reported earlier (Cvejic et al., 2009). The total phytoestrogen concentration was found to be between 2.24 and 3.79 mg/g dry bean weight. The average amount of daidzein and its conjugates in the analysed cultivars was the highest (45.6%), followed by genisteins (39.3%), while glyciteins were present in the least amount in all analysed samples (15.0%). Low percentage of genistein components in the analysed genotypes, in comparison with the genotypes examined in the USA and Korea (Wang & Murphy, 1994; Hoeck et al., Lee et al, 2003c) reflects the potentially less biological

quality of genotypes grown in this region. However, the analysis of Cvejic and collaborators showed that, considering isoflavone content and distribution, some specific genotypes could be distinguished. There was a group of genotypes, which exhibited a significantly higher content of isoflavones, in comparison to others. A group of genotypes with higher content of genisteins, biologically the most active phytoestrogens in soy, could also be clearly distinguished. The results suggested that analysed genotypes, especially 1581/99, BL-8 and Meli might be of interest to producers, plant breeders and phytopharmacists due to their elevated content of phytoestrogens.

Hybrid genotypes assayed in 2005 had concentration of total isoflavones from 1.56 to 3.66 mg/g of dried bean (unpublished results). Daidzein and its conjugates were the most abundant isoflavone forms (50.8%), followed by genisteins (39.5%), while glyciteins were less abundant (9.7%). The evaluation of the resemblance between hybrids and their parents was conducted according to the samples 21-30 (Table 1). These hybrids had one mutual parent (Alisa), while they differed in the other parent. There was a high correlation (r=0.9) between parents and corresponding hybrids, considering the values of their total isoflavone content (Fig. 3), content of daidzeins and content of genisteins. These results reflected the feasibility of breeding soybean genotypes with favourable characteristics and better healthpromoting potential.

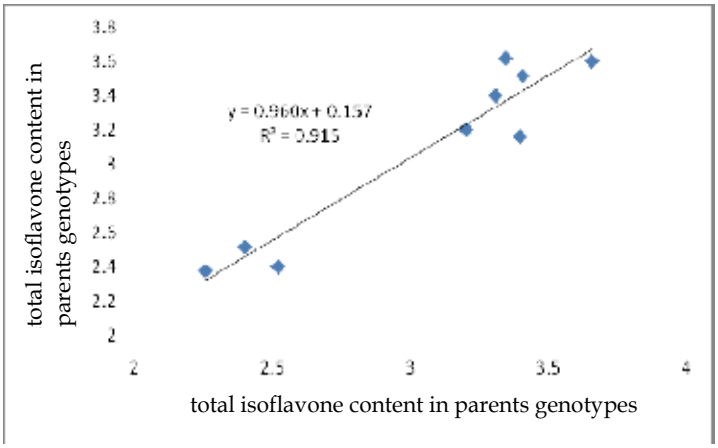


Fig. 3. Correlation of total isoflavone content between parent and hybrid genotypes (unpublished results)

The values of total isoflavone content in the samples from 2006 were between 1.45 mg/g and 4.59 mg/g of dry material (Tepavcevic et al, 2010). The amount of daidzein and its conjugates was the highest (47.2%), followed by genisteins (40.2%) while glyciteins were present in the lowest (12.6%) amount, considering the average of twenty analysed soybean varieties. There was a statistically significant difference among the examined genotypes in the contents of total isoflavones, daidzeins, glyciteins and genisteins ($p<0.05$). On the other side, the groups of genotypes formed on the basis of seed origin (American, Russian, Serbian and Chinese) did not differ significantly. The results suggest that isoflavone composition is a characteristic mainly determined by the genotype and not by the origin of soybean seeds.

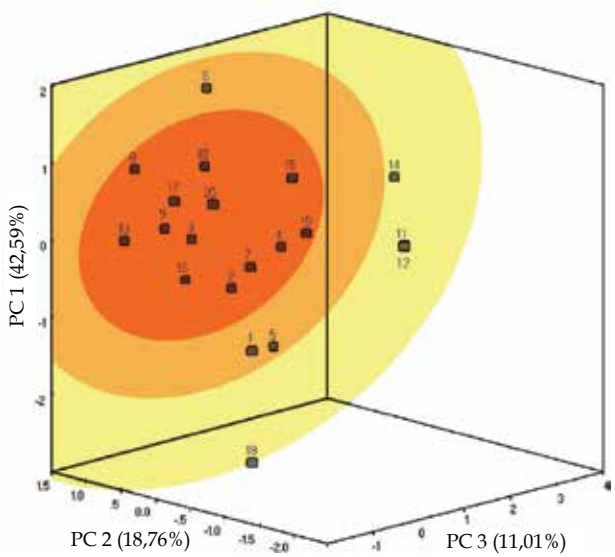


Fig. 4. Variance of cultivars explained by Principle Component Analysis (Tepavcevic et al., 2010).

In the research by Tepavcevic and collaborators (2010), principal component analysis (PCA), as statistical tool, was applied for the interpretation of the results about isoflavone content and composition in soybeans. To the best of author's knowledge, PCA was used in this purpose for the first time. Method of PCA enabled more comprehensive observation of the isoflavone profiles, simultaniously in twenty different genotypes of soy. The data about the content of twelve different isoflavones in the examined soy samples were abstarcted into three principal components which described 72.26% of total variance of the original data. These three principal components (PC1, PC2, and PC3) have been represented as coordinates of a three dimezional graph within which the samples are allocated according to their features, Fig 4. The resembling samples are grouped together, whereas the samples that are distinct are abstracted from the others in graph. For example, it is noticeable that genotypes 11, 12, 14, and 18 are clearly abstracted from others (numbers 1-20 in Fig.4 correspond to samples 41-60 in Table 1). This distinction is a result of dissimilarity in content and composition of isoflavones in these cultivars, comparing to the others: sample 18 (Ba-28) had a very low content of all isoflavones, and samples 11 (Sargent) and 14 (Barnes) had much higher content of malonyl genistin in relation to the content of malonyl daidzin.
The use of Principal Component Analysis in the research by Tepavcevic and collaborators (2010) enabled selection of genotypes with favourable characteristics, from the sample that contained twenty soybean varietes of different origin. Hence, the same method was applied within this chapter on a larger sample size, which included data on isoflavone contents in sixty previously analysed soybean genotypes (Table 1), with the aim to draw conclusions about factors that influence isoflavone distribution in soybeans.

3. Methods

Principal Component Analysis (PCA) is a mathematical procedure which is used to decrease the number of variables (features of the analysed objects), i.e. to decrease the size of data

matrix X (Brerton, 2003). In chemometrics of medical chemistry it is usual that rows of a matrix represent objects (in this case different genotypes of soy), while columns of the X matrix represent variables (concentartions of twelve different isoflavones). The aim is to group the observed objects by the similarity of variables of the X matrix, or to group the observed variables by the similarity of objects of the X matrix, and to visualise the mutual similarity of objects or variables. The PCA method gives eigenvectors, which are the vectors of principal components, PC (loadings). Loading vectors of principal components have the dimension that corresponds to the total number of variables of the X matrix, so these vectors determine orientation of the principal component axis in the original space of variables. If the coordinates of the principal components loadings are showed in 2D (first two loadings) or 3D (first three loadings) space, the information about the direction and the strength of mutual correlation between variables can be obtained easily according to the mutual position of variables. Eigenvectors (vectors of principal components, PC) are ordered according to the falling eigenvalues - a part of variation that they explain in the X matrix. The principal component PC1 explains most of the variation, while PC2 explains most of the variation which has not been explained by PC1. The principal component PC3 explains most of the total variation of the X matrix which has not been explained by PC1 and PC2 (Szepesvári, 2001; Posa, 2010). However, usually the first three principal components explain most of the variation in the X matrix, so other principal components are not used. According to Wold (1975) principal components with small eigenvalues explain the error of measurement. The product of the data matrix X and the matrix of loading vectors of principal components gives a score matrix of principal components (columns of this matrix are the vectors of the principal components values). In the plane (PC1 and PC2) or in the space (PC1, PC2, and PC3) scores of the principal components are visualised objects (Atanackovic et al., 2009). Similar objects form clusters (groups); the more resembling some objects are the closer they stand in the graph.

4. Results and discussion

The Principal Components Analysis method, using cross validation procedure according to Krsanowski, has been applied to the data matrix (Table 2) which is standardised to the unit variance of variables (objects: soybean seeds – 60, variables: isoflavones – 12). Screen test shows that the first two components (PC) are sufficient for modelling the twelve variables, because they explain 68.26% of the total variance of the original data, Fig.5.

In the plane of PC1 and PC2 loading vectors, the coefficients of the principal components are presented, Fig. 6. Variables mDI, aDI, mGI, GI, GYI, DI, GY, mGY are mostly present in the principal component PC1 (the coefficient of their correlation with PC1 is higher than 0.5). Concerning the fact that PC1 carries 50.83% of information about the variance of individual isoflavone contents, the above mentioned isoflavones have a crucial role in the defining of isoflavone profile in soybean. On the other hand, variables DE and GE are mostly present in component PC2, whereas variables aGI and aGYI are equally present in both principal components, which reflects minor importance of these isoflavone forms in determination of the isoflavone profile.

In the PC1-PC2 plane (Fig. 6) there are five groups of variables which are significantly correlated in the same direction: (GE, DE), (aGI, aGYI), (mDI, aDI, mGI), (GI, GYI) and (DI, GY, mGY). Their vectors mutually close an angle whose cosinus is close to 1, providing the ability for each variable within the brackets to be represented by the other from

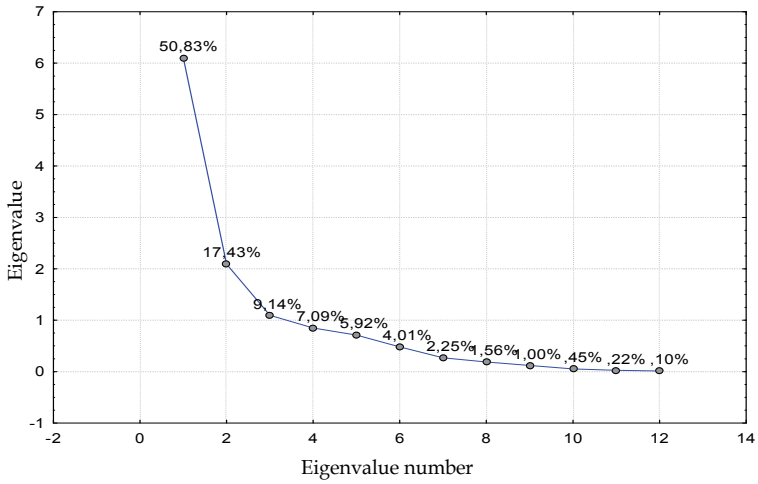


Fig. 5. Screen plot

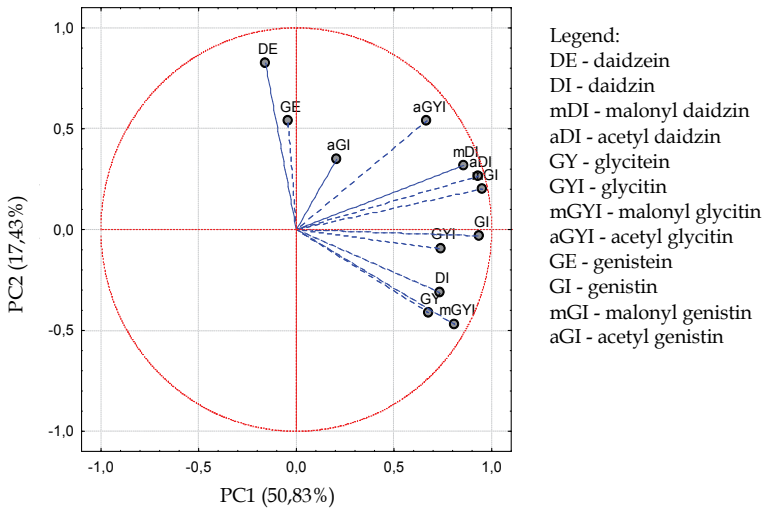


Fig. 6. Loadings of principal componets

the same group. Variables from the groups (GE, DE) and (mDI, aDI, mGI) are mutually orthogonal; they do not share common information (cosinus of the angle between their vectors is 0), whereas variables from the groups (GE, DE) and (DI, GY, mGY) are correlated in opposite directions (angle between their vectors is bigger than 90°).

Observing the variables that are most present in pricipal component PC1 and their mutual correlations (Fig. 6), some distinctive parameters of soybean genotypes may be defined. Wang and Murphy (1994) had suggested that, ratios of malonyl daidzin to daidzin and malonyl genistin to genistin in soybeans may be the characteristic of different genotypes, but in the study by Xu and Chang (2008) it was found that these ratios are not the same in one genotype across different locations. According to the results obtained in this study, ratios of malonyl daidzin to malonyl genistin, genistin to gliticin and malonyl daizdin to acetyl daidzin could be specific characteristics of a particular genotype.

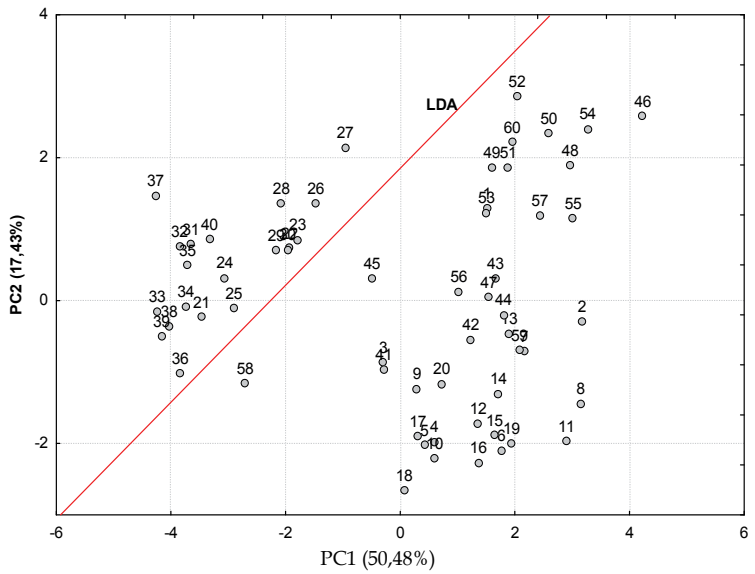


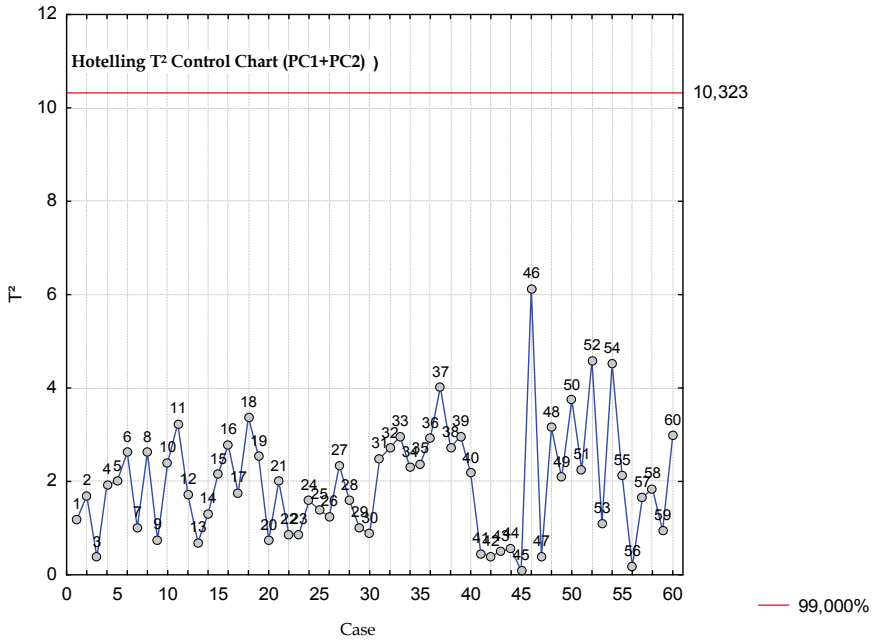
Fig. 7. Principle component scores in the PC1 – PC2 plane

In the score plane of the principal components PC1-PC2, sixty different soybean genotypes have been analysed (Fig. 7). The linear discriminative analysis (LDA) has been applied in vectors space of the principal components and a straight line of the linear discriminant function (red borderline in the graph) has been obtained. This borderline divides the examined sample into two groups: group I (1-20 + 41-60) and group II (21-40). Group II (Fig. 7) contains twenty hybrid soy genotypes grown during 2005, while group I is consisted of forty soybean genotypes, grown during 2004 and 2006. In comparison with the samples from the group I, samples from the group II have considerably lower content of total isoflavones, content of daidzein with its conjugates, content of glycitein with its conjugates and content of genistein with its conjugates. Despite the resemblance in isoflavone composition between parent and hybrid genotypes grown in 2004 and 2005 (Fig. 3), there is a significant ($p<0.05$) difference in the total and individual isoflavone contents between them ($p<0.05$, except for acetyl glycitin, genistein and acetyl genistin).

The importance of applied PC1-PC2 model has been established by Hotelling T^2 statistics for finding of strong outliers among the analysed objects and D-to-Model diagnostics for finding of moderate outliers. In Fig. 8 (A) it is noticeable that strong outliers do not exist among the examined samples. The samples 37, 50 and 52 are distinguished as moderate outliers, Fig. 8 (B). This confirms that PC1-PC2 model fits well the examined samples, and that the division into groups is the result of differences in the individual isoflavone contents among different genotypes.

Obtained results point out the relevance of the seeding year, as factor that influence isoflavone content in soybeans, which is in the agreement with the previous reports (Wang & Murphy, 1994; Hoeck et al., 2000; Lee et al., 2003c). On the other hand, the resemblance of isoflavone composition between parents and corresponding hybrids (Fig. 3) determines the crucial influence of genotype in the formation of isoflavone profile in soy.

(A)



(B)

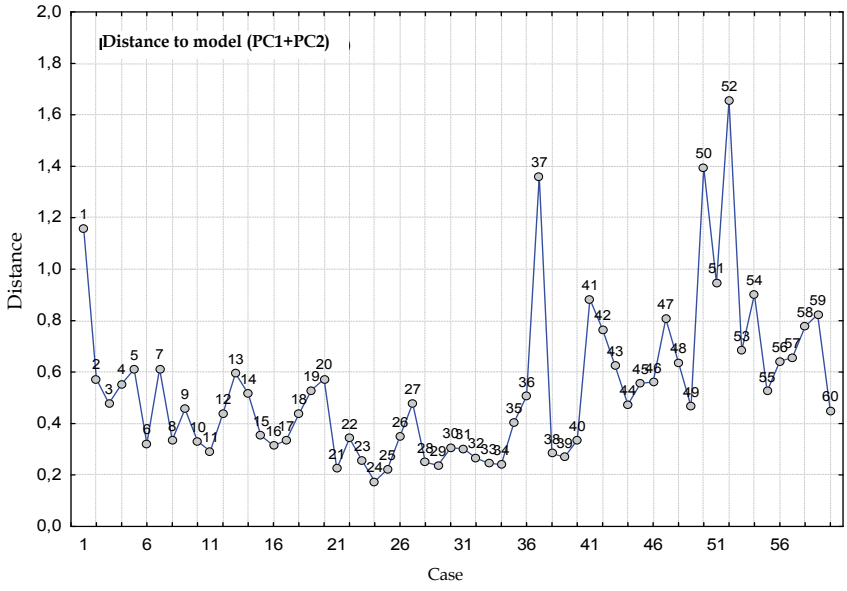


Fig. 8. (A) Hotelling T² statistics for the model PC1 – PC2 finding strong outliers among the objects). (B) D-to-Model diagnostics of moderate outliers.

No	DE	DI	m-DI	a-DI	GY	GYI	m-GYI	a-GYI	GE	GI	m-GI	a-GI
1.	0.02	0.58	1.76	0.27	0.03	0.15	0.47	0.07	0.07	0.47	1.82	0.01
2.	0.02	0.69	2.00	0.37	0.04	0.15	0.54	0.09	0.02	0.66	2.32	0.00
3.	0.02	0.45	1.16	0.21	0.03	0.13	0.42	0.07	0.01	0.35	1.18	0.00
4.	0.00	0.42	1.13	0.24	0.03	0.20	0.62	0.07	0.01	0.36	1.29	0.00
5.	0.00	0.45	1.17	0.21	0.02	0.29	0.57	0.06	0.01	0.35	1.18	0.00
6.	0.00	0.71	1.91	0.25	0.03	0.19	0.59	0.07	0.00	0.48	1.55	0.00
7.	0.02	0.88	2.20	0.27	0.02	0.15	0.61	0.09	0.01	0.53	1.62	0.00
8.	0.00	0.77	1.78	0.35	0.03	0.20	0.57	0.09	0.01	0.72	2.02	0.00
9.	0.00	0.53	1.15	0.23	0.02	0.13	0.38	0.08	0.01	0.50	1.47	0.00
10.	0.00	0.53	1.21	0.22	0.02	0.23	0.58	0.06	0.00	0.44	1.26	0.00
11.	0.00	0.82	1.83	0.31	0.03	0.19	0.65	0.07	0.01	0.69	1.96	0.00
12.	0.01	0.83	1.68	0.24	0.03	0.15	0.51	0.06	0.01	0.55	1.41	0.00
13.	0.03	0.85	1.81	0.28	0.02	0.15	0.50	0.07	0.01	0.71	1.69	0.00
14.	0.02	0.87	1.78	0.27	0.03	0.16	0.52	0.06	0.01	0.59	1.47	0.00
15.	0.01	0.76	1.55	0.28	0.03	0.21	0.50	0.04	0.01	0.63	1.59	0.00
16.	0.00	0.71	1.39	0.27	0.02	0.22	0.57	0.04	0.01	0.63	1.44	0.00
17.	0.00	0.62	1.13	0.22	0.02	0.15	0.42	0.05	0.01	0.61	1.29	0.00
18.	0.00	0.63	1.14	0.19	0.03	0.21	0.42	0.02	0.01	0.52	1.12	0.00
19.	0.01	0.91	1.68	0.26	0.03	0.21	0.47	0.04	0.01	0.71	1.58	0.00
20.	0.02	0.80	1.26	0.23	0.02	0.16	0.36	0.05	0.01	0.71	1.32	0.00
21.	0.02	0.21	0.78	0.13	0.00	0.09	0.14	0.04	0.01	0.12	0.72	0.00
22.	0.02	0.35	1.10	0.22	0.00	0.10	0.15	0.07	0.01	0.23	1.15	0.00
23.	0.03	0.35	0.93	0.23	0.00	0.11	0.16	0.07	0.02	0.31	1.13	0.00
24.	0.03	0.24	0.70	0.13	0.00	0.12	0.18	0.05	0.02	0.18	0.76	0.00
25.	0.02	0.30	0.74	0.16	0.00	0.09	0.12	0.05	0.01	0.24	0.79	0.00
26.	0.03	0.37	1.07	0.21	0.00	0.11	0.14	0.09	0.03	0.33	1.27	0.00
27.	0.05	0.52	1.37	0.24	0.00	0.11	0.14	0.09	0.03	0.37	1.27	0.00
28.	0.04	0.39	1.09	0.19	0.00	0.11	0.14	0.07	0.03	0.27	0.97	0.00
29.	0.03	0.40	1.06	0.16	0.00	0.11	0.14	0.07	0.02	0.27	0.94	0.00
30.	0.03	0.46	1.15	0.18	0.00	0.09	0.15	0.07	0.02	0.29	0.96	0.00
31.	0.04	0.29	0.85	0.12	0.00	0.05	0.08	0.04	0.02	0.17	0.68	0.00
32.	0.04	0.25	0.78	0.11	0.00	0.06	0.08	0.04	0.02	0.15	0.58	0.00
33.	0.03	0.22	0.56	0.05	0.00	0.07	0.10	0.03	0.01	0.15	0.54	0.00

34.	0.03	0.29	0.77	0.10	0.00	0.07	0.11	0.03	0.01	0.17	0.60	0.00
35.	0.03	0.23	0.60	0.09	0.00	0.06	0.08	0.07	0.01	0.16	0.54	0.00
36.	0.00	0.19	0.73	0.11	0.00	0.06	0.07	0.03	0.01	0.14	0.69	0.00
37.	0.02	0.17	0.55	0.10	0.00	0.07	0.10	0.03	0.01	0.14	0.36	0.00
38.	0.02	0.24	0.72	0.11	0.00	0.08	0.09	0.03	0.01	0.01	0.63	0.00
39.	0.02	0.24	0.59	0.10	0.00	0.05	0.06	0.02	0.01	0.17	0.56	0.00
40.	0.04	0.34	0.69	0.12	0.00	0.08	0.07	0.04	0.03	0.28	0.72	0.00
41.	0.02	0.30	1.37	0.22	0.01	0.34	0.39	0.03	0.01	0.39	1.16	0.00
42.	0.00	0.59	1.36	0.32	0.00	0.20	0.29	0.09	0.00	0.63	1.94	0.00
43.	0.02	0.45	2.48	0.34	0.00	0.23	0.47	0.07	0.01	0.55	1.90	0.00
44.	0.02	0.46	2.20	0.33	0.03	0.24	0.36	0.07	0.01	0.51	1.82	0.00
45.	0.02	0.23	1.20	0.27	0.00	0.19	0.36	0.08	0.01	0.36	1.48	0.00
46.	0.05	0.63	3.32	0.45	0.02	0.24	0.44	0.12	0.03	0.71	2.49	0.00
47.	0.00	0.39	1.78	0.33	0.00	0.21	0.40	0.11	0.01	0.59	1.92	0.00
48.	0.04	0.66	2.90	0.36	0.00	0.25	0.41	0.11	0.02	0.68	2.16	0.00
49.	0.04	0.59	2.23	0.32	0.00	0.20	0.33	0.10	0.03	0.64	1.82	0.00
50.	0.02	0.58	3.21	0.37	0.02	0.21	0.38	0.08	0.11	0.51	2.07	0.00
51.	0.02	0.32	1.42	0.39	0.02	0.18	0.25	0.11	0.02	0.65	2.06	0.01
52.	0.04	0.46	1.79	0.32	0.03	0.23	0.27	0.09	0.03	0.59	1.68	0.02
53.	0.04	0.68	2.54	0.28	0.00	0.26	0.33	0.08	0.02	0.57	1.56	0.00
54.	0.03	0.41	1.85	0.47	0.03	0.19	0.27	0.12	0.02	0.72	2.42	0.01
55.	0.03	0.50	2.09	0.41	0.02	0.21	0.33	0.11	0.02	0.74	2.19	0.00
56.	0.02	0.44	2.17	0.28	0.00	0.27	0.47	0.08	0.01	0.43	1.51	0.00
57.	0.03	0.50	2.55	0.36	0.00	0.30	0.43	0.09	0.02	0.61	2.06	0.00
58.	0.02	0.23	0.76	0.14	0.00	0.25	0.25	0.00	0.01	0.25	0.73	0.00
59.	0.02	0.28	1.67	0.34	0.03	0.32	0.62	0.07	0.02	0.47	1.89	0.00
60.	0.05	0.50	2.37	0.35	0.01	0.21	0.36	0.10	0.03	0.62	1.94	0.00

Abbreviations: No-number of sample, DE-daidzein, DI-daidzin, mDI-malonyl daidzin, aDI-acetyl daidzin, GY-glycitein, GYI-glycitin, mGYI-malonyl glycitin, aGYI-acetyl glycitin, GE-genistein, GI-genistin, mGE-malonyl genistin; aGE-acetyl genistin

Table 2. Data matrix

5. Conclusion

The content of the twelve isoflavones in sixty genotypes of soybean, planted during three different years, has been analysed using the principal component method, with the aim to examine if there is any correlation among different isoflavone forms in soybeans and to

investigate the relations between different soybean genotypes, according to their isoflavone profiles. The obtained results show that malonylglucoside and glucoside isoflavone forms determine to a great extent the isoflavone composition in soy. The mutual position of the variables in the plane of PC1 and PC2 loading vectors suggest that ratios of malonyl daidzin to malonyl genistin, genistin to glycitin and malonyl daizdin to acetyl daidzin could be specific characteristics of a particular soybean genotype. The division of the examined genotypes into two groups according to the PC1-PC2 model has showed that cultivation year significantly influences the total and the individual isoflavone contents in soybeans.

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18

Fermented Tofu, Tofuyo

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1. Introduction

Fermented tofu (soybean curd) is widely distributed in East and Southeast Asia. It is known in mainland China and Taiwan as *toufu-ru*, *toufu-ju*, *furu*, *rufu*, *sufu*, *funan*, *fuyu*, *tou-yu*, *tau-zu*, in the Philippines as *tafuri*, in Malaysia as *tau ju*, in Thailand as *tau-fu yee*, and in Vietnam as *chao* or *dau-phu-nyu*. In Japan, fermented tofu, known as *tofuyo*, is found only in Okinawa Prefecture (Shurtleff & Aoyagi, 1975; Su, 1986; Yasuda, 1983b, 1994). Surprisingly, fermented tofu is not found in mainland Japan and Korea, which are located in East Asia. In most Western scientific literature, Chinese fermented tofu has been introduced as *sufu*. However, the term *sufu* is not familiar to most Chinese people, while *furu* or *toufu-ru* seems to be more commonly known. In Japan, fermented tofu has been introduced as "nyufu" (*rufu*) or "funyu" (*furu*) (Ohta, 1965). According to Su (1986), sufu is prepared by overgrowing soybean curd with a mold of the genus *Actinomucor*, *Mucor*, or *Rhizopus* and further fermented in a salt-brine/rice-wine mixture. During fermentation, the mold and rice wine mixture imparts additional flavor to the product. Sufu is usually comprised of red, pale yellow or white colored blocks (2 to 4 cm^2 and 1 to 2 cm thick). Commercial products of both white sufu (furu) and red sufu (rufu) made in Taiwan are shown in Fig. 1. Pale yellow or white sufu are untreated, whereas red sufu is colored with red koji which is prepared by growing the mold *Monascus anka* or *Monascus purpureus* on cooked rice. Other additives are frequently incorporated into the brine solution, imparting either additional color or flavor to the various types of sufu. Sufu is consumed directly as a condiment (for example, as a seasoning for hot breakfast rice: gruel or Chinese bread) or is cooked with vegetable or meats. Sufu adds zest to the bland taste of a rice-vegetable diet. These descriptions are very interesting in comparison with the following description of tofuyo from Okinawa.

Tofuyo is a fermented tofu indigenous to Okinawa Prefecture, Japan. It is a low-salt vegetable cheese product prepared from tofu by the action of microorganisms. It is nutritionally rich, with high quality of protein, fat, and other nutrients. Tofuyo is a creamy cheese-like product with a mild flavor, fine texture, and good taste. Traditionally, tofuyo is directly consumed as a dish eaten with awamori, a traditional distilled liquor in Okinawa, or as cakes served at teatime for ladies (Fig. 2).

Red furu was brought to Okinawa from Fujian, China, and was introduced during the period of the Ryukyu dynasty, around the 18th century. Because the product was brined and had a strong taste, it was initially unpalatable to the Okinawan people in its original form. Therefore, it was re-created using awamori instead of salt by cooks in the dynasty for a more palatable taste that was milder in flavor. It was named tofuyo. Its other beneficial

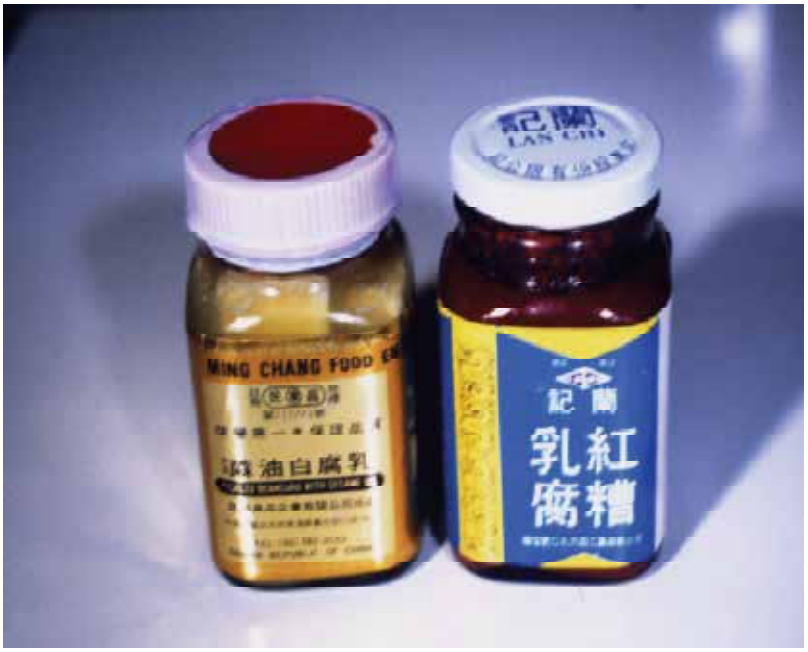


Fig. 1. Commercial products of sufu made in Taiwan Left hand side; white sufu (furu), Right hand side; red sufu (rufu)

Fig. 2. Tofuyo and awamori

properties, such as the smooth cheese-like texture, being a valuable source of protein, and improving blood circulation have made tofuyo treasured, not only as a nutritious side dish but also as a health food that has been eaten after illness for centuries. Improvement in

blood circulation is considered to be caused by the ethyl alcohol contained in awamori, which is used as one of the raw materials. During the Ryukyu dynasty, the royal family and high society relished tofuyo as a health food and high-level gourmet food; however, tofuyo was hardly known to commoners (Yasuda, 1983b).
As the secret techniques of tofuyo production have been passed on for generations only in select homes, few people have knowledge of this food. In order to develop tofuyo manufacturing, we have investigated the features of its production and scientifically analyzed its characteristics. The outcomes were transferred to the local industry and the market for tofuyo is now developing.
The scientific aspects of tofuyo have yet to be fully clarified. In this chapter, the history of tofuyo, its production, chemical characteristics and physiologically functional properties are described, as well as the role of the fungus *Monascus* in its production.

2. History of tofuyo

There have been few reports on the history of tofuyo since the one written in Japanese by Yasuda in 1983 (Yasuda, 1983b; 1994b). Shurtleff & Aoyagi introduced the tofuyo that was described in 1983 in their special report on the history of traditional fermented soy foods, written in English (Shurtleff & Aoyagi, 2007). In this section, the history of tofuyo from these literatures is outlined.

2.1 History of fermented tofu

Fermented tofu is considered to have originated in China; however, the exact time and place of its origin remain uncertain. Hong (1985) reported that the first description concerning fermented tofu was found in "*Peng Long Ye Hua*" written by Li Ri-Hua in the Ming dynasty (1368-1644), where it was prepared with molded tofu. Reference to furu appeared in the famous book on Chinese medicinal and herbal materials, "*Pen Ts'ao Kang Mu*" (*Honzo Kou Moku*, in Japanese) published by Li Shih-Chen in 1596. In this book, furu was prepared without the molding procedure. From these old records, it appears that there were two techniques involved in furu production, treatment with and without mold. Although the name rufu appears in the section of animals of this book, it is not fermented tofu, but refers to milk-curd, which is produced from the coagulation of protein in milk under acidic conditions.
During the Ch'ing dynasty (1644-1912), many records on fermented tofu production appeared, indicating that the molding procedure had become mainstream. The making of fermented tofu with the molding procedure is described in the book, "*Shi Xian Hong Mi*" written by Wang Zi-Zhen, in the middle of the Kang-Xi period (1681-1706). Interestingly, the author found that the fermented tofu was made with the "red koji" in this record. From this description, it is strongly suggested that the origin of tofuyo is the fermented tofu described herein. References to rufu often appear in documents of that time. Rufu seemed to be one of the finest products in the district of Jiang Nan, China, and was introduced in a kind of guidebook on local production, "*Jiang Nan Tong Zhi*" (1736). In this record, rufu was not milk-curd, but fermented tofu. It was revealed that the methods of making fermented tofu recorded in "*Xing Yuan Lu*" written by Li Shi-Ting in the middle of the Quianlong period (1757-1776) are similar to present-day methods. Fermented tofu, rufu, was also described in "*Sui Yuan Shi Dan*" written by Yuan Mei (1782), and the white type of rufu (containing

shrimp eggs) or famous production sites, and other aspects, were introduced in this document. From a variety of literature, it can be concluded that the development and spread of fermented tofu occurred in the Ch'ing dynasty, and became popular in the diets of people in mainland China and Taiwan, and continues to be enjoyed even today. As the Chinese fermented tofu spread to other countries in East and Southeast Asia, it was given its own name in each country, as described in the Introduction. From that time to the present, the fermented tofu of each country has been traditionally made in the home or small cottage industries.

2.2 History of tofuyo

Although fermented tofu has never been widely known nor consumed in mainland Japan, it has a long and interesting history in Okinawa Prefecture, where a mellow, delicious product named tofuyo has been enjoyed for nearly 200 years. Okinawa is one of Japan's southern prefectures, and consists of hundreds of the Ryukyu Islands in a chain over 1,000 km long, extending southwest from Kyusyu to Taiwan. The Ryukyu Kingdom existed in this area before the Japanese Meiji Period (1868). Since the islands are located in the center of the East China Sea and are relatively close to Japan, China and Southeast Asia, the Ryukyu Kingdom became a prosperous trading nation. However, four years after the beginning of the Meiji period (1872), the kingdom was officially annexed by Japan.

The earliest known reference to fermented tofu in Japan comes from Osaka. In 1883, Ka Hitsu Jun published the famous book, "*Tofu Hyaku Chin Zokuhen*" (The Sequel to One Hundred Favorite Tofu Recipes). In this book, "red tofu" and the other fermented tofu, "tofu-ji", were introduced. According to the book, how to make the red tofu was a family-secret, and few details on its production were provided. The other description was clearly of Chinese style red furu, because the materials not only included red koji from China, shiro zake (white sake, Chinese distilled liquor named "Bai-Jiu"), *sansho* (Japanese spice, this spice seems to be used instead of chili), but also refer to it as tofu-ji and use the same method of preparation. However, this fermented food subsequently disappeared from and can no longer be found in mainland Japan. On the other hand, because relations between the Kingdom of Ryukyu and China were close at that time, it strongly suggests that furu production methods were brought to Ryukyu from China (probably from Fujang) in cultural exchanges between the two countries at that time. However, there is very little information available on fermented tofu in Ryukyu. Red tofu, fermented tofu, furu or tofu-ru were not described in the Ryukyu's Old Language Dictionary "*Kongo Kensyu*" (1711), or in the book on the History of Ryukyu "*Ryukyu Koku Yurai Ki*" (1713) edited by the Government of Ryukyu Ohfu.

Red furu was likely introduced to Ryukyu during the late 1700s. Since the product was brined and had a strong taste and smell, it was not immediately accepted in its original form by the people. Therefore, it has been re-created using awamori. Namely, the processes of preparing molded tofu and fermenting the mold-overgrown curd in salt-brine were eliminated by cooks in the dynasty, resulting in a more palatable taste that was milder in flavor. Thereafter, they were able to decrease the amount of salt in the original recipe, and increase the shelf life without salt. Thus, an elegant, mellow and delicious fermented food, dubbed *tofuyo*, was newly created.

The earliest known indirect reference was in the book "*Account of a Voyage of Discovery to the West Corea and the Great Loo-Choo Island*" written by Basil Hall in 1818. In this book, "Corea"

refers to "Korea". Interestingly, Ryukyu was called the great Loo-Choo islands on the old map at that time. The Englishman Hall and his party visited Naha harbor in 1816, on route from China, where they were entertained by the Government of Ryukyu Kingdom and served the local cuisine. They were served "hard boiled eggs, cut into slices, the outside of the white being colored red…sackee (the liquor; awamori)…and something like cheese." The red color is thought to be the red koji-pigments produced by the genus *Monascus,* and the cheese-like food may be the fermented tofu, tofuyo, which is often made with red koji.
The earliest known direct reference to tofuyo and red koji was in "*Gozen Hon Sou*" written by the physician Tokashiki Tsukan Peichin in 1832. Peichin is a term for a high-ranking officer. In this book, foods, and medicinal and herbal materials in Okinawa were described in the context of medicine and pharmacy. He describes that "Tofuyo has a delicious flavor and is good for the stomach. It makes eating a pleasure and is good for various types of sickness". From these descriptions, it was considered that tofuyo was consumed by the royal family and the upper classes as a medicinal food and as a side dish. This group established the methods of making tofuyo, but the secret of its preparation has been a stubbornly guarded family-secret. In fact, there were no references to it from 1832 until 1938. It was not mentioned in the accounts of visits to Ryukyu by the English captain Frederick W. Beechey (1831) and the famous American Commander Mathew C. Perry (1857), nor in any documents from the Meiji period (1868-1912). This may explain why its use did not spread and why so few references have been made to it. During the early Showa period (1926-1989), home-made tofuyo was popular only among the upper classes in the cities of Shuri (the former capital) and Naha (the new capital) in Okinawa. However, its production was not clarified at all. Ladies ate it with tea, while men took it with awamori. Typically, a small cube was placed on a small dish and eaten with chopsticks or a tooth-pick (Fig. 2). The product was never widely known by regular people because it was only rarely made at home. In 1938, Shojyun Danshaku, who was a descendant of the Ryukyu Royal Family and a well-known connoisseur, wrote an article entitled "In Praise of Tofu" in the magazine, *Monthly Ryukyu*. He noted that tofuyo is one of the best rare and tasty foods in the world, if not the best.
After World War II, the special family-secret of making tofuyo techniques was, for many years, inherited only at select homes; few people are aware of the food. As it is a unique low-salt soybean cheese, its industrialization has been greatly expected. However, in order to develop its production, it is necessary to clarify features of the food making that depend upon intuition and experience, and to establish the manufacturing technology in proportion to mass production. Thus, we revealed its features, and scientifically established the basic technology involved (Yasuda, 1990). The outcome was transferred to the local industry during the mid 1980s. Subsequently, tofuyo that reflects the gastronomic culture in the age of the Ryukyu dynasty has been marketed. Furthermore, the red type of tofuyo is popular, and its production been greatly developing. Nowadays, attention is being paid to this food as a health food that is cholesterol free, and is a low-salt fermented soybean cheese. Therefore, it would also be suitable for western people because it could be utilized in almost the same way as cheese.

3. Production of tofuyo

Three steps are normally involved in making tofuyo: preparing tofu and dehydrated tofu-cubes (Yasuda & Hokama, 1984a; Yasuda et al., 1992), making koji (Yasuda et al., 1981;

1983a), and soaking and ripening (Yasuda et al., 1993; 1994a, 1995). A flowchart for tofuyo preparation is shown in Fig. 3.

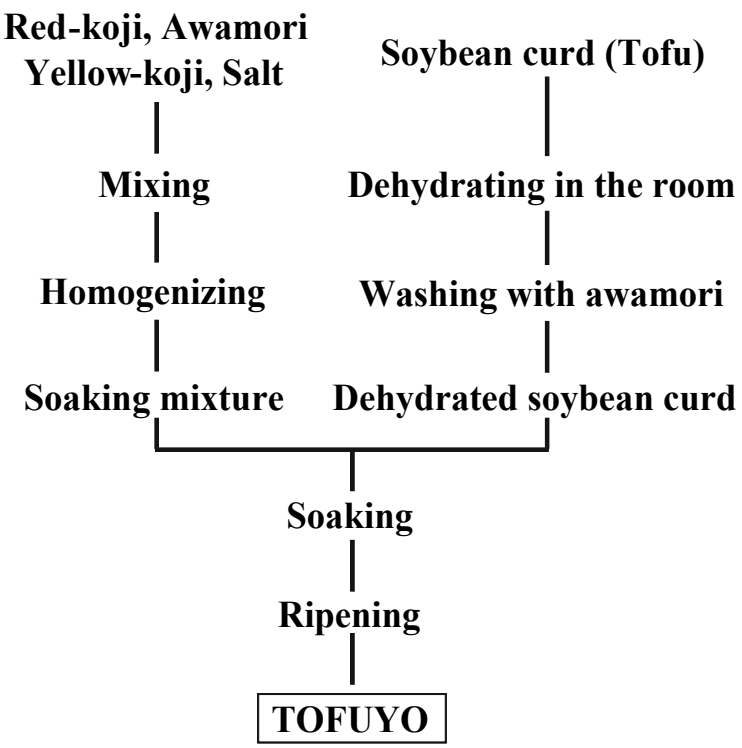


Fig. 3. Process for making tofuyo

3.1 Processes for making tofu and dehydrated tofu-cubes

To make tofu, soybeans were first washed, soaked in water, and then ground with water. The ground mixture was heated and then strained through a coarse cloth to separate the soybean milk from the insoluble residues. The soybean milk was then heated, and a coagulant was added to coagulate the protein. The coagulated milk was then transferred to a cloth lined wooden box, and pressed with a weight on top so as to remove the whey. In this way, tofu was formed. In general, tofu has a bland taste and high water content. The water content of regular tofu in Japan is 86%. It is suggested that the texture of tofu affects the quality of tofuyo. It is preferable that the water content of tofu used for making tofuyo be lower than that of regular tofu. Hardness of the tofu was from 10 to 11 $\times$ 10^3 Pa and the moisture was approximately 76%. The product has a suitable elasticity and fine texture (Yasuda & Hokama, 1984a).

To prepare dehydrated tofu, the tofu was cut into 2-cm cubes, and the cubes were dehydrated at room temperature for between 24 to 48 h. The dehydrated cubes were washed with awamori, and subsequently used as the material for tofuyo. The physical properties of dehydrated tofu-cubes are also important factors in determining the quality of tofuyo. If the dehydrated tofu-cubes are too soft, they will fall apart during maturation. On

the other hand, if they are too hard, it will be difficult to impart a good texture and taste to the tofuyo.

In order to establish a suitable texture of the cubes for making tofuyo, they were prepared using tofu with different values of hardness. It was found that the product of the best quality showed a value of 17.8×10^3 Pa and was obtained using tofu-cubes dehydrated to 28.9×10^3 Pa. This product was bland, smooth, and had a fine texture, like soft cheese. Thus, tofuyo with a value between 14 to 18×10^3 Pa was preferred. The preferred hardness of the dehydrated cubes was between 20 to 30×10^3 Pa (Yasuda & Hokama, 1984a). The relationship between tofuyo hardness and dehydration method is shown in Table 1. Tofuyo was made with dehydrated tofu-cubes prepared following methods and having the same value of hardness. The hardness of the product prepared at room temperature for between 24 to 48 h had a value of 17.5×10^3 Pa, agreeing with the preferred value as mentioned above. This product had a bland taste. However, the tofuyo prepared with tofu-cubes dehydrated by mechanical methods, such as by warm-wind, in the oven, or under vacuum, were not palatable and did not progress in maturation. Therefore, the best quality of tofuyo was obtained with tofu-cubes (hardness: from 20 to 30×10^3 Pa) that were gradually dehydrated at room temperature, and had a hardness of 14 to 18×10^3 Pa.

Method	Hardness (10^3 Pa)	
	Dehydrated tofu-cube	Tofuyo
A	28.6	27.8
B	25.9	25.0
C	24.9	22.4
D	26.5	17.5

Tofu-cubes were dehydrated in warm wind-drying box (Method A), oven-drying box (Method B) or vacuum-drying box (Method C) at 50°C for 4-6 h. Tofu-cubes were also dehydrated at room temperature (Method D) for 24-48 h. Tofuyo was prepared by these dehydrated tofu-cubes.

Table 1. Relationship between hardness of tofuyo and dehydration methods

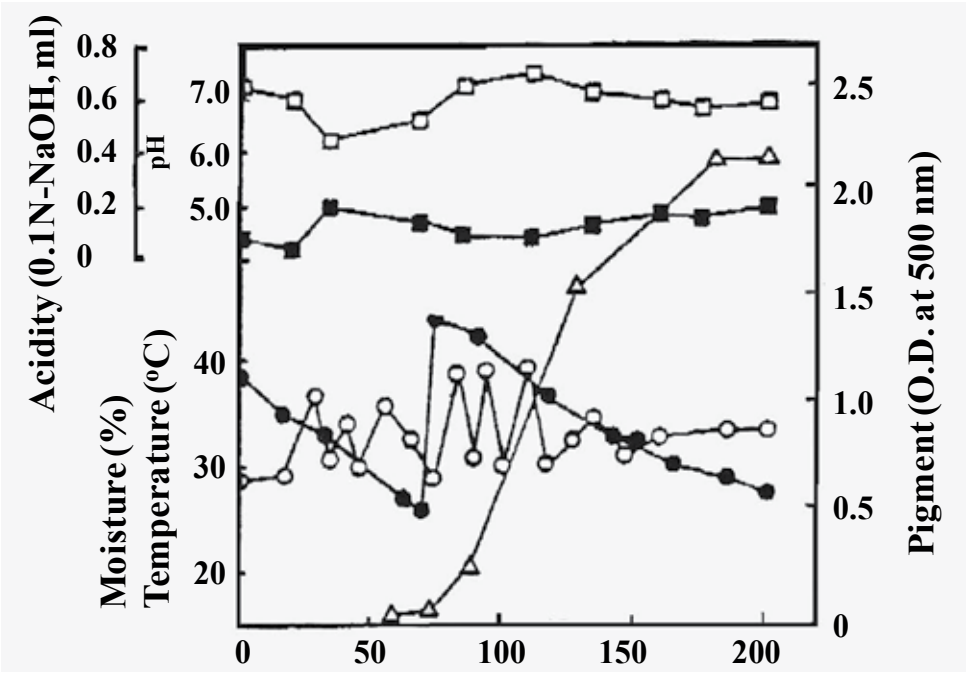
During dehydration, microorganisms such as bacteria grew on the surface of the tofu-cubes. The microorganisms may develop enzyme systems having higher proteolytic and lipolytic activities, because the bacteria grew on tofu which was protein and lipid rich. Furthermore, the microorganisms may play an important role in "pre-fermentation", which affects the quality of tofuyo by partially degrading the soybean protein of tofu. We selected *Bacillus* sp. TYO-67, which has high proteinase activity, for examining the above hypothesis by the use of a pure culture (Yasuda et al., 1992). The protein content of the cubes inoculated with the bacterium during dehydration was lower than that of those un-inoculated. Thus, it was confirmed that the former was more degraded than the latter. The ripening time of tofuyo prepared using the dehydrated tofu-cubes that were inoculated with the isolated bacterium

was shorter than that prepared with un-inoculated cubes. Therefore, the tofu-cube dehydration process at room temperature was characterized as being "pre-fermented" by bacteria (Yasuda et al., 1992). Later, the microorganism was identified and named as *Bacillus pumilus* TYO-67. An alkaline serine proteinase produced by this strain was purified and characterized (Yasuda et al., 1999; Aoyama et al., 2000a; 2000b).

3.2 Processes for making koji

Rice koji was prepared from polished rice, since it was essential that the mold quickly penetrate the rice kernel. Polished rice was soaked in water over night and the excess water was drained off. The swollen rice was cooked with steam at atmospheric pressure for 60 min, cooled to 35°C, and inoculated with a starter of the genus *Monascus* or *Aspergillus oryzae*. The incubated rice was placed in wooden trays. During the development of the koji, temperature, moisture, and aeration are extremely important factors and must be rigidly controlled. After incubation at 32°C for 7 days, the red koji, prepared using the genus *Monascus*, was harvested (Yasuda et al., 1983a). After incubation at 32°C for nearly 48 h, the yellow koji prepared using *A. oryzae* was harvested (Yasuda et al., 1981).

The data concerning the changes in temperature, moisture, pigment, pH, and acidity of the koji during cultivation are shown in Fig. 4. After 40 h of inoculation, the temperature of the



Cultivation was carried out at 32°C for the time indicated. Steamed rice was inoculated with seed containing *Monascus purpureus*, and cultured in an incubation box. Koji samples were taken for analysis at the indicated time.

-○-; Temperature, -●-; Moisture, -Δ-; Pigment, -□-; pH, -■-; Acidity

Fig. 4. Changes in temperature, moisture, pigment, pH, and acidity of red koji during cultivation

material increased to around 40°C. The temperature was controlled by hand mixing. The level of moisture decreased during incubation. After 50 h of incubation, the level of moisture was reduced to 30% or less, and the koji materials were moistened by water spray. These treatments resulted in marked enhancement of microbial growth and koji-pigment formation. The pH and acidity of the koji were not greatly affected during incubation. Although it is not shown in this figure, proteinase, α-amylase and glucoamylase activities were maximal at 150 h (Yasuda et al., 1983a). Thus, the optimum incubation period of the koji production was 150 h after the inoculation of the seed culture.

Koji is an important material for making tofuyo. It is a source of enzymes for converting starch into fermentable sugars and proteins into peptides and amino acids. The characteristics of suitable koji for making tofuyo are as follows. One is that it has high enzymatic activity, such as for proteinase, α-amylase and glucoamylase. Another is the provision of a good flavor and taste to the product. In order to enhance red koji enzyme activity, we examined the effect of steaming methods and rice varieties on the enzyme and pigment production (Table 2). Enzyme activities of the varieties of koji were indicated against relative activity of the koji prepared using non-glutinous rice by steaming under atmospheric pressure. *Monascus*-pigment was also indicated. In the system of steaming under atmospheric pressure, enzyme and pigment production of the koji prepared with glutinous rice was more abundant than that of non-glutinous rice. Traditionally, glutinous rice is used as a raw material for koji. α-Amylase and proteinase of the koji prepared with broken rice was the same as non-glutinous rice, but glucoamylase and pigment showed low production levels. However, with the steaming under high-pressure system, enzyme and pigment production of the koji prepared with non-glutinous rice was, in general, more abundant than that of broken rice from Thailand or glutinous rice. Thus, it was found that the highest production of enzymes and pigment in koji was obtained by employing autoclaved non-glutinous rice.

	Relative activity (%)					
	Steaming under atmospheric pressure			Steaming under pressure		
	A	B	C	A	B	C
α-Amylase	100	94	106	124	108	84
Glucoamylase	100	74	124	140	139	96
Proteinase	100	102	123	167	179	94
Pigment	100	68	138	417	183	61

A non-glutinous rice. B broken rice. C glutinous rice.

Table 2. Effect of steaming methods and varieties of rice on the production of enzyme and pigment in red koji

High enzyme activities in yellow koji by *A. oryzae* were also obtained in the same manner as in the case of red koji described above (Yasuda et al., 1981).

3.3 Soaking and ripening

The last step in making tofuyo was soaking and ripening. To make the moromi (soaking mixture; fermentation broth), red koji and/or yellow koji, awamori (43% of ethyl alcohol concentration) and a small amount of salt were mixed, and kept at 4°C for 24-48 h. The mixture was then ground and the result is known as moromi. The dehydrated cubes were immersed in the moromi and allowed to ripen at 25 to 30°C for 5 months. The ethyl alcohol concentration in the moromi was around 20% and the pH was from 5.6 to 6.0. It is unique and characteristic that the ripening was carried out under the presence of a high alcohol concentration. The quality of the product was affected by the variety of koji and the liquor in the moromi. According to our investigations, the product with red koji or the mixed koji with red koji and yellow koji was superior to that of yellow koji with respect to color and flavor (Yasuda et al., 1995). Awamori was the most important liquor for making tofuyo. Other liquor, such as whisky and shochu, which is distilled liquor from sweet potato or grains in Japan, were not suitable for the flavor of the product.

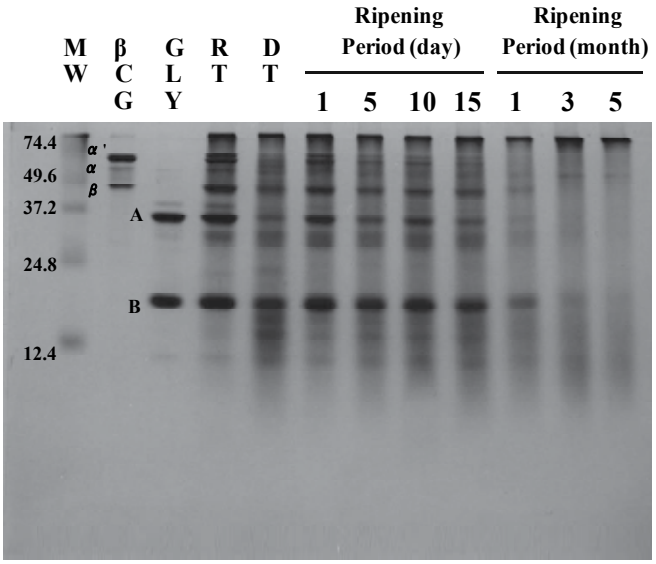
4. Characterization of tofuyo

4.1 Chemical changes in tofuyo during the ripening period

Although we have compared the characteristics of tofuyo made with red koji, yellow koji and mixed koji (Yasuda et al., 1993; 1994a; 1995; 1996), here the chemical characteristics of tofuyo prepared with the red koji is mainly described (Yasusda et al., 1993a). Chemical changes in tofuyo during the ripening period were examined. Crude protein and crude fat contents of tofuyo decreased, but reducing sugar content increased during the ripening period. Crude fiber was not detected. Sodium chloride content of the product was considerably constant (around 3%) throughout the ripening period. The sodium chloride content of tofuyo was lower than that of Chinese sufu (Wang & Hesseltine, 1970). Therefore, tofuyo is a low-salt fermented tofu.

Proteinase is an enzyme that plays a pivotal role in the maturation of tofuyo. Changes in proteinase activity in the moromi due to the koji during the ripening period were examined. Proteinase activity decreased during the ripening period due to the effect of the high alcohol concentration (20%; v/v) in moromi. Thus, it was suggested that the proteinase activity was controlled by the presence of ethyl alcohol originating in awamori, and the ripening might be gradually carried out. Therefore, soybean proteins may be degraded to a limited extent, and may also result in the formation of good physical properties, such as a smooth texture and optimal viscoelasticity.

In order to confirm its biochemical degradation, digestion of soybean protein during fermentation was examined by slab polyacrylamide gel electrophoresis (Fig. 5). Some bands originating from polypeptides of soybean globulin (such as the α'-, α-, and β-subunits of β-conglycinin, and the acidic subunit in glycinin) in the water-insoluble faction of tofuyo disappeared after the 3-month ripening in the presence of ethyl alcohol from awamori, whereas the basic subunit in glycinin was recognized as a thin band. Furthermore, those of the other polypeptides (*Mr.* 30-32 kDa, 10-15 kDa) were also recognized. These polypeptides and the remaining glycinin, which has strong gel-forming ability, are closely related with the body of tofuyo (Yasuda et al., 1993; 1994a; 1995). Therefore, it is considered



MW : Standard proteins (MW-marker, kDa)
β CG : β-Conglycinin, GLY : Glycinin
RT : Raw Tofu, DT : Dehydrated Tofu
α', α, β : α', α and β-subunits in β-Conglycinin
A, B : Acidic and Basic subunits in Glycinin

Fig. 5. SDS-PAGE pattern of the water-insoluble fraction of tofuyo prepared by red koji at various ripening stages

that the glycinin greatly contributes to preserving the desired texture of tofuyo (Yasuda et al., 1996), and is partially converted to peptides and amino acids. On the other hand, some polypeptides in the water-soluble fraction of tofuyo, with molecular masses of 31, 25, 23, 21, and 10-12 kDa, were leveled off by proteinases during the fermentation (0-3 months). The nitrogen components of tofuyo during the ripening period were examined. The ratio of water-soluble nitrogen to the total nitrogen (called the protein solubility ratio or ripening ratio) reached 36.3% after the 3-month ripening. The ratio of 4%-trichloroacetic-acid soluble nitrogen to the total nitrogen reached 34.0% after the 3-month ripening. Thus, 94% of non-protein-nitrogen compounds, such as amino acids and peptides, in the water-soluble fraction of the product were converted from soybean protein by proteinases and peptidases during the fermentation. Free amino acids, such as glutamic acid and aspartic acid, may contribute to the formation of flavor, and released peptides may contribute to the physiological function of tofuyo. These phenomena were also observed in the products prepared using yellow koji and mixed koji (Yasuda et al., 1994a; 1995; Katsura, 1996).

Both glucoamylase and α-amylase had high activities throughout the ripening period. Starches in koji were mostly converted to glucose by these amylases, and this can provide a desirable taste to the tofuyo. These phenomena were also observed in the products prepared using yellow koji and the mixed koji (Yasuda et al., 1994a; 1995).

Amino acids, peptides, and other taste-associated compounds, including saccharides, in tofuyo are discussed in more depth in other sections (4.3 and 4.4). Moreover, soybean lipids were digested to some extent into fatty acids by lipases in the moromi. The added awamori

can react with these fatty acids chemically or enzymatically to form esters, which may provide the pleasant aroma of tofuyo (Yasuda, 2010).

4.2 Physical changes in tofuyo during the ripening period

Tofuyo is a unique fermented soybean protein food that possesses a cream-cheese-like texture. Its texture is very different from that of the tofu that is the raw material for tofuyo. Therefore, textural changes in tofuyo during fermentation were investigated (Yasuda et al., 1996). Breaking stress and breaking energy values of the product decreased during fermentation. Creep analysis of tofuyo revealed that the values of each viscoelasticity coefficient also decreased during the ripening period. Interestingly, creep analysis of tofuyo, ripened for 3 months, showed that each viscoelasticity coefficient was similar to that of commercial cream cheese or soft-type processed cheese. Thus, it was scientifically confirmed that tofuyo has a cheese-like texture.

In order to understand the ripening of tofuyo, changes in the microstructure of the product were also examined. Electron microscope observation revealed that fat globules seemed to bind with proteins. Moreover, the results of electron microscopy showed that the structure of the soybean protein forming the body of tofuyo changed from fibriform to small particles, which connected to each other during the ripening period (Fig. 6). These phenomena were good agreement with the results of slab polyacrylamide gel electrophoresis, as shown in Fig. 5. It was thus considered that these phenomena contributed to maintaining the shape and unique texture of this product.

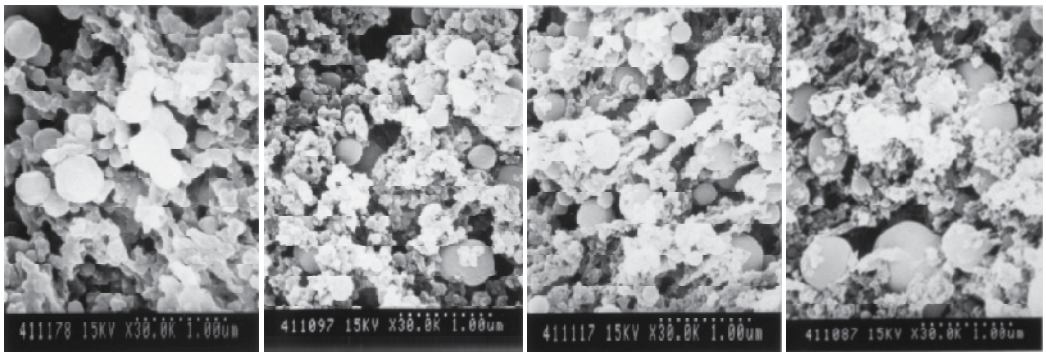


Fig. 6. Changes in form of soybean protein particles of tofuyo prepared by red koji during fermentation observed by a scanning electron microscopy

4.3 The flavor compounds in tofuyo

The chemical composition of tofuyo, after 3-month ripening, was as follows: 29.2% crude protein, 21.2% crude fat, 7.4% crude ash, 0% crude fiber, 24.2% reducing sugars, and 3.2% salt (on a dry weight basis). Thus, tofuyo is a fermented low-salt soybean food that is rich in proteins, lipids, and carbohydrates.

Amino acids greatly contribute to flavor formation. Changes in the free amino acids of tofuyo, prepared using red koji, during the ripening period are shown in Table 3. The data clearly indicate an increase in and the involvement of free amino acids after fermentation,

especially glutamic acid, alanine, aspartic acid, glycine and serine, which, after a 3-month ripening, may contribute to the pleasant taste. It is well known that glutamic acid and aspartic acid contribute to the pleasant umami taste or savory enhancement in foods. Phenylalanine is also rich in tofuyo. Recently, Lioe & Yasuda et al. (2004; 2007) carried out chemical and sensory studies on savory fractions obtained from soy sauces, and the presence of phenylalanine, as well as NaCl with glutamic acid, were found in the fractions. A potential synergistic effect of umami among free glutamic acid, salt and phenylalanine (the so called bitter amino acid) were observed (Lioe et al., 2010). Furthermore, this phenomenon of umami or savory enhancement by subthreshold aromatic amino acids in the soy sauce system has been established by the model experiment system (Lioe et al., 2005). Recently, we confirmed that l-phenylalanine also plays an important role in umami taste enhancement of glutamic acid in the presence of NaCl, both in tofuyo and soy sauce.

Amino acids (mg/100 g)	Raw tofu	Dehydrated tofu	Ripening period (month)			
			0.5	1	3	5
Aspartic acid	**15**	**29**	**317**	**511**	**673**	**773**
Threonine	**23**	**26**	**33**	**78**	**143**	**148**
Serine	**10**	**53**	**78**	**209**	**410**	**481**
Glutamic acid	**20**	**56**	**283**	**609**	**864**	**881**
Proline	**9**	**49**	**85**	**83**	**86**	**147**
Glycine	**14**	**67**	**73**	**302**	**422**	**543**
Alanine	**24**	**90**	**219**	**414**	**676**	**702**
Cysteine	**0**	**0**	**3**	**5**	**6**	**9**
Valine	**15**	**19**	**38**	**88**	**145**	**124**
Methionine	**0**	**4**	**23**	**60**	**51**	**45**
Isoleucine	**3**	**15**	**40**	**85**	**126**	**137**
Leucine	**11**	**65**	**117**	**246**	**403**	**477**
Tyrosine	**0**	**10**	**51**	**114**	**153**	**150**
Phenylalanine	**14**	**50**	**65**	**128**	**200**	**173**
Histidine	**7**	**5**	**17**	**29**	**34**	**39**
Lysine	**8**	**4**	**13**	**78**	**152**	**216**
Arginine	**1**	**2**	**44**	**137**	**176**	**109**
Total amino acids	**174**	**544**	**1499**	**3176**	**4720**	**5154**

Table 3. Changes in free amino acids of tofuyo prepared by red koji during the ripening period

Glucose is one of the major compounds, besides amino acids, involved in the taste component of tofuyo, with the other compounds being organic acids (e.g., malic, citric, lactic, and acetic acids), nucleotides and NaCl. It is strongly suggested that the desirable taste of tofuyo is formed by interactions between these components.

4.4 Physiologically functional properties of tofuyo

4.4.1 Angiotensin I-converting enzyme inhibitory peptides in tofuyo

Lifestyle-related diseases are a serious social problem, along with the current changes in peoples' dietary habits. Recently, much attention has been paid to the role of health-promoting foods in disease prevention. Hypertension is a serious risk factor for cardiovascular disease, and it is known to have high morbidity. It is well known that angiotensin I-converting enzyme (ACE) inhibitors have an excellent effect on hypertension. ACE is a dipeptidyl carboxypeptidase associated with the regulation of blood pressure. It converts angiotensin I to the potent pressor peptide, angiotensin II, and also degrades the depressor peptide bradykinin (Yang et al., 1971). ACE inhibitors from various foods have been recently studied in terms of their ability to prevent and alleviate hypertension. Physiologically functional foods enriched with ACE inhibitors, such as tripeptide (Val-Pro-Pro, Ile-Pro-Pro) from sour milk (Takano, 1998), dipeptide (Val-Tyr) from sardine muscle hydrolysate (Kawasaki et al., 2000) and dodecapeptide (Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys) from casein hydrolysate (Maruyama et al., 1987), are used as supplements to improve hypertension.

As it was of interest whether ACE inhibitors were present in tofuyo or not, ACE inhibitory activities were examined in our laboratory (Kuba et al., 2003). As a result, inhibitory activity was found in tofuyo extracts, with an IC_{50} value of 1.77 mg/ml. The IC_{50} value shows the concentration of inhibitor needed to inhibit the ACE reactions to 50%. That is to say, the ACE inhibitory activity is higher as the IC_{50} value is lower. Two ACE inhibitors were isolated to homogeneity from the extract by absorption and gel filtration column chromatographies, and by reverse-phase high-performance liquid chromatography (HPLC). Since both substances reacted with 2,4,6-trinitrobenzensulfonic acid sodium salts, which bind only to primary amines and the sulfhydryl group, they both appeared to be peptide-like substances. The amino acid sequences of the substances, determined by Edman degradation, were Ile-Phe-Leu (IFL; IC_{50} value, 44.8 μM) and Trp-Leu (WL; IC_{50} value, 29.9 μM). A computer search of the SWISS-PROT protein sequence data base showed that the amino acid sequence of IFL was present in the primary structures of the α- and β-subunits of β-conglycinin, whereas WL was present in the primary structures of glycinin. Since the α'-, α-, and β-subunits in β-conglycinin and the acidic subunit in glycinin were degraded to low-molecular-weight elements during 3-month tofuyo fermentation, it is likely that IFL was liberated from β-conglycinin by proteinases produced by *M. purpureus* and/or *A. oryzae* used in the fermentation process. Although the basic subunit in glycinin cannot be easily degraded by these enzymes, as compared with each subunit in β-conglycinin or the acidic subunit in glycinin, WL might have been liberated from the basic subunit during long-term fermentation (3 months).

In order to examine the resistance of both IFL and WL peptides in tofuyo to digestion *in vivo*, changes in their IC_{50} values before and after treatment with gastrointestinal proteinases *in vitro* were examined. As shown in Table 4, the ACE inhibitory activities of both peptides were completely preserved after pepsin treatment. Although the ACE-inhibitory activity of WL was also completely preserved after pepsin, chymotrypsin, or trypsin treatment, that of IFL decreased to 62% and 75% of the original value following chymotrypsin and trypsin treatments, respectively. In spite of the successive digestion with pepsin, chymotrypsin and trypsin, the inhibitory activity of IFL was found to be 38%, and that of WL was 29%, of the original value. It has been reported that the IC_{50} values of "Ile-Phe" (Cheung et al., 1980,

Seki et al., 1995) and "Phe-Leu" (Eto et al., 1998), which are parts of Ile-Phe-Leu, were 930 and 16 μM, respectively. Thus, IFL obtained from tofuyo is likely to preserve its activity until it is degraded to it individual amino acids.

Digestion	IC_{50} (μg/ml)	
	IFL	WL
None	18	10
Pepsin	18	10
Chymotrypsin	29	11
Trypsin	24	10
Pepsin → chymotrypsin and trypsin	47	35

Table 4. Digestive stability of IFL and WL toward ACE inhibition

It is well known that di- and tripeptides are more rapidly absorbed and reach a higher concentration in the blood than individual amino acids (Claft et al., 1968; Chun et al., 1996). Short peptides (mean residue length of 3.2) in a soybean hydrolysate were more rapidly absorbed than the long ones (mean residue length of 5.2) when using a rat intestinal everted sac (Chun et al., 1996). Although it has not demonstrated in the case of tofuyo, IFL and WL isolated from tofuyo are expected to be easily absorbed and to contribute to the antihypertensive effect via a transport system *in vivo.*

4.4.2 Antihypertensive and hypocholesterolemic effects of tofuyo in spontaneously hypertensive rats

Although ACE inhibitory activity was confirmed in tofuyo *in vitro*, the antihypertensive activity and other physiological functions of tofuyo *in vivo* remain to be clarified. We investigated the antihypertensive effects of tofuyo *in vivo* (Kuba et al., 2004). Four-week-old male spontaneously hypertensive rats (SHR) were fed a commercial diet for 4 weeks. The rats were housed individually at 25°C and 70% humidity under a 12-h light-dark cycle. At 8 weeks of age, the rats were divided into a control group and tofuyo group. The chemical composition, such as protein, carbohydrate, lipid, sodium chloride, and vitamins, of each diet was adjusted. Each group was fed a different diet for 6 weeks. The tofuyo group showed a similar growth rate to that of the control group. Diet intake during the experimental period did not differ greatly between two groups. The systolic blood pressure (SBP) of rats gradually increased with body weight. At 11 and 12 weeks of age, the SBP of rats in the tofuyo group tended to be lower than that in the control group, and at 13 weeks of age, the SBP of the tofuyo group (199.2 ± 4.4 mmHg) was significantly lower than that of the control (207.6 ± 4.4 mmHg). Several studies have examined the antihypertensive effect of ACE inhibitors in SHR by long-term feeding. The SBP of SHR fed sour milk (Nakamura et

al., 1996) and chicken essence hydrolyzate (Chen et al., 2002) had decreased 19 mmHg at 23 weeks of age and 26 mmHg at 24 weeks of age, respectively. The experimental period of our study seems to be shorter than those reports, and with a smaller decrease in SBP.

After feeding the experimental diets (for 14 weeks), ACE activities of various tissues were examined. The ACE activity of kidney was significantly lower in the tofuyo group (2.6 ± 0.8 mU/mg of protein) than that in the control group (4.9 ± 1.8 mU/mg of protein). It is known that persistent ACE inhibition in peripheral tissues, especially in the vascular wall and kidney, might be important for the prolonged antihypertensive effects of ACE inhibitors (Unger et al., 1985). As the elevation in SBP had been reversed by 14 weeks age, the ACE inhibition in kidney of the tofuyo group might be related to this antihypertensive effect. The authors also confirmed the presence of γ–amino butyric acid in tofuyo extracts. Thus, tofuyo is expected to have antihypertensive effects. Further investigations are required to clarify the antihypertensive mechanism of tofuyo and the dominant substances involved in its effect.

Serum lipids and total cholesterol in the tofuyo group were significantly lower than those in the control group. High density lipoprotein (HDL) cholesterol was significantly decreased in the tofuyo group. However, the ratio of HDL to total cholesterol in the tofuyo group tended to be higher than that in the control group. Triglycerol and phospholipid in the tofuyo group tended to be lower than that in the control group. These results indicate that tofuyo had hypocholesterolemic activity in serum. In addition, there were no differences in body weight and tissue weights between the groups, and no macroscopic liver lesions, such as fatty liver, were observed in either group. Thus, the hypocholesterolemic effect of tofuyo was probably not due to accumulation of lipids in the liver (Kuba et al., 2004). Inoue et al. (2006) also observed the influence of tofuyo on serum composition in high-fat-fed mice. Triglycerol values of the tofuyo group fed a high-fat diet plus tofuyo decreased by 1/2, while those of the HDL-cholesterol group increased over 2.6-fold as compared to the control group (fed a high-fat diet without tofuyo). From these results, it is expected that tofuyo contributed to the improved lipid metabolism in the mice.

4.4.3 Effect of tofuyo on erythrocyte deformability in high-fat-fed mice and other bioactivities

Inoue et al. (2006) examined the influence of tofuyo on erythrocyte deformability in high-fat-fed mice. After ICR mice were fed a high-fat diet (HFF), tofuyo paste was given to the animal over a 3-week period. In this experiment, "HFF + physiological saline solution" or "normal diet + physiological saline solution" were used as control groups. After 3 weeks, erythrocyte deformability was measured. The group given "HFF + tofuyo" showed a higher erythrocyte deformability value (0.532 ± 0.04 at 30 Pa) than the control group given "HFF + physiological saline solution" (0.498 ±0.01). On the other hand, this value was almost similar to that of the control group given "normal diet + physiological saline solution". Thus, tofuyo might contribute to the improvement in erythrocyte deformability.

Antioxidative activity and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity were found in the water-soluble fraction of tofuyo, which we are now purifying for identification of the compounds. On the other hand, we confirmed the presence of isoflavones that have DPPH radical scavenging activity in 70% ethyl alcohol soluble fractions of tofuyo. Further studies are needed to identify the chemical compounds that showed antioxidative activity and to clarify the physiological effects *in vivo*.

It is suggested that other interesting physiologically bioactive compounds are present in tofuyo. Of note are the microbial metabolites formed during fermentation, such as bioactive peptides and oligosaccharides, as well as bioactive substances originating in soybean, such as isoflavones, saponins, soybean proteins, and essential fatty acids.

5. Microbial biochemistry in tofuyo production

Microorganisms commonly used to the manufacturing of tofuyo belong to the genera *Bacillus*, *Monascus*, and *Aspergillus*. The genus *Bacillus* grows on the surface of tofu-cubes during the dehydration process, and plays an important role in the pre-fermentation of tofuyo, as described in section 3.1. The microorganisms involved in the main fermentation are molds. Mold belonging to the genus *Monascus* produces a red color, and is known as the *Redkoji* mold.

The mold *Monascus* was inoculated onto steamed rice, resulting in the production of red molded rice called red koji or *Beni koji.* This red koji has been historically used for many years in the fermentative production of red sufu, red rice wine and for Chinese medicine. This koji has also been traditionally used for tofuyo production and as a colorant used in the cooking of celebration foods, such as red cooked rice, red colored boiled eggs, red squid, red kamaboko (fish cake), and so on, of Okinawa Prefecture, Japan (Yasuda, 1983b). *A. oryzae* is a very important industrial microorganism for the production of miso paste and Japanese sake, for example. The fungal proteinases, peptidases, amylases and lipases play important roles in tofuyo fermentation. Especially, proteinases are very important enzymes for the maturation of the product (Yasuda & Sakaguchi, 1998; Yasuda, 2004).

5.1 Characterization and application of aspartic proteinases from *Monascus* fungus

Proteinases cleave peptide bonds at internal points within proteins/peptides to produce peptide fragments. They are classified based on their catalytic mechanisms as (1) serine proteinases, (2) cysteine proteinases, (3) aspartic proteinases, and (4) metallo proteinases. Although proteinases produced by the genus *Monascus* are important enzymes for tofuyo production, its enzymatic properties have not yet been fully clarified. Tsai et al. (1978) purified acid proteinases from *M. kaoliang*, and we purified aspartic proteinases from *M.* sp. No. 3403 (*M. purpureus*) (Yasuda et al., 1984b) and *M. pilosus* (Lakshman et al., 2010) as homogenous preparations using SDS-polyacrylamide gel electrophoresis. The molecular mass of the enzyme from *M. purpureus* was estimated to be 40 kDa by gel-filtration and 43 kDa by sedimentation method; therefore, it was determined to be a monomeric structure. The enzyme was active in acidic regions, with the maximum at around pH 3.0. The inhibition of pepstatin A was competitive with Hammarsten milk casein (The *Ki* value was 26 nM). On the other hand, two acid proteinases, MpiAP1 and MpiAP2, were purified to homogeneity from *M. pilosus*. Both purified enzymes were monomeric structures, with molecular masses of around 43 and 58 kDa, respectively. The former was an acidic non-glycoprotein, whereas the later was an acidic glycoprotein with 27% carbohydrate content. Although amino acid sequence analysis of both enzymes (MpiAP1 and MpiAP2), 20 amino acids in length, showed over 90% similarity, their amino-terminal amino acids differed from each other. Both enzymes were optimally active at 55°C and pH 2.5-3.0 against casein or human hemoglobin. The $T_{1/2}$ values of MpiAP1 and MpiAP2 were 65 and 70°C, respectively. Both enzymes were completely inhibited by pepstatin A. Milk casein and

hemoglobin were good substrates for these enzymes. Eleven cleavages were detected using the oxidised insulin B-chain as a peptide for the proteolytic specificity test of MpiAP1, while seven cleavages were detected for MpiAP2.

Degradation of soybean protein by *Monascus*-proteinase was investigated in order to reveal the role of the enzyme in the process of tofuyo ripening (Yasuda & Sakaguchi, 1998). It was found that the digestion of soybean protein by this enzyme progressed as follows: initially the α'-, α- and β-subunits in β-conglycinin, and then the acidic subunits in glycinin, were degraded. However, the basic subunit of glycinin still remained, and some polypeptide bands (around 10 kDa) were formed during the enzyme reaction. It is considered that the difficulty in degrading the basic subunit depends upon the substrate specificity of the enzyme. The degradation rate of soybean protein by this enzyme was affected by the ethyl alcohol concentration in the reaction mixtures. Namely, *Monascus*-proteinase gradually degraded the tofu protein (especially each subunit in β-conglycinin and the acidic subunit in glycinin) in the presence of ethyl alcohol in the moromi and formed polypeptides (around 10 kDa), peptides, and amino acids during the ripening. However, the enzyme could hardly degrade the basic subunit in glycinin, as shown in Fig. 5, section 4.1. Thus, it was concluded that *Monascus*-proteinase is a key enzyme for tofuyo ripening.

Proteinases, such as pepsin, chymotrypsin and trypsin, are frequently used in protein hydrolysis to obtain ACE inhibitory peptides. Microbial alkaline proteinases are also utilized in the production of ACE inhibitors from food proteins (Matsufuji et al., 1994; Matsui, 2003); however, there are few reports on the use of microbial acid proteinases for the production of ACE inhibitors. We investigated the production of ACE inhibitory peptides from soybean protein using *Monascus*-acid proteinase, prepared in our laboratory. Soybean proteins, β-conglycinin and glycinin were hydrolysed by the acid proteinase (Kuba et al., 2005). The degree of hydrolysis and ACE inhibitory activities increased with increasing proteolysis time. After 10 h of incubation, the IC_{50} values of β-conglycinin and glycinin hydrolysates were determined using ACE from pig lung as 0.126 mg/ml and 0.148 mg/ml, respectively. Four ACE inhibitory peptides were isolated; those from β-conglycinin hydrolysis were identified as LAIPPVNK and LPHF, and those from glycinin hydrolysis as SPYP and WL. It should be noted that WL has been purified from tofuyo extract described in section 4.4.1. Therefore, the result from this study suggested that ACE inhibitory peptides in tofuyo might have been derived from soybean protein by the action of *M. purpureus* acid proteinase. The inhibitory activity of SPYP markedly increased after successive digestion by pepsin, chymotrypsin and trypsin *in vitro*. The activity of the split peptide SPY, with tyrosine at the C-terminus, was expected to increase after digestion. However, that of LAIPVNKP markedly decreased after successive digestion by these gastrointestinal proteinases. Susceptibility to absorption, as well as resistance to digestion by gastrointestinal proteinases, is essential for the anti-hypertensive effect of ACE inhibitory peptides *in vivo*. Matsui (2003) found that 18 di- and tri-peptides derived from β-conglycinin, including WL, were absorbed intact via small intestine membrane transport in rats. WL, isolated from glycinin hydrolysis and tofuyo, is expected to exhibit an antihypertensive effect *in vivo*. The effect of tofuyo ingestion on ACE inhibitory activity is due to the relationship between peptide chemical structure and tofuyo metabolism. This is especially important from the viewpoint of bioactive peptide design, which must take metabolism into account. This enzyme not only contributes to the formation of ACE inhibitory peptides, but also has the potential to produce various bioactive peptides from food proteins.

5.2 Characterization and application of serine carboxypeptidases from genus *Monascus*

Carboxypeptidases are a kind of exopeptidases. The enzymes cleave peptide bonds at points within the proteins and remove amino acids sequentially from the C-terminus. We purified serine carboxypeptidases from *M. purpureus* (Liu et al., 2004a) and *M. pilosus* (Liu et al., 2004b) as homogenous preparations using SDS-polyacrylamide gel electrophoresis. The purified enzyme from *M. purpureus* was a heterodimer with a molecular mass of 132 kDa, consisting of two subunits of 64 kDa and 67 kDa. It was characterized as an acidic glycoprotein, with an isoelectric point of 3.67 and 17% carbohydrate content. The enzyme was strongly inhibited by piperastatin A, diisopropylfluoride (DFP), phenylmethylsulfonylfluoride (PMSF), and chymostatin, suggesting that it is a chymotrypsin-like serine carboxypeptidase. Benzyloxycarbonyl-l-tyrosyl-l-glutamic acid (Z-Glu-Tyr) was the best substrate for the enzyme. On the other hand, two serine carboxypeptidases, MpiCP-1 and MpiCP-2, were purified to homogeneity from *M. pilosus*. MpiCP-1 is a homodimer with a native molecular mass of 125 kDa, composed of two identical subunits of 65 kDa, while MpiCP-2 is a high-mass homooligomer with a native molecular mass 2,263 kDa, composed of 38 identical subunits of 59 kDa. This is unique among carboxypeptidases and distinguishes MpiCP-2 as the largest known carboxypeptidase. The purified enzymes were both acidic glycoproteins. MpiCP-1 has an isoelectric point of 3.7 and a carbohydrate content of 11%, while for MpiCP-2 these values were 4.0 and 33%, respectively. The optimum pH and temperature were 4.0 and 50°C for MpiCP-1, and 3.5 and 50°C for MpiCP-2, respectively. PMSF strongly inhibited MpiCP-1 and completely inhibited MpiCP-2, suggesting that they are both serine carboxypeptidases. Of the substrates tested, Z-Tyr Glu was the best for both enzymes. Substrate specificities of carboxypeptidases from *M. purpureus* and *M. pilosus* described above are consistent with the findings that glutamic acid is the one of the most abundant free amino acids found in matured tofuyo (Yasuda et al., 1993; Yasuda, 2010). Thus, carboxypeptidases from the genus *Monascus* are concluded to play an important role in the formation of taste-associated compounds (amino acids) from soybean protein during the maturation of tofuyo.

The action of pepsin and the admixture of pepsin and carboxypeptidase from the genus *Monascus* on the hydrolysis of soybean protein were studied (Liu & Yasuda, 2005). The results showed that the pepsin hydrolysates of soybean protein were much more bitter and contained relatively smaller amounts of total free amino acids than the hydrolysates obtained with the admixture of pepsin and the enzyme. In addition, hydrophilic and hydrophobic amino acids were present in almost equal proportions in the pepsin hydrolysates, while mainly hydrophobic amino acids made up the hydrolysates obtained with the admixture of pepsin and the enzyme. Thus, this enzyme not only plays an important role in the formation of taste-associated compounds, but also in the elimination of bitterness during the ripening of tofuyo.

The information obtained from the investigation may provide clues to the applicability of *Monascus* carboxypeptidase in the modification of soybean protein, and how bitterness is eliminated.

5.3 Characterization of glucoamylases from genus *Monascus*

Glucoamylases are exo-hydrolyzing enzymes that liberate glucose units from the non-reducing ends of amylose, amylopectin, and glycogen by hydrolysis of α-1,4-linkages in

consecutive monomers, producing d-glucose as the sole product. In tofuyo production, glucoamylases play an important role in providing taste to the product. Two forms of an extracellular glucoamylase, MpuGA-I and MpuGA-II, were purified to homogeneity from *M. purpureus* in our laboratory (Tachibana & Yasuda, 2007). The properties of the enzyme were summarized as follows. The molecular masses of these enzymes were estimated to be 60 kDa (MpuGA-I) and 89 kDa (MpuGA-II). These enzymes were characterized as glycoproteins, with a carbohydrate content of 15.0% in MpuGA-I, and 16.2% in MpuGA-II. The optimal pH was 5.0 for both enzymes, and the optimal temperatures were 50°C (MpuGA-I) and 65°C (MpuGA-II). The relative hydrolysis rates of various substrates by the purified enzymes were examined. Each final preparation showed the highest hydrolysis activity toward short polymerized amylose Ex-I having only the α-1,4-linkage. Amylopectin was moderately hydrolyzed by both the enzymes. The *K*m values for soluble starch were calculated to be 4.0 mg/ml (MpuGA-I) and 1.1 mg/ml (MpuGA-II). The primary structure of the N-terminal amino acid sequence of MpuGA-I shows 72.7% homology with that of MpuGA-II, and the N-terminal amino acid sequence of MpuGA-I showed high homology and similarity with other fungal glucoamylases that belong to the glucoside hydrolase family 15.

6. Conclusions

Tofuyo is a fermented tofu indigenous to Okinawa Prefecture, Japan. The origin of tofuyo is considered to be red furu, from China. It was created as a mellow and delicious food, as well as a low-salt health food. Various aspects of tofuyo production, including optimal conditions, tofu and koji preparation, as well as soaking and ripening, were clarified in our laboratory.

The main components comprising the body of tofuyo consist of basic subunit in glycinin and other polypeptides (*Mr.* 55 kDa, 11-15 kDa). The soybean proteins were digested into peptides and amino acids during maturation. Free glutamic acid and aspartic acid were strongly related to the desirable taste (*umami*-taste) of the product. The other hand, some of the peptides released from soybean protein in tofuyo have bioactivity. Angiotensin I-converting enzyme (ACE) inhibitors have an excellent effect on hypertension. Some of the peptides (IFL and WL) liberated from tofuyo inhibited the ACE activity that produces the vasopressor peptides. The systolic blood pressure of rats in the tofuyo group was significantly lower than that in the control group. Additionally, tofuyo might contribute to an improvement in erythrocyte deformability in mice.

Homogenous preparations of proteinase from *M. purpureus* and *M. pilosus* were characterized as aspartic enzymes. The enzyme plays an important role as a key enzyme for ripening of tofuyo. Furthermore, this enzyme not only contributes to the formation of ACE inhibitory peptides, but also has the potential to produce various bioactive peptides from food proteins. Homogenous preparations of carboxypeptidases from *M. purpureus* and *M. pilosus* were characterized as serine carboxypeptidases. These enzymes are involved to the release of amino acids from soybean protein and impart a pleasing taste to tofuyo. Furthermore, the enzymes also contribute to the removal of bitterness during the hydrolysis of soybean protein. Homogenous preparations of glucoamylase from *M. purpureus* were characterized. The enzymes contribute to the production of d-glucose from starch and play a role in the desirable taste of tofuyo.

Tofuyo is a health food that is cholesterol free, and is a low-salt fermented soybean cheese. The potential health benefits of tofuyo such as antihypertensive and hypocholesterolemic effects, erythrocyte deformability, antioxidative and DPPH radical scavenging activities are clarifying. Therefore, tofuyo is expected to widely spread as a health food. It would also be suitable for western people because it could be utilized in almost the same way as cheese. In order to spread tofuyo as health food, it is necessary to clarify the details of functional properties in this product. The author expects that tofuyo will develop into the health food of the world in the future.

7. References

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19

Signals in Soybean´s Inoculants

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1. Introduction

Legumes are an important component of all agricultural systems because of the nitrogen fixation provided by their root nodule bacterial symbiont, rhizobia. Among the legumes, the Soybean, also classed as an oilseed, is pre-eminent for its high (38-45%) protein content. Production of high-quality, protein-rich food is extremely dependent upon availability of sufficient N. Even though N is among the most abundant elements on Earth, it is the critical limiting element for growth of most plants due to its unavailability (Graham & Vance, 2000). Plants acquire N from two principal sources: the soil, through commercial fertilizer, manure, and/or mineralization of organic matter; and the atmosphere through symbiotic N_2 fixation. Symbiosis between leguminous plants and rhizobia, under conditions of nitrogen limitation, leads to the development of new plant organs, the N2-fixing nodules, which are usually formed on roots but also on stems in a few plants after the diazotroph and its host positively recognize each other. The root nodule is the site where N2 gas is reduced to ammonia, which is assimilated into amino acids; these are then used to synthesize other nitrogen-containing compounds. Inside the nodule the differentiated form of rhizobia, the bacteroids, fix molecular nitrogen, which is then used by the plant partner. Effective, nitrogen-fixing rhizobium–legume symbiosis requires an intricate molecular dialogue between the two interaction partners before and during invasion by the microsymbiont (Broughton et al., 2000; Perret et al., 2000). Host specificity is determined by several factors. From the bacterial side, the main signalling molecules are Nod factors, surface polysaccharides and secreted proteins. This chapter outlines an overview of the role of major determinants and signals in the symbiotic process, as well as some results obtained by the authors using *Bradyrhizobium* induced inoculants and its effect on soybean development.

2. Nod factors

The symbiotic interaction starts when the bacteria colonize the root surface and induce curling of the root hair tips (Long, 1996; Schultze et al., 1994). This is followed by cell wall invagination and the formation of an infection thread that grows within the root hair. The infection thread traverses the outer cell layers to reach the nodule primordium, which is

initiated by the reactivation of differentiated cells of the root cortex for division. Within the infection thread the rhizobia multiply but remain confined by the plant cell wall. As the primordium develops to a nodule, bacteria are released from the tip of the infection thread by endocytosis and differentiate into bacteroids surrounded by the peribacteroid membrane. That symbiotic interaction involves an exchange of complex molecular signals that confer specificity. Legume roots and seeds exude different substances: sugars, amino acids, dicarboxilic acids and various aromatic compounds such as some flavonoids (Brencic & Winans, 2005), in mixtures that differ between species. Rhizobia respond to these because they have one or more *nodD* genes, which encode regulator proteins that activate the other *nod* genes when they interact with appropriate plant signal compounds. Once activated, the *nod* genes direct the synthesis of Nod Factors (NF), a family of lipochitin oligomers (LCO), which acting as morphogens, initiating the nodulation program of the host plant (Schultze & Kondorosi, 1998; D'Haeze & Holsters, 2002). Structurally, all Nod factors are based on short chains of β 1,4-linked N-acetylglucosamine residues. The distal or non-reducing glucosamine residue is N-acylated. This common lipo-chitooligosaccharide core (produced by the enzymes encoded by the *nodABC* genes) may be modified by various specific substituents on the distal or reducing sugars. These modifications are governed by additional *nod* genes. Rhizobial species and genotypes differ in their complement of *nod* genes and in allelic forms of shared *nod* genes, and these differences lead to predictable differences in the structure of the Nod factors. NFs from several rhizobial species have been characterized and their structures have been determined. *Bradyrhizobium´*s LCO are produces mainly in response to flavonoids. For instance, the isoflavones daidzein and genistein, the main components present in soybean root extracts, are responsible for inducing the *nod* genes of *Bradyrhizobium japonicum* (Kosslak et al., 1987). Nodulation leads to the colonization of plant cells by invading bacteria. Although many host plants and effective rhizobia have the ability to enter into symbiosis with more than one partner, only certain combinations of symbionts result in the formation of nitrogen-fixing nodules. Ineffective associations lead to empty or nonfixing bacteroid-containing nodules. Specificity among compatible partners minimizes the chances of infection by pathogens and the formation of ineffective associations that are detrimental to both symbionts. Experimental evidence suggests that the progression of invasive rhizobia towards nodule primordia is challenged at various "doors". Codes contained in molecular signals open these checkpoints. During the initial phases of nodulation (root hair curling and bacterial entry), these codes are given by flavonoids (from the plant) and Nod factors (from the bacteria). In both cases, NodD proteins are the chief interlocutors of molecular traffic in the rhizosphere. Many studies have implicated Nod factors as a possible candidate in the host specificity of rhizobia, inducing several responses in plant until complete nodule (Vijn et al., 1993; Stokkermans & Peters, 1994; Heidstra et al., 1997; Spaink, 2000; Geurts et al., 2005). They invoke multiple physiological responses in the host, such as: root hair deformation (Lhuissier et al., 2001), induction of nodulin genes essential for infection thread formation and cortical cell division (Schlaman et al., 1997). Other authors have been found that Nod factors reduces the salicylic acid (SA) level in roots and this might help in the suppression of host defense responses, thus ensuring successful infection by rhizobia (Martinez-Abarca et al., 1998). Similar decreases in SA level in the leaf tissues occurred when soybean plants were sprayed with Nod factor (Prithiviraj et al., 2000). On the other hand, Nod factors from *Bradyrhizobium* have been shown to enhance seed germination and early seedling growth of its target plant, soybean, but also enhances these parameters in non-target plants from

diverse botanical families (Prithiviraj et al., 2003; Miransar & Smith, 2009). This way, nodulation factors or LCO have been considering the key to open door legumes, the first and main determinant (Relic et al., 1994, Long, 1996; Broughton et al., 2000; de Haeze et al., 2002; Gage, 2004).

3. Polysaccharides

Together with Nod factors, other bacterial components have been involved in bacterial adhesion, formation and extension of the infection thread, releasing of bacteria into the nodule cells and differentiation into bacteroids. Between these components, polysaccharides have a relevant role. Rhizobia synthesize different classes of polysaccharides: exopolysaccharide (EPS), capsular polysaccharides (KPS), lipopolysaccharides (LPS) and the cyclic glucan. Some of them are secreted to the media, others are exposed on the surface or present in the periplasmic space (Lepek & D´Antuono, 2005). EPS appears to be essential for the successful invasion of indeterminate nodules (Pellock et al., 2000), whereas LPS are involved principally in the formation of determinate nodules, especially during the initiation and elongation of the infection thread (Lerouge & Vanderleyden, 2001; Noel et al., 2000). Both, in determinate and indeterminate nodule formation, the absence of cyclic glucan synthesis affect the invasion capacity of the bacteria (D´Antuono et al., 2005). *Bradyrhizobium japonicum* strain 2143 and two derivative strains are capable of producing three exopolysaccharides that appear to be involved in the efficiency of their symbioses with *Glycine max* (Karr et al., 2000), and *B. japonicum* strain USDA 123 produces two structurally distinct polysaccharides, one when outside the nodule and the second when inside the nodule (An et al., 1995). Additionally, the symbiotic defects of EPS-deficient mutants of *B. japonicum* strain 110*spc*4 are host dependent, differing markedly on the hosts *Glycine max* and *Glycine soja* (Parniske et al., 1994). The results obtained by different groups suggest a possible role in two main points in the process, the bacterial relation with the defense response generated in the plant and the intimate interaction between bacterial and plant cell membranes. Therefore, polysaccharides are critical for the establishment of a productive plant-bacterium symbiosis, what become it in the second determinant of this symbiosis.

4. Protein secretion systems

Besides to previous host specificity determinants described (Nod factors and surface polysaccharides); there is a third class of rhizobial signals that can affect symbiosis between *Rhizobium* and Legume. It consists of secreted proteins. Cells from prokaryotes and eukaryotes alike must transport proteins across the membranes that envelop them (Economou & Dalbey, 2004). In Gram-negative bacteria, as *Rhizobium*, the transport is more complicated due to the presence of two barriers, the inner and outer membrane (IM and OM). Several secretion systems seem to have specialized in mediating *Rhizobium*-legume interactions, with the ability to translocate effector proteins into the host cell cytoplasm as a defining feature. These systems include the T3SS, T4SS and T6SS (Papanikou et al., 2007). T3SS are complex macromolecular structures that span not only the IM and OM, but the host cellular membrane as well. They allow direct translocation of secretion substrates into the host cell cytoplasm. The secretion signal is poorly defined and located in the amino-terminus of secretion substrates (Ghosh, 2004; He et al., 2004). Proteins secreted through rhizobial T3SS are called nodulation outer proteins or Nops. The first secreted rhizobial

protein for which a role in symbiosis could be shown was *Rhizobium leguminosarum* bv. viciae NodO (de Maagd et al., 1989). NodO was detected in spent medium of cultures grown in the presence of flavonoids and expression was found to be NodD dependent (de Maagd et al., 1988). These are also found in promoter regions of genes unrelated to T3SS, such as those involved in the biosynthesis of rhamnose-rich polysaccharides (Marie et al., 2004). This led to the discovery of a complex interplay between the T3SS and surface polysaccharides in the molecular dialogue of the rhizobium–host interaction (Broughton et al., 2006). In *Rhizobium* species strain NGR234 at least six T3SS-secreted Nops have been identified: NopA, NopB, NopC, NopL, NopP, and NopX (Ausmees et al., 2004; Deakin et al., 2005; Marie et al., 2003; Saad et al., 2005; Viprey et al., 1998). Depending upon the legume host, abolition of Nop secretion by NGR234 can improve or block symbiotic interactions. T3SS genes were subsequently identified in *B. japonicum* USDA110, *Sinorhizobium fredii* strains HH103 and USDA257, *Mesorhizobium loti* MAFF303099 and *R. etli* CNPAF512 (Kaneko et al., 2000; Krause et al., 2002; Krishnan et al., 2003; Hubber et al., 2004; de Lyra et al., 2006). In each case, the T3SS affects symbiosis in a host-specific manner. Taking into account that T3SS have previously related to microbe-host interaction could be possible that rhizobia use the exact same secretion mechanisms as their pathogenic counterparts in trying to persuade prospective hosts to allow rhizobial invasion (Fauvart & Michiels, 2008).

5. Inoculants to soybean

Dinitrogen fixation provides more N to the agricultural ecosystems worldwide than the total amount of fertiliser N applied. Soybean plants can fix nitrogen at rates of up to 200 $kg.ha^{-1}.year^{-1}$, eliminating the need for environmentally and economically costly nitrogen fertilizers (Ip et al., 2001). When soybeans are cultivated for the first time, inoculation with bradyrhizobia is essential for high yields. A number of different types of soybean inoculants are available. Advancements in technology have provided inoculant types with higher rhizobia concentrations and more options for planting systems. Due to the importance of nodulation for agriculture, intensive researching is being carried on in this area in order to understand the molecular bases of this process. The new knowledge could be used to obtain more efficient nitrogen fixation process, modification of the host range or increased competitiveness that may influence its capacity to compete in the rhizosphere with other bacteria. Better N2-fixing symbiosis may be brought about by manipulating both rhizobia and plant hosts and by eventually creating an artificial rhizosphere. An important aim is also to improve the symbiotic relationship in suboptimal environmental situations related to environmental stress. Plant nodulation and nitrogen fixation processes in nature are affected by the micro-ecology of the plant rhizosphere. Soil temperature, pH, texture, moisture, salinity, and deficiencies in essential elements inhibit all stages of symbiotic establishment investigated to date (root hair curling, infection thread formation and penetration, nodule formation and function) (Zahran, 1999). The infection and early nodule development processes are most sensitive to stressful environmental conditions. Although combinations of rhizobia and plants may be compatible, nodulation failure can still occur in the field (Robson & Bottomley, 1991). For example, the exudation of flavonoid compounds from clover roots required for *nod* gene induction in *R. leguminosarum* bv. *trifolii* was reduced when the plants were grown at pH<5 (Richardson et al., 1988). The presence of nitrogen in the root rhizosphere also limits the nodulation of legumes (Streeter, 1988), while nitrogen (as ammonia) has been shown to limit the induction of the *nodABC* genes (Dusha et al., 1989). In

the case of soybean, the time between inoculation and onset of nitrogen fixation is delayed by 2–3 d for each degree decrease in temperature from 25°C to 17.5°C. However, when the root zone temperature drops below 17.5°C, the onset of nitrogen fixation is sharply delayed by 7 d for each degree decrease (Zhang et al., 1995). Low temperature was also found to decrease both the biosynthesis of isoflavonoids and the excretion of these signal compounds from plant root cells to the rhizosphere (Zhang et al., 1995). Inoculating soybean with preinduced *B. japonicum* improved soybean nodulation and shortened the time between the onset of nitrogen fixation under low root zone temperature conditions (Smith & Zhang, 1999).

5.1 The culture media

Every organism requires finding all necessary substances for cellular biosynthesis and energy generation during its life. The culture media constitute the micro world of microorganisms in laboratory conditions. The design of a culture medium must answer the exigencies of specific bacteria and the finality we followed with it reproduction, since the composition may influence different microbial physiologic aspects: nutrition, multiplication and primary or secondary metabolites production (Bernal et al., 2002). Decreased legume grain yields, as compared with growth of the same crop with N fertilizer addition, have been reported. This is generally associated with poor-quality inoculants, with low numbers of bacterial cells (Hume & Blair 1992; Singleton et al., 1997; Lupwayi et al., 2000; Catroux et al., 2001; Supanjani et al., 2006). Also, abiotic stress factors can cause poor nodulation in the presence of otherwise compatible symbionts. Early events in the symbiosis such as signal production and excretion, rhizobial attachment, root hair curling, infection thread formation, and nodule initiation, are particularly sensitive to some stresses (Duzan et al., 2004). The fact that commercial inoculants generally compete poorly against indigenous strains (Loh & Stacey, 2003), have incited the continuous work of commercial industrial to provide high quality rhizobial inoculants for agricultural production. In that sense, our group has been working in the obtainment of soybean´s inoculants which contain as base a good culture media to guarantee the optimal multiplication of bradyrhizobia, the specific inducers to assurance the activation of all determinants in the symbiosis success and as result a product with good quality to be translated into agronomical sustainable benefits.

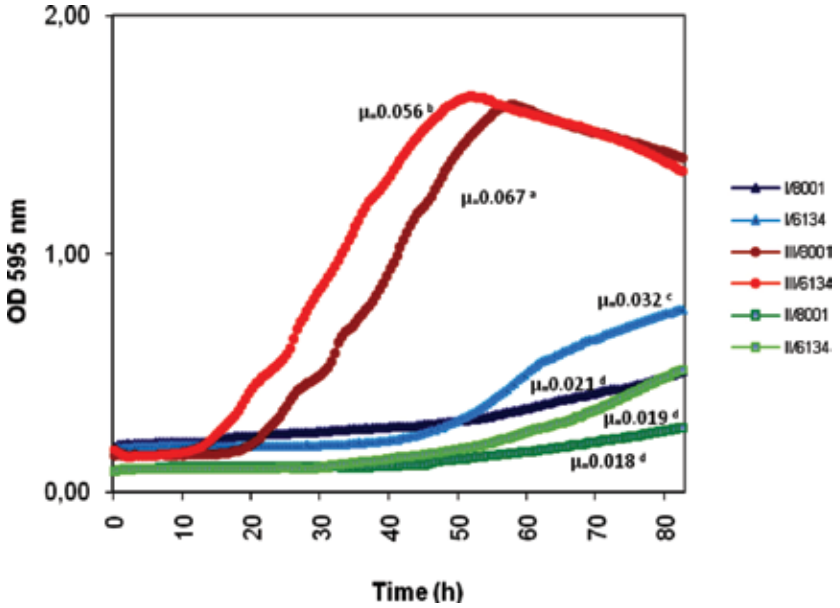
5.1.1 The culture media in multiplication

Our first studies evaluated different culture medium composition on multiplication of two *Bradyrhizobium elkanii* strains (*B. elkanii* ICA 8001 and *B. elkanii* LMG 6134). We use traditional media reported to bradyrhizobia (Vincent, 1970, López, 1990) and a new composition registered as Cuban Patent No. 22 797 (Nápoles, 2002). The strains were cultivated during 6 days at 30ºC and 230 rpm in orbital shaker conditions. The multiplication rate was measured by Bioscreen C (Labsystems, Helsinki, Finlandia), at 595 nm. The optical density was calculated for each time and the growing specific velocity was determined in lineal phase of growing by:

$$\mu = \frac{\ln(OD_2 / OD_1)}{(t_2 - t_1)}$$

The results shown clear differences between media compositions used for the two strains (Nápoles, et al., 2006). Not only cell density, but growth specific rates were higher with the

new medium proposed in both strains (figure 1). The components of a new culture medium for *Bradyrhizobium*, which contain different carbon, nitrogen and other nutrient sources, allow obtaining more cells at the same period of time.



ES = 0.0027, p≤ 0.001, n=5
μ: growth specific velocity

Fig. 1. Growth dynamic of *B. elkanii* ICA 8001 and *B. elkanii* LMG 6134 in three culture media (I and II traditional media, III new composition of culture media)

5.1.2 The culture media in Nod factors induction

Nod Factors biosynthesis by rhizobia is dependent on several factors. Therefore, the composition of the medium in which the rhizobia are grown is likely to affect NF production in a qualitative and quantitative manner. To compare the three culture media on the *nod* genes activation in *Bradyrhizobium elkanii* ICA 8001, we use two ways: β glucuronidase assay and Nod factor production. Triparental mating was carried out using the donor strain DH5α/pGUS and the helper strain HB101/pRK2013 from *E. coli* and the *B. elkanii* wild-type strain as an acceptor as described in (Hahn & Hennecke, 1984). *B. elkanii* conjugation mixtures were plated out on peptone-yeast extract medium with Km and Nal (30μg.ml^{-1}) to select for the *Bradyrhizobium* colonies harbouring pGUS32Km. Quantitative analysis of GUS A activity was then carried out with *p*-nitrophenyl-ß-D-glucuronide (pNPG) as the substrate in microtiter plates and GUS A activity was examined in VERSA max microplate reader (Molecular Devices). To determination of Nod factors profile, the nodulation factors were radioactively labelled and they were isolated by following a slightly modified protocol of (Laeremans et al., 1998). 100μL from *Bradyrhizobium* cultures, growth for two nights, were inoculated in 900μL of each fresh culture medium and the concentration was adjusted to 5x10^{8} CFU per medium milliliter. They were pre-incubated to 30°C with agitation, during 1h. Each sample was supplemented with genistein 10μM as inducer and incubated during 2 hours at the same temperature and agitation. After the

induction the isotopic label was carried out adding 125 µL of ^{14}C [2-^{14}C] acetic acid as sodium salt. The cells were labelled for 36h. The nodulation factors were isolated twice with 500µL n-butanol and washed with ethyl acetate. The solution was vacuum-dried and samples were applied on reverse-phase TLC plates (RP-18 F_{254s}, Merck). H_2O/acetonitrile (1:1, vol/vol) was used as the mobile phase. The radioactivity was visualized by autoradiography using Hyperfilm- β max (Amershan Life Sciences) after 4 days of exposure. The results show a correspondence between the *nod* genes expression by ß-glucuronidase activity and by Nod factors production determinate in TLC and autoradiography (Table 1).

Culture Media	Medium I		Medium II		Medium III	
	(A)	(B)	(A)	(B)	(A)	(B)
Miller units	-6.384		1.669		17.676	
	-5.407		1,360		22.209	
	-6.138		1.891		18.433	
	-5.990		1,428		17.831	
	-5.949		1.241		48.96	
	-6.006		1.507		33.619	
	-5.381		1.554		32.685	
	-5.778		1.482		33.222	
	0		1.587		19.037	
	0		1.446		37.121	
AV	**0**		**1.517**		**28.079**	

Table 1. Effect of different medium compositions on ß-glucuronidase activity (A) and Nod factors synthesis (B).

Medium I did not expressed GUS activity and the TLC showed no spots of Nod factors produced. Medium II induced a low enzymatic activity and two or three spots of Nod factors, while Medium III induced a major ß-glucuronidase activity and correspondently a high Nodulation factors production with at least five different structures of this biomolecules (Nápoles, et al., 2003). Then, another important characteristic of a culture medium is the possibility to contain natural substances which induce the *nod* genes in the bacteria to increase the nodulation factors concentration in the inoculum. A positive effect of genistein addition, as a *nod* gene inducer to *B. japonicum* inoculants on soybean grain and protein yield (Zhang & Smith, 1996), nodulation efficiency (Pan et al., 1998), N2 fixation and total N yield at low root zone temperatures (Zhang & Smith, 1997) has been reported.

5.2 The inoculants activity in plants

To corroborate the efficiency of different inocula derivated from culture in different media assays in plants were performanced. In the experimental set-up for *in vitro* evaluation, plants were grown in the plant growth room with a 12h photoperiod (day/night temperature 26ºC/22ºC; relative humidity 70%) as described by (Michiels et al., 1998). Four weeks after inoculation, nitrogen fixation capacity of the inoculated plant was determined by means of the acetylene reduction assay (ARA) using a gas chromatographer (5890 A; Hewlett-Packard, equipped with a "PLOT fused silica" column). Other parameters such as the number of nodules and fresh and dry weights of nodules per plant were determined (Nápoles et al., 2005). The obtained results favor inoculants grown in medium III since not only more nodules were formed, but also a higher ARA was measured (table 2).

Culture medium	Number of nodules per plant	Fresh weight of nodules per plant (g)	Dry weight of nodules per plant (g)	ARA (µmolethylene. $plant^{-1}.h^{-1}$)
Medium I	13.3 c	0.16 c	0.02 b	1.9 c
Medium II	26.3 b	0.21 b	0.05 b	4.1 b
Medium III	55.0 a	0.37 a	0.09 a	7.8 a
Control	0 d	0 d	0 c	0 d
SEx	2.87***	0.03**	0.007***	1.07**

Values followed by the same letter are not significantly different ($p \leq 0.05$).

Table 2. Effect of different media on nodulation and nitrogen fixation.

For experimental set-up of field trials, at harvest (90 days after sowing), plant heigh, number of pods, overall yield and weight of 100 grains were determined in two different soils.

Treatments	Height (cm)	Pod number per plant	100 grains weight (g)	Yield ($t.ha^{-1}$)
Medium II	70.67	30.40	12.0 b	1.26 b
Medium III	76.47	40.87	16.0 a	1.46 a
SEx	1.85 ns	3.29 ns	0.86**	0.04**

Values followed by the same letter are not significantly different ($p \leq 0.05$).

Table 3. Effect of different culture media on soybean yield, analyzed in a red ferralitic, compact and saturated soil.

The superior effectiveness of medium III was corroborated. Table 3 shows a 15.8% yield increase when medium III was applied in the saturated soil type. In the case of unsaturated soil, a 14.4% yield increase was observed (table 4). Clearly, the positive effect of the culture medium on nodulation resulting in higher N2-fixation per plant is reflected by a higher yield in field trials.

Treatments	Height (cm)	Pod number per plant	100 grains weight (g)	Yield ($t.ha^{-1}$)
Medium II	109.92 b	53.40 b	11.75 b	2.08 b
Medium III	114.12 a	66.82 a	15.5 a	2.38 a
SEx	1.05*	2.92**	1.01*	0.07*

Values followed by the same letter are not significantly different ($p \leq 0.05$).

Table 4. Effect of different culture media on soybean yield, analyzed in a red ferralitic, compact and unsaturated soil.

5.3 The inocula optimization

Bradyrhizobium elkanii ICA 8001 is used in Cuba for soybean inoculants production. The employ of *Bradyrhizobium* inoculants have been supplied between 80 and 100% of nitrogen requirement of this crop (Treto et al., 2005). After many years of research, (Pijeira & Treto, 1983; Pijeira et al., 1988) recommended the inoculation of this strain for numerous Cuban and introduced commercial soybean varieties. The culture medium design plays a crucial role for high cell density inoculants obtaining. The presence of specific nutrients in the culture medium, as well as their concentrations determine the good cells functioning and

influence on cellular metabolism, promoting the biomass or others specific metabolites production. On fermentative microbial process it is necessary culture medium and environmental conditions optimization to exploit completely the potential of the selected strains (Parekh et al., 2000). The choice of the adequate statistical tools is very important to save time and resources. Single variable optimization methods are not only tedious, but can also lead to misinterpretation of results, especially taking into account that the interaction between different factors is overlooked (Abdel-Fattah et al., 2005). Statistical experimental designs have been used for many decades and can be adopted on several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response (Kim et al., 2005; Lee & Gilmore, 2005; Nawani & Kapadnis, 2005; Senthilkumar et al., 2005; Wang & Lu, 2005; Gómez & Batista, 2006). The carbon, nitrogen and phosphorus sources are macronutrients of vital importance on cellular growth and maintenance, for this reason the influence of these compounds individually and their combinations on *B. elkanii* ICA 8001 cellular multiplication was evaluated by using Response Surface Methodology (RSM), a central composite design (CCD) and statistical analysis. The experiments were carried out at orbital shaker level and the cellular growth was expressed in colony forming units per ml of culture sample. The optimization process finished with the obtaining of a new optimized culture medium for *B. elkanii* ICA 8001 cellular multiplication. A value of 10^{11} CFU/ml with the optimum medium was reached, increasing in one level the value of viable cells obtained with the reference medium (figure 2). The new concentrations varied remarkable respect to reference medium, carbon source 2 concentrations increased in a 43%, phosphorus quantity increase in a 35% and carbon source 1 concentrations in a 5.2%, nevertheless the nitrogen concentration decreased in a 30%. This increase on carbon 2 source concentration promoted the growth of this bacteria, explained by its high contribution of assailable compounds to the cells, in the same way the phosphorus sources increasing, determinating a higher biomass production. The CCD method was efficient, because the optima concentrations of the three independents variables were determined with only 17 trials, which allowed the increasing of bacterial growth in a 10%. Besides, the design evaluated the concentrations interaction effect on the variable response.

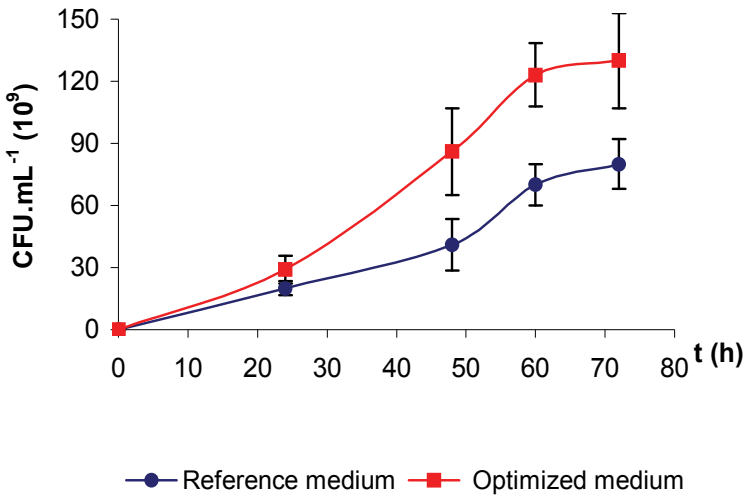


Fig. 2. Time course of cell density of *Bradyrhizobium elkanii* ICA 8001 in Medium III (Reference) and optimized medium, at 28 ± 2º C and 100 rpm.

Numerous are the works that demonstrated the Central composite design affectivity in the culture media optimization for microorganisms. (Lee et al., 1998) used this design for cholesterol oxidase enzyme production by *Rhodococcus equi* no. 23 and (Bhosale & Gadre, 2001), for the growth and carotenoids production by mutant 32 of *Rhodotorula glutinis*. The combination of complete and fractional designs is an effective tool in the optimization process, because they complement each other and permit to achieve significant response variables enhancing. For example, (He et al., 2004) employed a fractional factorial design to evaluate the effect of glucose, pectin, soybean cake extract, casein, corn flour, ammonium sulphate, sodium bicarbonate concentrations and initial pH on the growth of the probiotic strain *Clostridium butyricum*. Then the optimal concentrations of these compounds were found by a CCD. After 24 h of fermentation in the optimized culture medium a viable bacteria population of 10^9 CFU/ml was reached.
It is possible also to optimize a culture medium composition in order to achieved higher cell concentration, as well as, higher Nod factor production (figure 3).

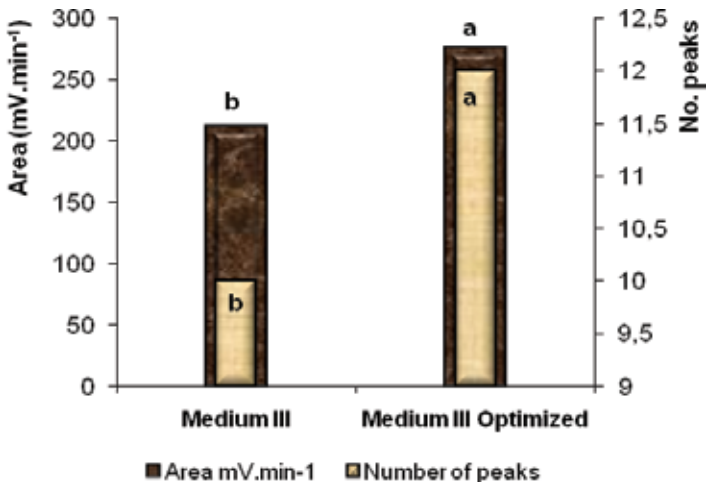


Fig. 3. Nodulation factor values of area under curve and number of peaks for Medium III before and after optimization. (Duncan, $p<0,05$). $SE_{picks}= 0.036$; n=3. $SE_{Area}= 36,45$; n=3.

In this sense, each inducer component of medium III and it concentration was optimized using similar methods to obtain a higher Nod factor production, this time evaluated by High Performance Liquid Chromatography (HPLC). The Nod factors purified were dissolved in 100% acetonitrile and then injected into a normal phase column Ultropac TSK OH-120 5um LKB with dimensions of 4.6 x 250 mm. The flow rate 1 $mL.min^{-1}$; as solvent were used: acetonitrile (A): water (B); the detector: an UV spectrophotometer at 206 nm and a cell of 10 mm, the Gradient in a Knauer pump: 0/0 10/0 70/20 t/%B and with an Injection of 250 µL in 100% acetonitrile. The run time in all cases was 70 minutes. The chromatographic profile of the Nod factors was analyzed according to the number, distribution and relative intensity of the obtained peaks. The number of peaks, corresponding to Nod factors and their area were higher when medium was optimized. The use of the optimization strategy allowed the obtaining of a new culture medium, with a nutrients and inducers balance, which guarantied the obtaining of high density cellular inoculums and the production of high Nod factors concentration. The fact that this

composition medium improves the growth and induction of this slow-growing rhizobial bacterium makes the *Bradyrhizobium* inoculants production a cheaper and efficient fermentative process.

5.4 Inoculants induced and the effect on drought stress

The soybean is a crop mainly grown under rain fed conditions although irrigation is increasingly being used. As with other grain legumes, soybean is very sensitive to drought stress which leads to reduced yield and seed quality (Bosniols et al., 1986; Frederick et al., 2001; Purcell et al., 2004). Negative effects of water stress on growth, photosynthesis, and photoassimilate translocation in soybean were demonstrated by (Ohashi et al., 2000) and (Fulai et al., 2004). The symbiosis process is also negatively affected by water stress, leads to decreased nodule formation, reduced nodule size and N fixation (Serraj et al., 1999; King and Purcell, 2001; Serraj, 2003; Streeter, 2003; Tajima et al., 2004). Negative effects of water stress on growth, photosynthesis, and photoassimilate translocation in soybean were demonstrated by (Ohashi et al., 2000) and (Fulai et al., 2004). The symbiosis process is also

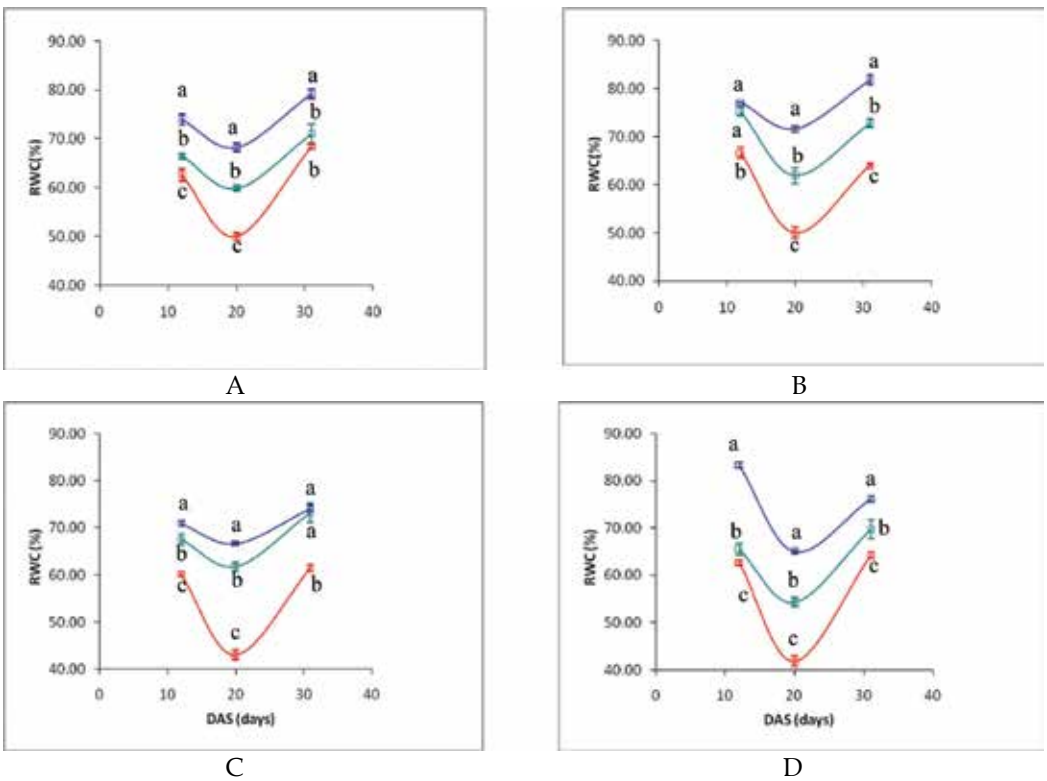
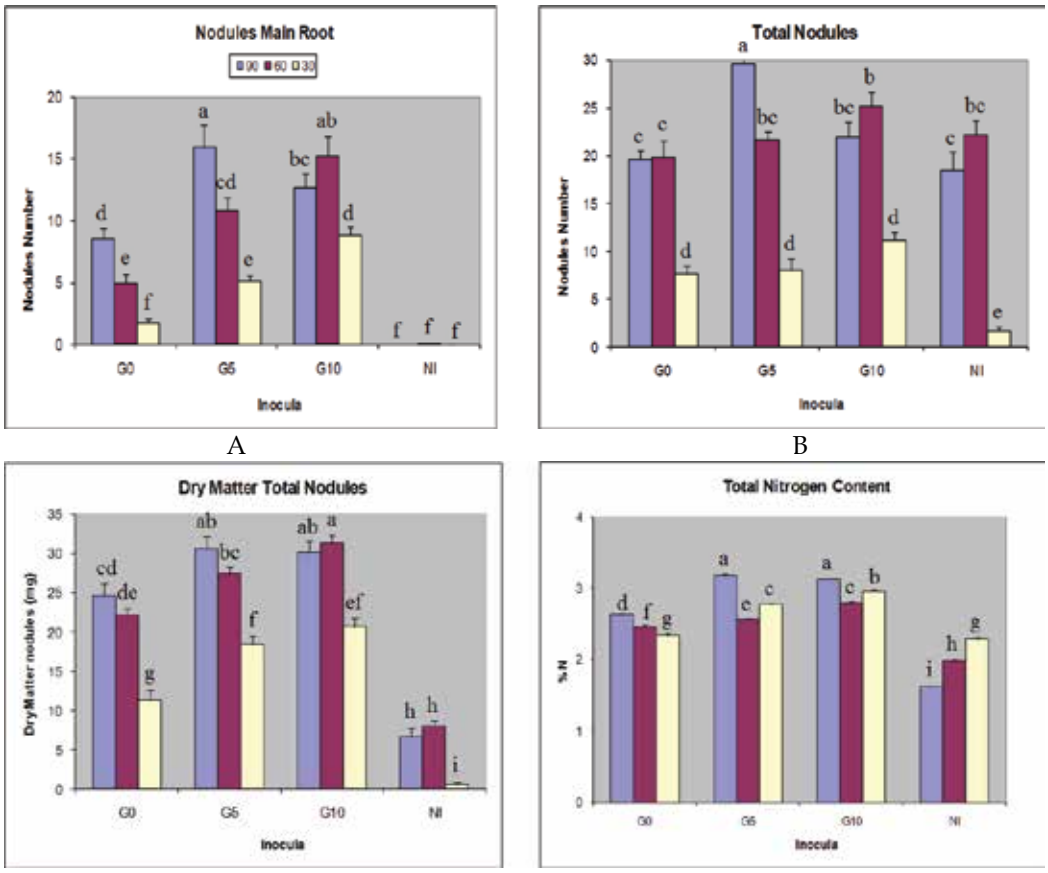


Fig. 4. Relative Water Content in plants inoculated (A: medium without induction, B and C: medium supplemented with genistein 5 and 10 µM, respectively) and non inoculated (D) with three irrigation levels (red: 30% field capacity, green: 60% field capacity, blue: 90% field capacity) during 12, 20 and 31 days after sowing (DAS). Vertical bars indicate ± standard error (SE). Treatments with different letters are significantly different, ANOVA (p<0.05) $LSD_{0.05}$.

negatively affected by water stress, leads to decreased nodule formation, reduced nodule size and N fixation (Serraj et al., 1999; King and Purcell, 2001; Serraj, 2003; Streeter, 2003; Tajima et al., 2004).

The isoflavone genistein have been recognized as a powerful inducer of Nod factors production by *Bradyrhizobium* and its addition to inocula has been shown to increase nodule number and promote soybean nitrogen (N) fixation at low temperatures. The impact of lipochitinoligosaccharids spray application on the physiology and productivity of water stressed soybean plants was evaluated by (Atti et al., 2005). Foliar application of Nod factors affected plant physiological activity, increased flower and pod numbers, and accelerated leaf senescence of soybean plants under water stress. Our study looks for answers about the possible role of genistein in countering the stress on nodulation produced by water deficit in soybeans.



G0: medium without induction, G5 and G10: medium supplemented with genistein 5 and 10 μM, respectively. NI: control non-inoculated.
Treatments with different letters are significantly different, ANOVA (p<0.05) $LSD_{0.05}$.

Fig. 5. Effect of different inocula in plants growing at three humidity conditions on nodulation and nitrogen accumulation.

We study the influence of three levels of water content on plants inoculated with different treatments: a conventional inoculum and two other previously induced with genistein. The experiment guaranteed severe and moderate drought stress condition in some plants (figure 4). The lowest values of Relative Water Content to 20 days to every treatment suggest that period when nodules were formed corresponded to the most critic phase of stress. The highest soil moisture level guaranteed better nodulation and a higher efficiency of this process were modulated by the inducer. (Williams & De Mallorca, 1984) demonstrated that the magnitude of stress effects and the rate of inhibition of symbiosis usually depended on the growth and development phase, as well as stress severity. In their results, mild water stress only reduced nodule number on soybean roots. Moderate and severe water stress reduced both nodule number and size. Our results showed a positive effect of genistein on nodulation, its efficiency and contribution to plant N nutrition at all soil moisture levels and was specially marked under the adverse conditions of drought stress (figure 5). Extensive research has focused on decreasing yield losses during soybean crop production. (Atti et al., 2005) found that foliar application of lipochitinoligosaccharides on soybean gave a positive effect on growth under moderate stress. Their results agree with ours, considering that they used LCO direct. In our work it was used as a *nod* gene inducer, which led to synthesis of Nod factors in the inocula. The effect of water lack on nodulation has been extensively documented (Franson et al., 1991; Sellstedt et al., 1993; Serraj et al., 1999). It is important to produce inoculants which have been obtained from induced media, because they will not only increase nodulation and N fixation, but can also help under adverse conditions of water stress. Other factors may be considered, such as plant growth stage. (Peña-Cabriales & Castellanos, 1993) found that water stress during vegetative growth was more detrimental to nodulation and N fixation than at the reproduction stage. In conclusion, after evaluating the effect of genistein as an inducer of *Bradyrhizobium japonicum* inoculants under water stress it was possible to show an important influence of this isoflavonoid on reducing the effect of water stress on nodulation (Nápoles, et al., 2009).

Many works have been conducting to understand the physiological mechanisms involved in soybean plants subjected to drought stress: leaf photosynthetic rates, carbohydrate concentrations, soluble invertase activities (Fulai et al., 2004), proline accumulation (Kolh et al., 1991; Curtis et al., 2004) among others. (Serraj et al., 1999) established that drought stress leads to a decrease of nitrogen fixation capacity, mainly as a consequence of ureides accumulation in shoots and asparagine in nodules. Several mechanisms have been reported to be involved in the physiological response, carbon shortage and nodule carbon metabolism, oxygen limitation, and feedback regulation by the accumulation of N fixation products, which results in poor nodulation and reduced amounts of fixed N (Zahran, 1999; Kurdalai et al., 2002; Serraj, 2003). Modifications in the activity of key nodule enzymes such as sucrose synthase and isocitrate dehydrogenase and in nodular malate content also occur. The decline in nodule water potential results in a cell redox imbalance (Marino et al., 2007). (Ladrera et al., 2007) demonstrated that drought reduced carbon flux and N accumulation in nodules, but not in shoots. Our group studied the ureides level in soybean treated with different inoculants in response to water deficit (Freixas et al., 2010). Soybean plants were firstly grown in nutrient solution for 20 days. Afterward, they were drought stressed for a 20 days period adding 10% polyethyleneglicol (PEG) 6000. *Bradyrhizobium elkanii* ICA 8001 was the strain used to inoculate soybean plants on this experiment, which was separately grown on three culture media, two of them induced in nodulation factor production. A statistically significant increase of ureides level in leaves and nodules was observed in plants with water

deficit and inoculants without nodulation factor induction (NFI). However, this increase was not observed in plants with water deficit and inoculants with NFI. These results suggest an important role of Nod factors also on ureides level regulation in soybean under drought conditions.

5.5 Inoculants and oligosaccharines

Oligosaccharines exert proven biological effects on the growth and development of plants and induce the expression of a variety of genes involved in defensive responses. The oligosaccharide part of the Nod factor structure from Rhizobium is also responsible for inducing cortical cell divisions of the root leading to form the nodular primordium. We study the influence of two oligosaccharines (partially hydrolyzed chitosan and a mixture of oligogalacturonides) on *Bradyrhizobium elkanii* ICA 8001 multiplication and on nodulation in soybean. The chitosan polymer was obtained by basic deacetylation of lobster chitin (Ramírez et al., 2000) and hydrolyzed for 24 h with Pectinex Ultra SP-L. The mixture of oligogalacturonides with a degree of polymerization between 7 and 16 residues of galacturonic acid was obtained from citrus pectin (Sigma), according to the methodology of (Cabrera, 2003). The partially hydrolyzed chitosan did not inhibit the *Bradyrhizobium* multiplication, whereas oligogalacturonic acid reduced the viability of the strain. The number of nodules developed showed the best results with the chitosan partially hydrolyzed at 10 and 100 mg L^{-1} (Costales et al., 2007). The positive effect of the hydrolyzed chitosan on the nodulation could be explained by several ways. It is known that the chitosan derivatives favor the plant growth and the radical system of several crops (Bitelli et al., 2001). As a result of these effects, the number and weight of the nodules could be benefit indirectly. On the other hand, the structure of nodulation factors contains basically a chitin oligosaccharide, which starts the process of nodule formation by inducing the cortical cell divisions on the roots leading to the formation of the nodular primordium. These signals mediate the entry of the microsymbiont and the process of nodulation (Macchiavelli & Brelles-Marino, 2004). Besides, foliar and seed application of chitosan oligosaccharides increase the isoflavonoids (genistein and daidzein) content in soybean seeds (Mal-Tawaha et al., 2005). These compounds constitute chemoatractans to *Bradyrhizobium* and specific inducers of *nod* genes activation in the bacteria. It would be valid the use of chitin derivatives as inoculant additives to increase the nodulation and soybean development. Further studies will be conducted to deepen into the structural requirements of oligosaccharines in the effect on symbiotic nodulation.

5.6 Inoculants as a product. Impact on agriculture

It is necessary to translate all of this knowledge in a product to be applied in big planting, to face the adverse condition, the competition, and then we can think our results contribute in a little way to perform a sustainable agriculture. Testing traditional and new induced inoculants on big extension of soybean, in different sites of Argentina, we can distinguish different results in yield. 78% of sites shown a positive response with the new product, with 243,12 $kg.ha^{-1}$ as average of yield´s increase and 188,07 $kg.ha^{-1}$ higher to the traditional inoculants (figure 6).

We evaluated the effect of some soil factors as pH, available phosphorus content, organic matter content, nitrates and *Bradyrhizobium* population, as well as the two inoculants on soybean yield (Nápoles, et al., 2009). The analysis of factors proved that, despite everyone

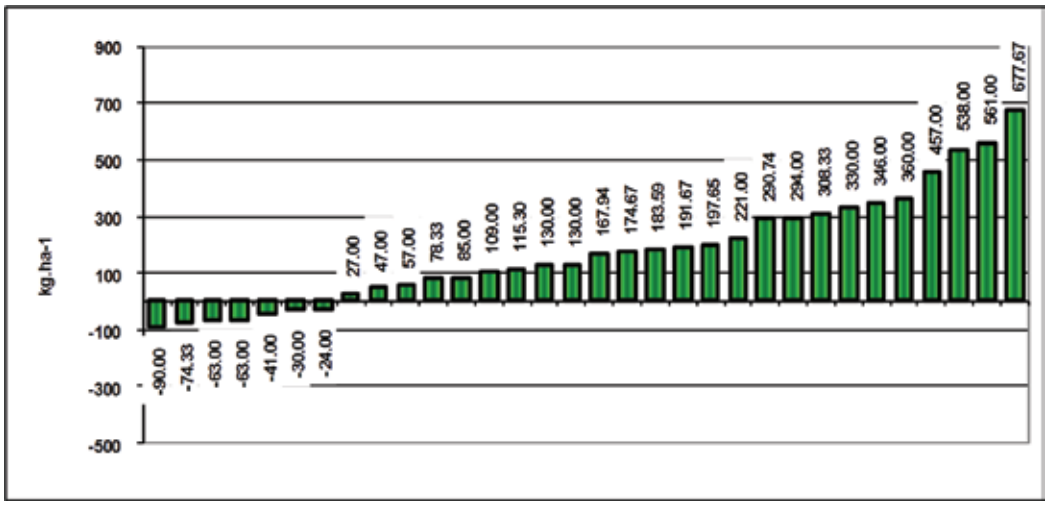


Fig. 6. Negative (Decrease) and Positive (Increase) effect of new inoculant on soybean yield in different sites.

influenced on it, only pH, *Bradyrhizobium* population existing in the soils tested and inoculant quality had a significant effect on yield. Plants growing on acid soils with a low bacteria population, which had been inoculated with the new induced biopreparation, showed higher yields. New products containing *Bradyrhizobium* as biological component in a good physiological state, increase competitiveness, assurance excellent nitrogen nutrition to the plant and guarantee higher yields.

6. Conclusions and future work

The outcome of the interaction *Bradyrhizobium*-soybean, as other legumes-rhizobium interaction, is dependent on an elaborate signal exchange that continues throughout the entire symbiotic process and has been likened to matching locks and keys (Broughton et al., 2000), with only the correct combination giving rise to efficient symbiosis. Taking into account that this symbiosis is in great measure responsible of nitrogen required by world agriculture (de Hoff & Hirsch, 2003), big efforts have done to improve this relation, specially related to the bacteria. It is very important that selective strains are effective, competitive. Our efforts have been focus on improve the physiological bacteria state to produce or activate the symbiosis determinants through culture media design, according to that purpose. We think that including natural sources of inducers it is possible not only activate the nodulation factor production, but also the protein secretion system and polysaccharides, all necessary to the symbiosis success. Nevertheless, we need to keep in mind all the factors related in that complex process, not only the bacteria, the plant, their genetic, physiology, also the soil and all the environmental factors biotic and abiotics, which can act on the system.

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Functional Properties of Soybean Food Ingredients in Food Systems

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1. Introduction

The food system is the transformation of raw materials into healthy food products within biophysical and socio-cultural contexts which results in production, processing, distribution, preparation and consumption of food. Food systems include components of food availability, access and utilization which underpin food security (Gregory et al., 2005). The expanding world population has resulted to a greater pressure for the consumption of plant products in foods with aesthetic and organoleptic appeal; consequently resulting in a great emphasis on the need for food ingredients with multiple functional properties. The role of soybean as a traditional food item in Far East is well recognized where it is used to make tofu, tempeh and soymilk. Advances in food technology resulted in the development of a variety of edible soy products including concentrates, isolates and extruded-expanded products; the consequence of which is increased soy consumption by populations of technically developed regions of the world (Young and Scrimshaw, 1979). Soybean is crushed into oil and defatted meal. The meal is usually used as an animal feed; a smaller percentage is further processed into food ingredients including soyflour, concentrates, isolates and textured protein. These are soy protein products used as food ingredients because of their multiple functional properties. Functional properties have been defined as "those physical and chemical properties that influence the behavior of proteins in food systems during processing, storage, cooking and consumption" (Kinsella 1976). The functional behavior of proteins in food is influenced by some physicochemical properties of the proteins such as their size, shape, amino acid composition and sequence, net charge, charge distribution, hydrophobicity, hydrophilicity, type of structures, molecular flexibility/rigidity in response to external environment such as pH, temperature, salt concentration or interaction with other food constituents (Damodaran 1997). The functional properties are the intrinsic physicochemical characteristics which affect the behavior of a food ingredient in food systems during processing, manufacturing, storage and preparation. Such functional properties include water holding, oil binding, emulsification, foam capacity, gelation, whipping capacity, viscosity and others. Functional properties are important in determining the quality (nutritional, sensory, physicochemical and organoleptic properties) of the final product as well as facilitating processing such as improved machinability of cookie dough or slicing of processed meats (Kinsella, 1979). Therefore functional properties

of food proteins are important in food processing and food product formulation (Wu et al. 2009). However, these properties vary with the type of food products; for example, proteins with high oil and water binding are desirable for use in meats, sausages, bread and cakes, while proteins with high emulsifying and foaming capacity are good for salad dressing, sausages, bologna, soups, confectionery, frozen desserts and cakes (Ahmedna et al. 1999).
This chapter presents a compilation of the functional food ingredients from soybean, their composition and structure and conformation in order to understand the mechanism of particular functional traits. The role of sprouting in soybean functionality will also be discussed.

2. Food ingredients from soybean

Foods made from soybean may be divided into four classes namely, soy ingredients, traditional soy foods, second-generation soy foods, and foods where soy is used as a functional ingredient (US Soybean Export Council 2008). These food products are outlined in Figure 1. Traditional soy foods include soy milk, tofu, tempeh, natto, miso and soy sauce. Soy milk is a protein-rich, milky liquid obtained from the soaking and grinding of whole soybeans with water, or from hydrating whole, full-fat flour, cooking the resultant slurry, and filtering all or part of the soy pulp or fibre from the cooked liquid. Second-generation soy foods include meat extenders, soy burgers, soy sausage, imitation chicken and soy cheese. Foods in which soy is used as functional ingredient include baked goods to which soy flour is added. Soy ingredients are the processed soybean protein products which include soy flour (defatted and full fat), soy concentrates, soy isolates, texturized vegetable soy protein and hydrolyzed soy protein. These soy ingredients (Figure 2) are the object of our discussion.

Soy Flour

Mature, whole, yellow soybeans are used for human food production. Soy flour is flour produced from hulled soybean seeds and is then ground into a fine powder; at least 97% of the product must pass through 100-mesh standard screen and contain 50% protein (Berk, 1992). The production of edible soybean flours and grits may take place either as an independent industrial activity or as a by-product of oil-mill operations. Soy grits are similar to soy flour except that the soybeans have been toasted and cracked into coarse pieces (US Soybean Export Council, 2010; Pyler, 1994; Lusas and Riaz, 1995). They are usually classified into three groups according to particle size: coarse 10 to 20 mesh; medium 20 to 40 mesh and fine 40 to 80 mesh (Berk, 1992). There are four forms: enzyme-active, natural or full-fat, defatted, and lecithinated (Figure 2). Further classification of the commercial soy flours and grits is in terms of their Nitrogen Solubility Index (NSI) or Protein dispersibility Index (PDI). Each of these tests indicates the percentage of total soluble nitrogen in water, with a range of values from 0 to 100 (Dubois, 1980). These parameters indicate the extent of protein denaturation and hence the intensity of heat treatment which has been applied to the starting material. The PDI decreases with higher levels of heat treatment. The NSI method gives lower values and has been related to PDI by the formula PDI = 1.07(NSI) + 1 (Lusas and Riaz 1995; Central Soya Company 1988). Flours made from white flakes have NSI of about 80%, while those from toasted flakes show NSI values of 10-20%. High PDI soy ingredients are more soluble and have highly active enzymes and anti-nutritional factors (Lusas and Riaz 1995).

Enzyme-active flours

Enzyme-active soy flour is produced from defatted soybeans that have been processed in such a manner as to preserve the activity of the enzyme lipoxidase. As early as 1934 it was reported that ground soybeans could be used for bleaching the carotene pigments of wheat flour during bread making (Wolf 1975). One method of preparing the bleaching agent involved grinding washed beans, removing the hulls, and mixing the resulting full-fat flour with four parts of corn flour; between 0.75 and 2% of the soy-corn flour mixture was sufficient for bleaching. This action is as a result of the activity of lipoxygenase in soybean. However, many studies have reported that increased addition of raw soybean in food products results in undesirable bean flavor. In addition to bleaching the pigments, lipoxygenase is also reported to cause oxidation of gluten proteins resulting in finer crumb. In the US enzyme active soy flour preparation are used at 1% level on wheat flour basis. In some continuous bread baking process, the lipoxygenase preparation is incubated with cottonseed or soybean oil and the peroxidised oil is then used in the dough formula resulting in improved bread flavor.

To obtain the enzyme active flour soybean seeds are cleaned, sorted into uniform size to minimize variations in processing, equilibrated to 10-12% moisture, preheated to 55^{o}C (enhances loosening of hulls from the cotyledons during cracking and aids the removal by aspiration) and cracked into six to eight pieces by using corrugated rolls. The raw cotyledons are milled into full-fat enzyme active flours. Active-defatted soy flour could be obtained by defatting the obtained flour with hexane at 66-71^{o}C (Lusas and Riaz 1995).

Full-fat flours and grits

Conventional processing method for full-fat flours and grits is shown in Figure 3. Natural or full-fat flour contains natural oils (18-20%) found in the soybean. Inactivation of lipoxygenase is a key step in the preparation of good flavored full-fat soy flours. Methods that have been reported include (1) conditioning of soybeans at 13-14% moisture in hot air (100^{o}C) or steaming of dehulled beans and subsequent drying and grinding to full-fat flour; such flour; (2) microwave heating before cracking and dehulling and (3) extrusion cooking in which the soybeans were cracked and dehulled followed by a dry-heat preconditioning to inactivate lipoxygenase and other enzymes (Wolf, 1975; Kanzamar et al. 1993; Lusas and Riaz 1995). The treated seeds are tempered to 15-20% moisture after cooling and extrusion cooked. The extruded material is dried, cooled and milled into flour (Mustakas et al 1971).

White flakes

White flakes are produced by cleaning, heating and cracking soybeans, removing the hulls by aspiration; flaking the chips to about 0.25-0.30 mm thickness and extracting the oil by hexane to 0.5-1.0% oil. While the oil is removed by extraction, the carotenes are removed as well and the extracted residue gets a typical white colour, hence the name white flakes (Anon, 2008a). Processors produce white flakes with PDIs of 20, 70 and 90. Flaking ruptures the cotyledon cells allowing the oil to flow together, becoming accessible to lipases in the presence of heat and moisture. Beany flavor in defatted soy flours has been reduced significantly by preheating the soybeans before dehulling or flaking for the PDI objective of the specific product where appropriate (Lucas and Riazs 1995). White flakes have uses and is sold as an intermediate form for other products. White flakes and defatted soy flour with a high PDI serve as raw material for the manufacture of most protein concentrates, isolated

soy protein and texturised soy flour. They are also used alone or in combination with whole soybeans, as a starting material for the production of soy sauce (Berk 1992).

Defatted flours and grits

Defatted flour and grits have the oils removed during processing, contains less than 1% oil (Berk, 1992). The defatted flour or grits actually consists of a range of products rather than a single one. The important variable in the processing is the toasting step- a moist cooking with steam under atmospheric or pressurized conditions (Figure 2). Toasting is omitted if the soy flour is to be used as a bleaching agent in bread where activity of the oxidative enzymes is required. For some food uses, toasting improves the flavor and destroys anti-nutritional factors. However, despite the advantages of toasting, intermediate degrees may be desirable in order to retain adequate levels of functional properties since some of which are destroyed progressively on toasting. Therefore, the particular potential uses of the product determine the degree of toasting required.

Lecithinated soy flour

Lecithinated is flour whose normal lecithin content of 0.5 to 1.5% has been augmented to 15% in order to increase its emulsifying properties. It is obtained by spraying lecithin and vegetable oil on 70 and 20 PDI soy flour resulting to a good emulsifier that can be used for partial or total replacement of egg (Riaz, 2006).

Soy protein concentrate

Soy protein concentrate (SPC) is protein (70%) produced by extraction of sugars, soluble carbohydrate material, mineral matter and other minor constituent from defatted soy flour (US Soybean Export Council, 2008). The objective in producing SPCs is to remove strong-flavour components and the flatulence sugars (stachyose and raffinose). At the same time both the protein and dietary fibre contents are increased (Lucas and Riaz 1995). Three processes may be used: (1) heating the white flakes resulting in denaturation of the protein and they become water insoluble before the extraction process begins. The drawback is that the protein has lost most of its functional properties; this method is no longer used today; (2) extraction with water at isoelectric pH (4.5) where the soy protein has its lowest possible solubility characteristics. The neutral pH can be restored after extraction by neutralization and the protein regains its original solubility characteristics and functional properties. This process results in the best tasting and most functional SPCs that have found applications in the preparation of fat emulsions; (3) extraction with aqueous (70-90%) ethyl alcohol for the extraction of the oligosaccharides. This is the most popular process because it results in the most bland tasting and nutritionally most attractive SPCs. This process is based on the (irreversible) alcoholic denaturation of the protein. Mild heat drying conditions are used to retain high PDIs and NSIs (Lusas and Riaz 1995; Anon 2008a). A variety of granulations (from grits to ultrafine flours) may be obtained if the SPC was made from white flakes, extracted and dried with minimum breakage (Beery 1989).

Soy protein isolate

High PDI (70-90%) white flakes and flour milled to 200 mesh are used in making soy protein isolates (SPIs) (Lusas and Riaz, 1995; US Soybean Export Council, 2010). The production consists of an aqueous solubilization of protein and carbohydrates at neutral or alkaline pH and the recovery of the solubized protein, separation and, optionally washing and neutralization before drying (Moure et al., 2006). Three steps are involved in the process of

making SPIs. (1) the soy flakes or flour are slurried with water under alkaline conditions (pH 6.8-10 at 27-66°C using sodium hydroxide and other alkaline substances approved for food use) so that the protein as well as the oligosaccharides can go into solution. The protein solution is then separated from the insoluble residue by centrifugation (Lusas and Riaz, 1995; Anon, 2008b); (2) the supernatant containing the protein and the sugars in solution is acidified to pH 4.5 (isoelectric point of proteins where their solubility is minimal), by using hydrochloric acid or phosphoric acid. This results in the precipitation of the protein as a curd. Typical process includes several extractions of white flakes or flour and washing of the curd; (3) The solubility of the precipitated protein is restored by neutralizing to alkaline pH 6.5-7.0 after re-dilution with fresh water or spray dried in its acidic form and packed in multilayer paper bags (Anon 2008b; Lusas and Riaz, 1995).

Textured soy protein

Texturization means the development of a physical structure that will when eaten provide a sensation of eating meat (Berk, 1992). Textured soy protein product is obtained either by spinning in into a fibre and then combining the fibre in layers to achieve the desired texture, or a thermoplastic extrusion process (US Soybean Export Council, 2010). Spun fibre is obtained using soy protein isolate as starting material; extrusion or steam texturised products from flour, concentrate or isolated protein (Berk, 1992). These products known as textured vegetable protein can simulate meat fibre structures (Anon, 2008b). Another possibility is the hydrolysed soya protein. The degree of hydrolyzation determines the functionality of the end products. Low degree of hydrolyzation results in highly functional foaming agents and high degree of hydrolyzation results in hydrolysed vegetable protein (HVP) which are used in soups and sauces as flavor enhancers (Anon, 2008a).

3. Classification of functional properties of food ingredients

Functional properties may be classified according to the mechanism of action on three main groups (Figure 4). These groups are (1) properties related with hydration (absorption of water/oil, solubility, thickening, wettability); (2) properties related with the protein structure and rheological characteristics (viscosity, elasticity, adhesiveness, aggregation and gellying), and (3) properties related with the protein surface (emulsifying and foaming activities, formation of protein-lipid films, whippability) (Kinsella, 1979; Moure et al. 2006). These properties vary with pH, temperature, protein concentration, protein fraction, prior treatment, ionic strength and dielectric constant of the medium as well as other treatments such as interactions with other macromolecules in the medium, processing treatments and modifications, physical, chemical and enzymatic methods (Kinsella, 1979).

Functional properties related with hydration mechanisms

Some functional properties can be interpreted as a result of the thermodynamically favourable protein-water interactions (wettability, swelling, water retention, solubility) or unfavourable (foaming, emulsification) (Moure et al., 2006). The interactions of protein with water are important in relation to dispersibility, water absorption and binding, swelling, viscosity, gelation and surfactant properties as these properties influence the important functions of proteins in meat, bakery and beverage systems (Moure et al., 2006). Ease of dispersibility or wettability is important in food formulations and is affected by surface polarity, topography, texture and area, and by the size and microstructure of the protein particles (Kinsella, 1979).

Bound water includes all hydration water and some water loosely associated with protein molecules following centrifugation, ranging from 30 to 50 g per 100 g protein (Riaz, 2006). Soy isolate having the highest protein content among soy protein products has the highest water-binding capacity, approximately 35 g/ 100 g (Hettiarachchy and Kalapathy, 1998). Soy concentrates contain polysaccharides, which absorb a significant amount of water. Generally, processing conditions can affect the amount of water that can be absorbed; these conditions can be varied to influence how tightly the water is bound by the protein in the finished food product (Endres, 2001).

Water holding capacity is the ability to retain water against gravity, and includes bound water, hydrodynamic water, capillary water and physically entrapped water (Moure et al., 2006). The amount of water associated to proteins is closely related with its amino acids profile and increases with the number of charged residues (Kuntz and Kauzmann, 1974), conformation, hydrophobicity, pH, temperature, ionic strength and protein concentration (Damodaran, 1997; Kinsella, 1979). Defatting increases the protein solubility and water and oil absorption capacities of the meals. Germination, fermentation, soaking or thermal treatments (toasting/autoclaving) significantly improves water absorption capacity (Moure et al, 2006).

Proteins swell as they absorb water and it is an important functional property in foods like processed meat, doughs and custards where the proteins should imbibe and hold water without dissolving and concurrently impart body, thickening and viscosity. Viscosity and swelling are closely related important properties in processed meats (Kinsella, 1979). Factors which affected swelling also influences viscosity; protein concentration, pH and temperature positively affects swelling and viscosity whereas sodium chloride depresses both (Kinsella, 1979).

Protein solubility is influenced by the hydrophilicity/hydrophobicity balance, which depends on the amino acid composition, particularly at the protein surface (Moure et al., 2006). The presence of a low number of hydrophobic residues; the elevated charge and the electrostatic repulsion and ionic hydration occurring at pH above and below the isoelectric pH favour higher solubility. Protein solubility is also influenced by production method and in particular by denaturation due to alterations in the hydrophobicity/hydrophilicity ratio of the surface. A highly soluble protein is required in order to obtain optimum functionality required in gelation, solubility, emulsifying acitivity, foaming and lipoxygenase activity (Riaz, 2006). Soluble protein preparations are easier to incorporate in food systems, unlike those with low solubility indices which have limited functional properties and more limited food uses.

Functional properties related with protein structure and rheology

Solubility, hydrodynamic properties, hydrophobicity and microstructure of proteins have been reported to play an important role in the rheological properties of proteins (Krause et al., 2001; Krase et al., 2002). Apparent viscosity of soybean isolates depends on interaction between soluble and insoluble proteins with water and between the hydrated particles (Añón et al., 2001). Due to the increased interactions of hydrated proteins, the water absorption and swelling, viscosity increases exponentially with protein concentration (Kinsella, 1979). Knowledge of the viscosity and flow properties of protein dispersions are of practical importance in product formulation, processing texture control and mouthfeel properties and in clarifying protein-protein interactions and conditions affecting conformational and hydrodynamic properties (Kinsella, 1979).

Protein gels are three-dimensional matrices or networks of intertwined, partially associated polypeptides with entrapped water; and are characterized by a relatively high viscosity, plasticity and elasticity (Kinsella, 1979). The ability of protein to form gels and provide a structural matrix for holding water, flavours, sugars and food ingredients is useful in food applications, and in new product development and provides an added dimension to protein functionality. Gelling property is important in comminuted sausage products and is the basis of many Oriental textured food e.g. tofu. Factors known to affect gelation include pH, ionic strength, reducing agents, urea, temperature, the presence of non-protein components and the mechanical forces applied to the system (Sathe, 2002). Properties of the gel are determined by the interactions between solvent and the molecular net resulting in transparent or coagulant gels. Coagulant gels are formed by proteins containing non-polar residues (Shimada and Matsushita, 1980), while those containing hydrophilic amino acids form transparent ones (Moure et al., 2006). Soy flour and concentrates form soft, fragile gels, whereas soy isolates form firm, hard, resilient gels. Protein gelation is concentration dependent; a minimum of 8% protein concentration is necessary for soy isolates to form a gel. The general procedure for producing soy protein gel involves heating the protein solution at 80 to 90^{o}C for 30 minutes followed by cooling at 4^{o}C (Riaz, 2006). The ability of the gel structure to provide a matrix to hold water, fat, flavour, sugar, and other food additives is very useful in a variety of food products (Kinselle, 1979; Hettiarachchy and Kalapathy, 1997).

Functional properties related to protein surface

Important properties of foods involve the interaction(s) of proteins and lipids, e.g. emulsions, fat entrapment in meats, flavor absorption, lipoprotein complexes in egg yolk, meats, milk, coffee whiteners, dough, and cake batters (Kinsella, 1979). Emulsions and foams are two phase systems commonly found in food systems, whose formation is significantly affected by protein surface activity (Moure et al., 2006; Kinsella, 1979). Emulsions are generated by mixing two immiscible liquids e.g. oil and water. The liquids are immiscible because of their relative polarities. When liquid of low polarity such as fat is mixed with water a strong driving forces limits the contact between the two liquids resulting to phase separation. Droplet size of emulsion significantly affects the stability of emulsions; emulsions with precisely controlled droplet size exhibit better stability. Reduction in droplet size also improves stability of an emulsion to separation due to gravity (McClements, 1999). The goal in food processing is to stabilize the emulsion thereby giving it a reasonable lifetime. The dispersed system can be stabilized against coalescence and phase separation by adding a component that is partially soluble in both phases. Such components are phospholipids (emulsifiers) which when mixed with lipid in an aqueous environment; the fatty acid portion of the molecule is inserted into the oil phase, while the phosphate ester head group remains in contact with the aqueous phase. The result is that the two immiscible phases are not in contact with each other and the total energy of the system is lower. Emulsifiers or foaming agents therefore reduce the interfacial tension and help to stabilize the oil-water and air-water interfaces (Moure et al., 2006). In decreasing the interfacial tension of emulsions low molecular weight surfactants (phospholipids, mono and diglycerides or monoesterates) are more effective than high molecular weight ones (proteins and hydrocolloids) (Damodaran, 1997). Despite the lower efficiency of proteins the emulsions and foams formed with proteins are more stable, hence proteins are preferred

over low molecular weight surfactants for emulsification purposes in foods (Moure et al, 2006). Surface activity of proteins is related to their conformation and ability to unfold at interfaces determined by molecular factors (flexibility, conformational stability, distribution of hydrophilic and hydrophobic residues in the primary structure) and external factors (pH, ionic strength, temperature, possible competitive adsorption of other proteins or lipids in the interface) (Vliet et al., 2002). Highly insoluble proteins are not good emulsifiers as they can generate coalescence (Kato and Nakai, 1980). Emulsion stability is not only influenced by the presence of salts and pH (Tsaliki et al., 2004), but also by several physical interdependent processes such as cream formation, flocculation or aggregation and coalescence (Damodaran, 1997) resulting in phase separation.

Foams are gaseous droplets encapsulated by a liquid film containing soluble surfactant protein resulting in reduced interfacial tension between gas and water. Capacity of proteins to form stable foams with gas by forming impervious protein films, is an important property in cakes (angel, sponge), soufflés, whipped toppings, fudges, etc (Kinsella, 1979). Properties of good foaming proteins include (1) solubility in the aqueous phase and rapid adsorption during shipping and bubbling; (2) concentrate at the air-water interface and unfolding to form cohesive layers of protein around air droplets with reduction of surface tension; and (3) possess sufficient viscosity and mechanical strength to prevent rupture and coalescence (Kinsella, 1979; Hettiarachchy and Ziegler, 1994).

Functional properties related to organoleptic/kinesthetic

The contribution of proteins to food flavours is known as it affects the sensory properties (appearance, colour, flavor, taste and texture) of foods. Proteins may modify flavor by binding flavours and off-flavours to generate flavors on cooking and to release reactants that may produce flavours, especially following hydrolysis or proteolysis (Kinsella, 1979). These are important factors considered in fabricating foods from soy proteins.

4. Functional properties of soybean food ingredients in food systems

The food systems where soy protein products find application are outlined in Table 1. These include (1) comminuted meat sausages, such as frankfurters and bologna; (2) low-viscosity emulsions, such as milk, coffee whitener and liquid whipped topping; (3) high-viscosity emulsions, such as mayonnaise or salad dressings; (4) Bakery, pasta and food bars; (5) Confectionery and (6) Infant formulas.

Meat and dairy based systems

Solubility, water binding, swelling, viscosity, gelation and surfactant properties are important functional properties of soy proteins in meat and dairy based systems. Comminuted meats (sausage, bologna, luncheon meats) usually contain more fat than normal meat, hence soy proteins are used to enhance and stabilize fat emulsion, improve viscosity, impart texture upon gelation following cooking and improve moisture retention and overall yields (Table 2). Heat-treated soy flour is commonly used as well as soy concentrates. However, there use results in poor flavor and mouthfeel as well as poor texture, dryness and flavour associated with flours and concentrates added above 10% (Kinsella, 1979). This is resolved by using soy isolates in meat loaves, sausage-type products for their emulsion-stabilizing effects, gelation, moisture retention and improved effects on texture.

Textured protein products in use both in the meat and vegetarian meat analog industry include texture soy flours, textured soy protein concentrates, and textured soy protein materials comprised of various blends of isolated soy protein, soy protein concentrate, soy flour, wheat flour, vital wheat gluten, rice flour, soy fibre, assorted starches etc. Textured soy flours are less functional with regard to water-holding capabilities compared to other textured vegetable protein ingredients (Riaz, 2006).
Texturized soy protein products are used as meat extenders in comminuted meat products such as patties, fillings, meat sauces, meat balls, etc. As much as 30% of the meat can be replaced by hydrated texturized soy products without loss of eating quality. In addition to offering economic savings, textured soy products offer certain product improvement- water and fat absorption resulting in increased product juiciness and use of meat with higher fat content (Berk, 1992).

Low-viscosity systems

In a low-viscosity, low fat emulsion, creaming is the usual form of breakdown and the contribution of protein to the whitening effect of the emulsion is important (Puski, 1976). Solubility is the most important criterion of soy protein product for beverages (Kinsella, 1976). Other requirements of the soy protein product include: formation of a clear or translucent solution that is bland, low viscosity, stability over pH range, ionic strength and temperature conditions and storage in liquid, concentrate or powder form. Solubility of the protein in acidic range is necessary in carbonated beverages. This is achieved by using protein hydrolysates (Kinsella, 1976), although the presence of bitter peptides is a problem with hydrolysate.
Beverages containing soy protein can be classified according to their position in the marketplace (Table 3). Soy protein products in ready-to-drink beverages affect sensory attributes in diverse ways. That is the type of protein is related to certain functionality characteristics in beverages. In general, soy proteins with good solubility will produce better beverage mouthfeel and suspension stability. The relationship between protein physical properties and beverage functional attribute are detailed in Table 4 (Riaz, 2006).
Physical properties of soy protein products differ from each other as a result of differences in component composition (protein, fat and fibre content), preparation, and processing. In addition, the physical properties differ within the same group to a great extent. Good functional proteins in beverage system are those with high solubility, high emulsification, and proper viscosity for the targeted market (Riaz, 2006). Proteins with high foaming and high gelling properties would have a negative impact on beverage quality.

High viscosity systems

The role of soy proteins in high-viscosity products includes emulsification and colloidal stability to heat. Soy isolates show a greater (6-fold) emulsifying capacity compared to soy protein concentrate (Kinsella, 1979) often depending on method of preparation. There is a close relationship between emulsifying properties and solubility of soy protein products especially in low viscosity emulsions (milk, salad dressing, coffee whitener). However, this is less important in viscous emulsions for example in comminuted meats where soy proteins with 50% solubility can ensure adequate emulsifying capacity and the thermal stability in preventing fat separation. Creaming is less important in high viscosity emulsions. However, coalescence and inversion into water-in-oil emulsions are the most likely breakdown problems.

Bakery, pasta and food bars

Soy ingredients that have unique functional applications in bakery products include soy flours, soy grits, soy protein concentrates, soy protein isolates, textured soy protein, soy brans, and soy germs (Riaz, 2006). Soy bran is obtained by toasting and grinding the seed coat portion of the soy bean (US Soybean Export Council, 2008; Dubois, 1980). Soy germ comprises 2% of the total soybean and is used in baked and extruded products as well as in cereal-based products as adjunct to other soy ingredients to increase isoflavone levels. Inclusion rates are usually 1 to 2% of the total formulation of the product because of its high isoflavones content (Riaz, 2006).

Soy protein ingredients are important in determining the quality of the product as well as facilitating such processing requirements as in improving machinability of cookie dough for instance. The extent of heat treatment during processing determines the use of soy proteins in bakery products (Table 5).

Soy proteins are rapidly insolubilized by heat, moist heat in particular during processing. However, heat is necessary during soy protein production as it is needed to desolventize, inactivate anti-nutrient compounds and to improve soy flour flavors. On the other hand, non-heated soy flours have bitter, beany flavour and limited applications while containing high lipoxygenase activity. To balance enzyme activity, flavor quality and solubility requirements, defatted soy flours with a range of solubilities as well as concentrates and isolates from minimally heat-treated flours possessing good solubility are produced (Riaz, 2006). Protein solubility is a measure of the percentage of total protein that is soluble in water under controlled conditions and is a measure of the degree of heat treatment to which the soy flour has been subjected (Riaz 2006). Soy flours with high NSI or PDI are used in bakery and cereal products and are added directly to the dough (Endres, 2001). Enzyme-active soy flour has a minimum water solubility of 70% (Schryver, 2002). Soy flours subjected to minimal heat treatment (PDI 80) show high lipoxygenase activity and are used at 0.5% to bleach flour and improve the flavour of bread. Flours with a PDI of approximately 60 are commonly used for improving water-binding capacity (1 to 2% in bread, 10% in waffles and pancakes). Some functional properties of soy protein products in baked goods are detailed in Table 6.

Soy protein ingredients are used to aid formation and stabilization of emulsion for many food products including cake batters. Generally, the emulsifying capacity of soy protein products increases with increasing solubility and interfacial tension is progressively reduced as concentration is increased (Riaz, 2006).

The capacity of proteins to form stable foams with gas by forming impervious protein films, is an important property in some food applications including angel and sponge cakes (Riaz, 2006). Soy protein products differ in their foaming ability, with soy isolates being superior to soy flour and concentrates. Soy protein foaming ability is closely correlated to its solubility.

Soy proteins addition to wheat flour dilutes the gluten proteins and starch while exhibiting a strong water-binding power that provides some resistance to dough expansion, the effect being proportional to quantity of soy flour. The water-binding power of soy flour is related to its high water absorption capacity, 110% by weight in defatted product. That is defatted soy flour will absorb an amount of water equal to its weight when mixed with wheat flour to normal dough consistency. However, with full-fat flour, no measureable increase in dough absorption results from normal use levels of the soy product (US Soybean Export Council, 2008). Water holding capacity of protein is very important as it affects the texture, juiciness, and taste of food products and in particular the shelf-life of bakery products. All

SPCs, irrespective of the processing method, have certain fat and water- holding characteristics (Endres, 2001). There is no reason for using SPC in bakery products, unless higher protein fortification levels are necessary. Soy flour does the same job of nutritional and functional improvement more economically (Berk, 1992).
Food bars are combinations of ingredients that provide food in a solid, low-moisture form and are consumed as a source of nutrients, as opposed to confectionery bars, which are consumed as sweet products (Raiz, 2006). The basic formulations of food bars are outlined in Table 7. All food bars use similar combinations of ingredients but their positioning varies widely as well as the nutrient profile. The largest proportion of soy protein in bars is in the form of isolates. Two main functional types are used in food bars. The first type has high gel strength, with its solution having high viscosity forming rigid, themostable gels at moderate concentrations. The highly gelling soy isolate makes firmer bars with a drying mouthfeel, with shorter texture, similar to that of a cookie. The second forms solutions with much lower viscosities and will not form gels at any concentration. The low viscosity soy proteins produce much softer, chewier nutritional bars. Water-holding capacity, viscosity and wettability all play a role in determining the texture of the end product (Riaz, 2006).

Confectionery

Lecithinated soy flour improves the dispersion of the flour and other ingredients in confections and cold beverage products as well as improving water retention in baked goods and extending their shelf lives (Riaz, 2006). Paritally hydrolysed soy proteins possess good foam stability properties and can be used as whipping agents in combination with egg albumen or whole eggs in confectionery products and deserts (Berk, 1992).

Infant formula

Soy protein isolate is the preferred ingredient in infant formulas where milk solids are replaced. This is because of its bland taste, absence of flatus-producing sugars and negligible fibre content (Berk, 1992).

5. Effect of sprouting on functional properties of soybean food ingredients

Sprouting is the practise of soaking, draining, and leaving seeds or grains until they germinate or sprout. The increasing interest in functional and healthy food products has promoted the use of germinated soya bean flour in the manufacture of foods for human consumption (Farrera-Rebollo & Calderon-Dominguez, 2007). It is known that germination induces increase in free limiting amino acids and available vitamins with modified functional properties of seed components (Akpapunam, 1996; Jideani and Onwubali, 2009). The nutrient composition of soy bean sprout is affected by sprouting conditions such as times and temperatures, presence or absence of light during the sprouting process, the composition of the soaking and rinsing water as well as post-sprouting handling. Sprouts in general are high moisture and low calorie products and good sources of protein and other nutrients (Jideani and Onwubali, 2009). On the basis of mineral bioavailability, the significant reduction of phytic acid during sprouting makes sprouts nutritionally more attractive than their non-sprouted seed.
Jideani and Onwubali (2009) reported that sprouted soy bean flour resulted in increased loaf volume, a firmer, spongy and more elastic loaf in wheat bread. A combination of yeast (2.4%) and sprouted soy bean flour (10.6%) produced an optimal loaf.

In our research we observed that sprouting of soybeans seeds at 25°C resulted in maximum values of vitamin C and lipase at 48 h; amylase activity 36 h. Equal mixtures of the flour obtained at these times were studied for its functional properties. The flour possessed a higher emulsification capacity compared to whole egg powder. There was no significant difference between the whole egg powder and the sprouted flour in oil absorption. Hence, sprouted soybean flour could find use in fried foods and food systems where egg is required to minimize oil absorption (Murevanhema, 2009). Soy bean are rich source of phenolic antioxidants, which occur naturally bound to carbohydrates (McCue and Shetty, 2004). Free phenolic antioxidants have higher antioxidant activity than their carbohydrate-bound forms. McCue and Shetty (2004) reported that water extracts of dark-sprouted soybean contained high levels of free phenolics and antioxidant activity, alpha-Amylase being the major enzyme responsible for driving carbohydrate metabolism to phenolic synthesis in dark-sprouted soybean. Therefore, sprouted soybean flour in addition to having improved functionality may have important antioxidant effects in stabilizing lipids in formulated foods.

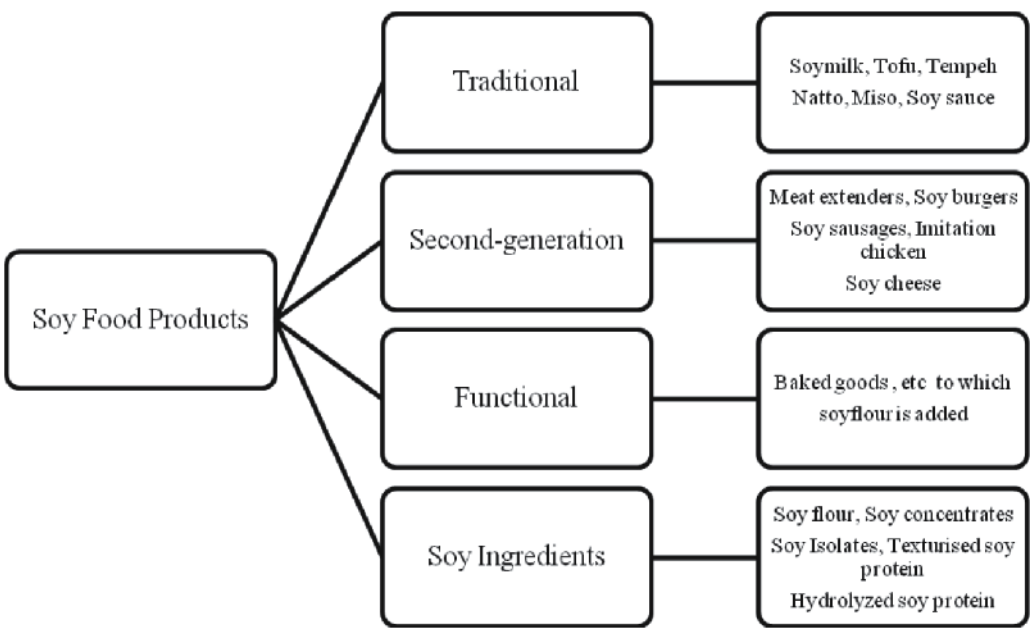

Fig. 1. Categories of soy food products (Adapted from US Soybean Export Council, 2008)

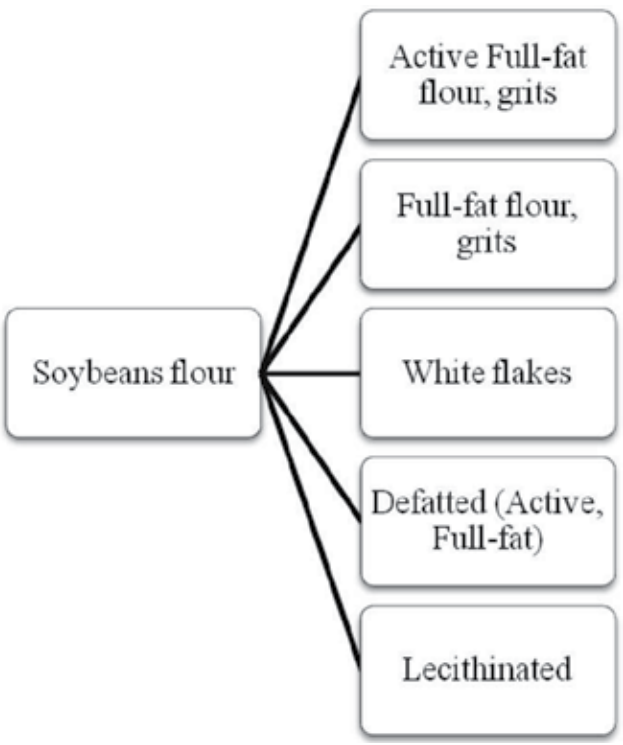

Fig. 2. Flour products from soybeans

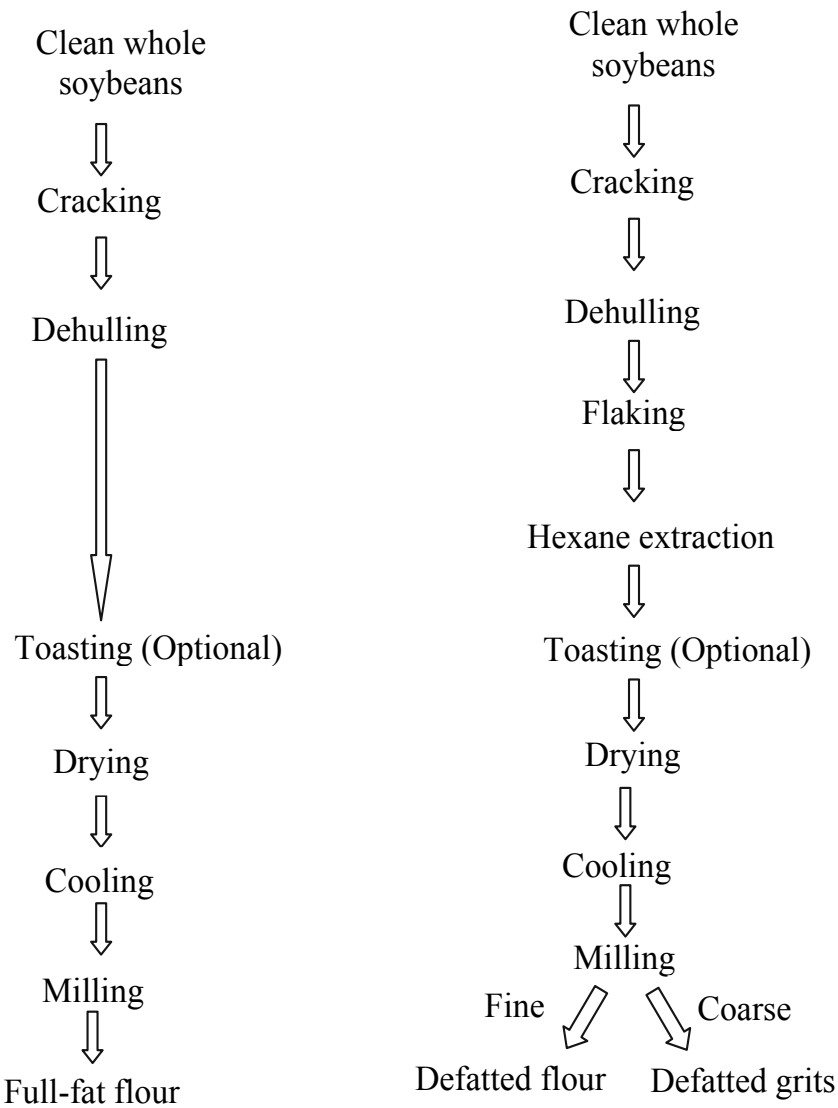

Fig. 3. Conventional processes for producing full-fat and defatted soybean flour/grits (Mutakas, 1971).

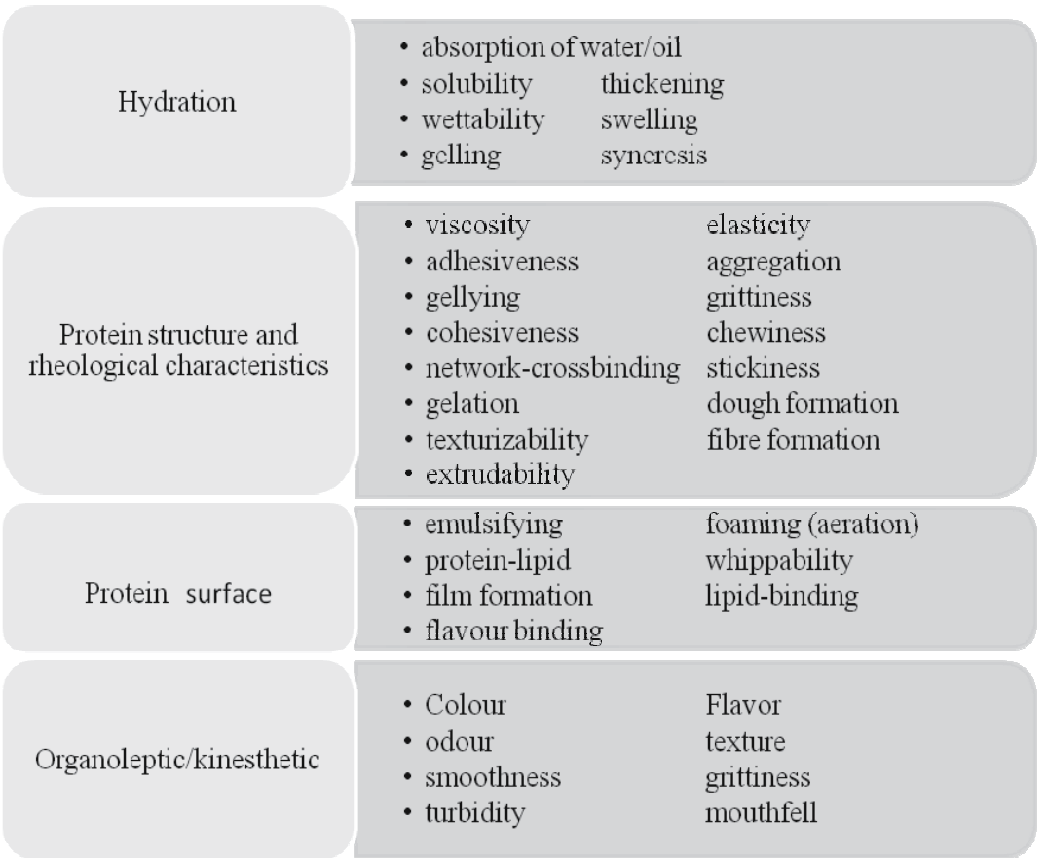

Fig. 4. Functional properties of food ingredients in food systems (Adapted from Moure et al. 2006; Kinsella, 1979)

	Food system	Functional property for all products	Soy ingredients[1]
1.	Meat and dairy		
	Meat, sausages, bologna	Water absorption, binding, elasticity, cohesion-adhesion, emulsification, fat adsorption	F, C, I
	Simulated meats	Flavour-binding	C, I,H
	Cheese, curds	Gelation	C, I
2.	Low viscosity		
	Beverages	Solubility, emulsifying, gelation	F, C, I, H
	Whipped toppings, frozen desserts	Foaming, emulsifying, dispersibility	I, W, H
	Imitation dairy	Emulsifying, colloidal stability	
3.	High-viscosity		
	Retortable sauces Mayonnaise and salad dressing	Emulsifying, colloid stability to heat	
4.	Bakery and pasta	Solubility, emulsifying, gelation, colour control, cohesion-adhesion	F, C, I
5.	Confectionery	Foaming, solubility	I, W
6.	Infant formula	Nutrition, solubility, emulsification, colloidal stability to heat	

[1]F, C, I, H, W are soy flour, concentrate, isolate, hydrolysate and soy whey, respectively.

Table 1. Functional properties of soy protein products in food systems

Improves uniform emulsion formation and stabilisation.
Reduces cooking shrinkage and drop by entrapping-binding fats ans water.
Prevents fat separation.
Enhances binding of meat particles without stickiness.
Improves moisture holding and mouthfeel.
Gelation improves firmness, pliability and texture.
Facilitates cleaner, smoother slicing.
May impart antioxidant effects.
Improves nutritional value.

Source: Kinsella (1979)

Table 2. Functions of soy protein in meat-based product

Ready-to-drink product	Description	Market
Milk-plus	Contain cow's milk plus soy protein.	Mainstream consumer
Milk alternatives	Contain either no diary ingredients or at least no lactos, are possess approximate composition of cow's milk.	Lactose-intolerant consumers
Soymilks/Soy beverages	Based on all-vegetable ingredients, with soy as the sole protein source.	Ethnic and religious groups, vegetarians, and health conscious consumers, have become a widening part of the mainstream consumer.
Pharmaceutical/nutritional	Scientifically formulated and clinically tested for specific human diseases or developmental statese.g. infant formula, adult nutritional supplements, and enteral feeding formula.	Sold via medical referral within pharmacies
Meal replacers/weight loss	Provide balanced nutrition as par to an overall diet plan.	Weigh conscious or obese consumers
Cream alternatives	e.g coffee whiteners and whipped toppings dairy-like cream alternatives without casein.	Religious, ethnic, and dietary needs demanding replacement of casein in toppings.
Fortified juice	Fruit-flavoured drink with soy protein included (<1.5%) for nutrient fortification.	Children, women, and helath-conscious adults
Fruit smoothies	Fruit-type drink with a higher protein addition (1.5 to 3%). Two subsets: semblance of drinkable yogurts; blended fruit shakes popular at smoothie bars.	Health conscious and active adults seeking added nergy and more nutrition in tasty, fruity flavours.

Adapted from Riaz (2006).

Table 3. Beverages containing soy protein products

Physical property	Beverage functional attribute
Solubility	Appearance, mouthfeel, sediment, suspension stability
Emulsification	Suspension stability, mouthfeel, appearance, colour
Viscosity	Mouthfeel, stability, flavour
Flavour binding	Flavour
Particle size	Mouthfeel, colour and appearance
Heat stability	Colour, suspension

Source: Riaz (2006)

Table 4. Relationship between physical properties of proteins and beverage attributes

Application	Protein dispersibility index (PDI)
Emulsifying, foaming	>90
Lipoxygenase bleaching of flour and bread	>85
Water absorption in bakery products	60
Waffles	30
Crackers, cereals	15

Source: Riaz (2006).

Table 5. Soy protein solubility requirement for selected bakery applications

Functional property	Mode of action	Baking system	Protein form
Emulsification			
Formation	Formation and stabilization of fat emulsions	Breads, cakes	Flour, concentrates, isolates
Fat Adsorption			
Prevention	Binding of free fat	Doughnuts, pancakes	Flour, concentrates
Water Absorption and Binding			
Uptake	Hydrogen bonding of water, entrapment of water, no drip	Breads, cakes	Flour, concentrates
Retention	Hydrogen bonding of water, entrapment of water, no drip	Breads, cakes	Flour, concentrates
Dough formation	-	Breads, cakes	Flour, concentrates, isolates
Cohesion-adhesion	Protein acts as adhesive material	Breads, cakes	Flour, concentrates, isolates
Elasticity	Disulphide links in deformable gels	Breads, cakes	Flour, concentrates, isolates
Flavour-binding	Adsorption, entrapment, release	Breads, cakes	Concentrates, isolates, hydrolyzes
Foaming	Forms stable films to trap gas	Whipped toppings, chiffon desserts, angel cakes	Isolates, soy whey, hydrol
Colour control			
Bleaching	Bleaching of lipoxygenase	Breads	
Browning	Maillard, caramelization	Breads, pancakes, waffles	

Source: Riaz (2006).

Table 6. Selected functional characteristics of soy protein in baking systems

Category		Description
1.	Athletic bars	Includes energy bars.
2.	Lifestyle or wellness bars	Includes the 40:30:30 concept of balanced calorie intake from carbohydrates, protein and fat)
3.	Diet bars	Includes meal replacement bars and high-protein, low-carbohydrate bars
4.	Carbohydrate energy endurance bars	Used by backpackers, climbers, cyclists, etc. for an energy boost.

Adapted from Riaz (2006)

Table 7. Basic formulations of food bars

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Soybean Polyunsaturated Fatty Acids Exert Potential Effects on the Natural Course of Inflammatory Diseases

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Brazil

1. Introduction

Soybean yields about 18.5% oil in weight (Soyatech, 2008), a characteristic that makes it an important source of vegetable oil. The majority of soybean oil production is used by food processors and food service operators as an ingredient for baked and fried food products, or it is packaged for sale as a cooking oil (Soyatech, 2008). The importance of soybean oil in human nutrition can be expressed in numbers; it accounts for about 50% of all the vegetable oil and for about 30% of all oils and fats produced around the world (USDA, 2010).

As the soybean oil is primarily used for human nutrition, constituting an important fraction of the lipids present in the diet of the majority of the population, it raises great economic and scientific interests. A quick search in the web for the keywords "soybean" and "oil" give out a list of about 30,000 articles published only in the last 10 years and the number of annual publications in this area is growing up about 10% per year. The aims of the majority of these scientific works are born in the lipid profile of this oil. But, what about the lipid profile of the soybean oil? It contains an impressive amount of PUFAs. Typically, it contains about 60% polyunsaturated fatty acids (PUFAs), 25% monounsaturated fatty acids (MUFAs) and 15% saturated fatty acids (SFAs). The high content of PUFAs in the soybean oil raises some questions which are the basis of the following discussion.

Lipids are not simply a source of energy in human nutrition as they do exert other important functions. They compose biological membranes, serve as precursors of inter/intracellular signaling molecules, carry hydrophobic molecules in the bloodstream, functionalize biomolecules such as enzymes and receptors, participate in the digestive process and are involved in several other aspects of the mammalian physiology. Particularly their hydrophobicity was intensively explored in biological evolution and the life as it is known would not be possible in the absence of lipids. Every cell in the human body contains lipids and is sensible for lipid-derived signaling molecules. The majority of the lipids required by the human cells are synthesized by the human body and, as expected for any physiological process, the control of the lipid metabolism is fine-tuned by feedback mechanisms. However, not every kind of lipids needed for the physiological processes to work properly is totally synthesized by the human cells.

Some lipids cannot be synthesized by the human cells by a *de novo* process, i.e., they are not synthesized by the cells from simple and freely available substrates. In this case, the dietary

intake of this kind of lipid is not only recommended but in fact essentially necessary. A nutrient that cannot be synthesized from simple substrates and then should be obtained by means of dietary intake is called "essential nutrient", a designation that summarizes the importance of including this nutrient in a diet. There are classical essential lipids, which are the ω3 and ω6 PUFAs. These fatty acids contain carbon-carbon double bonds (unsaturations) at the extremity of the acyl hydrocarbon chain – the first unsaturations at the third and at the sixth carbon atom counting from the methyl extremity, respectively – that cannot be achieved with the enzymes of the human cells. Although the human cells are not able to synthesize these lipids from simple substrates such as acetyl-CoA, they are necessary for several physiological processes, and this is the reason why these PUFAs are essential nutrients.

An example of a process dependent on essential lipids is the synthesis of the eicosanoids, a group of signaling molecules that participate in several physiological processes such as immunity, digestion, reproduction and neurotransmission. When the cell consumes the ω3 and ω6 PUFAs, restoration of the supply of these lipids must be done by means of the dietary intake. For example, when the synthesis of an eicosanoid is activated in a cell, the long chain ω3 and/or ω6 PUFAs are consumed. These lipids cannot be restored by the human cell by a *de novo* synthetic pathway. Instead, the cell must receive new ω3 or ω6 PUFAs from the diet. Therefore, if an individual consume little or no food containing all the essential lipids, the cell is not able to exert its proper function and this can lead to a physiological malfunction. Besides this, not only the quantity is important but also the dietary ω3-to-ω6 PUFAs ratio. These two kinds of lipids can be used in the same metabolic pathways but the products obtained may present quite different actions in some cases (James et al., 2000). Moreover, the human cells incorporate not only PUFAs but also MUFAs and SFAs, and the diet is an important determinant for the lipid composition of the cellular structures, which affect several cellular processes as discussed here.

Why the human organism is not able to *de novo* synthesize these kinds of lipids? The rational answer – and probably the right answer – is that the development of the biochemical machinery for producing them was not necessary because the human organism has evolved in an environment where the food itself offered a rich supply of these lipids. But nowadays a great part of the human population is living in an industrialized world. The food does not come directly from natural sources anymore but instead it is industrially processed before reaching the homes. Besides this, the great variety of nutrient sources for human nutrition available before agriculture was substituted by the large scale cultivation of some few very productive cultures (oligoculture), such as soybean. In this context, the arbitrary manipulation of the diet can give higher or lesser amounts of certain essential nutrients than the required by the organism (Bourre et al., 1989; Rapoport et al., 2007). Since the diet is the only source of these essential nutrients, this dietary imbalance can lead to physiological malfunction and exert health-deleterious effects.

As some lipids are provided only or mainly by the diet and cells are specialized in taking up these lipids, the diet profoundly affects the lipid profile of biological structures (James et al., 2000; Pan & Storlien, 1993). The relative excess intake of PUFAs – and not only the most discussed imbalanced intake of ω3 and ω6 PUFAs – can exert important health-deleterious effects in humans and this fact is closely related to both the essential nature of these lipids and to their higher susceptibility to oxidation. When PUFAs are oxidized, the pathogenesis and the natural course of an inflammatory disease can be affected. Therefore, this review shows that some facts point to the need of a rational consumption of the PUFAs-containing aliments, such as the soybean oil. Several inflammatory diseases can be influenced by

dietary PUFAs and the allergies are among them and are used in this chapter as a representative example. The dietary ω3-to-ω6 PUFAs ratio, which is another well discussed subject in the specific literature, will not be minutely discussed here. Above all, this text stresses the rational basis upon which new dietary studies and health-beneficial improvements in soybean products can be performed.

2. Changes in the dietary lipid profile in the industrialized world

The soybean oil has high contents of PUFAs and MUFAs, which are far known to exert several health-beneficial effects on the metabolism of lipids in the human body. This oil is one of the main sources of these fatty acids for the most of the population around the world and the importance of this food as a lipid source in human nutrition is increasing year after year.

Both the MUFAs and PUFAs are generally known as the "good fat" based on their health-beneficial effects in the context of the cardiovascular system. On the dark side of fatty acids are the bad fats, or the saturated fatty acids (SFAs) which, along with cholesterol, are known by most of the population to exert deleterious effects on human health when consumed in large amounts. This kind of judgment was based on the findings of several epidemiological and laboratory studies which have shown several correlations between lipids and cardiovascular diseases. These studies laid the basis for general recommendations for a healthy diet, which included a liberal intake of unsaturated fatty acids and a low SFAs dietary content.

These recommendations took effect mainly in the industrialized world, which includes both the western countries and some countries with westernized lifestyle. In these countries, the media-driven scientific discussions on health and the health-appeal in food marketing led to a quite interesting phenomenon: a fast rise on the dietary P/S ratio on the population living under the western lifestyle. Along with this health-appeal other factors such as some economic influences probably exerted an important role, but surely the first cited factor is one of the main events behind rise on the dietary P/S ratio.

Now it is worth analyzing the trend in fat consumption in the industrialized world. The data concerning the period between the years of 1966 and 2000 in the United Kingdom (UK) is shown in the graph below (Fig 1a). By analyzing this graph it is possible to see a markedly significant decrease in the SFAs consumption and a concomitant increase in the PUFAs consumption which taken together led to a significant rise in the dietary P/S ratio. The Fig 1 shows that in 2007 the P/S ratio was about 0.5, while at the middle 1960' it was about only 0.2, i.e., there was a increase of about 150 % in the P/S ratio in only four decades. The UK is a representative example for the industrialized countries in the context of the dietary lipids, as the trend in lipid consumption showed in the figures 1 and 2 was observed in other industrialized countries too.

The Fig 1a shows that, although the PUFAs consumption increased significantly, the rise in the dietary P/S ratio was mainly due to a decrease in the SFAs consumption. The trend of reduction on the dietary contribution of lipids for the total energy intake is the main responsible for the reduction in absolute SFAs consumption observed in the UK and in other industrialized countries. One of the strategies for reducing the intake of lipids was the substitution of fatty products by low-fat or fat-free products in the diet. In fact, over the last decades, SFA-rich products were substituted by low-fat or PUFAs-rich products and this trend is still observable in the last years (Bates et al., 2010). For example, in the beginning of the last century, butter (SFAs-rich) was the most consumed fat spread; nowadays,

margarines (PUFAs-rich) and low-fat spreads are the most common type of fat spread consumed (Bates et al., 2010). But the most important example of a decrease in SFAs consumption is related to the milk and milk products, which account for about 25 % SFA consumed by the UK population (fig 2). The consumption of milk was reduced in the period between 1997 and 2007 by 18 % in the UK population aged 11 to 18 years. For adults this decrease was larger, as the consumption fell by 32 % (Bates et al., 2010).

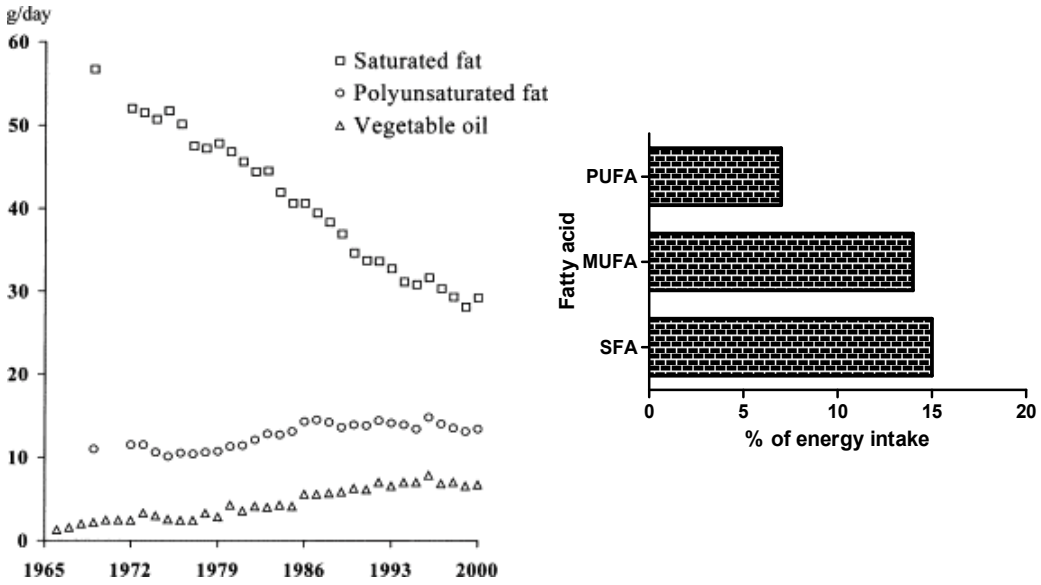


Fig. 1. a) Consumption of saturated fats, polyunsaturated fats and vegetable oils in the UK between 1966 and 2000 (DEFRA(UK), 2000; Devereux & Seaton, 2005); b) Dietary lipid profile in private UK households (Bates et al., 2010; DEFRA(UK), 2010).

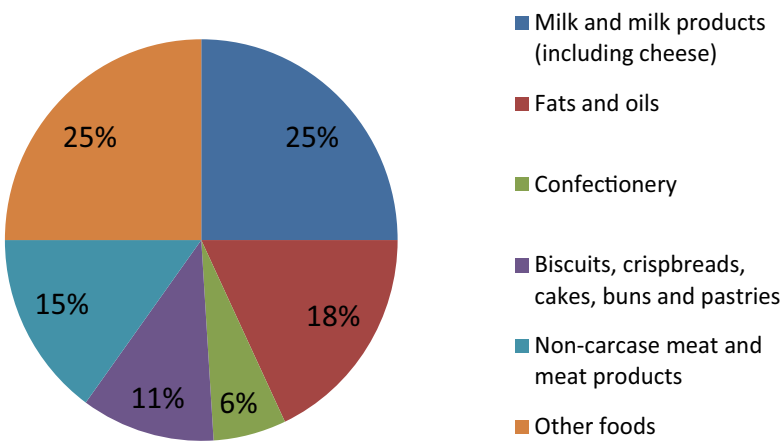


Fig. 2. Contributions of different sources of SFAs in household supplies in the United Kingdom in 2007 (DEFRA(UK), 2010).

Another interesting event concerning the change in dietary P/S ratio is that the rise in PUFAs consumption follows the rise in vegetable oil consumption (Fig 1a). This fact is easy to explain, as the vegetable oils are the main sources of PUFAs in human nutrition. The Fig 1a gives other relevant information about the PUFA consumption; the curves for both PUFAs and vegetable oils consumption are not perfectly parallel but instead they are convergent, evidencing that in the course of time the vegetable oils are contributing with an increasing fraction of the PUFAs in the westernized diet.

As soybean oil is increasingly the main vegetable oil used for human feeding purposes, its lipid profile is of great importance in human nutrition. For example, the linoleic acid (a ω6 PUFA) accounts for about 80% of the total PUFAs in soybean oil (Sanders, 2000). The linoleic acid is the main source of PUFAs in the diet of the UK population and the consumption of this fat increased about 50 % (from about 10g/day to 15g/day) between the 1970s and the 1990s in the UK (Sanders, 2000). The worldwide consumption of the soybean oil – as in the UK – has significantly increased over the last decades turning this oil one of the main responsible for the high content of linoleic acid in the diet of the population in the industrialized world (Sanders, 2000). These facts point to the important role played by soybean oil as a source of PUFAs (Sanders, 2000) in a population who is consuming more and more vegetable oils-derived PUFAs and less SFAs.

Now the discussion reaches a point where the trend in dietary lipid profile observed in the last decades should be paralleled to some events observed in the population under these drastic dietary changes. The increase in the dietary P/S ratio was accompanied by a concomitant decrease in the prevalence of several cardiovascular diseases, but could the dietary P/S ratio increase indefinitely with no deleterious effects on the human health? The next topics will stress why the optimal diet should include controlled amounts of PUFAs, MUFAs and SFAs instead of a higher as possible P/S ratio.

3. The western lifestyle-associated increase in allergy susceptibility

Several studies showed that, since the second half of the 20th century, the prevalence of allergic diseases is rising. This is not simply due to better diagnostic tools or to changes in diagnostic criteria. This could not also be due to a change in the genetic composition of the population, such as an increase in the percentage of the genetically susceptible individuals in the population. The common conclusion made by the authors at the time of the germinal studies in this area was that there should be an environmental change associated with allergy pathogenesis that could be responsible for the rise in allergy prevalence. It would not be so interesting if this effect was observable worldwide; instead, surprisingly, this increase had a geopolitical frontier! In 1993, the study by Nina and Russel showed that, between the years of 1964 and 1989, the prevalence of asthma and other allergies had doubled in Aberdeen, Scotland (Ninan & Russell, 1992). Still Another study in UK also showed that the prevalence of asthma had significantly risen over the same period (Burr et al., 1989). Similar results were found for different types of allergies and in different countries, like Australia, New Zealand, Finland, Sweden, United States and Canada (Seaton et al., 1994). On the other hand, other study showed an interesting finding regarding to asthma prevalence in Germany: the prevalence of this allergic disease was not the same in the areas of the two former German Republics; instead, it was significantly higher in West Germany in comparison to the East Germany (Magnussen et al., 1993). What was the difference between these two populations under the same flag? The population of the West Germany was under

a typical western lifestyle, which was very different from that found in the former eastern neighbor.

Further studies had shown similar results. The rise in the prevalence of allergies was restricted to the countries where the population was living under the western/westernized lifestyle. For example, the prevalence of asthma in three countries of the African region of Maghreb (Algeria, Morocco and Tunisia) is significantly lower than that found in industrialized countries, but in the urbanized regions (coastal cities) of these African countries this prevalence is higher than in the rural regions. Again, this event was associated with the westernized lifestyle adopted by the urban population (Bourdin et al., 2009). Therefore, there are plenty of solid evidences showing that the industrialized world shares its frontiers with the areas where the allergy prevalence has significantly increased.

Therefore, as the increase in allergy prevalence is somehow restricted to the industrialized countries (Devereux & Seaton, 2005; Grundy et al., 2002; Ramsey & Celedón, 2005), some issues can be raised. How could industrialization affect the susceptibility to allergy? What characteristics do the industrialized countries share that made its population more prone to develop allergy? Is the answer to this question a simple one or, alternatively, could this answer be complex, involving different and not necessarily correlated factors? Actually we do not know what the right answer is. Most probably a complex answer will be revealed as more studies are performed. Anyway, at the time these questions began to be raised, some hypotheses were formulated based on the knowledge on allergy pathogenesis and immune system available at that moment. These hypotheses did not ignore the fact that the rise in allergy prevalence was related to the westernized lifestyle, characteristic of the population of the industrialized world (Busse, 2000). The main first hypotheses presented below were based on the behavioral and environmental changes associated with the western lifestyle. Some of these hypotheses partially helped to explain the epidemiological data and opened new areas of interest in immunological researches.

3.1 Pollution?

The early industrial revolution increased the levels of airborne pollutants, including some environmental allergens. Therefore, a group of hypotheses was based on a supposed increase in the exposure of the population to allergens and other pollutants, which occurred mainly in the industrialized world. Indeed several air pollutants associated with the industrial activity characteristic of the developed world, such as sulphur dioxide and ozone, are known to increase the susceptibility to allergies and airway diseases. The logical basis of this kind of hypothesis is quite simple and therefore it initially raised some interest. However, it failed to explain why the allergy prevalence was increasing when the level of some important airborne pollutants (such as ozone) were stable (Seaton et al., 1994). Moreover, some pollutants (such as sulphur dioxide) had even decreasing atmospheric levels at the same period when the allergy prevalence was increasing (Seaton et al., 1994). If the airborne pollutants were the main responsible for the increased allergy prevalence in developed countries it would be expected that the increase in the level of these pollutants would parallel the increase in the prevalence of allergies; but this was not observed.

3.2 Hygiene?

Another hypothesis referred to a more complex characteristic of the westernized countries. This hypothesis proved to be solid as several evidences support it. This is the so called

"Hygiene Hypothesis", which was firstly proposed by Strachan who observed that the number of hay fever cases was inversely proportional to the household size (Okada et al., 2010; Strachan, 1989). This hypothesis proposes that the lower incidence of infections early in life due to better hygiene conditions, to the lower exposure of healthy individuals to infected ones (less siblings), and to the availability of new and better health care methods, – characteristics of the developed countries – could affect the whole development of the immune system, leading to an increased susceptibility to allergy. Indeed, the development of a proper immune response against antigens involves a complex network of cells and molecules, which are engaged in destroying harmful while tolerating innocuous molecules/cells. As allergy is related to an inadequate immune response to exogenous innocuous antigens, the inadequate development of the immune system early in life could in fact lead to an increased susceptibility to allergy. In this context, it is not surprising that the Hygiene Hypothesis is also applied to autoimmune diseases (Okada et al., 2010). The Hygiene Hypothesis is a good hypothesis for the purpose of explaining why the westernized lifestyle increases the susceptibility to allergies. However, it is very probable that this is just a partial and not the only explanation as other aspects of the westernized lifestyle have been associated to an increased susceptibility to allergy development. The dietary P/S ratio is one of them and it is minutely discussed below.

3.3 Dietary lipids?

There are plenty of evidences pointing to a correlation between diet and susceptibility to allergies (Devereux & Seaton, 2005). The changes in the westernized diet are increasingly being associated to the rise in the allergy prevalence seen in the industrialized world (Devereux & Seaton, 2005; McKeever & Britton, 2004). The dietary influence is not restricted to the most known sensitization to food allergens itself; it is notably related to the influence of some essential nutrients mainly on inflammation and on cell redox status. The investigation of the influence of vegetable oils consumption on the natural course of inflammation became increasingly important as epidemiological and laboratory studies showed some interesting facts linking the biosynthesis of proinflammatory mediators with the dietary lipid profile. Interestingly, dietary lipids can influence inflammation due to their interaction with oxidant species, which is closely related to the chemical characteristics of these lipids.

Since the association between oxidative stress and allergic inflammation was evidenced, some investigations focused on the potential effects of antioxidant nutrients on the asthmatic inflammation, as asthmatic patients are deficient in antioxidants such as vitamin C, vitamin E (Kalayci et al., 2000; Shanmugasundaram et al., 2001) β-carotene, α-carotene, lycopene, lutein e β- cryptoxanthin (Wood et al., 2005). The main rationale of these investigations was that the allergy-associated oxidative stress along with the deficiency in essential antioxidant nutrients could increase the risk of developing more intense allergy symptoms. Moreover, it was demonstrated that, in asthmatics, dietary supplementation with vitamin E attenuates the oxidative stress (Roberts, 2007), improves the pulmonary ventilation (Gilliland et al., 2003) and reduces both the production of IgE and the allergen sensitization (Fogarty et al., 2000). Besides these effects, the finding that the ratio between plasmatic oxidized/reduced tocopherol is directly proportional to the gravity of the asthmatic inflammation (Wood et al., 2008) is another evidence of the important role played by antioxidants in reducing the severity of allergic inflammation. Therefore, some authors suggest that the decreased intake of antioxidant nutrients due mainly to a decreased

consumption of fresh vegetables verified in the western diet in the last decades (Devereux & Seaton, 2005; Fogarty et al., 2000), increase the biological susceptibility to oxidative damage and lead to a higher susceptibility to allergy (Baker & Ayres, 2000). The influence of the oxidative stress in other inflammatory diseases is also very significant and the dietary intake of antioxidants can influence the inflammation in these cases.

Along with the antioxidants, the lipids figure among the nutrients which have been most extensively studied in the context of allergies. Black and Sharpe were the first authors to suggest that the dietary lipid profile could be associated to the increase in allergy prevalence in the industrialized world (Black & Sharpe, 1997). They noticed an apparent and interesting correlation between the trend in lipid consumption and the prevalence of allergies in developed countries (Black & Sharpe, 1997). According to these authors, the increase in dietary P/S ratio preceded and then accompanied the rise in the incidence of allergic diseases. This is a very interesting and intriguing conclusion, since it put a close correlation between dietary lipid profile and allergies.

Since the study by Black and Sharpe was published, several investigations support that the dietary lipid profile associated with the westernized lifestyle – high dietary P/S ratio – increase the susceptibility to allergies. In 2001, Bolte and cols demonstrated that the consumption of margarine – a source of vegetable PUFAs – was positively associated with allergic sensitization in children, as the allergy prevalence in this group was found to be significantly higher in comparison to the control group composed by children consuming butter – source of SFAs (Bolte et al., 2001). In a study published in 2003, Wijga and cols associated the consumption of milk and butter – again, important sources of SFAs – by almost 3000 children aged 2 years to a lower susceptibility to allergy when these children reached the age of 3 years (Wijga et al., 2003). In other study, in 2001, Haby and cols concluded that a PUFAs-rich diet is a significant risk factor for the development of asthma in children aged from 3 to 5 years (Haby et al., 2001). Interestingly, these authors even pointed that about 17 % of the asthma cases studied were directly associated only to a high intake of PUFAs and suggested that PUFAs consumption is a factor with a great potential to be modified in order to reduce the susceptibility to asthma in children [29].

Both the intake of antioxidant nutrients and the dietary lipid profile are behind the main mechanism by which the diet influences the natural course of inflammation, although the following discussion will be centered in the lipids themselves and in the potential effects of the soybean oil on allergy. Now it is worth discussing the reason why the lipids present in a tissue under oxidative stress – such as the lungs of an asthmatic individual – can be modified in such an extent that they can affect the inflammation and why the diet can influence the intensity of this event.

4. Mechanisms behind the influence of dietary lipids on allergies

Resuming the germinal study by Black and Sharpe, the mechanism pointed by these authors for the correlation between the dietary lipid profile and allergy risk was based mainly on a lower ω3 intake (Black & Sharpe, 1997). The main suggestion was that the dietary lipid profile in the western diet could favor the production of prostaglandin E_2 (PGE_2), a mediator of inflammation, which would then increase the susceptibility to allergic sensitization (Black & Sharpe, 1997). As the substrate for the PGE_2 synthesis is the arachidonic acid, a ω6 PUFA, the hypothesis suggested by Black and Sharpe was not simply based on an increase on the dietary P/S ratio, but it highlighted an increase of the ω6-to-ω3 PUFAs ratio – which was

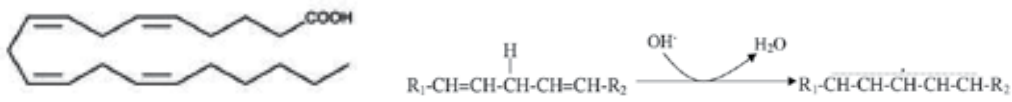
also inferred by the authors based on the available data. In fact, by the time this study was published it was known that dietary ω6-to-ω3 PUFAs ratio significantly affects inflammation and there is a still growing huge mass of researches on this subject. Generally, these researches have focused on the anti-inflammatory effects of ω3 PUFAs which are mainly – but not fully – due to the competition of the ω3 with the ω6 PUFAs for the same enzymes in the eicosanoid biosynthesis pathways, leading to the production of a less pro-inflammatory mixture of eicosanoids in comparison to that generated by ω6 PUFAs.
There are studies on diet and allergy showing that the consumption of ω3 PUFAs, such as the docosahexaenoic acid (DHA) found in fish oil, reduces the risk for developing asthma (Hodge et al., 1996). However, a systematic review on the effects of the dietary supplementation of asthmatic patients with fish oil shows that this procedure is not associated with beneficial effects and furthermore there is a lack of evidences supporting this approach for helping controlling asthma (Woods et al., 2003). Therefore, the dietary ω6-to-ω3 PUFAs ratio is not the only factor behind the effects of dietary PUFAs on allergy (Devereux & Seaton, 2005).

4.1 PUFAs are better substrates for non-enzymatic oxidation reactions in comparison to MUFAs and SFAs

Dietary PUFAs are incorporated by cells and can affect several cell processes and characteristics (Nishiyama et al., 2000). These lipids can be used as substrates for enzymatic reactions which produce lipid-derived molecules that serve as messengers in several physiological processes, such as inflammation. In comparison to SFAs and MUFAs, the PUFAs have quite different chemical features and this fact must be stressed on a hypothesis linking dietary P/S ratio to allergy susceptibility. A characteristic of all the PUFAs (including ω6 and ω3) is their higher susceptibility to oxidation in comparison to both SFAs and MUFAs (Frankel, 1984). In biological systems, this characteristic is exploited by biosynthetic enzymatic pathways, such as those leading to eicosanoid production. However, in some situations, such as inflammation, this oxidation can be non-enzymatic and involves oxidant molecules such as free radicals that evade the antioxidant system.
Obviously, due to the potential deleterious effects following oxidation of biomolecules, biological evolution has led to the development of a complex and efficient antioxidant system in order to neutralize oxidant species. In healthy tissues, the antioxidant system is capable of neutralizing oxidant species, avoiding them to react with other important biological molecules. For example, the group of molecules generically named as vitamin E is an important component of the antioxidant system and is mainly found in biological lipid membranes, where it reacts with oxidant species, such as hydroxyl or lipid radicals (see "lipid radicals" in Figure 4), avoiding the continuation of the lipoperoxidation reaction (Matés et al., 2000). Another important component of the antioxidant system is the catalase, an enzyme responsible for degrading the hydrogen peroxide (Rahman et al., 2006), the main source of hydroxyl radical in biological cells.
It is just because of the existence of this antioxidant system that the non-enzymatic oxidation of PUFAs in healthy tissues is strictly limited and it has little or no biologically significant effects. But in some situations the antioxidant system is not capable of neutralizing all the oxidant species. This may occur when there is an increased production of oxidant species or when the antioxidant capacity itself is decreased. Anyway, when the level of oxidant species in a certain biological tissue or cell is high enough to surmount the antioxidant system, a phenomenon named "oxidative stress" occurs. Oxidative stress is often accompanied by

oxidative damage in biomolecules – non-enzymatic oxidation – leading to cell malfunction. As discussed below, the non-enzymatic radical-mediated oxidation of PUFAs that occur in tissues under oxidative stress can have deleterious consequences that significantly affect the pathogenesis of allergies, the inflammatory disease discussed here.

In the context of fatty acid oxidation the PUFAs are by far the preferential substrates compared with MUFAs and particularly with SFAs. The molecular structures presented in the figure 3 help understanding this characteristic. In this figure both the structure of a PUFA and of a bisallylic group are presented; the presence of two carbon-carbon cis-double bonds (unsaturations) spaced by a single carbon atom is the distinctive characteristic shared by the PUFAs and this type of unsaturated system is called the bisallylic group (figure 3b). This system is especially susceptible to radical attack and is present neither in MUFAs nor in SFAs. The reaction diagramed in the figure 3b is one of the possibilities for the production of a lipid radical, the main mechanism by which the lipids are non-enzymaticaly oxidized. In this reaction, the hydroxyl radical (·OH) abstracts a hydrogen atom from the bisallylic group, producing a radical in which the unpaired electron is shared by 3 carbon atoms of the system. This phenomenon is called electronic resonance and the more the unpaired electron is dispersed in the resulting radical by resonance the more stable is the radical. This high stability renders this lipid radical sufficiently long-lived to react with other molecules – mainly with oxygen, as discussed ahead.



(a) (b)

Fig. 3. a) The structure of a polyunsaturated fatty acid (PUFA), the arachidonic acid; b) the bisallylic system is particularly prone to radical abstraction of a hydrogen atom from its central carbon due to the stabilization by resonance in the resulting radical.

The formation of the lipid radical is the key event for the initiation of the biologically relevant process known as lipoperoxidation (Frankel, 1984). In lipid membranes, the lipid radical is formed when the PUFA is esterified in the phosphatidic acid backbone of a phospholipid (Cracowski et al., 2002). It can then react with an oxygen molecule, generating another radical named the lipid peroxyl radical, which is able by its turn to react with another PUFA molecule (Frankel, 1984), producing another lipid radical (see figure 4). Therefore, when a peroxyl radical is generated in a PUFA-rich environment the lipoperoxidation can virtually continue indefinitely if the peroxyl radicals are not neutralized by some antioxidant. Such a PUFA-rich environment is found in biological lipid membranes and thus these structures are particularly prone to oxidative damage. When the cell is under oxidative stress the lipoperoxidation of PUFAs can persist sufficiently longer to produce deleterious effects.

4.2 Non-enzymatic oxidation of PUFAs can produce modified fatty acids with biological activity

Neither MUFAs nor SFAs present bisallylic systems. Due to this feature, MUFAs and SFAs are markedly less prone to the non-enzymatic radical-mediated oxidation in comparison to

PUFAs. Therefore, the higher the relative PUFA content of a certain biological structure – such as the cell membrane – the higher its susceptibility to the effects of oxidation. But does the higher dietary P/S ratio, observed in the westernized diet, affect the oxidability of biological structures? Several studies support an affirmative answer, indicating that the dietary lipid profile is the main variable behind the oxidability of biological structures (Aguilera et al., 2002; Berry et al., 1991; Cicero et al., 2008; Kratz et al., 2002; Mata et al., 1997; Muehlmann et al., 2009). However, how could the non-enzymatic oxidation of PUFAs affect the health? The answer for this question is as complex as the lipid metabolism itself. At one hand are the oxidation-mediated structural damages, on the other hand are the effects on the inter/intracellular signaling (Montuschi et al., 2004).

Regarding the cell signaling, several PUFA-derived molecules are produced by enzymatic pathways in normal physiology, which are under a fine-tuned feedback control. However, when an inflammatory disease generates the oxidative stress, the non-enzymatic radical-mediated fatty acid oxidation occurs in a significant extent (Talati et al., 2006) and, interestingly, this type of reaction is able to produce several molecules that mimic the physiological inflammatory mediators. Resuming the discussion on the allergy prevalence – the example used here in order to stress the health effects of a higher dietary P/S ratio – it is known that several PUFAs-derived molecules generated by enzymatic pathways are involved in allergy pathogenesis. It is not different when considering the inflammatory mediators generated by non-enzymatic oxidation of PUFAs. These mediators are produced after lipoperoxidation without the conventional feedback regulation (Marathe et al., 2000), having potential effects on the pathogenesis of allergies.

As some authors observed before, lipoperoxidation exerts deleterious effects in asthma (Wood et al., 2003), such as enhancement of airway hyperresponsiveness (Held & Uhlig, 2000; Talati et al., 2006), smooth muscle constriction (Fukunaga et al., 1993; Kawikova et al., 1996), airway obstruction, plasma exudation (Okazawa et al., 1997) and vascular constriction (Kromer & Tippins, 1996; Möbert et al., 1997). These references related these effects to the lipoperoxidation although these effects are classically known to be due to the activity of some proinflammatory mediators generated physiologically by enzymatic pathways in inflamed tissues. How could lipoperoxidation affect inflammation as it was due to the physiological pro-inflammatory mediators? The effect can be indirect, i.e., the lipoperoxidation damages the cells, an event that activates the production and release of pro-inflammatory mediators. But it also happens because some products of PUFAs lipoperoxidation possess biological activity analogous to that of the enzymatically-generated pro-inflammatory mediators. These biologically active PUFAs-derived molecules are named accordingly to the mediator molecule with which they share activity. In this context, two main types of inflammatory mediator-like molecules generated by peroxidation of PUFAs will be discussed: the isoeicosanoid and the PAF-like molecules, which have activity of eicosanoid and platelet-activating factor (PAF), respectively. These two kinds of non-enzymatically generated inflammatory mediators are known to exert biological actions that are important in several inflammatory diseases.

4.2.1 Isoeicosanoids: isoprostanes, isoleukotriene B4 e isotromboxanes

After lipoperoxidation (figure 4), the fatty acid peroxyl radical can undergo chemical rearrangements and the addition of further oxygen molecules. These modifications can produce molecules with prostaglandin, leukotriene B_4 or thromboxane-like activity

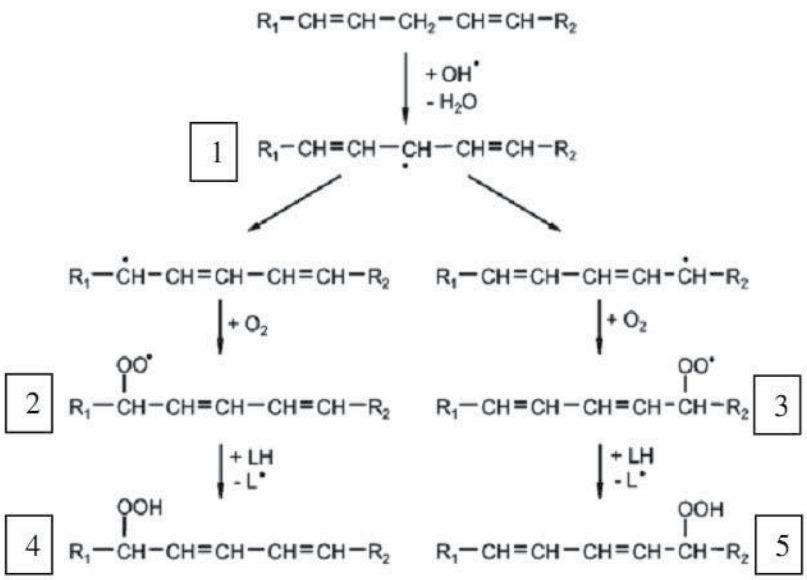


Fig. 4. The main steps involved in the formation of lipid hydroperoxides (4 and 5) from a lipid radical (1). Note that the lipid peroxyl radicals (2 and 3) are capable of producing additional lipid radicals (L·), leading to the continuation of the lipoperoxidation process. Adapted from Spitteler (2001) (Spiteller, 2001).

(Kayganich-Harrison et al., 1993; Wood et al., 2003) which are named, respectively, isoprostanes (iP), isoleukotriene B_4 (iL) and isothromboxane (iT) (Harrison & Murphy, 1995; Morrow & Roberts, 1996). The figure 5 schematically shows the reactions for the production of four types of iP-F_2 molecules. All the reactions presented in this diagram are initiated when a PUFA (arachidonic acid) is firstly converted into a radical by some oxidant species.

Note the reaction where the PUFA radical is converted into a peroxyl radical; this radical is able to produce more PUFAs radicals by abstracting a hydrogen atom from the bisallylic system. In the sequence, further rearrangements and additions of oxygen molecules produce the molecular complexity necessary for conferring these molecules an iP activity. The rationale for the production of iL and iT is basically the same, with some differences in the steps after the formation of the peroxyl radical.

The iPs are the most studied isoeicosanoids. The generation of these molecules is strongly associated with oxidative stress, so that they are used as markers of both oxidative stress and lipoperoxidation (Cracowski et al., 2002). The iPs are formed by the peroxidation of the PUFA arachidonic acid (Morrow et al., 1990) generally esterified in phospholipids, from which they can be released by phospholipase-mediated hydrolysis (Cracowski et al., 2002; Morrow et al., 1990). Some iPs exert important roles in the asthma pathogenesis, such as bronchiolar (Cracowski et al., 2002) and vascular (Morrow et al., 1990) constriction, alveolar plasma exudation (Okazawa et al., 1997) and airways hyperresponsiveness (Sametz et al., 1999). These effects are related to the prostaglandin-like activity conferred to these oxidized PUFAs by the structural similarity with the prostaglandins. The structures of both the prostaglandin $F_{2\alpha}$ and the iP F_{2a} generated by the non-enzymatic PUFA oxidation can be observed in the figure 6; their structural similarities are reflected in their similar biological activities.

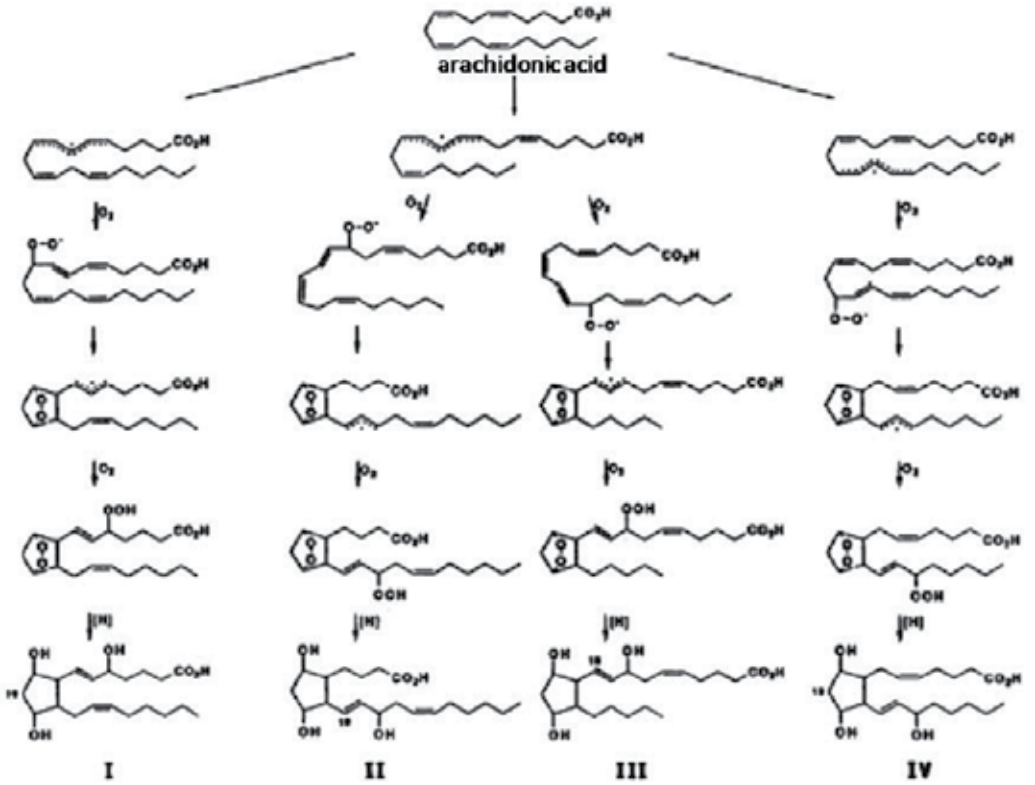


Fig. 5. Steps in the formation of isoprostanes by means of lipoperoxidation. Four possible isoprostane F_2 isomers are formed from arachidonic acid (Morrow et al., 1990).

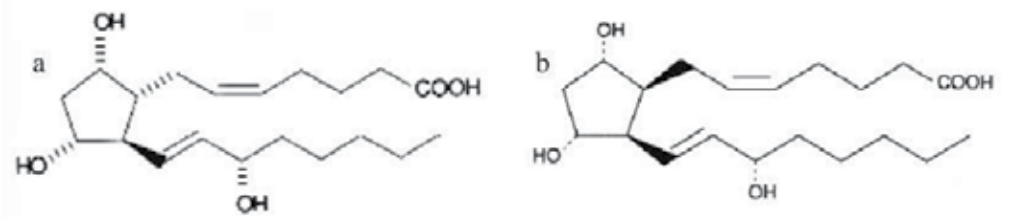


Fig. 6. a) Prostaglandin $F_{2\alpha}$ and b) one of its analogous molecules generated by means of lipoperoxidation, the isoprostaglandin $F_{2\alpha}$ (8-isoPG$F_{2\alpha}$).

PAF-like phospholipids

The molecules with biological activities shown in the precedent topic are generated by non-enzymatic oxidation and rearrangement occurring in PUFAs in an oxidant environment. Another important non-enzymatic oxidation reaction leading to the generation of some molecules with pronounced biological activity is the hydrocarbon chain cleavage that can occur in PUFAs. As in the formation of isoeicosanoids, the reactions leading to the formation of PAF-like lipids are initiated by lipoperoxidation. The schematic reaction steps are presented in the figure 7.

Both the PUFAs peroxyl (LOO·) and alkoxyl (LO·) radicals can undergo hydrocarbon chain cleavage (figure 7 and 8) producing shortened carbon-chains and aldehydes (Marathe et al., 2000). These radicals are easily formed by a reaction involving lipoperoxide and transition metallic cations (Coffey et al., 1995), such as the ferrous (figure 8).

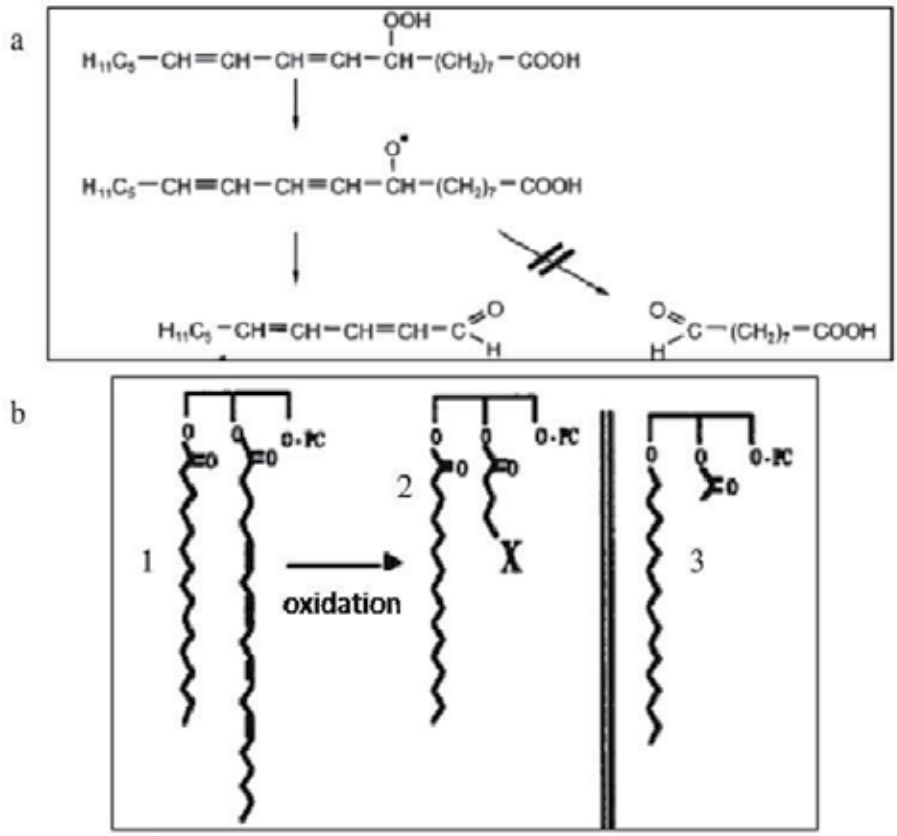


Fig. 7. a) Cleavage of a carbon-carbon bond in the hydrocarbon chain of a polyunsaturated fatty acid hydroperoxide; adapted from Spitteler (2001) (Spiteller, 2001). b) The shortening of the acyl hydrocarbon chain in the sn-2 position of a phosphatidylcholine (1) yields a PAF-like lipid (2), whose structure and activity resemble those of the authentic PAF; based on Zimmerman and cols (1995) (Zimmerman et al., 1995). The phosphatidylcholine precursor of a PAF-like molecule can have an acyl or an alkyl group in the sn-1 position (Stremler et al., 1991).

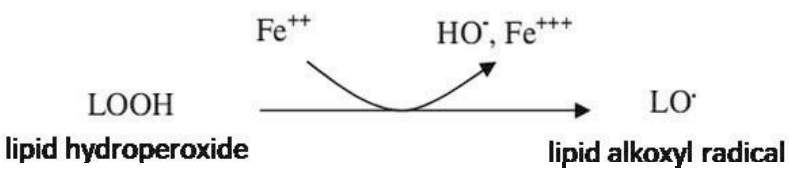
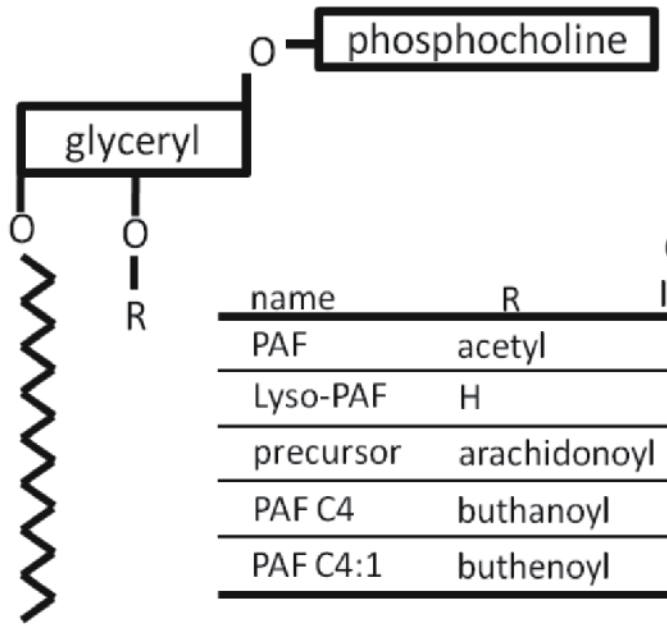


Fig. 8. Formation of a lipid alkoxyl radical by the cleavage of the peroxide bond in the presence of ferrous cation.

When a phosphatidylcholine contains a oxidatively shortened acyl group esterified in the sn-2 position, the resultant phospholipid can present PAF-like biological activity (Marathe et al., 2000). Once more, this phospholipid with a shortened sn-2 hydrocarbon chain has biological activity just because it has a close structural similarity to a biologic messenger, in this case the PAF (figure 7). It was shown that the formation of PAF-like phospholipids can take place in cell membranes (Patel et al., 1992), lipoprotein particles such as the LDL (Heery et al., 1995) and in synthetic phosphatidylcholines (Smiley et al., 1991).

Even if produced in low amounts, the PAF-like phospholipids derived from the non-enzymatic oxidation of PUFA-containing phospholipids exert several important actions in inflammatory diseases. The PAF is a highly potent and versatile proinflammatory mediator known to exert several effects in different cells (Uhlig et al., 2005). PAF is biosynthesized from the precursor sn-1 alkyl phosphatidylcholine and it is structurally named 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (Marathe et al., 2001). Physiologically, PAF is produced by the transesterification of the sn-2 position of the lysophosphatidylcholine glycerol backbone, where an acetyl group substitutes the usual long-chain fatty acyl group in a fine-tuned enzymatic reaction (Marathe et al., 2001). The pathogenesis and the pathophysiology of several inflammatory diseases involve the PAF actions and several studies demonstrate that this pro-inflammatory mediator has important roles in the asthmatic inflammation, such as leukocyte chemotaxis and activation, increase in vascular permeability, vasoconstriction, and bronchial constriction and hyperresponsiveness (Chung, 1992; Hsieh & Ng, 1993; Uhlig et al., 2005; Zimmerman et al., 2002). The PAF-like phospholipids are about 10 times less potent than the PAF itself (figure 9) however, taking into account that PAF elicits proinflammatory effects even in subnanomolar concentrations



name	R	Chain length	% PAF activity
PAF	acetyl	2	100
Lyso-PAF	H	0	0
precursor	arachidonoyl	20:4	0
PAF C4	buthanoyl	4	10
PAF C4:1	buthenoyl	4:1	10

Fig. 9. PAF and its structural analogues. Phosphatidylcholine containing a shortened acyl group in the sn-2 position has PAF-like activity. Adapted from Marathe and cols (2001) (Marathe et al., 2001).

(Marathe et al., 2001), the non-enzymatic production of PAF-like phospholipids is an important event in inflammatory diseases (Marathe et al., 1999). In addition to the PAF-like phospholipids containing the fatty alkyl group in the sn-1 position, a characteristic of the PAF, it was shown that some PAF-like phospholipids can have a fatty acyl group in this position (Stremler et al., 1991), though these lipids are about 800 times less potent than their sn-1 alkyl analogs (Marathe et al., 1999).

5. The rational consumption of vegetable oils can help controlling inflammatory diseases

The PUFAs are essential nutrients and the consumption of these lipids is a necessary component in any healthy diet. However, there is a relative limit for PUFAs consumption. The arguments presented here lead to the conclusion that there is in fact a healthy limit for the dietary P/S ratio, especially when the subject is inflammatory diseases. The PUFAs are necessary for several physiological processes and the control of some cellular characteristics, such as the fluidity of cellular membranes, the functionality of membrane-associated proteins, the cell-cell communication (as messengers), the intracellular signaling, the neurotransmission, the fat metabolism, and so on. However, once the proportion of PUFAs in biological structures is above a certain limit, the oxidative damage that occurs in oxidative stress situations – such as during inflammation – can be very deleterious due to the high oxidability of these PUFAs-rich structures.

As the dietary PUFAs are incorporated in the cells, the oxidability of the cellular structures is affected by the dietary lipid profile. The great amount of PUFAs in soybean oil is an important feature of this widely consumed edible oil and the importance of this food as a lipid source is growing up as it is more and more used for human nutrition. PUFAs account for about 60% of the total fatty acids content in the soybean oil and linoleic acid, a ω6 PUFA, accounts for about 80% of the soybean PUFAs. The molecules derived from oxidized PUFAs – generated in inflamed tissues – can exert significant effects on the natural course of inflammation, an event that can be partially responsible for the worsening or for the developing of an inflammatory disease, such as allergy.

Indeed, a recent study showed that dietary supplementation with soybean lecithin (a soybean oil derivative) exerts deleterious effects on asthma by means of PAF-like molecules – a product of the oxidation of PUFAs-containing phosphatidylcholine, discussed above (Muehlmann et al., 2009). In this study, the diet of asthmatic rats was supplemented with soybean lecithin, increasing the dietary P/S ratio in comparison to the control group, receiving a conventional diet. The authors showed that both the PAF activity – which includes the activity of both PAF and PAF-like molecules – and lipoperoxidation were increased in the lungs of the group receiving soybean lecithin. Interestingly, these effects were totally reverted by the concomitant supplementation with vitamin E, evidencing that the dietary lipids and the cell redox status affect inflammation by means of the products of PUFAs oxidation possessing pro-inflammatory activity (Muehlmann et al., 2009).

There is a huge mass of data available in the literature on the association between dietary lipids and the risk of inflammatory diseases. The evidences and the rationale presented here can be used in order to improve the lipid profile of the soybean as well as for the design of diets with the optimal lipid profile. As the dietary SFAs increase the plasma LDL levels and

the risk of a cardiovascular disease, high MUFAs, instead of high SFAs, diet has already been recommended for reducing the oxidability of biological structures (Berry et al., 1991; Cicero et al., 2008). Anyway, it is becoming increasingly clear that the consumption of vegetable oils or even the lipid profile of the vegetable oils could be improved in order to help controlling the inflammatory diseases.

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22

Renin Inhibitor in Soybean

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1. Introduction

Renin-angiotensin-aldosterone system (RAS) is the most important blood pressure control system in animals (Fig. 1). Renin [EC 3. 4. 23. 15] is a highly specific aspartic proteinase that is mainly synthesized by juxtaglomerular (JG) cells in the kidney. The human renin gene encodes preprorenin consisting of 406 amino acids (1-23 signal sequence, 24-66 propeptide, and 67-406 mature renin) [Imai *et al.*, 1983]. The synthesized renin precursor is processed to mature renin by proteolysis and stored in renin granules in JG cells. The secretion of renin into the circulation is controlled by several stimuli. The enzyme catalyzes the release of angiotensin I from plasma substrate angiotensinogen. This conversion is the rate-limiting step in RAS. Angiotensin I is an inactive peptide activated by angiotensin converting enzyme (ACE) [EC 3. 4. 15. 11]. ACE cleaves C-terminus dipeptide from angiotensin I. The angiotensin II produced acts directly on arterial smooth muscle cells to maintain blood pressure and stimulate the synthesis and release of aldosterone. Hence, RAS is a major target in the treatment of hypertension. ACE inhibitor is commonly used in clinical treatment. In connection with the control of renin activity, renin-binding protein (RnBP), a cellular renin inhibitor, was first isolated from porcine kidney as a complex of renin, called high-molecular-weight renin [Takahashi *et al.*, 1983a, Takahashi *et al.*, 1983b]. The nucleotide sequences of porcine, human, and rat RnBP cDNAs were determined and the amino acid sequences consisted of 402, 417, and 419 amino acid residues, respectively [Inoue *et al.*, 1990, Inoue *et al.*, 1991, Takahashi *et al.*, 1994]. The co-expression of human renin and RnBP cDNAs in AtT-20 cells showed that RnBP regulates active renin secretion from the transfected cells [Inoue *et al.*, 1992].
ACE has been used to screen inhibitors from foodstuffs because of its simple assay method, but renin is a rate-limiting enzyme in RAS, so it was not used because of the complicated assay system. In this chapter we describe expression of recombinant human renin in *E. coli* and *Spodoptera frugiperda* (Sf-9) insect cells, development of a simple and rapid assay method for human renin, occurrence of renin inhibitor in fermented soybean, isolation of renin inhibitors from soybean, and structure-function relationship of saponins.

2. Expression of recombinant human renin in *E. coli* and Sf-9 insect cells

The isolation of human renin from the kidney was very difficult because of the starting materials and the extremely low concentration of renin in the kidney, although some groups have succeeded in purifying human kidney renin associated with juxtaglomerular cell

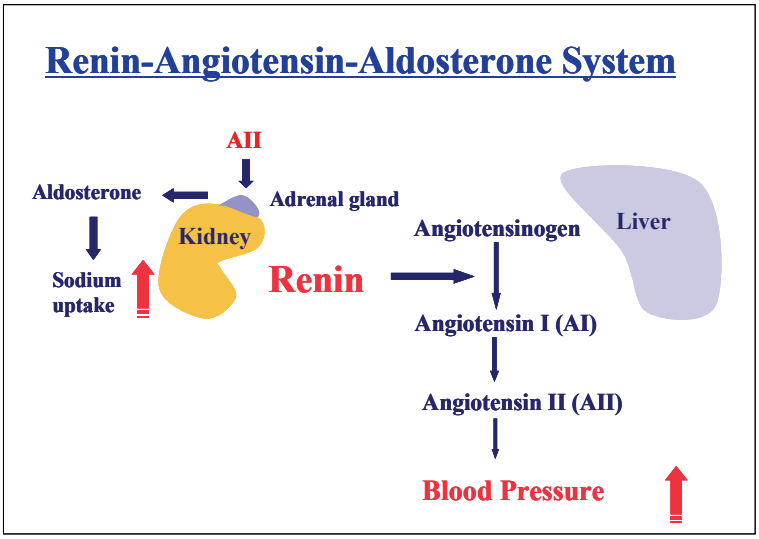

Fig. 1. Renin-angiotensin-aldosterone system

tumor [Galen *et al.*, 1979] and using Haas's preparation [Yokosawa *et al.*, 1980]. These human renins showed a heterogeneous electrophoretic pattern because of the variety of sugar chains and partial degradation. The expression of recombinant human prorenin in animal cells [Poorman *et al.*, 1986, Weighous *et al.*, 1986, Vlahos *et al.*, 1990] or *Escherichia coli* cells [Imai *et al.*, 1986] has also been reported. In the case of Chinese hamster ovary cells [Poorman *et al.*, 1986], recombinant human prorenin was secreted into the medium. However, the expression level was very low. On the other hand, with the expression of human renin in *E. coli* cells, the expressed human prorenin formed inclusion bodies and did not properly refold into active renin [Imai *et al.*, 1986].

We constructed thioredoxin-human prorenin fusion protein expression vector. The constructed expression vector, pETHRN1, was transformed into *E. coli* BL21 (DE3) cells [Takahashi *et al.*, 2006]. The addition of IPTG to the cells carrying pETHRN1 resulted in the highly efficient production of fusion protein. The SDS-PAGE analysis of whole cell extract showed the major protein in the *E. coli* cells to be the fusion protein. The expressed fusion prorenin formed inclusion bodies in *E. coli* cells. The inclusion bodies were purified by sonication and centrifugation. The purified inclusion bodies were solubilized with a 4 M guanidine hydrochloride solution. The gradual removal of guanidine hydrochloride by stepwise dialysis with the introduction of L-arginine and a non-ionic detergent Briji 35 resulted in efficient refolding of fusion prorenin. The refolded fusion prorenin was activated by trypsin, a model activator of prorenin. As shown in Fig. 2, the 58 kDa fusion prorenin disappeared with the emergence of 35-40 kDa mature renins. The 35-40 kDa mature enzymes are active species formed by limited proteolysis of trypsin. The active renin was used for the screening of renin inhibitor from various foodstuffs.

The expression of recombinant human prorenin and renin in mammalian cells has been reported. In these cases, the major secreted protein was inactive prorenin and trypsin treatment was essential for the activation of prorenin. We also expressed recombinant human renin in Sf-9 insect cells with recombinant baculovirus, vhpR, carrying human preprorenin cDNA in the polyhedrin locus of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) [Takahashi *et al.*, 2007]. Sf-9 cells were infected with

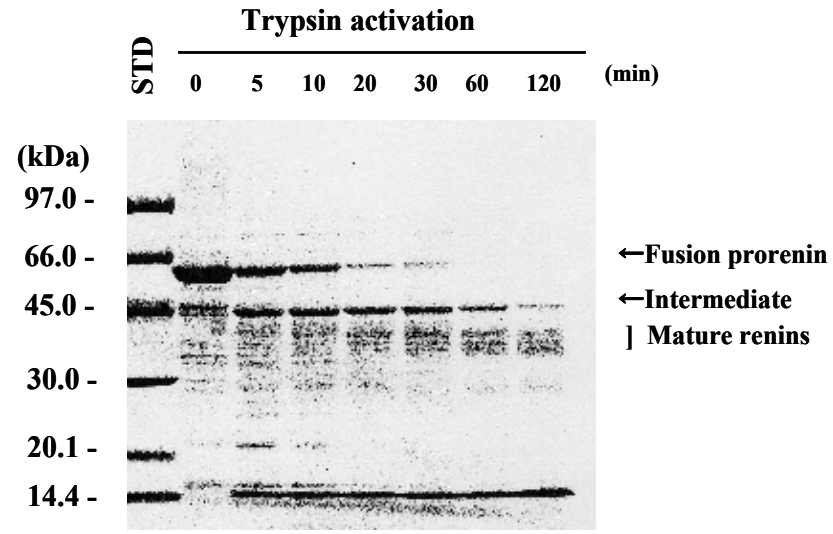

Fig. 2. Processing of fusion prorenin by trypsin.

recombinant baculovirus at a multiplicity of infection of 1.0 pfu/cell and cells were cultured in SF-900II serum-free medium using 250-ml shaker flasks on an orbital shaker at 100 rpm at 28°C. Cells grew continuously until day 3, but total cell numbers and viability decreased at days 4 and 5 of culture. Renin activity was not detected until day 3. A small amount of renin activity was detected at day 4 and increased dramatically at day 5. When the media were used for Western blotting, prorenin with a molecular weight of 43,000 was detected at days 3 and 4 of culture. On the other hand, only mature renin (molecular weight of 40,000) was detected in the day 5 culture. These results clearly show that the expressed prorenin was activated by proteinase appearing at the late stage of culture. This is the first demonstration of the accumulation of active renin in the baculovirus expression system [Takahashi *et al*., 2007]. Recently, we purified prorenin processing enzyme (PPE) from a medium of baculovirus-infected Sf-9 cells and revealed it to be a cysteine proteinase encoded by the AcNMPV gene [Gotoh *et al*., 2009, Gotoh *et al*., 2010a, Gotoh *et al*., 2010b].

We purified recombinant human renin in day 5 culture. Table 1 shows a summary of purification. Approximately 0.6 mg of purified preparation was obtained from 200 ml of culture with a yield of 35%. The quantity of renin production in the medium was estimated to be 8.7 mg/l from the yield. Previously, the amounts of recombinant prorenin produced by mammalian and insect cells were 2-15 mg/l of medium [Poorman *et al*., 1986, Weighous *et al*., 1986, Fritz et al., 1986]. However, the production of active renin was very low even in insect cells. Thus, our result is the highest production of active human renin in conventional reports.

Steps	Total protein (mg)	Total activity (U/mg)	Specific activity (%)	Yield
1. Medium	875	470	0.55	100
2. Pepsatin column	1.69	171	101	36.4
3 Mono Q	0.613	166	270	35.3

Table 1. Purification of recombinant human renin from Sf-9 medium.

The purified renin preparation showed a single protein band on SDS-PAGE with an apparent molecular weight of 40,000. The N-terminal amino acid sequence of the purified preparation was determined to be NH_2-Leu-Gly-(X)-Thr-Thr-Ser-Ser-Val-Ile-Leu-. The sequence agreed with the N-terminal sequence from +3 to +12 of mature human renin, except for a unidentified residue, which appeared to be a glycosylated Asn residue, as reported previously [Imai *et al.*, 1983]. The processing site of the renin expressed in Sf-9 cells was different from that of authentic renin because of the substrate specificity of PPE in Sf-9 cells [Gotoh *et al*., 2010b].

3. Development of internally quenched fluorogenic substrate for human renin

The internally quenched fluorogenic (IQF) substrate for human renin, *N*-methylanthranyl (Nma)-Ile-His-Pro-Phe-His-Leu*Val-Ile-Thr-His-N^{ε}-2, 4,-dinitrophenyl (Dnp)-Lys-D-Arg-D-Arg-NH_2 (*, scissile peptide bond) was custom-synthesized at Peptide Institute (Osaka, Japan). Hydrolysis of IQF substrate at the Leu-Val bond was spectrophotometrically determined. The reaction mixture contained 1 µl of 1 mM IQF substrate solution in DMSO, 44 µl of sodium phosphate buffer, pH 6.5, 0.1 M NaCl, 0.02% Tween 20, 0.02 % NaN_3, and 5 µl of renin solution in a total volume of 50 µl. The reaction mixture was incubated at 37℃ for 30 min and the reaction was terminated by adding 0.1 M triethanolamine, pH 9.0. The increase in fluorescence intensity was measured at an emission wavelength of 440 nm upon excitation at 340 nm. The k_{cat} and K_m values of recombinant renin for the IQF substrate at pH 6.5 and at 37℃ were 833 s^{-1} and 35.7 μM^{-1}, respectively (Fig. 3) [Takahashi *et al.*, 2007].

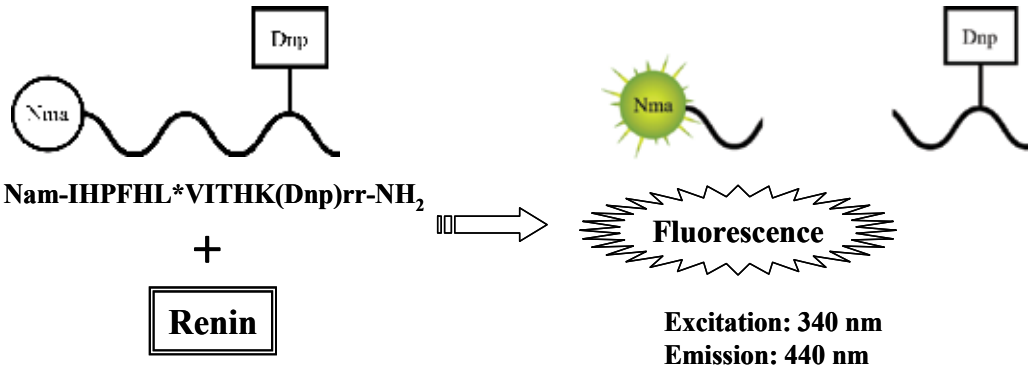

Fig. 3. Measurement of human renin activity using IQF substrate.

4. The occurrence of renin inhibitor in fermented soybean (miso)

Using recombinant human renin, we screened the inhibitory activity of desalted miso extract. Miso is a very common seasoning in Japan. The water extracts of miso were not suitable for the renin inhibition assay because of the high salt concentration. A high concentration of NaCl interrupted the renin activity. Thus, the water extracts of miso were desalted using a Sep-Pak C18 cartridge (Millipore). We tested commercially available miso and found that some miso exhibited renin inhibitory activity [Takahashi *et al*., 2006].

To understand the origin of the renin inhibitory activity in the miso samples, we studied the renin inhibitory activity during fermentation of miso. As shown in Table 2, young miso

showed high renin inhibitory activity. Seven-day fermented miso was more potent than 30-day fermented miso. These results suggest that the renin inhibitory activity in miso decreased during fermentation, so that soybean or koji may exhibit renin inhibitory activity. Thus, we prepared extracts of soybean, steamed soybean, and koji. Soybean and steamed soybean extracts showed high renin inhibitory activity (Table 2). On the other hand, koji had nearly no renin inhibitory activity. These results clearly show that miso exhibited the renin inhibitory activity derived from soybeans.

Samples	n	Renin activity (%)	
		Mean	Standard deviation
Control	9	100.23	4.68
7-day miso	5	67.10	13.28
30-day miso	5	83.52	4.87
Koji	5	90.01	3.70
Soybean	5	49.42	3.16
Steamed soybean	5	37.38	3.78

Table 2. Effects of miso, koji, and soybean extracts on renin activity.

5. Isolation of renin inhibitor from soybean

Before isolation of renin inhibitor from soybean, we investigated the localization of renin inhibitor in soybean. Soybean was separated into two parts, embryo and cotyledon, and then extracted and evaluated for renin inhibitory activity. Embryo extract contained about 3-fold-higher renin inhibitory activity than cotyledon extract. Hence, we used soybean embryo for isolation of renin inhibitor. The scheme for the purification of soybean renin inhibitor is shown in Fig. 4. Approximately 70 mg of purified inhibitor was obtained from 750 g of soybean embryo. Isolated soybean renin inhibitor (SRI) gave soyasapogenol moiety and sugar chain unit as rhamnopyranosyl (1→2) galactopyranosyl (1→2) glucopyranosiduronic acid for ^{1}H and ^{13}C NMR spectra [Kitagawa *et al*., 1982, Kitagawa *et al*., 1988, Tsunoda *et al*., 2008]. Finally, the soybean renin inhibitor was identified as soyasaponin I (Fig. 5) by direct comparison with standard compounds for $[\alpha]_D$, mixed melting point, ^{1}H NMR, and IR spectra [Takahashi *et al*., 2008]. Soyasaponin I is one of the major saponins in soybean [Gu *et al*., 2002].

The purified SRI inhibited renin activity in a dose-dependent manner. An IC_{50} value of 30 μg/ml was obtained. Kinetic studies with SRI indicated partial noncompetitive inhibition with a *K*i value of 37.5 μM. The inhibitory spectra of SRI were studied using various proteinases. SRI also inhibited porcine kidney renin activity with an IC_{50} value of 30 μg/ml. SRI had very little effect on porcine pepsin or cysteine proteinases (papain and bromeline), and had no effect on serine proteinases (bovine pancreatic trypsin and human urinary kallikrein) or metalloproteinases (rabbit lung ACE and porcine kidney aminopeptidase) [Takahashi *et al*., 2008]. Moreover, a significant decrease in systolic blood pressure of spontaneously hypertensive rats was observed when commercially available soyasaponin was orally administered at 80 mg/kg/day for 8 weeks [Hiwatashi *et al*., 2010].

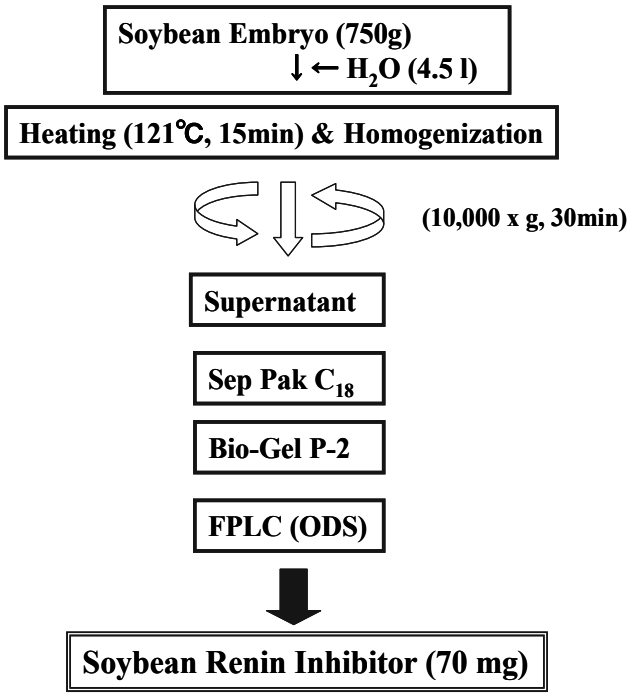

Fig. 4. Isolation of renin inhibitor from soybean embryo.

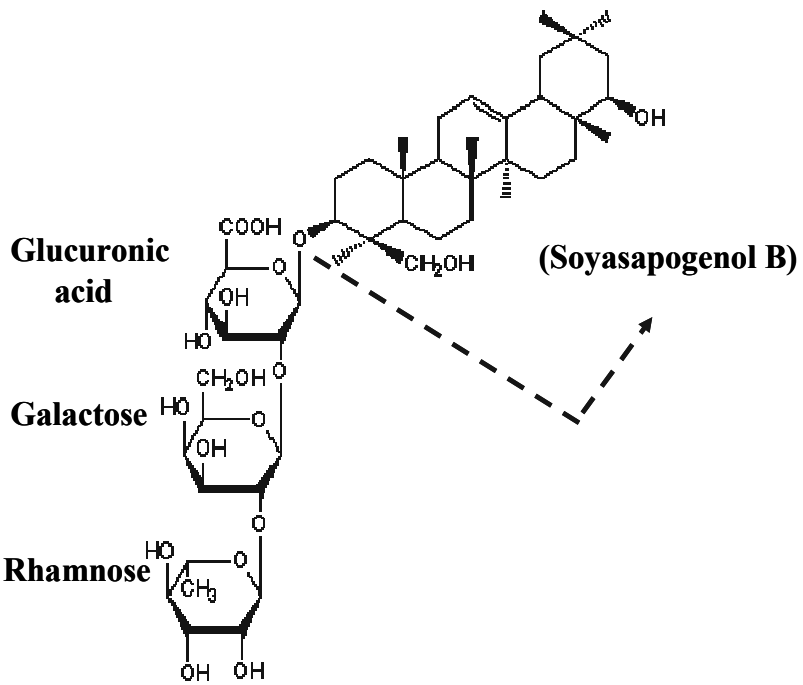

Fig. 5. Chemical structure of soyasaponin I.

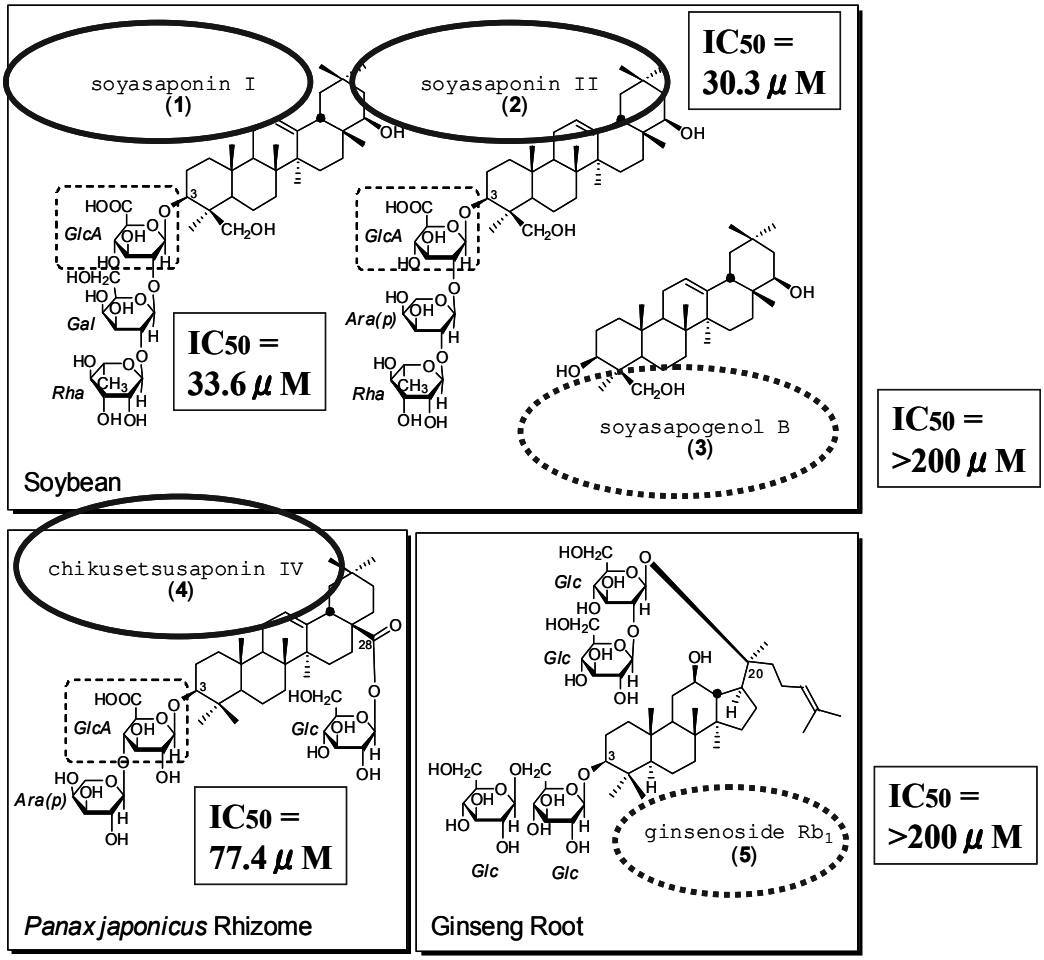

Fig. 6. Chemical structures and IC_{50} values of soyasaponin I (1), soyasaponin II (2), soyasapogenol B (3), chikusetsusaponin IV (4), and ginsenoside Rb_1 (5). Compounds 1, 2, and 4 had renin inhibitory activity. Compounds 3 and 5 had no effects on renin activity. *Ara(p)*, arabinose; *Glc*, glucose; *GlcA*, glucuronic acid; *Rha*, rhamnose.

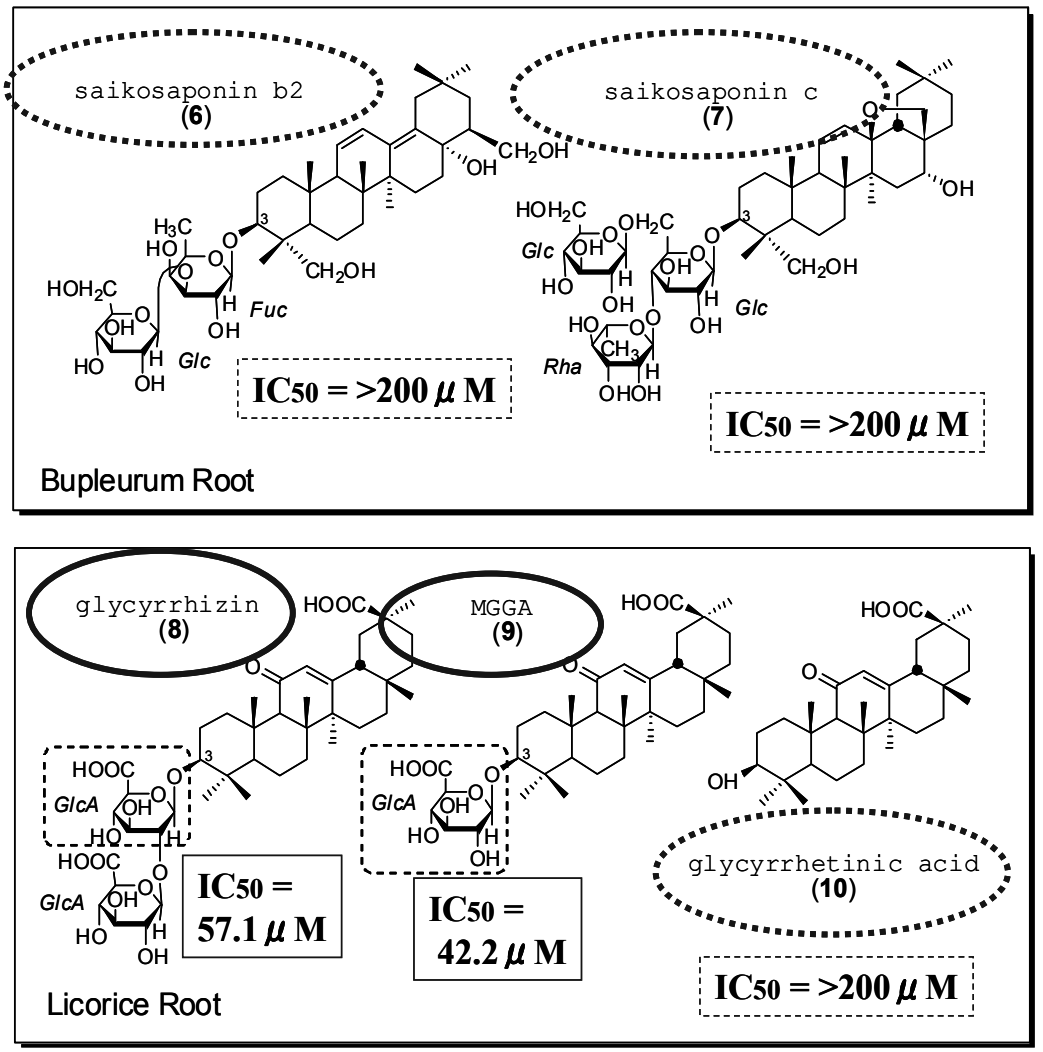

Fig. 7. Chemical structures and IC_{50} values of saikosaponin b2 (6), saikosaponin c (7), glycyrrhizin (8), monoglucuronyl glycyrrhetinic acid (MGGA) (9), and glycyrrhetinic acid (10). Compounds 8 and 9 inhibited renin activity. Compounds 6, 7, and 10 had no effect on renin activity. Fuc, fructose; *Glc*, glucose; *GlcA*, glucuronic acid; *Rha*, rhamnose.

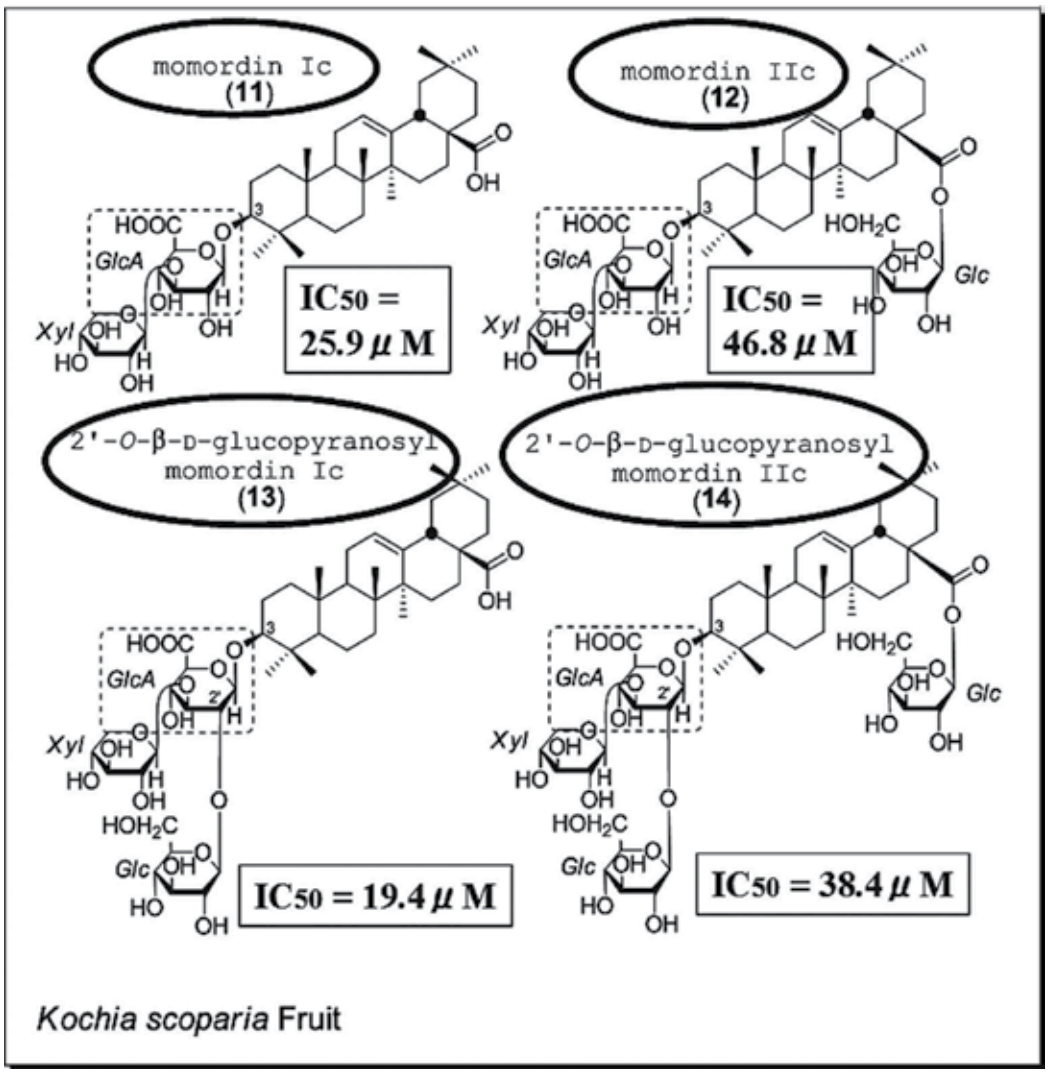

Fig. 8. Chemical structures and IC_{50} values of momordin Ic (11), momordin IIc (12), 2′-O-β-D-glucopyranosyl momordin Ic (13), and 2′-O-β-D-glucopyranosyl momordin IIc (14). Compounds 11, 12, 13, and 14 inhibited renin activity. *Glc*, glucose; *GlcA*, glucuronic acid; *Xyl*, xylose.

6. Renin inhibition by saponins

We investigated the effects of various saponins and sapogenols on human renin activity to elucidate the structure-function relationship of saponins [Takahashi et al., 2010]. Figures 6 to 8 show the chemical structure of saponins and sapogenols tested. Among them, soyasaponin I (**1**), soyasaponin II (**2**), saikosaponin c (**7**), 2′-*O*-β-D-glucopyranosyl momordin Ic (**13**), and 2′-*O*-β-D-glucopyranosyl momordin IIc **(14)** contain three sugar units attached at the 3β-hydroxyl position. Chikusetsusaponin IV (**4**), ginsenoside Rb_1 (**5**), saikosaponin b_2 (**6**), glycyrrhizin (**8**), momordin Ic (**11**), and momordin IIc (**12**) contain two sugar units at the same position. Compound **9** (monoglucuronyl glycyrrhetic acid: MGGA) contains one sugar unit at the same position. Soyasapogenol B (**3**) and glycyrrhetinic acid (**10**) are sapogenols.
Soyasaponin I (**1**), soyasaponin II (**2**), chikusetsusaponin IV (**4**), glycyrrhizin (**8**), MGGA (**9**), and saponins from *Kochia scoparia* fruit (**11-14**) inhibited human renin activity in a dose-dependent manner with IC_{50} values of 19.4-77.4 μM. These saponins have a glucuronide residue at the 3β-hydroxy position. On the other hand, ginsenoside Rb_1 (**5**), saikosaponin b_2 (**6**), saikosaponin c (**7**), and sapogenol compounds [soyasapogenol B (**3**) and glycyrrhetinic acid (**10**)] had no effect on renin activity. Compounds **5**, **6**, and **7** have glucose or fructose residues at the 3β-hydroxy sugar chain's first inner position. These results clearly indicate that glucuronic acid residues at the 3β-hydroxyl sugar chain's first inner position are essential for renin inhibition.

7. Conclusion

We developed efficient production of recombinant human renin in *E. coli* and *Spodoptera frugiperda* (Sf-9) insect cells. Using recombinant human renin and newly developed IQF substrate, we screened for renin inhibitor from several foodstuffs and found renin inhibitory activity in miso originated from soybean. The purified renin inhibitor from soybean was identified as soyasaponin I. Moreover, we investigated the effects of various saponins and sapogenols on human renin activity and showed that glucuronide saponins, glucuronic acid residues at the 3β-hydroxyl sugar chain's first inner position are essential for renin inhibition.

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23

Heat, Salinity, and Acidity, Commonly Upregulate A1aB1b Proglycinin in Soybean Embryonic Axes

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1. Introduction

All organisms respond to elevated temperatures and to chemical and physiological stress by increasing the synthesis of heat shock proteins. By definition, heat shock proteins (HSPs) are a group of functionally related proteins whose expression is increased when cells are exposed to elevated temperatures (De Maio, 1999). Brief exposure of cells to sub-lethal high temperature toughens the cells and provides protection from subsequent and even more severe temperature. In 1962, Ritossa reported that heat induced a characteristic pattern of puffing in the chromosomes of *Drosophila*. This discovery eventually led to the identification of the heat-shock proteins (HSPs) whose expression these puffs represented. Increased synthesis of specific proteins in *Drosophila* cells following heat shock was first reported in 1974 by Tissières *et al*. The regulation of heat shock gene expression in eukaryotes is largely mediated by the conserved heat shock transcription factors (HSFs). HSFs are present in a latent state under normal conditions. They are activated upon heat stress by induction of oligomerization and high-affinity binding to DNA and by exposure of domains for transcriptional activity (Wu, 1995). Experimental manipulation by molecular engineering of the heat response aims to select plant species resistant to hot weather, mainly in arid soils.

Stress factors other than heat also alter the cell physiology and induce stress responses comparable to that induced by thermal shock. Cellular energy depletion, extreme concentrations of ions, presence of uncommon osmolytes, rare gases, pollutants including many toxic substances, high or low temperatures, and drought, among others, all are stressing factors (Feder and Hofmann, 1999).

Beginning in the mid-1980's, researchers recognized that most stress-induced proteins, including HSPs, function as molecular chaperons. The chaperones are proteins that assist other proteins to fold, refold, travel to their place of residence (cytosol, organelle,

membrane, extracellular space), and translocate across membranes. Molecular chaperones participate in a variety of physiological processes and are widespread in organisms, tissues, and cells. Chaperone failure will have an impact on one or more cellular functions, which may lead to disease or even to death (Macario and Macario, 2007).

The principal heat-shock proteins with chaperone activity belong to five conserved families: HSP60, HSP70, HSP90, HSP100, and the small HSPs (HSP10, HSP20-30, and HSP40) (Schlesinger, 1990, 1994; Li and Srivastava, 2004). Heat-shock proteins are components of the stress response of eukaryotic and prokaryotic cells subjected to a variety of adverse conditions.

In plants, production of high levels of heat shock proteins can also be triggered by their exposure to different kinds of environmental stress conditions other than heat, including nitrogen deprivation, salinity in general and sodicity in particular, pollutants (heavy metals and pesticides), cold, and dryness, among others (Wang *et al*., 2003; Mahajan and Tuteja, 2005). Hence, HSPs are also known as stress proteins (Santoro, 2000). Hundreds of studies on plants of nutritious or economical importance subjected to a diversity of stress conditions, aimed to select resistant plant varieties, have been reported. *Glycine max* (LaFayette and Travis, 1990; Hsieh *et al*., 1992), *Arabidopsis thaliana* (Queitsch *et al*., 2000; Banti *et al*., 2008; Tonsor *et al*., 2008), *Oryza sativa* (Pareek, *et al*, 1995), *Nicotiana tabacum* (Usami *et al.,* 1995), *Triticum aestivum* (Hendershot *et al*., 1992; Blumenthal, *et al*., 1998; Efeoglu and Terzioglu, 2007; Sancho *et al*., 2008), *Zea mays* (Cooper and Ho, 1983; Lund *et al*., 1998, Nieto-Sotelo *et al*., 2002), *Prosopis chilensis* (Ortíz *el al*., 1995; Ortíz and Cardemil. 2001), *Pisum sativum* (Lee *et al.,* 1995), *Lycopersicon esculentum* (Polenta *et al*., 2007), *Sorghum bicolor* (Ougham and Stoddart 1986), *Cicer arietinum* (Bibi *et al*., 2009) and *Phaseolus vulgaris* (Nagesh-Babu and Devaraj, 2008) are within the many plants whose stress responses have been investigated.

Soybean subjected to thermal shock for brief periods respond by increasing the expression of certain proteins. The response is variable depending on several factors, mainly temperature, length of the shock, age of the crop at the time the thermal shock is applied, and source of the sample (embryonic axes, cotyledon, leaves, roots, etc.). Depending on these factors, several HSPs have been identified belonging to the five HSP families (Key *et al*., 1981; Mansfield and Key, 1987; Lin *et al*., 1984; Hsieh *et al*., 1992).

Stress response of soybean to high salinity has also been investigated; high salt concentrations have negative effects on growth, nodulation, seed quality and quantity, thus reducing the yield of soybean. To cope with salt stress, soybean has developed several resistance mechanisms, including: maintenance of ion homeostasis; adjustment in response to osmotic stress, restoration of osmotic balance, and other metabolic and structural adaptations (Phang *et al*., 2008).

As far as we know, the study of acidity and alkalinity as stressing factors on soybean has not been addressed. In this communication we report the experimentally-induced stress response of embryonic axes of soy to thermal shock (40ºC, 42ºC), salinity (200 mM NaCl), and acidity (pH 5.5) in a comparative manner. Hot soils and soils with high salt concentration, as well as acid- or alkaline- soils, certainly constitute inappropriate substrates for the satisfactory growth of soybean and other plant species.

2. Materials and methods

Chemicals

Except otherwise indicated, chemical reagents were from Sigma/Aldrich Chemical Co. (Branch in Mexico).

Soybean seeds

The variety Huasteca-100 of soybean (*Glycine max*) was used in this study; this variety was selected out of three other soybean varieties (Crystalline, UFV-1 and FT-0191) because of its good quality (Arce-Paredes *et al*, 2009) and because it is a prospect for high cultivation in certain regions in México (it is expected to cultivate up to 80 million square meters of land with soy Huasteca-100 by the year 2011). Soybean Huasteca-100 was contributed by Ing. Rafael Reza Alaman from Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), campo experimental at Iguala, Guerrero, México, and it was a recent crop.

Soybean minicrops

Healthy soybean seeds were selected, decontaminated for 10 min in 1% hypochlorite in water, rinsed once with distilled water (DW), let to hydrate for 4 h with DW, and planted on moisten cotton beds at 28°C. Moistening solutions depended on the experiment and were: 10 mM phosphate buffer, pH 7.0; 200 mM NaCl in 100 mM phosphate buffer, or 10 mM acetate buffer, pH 5.5.

Heat shock

Soybean seeds were grown on a cotton bed moistened with 10 mM phosphate buffer, pH 7.0, at 28°C for 48 h in the dark before they were subjected to thermal shock for 2 h. At this time, germinated embryonic axes measured about 1.0 cm in length. Three lots were prepared and subjected to no heat-shock (lot 1, control), 40°C (lot 2), or 42°C (lot 3). Lots were then reincubated at 28°C for 4 h. At this time, cultures were harvested and examined for changes in length, mass, and protein content profiles of embryo axes.

Saline shock

Lots of 50 soybean seeds were grown at 28°C on cotton beds moistened with 200 mM NaCl in 10 mM phosphate buffer, pH 7.0. From previous results from heat-shocked soybean seeds, crops were harvested at 54 h of cultivation, time at which embryonic axes were excised, measured, weighted, and subjected to protein extraction.

pH shock

Lots of soybean seeds were grown at 28°C on cotton beds moistened with 10 mM acetate buffer, pH. 5.5. Based on previous results, crops were harvested at 54 h of cultivation and embryonic axes were collected, measured, weighted and subjected to protein extraction.

Extract preparation

At each harvesting time, embryonic axes were excised from germinated soybean seeds, measured, and weighted. Extracts were prepared from equal amounts (1.0 gram) of embryonic axes collected from each stress-treated soybean lot. Embryonic axes were cut into small (2-3 mm) pieces and then they were ground in a mortar with 5.0 ml of 25 mM Tris, 192 mM Glycine, pH 8.3 solution in the presence of 1 mM ethyl maleimide and 1 mM phenylmethylsulfonyl fluoride (PMSF). After filtering trough Whatman No. 2 filter paper, extracts were centrifuged at 10,000 x g (Sorvall RC-5B, DuPont Inst., USA) for 15 min at 4°C and soluble extracts were sterilized through 0.2 µm filters (Millipore), separated into 0.5 ml aliquots, and stored frozen at -20°C until used.

Protein contents

The amount of protein in each extract was determined by a micro-adaptation to the Lowry's method (Lowry *et al*., 1951). Assay was carried out in ELISA plates containing 200 µl of

alkaline reagent per well. The alkaline reagent consisted of 0.2% sodium-potassium tartrate, 0.1% cupric sulfate and 2% sodium carbonate in 0.1N sodium hydroxide. Ten microliters of serially diluted samples or protein standard (BSA, 1 mg per ml) were added per well. The plates were thoroughly shaken and then left undisturbed for 15 min. Then, 10 µl of Folin-Ciocalteau's reagent were added per well. Well-contents were individually homogenized by pipetting and plates were left undisturbed for 30 min at room temperature. Finally, absorbance at 600 nm was measured in an ELISA-reader (Labsystems multiscan Plus, Finland).

Protein precipitation

One ml of soluble extracted protein was mixed with 8 ml of 100% ice-cold acetone and 1 ml of 100% TCA. The mixture was kept at -20ºC for 1 h and then centrifuged at 18,000 x g (Sorvall, RC-5B, DuPont Instr., USA) for 15 min at 4ºC. Supernatant was discarded and the precipitate was washed with 1 ml of ice-cold acetone and recovered by centrifugation as above. Finally, the precipitate was dissolved in lysis buffer as described below.

Protein solubilisation

Proteins were dissolved in 2-D lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4% CHAPS) and incubated at room temperature for 30 min on a shaker. Protein solutions were centrifuged for 30 min at 16,000 x g (Sorvall, RC-5B, DuPont Instr., USA) at room temperature, supernatants were collected and their protein content were measured using the micro-Lowry method already described.

CyDye labeling and Two dimensional differential in gel electrophoresis (2D-DIGE)

To label proteins, 30 µg of each protein samples were incubated with 0.7 µl of CyDye solutions (Cy2, Cy3 or Cy5) diluted 1:5 in dimethyl formamide (DMF) from 1 nmol/µl stock, (GE Healthcare, Piscataway, NJ) at 4°C for 30 min. Labeling was stopped by adding 0.7 µl of 10 mM L-Lysine and incubating at 4°C for 15 min. Then, labeled samples were mixed together, and equal volume of 2X 2-D sample buffer (8 M urea, 4% CHAPS, 20 mg/ml dithiotreitol (DTT), 2% pharmalytes and a trace amount of bromophenol blue) and 100 µl of destreak solution (GE Healthcare) were added. Total sample volumes were adjusted to 260 µl by adding Rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes and a trace amount of bromophenol blue). Samples were incubated at room temperature for 10 min on a shaker and centrifuged for 10 min at 16,000 x g (Sorvall, RC-5B, DuPont Instr., USA). Finally, supernatants were loaded onto 13 cm IPG strip holder (GE Healthcare).

Isoelectrofocusing (IEF) and SDS-PAGE

Thirteen cm IPG strips (pH 3-10) were put on the loaded samples and 1 ml of mineral oil was added on top of the strip. Isoelectrofocusing experiments (IEF) were run following the protocol provided by the manufacturer (GE Healthcare). Upon completion of IEF, strips were equilibrated in buffer 1 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), a trace amount of bromophenol blue and 10 mg/ml DTT) for 15 minutes and then in buffer 2 (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 45 mg/ml DTT) for 10 min with gentle agitation.
IPG strips were then rinsed once in the SDS-gel running buffer, transferred to a 10.5% SDS-gel prepared using low fluorescent glass plates) and sealed with 0.5% (w/v) agarose solution (in SDS-gel running buffer). Electrophoresis was performed at room temperature until dye fronts run out of the gels.

Image scan and data analysis

Upon completion of electrophoresis, gels were scanned using Typhoon TRIO (Amersham BioSciences) following the manufacturer's protocol. The scanned images were then processed by Image Quant software (version 5.0, Amersham BioScience). The quantitative analysis of protein spots was performed using DeCyder software (version 6.5).

Preparative gel

Unlabeled proteins (600-700 μg) were run in analytical gels and stained with Deep Purple total protein stain (GE Health care). Then, gels were scanned and images were processed by DeCyder software to generate a pick list.

Protein identification by Mass Spectrometry

Spot picking and Trypsin digestion

Spots of interest were picked up by Ettan Spot Picker (Amersham BioSciences) based on the in-gel analysis and spot picking design by DeCyder software. The gel spots were washed a few times then digested in-gel with modified porcine trypsin protease (Trypsin Gold, Promega). Digested tryptic peptides were desalted by Zip-tip C18 spin columns (Millipore) and peptides were eluted from the Zip-tip columns with 0.5 μl of matrix solution (5 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% trifluoroacetic acid, 25 mM ammonium bicarbonate), and spotted on a MALDI plate (model ABI 01-192-6-AB).

Mass Spectrometry Analysis

MALDI-TOF MS and TOF/TOF tandem MS/MS were performed on an ABI 4700 mass spectrometer (Applied Biosystems, Framingham, MA). MALDI-TOF mass spectra were acquired in reflectron positive ion mode, averaging 4000 laser shots per spectrum. TOF/TOF tandem MS fragmentation spectra were acquired for each sample, averaging 4000 laser shots per fragmentation spectrum on each of the 10 most abundant ions present in each sample (excluding trypsin autolytic peptides and other known background ions).

Database search

Both of the resulting peptide mass and the associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine (Matrix science) to search the database of National Center for Biotechnology Information non-redundant (NCBInr). Searches were performed without constraining protein molecular weight or isoelectric point, with variable carbamido methylation of cysteine and oxidation of methionine residues, and with one missed cleavage also allowed in the search parameters. Candidates with either protein score C.I.% or Ion C.I.% greater than 95 were considered significant.

3. Results

Protein profiles of soybean embryonic axes grown in different stress conditions obtained by two dimensional differential in gel electrophoresis (2D-DIGE)

Proteins extracted from the embryonic axes of soybean subjected to nil (control lot 1), HS40°C (lot 2), HS42°C (lot 3), acid shock (AS) at pH 5.5 (lot 4), or saline shock (SS) at 200 mM NaCl (lot 5) were fractionated by two-dimensional differential gel electrophoresis (2D-DIGE) as mentioned in Materials and Methods. In each case, matching of protein spots across the gels allowed us to calculate the relative volume of each spot (RV) as the ratio:

volume of a particular spot/ volume of all spots detected in the gel. Those protein spots that showed an increase or a decrease in their RV of at least 1.5 times in relation to control sample (lot 1) were qualified as stress proteins (SPs) or HSPs. Under this criterion, we detected 35 HSPs in lot 2 (HS40°C), 15 HSPs in lot 3 (HS42ºC), 31 ASPs in lot 4 (pH 5.5), and 29 SSPs in lot 5 (NaCl 200 mM). In addition, 11 protein spots showed a diminution in lot HS42ºC. In general, most stressing conditions induced changes in the same proteins. In other words, same proteins were affected by the several stressing factors, with some exceptions.

2D-DIGE maps

Figure 1 shows the 2D-DIGE map of proteins extracted for embryonic axis of soybean grown under normal conditions (Control lot 1, in green), and the protein map for embryonic axes of soybean subjected to HS of 40°C for 2 h (lot 2, in red). Over 2000 protein spots were detected in all lots. Thermal shock (HS 40°C/2 h) induced the over-expression of 35 proteins, the largest number of up-regulated proteins in the experiment. Those proteins over expressed more than 1.5 fold in relation to the control lot appear encircled and orderly numbered from top to bottom.

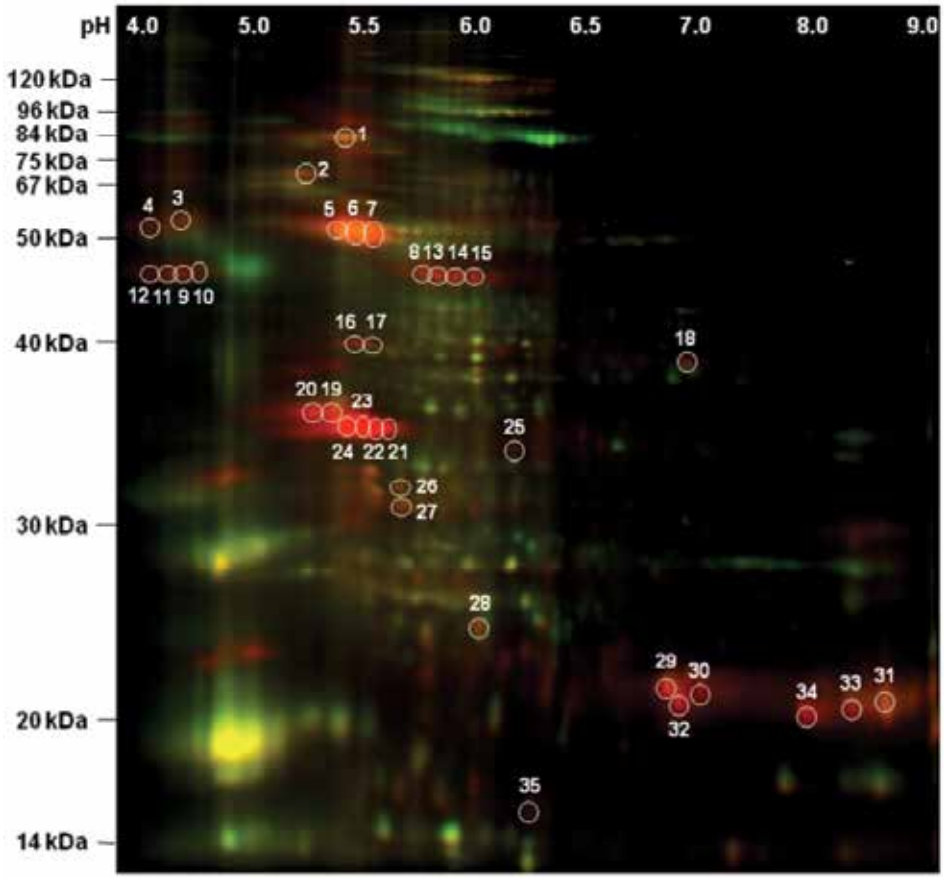


Fig. 1. 2D-DIGE expression map of the proteins extracted from embryonic axes of intact soybean (green) and soybean subjected to thermal shock at 40°C for 2 h (red). Thirty-five proteins (encircled and numbered) were over- expressed in relation to the control lot sample.

Figure 2 shows the 2D-DIGE map of proteins extracted from the embryonic axes of soybean subjected to thermal shock at 42 °C for 2 h (lot 3). In the image, proteins that were over-expressed (15) are encircled and numbered, proteins that were under expressed (11) are only encircled but not numbered, and proteins whose expression was not modified are not pointed at. All protein spots retained their number given in relation to spot proteins in lot 2.

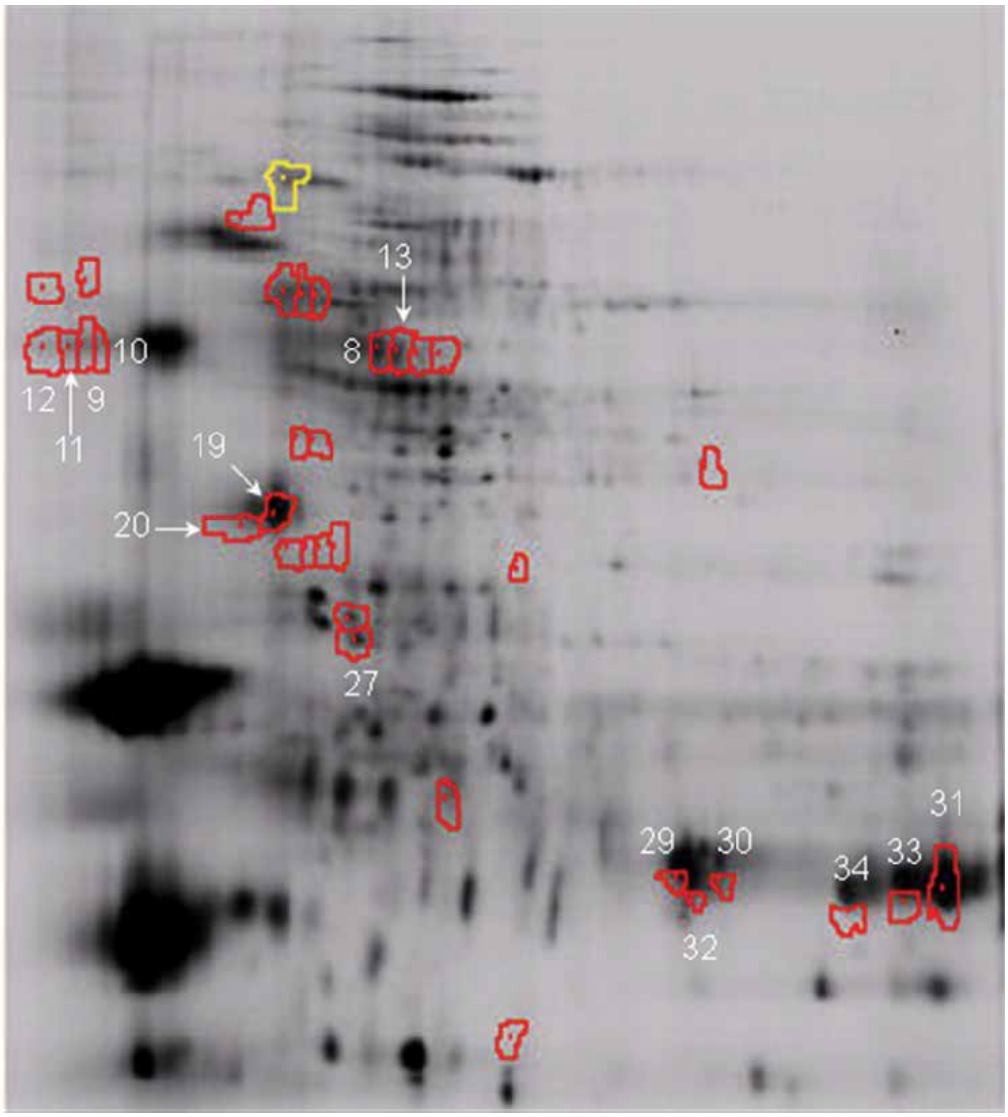


Fig. 2. 2D-DIGE map of the proteins extracted from the embryonic axes of soybean subjected at 42°C for 2 h. Fifteen proteins were over-expressed (encircled and numbered), 11 proteins were under-expressed (encircled but not numbered) and 9 proteins were not modified (not pointed at). Numbers given in reference to the protein map of soybean subjected to 40°C/2 h (lot 2). Arrows identify single spots within a tight group of spots. Yellow circle marks a specific spot not particularly relevant for this study.

2D-DIGE map of the proteins extracted from embryonic axes of soybean subjected to pH 5.5 is shown in Figure 3. Compared with the map for soybean subjected to thermal shock at 40°C (lot 2), 31 proteins were over-expressed (encircled and numbered spots). Protein spots are numbered in relation to spot proteins in lot 2.

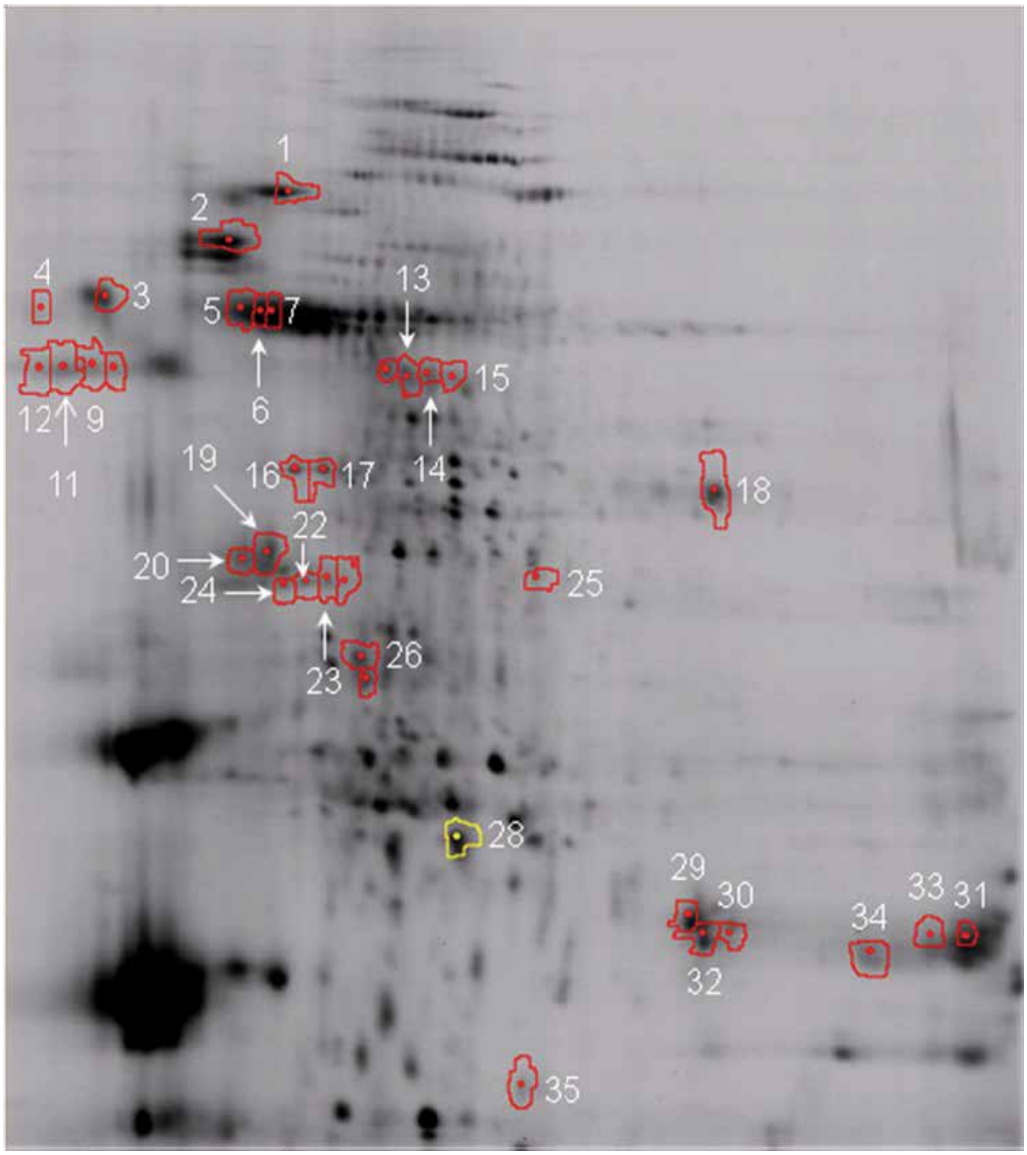


Fig. 3. 2D-DIGE image of proteins extracted from the embryonic axes of soybean grown at pH 5.5. Thirty-one proteins were over-expressed (encircled and numbered). Arrows identify single spots within a tight group of spots. Yellow circle marks a specific spot not particularly relevant for this study.

2D-DIGE spot map of proteins extracted from embryonic axes of soybean grown in the presence of 200 mM NaCl is shown in Figure 4. Twenty-nine proteins behaved as HSPs by increasing their expression beyond 1.5 fold (spots encircled and numbered) in relation to control sample.

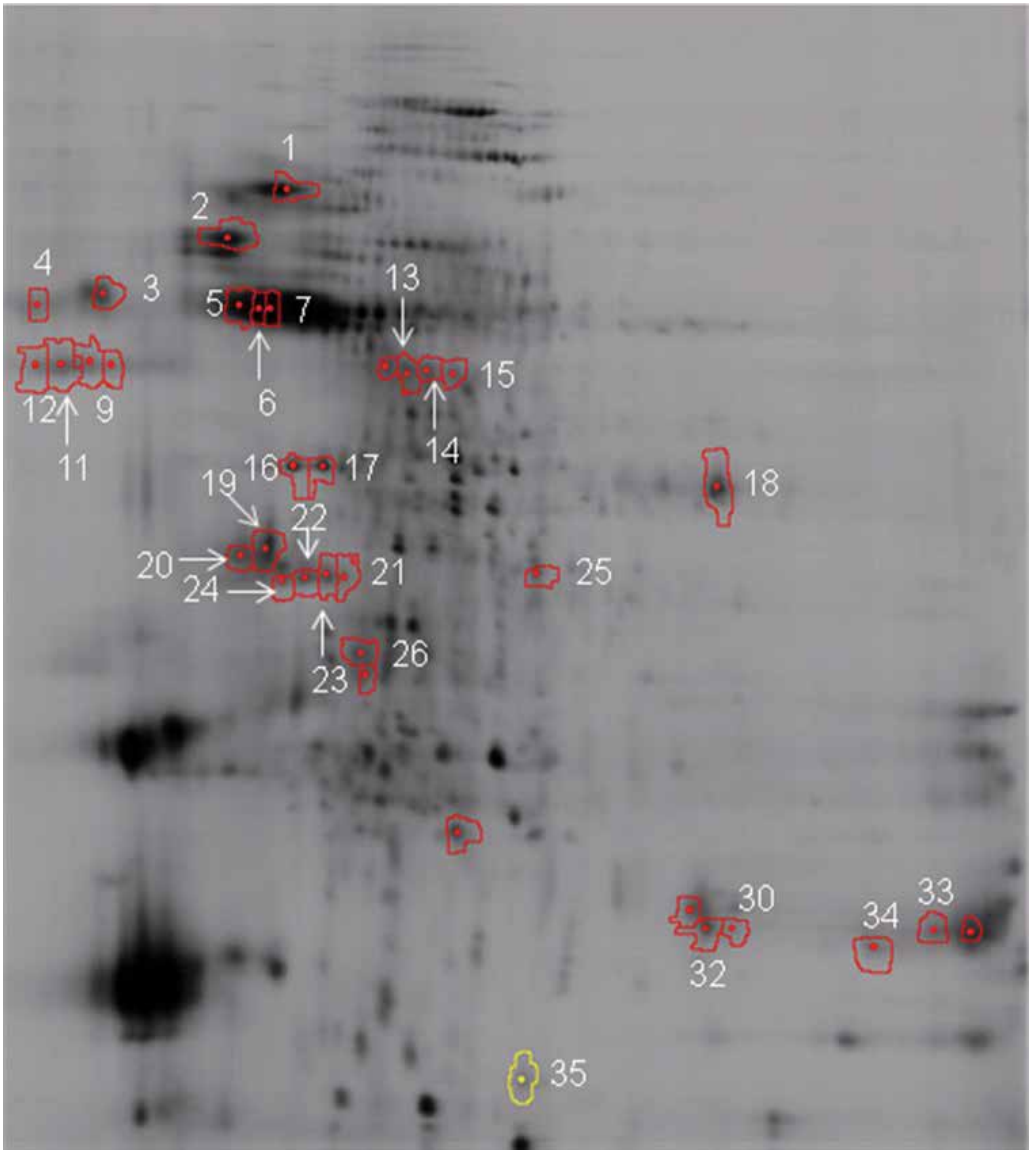


Fig. 4. 2D-DIGE spot map of proteins of embryonic axes of soybean cultured in the presence of 200 mM NaCl. Twenty-nine proteins were over expressed (encircled and numbered). Arrows identify single spots within a tight group of spots. Yellow circle marks a specific spot not particularly relevant for this study.

The relative volume ratio (stressing factor/control) of the proteins whose expression was modified in response to the several stressing factors tested is shown in Table 1. The strongest stressing, non-lethal factor was thermal shock of 40°C for 2 h. This stressing factor

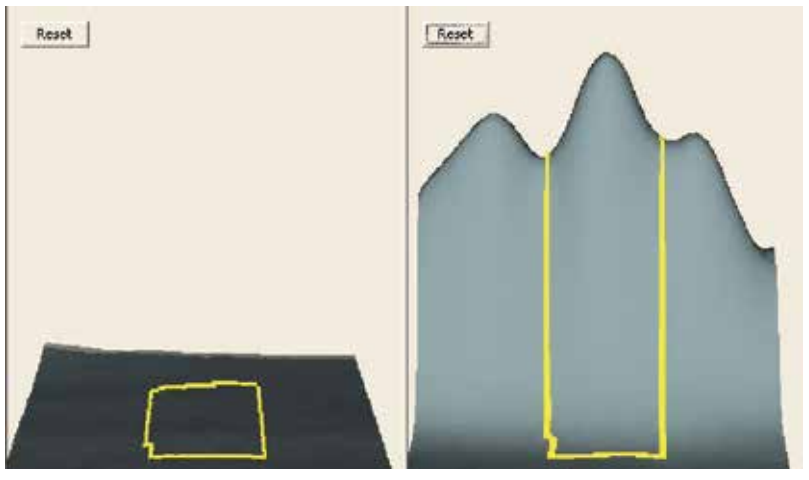
Protein ID	HS40 °C/Control Volume Ratio	HS42 °C/Control Volume Ratio	pH5.5/Control Volume Ratio	200mM/Control Volume Ratio
1	2,79	-1,63	1,63	2,36
2	2,65	-1,99	2,58	3,00
3	2,98	-5,84	2,33	2,33
4	2,17	-1,84	1,90	3,53
5	5,21	-2,03	2,54	5,63
6	5,99	-1,97	2,51	4,76
7	8,07	-2,20	2,73	5,06
8	6,99	3,59	1,04	1,22
9	4,68	1,68	2,18	2,55
10	4,28	1,80	1,39	1,34
11	4,75	1,82	3,77	4,63
12	4,00	1,73	3,40	3,71
13	5,34	2,89	2,10	1,79
14	4,95	1,08	2,18	2,17
15	5,50	-1,35	1,63	1,85
16	7,24	1,31	3,48	6,00
17	3,41	-1,07	1,66	2,61
18	1,50	-4,68	2,31	2,89
19	5,56	3,18	1,67	2,14
20	16,11	2,18	2,05	3,25
21	29,55	-1,59	1,34	2,10
22	**87,07**	1,01	**15,48**	**24,32**
23	50.00	-1,26	2,51	4,48
24	**78,67**	-1,06	**10,07**	**12,63**
25	1,90	-2,47	2,18	4,05
26	2,07	1,44	1,51	1,64
27	2,73	1,94	1,48	1,21
28	1,97	-1,50	1,87	1,05
29	7,55	3,30	1,93	1,23
30	10,40	4,52	3,32	2,32
31	3,70	4,33	1,88	1,46
32	15,86	4,43	3,57	2,10
33	6,78	3,17	1,72	1,56
34	13,02	2,83	2,83	1,98
35	3,42	1,01	3,01	2,94
Up-regulated	35	15	31	29
Down-regulated	0	11	0	0
Non affected	0	9	4	6

Table 1. Relative volume ratios (stress factor/ control) of 2D-DIGE spot proteins of soybean subjected to diverse stressing conditions.

induced the over-expression of 35 proteins that in the 2D-DIGE map are identified from top to bottom with a progressive identification (ID) number (spots ID-1 to ID-35). A similar effect was observed when pH 5.5 and 200 mM NaCl were the stressing factors: 31 proteins were over-expressed at pH 5.5 and 29 proteins were over-expressed at 200 mM NaCl. The most deleterious effect on the germination of soybean was observed when the stressing factor was thermal shock of 42°C for 2 h. Under this condition, of the 35 proteins identified, 11 were down-regulated, 15 proteins were over-expressed and 9 proteins were unaffected.
The most interesting observation that comes out from the results shown in Table 1 is that 2 protein spots, ID-22 and ID-24, maximally increased their expression under all of the stressing situations tested, excepting the heat shock at 42°C for 2 h, which was the most lethal stressing factor in this study.

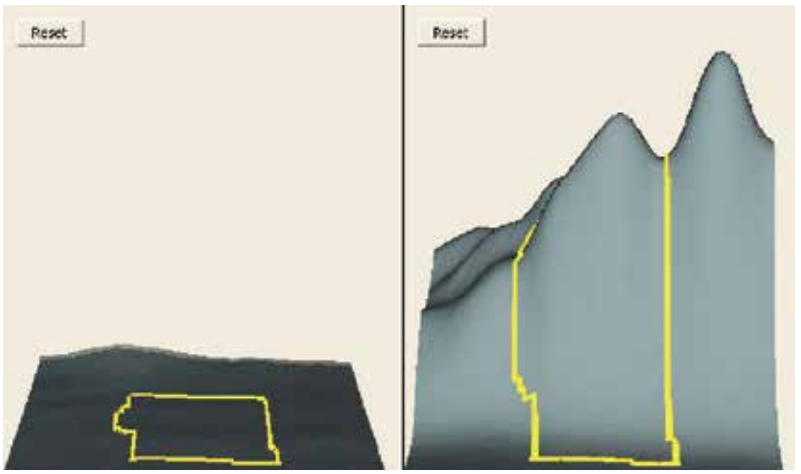
Volume graphs

The volume graphs for spot proteins ID-22 and ID-24 appear in Figures 5 and 6, respectively. The images, obtained with the DeCyder Differential Analysis Software version 6.5, show the spot number and position in the gel, the calculated relative volume, the peak height, and the area of each spot. Protein ID-22, increased 87.07 times at 40°C for 2 h in relation to the same protein in the control lot (lot-1) (Figure 5), while protein ID-24 increased 78.67 times at 40°C for 2 h over the same spot in the control lot (Figure 6).



Spot No. 1367	Spot No. 1367
Position 367,646	Position 367,646
Volume: 5.298e+004	Volume: 4.607e+006
Peak height: 125	Peak height: 14750
Area: 659	Area: 659

Fig. 5. Relative volume graph of 2D-DIGE spot ID-22 from soybean subjected to thermal shock of 40°C for 2 h (right panel) compared to the relative volume of the same spot in the control, intact lot (left panel).



Spot No. 1371	Spot No. 1371
Position 344,647	Position 344,647
Volume: 6.076e+004	Volume: 4.774e+006
Peak height: 84	Peak height: 11542
Area: 901	Area 901

Fig. 6. Relative volume graph of 2D-DIGE spot ID-24 from soybean subjected to thermal shock of 40°C for 2 h (right panel) compared to the relative volume of the same spot in the control, intact lot (left panel)

Protein identification by peptide mass fingerprint from two-dimensional gels

To identify the ID-22 and ID-24 spot proteins, we proceeded to analyze them by MALDI-TOF MS and TOF/TOF tandem MS/MS as described in Material and Methods.

The mass spectrum (MS) obtained for tryptic peptides eluted from 2-D gel spot ID 22 is shown in Figure 7. After baseline correction, background subtraction and peak deisotoping, 10 high-scored ions were submitted to Mascot. Four of the submitted ions matched to theoretical tryptic peptides from chain C, crystal structure of soybean proglycinin A1aB1b homotrimer (Accession No. gi|15988119). The sequences of these peptides are given together with the mass of monoisotopic, single charged ions. The peptide with the highest ion score (IS 42, CI 96.051 %) appears within a rectangle. The mass spectrum MS/MS for this ion is shown in Figure 8.

Figure 9 shows the mass spectrum obtained for tryptic peptides eluted from 2-D gel spot ID-24. The 10 high-scored ions were submitted to Mascot server. Four of the submitted ions matched to theoretical tryptic peptides from chain C, crystal structure of Soybean proglycinin A1aB1b homotrimer (gi|15988119). The sequences of these peptides are given together with the mass of monoisotopic, single charged ions. The peptide with the highest ion score (42, CI 96.051 %) appears within a rectangle. The mass spectrum MS/MS for this ion is shown in Figure 10.

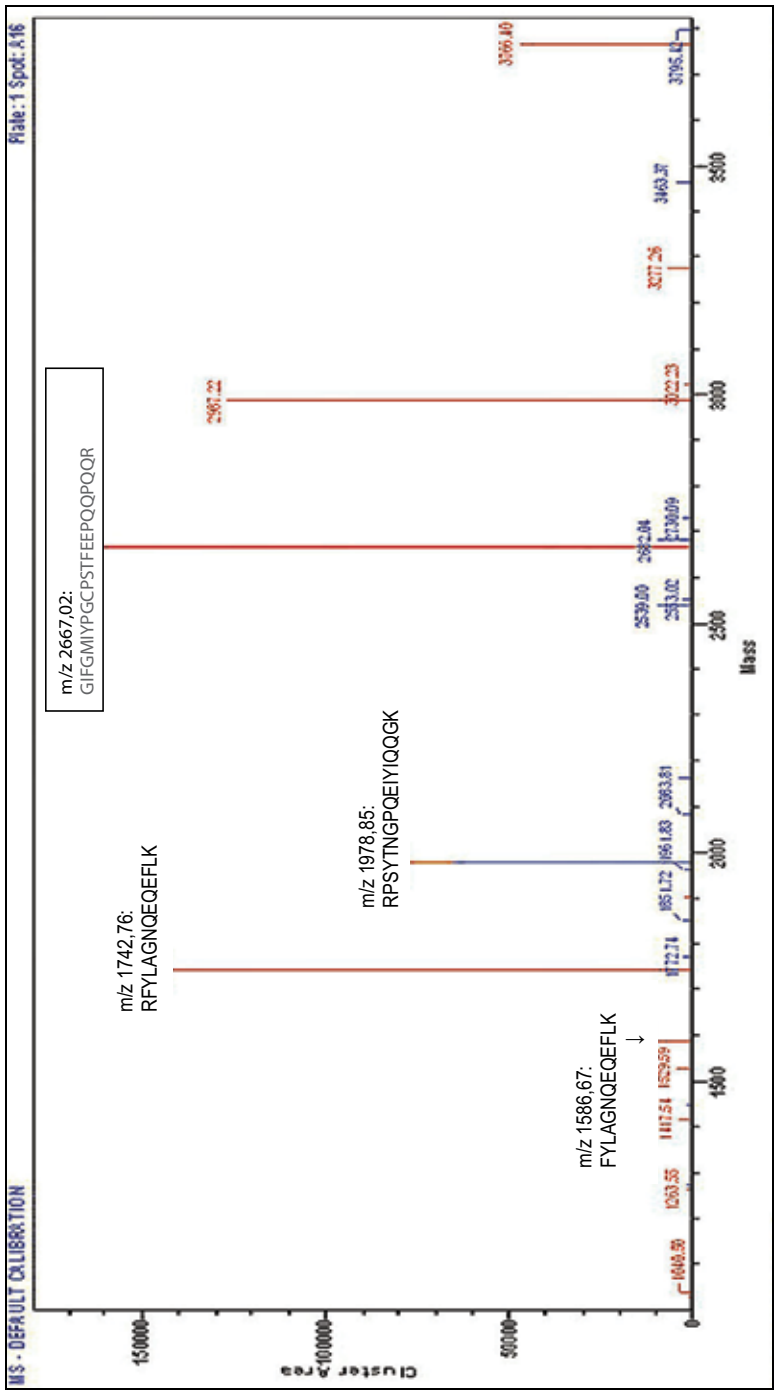


Fig. 7. Mass spectrum (MS) of tryptic peptides eluted from 2D-DIGE spot 22. The m/z value and sequence for each peptide is shown in the graph. The peptide with the highest ion score (42, CI 96.051 %) appears within a rectangle.

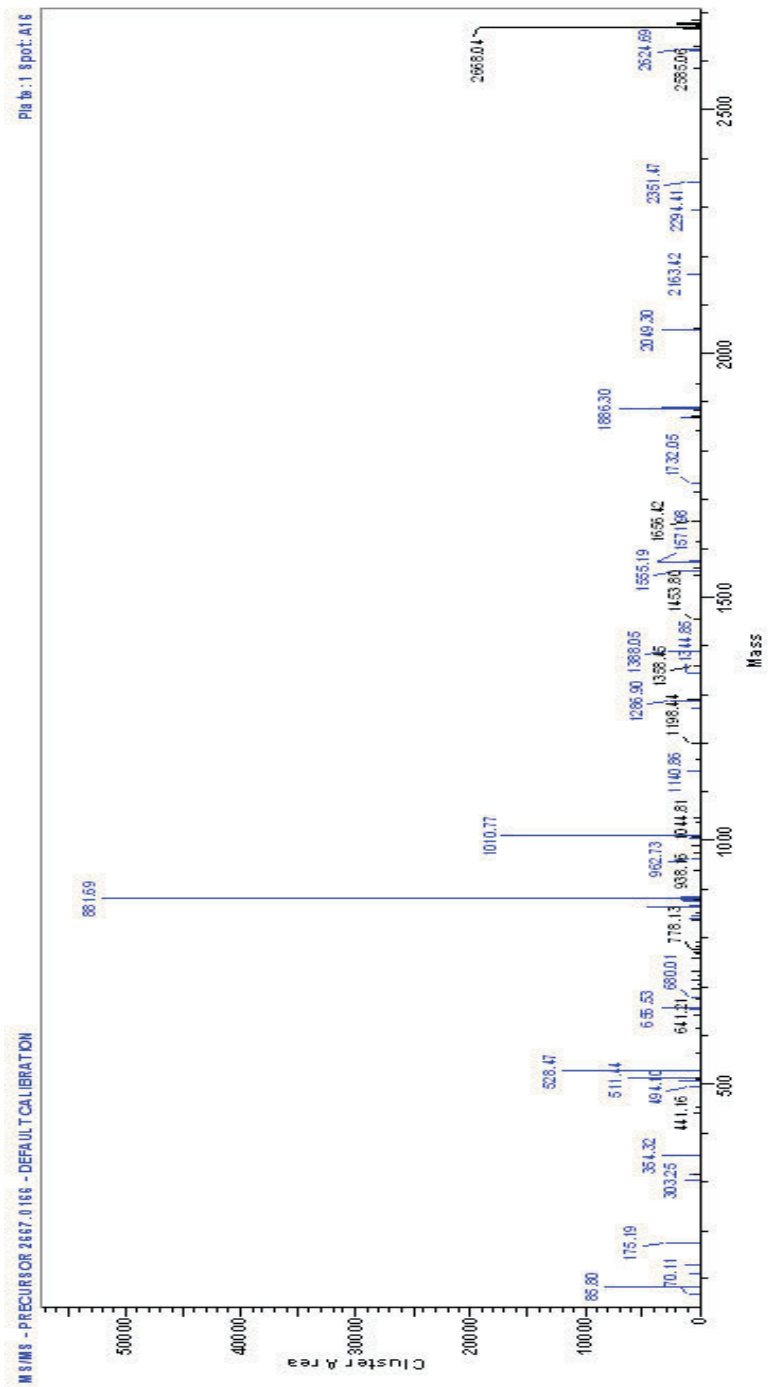


Fig. 8. MS/MS spectrum of peptide m/z 2667.02 (GIFGMIYPGCPSTFEEPQQPQQR) from protein spot ID 22. Sequences of these peptide ions fit within the sequence of peptide m/z 2667.02

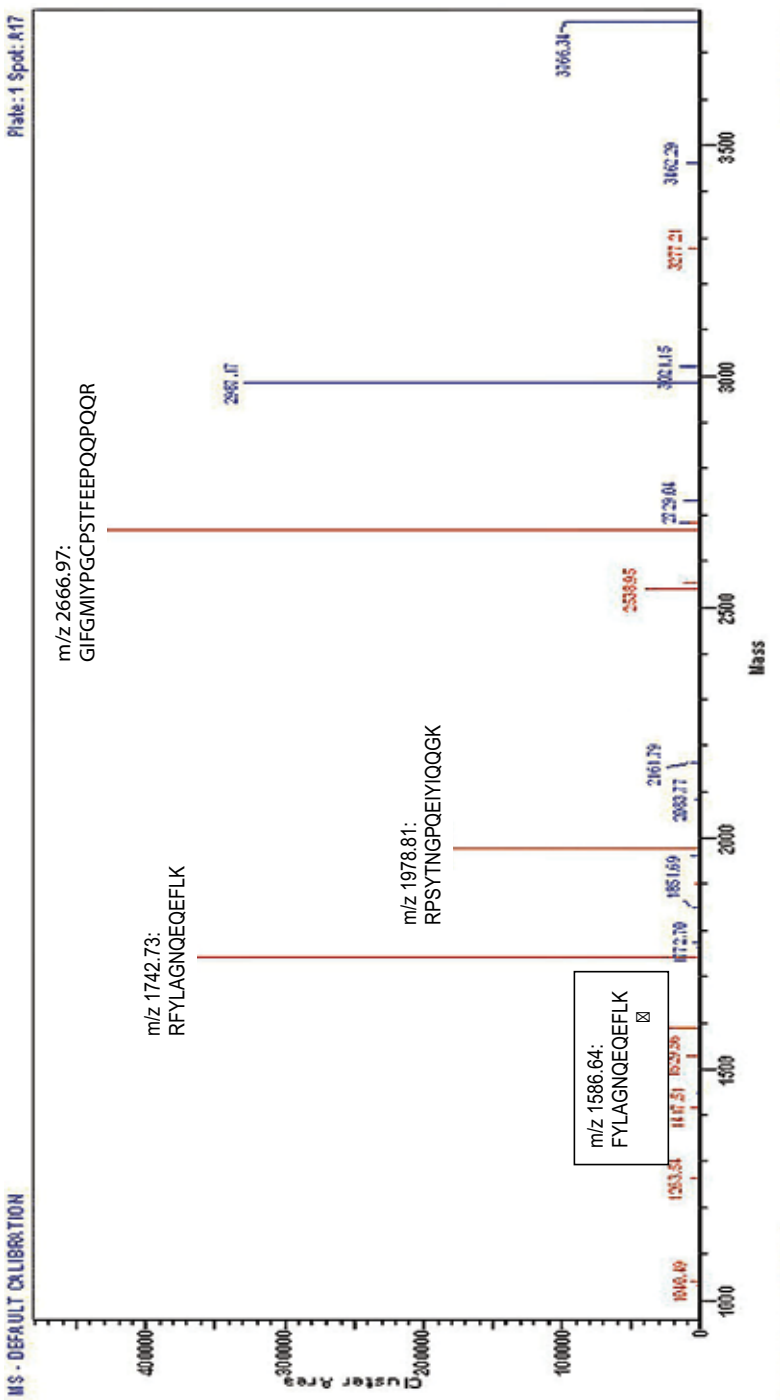


Fig. 9. Mass spectrum (MS) of tryptic peptides eluted from 2D-DIGE spot 24. The m/z value and sequence for each peptide is shown in the graph. The peptide with the highest ion score (42, CI 96.051 %) appears within the rectangle.

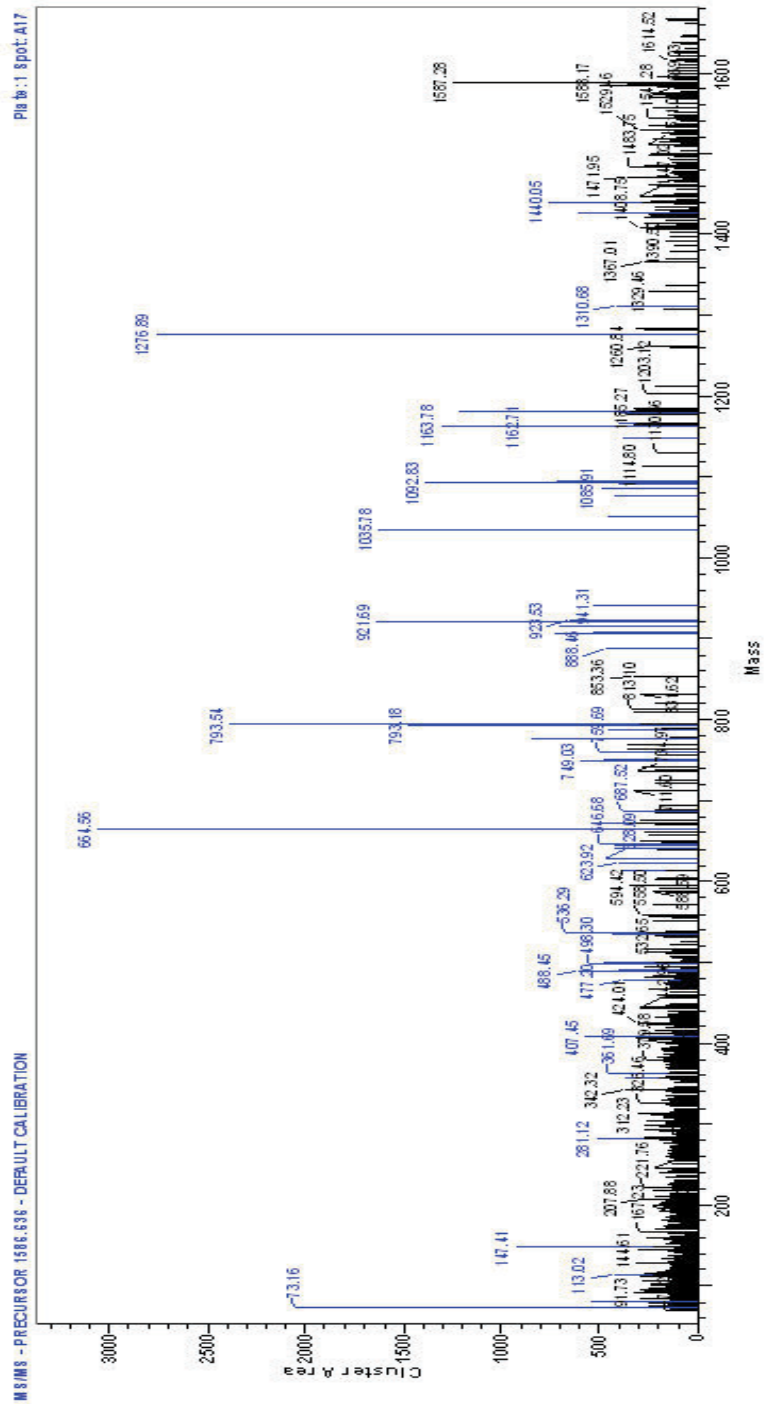


Fig. 10. MS/MS spectrum of peptide m/z 1586.64 (FYLAGNQEQEFLK) from protein spot ID 24. Sequences of these peptide ions fit within the sequence of peptide m/z 1586.64.

The physicochemical parameters relative to the molecular identification of proteins in spots ID22 and ID24 are shown in Table 2.

Spot ID	Accession No.	Identity	Molecular weight (Da)	Isoelectric point	Pep. Count	Protein Score	Protein Score C.I. %	Total Ion Score	Total Ion C.I. %
22	gi|15988119	Chain C, Crystal Structure Of Soybean Proglycinin A1aB1b Homotrimer	53590,6	5,78	12	198	100	89	100
24	gi|15988119	Chain C, Crystal Structure Of Soybean Proglycinin A1aB1b Homotrimer	53590,6	5,78	10	199	100	124	100

Table 2. Chemical characterization of proteins in 2D-DIGE spots 22 and 24 from the embryonic axes of soybean subjected to thermal shock at 40°C for 2 h.

The increment in the volume of spots 22 and 24 (proglycinin A1aB1b isomers) is shown in Table 3. The two protein isomers showed the highest expression under each stressing conditions tested (the exception was HS42°C/2 h).

Spot ID	Accession No.	Identity	HS/40ºC/2h	HS/42ºC/2h	pH 5.5	200 mM NaCl
22	gi|15988119	Chain C, Crystal Structure Of Soybean Proglycinin A1aB1b Homotrimer	87,07*	1,01	15,48	24,32
24	gi|15988119	Chain C, Crystal Structure Of Soybean Proglycinin A1aB1b Homotrimer	78,67	-1,0	10,07	12,63

*Upregulation or downregulation of proteins in relation to control lot (fold times).

Table 3. Increment in the expression of spot proteins ID-22 and ID-24 in the embryonic axes of soybean subjected to several stressing conditions.

Peptide localization.

Localization of peptides m/z 2667.02 (from 2D-DIGE spot ID 22) and m/z 1586.64 (from 2D-DIGE spot 24) within the Chain C, crystal structure of soybean proglycinin A1aB1b homotrimer (Accession No. gi|15988119) (Adachi et al. 2001), is shown below. Peptide m/z 2667.02 goes from a. a. 79 to 101 (bold, underlined sequence) and peptide m/z 1586.64 goes from a.a. 163 to 175 (underlined sequence).

1 FSSREQPQQN ECQIQKLNALKPDNRIESEG GLIETWNPNN KPFQCAGVAL SRCTLNRNAL

61 RRPSYTNGPQ EIYIQQGK **GI FGMIYPGCPS TFEEPQQPQQ** RGQSSRPQDR HQKIYNFREG

121 DLIAVPTGVA WWMYNNEDTP VVAVSIIDTN SLENQLDQMP RR FYLAGNQEQEFLK YQQEQ

181 GGHQSQKGKH QQEEENEGGS ILSGFTLEFL EHAFSVDKQI AKNLQGENEG EDKGAIVTVK

241 GGLSVIKPPT DEQQQRPQEE EEEEEDEKPQ CKGKDKHCQR PRGSQSKSRR NGIDETICTM

301 RLRHNIGQTS SPDIYNPQAG SVTTATSLDF PALSWLRLSA EFGSLRKNAM FVPHYNLNAN

361 SIIYALNGRA LIQVVNCNGE RVFDGELQEG RVLIVPQNFV VAARSQSDNF EYVSFKTNDT

421 PMIGTLAGAN SLLNALPEEV IQHTFNLKSQ QARQIKNNNP FKFLVPPQES QKRAVA

4. Discussion

Soybean (*Glycine max*), as a crop of upmost nutritional and industrial importance, has been the subject of an enormous amount of research; the effect of adverse environmental conditions on soybean germination, aiming to develop resistant varieties, has been one of the most investigated aspects. As a result, many proteins have been found to be modified in their expression, and also in their function.

Heat, was probably the first stressing factor studied in plants. Key *et al.* in 1981 showed that when the growth temperature of soybean seedlings was shifted from 28°C to 40°C the pattern of protein synthesis changed rapidly; normal protein synthesis decreased and a new set of proteins was induced. These "new" proteins were identified as heat shock proteins or HSPs. A strong positive correlation between HSPs and the acquisition of thermal tolerance was noticed thereafter (Lin *et al*., 1984). From the time when these initial publications appeared, the study of heat-induced HSPs has been continuous, aiming to identify particular molecular species. The most recent investigations have made use of the proteomic technology; most HSPs have been identified as stabilizing (refolding) proteins, storage proteins or respiratory-related proteins (Ren *et al*., 2009).

Although HSPs are meant to prevent, and even reverse, the molecular alterations induced by heat (Boston *et al*., 1996), they might be inefficient in cell protection when the temperature intensity is beyond the tolerance threshold (Schöffl *et al*., 1999). Direct effects of heat include denaturation and aggregation of proteins, changes in the structure and function of cell membranes, including lipid fluidization, enzyme inactivation in mitochondria and chloroplasts, inhibition in the synthesis of proteins and many other alterations (Howarth, 2005). The diminution in the expression levels of 11 proteins in the lot shocked at 42ºC in the present study (Table 1) indicated that this stressing condition overwhelmed the tolerance threshold in the soybean seeds.

The effect of salinity has also been studied in soybean. Protein changes similar to those noted in soybean subjected to thermal shock have been described in soybean subjected to

high salinity. High salinity may lead to rapid accumulation of reactive oxygen species (ROS) in the plant tissues (Zhu, 2001). In turn, ROS may produce degradation of photosynthetic pigments, lipid peroxidation, membrane permeability alteration, protein denaturation, and DNA mutations (Mittler, 2002). Salinity leads to reduction in photosynthesis, leaf area and biomass (Kao *et al*., 2006). To cope with salt stress, soybean has developed several tolerance mechanisms, including maintenance of ion homeostasis; restoration of osmolite balance, and the synthesis of antioxidants (phenols) and enzymes such as superoxide dismutase and peroxidase (Gossett *et al.,* 1994; Phang *et al*., 2008), among others. In addition, several metabolic proteins in soybean have been found to be down-regulated by high salinity (glyceraldehydes-3-phosphate dehydrogenase and fructokinase-2, among them) while others have been found to be up-regulated (stem 31 kDa glycoprotein precursor, for instance) (Sobhanian *et al.,* 2010). In our study, germination of soybean in the presence of 200 mM NaCl led to a significant increase in the expression of 29 out of the 35 proteins over expressed in soybean shocked at 40ºC. Connection between high salinity, extreme heat, dryness, and oxidative stress, as stressing factors in plants has been recognized (Zhu, 2001).

To our knowledge, acidity as a stressing factor has not been studied, at least not in soybean; therefore, our results are pioneers in this subject. As pointed out in the Results section, acidity at pH 5.5, induced the overexpression of 31 proteins, two of them being the same proteins over-expressed in response to the other stressing factors tested (HS40°C and 200 mM NaCl).

The importance and novelty of the present study is that the stress response of soybean was investigated in a comparative manner, in soybean-minicrops subjected to four different stressing conditions, namely: heating at 40°C (HS40°C/2h), heating at 42°C (HS42°C/2 h), acidic environment (pH 5.5), and high salt concentration (200 mM NaCl).

The finding that 35 proteins out of the ≈2000 proteins detected in soybean grown under normal conditions (10 mM phosphate, pH 7.0, 28°C) showed modification in their expression, most of them at increase, was very informative. These 35 proteins behaved as heat-shock proteins under the principle that they increased (the majority) or decreased their expression in ≥ 1.5 times in comparison with the control lot. Within those proteins that increased their expression there were two spot-proteins, ID-22 and ID-24 (by 2D-DIGE), that maximally increased under all of the stressing situations applied; the exception was HS42°C/2 h that resulted the most deleterious factor as this factor negatively affected not only the expression of proteins but also the germination of the grain. As proteins ID-22 and ID-24 maximally over expressed under the stressing factors HS40°C/2 h, pH 5.5, and 200 mM NaCl, we thought of these two proteins as vital elements for the growing of soybean exposed to all kinds of adverse environments. Therefore, these proteins deserved further analysis and on this base we decided to analyze the nature of these proteins by MALDI TOF and MALDI TOF/TOF. The analysis of peptides allowed us to identify the two proteins as isomers of the glycinin in its immature form, pro-glycinin. Seed proteins are usually synthesized as precursors having a leader pre-peptide sequence that contains the signal for transport, processing and targeting from the site of synthesis to the storage organelles. The signal sequence is involved in directing the nascent chain from the rough side of the endoplasmic reticulum (ER) to the ER lumen. After cotranslational cleavage of the signal peptide, the polypeptides are subjected to glycosylation and/or to folding through enzyme-catalyzed disulfide formation. Then, mature polypeptides are directed to storage protein bodies with the help of chaperones. Close to 90 % of the proteins in soybean exist as storage proteins, which mostly consist of β-conglycinin (7S) and glycinin (11S). Glycinin, the protein found maximally up-regulated under all stressing situations tested in this study, is a 300-380 kDa hexamer (Peng *et al,* 1984; Utsumi, 1992); each

subunit is composed of acidic (~35 kDa) and basic (~20 kDa) polypeptides linked together by a disulfide bond. Five subunits have been identified and classified within group I: A1aB1b (53.6 kDa), A2B1a (52.4 kDa) and A1bB2 (52.2 kDa), or within group II: A5A4B3 (61.2 kDa) and A3B4 (55.4 kDa) (Maruyama *et al*, 2006). Glycinin subunits are polymorphic (*i.e.*, there are some amino acid replacements in the same kind of subunit among different soybean cultivars). In addition, glycinin exhibits molecular heterogeneity, because the molecule is a hexamer with different subunit composition. Glycinin hexamers dissociate to their constituent polypeptide subunits, depending upon pH, ionic strength, and heating temperature (Adachi *et al*., 2001, 2003). The reason for the impressive over-expression of glycinin when soybean is subjected to heat, acidity or salinity (this study) is not yet understood. However, because this protein shows chaperone activity (Choi *et al*., 2004), it is possible that acting as a finely tuned sensor of stress, glycinin A1aB1b is efficiently up-regulated to cope with the adverse effects of environmental stressants in general, including heat, salinity and acidity (see text), waterlogging (Alam *et al*, 2010), Chilling (Cheng *et al*, 2010, and radioactivity (Danchenko *et al*, 2009). Reports on other proteins with hexameric structure and chaperone activity, reinforce this idea (Sauer *et al*., 2004; Lee *et al*, 2010).

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Part 2

Chemistry

24

Heavy Metals Uptake by Aerial Biomass and Grain of Soybean

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1. Introduction

All phases of crude oil exploitation, starting from drilling works to the construction of underground pipeline networks, including transport, processing and storage, are causing interventions and procedures hazardous to the environment. Although modern technical solutions and materials are used in all segments of petroleum industry warrant a high degree of safety, the occurrence of various incidents is unfortunately not fully excluded. Incidents leading to pipeline spillage and crude oil contamination of the environment constitute a hazard to natural resources, primarily soil and water, and depending on their severity can jeopardize, for a shorter or longer time, the intended use of land on which the incident occurred, namely make it unsuitable for plant production. Petroleum and gas fields are located in Pannonian area where cultivation of agricultural crops is dominated and among them the most represented are corn, wheat and soybean.
On the other side, these (petroleum and gas) activities generate waste - drilling fluids which contain different chemical compounds, some of which are ecologically hazardous (hydrocarbons), or toxic substances (heavy metals). As already mentioned, drilling fluids contain increased levels of some heavy metals (barium, zinc and mercury), so their possible application as liming material involves the risk of heavy metal accumulation in soil and plants (Agbogidi et al., 2007). To assess the extent to which such material may be useful or harmful to soil, and thereby to plants grown on it, since pollutants enter the animal and human food chain via soil and plants, investigations into this problem were undertaken (Nelson et al., 1984; Lengrand et al., 2005; Kabata-Pendias & Mukherjee, 2007).

2. Goals of investigation

The research objectives were to investigate the possibility of:

- Effects of different concentrations of crude oil and drilling fluids on the uptake of heavy metals in aerial biomass or grain of soybean, maize and winter wheat,
- Calculate enrichment coefficient for some parts of aerial biomass or grain of investigated crops.

3. Materials and methods

The trial was set up on Luvisols (FAO, 2006) and located near Popovaca (N 45º31'49'' – E 16º34'48'') in the Pannonian agricultural part of Croatia. The total trial field covered 1.25

hectare, the size of each trial plot was 96 m^2 (6.0 m x 4.0 m x 4 replications). At the beginning of investigations in May 2006, before preparing the seedbed layer, crude oil and drilling fluids were applied on the soil surface. The seedbed was prepared using a tractor-mounted rototiller to the depth of 25 cm. Experiment was set up as a randomized complete block design with four replications of each of the following treatments:

I. Control (unamended soil);
II. Soil contaminated by crude oil - 8 L m^{-2};
III. Soil contaminated by crude oil - 4 L m^{-2};
IV. Soil contaminated by crude oil - 2 L m^{-2};
V. Soil contaminated by drilling fluids - 30 kg m^{-2};
VI. Soil contaminated by drilling fluids - 20 kg m^{-2}, and
VII. Soil contaminated by drilling fluids - 10 kg m^{-2}.

Soybean (*Glycine max* L. *Merr*), Maize (*Zea mays* L.) and Winter Wheat (*Triticum aestivum* L.) were grown on the trial field. Composite soil samples (0-25 cm), in four replications, were taken before crops were sown. Composite plant samples (aerial biomass and grain) were taken after crop harvest. The observed results were analyzed using ANOVA analysis, SAS Institute 9.1.3. The significance level was set at 5 % in all statistical tests. Methods used to determine the studied parameters are given in Table 1.

Analysis	Method
Preparation of soil samples for physical and chemical analyses	ISO 11464:2004
Determination of particle size distribution in mineral soil material – Method by sieving and sedimentation	ISO 11277:2004
Determination of organic (TOC/OM) and total carbon (TC) after dry combustion (elementary analysis)	ISO 10694:2004
Soil organic matter (SOM)	Tjurin (wet digestion) - titrimetric
Determination of pH in $CaCl_2$, (1:2.5 (w/v))	ISO 10390:2004
Extraction of trace elements soluble in aqua regia	ISO 11466:2004
Determination of Zn, Pb, Cd, Co, Ni, Cr and Cu using AAS	ISO 11047:2004
Determination of As, Ba, Mo, V and Hg using ICP-MS	ISO 11885:1998 & ISO/DIS 22036:2006

Table 1. Methods used in investigations

4. Results

Particle size distribution of the studied soil is shown in Table 2. Major physical and chemical characteristics of crude oil and water-based drilling fluids (muds) applied in the trial are given in Tables 3 and 4.

		Particle size distribution, %					
Depth, cm	Soil horizon	Coarse sand 2-0.2 mm	Fine sand 0.2-0.05 mm	Coarse silt 0.05-0.02 mm	Fine silt 0.02-0.002 mm	Clay <0.002 mm	Texture class
0-15	Ap+Eg	2.0	5.40	46.25	27.75	18.60	Silty loam
15-30	Eg+Btg	1.5	6.40	41.80	30.00	20.30	Silty loam
30-45	Btg	0.5	4.30	43.25	29.80	22.15	Silty clay loam

Table 2. Particle size distribution of Luvisols

Components of crude oil	Sum, vol. %	Density at 15 °C, g cm^{-3}	Viscosity at 37.8 °C mm^2 s^{-1}
Light gasoline	6.53	0.69	
Light gasoline + heavy gasoline	32.00	0.75	
Kerosene	10.40	0.82	
Gas oil	17.57	0.85	
Low viscosity lubricant oil	12.08	0.86 - 0.89	7.5-20.6
Medium viscosity lubricant oil	6.75	0.89 - 0.90	20.6-43
High viscosity lubricant oil			> 43
Residue	20.51	0.96	
Loss	0.69		

Water, %	Total sulphur, %	Viscosity, 30 °C, mPa	Viscosity, 30 °C, mm^2	Pour point, °C	Coke, %	Ash, %	Paraffins, %	Asfalten, %	Total nitrogen, %
0.08	0.433	5.56	6.65	- 8	2.193	0.004	5.57	0.68	0.439

Table 3. Some characteristics of crude oil applied in the trial

	Cd	Hg	Pb	As	Ni	Cu	Cr	Zn	Ba	V	Co	Mo	Ca
Minimum	0.96	0.34	89	6.9	21.2	23	27	116	163	84	5.8	2.3	10 000
Maximum	1.03	37.70	209	9.0	57.5	188	54	755	2 333	132	12.0	5.2	156 000
Average	1.01	20.84	137	8.2	35.6	79	36	437	1 602	95	8.4	4.0	103 000

Table 4. Concentrations of measured elements in drilling fluids applied in the trial, mg kg^{-1}

4.1 Changes in soil pH, soil organic matter, total carbon and nitrogen

Research results show marked and expected heterogeneity of the studied parameters. In trial treatments where drilling fluids were applied (V, VI and VII) soil pH was significantly higher compared to other treatments (Table 5). This was expected because drilling fluids are very rich with $CaCO_3$, CaO, $Ca(OH)_2$, $Ca(SO)_4$ and other calcium compounds. In treatments where crude oil was applied, soil pH was not significantly different compared to the control treatment. These findings indicate that crude oil had no influence on the changes in soil pH. A highly significant difference in SOM concentrations compared to all other treatments was determined in the treatment where the largest amount of crude oil was applied (treatment II). In the control treatment and in treatment with drilling fluids, SOM is mainly composed

of humic compounds and plant debris. In the crude oil contaminated soils, part of the "SOM" is in fact carbon hydrides from crude oil.
Changes in total carbon follow trends and statistical differences that are almost identical to SOM. Compared to the control treatment significantly higher carbon content was recorded in treatments where crude oil was applied (treatments II, III and IV) as well as in treatment with the highest dose of drilling fluids (treatment V). Significantly higher nitrogen content compared to the control treatment, was determined in treatments IV and V. No significant differences in nitrogen content were determined in other treatments.

Treatment	I	II	III	IV	V	VI	VII
Soil pH	5.34 b*	5.41 b	5.23 b	5.26 b	6.58 a	6.59 a	6.92 a
SOM, %	1.83 bc	4.07 a	2.07 bc	1.30 c	2.07 bc	1.83 bc	2.53 b
Carbon, %	1.70 c	2.78 a	2.57 a	2.40 ba	2.42 ba	2.03 bc	1.99 bc
Nitrogen, %	0.184 c	0.200 bac	0.212 ba	0.230 a	0.222 a	0.182 bc	0.197 bac
C /N ratio	9.24 f	13.90 a	12.12 b	10.43 de	10.90 dc	11.15 c	10.10 fe

* Values are means of 4 replicates. For each parameter, values in the same row followed by an identical letter are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Table 5. Changes in soil pH, soil organic matter, carbon and nitrogen

4.2 Heavy metals in soil

The aim of this chapter is to show the degree to which crude oil or drilling fluids affect changes in heavy metal concentrations in soil. Cadmium concentrations in soil were lower than 0.3 mg kg^{-1} (Table 6). Regardless of the applied material (crude oil or drilling fluids), the detected changes in lead, molybdenum, arsenic, nickel, cobalt, cooper, chromium and vanadium concentrations cannot be associated with the application of the mentioned materials. Compared to the control treatment and treatments were crude oil was applied, significantly higher mercury, zinc and barium concentrations were recorded in treatments with drilling fluids.

Treatment	I	II	III	IV	V	VI	VII
Cd	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
Hg	0.03 c*	0.05 c	0.04 c	0.05 c	0.37 a	0.33 a	0.14 b
Pb	30 a	25 a	22 a	27 a	26 a	25 a	31 a
Mo	0.40a	0.34 a	0.33 a	0.33 a	0.47 a	0.34 a	0.31 a
As	8 a	9 a	9 a	9 a	9 a	10 a	8 a
Ni	20 a	20 a	20 a	20 a	20 a	22 a	21a
Co	12 a	12 a	12 a	13 a	11 a	12 a	11 a
Cu	17 a	17 a	19 a	19 a	20 a	19 a	19 a
Cr	26 a	25 a	25 a	27 a	26 a	27 a	28 a
Zn	62 b	62 b	64 b	65 ba	78 a	78 a	72 a
Ba	96 c	93 c	92 c	91 c	160 ab	194 a	144 b
V	33 a	32 a	34 a	36 a	34 a	35 a	36 a

* Values are means of 4 replicates. For each parameter, values in the same row followed by an identical letter are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Table 6. Total heavy metal concentrations in soil (mg kg^{-1} soil)

4.3 Plant uptake of heavy metals

Concentrations of heavy metals in aerial biomass and grain of soybean and other crops are presented in Table 7. Higher levels of cadmium, chromium, zinc and barium were recorded in soybean aerial biomass than in its grain almost in all treatments. Soybean grain contained more nickel and copper compared to its aerial biomass. Values determined for molybdenum show no regularity. Higher levels of molybdenum were determined in

Treatment		I	II	III	IV	V	VI	VII
		Soybean						
aerial biomass	Cd	0.34 b*	0.21 c	0.35 b	0.41 a	0.20 c	0.15 d	0.11 e
grain		0.12 b	0.14 ab	0.15 ab	0.16 a	0.01 c	0.14 ab	0.16 a
aerial biomass	Mo	0.65 e	1.29 a	1.17 b	0.69 e	1.11 c	0.64 e	0.79 d
grain		0.81 c	0.69 d	0.42 e	0.65 d	1.16 b	1.09 b	1.47 a
aerial biomass	Ni	4.02 d	14.00 a	8.56 b	5.74 c	5.56 c	5.18 c	4.84 cd
grain		8.71 cd	10.00 b	9.21 c	11.6 a	10.3 b	8.91 cd	8.34 d
aerial biomass	Cu	10.3 a	7.23 ed	7.12 ed	7.77 c	8.78 b	6.96 e	7.53 cd
grain		19.4 b	17.7c	18.4 bc	19.2 b	22.1 a	19.5 b	18.4 bc
aerial biomass	Cr	2.34 e	6.43 a	3.81 b	3.18 c	2.99 cd	3.14 c	2.65 ed
grain		1.43 a	1.41 a	1.31 ab	1.35 ab	1.26 ab	1.16 bc	0.99c
aerial biomass	Zn	94 c	98 b	98 b	109 a	81 d	60 e	61 e
grain		50 e	54 d	74 a	59 c	67 b	62c	50 e
aerial biomass	Ba	55 d	60 c	76 b	88 a	57 cd	56 d	42 e
grain		5.08 c	5.99 b	6.86 a	6.91 a	5.10 c	4.47 d	3.69 e
Maize								
aerial biomass	Cd	0.13 d	0.24 a	0.24 a	0.16 c	0.13 d	0.20 b	0.13 d
grain		0.09 b	0.09 b	0.05 d	0.07 c	0.09 b	0.08 bc	0.12 a
aerial biomass	Mo	0.65 f	0.74 d	0.42 g	0.68 e	1.01 b	1.14 a	0.98 c
grain		0.40 d	0.83 a	0.52 c	0.87 a	0.53 c	0.68 b	0.72 b
aerial biomass	Ni	4.19 f	5.03 c	4.95 d	4.47 e	4.96 d	5.84 a	5.46 b
grain		4.86 e	5.44 a	4.48 f	5.13 b	4.93 d	5.05 c	3.96 g
aerial biomass	Cu	19.9 e	18.3 f	20.7 d	24.8 a	23.1 b	21.9 c	17.1 d
grain		2.45 c	2.50 c	2.09 d	2.42 c	2.68 b	2.76 a	2.11 d
aerial biomass	Cr	3.61 c	5.00 a	4.07 b	3.72 c	3.28 d	3.94 b	3.18 d
grain		1.73 a	1.64 b	1.38 e	1.63 b	1.55 c	1.69 ab	1.46 d
aerial biomass	Zn	80 b	64 e	79 b	88 a	75 c	67 de	69 d
grain		21 b	21 b	25 a	22 b	21 b	24 a	20 b
aerial biomass	Ba	17.6 d	20.0 b	22.0 a	18.8 c	17.1 d	13.6 e	13.7 e
grain		9.70 b	10.40 a	7.65 e	2.28 f	9.42 c	9.43 c	8.74 d
aerial biomass	V	0.62 e	0.79 cd	0.75 cd	1.50 b	0.69 de	0.81 c	2.68 a
grain		3.98 a	1.17 c	1.78 b	1.03 c	0.63 d	0.50 d	0.60 d
Winter Wheat								
aerial biomass	Cd	0.13 bc	0.19 a	0.17 a	0.14 b	0.13 bc	0.12 c	0.08 d
grain		0.17 a	0.13 b	0.09d	0.11 bc	0.13 b	0.10 cd	0.18 a
aerial biomass	Hg	0.02 a	0.01 a	0.01 a	0.01 a	0.01 a	0.01 a	0.01 a
grain		0.01 a	0.02 a	0.01 a	0.02 a	0.01 a	0.01 a	0.01 a

Treatment		I	II	III	IV	V	VI	VII
		Winter Wheat						
aerial biomass	Mo	0.74 d	1.17 c	1.51 a	1.42 ab	1.23 bc	1.24 bc	1.28 bc
grain		0.92 cd	0.99 bc	1.14 b	1.41 b	0.90 cd	0.91 cd	0.72 d
aerial biomass	Ni	3.52 d	2.87 e	3.59 d	4.78 b	5.14 a	5.29 a	4.39 c
grain		5.15 b	5.62 a	5.13 b	4.78 de	4.92 cd	5.11 bc	4.64 e
aerial biomass	Cu	3.56 e	4.28 c	4.58 b	4.73 b	4.98 a	3.91d	3.54 e
grain		7.56 d	8.21 bc	8.47 a	8.17 bc	7.98 c	8.29 ab	8.04 c
aerial biomass	Cr	1.47 d	1.23 e	2.58 c	2.61 c	2.84 ab	2.71 bc	2.96 a
grain		1.48 d	1.53 d	2.14 c	2.18 c	2.56 b	2.72 a	2.69 a
aerial biomass	Zn	34 e	38 d	42 b	35 e	39 cd	48 a	41bc
grain		49 d	52 c	51 cd	55 b	58 a	52 c	56 ab
aerial biomas	Ba	12.47 b	13.28 a	12.78 b	11.05 c	13.63 a	12.47 b	11.23 c
grain		4.57 d	5.12 c	5.89 a	4.73 d	4.96 c	5.12 c	5.47 b

* Values are means of 4 replicates. For each parameter, values in the same row followed by an identical letter are not significantly different according to Fisher's LSD test ($P \leq 0.05$).

Table 7. Total heavy metal concentrations in aerial biomass and grain of investigated crops (mg kg^{-1} dry matter)

aerial biomass in some treatments while in other treatments higher levels were found in grain. The grains of soybean and wheat contained more cadmium than grain of maize. The higher content of barium was determined in grain of maize than in grains of soybean and wheat. The highest copper content was determined in aerial biomass of maize. The highest copper content was determined in grain of soybean. Compared to the aerial biomass of wheat the aerial biomass of soybean and maize contained a higher content of chromium and zinc.

The higher content of molybdenum was determined in aerial biomass and grain of wheat than in aerial biomass and grain of other two crops. The grains of wheat and maize contained higher content of mercury compared to soybean grain.

4.4 Enrichment Coefficients (EC)

In various literature enrichment coefficient is also called enrichment ratio; translocation coefficient; transfer factor; soil-plant transfer coefficient; accumulation factor; phytoaccumulation factor; bioaccumulation factor; biological adsorption coefficient and uptake coefficient (Zgorelec, 2009). Enrichment coefficient was calculated with equation: $EC = C_{aerial\ biomass\ or\ grain}/C_{soil}$, where $C_{aerial\ biomass\ or\ grain}$ and C_{soil} represent the metal concentration (mass ratio) in the aerial biomass or grain and in the soil on dry weight basis (mg/kg in DM), respectively. Malayeri et al., 2008 grouped plant species according to their heavy metal uptake capacities and sensitivity to metal pollution:

High accumulator plants	EC between 1 - 10
Moderately accumulator plants	EC between 0.1 – 1.0
Low accumulator plants	EC between 0.01 – 0.1
Non accumulator plants	EC < 0.01

The highest enrichment coefficient for all investigated crops was molybdenum (Table 8). In all cases, enrichment coefficient of molybdenum was higher for biomass than for grain.

According to the higher values of enrichment coefficient the next metal is zinc. However, in this case, content of zinc in maize and soybean is higher in biomass than in grain, while in winter wheat higher content of zinc in grain was determined. Investigated crops also had very high enrichment coefficient for cadmium. As for molybdenum, cadmium had higher content in aerial biomass than in grain. High content of copper was determined in maize aerial biomass, while for soybean and wheat higher content of copper was determined in grain than in biomass. Increased content of nickel was determined in biomass as well as in grain of all investigated crops. All other investigated metals and metalloids (Pb, Hg, As, Co, Cr, Ba and V) had very low enrichment coefficient.

Treatment		I	II	III	IV	V	VI	VII
		Soybean						
aerial biomass	Cd	1.1	0.7	1.2	1.4	0.7	0.5	0.4
grain		0.4	0.5	0.5	0.5	0.0	0.5	0.5
aerial biomass	Mo	1.6	3.8	3.5	2.1	2.4	1.9	2.5
grain		2.0	2.0	1.3	2.0	2.5	3.2	4.7
aerial biomass	Ni	0.2	0.7	0.4	0.3	0.3	0.2	0.2
grain		0.4	0.5	0.5	0.6	0.5	0.4	0.4
aerial biomass	Cu	0.6	0.4	0.4	0.4	0.4	0.4	0.4
grain		1.1	1.0	1.0	1.0	1.1	1.0	1.0
aerial biomass	Cr	0.1	0.3	0.2	0.1	0.1	0.1	0.1
grain		0.1	0.1	0.1	0.1	0.0	0.0	0.0
aerial biomass	Zn	1.5	1.6	1.5	1.7	1.0	0.8	0.8
grain		0.8	0.9	1.2	0.9	0.9	0.8	0.7
aerial biomass	Ba	0.6	0.6	0.8	1.0	0.4	0.3	0.3
grain		0.1	0.1	0.1	0.1	0.0	0.0	0.0
Maize								
aerial biomass	Cd	0.4	0.8	0.8	0.5	0.4	0.7	0.4
grain		0.3	0.3	0.2	0.2	0.3	0.3	0.4
aerial biomass	Mo	1.6	2.2	1.3	2.1	2.1	3.4	3.2
grain		1.0	2.4	1.6	2.6	1.1	2.0	2.3
aerial biomass	Ni	0.2	0.3	0.2	0.2	0.2	0.3	0.3
grain		0.2	0.3	0.2	0.3	0.2	0.2	0.2
aerial biomass	Cu	1.2	1.1	1.1	1.3	1.2	1.2	0.9
grain		0.1	0.1	0.1	0.1	0.1	0.1	0.1
aerial biomass	Cr	0.1	0.2	0.2	0.1	0.1	0.1	0.1
grain		0.1	0.1	0.1	0.1	0.1	0.1	0.1
aerial biomass	Zn	1.3	1.0	1.2	1.4	1.0	0.9	1.0
grain		0.3	0.3	0.4	0.3	0.3	0.3	0.3
aerial biomass	Ba	0.2	0.2	0.2	0.2	0.1	0.1	0.1
grain		0.1	0.1	0.1	0.0	0.1	0.0	0.1
aerial biomass	V	0.0	0.0	0.0	0.0	0.0	0.0	0.1
grain		0.1	0.0	0.1	0.0	0.0	0.0	0.0

Treatment		I	II	III	IV	V	VI	VI
		Winter Wheat						
aerial biomass	Cd	0.4	0.6	0.6	0.5	0.4	0.4	0.3
grain		0.6	0.4	0.3	0.4	0.4	0.3	0.6
aerial biomass	Hg	0.7	0.2	0.3	0.2	0.0	0.0	0.1
grain		0.3	0.4	0.3	0.4	0.0	0.0	0.1
aerial biomass	Mo	1.9	3.4	4.6	4.3	2.6	3.6	4.1
grain		2.3	2.9	3.5	4.3	1.9	2.7	2.3
aerial biomass	Ni	0.2	0.1	0.2	0.2	0.3	0.2	0.2
grain		0.3	0.3	0.3	0.2	0.2	0.2	0.2
aerial biomass	Cu	0.2	0.3	0.2	0.2	0.2	0.2	0.2
grain		0.4	0.5	0.4	0.4	0.4	0.4	0.4
aerial biomass	Cr	0.1	0.0	0.1	0.1	0.1	0.1	0.1
grain		0.1	0.1	0.1	0.1	0.1	0.1	0.1
aerial biomass	Zn	0.5	0.6	0.7	0.5	0.5	0.6	0.6
grain		0.8	0.8	0.8	0.8	0.7	0.7	0.8
aerial biomass	Ba	0.1	0.1	0.1	0.1	0.1	0.1	0.1
grain		0.0	0.1	0.1	0.1	0.0	0.0	0.0

Table 8. Enrichment coefficient for investigated crops

5. Discussion

In the presented study, the application of crude oil did not cause elevated levels of heavy metals in soil, but the application of drilling fluids resulted in higher mercury, zinc and barium concentrations. Increased zinc and barium concentrations in treatments involving drilling fluids are attributed to the use of zinc carbonate and barite ($BaSO_4$) in drilling (U.S. Congress, Office of Technology Assessment, 1992). Barium is insoluble, inert and non-toxic (Deuel, 2003), and therefore is not considered to be a great soil problem. Increased mercury concentrations in treatments where drilling fluids were applied are attributed to the source of drilling fluids. The reason why drilling fluids contain a larger amount of calcium compounds is its use as an additive for inhibiting corrosion of oil and gas pipes, or as an additive to increase drilling fluid density (Veil & Dusseault, 2003; Kisic et al., 2009). Also, material with increased calcium compounds is usually added to bind fluids when repairing a pipe break or other incidents.

The problem of increased concentrations of some heavy metals caused by the application of crude oil-based fluids was pointed out by Nelson et al., 1984. When crude oil-based fluids were used as liming material, the expected positive changes in soil pH occurred (Deeley & Canter, 1986).

As optimum soil reaction for the majority of cultivated plants is around the neutral pH value, liming is a desirable practice because under neutral to weakly alkaline conditions bioavailability of some heavy metals (except As, V, Mo, Co and Cr) decreases (Mathur et al., 1991; Dermatas & Meng, 2003). This reduces the possibility of their translocation from roots into the plant itself (Bolan et al., 2003).

Levels of heavy metals detected in aerial biomass or grain of crops in all trial treatments are within the ranges determined in some other studies (Adriano, 2001; Kabata-Pendias & Mikherjee, 2007). A number of authors (Darell et al., 1984; Khan et al., 2000; Lengrand et al.,

2005) report that soil pH, electrical conductivity, mechanical composition, organic matter concentrations, crop type and total heavy metals concentrations have a decisive influence on the heavy metal uptake by plants.

6. Conclusions

In soybean and wheat grain more nickel and copper were determined compared to soybean and wheat biomass. Also, higher content of zinc in wheat grain compared to biomass was determined. In all other cases, higher concentrations of heavy metals in aerial biomass than in grain of investigated crops were determined.
Regarding other investigated crops soybean had the highest enrichment coefficient for cadmium, copper, nickel and zinc.

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Effect of Refining Process and Use of Natural Antioxidants on Soybean Oil

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1. Introduction

Soybean (*Glycine max* L. Merril.) is an annual plant of Asian origin, adapted to temperate climates. This plant is a member of the family *Fabaceae*, subfamily *Papilionoideae*, tribe *Phaseoleae*, genus *Glycine* and subgenus *Soja* (Hymowitz, 2004). One of the most important agronomic characteristics of soybean is that it can take nitrogen from the air and fix it to be used as nutrient by the plant. The symbiotic relationship between the soybean plant and its modulation by bacterium (*Rhixobium japonicum*) is responsible for the conversion of atmospheric nitrogen into plant-available nitrogen. This also makes to soybean a good rotational plant for use with high nitrogen-consuming crops. Another important benefit of this nitrogen fixation is that it helps to keep the production cost of soybeans relatively low compared to other crops that competing for the same land area (Erickson, 1995).

Among the factors that allow the soybean to be a dominant crop could be mentioned some of the most important agronomic traits: good profits for producers and processors, as well as the possibility of obtaining high-protein quality meal, which are used as ingredient in food animal. These characteristics have allowed the soybean oil is consumed more than other vegetable oils. Soybean is the most important crop in the world because of its high quality protein and edible oil products. World production of oilseeds during 2009-2010 was 403.58 million metric tons (tons), from which 60% corresponded to soybean, 13.74% to rapeseed, 10.23% to cottonseed, 8.30% to peanut and 7.87% to sunflower (Soya & Oilseed Bluebook, 2010).

Others factors that have contributed to soybean worldwide importance are the high demand of meal and oil. The main consumer of these meals is the agricultural sector. World production of vegetable meals during 2009-2010 was 237 million tons; soybean meal represented 67%, rapeseed 13.6% and cotton 6.2% (Soya & Oilseed Bluebook, 2010). Oil is a byproduct of meals production, which has a great demand.

In this situation of soybean oil domain in fat total supplies, is extremely important to take advantage of its nutritional properties, such as its high content of polyunsaturated fatty acids essential for humans(n-6 and n-3), and their contents of tocopherols (vitamin E) (Emken, 1995).

2. Importance

The high polyunsaturated fatty acids (PUFA) content in soybean oil is very attractive for meeting the essential fatty acid requirements in human nutrition; however, they are highly

susceptible to oxidative reactions. Therefore, appropriate processing conditions should be used to eliminate or reduce impurities such as phospholipids, gums, metals, free fatty acids (FFA), oxidation products (peroxides, aldehydes and ketones) and pigments, in order to ensure the best oil quality. The impurities are eliminated through degumming (Farr, 2000), chemical or physical refining (Kellens & De Greyt, 2000), bleaching (Ortega-García et al., 2005; González-Tovar et al., 2005), and deodorization (Zehnder, 1995; Medina-Juárez et al., 2000). During this process the modification of essential fatty acids to *trans* isomers, and the loss of tocopherols and sterols, should be monitored (Medina-Juárez et al., 2000). To follow up this process, oils should be physicochemically characterized using the official methodology. By other hand, it is important to use additives such as natural antioxidants that ensure the stability and shelf life of PUFA (Gámez-Meza et al., 1999; Gámez-Meza et al., 2009).

This chapter presents relevant bibliographic information about soybean oil. It highlights the impact of stages of refining process and the importance of natural antioxidants to avoid oxidation. Also the official methods recommended for the physicochemical characterization of this oil, with a special emphasis on the determination of *trans* isomers, omega 3 and 6 and other bioactive compounds naturally found in this vegetable oil as tocopherols and sterols are discussed.

The authors consider these issues as the major areas of research for the present and future knowledge of vegetable oils.

3. Processing

3.1 Oil extraction

Two products are mainly obtained in the milling of soybean: meal and oil.

Soybean meal. The soybean meal is mainly used as ingredient of formulated feed for animal nutrition. This meal is the most extensively used among the oilseed meals and the most economic high-quality protein available for animal feed; hence it assumes a dominant role.

Soybean meal contains from 44 to 50% crude protein and from 2500 to 2800 kcal of metabolizable energy per kilogram, depending of amount of hull present and the species of animal to fed (Smith & Baldwin, 1986). Its nutritional quality and acceptance are widely known.

The process for producing meal for livestock feeding includes three steps: desolventizing-toasting, drying and cooling, and grinding and sizing.

Soybea oil. The three most common procedures to extract oil from oilseeds are: mechanical pressing, pressure-solvent and direct solvent extraction. The application of the extraction process depends primarily on the content of oil in the seed, the content of remaining oil, denatured protein allowed and environmental restrictions for the emissions of volatile organic compounds. Mechanical pressing is generally used for materials exceeding 20% oil content. In the case of soybean oil, the solvent direct extraction is more commonly used, because it is more efficient (<1% residual oil) that when mechanical pressing is used (>6 % residual oil) (Williams & Hron, 1996).

Solvent extraction processes include basically three steps: preparation, extraction, and desolventizing. The major differences in soybean oil solvent extraction processes occur in the preparation steps. One variation in the conventional system is the introduction of an expander after flaking or other method of size reduction (grinding).

The hexane is currently used as solvent, although others solvents have been evaluated (ethanol, isopropanol, acetone, isopentane, isohexane and trichloroethylene). The

advantages of hexane are the high oil solubility and low price. Some disadvantages include the regulation of their emissions and flammability (Johnson, 2000).

3.2 Chemical refining of soybean oil

The purpose of refining of soybean oil is to convert the crude oil that is not adequate for human consumption in a healthy and nutritious food. The crude oil obtained from seeds by mechanical pressing or solvent extraction has many undesirable components. The refining process removes some of those impurities from the oil, such as: free fatty acids (FFA), phospholipids, pigments and other minor impurities. After the refining, a pure oil with desirable properties for the consumers, such as odor, taste, light color, and stability is obtained. The refining term may means different processing steps. We will consider degumming, neutralization, bleaching and deodorizing, as steps of the refining process.

Degumming. Degumming may be considered the first step in the refining process. This process is designed to remove the phosphatides that interfere with subsequent processing and especially for processors with an integral disposal option of gums. The primary reasons for degumming are: to provide a crude-degummed oil suitable for storage or long transportation, to prepare oil for physical refining, or to produce lecithin. In the case of soybean oil, high quality, food grade lecithin can be produced. Lecithin is a product of commercial interest that permits cost reduction by wastewater treatment (Farr, 2000).

Crude soybean oil has high levels of phosphorus, which can be removed in degumming step. The oil has two kinds of gums, hydratable (phospholipids) and non-hydratable (partially hydrolyzed phospholipids, which form salts of calcium, magnesium and iron). The hydratables are easily separated by treating of crude oil with hot water (deionized water at 75°C, at a rate of 1-3%, of oil weight), followed by centrifugation. Contrarily, non-hydratable gums are more soluble in oil than the first and require an additional treatment with phosphoric acid (0.1-0.3% of 85% solution) and sometimes with citric acid (0.1-1.0% of 30% solution) in order to convert them to hydratable gums. During this operation, phosphorus levels are reduced from 800-1200 to 100 ppm when hydratable gums have been removed and they can be reduced to 30 ppm if non-hydratables are efficiently removed (Farr, 2000). It is important to know the amount of tocopherols that are lost with gums in order to maintain the nutritional quality of oil in this operation (Medina-Juarez et al. 2000).

Neutralization. The neutralization operation is usually performed by chemical method, with caustic soda; or by physical method, refining by steam distillation. The aim of this operation is to eliminate the free fatty acids (FFA) through the saponification reaction. To carry out this reaction can be used several alkaline compounds such as sodium hydroxide (NaOH), potassium hydroxide (KOH), sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3). However, in the refining of soybean oils is sodium hydroxide the most widely used. In some cases, despite being more expensive, in order to use the soaps as fertilizer potassium hydroxide is used (Erickson, 1995).

The reversibility of the saponification reaction by pressure and temperature is possible, therefore at atmospheric pressure and temperatures between 60 and 80°C the reaction is from left to right (Egan & Kirk, 1991).

$$R - COOH + NaOH \longrightarrow R - COONa + H_2O$$

Once the oil has been treated with caustic soda, to remove the soap to levels below 5 ppm it is washed with water (two washes with 10% water each). At this point the oil must have

levels of phosphorus not greater than 5 ppm and 0.05% FFA (Farr, 2000). Quantification of tocopherols should be made further, in order to determine the amount reduced during this operation.

Studies of the soybean oil neutralization with sodium silicate show an improved yield and quality, as well as, an efficient removal of soap and trace metals. This allows an efficient filtration to separate the gums and free fatty acids in the form of sodium salts. The advantage of this process is that the amount of soap after filtration can be reduced to 80 ppm without washing with water (Hernández & Rathbone, 1999; Farr, 2000).

Bleaching. The purpose of this operation is to remove all products of oxidation and oxidation promoters to obtain a stable oil to oxidation. The main oxidation products are: hydroperoxides (non-volatile compounds), aldehydes and ketones (volatile compounds). The promoters of oxidation are chlorophyll, phospholipids and traces of metal particles. The loss of oil at this stage is related to the amount of clays used.

The bleaching operation which is done with activated acidic clays (aluminum silicate) eliminates or reduces color besides primary products (peroxides) and secondary oxidation products (aldehydes and ketones), metals, gums and soap traces (Mag, 1990). Peroxides are chemically discomposed during bleaching (Kellens, 1997). The metals form ionic bonds with bleaching clays surface. Also, bleaching clays through forces of Van Der Waals absorbs colorful compounds as chlorophylls and carotenoids. The levels of peroxides, chlorophyll, and soap in bleached oil, should be zero. The *p*-anisidine value (presence of aldehydes and ketones) will be not greater than 5 mmol/kg, the iron content must be less than 0.1 ppm and the copper less than 0.05 ppm (Erickson, 1995; Zschau, 2000). It is very important to know the variation in the content of *trans* isomers and tocopherols in the bleached oil.

To understand the process of bleaching oils is required to know the bleaching clay properties and the variety of impurities present in oils. This process uses aluminum silicates, which are activated clays (bentonite, attapulgite, montmorillonite), containing a high amount of magnesium, calcium or iron. In the case of clays non-activated, they do not adsorb natural pigments (O'Brien, 2000; Mag, 1990; Bockisch, 1998). The granule size of the clays and the specific surface are parameters of great importance for the process (Bockisch, 1998).

The theories that have been established on the bleaching process are based on the Freundlich adsorption isotherms, which are an adaptation of the Langmuir equation. These equations are valid for constant temperatures and describe the dependence of the adsorbed amount of a substance (k) in relation to the residual amount (c) in the solvent (in this case oil).

$$k/k_0 = \alpha\ (c/c_0)^b \quad (1)$$

Taking these considerations into account equation (1) is represented in simplified form as follows:

$$k_r = \alpha\ (c_1)^b \quad (2)$$

That is equivalent to:

$$\log k_r = \log \alpha + (b)\ (\log c_1) \quad (3)$$

Where the indices 0 and 1 from equation (1) indicate the relative amounts of initial and final respectively, k_r is the ratio of adsorbed components, c is the number of components not

adsorbed and b are constants specific system. If an amount of adsorbent m (bleaching agent) is include then the result is:

$$k= (m)(\alpha) + (c^b) \quad (4)$$

which would be equal to:

$$\log k - \log m = \log \alpha + (b) (\log c) \quad (5)$$

With the development of various types of bleaching processes using different types of clays, α is a measure of the relative amount of bleaching clays that will be used to obtain a specific result. Keeping the product of *m* and α constant, for example α = 0.25, the amount of clays must be four times as higher as at α = 1.0. Many studies which have developed different values for α and *b*, depending on the equipment used in the process, type of clays and basically type of oil to be bleached (Bockisch, 1998).

Bleaching clay load (typically 0.1-2.0 percent) and temperatures depend of type and quality of oil processed. To minimize the oil oxidation during bleaching a vacuum of 50 mm Hg is recommended. Ortega-García et al. (2005), reported the following optimal bleaching conditions for soybean oil: temperature, 96ºC; time, 23 min; clay amount (Tonsil Optimum 320 FF), 1.4% w/w oil; stirring, 250 rpm, and partial vacuum, 60 mmHg. Under these conditions a bleached soybean oil with 0.1 meq/kg of PV, 91.74% of TOCR, and a colour of 1.53 Lovibond red value units was obtained Table 1. A response surface methodology (RSM) was used to find the parameters that produce bleached oil with minimum peroxide value (PV), maximum tocopherol retention (TOCR) and light colour.

Analysis	Neutralized soybean oil	Bleached soybean oil
Free fatty acids (% as oleic acid)	0.456±0.001	0.562±0.002
Peroxide value (meq/kg)	0.292±0.023	0.1±0.04
Colour	70Y 8.3R	1.53R
Tocopherol (ppm)	925.27	848.84

Table 1. Chemical analysis and colour of neutralized and bleaching soybean oil.

During bleaching process, suspended matter in the oil is adsorbed on clays at different adsorption rates. These adsorption rates are important for design of processes. For this reason, knowledge of bleaching kinetics is requires. Gonzalez-Tovar et al., (2005), reported the adsorption kinetic of pigments, peroxides and tocopherols during bleaching process of soybean oil. In this work, both empirical and theoretical mathematical models were applied (Table 2), in order to predict the adsorption kinetic parameters for tocopherols, peroxides and pigments. The experimental part consisted of mixing neutralized soybean oil with different concentrations of bleaching clays (0.16, 1.0 and 2.0% w/w) in a laboratory reactor at 96°C for 64 min. The results showed that the first order theoretical model predicted better (R^2>0.93) the adsorption kinetic for tocopherols, peroxides and pigments than the empirical model. The determined kinetic parameters suggest that these bleaching clays (Tonsil Optimum 320 FF, Süd-Chemie) presented a high capacity for pigment and peroxide adsorption. Adsorption occurred mostly at the first minutes (20 min) of the process. The tocopherols losses were directly related to the bleaching clay dosage: 16.16, 16.56 and 19.98% for bleaching clays concentration of 0.16, 1.0 and 2.0%, respectively.

Model	Equation
First Order	$q = 1 - e^{\left(\frac{k_0}{k_d}\left(e^{-k_d t} - 1\right)\right)}$
Nicol	$r = k_5(KC - q)$
Fleming	$q = k_6 C_0 t^n$
La Brooy	$q = k_7 C t^n$

q=amount of adsorbed material/g adsorbent; C = adsorbate concentration; r = adsorption rate; C_0 = initial concentration of adsorbate, t=time, k_0=initial velocity, k_d = equilibrium constant; K, k_5, k_6, and k_7 = characteristic constants of the models.

Table 2. Kinetics adsorption empirical and theoretical mathematical models.

Deodorization. Deodorization is the final step in the production of edible oils and fats. This step is basically a vacuum-steam distillation at high temperature and very low absolute pressures, where free fatty acids (FFA) and volatile odiferous components (aldehydes and ketones) are removed to obtain bland and odorless oil (Table 3). The deodorization process requires less stripping steam, and shorter residence time (Kellens & De Greyt, 2000). This process is feasible because of the oil low volatility (i.e. triacylglycerides) and the relative volatility of the odiferous compounds in the oil (Mattil et al., 1964; Zehnder, 1995).

Additionally during desodorization, certain carotenoids are reduced, because of their instability at temperatures in which the process is made (Mattil et al., 1964). Therefore, the purpose of deodorization is to obtain an oil with a free fatty acid content of lesser than 0.03%, maximum values of Lovibond color must be 10 Yellow, 1 Red, odorless and essentially bland-tasting (Erickson, 1995).

It is important to consider that during the deodorization, can occur other reactions such as thermal decomposition of triglycerides into free fatty acids, formation of *trans* isomers (Kellens, 1997) and loss of tocopherols and sterols (Zehnder & McMichael, 1967; Medina-Juárez et al., 2000). Therefore, it is very important to establish the optimal conditions to avoid formation of *trans* isomers and the loss of tocopherols.

Absolute pressures	1 – 6 mm Hg (1.3 – 8 x 102 Pa)
Temperature	250-260°C 482-500°F
Time	20 – 40 min
Sparge steam	0.5 – 2.0 %

Kellens and De Greyt, 2000.

Table 3. Deodorization conditions for soybean oil.

The fats, oils and volatile compounds that contain them, by the laws of Dalton partial pressures and Raoult vapor-liquid equilibrium are governed (Zehnder & McMichael, 1967). Dalton's law states pronounce that the molar ratio of volatile compounds is proportional to the ratio of partial pressure. In the case of the deodorizing system, the molar ratio of steam

(S) and molar ratio of volatile compounds such as free fatty acids and carboxylic compounds (V) are proportional to their partial pressures (p_s, p_v').

$$dS/dV = p_s/p_v' \quad (6)$$

Where:
p_s = partial vapor pressure
p_v' = partial volatile compounds pressure
But assuming that pv' is very small compared to p_s, then p_s will be similar to the total pressure (P). Therefore an approximation of equation 6:

$$dS/dV = P/ p_v' \quad (7)$$

Where: P= total pressure or $p_v' + p_s$
Raoult's Law states that the vapor pressure of volatile compounds is equal to its vapor pressure in pure form, multiplied by its molar concentration in the oil.

$$p_v = P_v (V/(O + V)) \quad (8)$$

Where: O = moles of oil
Since V is small compared to O, the equation can be written:

$$p_v = P_v (V/O) \quad (9)$$

Using the equation 10 to calculate the efficiency of vaporization

$$E = p_v '/ p_v \quad (10)$$

Where p_v is the pressure equilibrium, then combining equations 9 and 10, equation 11 is represent as follows:

$$P_v' = (E P_v V)/O \quad (11)$$

From equations 6 and 11 gives

$$dS/dV = PO/(E P_v V) \quad (12)$$

If it is integrate equation 12, it has obtain the amount of steam for deodorization

$$S = PO/E P_v (\ln (V_1 / V_2) \quad (13)$$

Where V_1 is the initial concentration of volatile compounds in the oil and V_2 the final concentration.
This equation applies only to ideal systems. However, in the practice, the system vegetable oil-fatty acid has a non-ideal behavior. Therefore, an activity coefficient (A) is introduced to the equation 13 for the case of non-ideal systems (Mattil et al., 1964), leaving this equation as follows:

$$S = PO/E P_v A (\ln (V_1/V_2) \quad (14)$$

Worldwide the deodorizing equipments can be classified into three groups: batch, semi-continuous and continuous. The selection of the most appropriate depends on several factors, such as: capacity, required investment, and operating costs.

The batch deodorizer equipment is simple and low cost construction. However, operating costs are high (high steam consumption) and long processing times (more than 8 hours). Other disadvantages include its small capacity (50 tons per day), and irregular production (Kellens & De Greyt, 2000).
Semi-continuous system is basically a semi-batch system, designed for larger capacities. The advantage of this system is the good quality oil it can produce and the possibility of frequent feedstock changes of oil per day. However, it requires a large investment, high operating costs and relatively long residence times (Zehnder, 1992).
The continuous deodorizer system is the most preferred by high capacity plants with few stock changes. The main advantages are the moderate investment cost, the short residence times (20-60 minutes), the possible heat recovery and easy maintenance (Kellens & De Greyt, 2000).
There are certain critical steps that's should be carried out during neutralized, bleaching and deodorization, in order to obtain a good-quality soybean oil for edible uses. For example, heating of soybean oil, after neutralized with alkali is an important step in the inactivation of trace metals (Pryde et al., 1980). Also, in order to improve the flavor stability, bleaching can be made more selectively for increase the ratio of carotenoids over chlorophylls. To reduce the problem caused by certain polymers, deodorization of soybean oil should only carryout after ensuring that the oil is free of peroxides or after their removal.

3.3 Physical refining of soybean oil

Another way to remove FFA from vegetable oil is by physical refining. This is not a new process; it was firstly used in the 30´s in order to process oils with low gums content (coconut oil, palm oil and animal fats) and high FFA content (> 1%). The physical refining process can offer important advantages to the refiner, including the use of fewer chemicals, reduction of handling of by-products (soaps, and acidified washed waters), and minimal loss of neutral oil. The physical refining of oils such as canola, sunflower and especially soybean oils has gained increasing interest (Zehnder, 1992). However, in the case of these oils, physical refining is suitable only for crude oils of a good quality (low level of oxidation and low phosphatides content after degumming). Chemically refined oils usually require less steam, shorter residence time, high pressure and low temperature than physically refined oil (Kellens & De Greyt, 2000).

4. Chemistry of soybean oil and its nutritional significance

Vegetable oils are insoluble in water but soluble in organic solvents such as hexane, chloroform and petroleum ether. They consist predominantly of triglycerides, which are characterized by three monocarboxylic fatty acids esterified to a glycerol molecule. These fatty acids generally have an even number of carbon atoms (C14-C22) and can be saturated and unsaturated.

4.1 Fatty acids

The most common saturated fatty acids are palmitic acid (C16) and stearic acid (C18), monounsaturated is oleic acid (C18:1), and the most common polyunsaturated are linoleic acid (C18:2) and α-linolenic acid (C18:3). Unsaturated fatty acids have geometric isomerism, *cis* and *trans* isomers.

Some studies have examined the effect of fatty acids (saturated and unsaturated) on health, concluding that there is sufficient evidence that lauric acid (C12), myristic acid (C14) and palmitic acid (C16), increase serum cholesterol concentration but not stearic acid (C18). In contrast, the consumption of mono and polyunsaturated fatty acids (*cis*), reduce serum cholesterol and low-density lipoprotein (Connor, 2000; Stanley, 2000).

Caggiula & Mustad (1997) reported the results of an epidemiological survey conducted in seven countries, in order to identify dietary factors that could be considered etiologic in the development of coronary heart disease. The study found a high correlation between intake of saturated fatty acids and the incidence of coronary heart diseases. With respect to mono and polyunsaturated fatty acids, they found a negative correlation with these diseases. Given this situation, is recommendable to reduce the intake of saturated fatty acids (animal fats) and *cis* fatty acids substituting them by mono and polyunsaturated (vegetable oils) (Eritsland, 2000).

All fatty acids are necessary for the normal physiological functions of human. The saturated are involved in producing and storing energy in lipid transport and synthesis of phospholipids. Monounsaturated fatty acids are also involved in these processes; however, the term "essential" does not apply to these saturated and monounsaturated fatty acids. This designation is reserved for polyunsaturated fatty acids that are not synthesized by the human body; therefore, they should be in the diet because they are required to maintain good health (Spector, 1999).

Among the polyunsaturated fatty acids, are especially important the linoleic acid (n-6) and α-linolenic acid (n-3). These fatty acids are considered essential for growth and development of the human body. They are precursors of prostaglandins and hormones that play an important activity in the regulation of some physiological and biochemical functions of the human body. When there is a deficiency of essential fatty acids, the growth, and synthesis of prostaglandins is reduced, and damage to the skin can occur. Soybean oil is a major source of these fatty acids, contains about 56% of n-6 linoleic acid and 8% α-linolenic acid, n-3 (Bockisch, 1998).

It has been suggested that an increasing intake of n-3 fatty acids produce beneficial physiological effects such as: a decrease on concentration of triglycerides in the blood, modulation of blood pressure, reduction on the risk of heart and kidney disease, regulation of neuromuscular disorders, control of hyperglycemia, and a beneficial influence on the immune system, allergies, cancer and growth (Chavarro et al., 2007; Sun et al., 2008; Ahren et al., 2009; Barbosa-Tinoco et al., 2009; Sioen et al., 2009; Yongsoon, 2009).

Trans fatty acids. The refining process applied to soybean oil to improve flavor and oxidative stability provokes chemical changes to some compounds with nutritional importance, mainly on deodorizing stage. These nutritional modifications include tocopherols (vitamin E) loss and *trans* isomerization of polyunsaturated fatty acids (Medina-Juarez et al., 2000). Highly polyunsaturated oils such as soybean and rapeseed oils are particularly sensitive to *trans* isomerization during deodorization. Medina-Juarez et al., (2000) evaluated the effects of different conditions of deodorization process (temperature, pressure, time, and steam) on the formation of *trans* fatty acids and tocopherols loss during chemical refining of soybean oil. The results showed that formation of *trans* fatty acids was significatively favored by temperature and time ($P<0.05$). Additionally, this work reported the optimal conditions in lab-scale batch deodorizing for soybean oil (temperature 220°C and 5 mm Hg pressure). Under these conditions, was obtained a deodorizing soybean oil with 1.0% of *trans* fatty acids, 70% of tocopherols retention, 0.03 % of FFA, color 0.7R, and 6

hours Rancimat (110°C) of oxidative stability (Medina-Juarez, 2000). For these reasons the modern deodorizers are operated at low temperatures 220-235/428-455°F, and vacuums of 2 mbar. The highest isomerization rate has been observed for linolenic acid and the lowest for oleic acid (Kellens & De Greyt, 2000).

Several population studies have examined the relationship between *trans* fatty acids intake and coronary heart disease risk. These studies show that high *trans*-fat intake has been linked to coronary heart disease, in which fatty plaques build up in the heart arteries, provoking heart attacks (Hunter, 2006; Mozaffarian et al., 2006; Chiuve et al., 2009). In addition, *trans* fatty acids inhibit desaturation of essential fatty acids (Phivilay et al., 2009).

The average intake of *trans* fatty acids in industrialized countries is estimated as 6-8 g/day, corresponding from 1.5 to 3% of the calories in the diet. The intake of *trans* acids in the diet of Americans is estimates that exceed 10 g/day, being the major source of intake of such products from fast food and snacks. Recent studies showed that the average intake of *trans* fatty acids in women from Turkey was 2.13 +1.03% of the calories in the diet, being the main source, margarine and butter (37.0%), bakery products and confectionery (29.6%) (Samur et al., 2009).

The reason because of *trans* fatty acids, as well as saturated fatty acids are involved in increasing coronary heart disease (Khosla & Hayes, 1996) is that both *trans* fat and saturated fatty acids produce similar effects on plasma proteins, increasing the cholesterol-low density lipoprotein (LDL-C) (Hunter, 2006; Mozaffarian, et al., 2006; Chiuve et al., 2009).

Similar results was reported by Caggiula & Mustad (1997), in a study conducted in seven countries with over 12,000 men from 16 towns, where they found a high correlation (r=0.81) between intake of saturated fatty acids and increased artery disease. Also, they found a strong correlation between the content of *trans* fatty acids in the diet and mortality from coronary artery disease (r=0.78).

Based on these facts and the scientific evidences about the negative effects of *trans* fatty acids on human health, the Food and Drug Administration (FDA) U. S., issued a regulation where, from January 2006 must be included on food labels the content of these *trans* fatty acids.

4.2 Phospholipids

Phospholipids are diacylglycerides (R1, R2) containing a phosphoric acid molecule attached to the glycerol (C3) by an ester bond. These compounds are part of biological membranes and have an active participation in biological processes such as cell permeability and transport of lipids and cholesterol. In vegetable oils are mainly three phospholipids: phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol. These phospholipids are the main components of lecithin, which are used in the food industry as emulsifiers, due to the ability to blend with the lipophilic hydrophilic phases.

Lecithin is a byproduct of the refining process of soybean oil. During the stage of degumming (before neutralization), phospholipids must be removed because the lecithin or phospholipids (gums) increase refining losses, form precipitates in storage tanks or pipes and they make more difficult the filtrate processes (Kellens, 1997).

4.3 Tocopherols

Tocopherols are the most important natural antioxidants in vegetable oils, and they have an important activity as vitamin E. Only plants synthesize these compounds, which are

important nutrients for humans and animals. Tocopherols are present in oilseeds, leaves and other green parts of higher plants. The content of tocopherols in vegetable oils is show in Table 4.

Oil	Tocopherols (ppm)	Iodine value	Unsaponifiables (%)
Soybean	1700-2000	120-143	0.6-1.6
Rapeseed	700-1200	110-126	0.5-1.2
Corn	1000-1500	127-133	0.4-1.2
Sunflower	600-800	110-144	0.3-1.3
Olive	150-200	75-94	0.4-1.1

Kellens, 1997.

Table 4. Tocopherols content in crude vegetable oils.

Tocopherols are found in four forms: α-tocopherol, β-tocopherol, γ-tocopherol and δ-tocopherol. There is another series of compounds with similar chemical structure to tocopherols; the tocotrienols, which differ structurally from tocopherols by having three double bonds in the aliphatic chain. Tocotrienols are found primarily in corn germ, rice bran, palm oil and coconut (Eitenmiller, 1997; Shahidi, 1997).

Vitamin E is known as mixed isomers of tocopherols and tocotrienols with a recognized efficient to inhibit lipid oxidation in foods and biological systems. Tocopherols are found in oilseeds, leaves and other green parts of higher plants. A marked variation on the content of tocopherols can be found in vegetable oils. Kamal-Eldin & Andersson (1997) evaluated oils from 14 plant species, and found a possible correlation between the content of linoleic acid (C18: 2) and α-tocopherol as well as between linolenic acid (C18: 3) and γ-tocopherol. Therefore, it is difficult to establish a correlation between tocopherol content and fatty acid composition.

The antioxidant activity of tocopherols depends on its chemical structure, which defines the ability to donate hydrogen from phenolic group of free radicals. The α-tocopherol isomer has a higher antioxidant activity, followed in descending order by the γ-tocopherol, and δ-tocopherol, in living systems. Contrarily, in the case of fats, the increased activity is δ-tocopherol followed by γ-tocopherol and finally α-tocopherol. The change in the antioxidant activity of tocopherol isomers *in vitro*, not only depends on its chemical reactivity, but also because of the effect of temperature, light and the presence of other compounds that act as pro-oxidants (Kamal & Appelqvist, 1996).

4.4 Sterols

Sterols are the largest proportion of unsaponifiable matter in vegetable oils. The basic structure of these compounds is alcohols derived from polycyclic ciclopentanofenantrene (Bockisch, 1998). These compounds are constituted mainly by β-sitosterol, and in lower concentrations campesterol, stigmasterol and brassicasterol (Frandsen, 1996). The ratio of sterols is specific for each oil. The molecular structure of sterols is similar to the structure of cholesterol. The minor components are used to determine the purity of oil (olive oil "virgin oil") (Gutierrez et al., 2000) or identify edible oil mixtures (Gordon & Miller, 1997, Alonso et al., 1997).

Sterols are also used in the cosmetics industry as emulsifiers. A 75% of the sterols that are currently marketed in the world come from soybean oil. These sterols are obtained from the

distilled products of deodorization of soybean oil and other vegetable oils (Clark, 1996). It has been demonstrated that the sterol consumption may benefit the human health. Recent studies have shown that sterols help in the reduction of LDL-cholesterol in human serum. This reduction is the result of a decrease in cholesterol absorption in the small intestine and an increasing of bile acid excretion (Normén et al., 2000; Vissers et al., 2000; Sanclemente et al., 2009).

4.5 Reactions of soybean oil

The terms for the types of characteristics reactions of oils, reported in the literature are: acidolisis, alcoholysis, esterification, interesterification and transesterification. However, the meaning of these terms has not been consistent in the literature.

Acidólisis. Reaction between an ester (triacylglycerides) and a fatty acid. The result is an exchange of acyl groups.

$$R_1COOR_2 + R_3COOH \longrightarrow R_2OH + H_2O \longrightarrow R_3COOR_2 + R_1COOH$$

Alcoholysis. Reaction between an ester and an alcohol, which can be a glycerol or methanol, resulting an exchange of alcoholic fractions.

$$R_1COOR_2 + R_3OH \longrightarrow R_1COOH + H_2O \longrightarrow R_1COOR_3 + R_2OH$$

Esterification. Reaction between a fatty acid and alcohol, or among compounds containing carbonyl and hydroxyl groups.

$$R_1COOH + R_2OH \longrightarrow R_1COOR_2 + H_2O$$

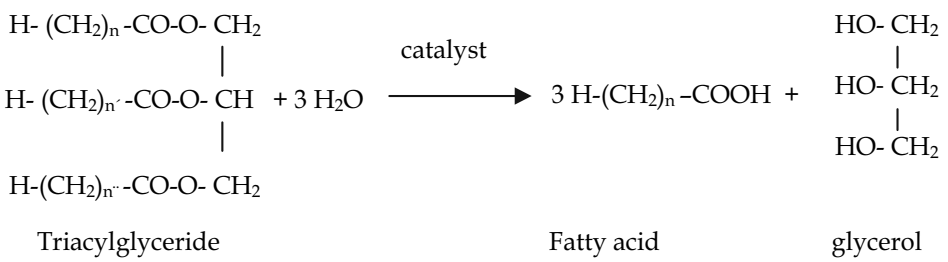
Transesterification. Term commonly used for the exchange of an ester (triacylglycerol) to another ester can be another triacylglycerides or ethyl ester. The result is an exchange of acyl groups between two esters.

$$R_1COOR_2 + R_3COOR_4 \longrightarrow R_1COOH + R_2OH + R_3COOH + R_4OH + H2O \longrightarrow R_1COOR_4 + R_3COOR_2$$

Interesterification. A general term used for the reactions between an ester and a fatty acid, an ester alcohol or other (includes acidólisis, alcoholysis and transesterification). Hydrolysis. Reaction between an ester (triacylglycerides) and a water molecule in the presence of a chemical catalyst or enzyme.

$$R_1COOR_2 + H_2O \longrightarrow R_1COOH + H_2O \longrightarrow R_1COOH + R_2OH$$

The following diagram shows the hydrolysis of a fat.



During these reactions, water is consumed (hydrolysis) or generated (esterification) or consumed and released at once (interesterification).

5. Digestion and absorption of triacylglycerides

Pancreatic lipase preferentially hydrolyzes ester linkages of triacylglycerides 1-3, producing free fatty acids and 2-monoacylglycerols, which are absorbed through the intestinal wall cells (Ikeda et al., 1991). The fatty acids released during digestion, are metabolized more rapidly if they are short and medium chain (<C10: 0), while long-chain fatty acids are absorbed directly as monoacylglycerols. The long-chain triglycerides are not effective as an energy source because they are metabolized more slowly than medium chain triglycerides. The short-chain fatty acids are incorporated into chylomicrons and transported by the lymphatic system to the liver to provide the necessary energy or adipose tissue for storage. The medium chain triglycerides are absorbed by enterocytes of the intestinal wall and enter the portal vein, they not depend of carnitine for transport through the mitochondria, and are not incorporated into chylomicrons, and therefore, they are not easily stored in adipose tissue (Mascioli et al., 1987). In people with malabsorption, essential fatty acids are used more efficiently in the form of 2-diacyl-glycerol (Xu, 2000).

6. Modulation of eicosanoid biosynthesis by n-3 and n-6 dietary fatty acids

Once the digestion, absorption and transport in the human body are done, polyunsaturated fatty acids n-3 and n-6 are introduced to fat cell in non-esterified form, and are used as substrates for the synthesis of eicosanoids (leukotrienes, prostaglandins and thromboxanes). Eicosanoids are synthesized by stimulation. The class of eicosanoids produced varies with the type of tissue. For example, thromboxane A_2, is an agglomerating agent of platelet, which is synthesized in platelets; whereas prostaglandin I_2, which is an inhibitor of the agglomeration of platelets, are synthesized in endothelial cells. Eicosanoids are not stored in the cells; their effects are manifested locally and perform various actions on the cardiovascular, reproductive, respiratory, renal, endocrine, nervous and immune systems. Excessive inhibition of eicosanoid synthesis involves a number of pathological conditions including thrombosis, inflammation, asthma, ulcers and kidney disease. Some studies have suggested that the amount and type of eicosanoids produced in the tissues can be modulated by manipulation of dietary fatty acids, since the type of eicosanoids produced depends on the substrate available.

The n-6 fatty acids such as linoleic acid (LA) or arachidonic acid (AA) act as substrates for the synthesis of prostaglandins of the series-2 (PCI_2thromboxane A_2 and prostacyclins), and leukotrienes of series-4; while, prostaglandin series-3 (PCI_3thromboxane A_3 and prostacyclins), and leukotrienes of series-5 are derivatives of n-3 fatty acids. Depending of enzyme activity in the tissue, the availability of cofactors, the specificity of each enzyme for fatty acid and the concentration of each acid on the value of Michaelis constant (K_m), there are three possible metabolic pathways PUFA: the way of acetyl-CoA, the route of cyclo-oxygenase and route of the lipo-oxygenase (Lands & Bimbo, 1983).

The route of acetyl-CoA regulates oxidation and esterification of fatty acid within lipidic cells, depending on energy demand.

The route of cyclo-oxygenase provides to human organism of prostaglandins, thromboxanes and prostacyclin, by oxidative cyclization of polyunsaturated fatty acids. The prostacyclins

are hormones that play an important role in the regulation of some physiological and biochemical functions of the body. The thromboxanes and prostacyclins, transformation products of prostaglandins, have the function of regulating the activity of platelets (Scott et al., 1982).
The route of the lipo-oxygenase provides to human organism of leukotrienes, which are potent eicosaenoics, which cause contractions and increase the permeability of smooth muscle (Lands & Bimbo, 1983).
Dietary fatty acids may modulate the biosynthesis of eicosanoids in two stages. The first is the desaturation and elongation of fatty acids. There is a competitive inhibition between linoleic acid (LA, n-6) and linolenic acid (n-3). n-3 PUFAs may reduce the conversion of LA to AA, modulating the biosynthesis of eicosanoids and consequently modifying the physiological response(Machlin, 1962). The second stage is the path of cyclo-oxygenase, the n-3 fatty acids competitively inhibit the oxidation of AA. Competitive inhibition between n-3 fatty acids and n-6 by desaturases and cyclo-oxygenases suggests that an increase in dietary n-3 fatty acids may reduce eicosanoids derived from AA (Hwang, 1992).

7. Physical and chemical analysis of soybean oil

Crude vegetable oils should be subjected to refining process in order to improve taste, appearance and oxidative stability. During this process color, free fatty acid, oxidation products (peroxides and aldehydes and ketones), the modification of essential fatty acids to *trans* isomers, and the loss of tocopherols and sterols, should be monitored.
The official methods used for physical-chemical analysis of oils and fats are containing in "Official Methods and Recommended Practices" published in 2009 by the American Oil Chemists' Society. This manual includes the following methods:

7.1 Humidity

The presence of moisture in oil samples indicates that they were inadequately processed (washing in the neutralization) or the oil containers were contaminated. The moisture determination is made in a vacuum oven, calculating the moisture and volatile material by difference in weight (AOCS, Ca 2d-25).

7.2 Color

The method determines color by Lovibond. This equipment uses color filters calibrated in accordance whit the Lobivond tintometer color scale (AOCS, Cc 13e-92).

7.3 Soap

This determination indicates the efficiency of soap removal (washing the oil with water) after neutralization operation. The method determines the alkalinity of the oil with sodium oleate (AOCS, Cc 17-79). The content of soap should be less than 30 ppm after neutralization and less than 1 ppm after the bleaching (Erickson, 1995).

7.4 Conjugated dienes

In the structure of polyunsaturated fatty acids is not common to find conjugated dienes. Therefore, conjugated dienes, are the result of a structural modification of polyunsaturated fatty acids during autoxidation (Banni & Martin, 1998). Quantification of conjugated dienes

is performed by a spectrophotometric method (AOCS, Ti la-64). Polyunsaturated fatty acids containing conjugated dienes have their maximum absorbance at 234 nm. This index is useful for determining the advance of the start of oxidation, since the increase is proportional to oxygen consumption and the concentration of hydroperoxides formed in the early stages of the reaction.

7.5 Free fatty acids

Is the number of milligrams of potassium hydroxide necessary for saponify free fatty acids from a sample of one gram of oil or grease and is expressed as percentage of oleic acid. This method is a measure of the degree of decomposition of the oil triglycerides (AOCS, Ca 5a-40).
This analysis is performed to determine the effectiveness of chemical neutralization in the refining process and to obtain rapid information of loss of oil in the same operation.

7.6 Peroxide value

The formation of primary products of oxidation (hydroperoxides) can be measured by peroxide value. This method quantifies the primary compounds of oxidation reaction in oils and fats (AOCS, Cd 8b-90).

7.7 *p*-anisidine value

Is a colorimetric measurement (350 nm) based on the color development produced by the reaction between *p*-anisidine reagent and the secondary oxidation compounds (aldehydes, ketones, alcohols and acids) (AOCS, Cd 18-90).
The use of peroxide value (PV) and *p*-anisidine value (AV) together provides a comprehensive overview of the oxidation process in oils. This is a mathematical prediction of oxidative stability and the value is calculated as: TOTOX = VA + 2VP (AOCS Cg 3-91).
Unfortunately, none of these methods can characterize the real oxidative grade of oil. A more effective method for understanding the oxidative state of oils is the determination of the composition and amount of volatile materials by solid-phase microextraction (SPME) and gas chromatography (Ho et al., 1996; Hinshaw, 2003). Among the main components, 2-pentilfurane, is a main product of auto-oxidation of linoleic acid and responsible of flavor reversion of soybean oil.

7.8 Oxidative stability

The Rancimat method (AOCS, Cd 12 b-92) has been used successfully to measure the oxidative stability index (OSI) of oils with synthetic and natural antioxidants (Hasenhuettl & Wan 1992; Gamez-Meza et al. 1999). The method measures the increases of the electric conductivity that arises when fats and oils are oxidized to short free fatty acids (chiefly formic acid) under accelerated conditions of heat and aeration (Kolb et al. 2002; Anwar et al. 2003).
OSI values of 5.24 (Anwar et al. 2003), 6.63 (Gámez-Meza et al., 2009) and 7 h (Frank et al. 1982; Judde et al. 2003) for soybean oil without antioxidant at 110ºC have been reported.
Lopez-Aguilar et al., (2006) found that differences in *p*-anisidine value and Rancimat were correlated to the difference in rancidity of soybean oils.

7.9 Minerals

The properly processed soybean oils must provide calcium and magnesium levels below 1 ppm and concentration of iron lower than 0.1 ppm (Erickson, 1995). These trace metals can

be analyzed by atomic absorption spectroscopy or optical emission spectroscopy inductively coupled to plasma (ICP), the latter method is recommended for use in edible oils with less than 1 ppm of trace metals (Perkin-Elmer, 2010).
Copper is not normally found in vegetable oils, however, it is recommended monitor it because it is a potent prooxidant (Coppin & Pike, 2001) and oils can be contaminated easily during processing or storage. The limit values for soybean oil must be below 0.05 ppm (Erickson, 1995).
Ahmad et al. (1983) found that the oxidative stability index of refine soybean oil without antioxidants (6.63 h) was significantly lower ($p< 0.05$) than soybean oil with grape peel extracts and citric acid at all concentrations tested. This suggests that citric acid chelated the iron and inhibited its prooxidant effect.

7.10 Fatty acid profile

The composition of fatty acids is one of the most important chemical characteristics to identify the oils, since each one has a distinctive profile. The method AOCS Ce 1h-05 provides a gas-liquid chromatography procedure for the determination of the fatty acid profile, including the *trans* fatty acids isomers of vegetable oils and fats. The samples are saponified and methylated according to AOCS procedure (Ce 2-66). Fatty acid methyl esters (FAME) are analyzed in a gas chromatograph equipped with flame-ionization detector. A fused-silica capillary column coated with 100% biscyanopropylpolysiloxane as the stationary phase (SP-2560, 100 m × 0.25 mm i.d. 20 µm thickness, Supelco, Bellefonte, PA), and an oven temperature of 180°C are recommended. FAME peaks are identified by comparison with the retention time of the respective standards. *Trans* isomers are identified from a methyl ester isomer mix of linoleic acid and linolenic acid (Figure 1). C21:0 or C23:0 as internal standard is used (Medina-Juarez et al., 2000). Results are expressed as weight percentage of oil. The specific conditions of the method not determined oxidized fatty acids or polymerized.

7.11 Quantification of glycerides

The quantification of mono-, di-and triacylglycerides is done through an equipment of high performance liquid chromatography (HPLC) with ELSD detector (Evaporative Light Scatering Detector) and a Lichrosorb Si60 column (250 x 4.6 mm, 50 um of particle size, Supelco, Inc.). The temperature must be maintained at 40°C, using a column jacket. The solvent system used is hexane (solvent A) and a mixture of hexane:2-propanol:ethyl acetate (80:10:10) with 0.1% of formic acid solution (10% in 2-propanol) (solvent B) (Liu et al., 1993).

7.12 Tocopherols

The quantification of tocopherols by HPLC with UV detector and a Lichrosorb Si60 column (25 cm x 4 mm, 5µ particle size, Supelco) is recommended. The mobile phase for this determination is a mixture of hexane: isopropanol (99.5:0.5) (Medina-Juárez et al., 2000, Ortega-Garcia et al., 2006). The analysis of tocopherols may also be performed with a reverse phase C18 column and an isocratic elution with 100% methanol, using a mass detector (Deselet al., 2007). The wavelength of detector should be set at 292 nm. It must be define the purity of the standards of tocopherols using their extinction coefficients (E 1%) (AOCS Ce 8-89).

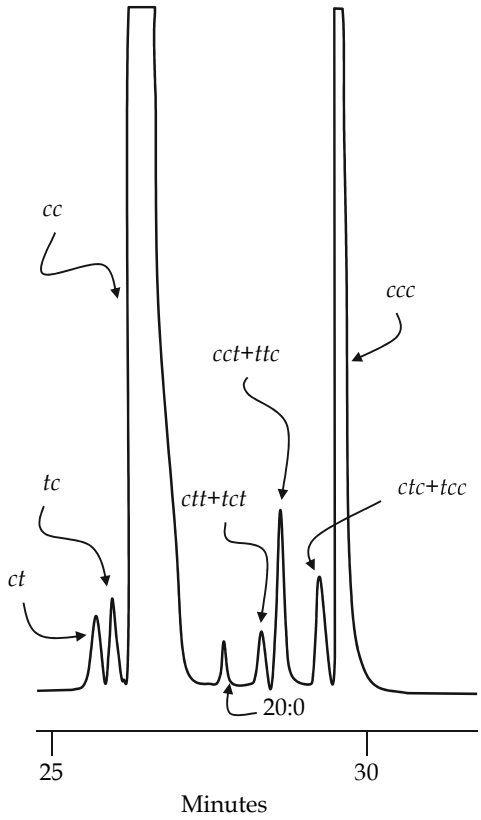


Fig. 1. Partial chromatogram of fatty acid methyl esters of refining soybean oil using a SP-2560 (Supelco, Belloefonte, PA) capillary column with a temperature program, and nitrogen as carrier gas at a flow rate of 20 cm/min. ct, 18:2Δ9c,12t; tc, 18:2Δ9t,12c; cc, 18:2Δ9c,12c; ctt + tct, 18:3Δ9c,12t,15t + 18:3Δ9t,12c,15t; cct + ttc, 18:3Δ9c,12c,15t + 18:3Δ9t,12t,15c; ctc + tcc, 18:3Δ9c,12t,15c + 18:3Δ9t,12c,15c; ccc, 18:3Δ9c,12c,15c. (Medina-Juárez et al, 2000).

7.13 Sterols

The first part of this technique consists to separate the saponified fatty acids from unsaponified fraction (mainly sterols and tocopherols) (Gutierrez et al., 2000). One gram of sample with 100 mL of 0.8 M KOH in methanol, is saponified for 30 min at 80°C. The unsaponified fraction with two fractions of 100 mL of ethyl ether is extracted. The ethereal fraction is washed with water and then this fraction in a rotary evaporator at 60°C and vacuum is concentrated. The sample is brought in ethanol (25 mL) and injected to an equipment of high performance liquid chromatography (HPLC) with a UV detector and a C18 column (15 cm x 4.6 mm, 5µ particle size, Varian) reverse phase. Methanol (100%) is the mobile phase. The measurement is carried out at 206 nm (Ortega-Garcia et al., 2006).

7.14 Sensory evaluation

The flavor characteristic of soybean oil and other linolenate-containing oils at early stages is "beany and grassy" and "fishy or painty" at the more advance stages and at low levels of oxidation (i.e., the peroxide value is 10 or below). The oxidation degree is so low that often it

cannot be measured chemically (Frankel, 1998). Flavor reversion, in contrast to oxidative rancidity is observed at lower levels of oxidation.
According to Sensory Evaluation Division of Institute of Food Technologists (IFT), the sensory evaluation is defined as a scientific discipline, which is responsible to measure, analyze and interpret results of characteristics of food and materials, in how they are perceived by the senses of sight, smell, taste, touch and hearing (Pedrero & Pangborn, 1989). The official method (Cg 2-83), is based on attribute intensity scales. Some researchers have shown that these techniques can generate biased answers due to forgetfulness of intensity from sample to sample, because the assessment is based on the memory of the judges (Kim & O'Mahony, 1999). In recent years, it has been increased research in this area, proposing innovative methods. One of these techniques is called n-AFC (n Alternative Forced Choice). This is a discriminatory technique that has shown to have higher probability of success than traditional discriminatory techniques, duo-trio and triangular (Angulo et al., 2007).
Medina-Juárez, et al., (1998) evaluated the oxidative stability and the sensory characteristics (Cg 2-83) of some samples of soybean oil, produced in Mexico, U.S. and Costa Rica. These authors found that 41% of the samples analyzed from 18 main Mexican refineries had similar flavor to the samples produced in the U.S. and Costa Rica. However, in 2006, Lopez-Aguilar et al. reported that Mexican soybean oils met quality control criteria and correlated with sensory evaluation by two methods (official method of AOCS, Cg 2-83 and forced-choice test of two alternatives, 2-AFC). The method most effective in determining small differences in rancidity was 2-AFC.

8. Oxidative stability of soybean oil: antioxidants

Refined soybean oil has been found to be one of the most unstable product, not only due to its high unsaturated fatty acids content but also due to the absence of natural compounds capable of provide a protective antioxidant effect. By other hand, the types of volatile products depend of triacylglyceride precursors. A complex mixture of volatile products is expected from decomposition of each one of the many of hydroperoxides found in oxidized soybean oil. Due to the improved analytical methodology, the number of flavor and odor compounds identified by different authors is continuously increasing. Therefore, this information should permit better understanding of influence of triacylglycerol structure on the relative oxidative stability of unsaturated glycerides. In spite of many studies in the control of oxidation of refining soybean oil, problems caused by light and thermal instability still await solution.
The effect of trace metals and light on flavor stability is well recognized. Treatment with citric acid is an effective practice to improve flavor stability.
Antioxidant addition during industrial food formulations is one of the most effective means to retard fat oxidation. It is a popular method for increasing the shelf life of oils and oils-containing foods (Halliwell et al., 1995). Synthetic antioxidants, such as butylatedhydroxytoluene (BHT), butylatedhydroxyanisole (BHA) and tertiary butyl hydroquinone (TBHQ) are widely used in many oils. However, their use has been questioned because of issues related to toxicity and carcinogenicity (Branen, 1975; Imaida et al., 1983). For this reason, considerable attention has been given to the application of natural antioxidants in foods, because of their potential nutritional and therapeutic effects.
There is a continuous need for technological innovations on safe antioxidant systems. The best known and most effective primary antioxidant among natural substances are polyphenols. Primary antioxidants interfere with auto-oxidation by interrupting the chain

propagation mechanism. The auto-oxidation proceeds when the antioxidant has been destroyed completely.

Phenolic antioxidant commonly used in foods, are highly reactive, have two hydroxyl groups or one hydroxyl and one substituted hydroxyl group in *ortho* or *para* positions. These substances are effective at low concentrations; at high concentration some of them may accelerate the rate of auto-oxidation. They are most effective in foods that contain little natural antioxidants, while they are much less effective in vegetable oils that may contain amounts of naturally antioxidant. However, the naturally occurring antioxidants in oils as soybean oil are readily destroyed by heat during refining process.

Some sources of natural antioxidants are: algae, browning products, protein hydrolysate, plant (extracts), cocoa powder, oat flour, herbs, spices, fruits pulp and peel.

The peel and seeds of grapes are important sources of phenolic compounds, which are considered antioxidant substances. Phenolic compounds at concentrations as high as 718-1060 mg/100 g have been found in peel grapes (Molina-Quijada et al., 2010).

Gamez-Meza et al. (1999) evaluated the antioxidant activity in soybean oil of an extract from Thompson grape peel by Rancimat and Schaal methods. At all concentrations tested (0.1, 0.3, and 0.5% of total phenols), the grape peel extract exhibited appreciable activity, which exceeded the activity of BHA. The highest induction period (Rancimat test) was achieved with the extract at 0.5% of total phenols; this activity exceeds significantly ($p< 0.05$) the antioxidant activity of both synthetic antioxidants (Table 5).

In other study with soybean oil (Gámez-Meza et al., 2009), the antioxidant activity of grape peel extract (GPE) with those of rosemary extract (RE), and tocopherols mix (TM) known as covi-ox T-70 was compared. Thompson GPE at 0.3 and 0.5% (w/w) exhibited greater antioxidant activity than TM. Soybean oil containing GPE or RE at 0.5% (w/w) showed an OSI higher than 48 h at 110°C. Also the synergistic effect of citric acid was evaluated. When citric acid was added, the RE exhibited a significantly greater antioxidant activity ($p<0.05$) than TM. A synergistic effect for TM at 0.02% (w/w) with citric acid at 1.0% (w/w) was observed. No synergistic effect of citric acid with Thompson grape peel extract was observed (Table 6). Banias et al. (1992) found an antagonistic effect when plant extracts (including RE) combined with citric acid were tested as antioxidants.

The results of studies suggest that it is important to pursue a characterization of the biologically active antioxidant compounds. Of the hundreds of substances that have been proposed to inhibit oxidation of oxidizable compounds, only a few can be used in products for human consumption. Antioxidants required to be approved for its food use by specialized agencies.

Treatment	Induction time[a] (110°C)
Soybean oil (control)	6.24 ± 0.20^{a}
Soybean oil + extract 0.1% TP	15.20 ± 0.56^{b}
Soybean oil + extract 0.3% TP	29.10 ± 1.84^{c}
Soybean oil + extract 0.5% TP	$> 48.00^{d}$
Soybean oil + BHA, 0.02% w/w	8.56 ± 0.37^{e}
Soybean oil + TBHQ, 0.02% w/w	21.70 ± 0.28^{f}

TP, total phenol; BHA, butyllatedhydroxyanisole; TBHQ, tertiarybutylhydroquinone.
[a]Values are mean + SD (n = 2). Mean with different superscript letters (a-f) are signicantly different ($p< 0.05$).

Table 5.Antioxidant activity (Rancimat test) of thompson grape peel extract and synthetic antioxidant.

Antioxidant %	Citric acid (%)					
	0.05		1.0		2.0	
	OSI (h)	SE	OSI (h)	SE	OSI (h)	SE
GPE						
0.00	7.50 (0.15)^{a}	-	8.24 (1.01)^{abc}	-	9.61 (0.48)^{bc}	-
0.02	7.70 (0.72)^{a}	0.45	7.87 (0.51)^{ab}	0.44	8.67 (0.66)^{bc}	0.45
0.10	10.24 (1.16)^{bc}	0.54	11.65 (0.75)^{d}	0.51	11.90 (0.40)^{d}	0.57
0.30	21.95 (0.95)^{g}	0.78	19.40 (0.30)^{f}	0.76	25.90 (3.10)^{h}	0.85
0.50	>48	-	>48	-	>48	-
TM						
0.02	8.67 (0.14)^{b}	0.56	17.00 (2.13)^{f}	1.04	17.25 (1.85)^{f}	0.97
0.10	7.87 (0.24)^{a}	0.48	13.60 (0.30)^{e}	0.79	17.50 (1.70)^{f}	0.94
0.30	8.76 (0.20)^{b}	0.50	13.65 (0.45)^{e}	0.75	14.60 (0.70)^{e}	0.75
0.50	9.66 (0.28)^{bc}	0.53	10.00 (0.90)^{c}	0.53	11.45 (0.76)^{d}	0.57
RE						
0.02	18.23 (1.89)^{fg}	0.86	19.60 (1.23)^{f}	0.89	29.00 (1.42)^{h}	1.24
0.10	27.00 (0.89)^{h}	0.81	34.65 (1.65)^{i}	1.04	42.50 (1.30)^{k}	1.20
0.30	37.80 (0.20)^{j}	0.96	37.87 (0.52)^{j}	0.94	43.80 (0.28)^{k}	1.06
0.50	>48	-	>48	-	>48	-

SE: values >1 define a synergistic effect between the implicated antioxidants, whereas values <1correspond to an antagonism.
Values represent the means (SD) (n = 3). Equal letters indicate equal means.
* Control oil (no antioxidants added) with an OSI = 6.63 (0.20) h at 110°C and 20 L/h air flow.
OSI, oxidative stability index; *SE*, synergistic effect; GPE, grape peel extract; –, not determined; TM, tocopherols mix; RE, rosemary extract.

Table 6. Synergistic effect of citric acid with thompson grape peel extract (GPE), tocopherols mix (TM), and rosemary extract (RE) on the oxidative stability index (OSI, in h Rancimat) in refined soybean oil*.

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26

Soybean Fibre: A Novel Fibre in the Textile Industry

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1. Introduction

Although natural protein fibres such as wool and silk have good physical properties and have been used extensively in the textile industry, they are relatively expensive to use and process. In silk, a large quantity of mulberry leaves is required for the production of a very small quantity of silk resulting in an increased cost of production. In addition, apart from the economic aspects, animal fibres are physically limited in several aspects. Firstly, both wool and silk fibre vary in diameter and their performance profile is limited. Secondly, morphologically, the presence of scales on wool surface results in felting shrinkage and difficulties in dyeing. In contrast, regenerated protein fibres, such as soybean fibre, do not have a theoretical limit in fineness to which fibres may be drawn. In addition, soybean is a competitive production material for fibres in the textile industry since it is abundant and cost-effective. Although the price of petroleum increased 21 times over the last fifty years, the price of soybean protein increased only 6.5 times (Agricultural Statistics Board, 1990; Monthly Energy Review, 1991).
Another advantage of soybean protein is its higher protein content (40%), compared to peanuts (25%) and corn (10%) and the environmentally friendly production claimed by the manufacturers (Huakang Ltd., 2005). Additionally, the possibility that a plant protein can be modified by molecular genetic techniques, provide the opportunity to improve the properties of the fibre in specific applications.

2. Soybean fibre: definition and morphology

Soybean fibre is a man-made regenerated protein fibre from soybean protein blended with PVA (Zhang et al., 2003). Soybean fibres have a cream colour and their diameter is around 20 μm (Vynias, 2006). Scanning Electron Microscopy (SEM) analysis of soybean fibre indicated longitudinal striations on the surface parallel to the axis, varying in length and depth, Figure 1 (Vynias, 2006). SEM micrographs of the cross-section of soybean fibre, Figure 2, showed a kidney bean-like cross-sectional shape. Recent research on the cross-section of soybean correlates well with the previous finding indicating kidney form shape (Jiang et al. 2004). Studies on cross-sectional shapes of wet-spun fibres have associated the coagulation rate with the cross-section (Tsai and Su, 1991). It was suggested that non-circular cross-sections occur due to high coagulation rate in wet spinning.

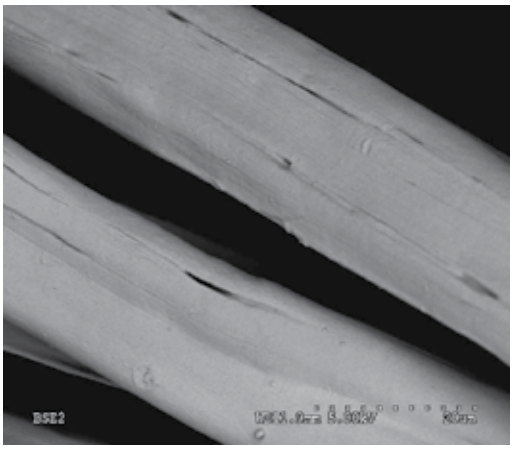


Fig. 1. SEM Micrograph of Untreated Soybean Fibre (Vynias, 2006)

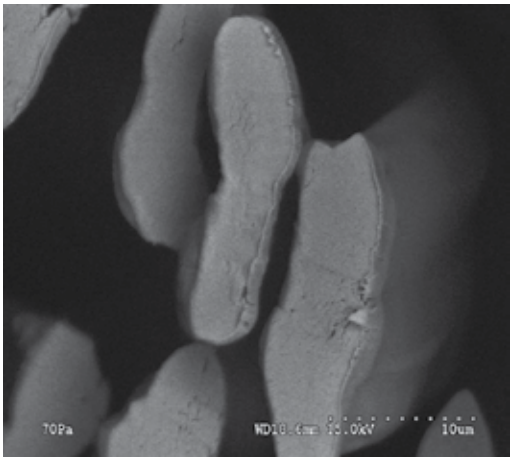


Fig. 2. SEM Micrograph of the Cross-Section of Untreated Soybean Fibre (Vynias, 2006)

3. Brief history of soybean fibre

The first attempts to manufacture textile fibres from soybean protein were carried out in Japan (Kajita and Inoue, 1940a; Kajita and Inoue, 1940b) and U.S.A. (Boyer et al. 1945). The wet-spinning process included the extraction of oil to achieve an oil-free meal, extraction of proteins from the meal with alkali, dispersion of the alkaline proteins, fibre formation by passage through a spinneret into an acid coagulating bath and post-spinning treatments. However, the fibres were never commercially produced due to the lack of functional characteristics. After the end of the World War II, petroleum became the major source of synthetic textile fibres due to its low cost and case of processing, marginalising the commercial outlook of man-made protein fibres. However, due to environmental concerns, during the last decade there has been a renewed interest in soybean fibre and a production line has already been established in China (Huakang Ltd., 2005).

3.1 Soybean: A globular protein

Soy proteins consist of several individual proteins and protein aggregates with a wide range of molecular sizes. However, the most important proteins in soybean are globulins. Globulins can dissolve above or below their isoelectric point (pI) and are insoluble near their pIs. Soy proteins demonstrate maximum solubility at pH 1.5-2.5 and above pH 6.3 whereas minimum solubility is obtained between pH 3.75 and 5.25 (Pearson, 1984). Soybean proteins are mainly composed of two storage proteins glycinin and β-conglycinin with their isoelectric point being between pH 4 and 5 are responsible for the insolubility of soybean proteins in that pH range (Nielsen, 1985). B-conglycinin is a heterogeneous group of glycoproteins composed of varying combinations of three subunits namely a',α and β with their Molecular Weights (MW) being 58,000, 57,000 and 42,000, respectively. The subunits contain hydrophobic regions and link to form compactly folded trimers (Kinsella et al., 1985).

Glycinin is a large oligomeric protein with a MW of approximately 350,000 Daltons. Electron miscoscopy and X-ray scattering techniques have shown that the quaternary structure of glycinin is a pair of identical face to face hexamers (Kinsella et al., 1985). In addition, Pearson (1984) proposed that these hexamers consist of two subunits, acidic or basic in relation to their isoelectric point. It was suggested that acidic subunits had a pI of 4.6-5.4 with a MW of approximately 40,000, whereas basic subunits had a pI of 8.0-8.5 and MW of approximately 20,000 (Nielsen, 1985). Utsumi and Kinsella (1985) suggested that the possible molecular forces involved in the formation of glycinin and β-conglycinin in aqueous solution are hydrogen bonding, hydrophobic associations, ionic interactions and disulphide linkages.

3.2 Amino acid analysis of soybean fibre

The amino acid analysis of soybean fibre is presented in Table 1, with the predominant amino acid being glutamic acid. Acidic amino acids (aspartic and glutamic acid) account approximately for 30% of the total amount of amino acids present in the fibre (Vynias, 2006). In contrast, the sulphur containing groups are present in low amounts, with the cystine content being low (0.1%).

3.3 Manufacturing process of soybean fibre

Soybean protein is a globular protein in its native stage and is not suitable for spinning. Therefore it has to undergo denaturation and degradation in order to convert the protein solution into a spinnable dope. The manufacturing process of soybean fibre is presented in Figure 3.

3.4 Denaturation

Denaturation of soybean protein can be achieved with either:

i. alkalis;
ii. heat;
iii. enzymes[1].

[1] athough denaturation of soybean with enzymes is documented in the literature it has not been applied in the spinning of soybean fibre and therefore not presented in this chapter.

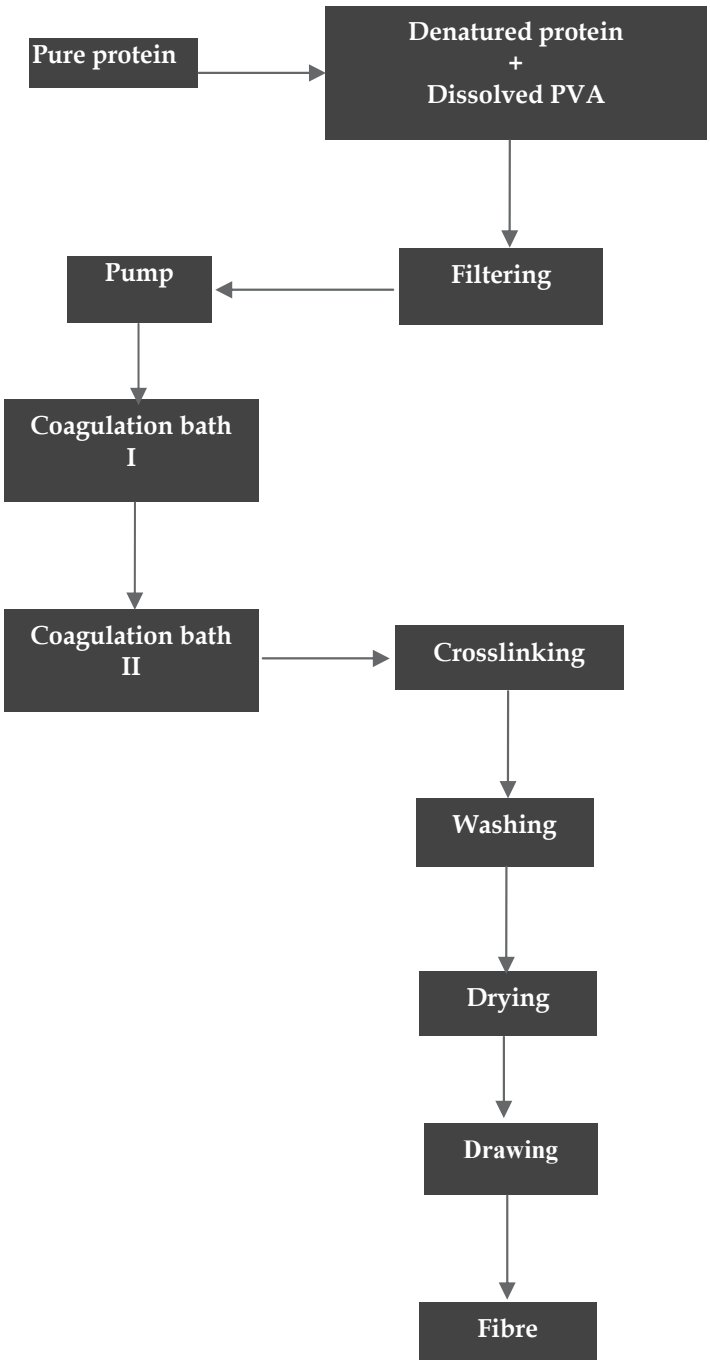


Fig. 3. Manufacturing Process of Soybean Fibre (Zhang et al., 2003)

Amino Acid	Abbreviation	Soybean	Nature of the Side Chain
Glycine	Gly	8.8	Hydrocarbon
Alanine	Ala	7.5	
Phenylalanine	Phe	4.4	
Valine	Val	6.3	
Leucine	Leu	9.8	
Isoleucine	Ile	4.8	
Serine	Ser	6.4	Polar
Threonine	Thr	4.3	
Tyrosine	Tyr	---	
Aspartic acid	Asp	12.8	Acidic
Glutamic acid	Glu	18.2	
Histidine	His	5.5	Basic
Arginine	Arg	0.8	
Lysine	Lys	3.9	
Methionine	Met	0.8	Sulphur-containing
Cystine	CysH	0.1	
Tryptophan	Trp	---	Heterocyclic
Proline	Pro	5.6	

Table 1. Amino Acid Analysis of Soybean Fibre (DWI, 2003)

i. Alkali Denaturation

Boyer et al. (1945) obtained a patent for the manufacture of soybean fibre. In this patent, it was suggested the use of xanthate in spinning of soybean fibre. According to the proposed formula ethyl alcohol reacted with sodium hydroxide forming sodium ethylate (ethoxide) in excess of sodium hydroxide. Further, carbon disulphide was incorporated into the sodium ethylate solution resulting in the formation of sodium ethyl xanthate, a compound with yellow- orange colour that was allowed to age from ten to thirty days. A spinning solution consisting of soy protein, sodium ethyl xanthate, sodium hydroxide and water was prepared with the protein content being approximately 18% of the solution. Xanthate was suggested to react with amino groups forming carbaminodithio groups (-NHCSSH) that prevent the gelation of the protein and improve its elasticity (Zhang et al. 1999). However, Motonaga et al. (1965) have also used sodium sulphite to break disulphide bonds in soybean, Figure 4.

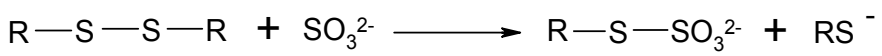


Fig. 4. Reaction Mechanism of Sodium Sulphite Breaking of Disulphide Bonds

Zhang et al. (1999) also utilised sodium sulphite to cleave disulphide bonds in order to denature the protein in the spinning of fibres from soybean protein and poly(vinyl alcohol) (PVA). However, little change was observed in the viscosity of the spinning solution. In contrast, the inclusion of urea was reported to decrease significantly the viscosity. The beneficial effect of urea on denatured soybean to prevent gel formation of the solution has been reported by Ishino and Okamoto (1975) and Ishino and Kudo (1980).

ii. Thermal Denaturation

It is known that heating denatures soy proteins and produces gels (Wolf and Tamura, 1969). When soybean protein is heated two irreversible processes take place:

i. The interchange and degradation of disulphide bonds and sulphydryl groups may lead to irreversible crosslinking;
ii. heating may result in fission of the protein polypeptide.

Gel formation is not beneficial in fibre spinning with a number of studies reported in the literature on the thermal denaturation of soybean (German et al., 1982; Petruccelli and Anon, 1995; Sorgentini et al., 1995). In addition, the denaturation temperatures of β-conglycinin and glycinin have been reported to be in the 72-80^{o}C and 85-95^{o}C range, respectively (Arrese et al. 1991; Berli et al., 1999).

Zhang et al. (2003) reported benefits in terms of viscosity stability when thermal denaturation was used compared to alkali denaturation in the spinning of PVA/soybean blend fibres. The viscosity of the spinning solution was thermally stable at 70^{o}C for approximately 3 hours. In addition, it was noted that the alkali-denatured solution was not suitable for the fibre spinning of soybean protein/PVA blends with only thermal denaturation providing benefits.

3.5 Coagulation

The solution consisting of soybean and PVA is filtered and forced through the spinneret where orientation of the molecular chains occurs. The chain molecules are then arranged into a structure consisting of crystalline regions and amorphous regions. In the crystalline regions the molecules are closely packed and responsible for the strength and rigidity of the material, whereas in the amorphous regions are less closely packed and less ordered, creating flexibility and accessibility (Cook, 1984).

The orientation is highly maintained in the two consecutive coagulation baths of sodium sulphate and ammonium sulphate in water with 1M sulphuric acid (Zhang et al., 2003). The temperatures of the initial and last coagulation baths are maintained at 50 and 70^{o}C, respectively, based on the spinning conditions of PVA (Sakurada, 1985). The cross-section of fibres produced is dependent on the coagulation rate and the blend of soybean/PVA used (Zhang et al., 2003). The next processing step followed is crosslinking in order to enhance the mechanical properties.

3.6 Crosslinking

In the production of soybean fibres, after winding the coagulated fibre is passed into a crosslinking bath that contained formaldehyde (Boyer et al., 1945). Formaldehyde was one of the first crosslinking agents used with proteins. It is known that formaldehyde can react with amino, amido guanidyl, hydroxyl, phenolic and indole groups and reduce disulphide bonds (Carter, 1971). In addition, it was reported that formaldehyde, at room temperature, forms methylene bridges between amino groups or primary amide/guanidyl groups in a wide range of pH (Fraenkelconrat and Olcott, 1948).

Although formaldehyde is cost effective and can crosslink protein fibres, it is an irritant, mutagenic in certain bacterial and animal species and has been classified as a probable human carcinogen (Petersen, 1987; U.S. Environmental Protection Agency, 1987; Monticello et al., 1989). Consequently, due to possible health risks crosslinking of soybean fibre following spinning has been carried out with reagents that do not contain formaldehyde and are still cost effective.

Acetylation is reported to improve the properties of regenerated protein fibres. Atwood (1940) showed that the water resistance of casein fibre was enhanced by acetylation. In addition, Evans et al. (1947) investigated the effect of acetylation on various properties of zein fibres and suggested good resistance to boiling in alkaline solutions following the modification. Acylation of soy proteins has been reported to change the conformation of glycinins and increase surface hydrophobicity (Barman et al., 1977). Huang (1994), reported an improvement in the wet-spun tensile properties of soybean fibres treated with acetic anhydride suggesting the reaction of ε-amino groups of protein by acetic anhydride, Figure 5.

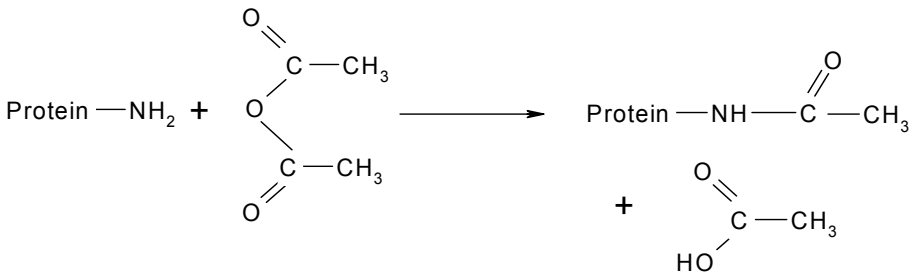


Fig. 5. Acylation of ε-Amino Groups of Proteins by Acetic Anhydride (Huang, 1994)

Enhanced fibre properties, in terms of tensile strength, were produced when glutaraldehyde was used as a crosslinking agent following spinning (Huang et al., 1995). Difunctional aldehydes can be used as cross-linking agents for protein molecules and modify the amino group of protein, forming a stable Schiff base. The chemical reaction of proteins and difunctional aldehydes such as glutaraldehyde is presented in Figure 6.

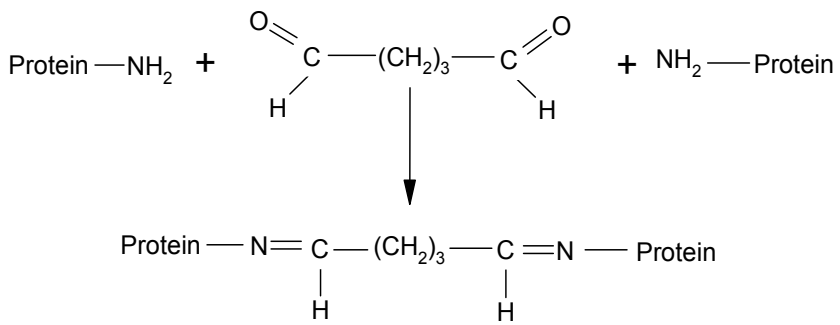


Fig. 6. Crosslinking Reaction of Glutaraldehyde with Proteins (Huang, 1994)

In agreement with the findings of Huang et al. (1995), Zhang et al. (2003) showed that the mechanical properties of PVA/soybean blend fibre can also be enhanced with cross-linking with glutaraldehyde.

3.7 Washing-drying-drawing

In the final stages of production the fibres are washed, dried and subsequently drawn in order to enhance tensile properties.

3.8 Physical properties of soybean fibre

Moncrieff (1975) summarised the properties of various regenerated protein fibres, Table 2. In all fibres, lack of wet strength can be observed with the tenacity of soybean fibres decreasing from 0.8 g/den to 0.25 g/den when wet. Generally, regenerated protein fibres have a tendency to be weak.

Properties	Casein	Groundnut	Zein	**Soybean**	Collagen
Tenacity (cN/tex)	8.0-9.7	6.2-8.0	10.6	**7.0**	nd
Elongation, % (dry)	60-70	40-60	25-35	**50.0**	nd
Specific gravity	1.3	1.31	1.25	**nd**	1.32
Moisture regain, %	14.0	15.0	10.0	**11**	nd
Effect of temperature	Softens on heating	No softening or melting on heating	Non-thermoplastic	**nd**	nd

where nd: not determined

Table 2. Properties of Regenerated Protein Fibres (Harris, 1954)

Cook (1984) suggested that the molecules in regenerated protein are not aligned with precision and regularity in order to form crystalline regions in the fibre. In addition, it was noted that molecules could not bond and align in order to achieve the tensile strength characteristics of fibres with crystalline structures.

3.9 Efforts to enhance the tensile properties of soybean fibres

A serious defect of soybean fibres is their poor tensile strength, especially in the wet state (Cook, 1984; Saltzberg, 1985). Vynias (2006) demonstrated that soybean fibre consists of high amounts of glutamic and aspartic acid. Both amino acids are highly polar amino acids that can attract water. Huang (1994) suggested that hydrogen bonding between water and protein is competing with the hydrogen bonding between molecules. In an attempt to explain the low wet tensile properties of soybean fibre, it was proposed that when soybean fibre is in an aqueous environment hydrogen bonding between molecules is replaced by hydrogen bonding between proteins and water. X-ray diffraction studies have shown little or no parallel orientation of soybean fibres (Harris, 1954).

A number of studies have been carried out to improve the tensile properties of soybean fibres. Kajita and Inoue (1940a) incorporated lecithin into the spinning solution in order to increase fibre strength. Sugar and tartaric acid were also utilised to reduce the brittleness of the fibres. Huppert (1943; 1944) treated fibres with nitrous acid whereas Croston et al. (1945) strain hardened fibres by stretching them up to 300%. The introduction of petroleum-based fibres during the 40's and 50's altered the commercial aspect of soybean fibres. However, lately, due to economic and environmental issues, soybean fibres are considered a competitive material in the textile industry. Zhang et al. (1999) used polyvinyl alcohol (PVA) in order to improve the drawability of soybean fibres. PVA is a synthetic polymer with high tensile strength and modulus (Sakurada, 1985), and has been used as reinforcement in keratin fibres (Katoh et al., 2004). Zhang et al. (2003) investigated the processing of blended

PVA/soybean fibre and demonstrated that the incorporation of PVA into the spinning process enhanced mechanical properties, Figure 7.

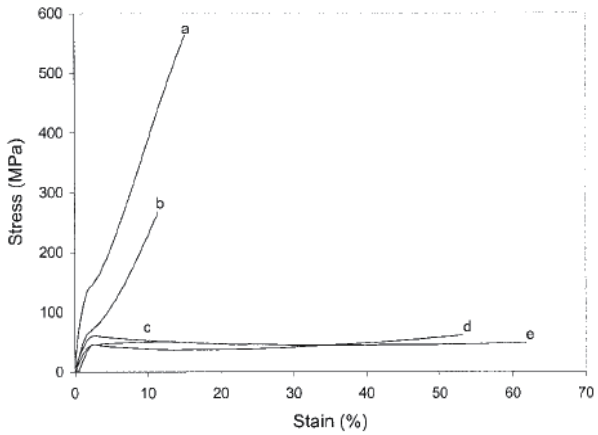


Fig. 7. Tensile stress–strain curves for PVA/soybean blend fibers at various compositions: (a) PVA; (b) PS9010; (c) PS4060; (d) PS2080; (e) pure soybean protein fiber [Zhang et al. 2003, *permission required*].

3.10 Aesthetic properties

The promotional marketing features of soybean fibre typically emphasised by the manufacturer (Huakang Ltd., 2005) are:

i. natural lustre and smooth surface;
ii. good physical and dyeing properties;
iii. breathability and comfort; and
iv. fine appearance with excellent drape.

3.11 Differences between soybean, silk and wool fibre

Since the amino acid composition of soybean fibre is significantly different from wool or silk, it was thought that a comparison of soybean wool and silk fibre would be informative and interesting. The amino acid compositions of soybean wool and silk fibre indicate that differences do not only occur in the macroscopic view (soybean-globular, wool/silk-fibrous) but also in the molecular scale. Acidic amino acids (glutamic and aspartic acid) are in much higher amounts than in wool, with the predominant amino acid being glutamic acid. However, sulphur-containing amino acids such as cystine are lower than in wool, indicating less cross-linkages through disulphide groups (Stevens, 1990).

4. Instrumentation and characterization of fabrics

4.1 Surface characterization of fabrics

Scanning Electron Microscopy (SEM)

The morphology of untreated and modified soybean fibers was examined by SEM and was carried out on a Hitachi S-300N instrument. An SEM instrument is presented in Figure 8.

Fig. 8. A Hitachi 3700N SEM instrument (www.hht-eu.com)

4.2 X-ray photoelectron spectroscopy (XPS)

XPS measurements were carried out with a Kratos Axis Ultra XPS instrument, Figure 9. The samples were analyzed with a monochromatic AlKa radiation source (1486.6eV) operating at base pressure of 3 $\times 10^{-9}$torr. Wide survey spectra were recorded at a pass energy of 100 eV to determine the surface chemical compositions. High-resolution spectra were obtained at a pass energy of 20 eV to determine the chemical state of phosphorus. All surface analyses were obtained in triplicate in order to ensure reproducibility and confirm uniformity of treatment.

Fig. 9. Kratos Axis Ultra XPS instrument (http://xps.web.its.man.ac.uk)

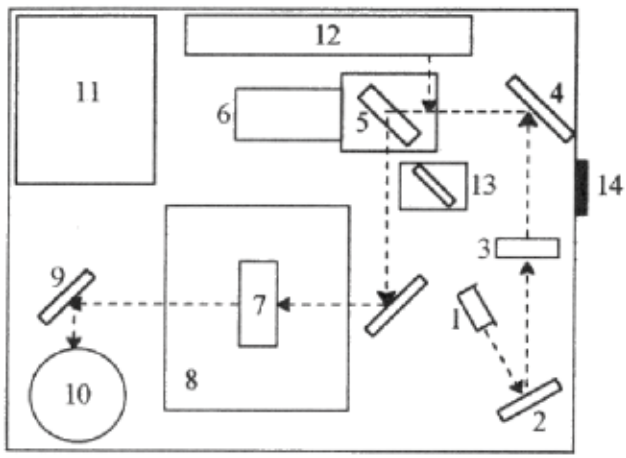
4.3 FT-IR Spectroscopy (Ferraro and Basile, 1978)

An FT-IR spectrometer basically consists of two parts: 1) an optical system which uses an interferometer and 2) a dedicated computer. The computer controls optical components,

collects and stores data, performs computations on data, and displays spectra. The real advantages of an FT-IR spectrometer result from the use of an interferometer rather than a grating or prism.

Compared to the conventional techniques FT-IR spectroscopy offers the following advantages (Frank, 1984): a) considerable saving of time; b) a better signal to noise ratio; c) high wave number precision; d) averaging of spectra produces better quality final spectrum. The schematic illustration of the Nicolet Magna-IR 750 spectrometer used in this study is shown in Figure 10.

The radiation from the infrared source is collimated and passed to the beam splitter of an interferometer. A part of the divided beam goes to the moving mirror whereas the other goes to the fixed mirror. The return beams recombine at the beam splitter resulting in interference. The reconstructed beam is then directed through the sample and focused onto the detector. The transmitted frequencies reach the detector in the form of an interferogram, which after Fourier transform, is converted to a normal spectrum.



1 Mid-infrared source; 2 Mirror; 3 Aperture; 4 Parabolic mirror; 5 Beamsplitter; 6 Interferometer; 7 Sample accessory; 8 Sample compartment; 9 Detector mirror; 10 Liquid nitrogen cooled detector; 11 Power supply; 12 HeNe Laser; 13 Slide mirror; 14 Beam port to microscope attachment.

Fig. 10. Diagram of Nicolet (750) FT-IR Spectrometer

4.4 Attenuated total (internal) reflectance (Ferraro and Basile, 1978; Hesse et al. 1997)

ATR Spectroscopy is a non-destructive and sensitive surface analysis technique. The substrate to be analysed is pressed into intimate optical contact with a prism (crystal) that is transparent over the range of IR wavelengths to be studied. The IR radiation enters the prism and is incident on the surfaces of the prism at angles greater than the critical angle, with multiple internal reflections occurring. By varying the angle of incidence, it is possible to vary the number of internal reflections within the ATR element. In practice, up to 100 internal reflections may be employed. The substrate surface is pressed against the ATR prism and at each reflection the electric vector of the IR radiation samples the surface in contact with the prism via the so-called 'evanescent' wave, which extends beyond the boundary of the prism. To obtain internal reflectance, the angle of incidence must exceed the 'critical' angle. This angle θ_c is a function of the real parts of the refractive indices of both the

sample and ATR prism, Equation 1, where n_2 is the refractive index of the sample and n_1 is the refractive index of the prism. The evanescent wave decays exponentially with distance from the surface of the prism into the sample over a distance on the order of a few microns. Thallium bromide/iodide (KRS-5), zinc selenide or germanium, are typical crystal materials.

$$\theta_c = \sin^{-1}(n_2/n_1) \quad (1)$$

4.5 TGA measurements

TG measurements were performed on a TA Q100 instrument with a refrigerated cooling system (RCS), Figure 11. The thermal analyser was operated at a heating rate of 10°C/min and an environment of 100% N_2 gas flow with the gas rate being 50 mL min^{-1}. The start temperature of measurements was 35°C and 0.1 mg of sample was utilized. The residual amount of char remaining after heating was determined after heating to 700°C. The maximum degradation rate point (MDRP) for each sample was determined as the temperature at, which each differential DTG showed a peak.

Fig. 11. Thermogravimetric analyzer (TA Q100 instruments)

4.6 Limited Oxygen Index (L.O.I.)

The L.O.I. of soybean fabrics was measured according to BS 4599-2:1999 on a Stanton Redcroft FTA flammability test unit. L.O.I. values were calculated based on the following equation.

$$\text{L.O.I.}(\%) = \frac{[O_2]}{[O_2] + [N_2]} \quad (2)$$

4.7 Vertical flammability test

The burning behavior of treated soybean fabrics was also assessed with the BS 5438 vertical flammability test method. Fabrics of 180 × 650 mm length and width were mounted on a

suitable clamp and placed in a standard cabinet with a 2 mm/sec airflow. The bottom edge of the fabric was exposed to a standard flame for 1 sec and after the removal of the flame, the flame spread speed (mm/sec) was determined by the average of 10 measurements for both warp and weft directions.

4.8 Yellowness Index

The Yellowness Index, YID, of the soybean fabrics was determined with a Datacolor International Spectrophotometer under illuminant D65/10° observer conditions.

4.9 Tensile strength of fabrics

The soybean fabrics were conditioned for 24 h at 20°C and 65% R.H. before testing on an Instron 5564 testing system. Tensile strength of the untreated and Pyrovatex CP modified fabrics were determined as the average of 10 measurements in the warp direction using an Instron instrument, Figure 12.

4.10 Evaluation of crease recovery

The crease recovery was evaluated by measuring the dry crease recovery angle. All samples were conditioned at 20 ± 2°C and 65 ± 5% R.H. for at least 24 hours prior to testing. The crease recovery angle was determined in accordance with the BS EN 22313:1992. A press was utilised to apply a load of 10 Newton on an area of 15mm ×15mm of the folded specimen. The specimen were loaded for 5 minutes and transferred to the holder using a pair of tweezers. Reading of the crease recovery angle was obtained 5 minutes after the removal of the load, with the mean value calculated for the warp and the weft direction for both sides of the samples.

Fig. 12. Instron 5560 series instrument (http://instron.itrademarket.com)

5. Wet processing of soybean fabrics

5.1 Flame retardancy

An important factor of the quality performance of fabrics is the level of flame retardancy of fibres required to improved safety for the consumer. An inherent deficiency of soybean fibre

is its poor creasing performance. The flame retardancy of soybean fabrics can be imparted by utilising phosphorus and non-phosphorus reagents.

5.2 Classification of flame retardants

The non-phosphorus containing flame retardants can be categorized into three groups:

a. compounds with low decomposition temperature that produce a foam that creates a barrier between the flame and substrate such as boric acid and its sodium salts;
b. compounds that sublime or release non-flammable vapours such as carbonates, halides, hydrated salts and ammonium salts
c. compounds that cause dehydration and char promotion, such as sulphamic acid or ammonium sulphate.

The last group is of significant importance, since sulphamic acid and sulphamates have been known for many years as flame retardants for protein fibres and in particular wool (Aarons 1960).

5.3 Mechanisms of flame retardancy

The development of flame-retardant finishing systems have been followed by intensive investigation into the mechanisms of flame retardancy with several theories being proposed due to the complexity and probable multiplicity of these mechanisms. A number of physical and chemical mechanisms have been developed in order to provide an insight into the flame retardancy of textiles fibres with the following being the most important (Benisek, 1978)

i. physical mechanisms
   - glasslike coating
   - evolution of non-combustible gases
   - adsorption of heat

ii. chemical mechanisms
   - modification of thermal decomposition
   - reducing the production of combustible gases
   - increasing char formation

According to the ***first physical mechanism***, the flame retardant is decomposed by heat to form a glass-like coating that performs as a barrier between the fibre, the flame and the atmospheric oxygen. Consequently, the solid layer entraps the highly flammable tars of decomposition leading to a reduction in the combustion reactions. Inorganic compounds such as borax, boric acid and diammnoium hydrogen phosphate behave in this manner.

The ***second physical mechanism*** involves selected reagents that decompose at elevated temperatures yielding inert or low oxidisable gases. Flame retardancy is imparted by dilution of the flammable gases during combustion or by blanketing the material with an inert atmosphere. As a result, the oxidising atmosphere is either reduced or eliminated the fuel-to-air ratio is altered with higher temperatures required for ignition. Inorganic carbonates and halides, ammonium salts and hydrated salts belong to this category. Flame-retardants of the ***third physical mechanism*** can maintain the temperature of the fabric below the minimum combustion temperature. In addition they have to dissipate large amounts of energy from the flame front at a rate equal or greater than at which it is supplied.

Chemical mechanisms have been widely used to explain the role of flame-retardants. It was proposed that the flame retardant agent altered the degradation of the substrate, increased char formation and/or reduced the flammable gas production. In addition, the effectiveness

of volatile halogen derivatives resulted in the recognition of a radical-trap mechanism. Halogens such as chlorine or bromine trap radicals that are formed during oxidation such as H^+, OH^- and HO^-_2, Figure 13.

$$RBr + H^{\cdot} \longrightarrow HBr + R^{\cdot}$$

Fig. 13. Entrapment of radicals during oxidation by halogens

5.4 Sulphamic acid as flame retardant reagent for soybean fabrics

Sandoz (1955) patented a process whereby protein fibres could be treated with sulphamic acid providing a high degree of resistance to acid dyes. The mechanism of reaction was investigated and it was found that sulphation occurs on the free amino and hydroxyl groups of serine and threonine, whereas the sulphation with sulphuric acid occurs merely on the hydroxyl groups (Elliott et al., 1958).

In addition, wool treatment with sulphamic acid increased substantivity for basic dyes with improved wash and light fastness (Cameron and Pailthorpe, 1987). Lewin et al. (1975) have reported that wool sulphation with sulphamic acid can impart benefits in terms of flame resistance. Examination of Table 3 indicates the soybean fabric has a low L.O.I. value, suggesting it has relatively poor flammability properties in comparison to other proteinaceous fibers such as wool which typically has an L.O.I. value of 25–26% (Vynias, 2006).

SA treatment level, (% w/v)	Curing time (min)	L.O.I (% ± SD)	Yellowness Index	Strength loss (%)
untreated		18.8 ± 0.1	41.9	
10	0.5	23.2 ± 0.1	44.5	nd[a]
10	1	22.9 ± 0.3	46.6	nd[a]
10	2	22.5 ± 0.2	47.3	nd[a]
10	5	22.0 ± 0.1	48.9	nd[a]
20	0.5	24.8 ± 0.2	46.9	12.5 (21.6)[b]
20	1	24.7 ± 0.1	46.9	13.6 (22.7)[b]
20	2	24.6 ± 0.3	48.9	16.5 (26.1)[b]
20	5	23.1 ± 0.1	52.6	19.3 (28.9)[b]
30	0.5	22.9 ± 0.1	47.1	nd[c]
30	1	22.6 ± 0.1	48.2	nd[c]
30	2	21.9 ± 0.2	49.4	nd[c]
30	5	21.7 ± 0.1	53.8	nd[c]

[a] 150°C, cure temperature, no urea included in pad formulation
[b] Strength loss of fabric cured at 170°C
[c] Not determined

Table 3. Effect of Sulphamic Acid (SA) Application Concentration on the L.O.I., Yellowness Index, and Strength of Treated Soybean Fabric

A 20% w/v sulfamic acid treatment level appears, however, appears to offer the most effective application level in terms of increasing the L.O.I. value, but that increased yellowing and strength loss is also associated with increasing the sulfamic acid application levels and the curing temperatures and time. The nature of this associated fiber degradation

must be related to the combination of elevated temperature and acidity progressively damaging the fiber and hence reducing the beneficial effect of the sulfamic acid treatments. Cameron and Pailthorpe (1987) similarly reported that the reaction of sulfamic acid with wool also resulted in an increase in fiber yellowness. Therefore, the need for short thermal exposure is apparent and SEM examination of the 30s treated fibers indicated little topographical change occurs in this period. Flame resistant finishing of wool with sulfamic acid in the presence of urea has been reported to increase weight gains, improve flame retardant performance, and enhance dissolution of the sulfamic acid in water.

Vynias and Carr (2006), similar to wool, found that application of 20% (w/v) urea with the sulphamic acid also increased the fibre modification/weight gain, increased the L.O.I. values, and lowered the increase in fabric yellowness. All these effects were achieved by probably increasing the uniformity of reaction throughout the fibre by increased accessibility. Elliot (1958) investigated the mechanism of the reaction and found that sulphation occurs at the free amino and hydroxyl groups in the wool. Vynias and Carr (2006) found with Fourier Transform Infrared (FTIR) analysis that the sulphamic acid treated soybean fibres also indicated that the hydroxyls were sulphated with an increase in the peak intensity observed at 1000 cm^{-1}, which has previously been assigned to sulfate esters (Carr and Lewis., 1993). While the incorporation of urea into the formulation increased the L.O.I. values, the effect of increasing the cure treatment time was still to reduce the L.O.I. value (Table 4). This behavior was also reflected in the BS 5438: 1989 vertical flammability test where the although the fiber modification initially reduced the flame spread speed, with increasing treatment time the associated degradation of the fibre resulted in the flame spread performance again deteriorating. Obviously in any treatment for soybean fibres the acceptable balance between serviceability and fibre damage has to be established commercially.

Urea Concentration	Curing time	Weight gain	Yellowness Index	L.O.I.	Flame spread speed
(% w/v)	(min)	(%)	-	(% ± SD)	(mm/sec)
-	-	-	41.9	18.8 ± 0.1	-
0	0.5	7.9	46.9	24.8 ± 0.2	3.0
0	1	8.4	46.9	24.7 ± 0.1	5.0
0	2	9.1	48.9	24.6 ± 0.3	7.0
0	5	9.3	52.6	23.1 ± 0.1	10.0
20	0.5	10.9	45.8	25.6 ± 0.1	2.0
20	1	11.6	46.7	25.5 ± 0.2	5.0
20	2	12.5	46.8	25.3 ± 0.1	6.0
20	5	13.2	48.9	24.1 ± 0.3	10.0

Table 4. Effect of Urea Concentration on the Weight Gain, L.O.I., Yellowness Indices, and Flame Spread Speed of Soybean Fabric Treated with 20% w/v Sulphamic Acid

The TG curves of sulphamic acid/urea modified soybean showed a similar behaviour to those of sulphamic acid modified soybean, however there is a shift of the onset point to a higher temperature of approximately 190^{o}C for all samples examined. In addition, the inclusion of urea, at comparable curing times, enhanced the char formation, Figure 15. It was observed that the addition of urea in the finishing bath enhanced the flame retardancy of all sulphamic acid treated soybean fabrics. However, low durability to laundering was

observed upon washing. A cationic agent (Matexil FC-ER) that was applied subsequent to the finishing treatment was found to be beneficial in terms of durability to laundering. Even though, in our work, sulphamic acid treatment of soybean fabric imparted improved flame-retardant properties, the requirement in textile industry for a cost effective finishing treatment without aftertreatment is still evident.

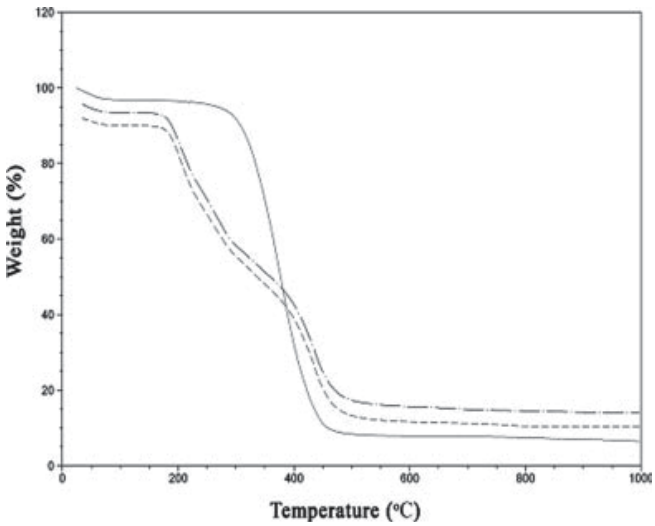


Fig. 14. TG curves of untreated and sulfamic acid treated soybean fabric. (—, untreated fabric, – –, sulfamic acid modified fabric, – ●–, urea/sulfamic acid modified fabric) (Vynias and Carr, 2006)

5.5 Pyrovatex CP as flame retardant reagent for soybean fabrics

Phosphorus-containing reagents may provide an alternative process to this prospect. It is well known that phosphorylation with phosphoric acids and derivatives results in hydrolytically unstable ester bonds in flame-retardant finishing. Therefore, to overcome this negative aspect, Jones and Noone (1962) prepared more stable flame retardants based on phosphoric acid esters. Although Tesoro et al. demonstrated that phosphonoacetamide derivatives of cellulose impart acceptable flame retardancy, it has been shown that the most commercially successful phosphonates are the N-methylol diakyl phosphonopropionamides. Pyrovatex CP (Ciba) has been successfully used as a flame-retardant reagent for textile fibers, such as cotton (Hebeish et al., 1994/Price et al., 1997), nylon (Yang et al., 1992), and lyocell (Hall et al., 1998). It is a phosphoruscontainingflame-retardant (N-methylol diakyl phosphonopropionamide) with the structure shown in Figure 15. Kapura (1994) found using NMR and HPLC analysis that Pyrovatex CP does not consist of an individual compound but of several species based on dimethoxyphosphorylpropionamide.

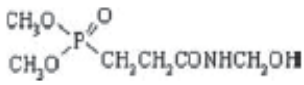


Fig. 15. Structure of Pyrovatex CP

The effect of Pyrovatex CP on the flame retardancy of soybean has been recently studied (Vynias, 2010). The results indicated, Table 5, a slight increase in the L.O.I. values with increasing concentrations of Pyrovatex CP. Similar to soybean, little benefit was observed in terms of flame retardancy for cotton fabrics treated with Pyrovatex CP alone (Nakanishi et al., 1999) suggesting that additives were required. Tesoro et al. (1969) have demonstrated the synergistic effect of nitrogen on phosphorus compounds, resulting in enhanced flame retardancy, with the application of a melamine formaldehyde compound in a phosphonate flame-retardant finishing. Therefore, an etherified methylolated melamine reagent (Lyofix MLF), was incorporated into the pad formulation with the expectation to enhance flame retardancy (Vynias, 2010).

Pyrovatex CP	Lyofix MLF	L.O.I	Flame spread speed	Tensile strength	Yellowness
(% w/v)	(% w/v)	(% ± SD)	(mm/sec)	(kN/m ± SD)	Index
untreated	-	18.8 ± 0.1	17	17.6 ± 0.1	42.1
0	3	19.3 ± 0.5	-.	13.6 ± 0.8	44.3
0	6	19.4 ± 0.3	16	15.0 ± 0.6	45.2
10	0	20.5 ± 0.4	10	14.5 ± 1.1	43.2
10	3	23.4 ± 0.5	6	16.2 ± 0.7	46.2
10	6	23.7 ± 0.3	5	16.8 ± 1.1	47.4
20	0	21.1 ± 0.3	9	14.4 ± 0.5	43.5
20	3	24.3 ± 0.4	5	14.9 ± 0.5	48.9
20	6	24.6 ± 0.2	3	16.1 ± 0.6	50.2
30	0	21.0 ± 0.1	9	13.9 ± 0.7	43.7
30	3	23.1 ± 0.3	9	15.1 ± 0.8	50.3
30	6	23.5 ± 0.1	8	16.3 ± 0.5	52.1

Table 5. Effect of Pyrovatex CP and Lyofix MLF on the L.O.I, Flame Spread Speed, Tensile Strength, and Yellowness Indices of Treated Soybean Fabrics

The inclusion of Lyofix MLF at 3% w/v application level enhanced flame retardancy compared to the Pyrovatex CP treatment alone, Table 5. This finding is not unexpected as melamine resins have been reported to be the most effective reagents for P-N synergism for cotton (Wu et al., 2003 /Wu et al., 2006). However, at higher concentration of Lyofix MLF (6% w/v), only a marginal improvement in the L.O.I. value was observed with a negligible beneficial effect on the flame-retardant properties. The vertical flammability test data support these findings where, although, the fibre modification with Pyrovatex CP initially reduced the flame spread speed, with increasing concentrations of Lyofix MLF an enhanced flame spread performance was achieved, presented in Table 5. However, at higher concentrations of Lyofix MLF an increase in yellowness was observed for all 10–30 % w/v concentrations of Pyrovatex CP examined, suggesting discolouration of the fabric. The surface of the fibre plays an important role in heat transfer within the material. Therefore, it was decided to investigate the effect of the chemical treatment on soybean surface utilizing SEM. SEM analysis of fibre indicated longitudinal striations on the surface parallel to the axis, varying in length, and depth, Figure 16(a).

In our trials, prolonged curing time (5 min) altered the structural characteristics of the fibre, Figure 16(b). Soybean became flatter with cracks appearing at the edges of the fibre, an indication of damage.

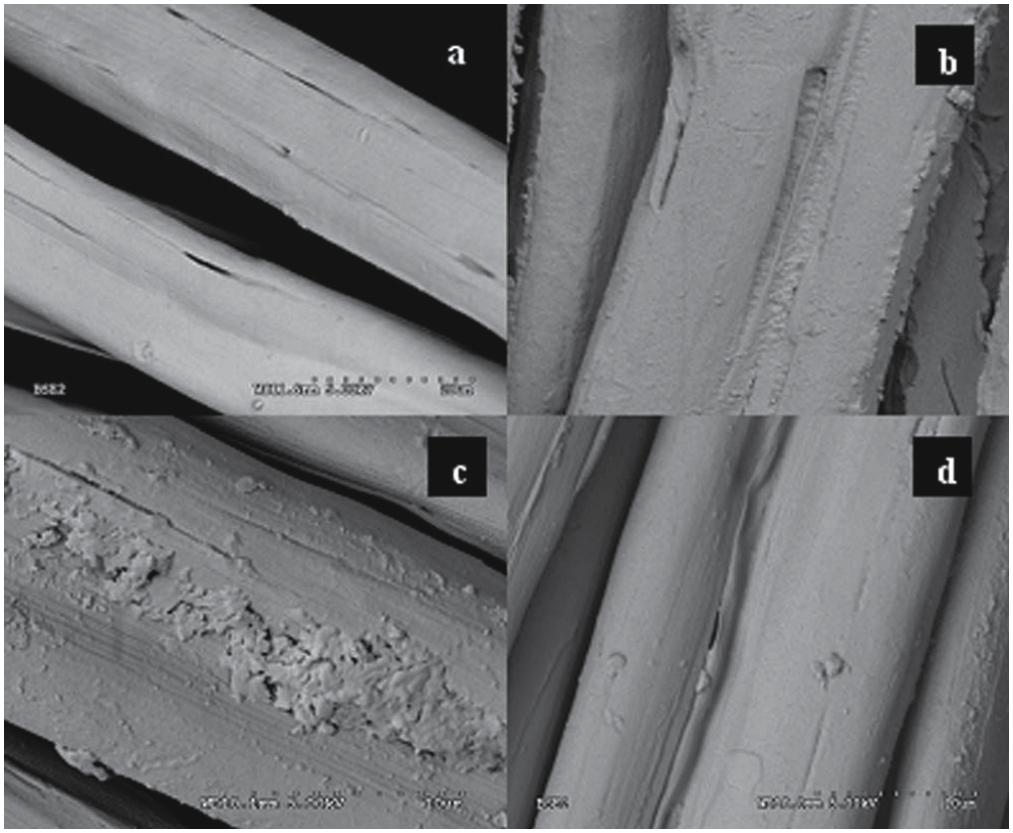


Fig. 16. SEM micrographs of (a) untreated soybean (b) cured at 150°C for 5min, (c) 20% w/v Pyrovatex CP, (d) 20% w/v Pyrovatex CP and 6% w/v Lyofix MLF.

Increasing concentrations of Lyofix MLF in the finishing bath improved the tensile properties of the treated soybean due to the rigidity caused by the melamine resin. Nevertheless, in all trials the tensile strength of the modified fabrics was still lower compared to that of untreated soybean.

5.6 XPS analysis

XPS was used to monitor the deposition of Pyrovatex CP at the fibre surface and to identify changes on the atomic composition following the inclusion of the melamine resin. Untreated soybean, Table 6, does not contain phosphorus. However, examination of the P(2p) spectrum of Pyrovatex CP modified soybean indicated the presence of phosphorus species located at 135eV, which may be attributed to the P^{+5} form of the phosphonopropionamide derivative, (Beamson and Briggs, 1992), Figure 17. Increasing the concentration of Pyrovatex CP resulted in an increase in the surface phosphorus, Table 6. Nevertheless, the incorporation of Lyofix MLF into the flame-retardant system, altered the surface atomic composition. An apparent increase in the N/P ratio was observed at all concentration levels of Pyrovatex CP examined. Raising the concentration of Lyofix MLF led to an increase in the surface nitrogen with the intensity of the N(1s) spectrum becoming higher with increasing concentration of Lyofix MLF, Figure 18.

Flame-retardant finishing with Pyrovatex CP/Lyofix MLF system was found to be more beneficial with regard to flame retardancy compared to Pyrovatex CP alone. However, in textiles, it is of great importance that the finishing treatment is durable to laundering. Therefore, the effect of washing on the flame retardancy performance was investigated. Successive washing cycles reduced the L.O.I. values for all treatments studied. However, after three washing cycles, the L.O.I. values for Pyrovatex CP were significantly reduced to the same value to that of untreated fabric, Table 7.

Pyrovatex CP (% w/v)	Lyofix MLF (% w/v)	C	O	N	S	P	C/N	N/P
unteated	-	74.7	19.8	3.2	0.3	-	23.3	-
20	0	71.6	22.9	5.1	0.2	0.5	14.0	10.2
x1	0	71.9	23.5	4.0	0.2	0.4	18.0	10.0
x3	0	72.4	23.7	3.3	0.2	0.4	21.9	8.3
20	3	68.8	23.7	7.2	0.2	0.5	9.6	14.4
x1	3	68.6	24.7	6.3	0.2	0.4	10.9	15.8
x3	3	69.6	24.9	4.9	0.2	0.3	14.2	16.3
20	6	68.7	22.9	7.9	0.3	0.5	8.7	15.8
x1	6	68.3	23.5	7.6	0.2	0.4	9.0	19.0
x3	6	68.6	23.9	6.9	0.2	0.3	9.9	23.0

Table 6. Effect of Pyrovatex CP and Lyofix MLF on the C/N and N/P Surface Atomic Ratios of Treated Soybean Fabrics

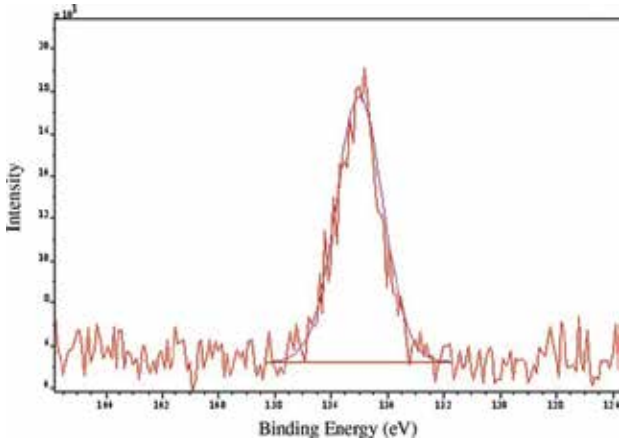


Fig. 17. P(2p) XP spectrum of 20% w/v Pyrovatex CP treated soybean, cured at 150°C for 30 sec.

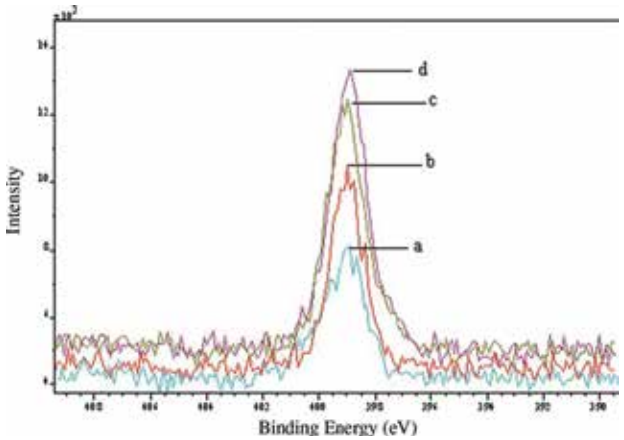


Fig. 18. N(1s) XP spectrum of (a) untreated soybean, (b) 20% w/v Pyrovatex CP modified soybean, (c) 20% w/v Pyrovatex CP, 3% w/v Lyofix MLF, (d) 20% w/v Pyrovatex CP, 6% w/v Lyofix MLF.

Pyrovatex CP	Lyofix MLF	L.O.I	Flame spread speed	Yellowness
(% w/v)	(% w/v)	(% ± SD)	(mm/sec)	Index
untreated		18.8 ± 0.1	17	41.9
20	0	21.1 ± 0.3	9	43.5
x1	0	19.1 ± 0.2	11	43.3
x3	0	18.8 ± 0.3	16	43.1
20	3	24.3 ± 0.4	5	48.9
x1	3	23.7 ± 0.1	7	48.7
x3	3	23.0 ± 0.3	10	48.5
20	6	24.6 ± 0.2	3	50.2
x1	6	24.1 ± 0.3	5	50.0
x3	6	23.8 ± 0.1	5	49.8

Table 7. Effect of Laundering on the L.O.I., Flame Spread Speed and Yellowness Indices of Treated Soybean Fabrics

The flame spread speed increased, from 9 mm/sec to 16 mm/sec, resulting in a fabric that had low protection against the flame. In contrast to Pyrovatex CP treatment, the Pyrovatex CP/Lyofix MLF treated sample provided higher L.O.I. values after three washing cycles, demonstrating wash-durable flame-retardant properties. It is likely that the bonding of Lyofix MLF with Pyrovatex CP and soybean fiber is more resistant to hydrolysis during laundering than that between Pyrovatex CP and soybean. The highest flame retardancy performance was obtained with 6% Lyofix MLF with the loss in L.O.I. values reaching 3.3%

after three washing cycles, Table 7. In addition, the flame spread was approximately 70% lower than that of untreated soybean.
The effect of laundering on the phosphorus and nitrogen content of treated cotton is well documented (Horrocks et al. 1992/Wu et al. 2007). In addition, it is known that Pyrovatex CP is bound to cotton by its methylol group with the bonding being highly resistant to hydrolysis (Wu and Yang, 2006). However, to our knowledge, the effect of laundering on the fiber surface of flame-retardant fabrics has not been investigated. Therefore, the enhanced durability to laundering imparted in the Pyrovatex CP/ Lyofix MLF soybean fabric was investigated by the XPS technique to probe changes at the fiber surface following hydrolysis due to laundering. In the light of XPS analysis it was revealed that for the Pyrovatex CP modified soybean fabric, washing after one and three cycles reduced the N/P ratio at the fiber surface, Table 6.
In contrast, after three washing cycles, the N/P ratio for Pyrovatex CP/Lyofix MLF treatment increased with increasing concentrations of Lyofix MLF. Although surface phosphorus content (% atomic) was reduced in all treatments, it is evident that nitrogen was retained on the surface. Therefore, the benefits imparted by Pyrovatex CP/ Lyofix MLF system compared to the Pyrovatex CP system alone can be attributed to nitrogen ''binding'' on the surface, complexing with phosphorus and delivering associated enhanced flame retardancy.

5.7 Crease recovery

Finishing techniques using physical or chemical approaches have been extensively used in textile materials with regard to improve functional properties. One of the most important functional properties is crease resistance with the consumer demanding garments manufactured to high specifications and crease-free appearance. Although synthetic fabrics demonstrate excellent crease resistance, in the case of natural fibres an inherent problem is the ease of creasing and poor crease shedding. Therefore a number of chemical treatments have been developed to overcome this drawback. In this section the mechanisms of crease resistance and a brief review of formaldehyde and non-formaldehyde based finishing treatments are discussed.

5.8 Mechanisms of crease recovery

Crease-resist agents react with the hydroxyl groups of cellulose in the presence of heat and catalysts to form covalent crosslinks between adjacent cellulose chains (Welch, 2000). Therefore a three dimensional networked is created at the interior of the fibre that imparts increased resilience to the fabric. Any bending or deformation imparted to the fabric during garment use or laundering causes return of the fabric to the flat or creased configuration it had when the crosslinks were originally introduced. Marsh (1962), Figure 19, used a model in order to explain the fibre recovery from the deformations in the collapse state to impart dry crease recovery and in the swollen state to impart wet crease recovery.
Crosslinks between cellulosic chains in the diagram are represented by solid lines, while hydrogen bonds between chains are represented by dotted lines. The hydrogen bonding network remains intact in the collapsed state due to the crosslinking, delivering dry crease recovery. However, in the wet state, hydrogen bonding is disordered with covalent crosslinks between polymer chains imparting crease resistance.

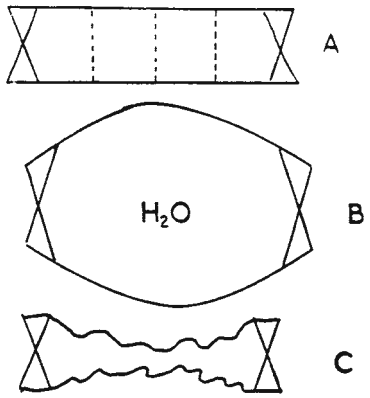


Fig. 19. Diagram representing the dry crease recovery of the collapsed fibre (Marsh, 1962)

5.9 Formaldehyde-free crease resistant finishing treatments

Marsh (1962) showed that the crease resistance of cellulosic fabrics could be enhanced with thermosetting resins such as formaldehyde and urea-formaldehyde. Formaldehyde is considered as the most efficient crosllinking agent for cellulose and protein fibres (Welch, 2000). However, despite its low cost, it is an irritant and mutagenic in certain bacterial and animal species and is classified as possible human carcinogen (Petersen, 1987; U.S. Environmental Agency, 1987; Monticello et al. 1989).

Therefore, the need to develop formaledehyde-free durable press finishing agents has become evident. A formaldehyde-free crosllinking agent in order to impart crease recovery should fulfil the following criteria (Welch, 2000):

- Stable in hard water
- Colourless and non-volatile during crosslinking
- Suitable for high-speed continuous fabric processing
- Less irritating, odorous, toxic or mutagenic than formaldehyde
- Available and low cost

Although a number of different reagents have been used as crosslinking agents to cotton to impart crease recovery such as glyoxal (Welch and Peters, 1987) and glutaraldehyde (Frick and Harper, 1982), N-methylol agents are the most common DP finishing agents applied to cotton with 1,3-dimethylol-4,5-dihydroxyethyleneurea (DMDHEU) being the major formaldehyde-based DP reagent (Welch, 2000).

Petersen (1983; 1987) examined the mechanism of reaction of N-methylol and N-allkoxymethyl compounds with cellulose under acidic conditions and the relationship between the chemical constitution of these compounds and their reactivity towards cellulose. A number of substances could be classified with respect to their reactivity with cellulose and their hydrolytic stability following reaction with the fibre. Compounds such as methylol and alkoxymethyl derivatives of urea and of ethylene and propylene ureas are highly reactive but are moderately stable when reacted with the fibre. In contrast, low reactivity was demonstrated for 4,5-dihydroxy- and 4,5-dialkoxyethylene ureas and 4,6 dihydroxy- and 4,6-dialkoxy-5,5-dimethylpropylene ureas containing $–NH_2OH$ and $–N-CH_2OR$ groups. Nevertheless, the former substances are covalently bound to cellulose and show excellent resistance to hydrolysis.

5.10 Crease-resistant finishing of soybean with polycarboxylic acids

One alternative development aimed at overcoming the formaldehyde release problem has been based on polycarboxylic acids. Compounds that possess two or more carboxyl groups in each molecule are referred to as polycarboxylic acids. The former reagents can form crosslinks in cotton through esterification of hydroxyl groups of adjacent cellulosic chains. However, it has been demonstrated that tricarboxylic acids are more beneficial with regard to crease recovery than dicarboxylic acids. Among the new crosslinking agents being investigated polycarboxylic acids appear to be the most promising agents (Welch, 1992; Welch 1994). Rowland et al. (1967) were the first to provide evidence that carboxylic acids could impart crease recovery to cotton coupled with strength retention.

In addition, Welch (1988) demonstrated that 1,2,3,4-butanetetracarboxylic acid (BTCA) was the most effective crosslinking agent for cotton cellulose, imparting high crease recovery values and durability to laundering. However, the relatively high cost of BTCA and the high application temperatures it required has prevented its use in the textile industry on a commercial scale.

5.11 Effect of polycarboxylic acids on the crease recovery of soybean fabrics

Fixapret CP is a low concentration 1,3-dimethylol-4,5-dihydroxyethyleneurea (DMDHEU) resin that has been widely applied to cellulosic fibres in order to improve end-use performance (Heywood, 1995). DMDHEU resins are based on the reaction of glyoxal, urea and formaldehyde and reacts with cellulose by etherifying the hydroxyl groups in the amorphous phase. The concentration of Fixapret CP had a beneficial effect on crease recovery resulting in higher values of crease recovery angle, Table 8. However, at concentrations (>100g/l) Fixapret CP had an adverse effect on crease resistance coupled to a marginal increase in yellowness. Nevertheless, the crease recovery obtained was higher compared to the untreated soybean. After the treatment with Fixapret CP it was revealed that curing times (>2min) offered little benefits in terms of crease resistance. In addition, the fabric became stiffer with a discolouration of the substrate being evident.

The effectiveness of maleic acid as a cellulose crosslinking agent has been examined by Welch and Peters (1997). Maleic acid contains two carboxyl groups per molecule and provides in the presence of phosphoric acid and sodium hypophosphite moderate to fair durable press properties for cotton fabrics (Welch, 2000). In soybean fabric, crease resist finishing of soybean fabrics with maleic acid did not impart any benefits in crease resistance regardless of the concentration used or the presence of the persulphate free radical initiator. The crease performance of fabrics was worse than that of the unmodified soybean.

Maleic acid (MA) and Itaconic acid (IA) are cheap dicarboxylic monomers that can undergo an esterification reaction with cotton cellulose. At elevated temperatures the molecules lose water prior to the formation of anhydrides (Marvel and Shepherd, 1959; Traskmorrell et al., 1990). Choi (1992) investigated the effectiveness of MA/IA system as a non-formaldehyde durable press finishing for cotton. It was found that treatment of cotton with MA and IA can improve durable press rating and crease recovery angle. In addition, it was suggested that a copolymer system of maleic acid and itaconic acid can be more efficient than MA alone through the formation of 5 or 6 membered anhydride rings by the dehydration of carboxyl groups in the polymeric backbone. The persulphate initiates free radical polymerisation of the unsaturated acids in situ, so forming a polycarboxylic acid species.

The 1:1 mole ratio of MA and IA, Table 8, improved marginally the crease recovery of soybean fabrics. However, when citric acid was used as polycarboxylic acid, an improved

crease resistance was observed in the treated fabrics. The best crease recovery value was obtained with a 100g/l citric acid concentration for 30 seconds. The effect of increasing curing time was to reduce slightly crease resistance. It seems that longer exposure times damage the fibre, resulting in a stiffer and harsher fabric.

Reagent	Curing temperature	Time	Yellowness	Recovery
g/l	^{o}C	min	Index	Angle ($^{o}\theta$)$^{+}$
untreated	---	--	41.9	172
Fixapret CP[a]				
100	150	0.5	44.0	205
100	150	1	44.6	194
100	150	2	45.2	190
Maleic acid[b]				
50	150	0.5	43.9	170
50	150	1	44.0	165
50	150	2	44.1	160
Maleic-Itaconic acid[c]				
1 :1	150	0.5	43.5	183
1 :1	150	1	44.0	180
1 :1	150	2	44.4	175
Citric acid[d]				
100	150	0.5	44.7	227
100	150	1	46.0	217
100	150	2	48.1	210

[a]: 12 g/l $MgCl_2$ were incorporated into the finishing treatment
[b]: 20 g/l $K_2S_2O_8$ were incorporated into the finishing bath
[c]: 1:1 refers to the ratio of reagents in the pad formulation with a 20 g/l $K_2S_2O_8$ added to the treatment
[d]: 40g/l formic acid and 60g/l sodium hypophosphite were added in the formulation bath
[+]: the sum of the warp and weft of the treated fabrics

Table 8. Effect of different chemical treatments on the dry crease recovery and yellowness indices of soybean fabrics (Vynias, 2006)

5.12 Durability to laundering of citric acid treated soybean fabric

Citric acid was found to be the most beneficial among the polycarboxylic acids examined. In this study, the durability of citric acid treatment on the crease resistance performance of soybean was examined with the results presented in Table 9. It was showed that washing resulted in a decrease in the crease recovery angles. After three washing cycles the crease recovery angle for citric acid modified soybean was reduced from 227^{o} to 195^{o}, respectively. However, the crease recovery angle was still higher than the untreated. Nevertheless, further washing (x5 cycles) led to a crease resistance performance similar to that of the untreated.

Sample	Recovery Angle ($^o\theta$)	Yellowness Index
untreated	172	41.9
CA*	227	44.5
CA, x 1	210	44.4
CA, x 3	195	44.2
CA, x 5	175	44.0

*all samples were treated with 100g/l citric acid, 40g/l formic acid at 150^{o}C for 30 seconds
x: number of washing cycles

Table 9. Effect of laundering on the recovery angle and yellowness index of citric acid modified soybean fabrics

5.13 Fourier Transform Infrared (FT-IR) analysis of soybean fabric

FT-IR is a complementary technique able to probe the vibrational behaviour of molecules. FT-IR spectroscopy using Attenuated Total Reflectance (ATR) mode is now an established technique for the surface analysis of protein fibres (Sibilia, 1975). For example, in wool, the intermediate oxidation products of cystine can be quantitatively determined when FT-IR (ATR) technique is coupled with second derivative spectroscopic analysis (Schumacher-Hamedat, Fohles and Zahn, 1985; Bridge, Fell and Wardman, 1987; Carr and Lewis, 1993). With regard to crease resistance, FT-IR technique has been successfully used to study the ester crosslinking mechanism of cotton with polycarboxylic acids (Yang, 1991; Yang and Wang, 1996a; Yang and Wang, 1996b). Vynias (2006), used FT-IR to investigate qualitatively and quantitatively the extent and nature of the modification of soybean with citric acid and to examine the conformational changes occurring in soybean.

5.14 FT-IR analysis of untreated soybean fabric

The FT-IR (ATR) absorbance spectrum of untreated soybean is shown in Figue 20, and the main band assignments are listed in Table 10.

Frequency (cm^{-1})	Assignment
3267	N-H (s) secondary amides
	O-H (s) hydrogen bonded (broad)
2937	CH_2 (as)
2905	CH_3 (ss)
2855	CH_2 (ss)
1636	Amide I C=O (s), C-N (s), C-C-N (d)
1531	Amide II N-H (ib), C-N (s), C-C (s)
1434	CH_2 and CH_3, C-H (d)
1407	CH_3 C-H (d)
1236	Amide III N-H (ib), C-N (s)

Table 10. Infrared Band Assignments for Soybean Fabric (Harrick, 1967; Hesse, Meier and Zeeh, 1997)

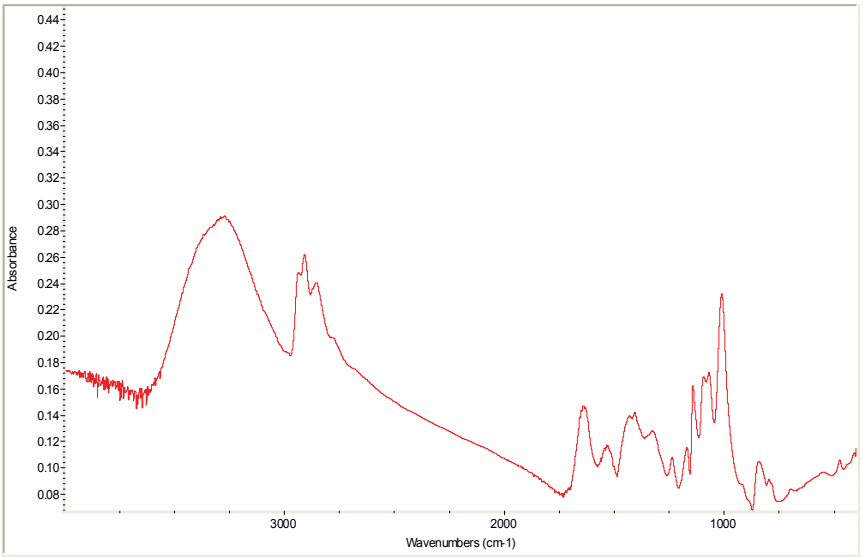


Fig. 20. FT-IR (ATR) Spectrum of Untreated Soybean Fabric

5.15 FT-IR analysis of citric acid treated soybean fabric

Surface analysis of soybean to a depth of 2.0-2.5 µm in the ester/carboxyl region between 1740 and 1710 cm^{-1} can be achieved with the ATR technique. Taking into consideration the "surface" modification of the citric acid treatment the ATR technique would certainly be useful in probing any changes on the surface due to the reagent. Using the second derivative, the most prominent change after citric acid modification was a well-defined peak appearing at 1720 cm^{-1} in contrast to that of untreated soybean, Figure 21. This is attributed

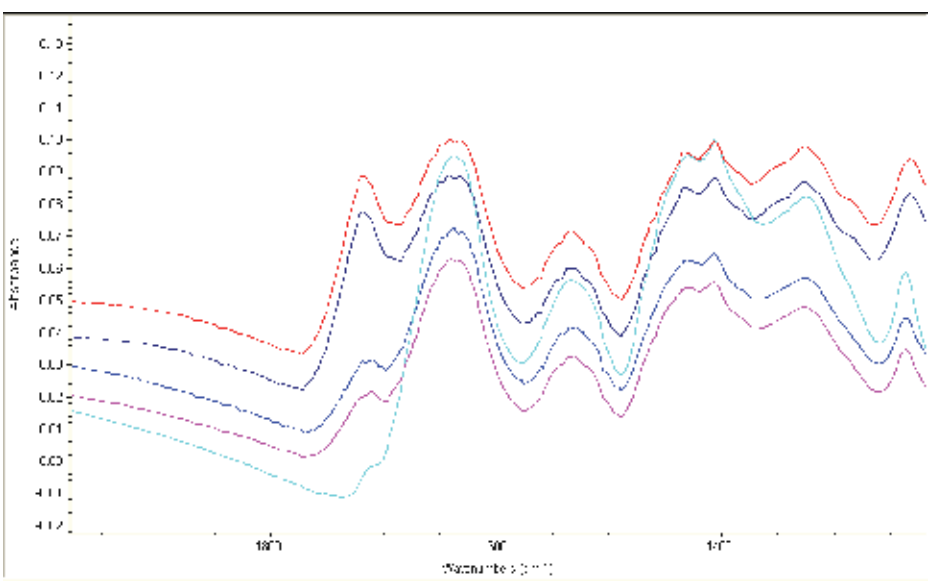
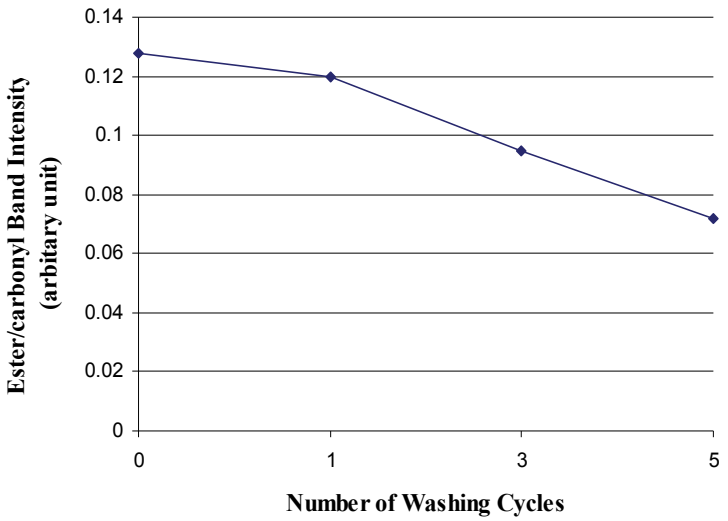


Fig. 21. Second Derivative FT-IR Absorbance Spectra of Citric Acid Treated Soybean

to the carbonyls of the carboxylic acid and the ester overlapping at 1720 cm^{-1} (Hesse et al., 1997). Yang (1991), made the same observation when he characterised ester crosslinkages in cotton with FT-IR Photoacoustic Spectroscopy. The ester/carbonyl signal at 1720 cm^{-1} increased with increasing citric acid concentration.

5.16 FT-IR analysis of washed citric acid treated soybean fabric

The wash durability of citric acid modification on the soybean fabric was studied with the FT-IR (ATR) technique. After the first washing cycle, a decrease in the carboxyl carbonyl band was observed (0.128 to 0.120) indicating the relatively poor stability of the finish under perborate-based washing conditions. Further washing to three cycles reduced the carboxyl carbonyl signal to 0.095, Figure 22. However, the signal obtained was still higher compared to the untreated. Nevertheless, after five cycles, the surface carboxyl groups attributed to citric acid were significantly reduced to a value similar to the untreated. Therefore, it can be suggested that, for the citric acid modified soybean fabrics wash durability can be imparted up to three washing cycles. This suggestion correlates well with the crease resistance performance of washed fabrics evaluated by the crease recovery angle presented previously.



a: untreated; b: 20g/l citric acid; c: 50 g/l citric acid;
d: 100 g/l citric acid; e: 150 g/l citric acid

Fig. 22. Effect of Laundering on the Second Derivative Band Intensity at Ester/Carbonyl Signal (1070cm^{-1}) of Citric Acid Treated Soybean Fabrics

6. Future work

The performance properties of the soybean fibre still need to be improved in order to achieve wider commercial usage. As the fibre is based on protein and PVA there are

opportunities for fibre modification to improve the performance, however there are associated limitations due to the chemical reactivity and sensitivity of the soybean proteins and protein aggregates.

An area of future research would therefore be to further characterize the distribution of the components within the bulk of the fibre and at the surface, and their chemical accessibility. Though this detailed information selective treatments could be developed to improve basic performance and maintain properties during wet and dry processing. Directly related to this improvement could be a better understanding of the nature of the fibre fracturing process during bleaching and its associated impact on handle and wear. In addition, it would be important to examine the flat abrasion behaviour and the fibre fibrillation performance.

In future studies the effect of softeners, elastomeric silicones and alternative polymeric crosslinkers should be examined with a view to improving crease resistance, while still maintaining fabric strength and handle. Both sulphamic acid/urea and Pyrovatex CP treatments imparted improved flame retardancy to the soybean fabric. However, the processing still needs to be improved, particularly in terms of fabric handle and wash durability. The role of alternative fixing agents, which could improve fastness as well as flame retardancy should be further examined. Similarly the range of flame retardant agents should be extended, including non-durable finishes. Underpinning all of these developments, a further investigation of the decomposition mechanism of the treated soybean could be carried out in order to potentially engineer more durable and cost-effective flame retardant finishes.

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Residue Analysis of Glyphosate and Aminomethylphosphonic Acid (AMPA) in Soybean Using Liquid Chromatography Coupled with Tandem Mass Spectrometry

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1. Introduction

Glyphosate (N-phosphonomethyl glycine) is one of the most widely used pesticide around the world. It is very well known that glyphosate compound rapidly degrades into aminomethylphosphonic acid (AMPA) metabolite (Fig. 1). Glyphosate is a herbicide with a broad spectrum of activity, very effective even on plant roots with little harmful effects on mammals. Its high efficacy, low toxicity and affordable price, when compared with other pesticides, justify the wide utilization in several crops. Due to the low toxicity of glyphosate, the maximum residues levels (MRLs) established around the world are generally greater than the limits for other pesticides. For example, the Codex Alimentarius Commission and the Environmental Protection Agency/USA established the MRL at 20 mg kg^{-1} for glyphosate in soybean and the National Health Surveillance Agency (ANVISA) in Brazil set the MRL at 10 mg kg^{-1}.

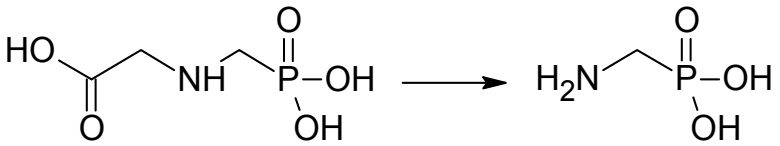

Fig. 1. Degradation of glyphosate (M.W. 169.07 g mol^{-1}) into its metabolite AMPA (M.W. 111.04 g mol^{-1}).

The glyphosate and AMPA's physical and chemical properties such as no volatility, high water solubility, low molecular weight and absence of chromophore groups, make them very difficult to analyze using gas or liquid chromatography techniques with traditional detectors without derivatization. On the opposite, glyphosate and AMPA may be analyzed

by mass or tandem mass spectrometry, especially by using electrospray ionization, because they are very polar and easily ionized on this technique. However, just a few methods using mass or tandem mass spectrometry coupled with liquid chromatography have been found in the literature for glyphosate and AMPA analysis. In most of published papers, pre and post-column derivatization procedures were employed to analyze the compound derivative by fluorescence detection (Sancho et. al. 1996; Hogendoon et. al. 1999). Vreeken and co-workers developed an analytical method to analyze glyphosate, AMPA and glufosinate in water samples using reversed phase liquid chromatography separation after pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) and detection by tandem mass spectrometry (Vreeken et. al. 1998). Bauer and co-workers detected glyphosate and AMPA in water samples using ion chromatography followed by electrospray with single quadrupole mass spectrometry detection. (Bauer et. al. 1999). The ion chromatography separation without derivatization was also used by Granby and co-workers after clean-up on reversed phase column to analyze glyphosate and AMPA through electrospray ionization/tandem mass spectrometric analysis (Granby et. al. 2003). Goodwin and co-workers studied the electrospray negative ion fragmentation pathways of glyphosate and AMPA using an ion-trap mass spectrometer (Goodwin et. al. 2003). Some other methods found in the literature to analyze glyphosate and AMPA include: capillary electrophoresis (Cikalo et. al. 1996), ion chromatography with conductivity detection (Zhu, et. al. 1999), ion chromatography with fluorescence detection (Patsias et. al. 2001), gas chromatography (Hudzin et. al. 2002), immunoassays (González-Martinez et. al. 2005), nuclear magnetic resonance (Deen et. al. 2002) and integrated pulse amperometry (Ji-Ye et. al. 2001).

In this work, we investigated the potential of reversed phase liquid chromatography coupled with electrospray tandem mass spectrometry (LC-ESI/MS/MS) for the quantification of glyphosate and AMPA in soybean spiked samples. In this approach, the compounds were analyzed without derivatization procedures using calibration curves prepared in the matrix, after a simple sample extraction and liquid-liquid partition followed by protein precipitation step with organic solvent to minimize the complexity of the sample. The mobile phase composition and the matrix effects were also investigated to validate the method using a high flow gradient program in a total run time of four minutes for each analyte.

2. Experimental

2.1 Chemicals and standards

Glyphosate and AMPA standards were obtained from Sigma-Aldrich (Steinheim, Germany). Methanol and dichloromethane HPLC-grade solvents were purchased from J. T. Baker (Deventer, The Netherlands) and ammonium carbonate P.A. was obtained from Merck (Darmstadt, Germany). Purified water was obtained on EASYpure RF System from Barnstead (Dubuque, IA, USA).

The stock solutions of glyphosate and AMPA at 500 mg L^{-1} were prepared by dissolution of the standards in water. The solutions were maintained at 4 ^{o}C away from light and stocked in polypropylene tubes to avoid adsorption to glass. The calibration standards were prepared in water or blank soybean extracts for the calibration curves.

2.2 High-performance liquid chromatography

An Agilent 1100 series (Agilent Technologies, Waldbronn, Germany) system was operated at flow rate of 1.2 mL min^{-1} without split using a Zorbax Eclipse® RDB C8 (Agilent Technologies,

Waldbronn, Germany) analytical column of 150 mm x 4.6 mm (i.d.) and 5 µm of particle size, maintained at 25 °C during the experiments. The injected volume into the LC-MS/MS system was 40 µL. The binary mobile phase consisted of an aqueous solution with 1.5 mmol L^{-1} of ammonium carbonate (phase A) and a solution of methanol/water (95/5%, v/v) with 1.5 mmol L^{-1} of ammonium carbonate (phase B). The mobile phase initial composition of 65% A and 35% B (v/v) was held for 0.5 min, followed by linear ramping of 90% of B over 1.75 min. After the gradient ramping, the mobile phase returned to the initial composition and was held for 1.75 min. The total chromatographic run time was 4.0 minutes.

2.3 Tandem mass spectrometry

The experiments were performed using a triple quadrupole mass spectrometer API 4000™ (AB Sciex, Concord, Canada) operated in TurboIonSpray® (electrospray) ionization. The capillary voltage was maintained at 5500 and –4500 V for positive and negative ion modes, respectively, and the temperature of the turbo heaters was set at 750 °C. Ultrapure air was used as nebulizer gas (GS1) at 55 p.s.i. and heater gas (GS2) at 55 p.s.i. Nitrogen was used as Curtain Gas™ at 12 p.s.i. in the interface and collision gas (CAD Gas™) at 10 arbitrary units in the LINAC® collision cell (Q2). The declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) parameters used in the multiple reaction monitoring (MRM) mode are presented in Table 1. The dwell time was set at 150 ms for each MRM transition, using a pause time of 5 ms. The data were acquired and processed using the Analyst software version 1.4.1 (AB Sciex).

Analyte	Transition (*m/z*)	DP (V)	CE (V)	CXP (V)
Glyphosate	170>42	50	37	8
	170>60	50	25	10
	170>88	50	15	14
AMPA	110>63	-41	-26	-1
	110>79	-41	-38	-3
	110>81	-41	-18	-3

Table 1. Optimized MRM detection parameters.

2.4 Sample preparation

Organic soybean was purchased in a local market in São Paulo and used as blank samples in recovery experiments. The soybean sample was blended and extracted immediately or stored at –4 °C. A quantity of 2.0 g of blended sample was weighted in a 50 mL polypropylene tube and the extraction was carried out with 20.0 mL of water and 5.0 mL of dichloromethane during 60 min by mechanic agitation with a shaker. After the extraction, the sample was centrifuged during 15 min at 3000 rpm and an aliquot of 1.0 mL was transferred into a 15 mL polypropylene tube followed by addition of 1.0 mL of methanol for

protein precipitation. The tube was mixed by vortex during 1 min and centrifuged at 3000 rpm during 15 min. Prior to analysis, the samples were 50 times-fold diluted by mixing 20 µL of the aqueous extract into a 2 mL chromatographic vial with 980 µL of water. An injection volume of 40 µL of sample was injected into the LC-MS/MS system.

2.5 Validation of the method

The method was validated according to the European Community guidelines (2002/657/EC). Four experiment batches of spiked and blank samples were analyzed to evaluate the performance of the method. These batches were analyzed in four consecutive days with a total of 83 soybean samples. The spiked levels were 0.2 and 0.6 mg kg^{-1} (n = 18), 0.4 mg kg^{-1} (n = 28) and, 0.8 and 2.0 mg kg^{-1} (n = 3). Thirteen blank samples were also analyzed. The results for glyphosate and AMPA recoveries are shown in the Tables 2 and 3, respectively. In the Table 4, the results from the four validation days were pooled to evaluate the interday recovery and precision.

Batch/Day	Spike Level (mg kg^{-1})	Recovery (%)	SD (%)	RSD (%)	*n*
1	0.2	88.1	11.5	13.0	6
	0.4	93.0	8.3	9.0	6
	0.6	94.9	5.7	6.0	6
	0.8	102.4	-	-	1
	2.0	91.6	-	-	1
2	0.2	76.9	9.9	12.9	6
	0.4	89.2	8.1	9.0	6
	0.6	85.8	6.1	7.1	6
	0.8	89.7	-	-	1
	2.0	89.9	-	-	1
3	0.2	73.9	7.9	10.8	6
	0.4	80.9	7.4	9.2	6
	0.6	85.1	4.5	5.3	6
	0.8	84.2	-	-	1
	2.0	85.7	-	-	1
4	0.4	109.1	6.6	6.1	10

Table 2. Recovery results of glyphosate in spiked soybean samples.

Batch/Day	Spike Level (mg kg^{-1})	Recovery (%)	SD (%)	RSD (%)	*N*
1	0.2	93.1	7.7	8.3	6
	0.4	95.5	5.5	5.8	6
	0.6	87.9	7.5	8.5	6
	0.8	78.3	-	-	1
	2.0	91.1	-	-	1
2	0.2	87.7	12.8	14.5	6
	0.4	85.8	8.0	9.3	6
	0.6	95.6	8.3	8.7	6
	0.8	85.7	-	-	1
	2.0	91.8	-	-	1
3	0.2	88.2	4.9	5.5	6
	0.4	73.9	4.2	5.7	6
	0.6	81.3	6.1	7.5	6
	0.8	93.1	-	-	1
	2.0	89.9	-	-	1
4	0.4	94.0	5.6	6.0	10

Table 3. Recovery results of AMPA in spiked soybean samples.

3. Results and discussion

Glyphosate and AMPA might be ionized in both positive and negative ionization modes, by addition or loss of proton, respectively, with electrospray technique. However, for quantitation purposes using the MRM experiments, glyphosate and AMPA's higher sensitivity were achieved at positive and negative ion modes, respectively. For this reason, the samples were injected twice into specific methods for each analyte. The Figure 2 shows the positive and negative MS/MS scans acquired for glyphosate and AMPA, respectively. The MRM methods were optimized using three MRM transitions for each analyte, where, one transition was employed for quantification purposes and the two other transitions for confirmation. For quantification, the transitions of *m/z* 170>88 and 110>79 were used for glyphosate and AMPA, respectively. The selected confirmation transitions for glyphosate were *m/z* 170>60 and 170>42, and the transitions of *m/z* 110>63 and 110>81 were used for AMPA.

Due to the strong interactions of glyphosate and AMPA with the free silanol groups from stationary phase, the better buffers used in the chromatographic elution were the ones at high pH. The Figure 3 shows the final chromatographic condition obtained by using ammonium carbonate as mobile phase buffer.

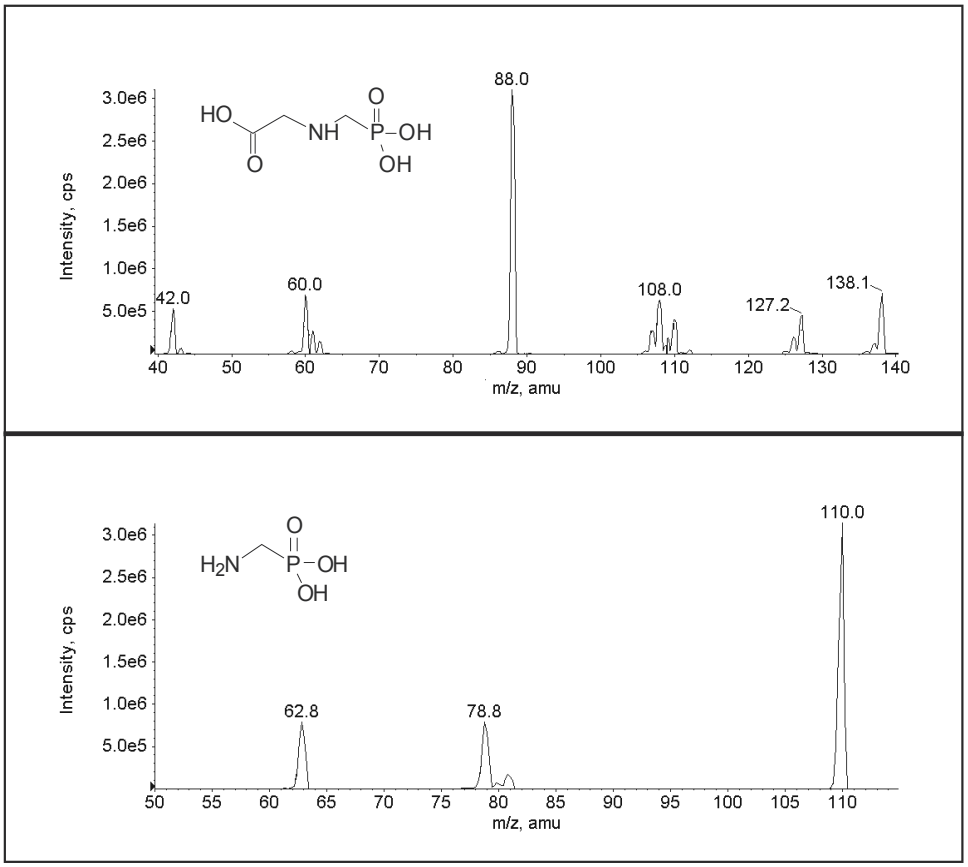

Fig. 2. Positive and negative product ion scan spectra (MS/MS) obtained for glyphosate (on the top) and AMPA (on the bottom).

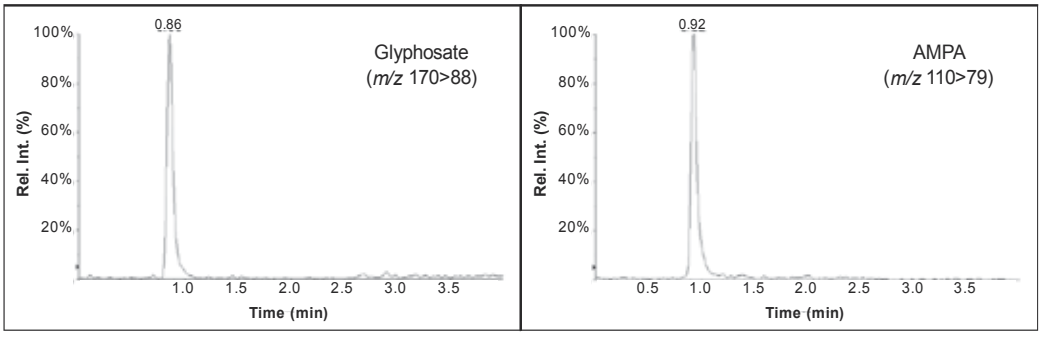

Fig. 3. Chromatographic condition used for glyphosate and AMPA determination.

The sensitivity of the analytes was better when high chromatographic flow was applied. Even when the chromatographic start to run with 65% of aqueous phase, no loss of ionization efficiency was observed and this solvent composition provided the best analytes peak shape. This better compromise obtained between sensitivity and consumed time of analysis was

reached producing column pressure smaller than 200 bar. Because of the low retention time of the analytes on the reversed phase some samples were also analyzed using direct flow injection analysis (FIA) into the tandem mass spectrometer without the C8 analytical column to verify a possible ionization signal's suppression or enhancement. The results depicted worse peak shapes and lower recoveries because the matrix signal suppression. Therefore the reversed phase C8 column used was important to remove some non-polar background compounds from the mobile phase and matrix and enhance the performance of the method.

3.1 Matrix effect

Three different solvents were applied to protein precipitation of the dirty extracts obtained from the soybean aqueous extraction: acetone, acetonitrile and methanol. The use of acetone and acetonitrile solvents decreased the recovery of the analytes probably because of the co-precipitation of the analytes with the soybean proteins. The best recoveries for glyphosate and AMPA were obtained using methanol as solvent, however, the application of protein precipitation was not enough to eliminate the matrix interference and a little signal suppression was observed in both analytes detection. The solid phase extraction and the liquid-liquid partition were also unsuccessfully applied to samples clean-up but the ion suppression was not completely avoided. The Figure 2 shows a blank sample extracted ion chromatograms obtained using the post-column infusion method for matrix effects evaluation.

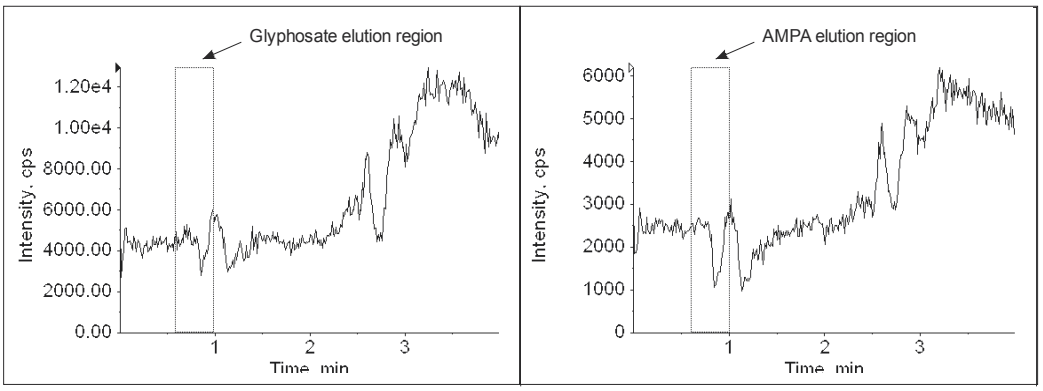

Fig. 4. MRM signals obtained for post-column infusion of glyphosate (left) and AMPA (right). The shaded portions shows the signal suppression for the compounds at their retention times.

Although glyphosate and AMPA detection have been showed noticeable signal suppression effects even after liquid-liquid partition and protein precipitation as showed in Figure 4, the large dilution volume applied to the samples was effective to minimize this effect in the electrospray ion source. In order to solve the matrix effect interference, the analytes were quantified using matrix-matched calibration standards. The standards of the compounds were prepared by dilution of the analytes in soybean blank sample extracts at the concentrations of 0.1, 0.2, 0.5, 1.0, 1.5 and 3.0 $\mu g\ L^{-1}$.

3.2 Recovery and performance

Although glyphosate and AMPA with their low molecular weight and high polarity provide less sensitivity than other pesticide compounds analyzed in LC-MS/MS, low limits of

detection were reached. As glyphosate and AMPA have high solubility in water (up to 12 mg L^{-1}), the aqueous extraction was applied successfully to extract these analytes from the soybean spiked samples. The liquid-liquid partition with dichloromethane and the protein precipitation were used to purify the sample extracts yielding the recovery results presented in the Tables 2, 3 and 4.

Compound	Spike Level (mg kg^{-1})	Recovery (%)	SD (%)	RSD (%)	*n*
Glyphosate	0.2	79.6	9.8	12.2	18
	0.4	109.1	6.6	6.1	28
	0.6	88.6	5.4	6.1	18
	0.8	92.1	-	-	3
	2.0	89.0	-	-	3
AMPA	0.2	89.7	8.5	9.4	18
	0.4	94.0	5.6	6.0	28
	0.6	88.3	7.3	8.2	18
	0.8	85.7	-	-	3
	2.0	90.9	-	-	3

Table 4. Pooled recovery results for glyphosate and AMPA in spiked soybean samples.

Considering the pooled results presented in the Table 4, the recoveries data for 0.2, 0.4 and 0.6 mg kg^{-1} were between 79.6 and 109.1% with relative standard deviation (RSD) smaller than 12.2% for both analytes. According to the European Community Directive 2002/657/EC, for analyte recovery level higher than 0.01 mg kg^{-1}, the recovery percent level should be between 80 to 110%.

The decision limit (CCα) and detection capability (CCβ) established by the EC/657/2002 were, respectively, 0.03 and 0.05 mg kg^{-1} for glyphosate and 0.03 and 0.06 mg kg^{-1} for AMPA. These limits were calculated using the following equations based on the interday data set results presented in the Table 4:

$$CC\alpha = \frac{[(y\_intersec + 2.33\ stdev\ y\_intersec) - y\_intersec]}{slope} \quad (1)$$

$$CC\beta = \frac{[(y\_intersec + 2.33\ stdev\ y\_intersec + 1.64\ stdev\ y\_intersec) - y\_intersec]}{slope} \quad (2)$$

The recovered samples curves showed correlation coefficients higher than 0,9885 for both analytes at the studied spike levels.

Considering the low retention time of the compounds in reversed phase columns, three *m/z* transitions were monitored to enhance the reliability of the data and avoid false positives. The Figure 5 shows the zoomed in extracted ion chromatograms for glyphosate and AMPA at three MRM transitions in spiked sample of 0.4 mg kg^{-1}.

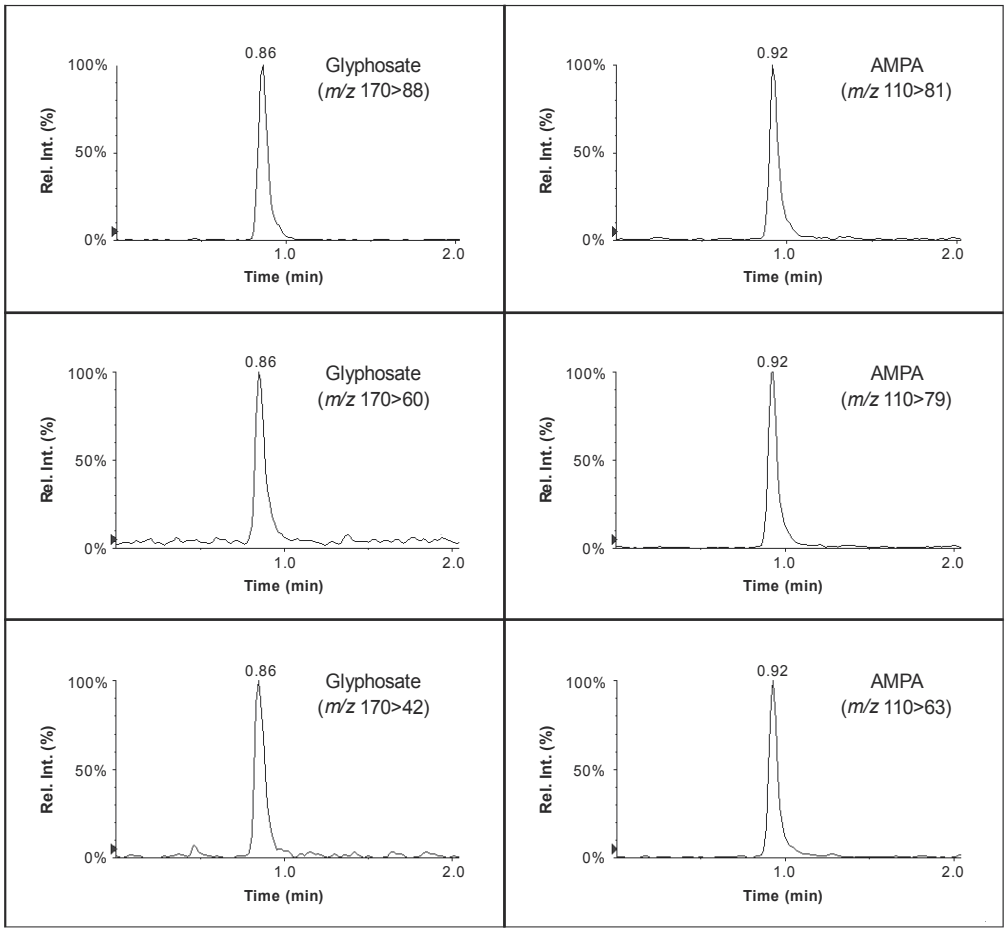

Fig. 5. Extracted ion chromatograms for glyphosate (left) and AMPA (right) for the three MRM transitions used in the method.

The calibration was performed using external matrix-matched calibration solutions at concentration range from 0.1 to 3.0 µg L^{-1}. For glyphosate (*m/z* 170>88) and AMPA (*m/z* 110>79), the correlation coefficients (r^2) calculated by weighted regression ($1/x$) were 0.9991 and 0.9998, respectively, and the slopes of the calibrations performed on the matrix-matched standards were smaller than those obtained on the solvent standards.

4. Conclusion

An alternative methodology for the determination of glyphosate and AMPA residues in soybean with simpler sample preparation, fast chromatographic analysis and sensitive detection was presented. The method's sensitivity and specificity is suitable to meet the limit of residues established in most of the countries as a reliable strategy to evaluate the presence of glyphosate and its major metabolite in soybean samples. The method was developed and validated to spiked organic soybean samples according to the 2002/657/EC.

The methodology presented in this work improves and simplifies the throughput in glyphosate and AMPA routine analysis, especially by exploring the power of liquid chromatography for quantitation analysis with the sensitivity and confidence of electrospray tandem mass spectrometry. These presented results suggest that this technique may be used with the same purpose for analyzing these compounds residues in other matrices such as: corn and cotton, due to the wide application of the glyphosate on the several crops.

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28

Estimation of Fatty Acid Composition in Soybean Powder by Examining Near Infrared Spectroscopic Patterns

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1. Introduction

Soybean (*Glycine max* L.) is one of the major oilseed crops. The fatty acid composition in oilseeds is an important consideration for breeding programs (Daun, 1998). Proper levels of unsaturated fatty acid moieties in vegetable oils have been recognized as good nutritional characteristics for health. One goal of breeding projects is the alteration of the fatty acid composition, for example, the reduction of the level of linoleic acid moiety. Then, its analytical method is of an important concern. However, the conventional method for the fatty acid composition is very time-consuming and much labor intensive. It includes milling samples, oil extraction from it, chemical-reaction, and gas-chromatographic (GC) or high performance liquid-chromatographic analysis. A simple and rapid method for the determination of the fatty acid composition is necessary for screening soybean varieties on the demands in breeding projects, where useful individuals are selected from a lot of samples to be tested. Furthermore, in this case, an individual seed is sometimes to be selected from many samples to be tested, and because of this situation, an analysis on a very small amount of sample is also demanded.

Near infrared (NIR) spectroscopy has been recognized as one of the most powerful analytical tools (Osborne et al., 1993a; Panford, 1988; Osborne, 2007), and is widely used for the simple and rapid analyses of various agricultural and food products. NIR spectroscopy has also been used for the analysis of soybean constituents (Sato et al., 1994b; 2008). The fatty acid composition is an important index of fat or oil quality from nutritional point of view. Therefore, if NIR spectroscopy can be used for the analysis of the fatty acid composition, it would become an even more useful technique for soybean analysis. NIR analyses for the fatty acid composition have been carried out using a statistical method, i.e., a multivariate analysis (Pazdermik et al., 1997; Roberts et al., 2006; Patil et al., 2010). However, in this chapter, the author took another approach. The feasibility of NIR spectroscopy for estimating the fatty acid composition in soybean oil was examined according to the spectral patterns or assignments. Since the NIR spectral patterns were reflected by the fatty acid compositions, the absorption bands of cis-unsaturation and the carbon chain length of the fatty acid moieties in oil appear in the NIR wavelength region, especially around the 1600-1800 nm region (Sato et al., 1990, 1991, 1994a, 1995ab, 1998,

2002abc, 2003; Karen et al., 1994). In this region, the absorption band around 1720 nm is shifted to the shorter wavelength when the unsaturation in the fatty acid moieties increases, and it is shifted to the longer wavelength when the carbon chain length increases. Then, the author examine this wavelength range to determine the fatty acid composition in oil.

2. Materials and methods

2.1 Samples

The thirty-one samples were used in this study, and they were cultivated, harvested, and collected from various areas of Japan in 1999. They were Ohsodenomai, Kariyutaka, Kitamusume, Toyokomachi, Toyohomare, Toyomusume, Hayahikari, Yuhzuru, Otofuke'ohsodefuri, Suzumaru, Ohsuzu, Miyagishirome, Ryuhou, Suzuyutaka, Enrei, Ohtsuru, Tamahomare, Akishirome, Tachinagaha, Tama'urara, Nattoshouryu, Hatayutaka, Nakasen'nari, Hou'en, Ayakogane, Tamamasari, Sayanami, Suzukogane, Fukuyutaka, Murayutaka, and Ichihime (Sato et al., 2002a). The other twenty-nine samples were also used for this study, and they were cultivated, harvested, and collected from various areas of Kyushu, the southern part of Japan, in 2000. Their varieties were Fukuyutaka, Suzuotome, Kiyomidori, L-star, and Sachiyutaka (Sato et al., 2002b).

These samples were sent to our laboratory and were milled by a Cyclone Sample Mill (Udy Corporation, Colorado, USA) through a screen (φ=1.0mm). Plural whole seeds, a single seed, and soybean powder were measured. Peeled single soybean seeds, a piece of a particle cut from a soybean seed with a knife, and a small amount of powder obtained by a drill (a rotary blade: φ=1.0mm) were also prepared from the seeds of the samples collected in 2000. There were no damages on its hypocotyl. All the samples were packed in a sealed polyethylene bag (Unipack; Seisan Nihon Co., Tokyo, Japan), and stored at 5°C until being analyzed.

2.2 Chemical measurements

The fatty acid composition was determined by the conventional GC method: The oil was extracted from about 2 g of sample powder with diethyl ether by the Soxhlet method using an instrument: Soxtec System HT 1043 Extraction Unit (Tecator, Sweden). The extracted oils were then used for the GC analysis after trans-esterification with sodium methoxide/methanol solution according to the conventional method (Chikuni et al., 1989).

The gas chromatograph was equipped with an FID detector (GC-17A, Shimadzu Co., Kyoto, Japan) (Sato et al., 2002a). Each sample was analyzed twice. The average was used for the following analyses. As for the calculation of the fatty acid composition, the sum of the percentages of the major five fatty acid moieties, palmitic (C16:0) + stearic (C18:0) + oleic (C18:1) + linoleic (C18:2) + linolenic (C18:3), was converted to 95.0%.

2.3 Near infrared spectroscopic analysis

An InfraAlyzer 500 (Bran + Luebbe Gmbh, Norderstedt, Germany) was used to measure the NIR reflectance spectra in the wavelength range of 1100 to 2500 nm at 2-nm intervals on each sample a few times. The average spectra were calculated, and the analysis was carried out. The sample presentation methods for the NIR measurements were depending upon the sample types as follows:

1. whole plural soybean seeds (about 60g) in a whole grain cell (Photo 1-a)) on a rotating drawer.

2. a whole single soybean seed, a husked single one, or a crashed particle in a single grain cup (center hole diameter = 24 mm, Bran+ Luebbe Co.) (Photo 1-b)).
3. a small amount of soybean powder in a modified single grain cup (Photo 1-c)) : a small amount of soybean powder (about 8 mg) was taken into a modified single-grain cup (center hole diameter = 20 mm, Bran+ Luebbe Co.) with a small spatula, and the cup was jiggled slightly until the surface of the flour was arranged to the same or the horizontal level in the center of the cup, then the NIR spectra were measured. This cup was developed for measuring NIR spectra of a small particle and was successfully used for the analysis of the fatty acid composition of a single rapeseed as previously reported (Sato et al., 1998). It has a smaller and deeper hole in it, and it has a sharper parabolic curve to the base than a normal one. This cup was also used for a small amount drilled soybean powder.
4. a standard amount of soybean powder (about 3g) in a standard cell (Photo 1-d)) .
5. extracted oil sandwiched between two slide glasses on a British cup (Photo 1-e)).

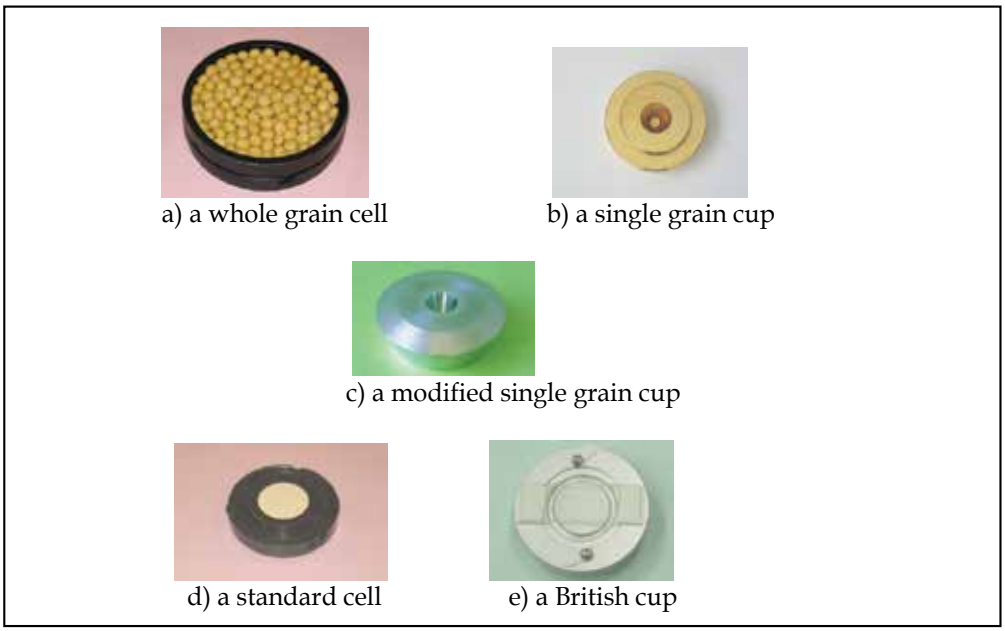

Photo 1. The sample presentation methods for the NIR measurements

2.4 Mathematical treatment on NIR spectral data for the calculation of the second derivative spectra and that of the standardization.

The NIR wavelength range from 1600 to 1800 nm includes the information concerning the carbon chain length and the unsaturation of the fatty acid moieties (Sato et al., 1990, 1991, 1994, 1995ab, 1998, 2002abc, 2003). However, in the original NIR spectra, the difference due to the fatty acid composition could not be detected, and the second derivative spectra were calculated. The default parameters for calculating the second derivative spectra were as follows: the size of the moving average was 24 nm; the size of the derivative segments was 24 nm; and the gap between the derivative segments was 30 nm. However, with this condition,

the differences of the spectral characteristics do not appear. In order to strengthen this information and to make their differences clearer, the following parameters for calculating the second derivative spectra were adopted: the size of the moving average was 4 nm; the size of the derivative segments was 12 nm; and the gap between the derivative segments was 12 nm. Then, since the intensity level of the obtained second derivative spectral values were different, these NIR spectral data were further standardized in order to make the comparison easier by making the spectral value at 1600 nm as 0.0 and the spectral value at the minimum around 1724 nm as -1.0 (Sato et al., 2002c) as in the following equation:

The corrected or standardized 2nd NIR spectral data at xxxx nm

= (-1)*(d2L(xxxx) - d2L(1600))/ (d2L(around 1724) - d2L(1600))

3. Results and discussion

3.1 A general NIR method

NIR spectra have information of constituents, and the information is extracted from the NIR data using a computer, i.e., the relationship between NIR spectral values and the contents of the constituents or the property values is calculated using the statistical techniques of the multivariate analysis by comparing their variations with a computer. On the other hand, as for a colorimetric method (Fig. 1-A)), the dilution series of some fixed quantity or concentration of the target substance is prepared as a standard in advance. Next, a target substance in the sample is extracted, refined, and is made in the chemically same condition as this dilution series. Then, they are compared at the same unit. The substances both in the dilution series and in the extract are compared with the colorimetry. Also in the NIR method, there is surely a similar procedure for moisture content analysis (Osoborne & Fearn, 1986). However, as for the general NIR method (Fig. 1-B)), the contents values and the spectral values are compared directly without any chemical treatments but with the mathematical conversions for emphasizing to make the relationship linear. The data are compared directly at the remained different unit of their own, or in the chemically different situation. The fluctuations of information both in the chemical data and in the NIR spectra are compared by a computer. The purpose of the NIR method is not only making the calibration equations (Fig. 2, Step I), but using them on the mother population to obtain individual numerical values (Fig. 2, Step II) for the decision-making (Fig. 2, Step III) such as the screening varieties in breeding projects, the quality control in the producing plant, the sample selection or rejection and so on. If you stop at the step I, you are suspected to only „make up the cover story". An NIR method is that this grand design of Fig. 2 is made work well. The NIR method is the tool for practical use. One's demand of the precision of the analysis depends upon one's purpose: sometimes precise, and sometimes rough. The relationship is one of the following things; a direct, an indirect, and a pseudo one. If you can take a risk judging from the SEP, RPD and so on, you can use any calibration equations you developed, sometimes not for an official use, but for your internal or private use. This might be the use in the unintended direction by a founder of NIR analysis. Genuine spectroscopists dislike this situation. However, you can use them with your self-responsibility. The statistics describes the events happened in the past. Then, if it grasps the principle, it can predict the events that happen in the future?! In general, when using an instrument, at least the zero-adjustment calibration is needed. As for the NIR method, the degree of the calibration

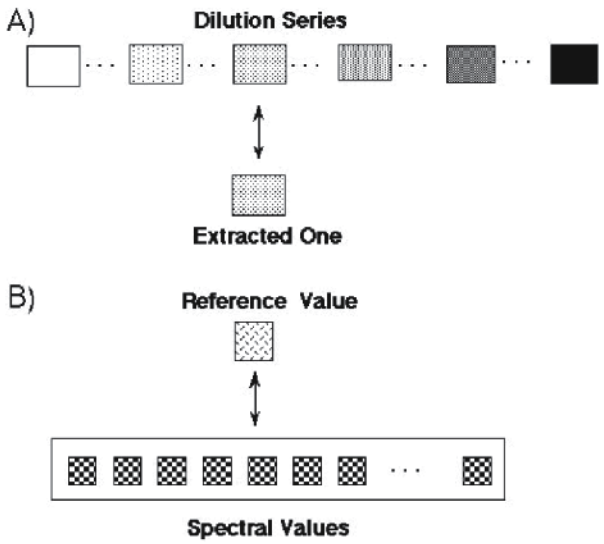

Fig. 1. Comparing A) colorimetric method vs. B) NIR method.

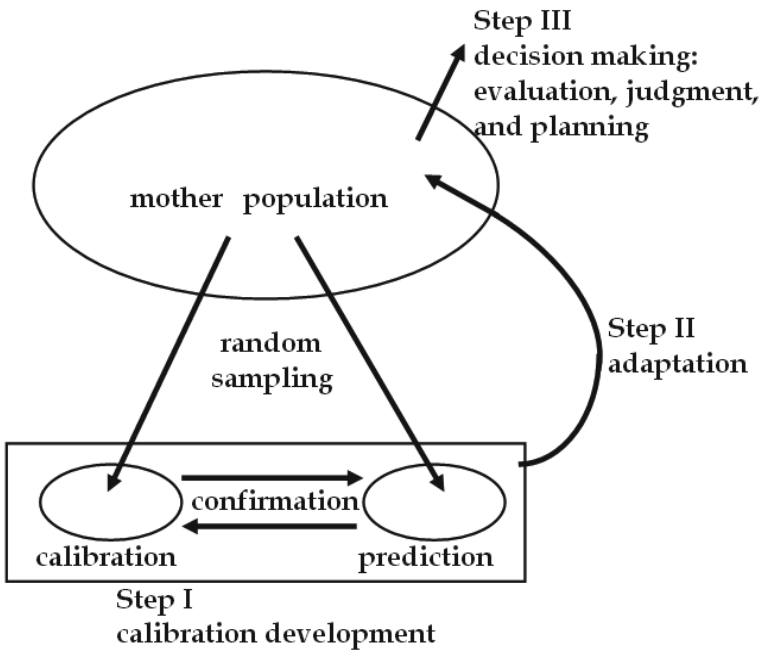

Fig. 2. The outline of the NIR method

becomes to the extreme extent. An NIR method is adjusted to the respective official method, and when the latter assumed to be stable and steady, the former demonstrates a very good performance. In NIR spectroscopy, multiplevariate analysis is mainly carried out for the analysis of fatty acid composition (Kovalenkoet al., 2006 ; Roberts et al., 2006). The author takes another approach of not using multivariate analysis but examining the NIR spectral patterns or the assignments as in the following.

3.2 Characteristics of NIR spectra

Firstly, the spectra were examined. Figure 3-a) and 3-b) show the original NIR spectra. As for powdered soybean(Fig. 3-b)), there are absorption bands due to moisture (around 1960 nm), oil (1600-1800, 2120-2170, and 2200-2400m), carbohydrate (around 2100 nm), and protein (around 2180nm). However, as for extracted oil, only the absorption bands due to oil were clear (1600-1800, 2120-2170, and 2200-2400m). On the other hand, as for plural whole seeds (Fig3-a)), oil and protein absorption bands are weak.

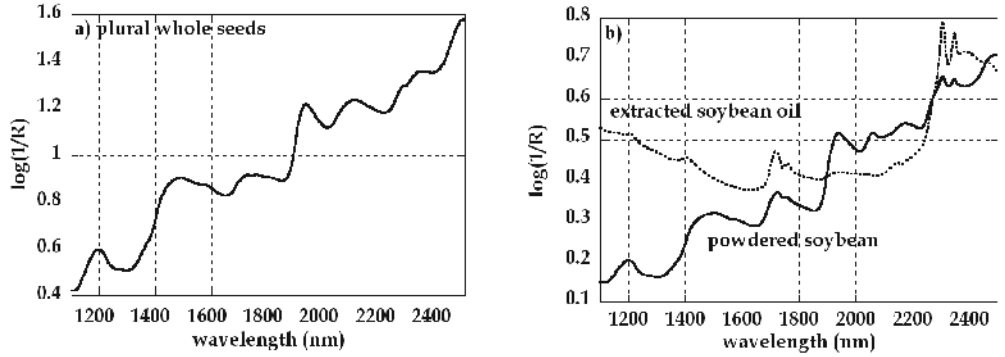

Fig. 3. The original NIR spectra of a) plural whole soybean seeds, and b) powdered soybean and extracted soybean oil.

A single seed, a peeled single seed, and a crashed peeled particle have similar spectral pattern as plural whole seeds, but small fluctuations. A small amount of powdered soybean has similar spectral pattern as a standard amount of soybean powder, but small fluctuations. In the following sections, discussion is proceded with soybean powder because of the clearer appearance in oil or fat absorbance region.

3.3 Examination of the conditions of calculating the second derivative spectra.

Figure 4 shows the default second derivative spectra of soybean powder in the standard cup. It was calculated with the default parameter condition. A part of the spectra concerned (1600 – 1800 nm) were also shown in Fig. 5. Their fatty acid compositions are described also in Fig. 5. No differences appeared. On the other hand, Figure 6 and 7 shows the second derivative spectra calculated with the other parameter condition. In this case, the differences were clearer. Their fatty acid compositions are described also in Fig. 7. They were characteristic especially in the ratio of oleic acid moiety (C18:1) and linoleic acid moiety (C18:2). Using the parameters described above, a clearer difference was obtained as shown in Fig. 7. Further, in order to make the spectral patterns clearer and to arrange the fluctuation level of the absorption to the same, the standardized second derivative NIR spectral values were calculated (Fig.8). As the percentage of the linoleic acid moiety increased, the absorption band around 1708 nm was stronger downward in the corrected second derivative NIR spectra, because the spectral phase was reversed in the second derivative spectra.

As for a small amount of soybean powder analysis, the clear NIR spectra of it were obtained using a modified single-grain cup, because the modified cup had been improved for a better collection of the reflection light from a small amount of sample due to the sharper parabolic curved surface than normal single seed cup. A single-grain cup itself has a very small artificial effect around 1400 nm, but it does not affect the absorption bands due to oil. As for a small amount analysis, its spectra have small fluctuations, but have similar characteristics. Further, standardization makes them clearer.

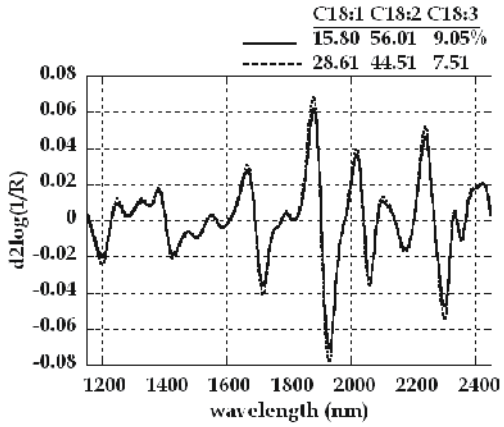

Fig. 4. The default second derivative NIR spectra of soybean powder in the standard cup.

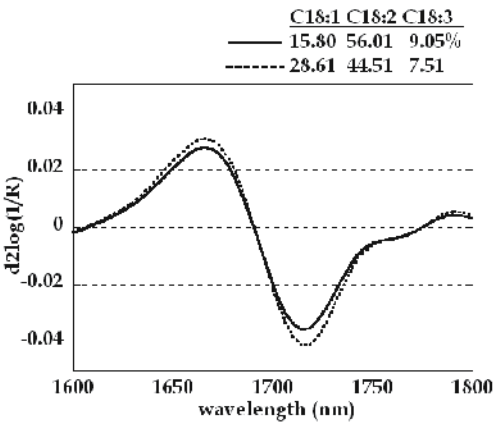

Fig. 5. A part of the wavelength region (1600-1800nm) of the default second derivative NIR spectra (Fig.4).

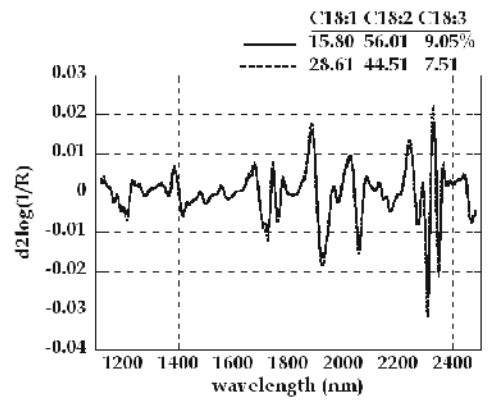

Fig. 6. The second derivative NIR spectra, calculated with the other parameter condition, of soybean powder in the standard cup.

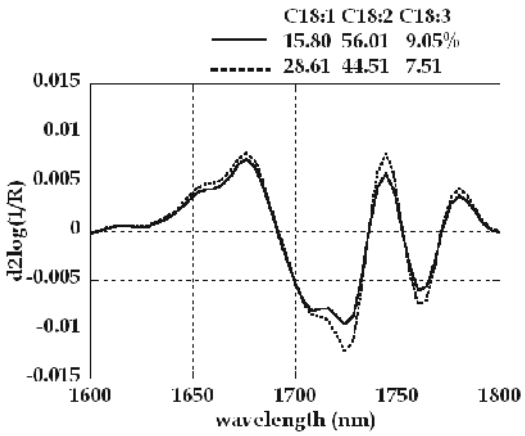

Fig. 7. A part of the wavelength region (1600-1800nm) of the other second derivative NIR spectra calculated with the other parameter condition (Fig.6).

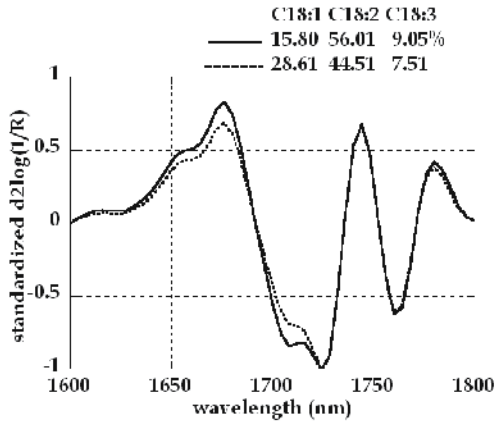

Fig. 8. The standardized second derivative NIR spectra of powdered soybean.

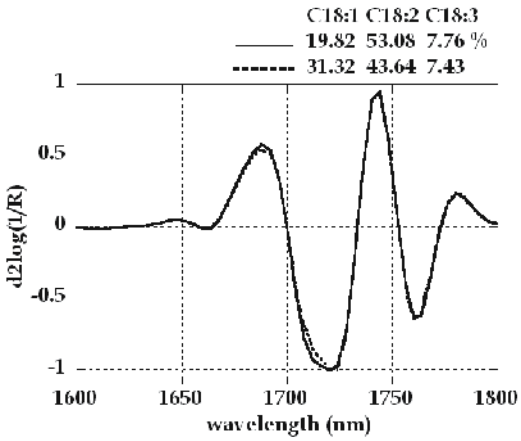

Fig. 9. The standardized second derivative NIR spectra of extracted oil.

As for the NIR spectra of extracted oil, Two varieties were adopted as examples for the explanation. Their fatty acid compositions are described in Fig. 9. They were characteristic especially in the ratio of oleic acid moiety (C18:1) and linoleic acid moiety (C18:2). Using the other parameters, a clearer difference was obtained as shown in Fig. 9. The spectral NIR pattern was different from that of soybean powder. However, as the percentage of linoleic acid moiety increased, the absorption band around 1708 nm was stronger downward in the second derivative NIR spectra because the spectral phase was reversed in the second derivative spectra.

3.4 Relationship between the NIR spectral patterns and the fatty acid composition.

The standardized spectral value at 1708nm is adopted as an index of linoleic acid moiety in oil for further discussion. As for soybean powder in the standard cup, which was harvested in 1999, the correlation between the percentage of the fatty acid moiety and the standardized reading at 1708 nm was shown in Fig. 10. Figure 10-a) shows the relationship of the percentages of linoleic acid moiety (C18:2) vs. the readings at 1708 nm, and the correlation coefficient was -0.803. The scattering plot shows: the stronger the standardized second derivative spectral value at 1708 nm downward, the higher the percentage of the linoleic acid moiety (C18:2) as mentioned above. The results showed that the rough estimation of the percentage of the linoleic acid moiety (C18:2) was possible. However, in this region, linolenic acid moiety (C18:3) has a little influence on the absorption band. There is a tendency for the linolenic acid (C18:3) to increase as the amount of linoleic acid (C18:2) increases. This is why a discrepancy from the regression line emerged when the linoleic acid (C18:2) moiety ratio increased as shown in Fig. 10-a). Then, the correlation of the percentages of linoleic (C18:2) + linolenic acid moiety (C18:3) vs. the readings at 1708 nm was examined (Fig. 10-b)). The correlation coefficient improved dramatically. The discrepancy from the regression line diminished. Incidentally, the correlation coefficient between the linolenic acid moiety (C18:3) vs. the readings at 1708 nm was -0.717, because its ratio and the variation were small. On the other hand, in Fig. 10-c), the correlation of the percentages of oleic acid moiety (C18:1) vs. the standardized readings at 1708 nm was shown. This shows the reverse correlation.

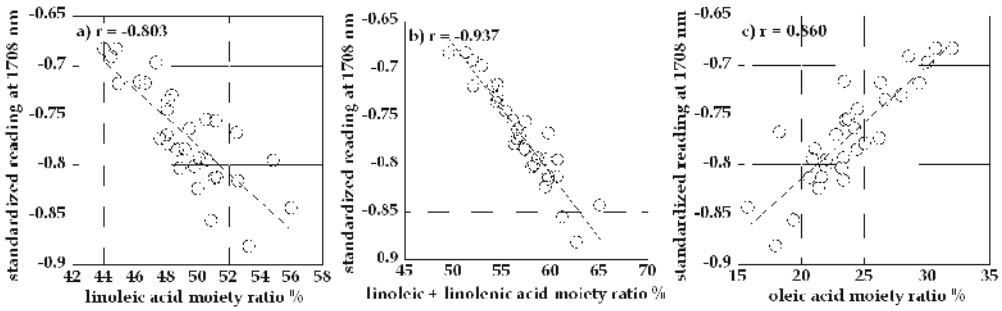

Fig. 10. The scattering plots between the standardized second derivative NIR spectral reading at 1708nm vs. a) linoleic acid moiety ratio, b) linoleic + linolenic acid moiety ratio, and c) oleic acid moiety ratio. (soybean powder from samples cultivated in 1999).

On the other hand, as for a small amount of soybean powder from samples cultivated in 1999, the correlation coefficiencts between the standardized second derivative NIR spectral reading at 1708nm vs. linoleic acid moiety ratio, linoleic + linolenic acid moiety ratio, and

oleic acid moiety ratio were -0.853, -0.947, and 0.877, respectively. In spite of a small amount analysis, clear results were obtained due to the standardization.

3.5 Relationship between the NIR spectral patterns and the fatty acid composition of the other materials harvested in 2000.

The correlation coefficients between the standardized second derivative NIR spectral reading at 1708 nm vs. oleic acid moiety ratio, linoleic acid moiety ratio, linolenic acid moiety ratio, and linoleic + linolenic acid moiety ratio (soybean samples harvested in 2000) described in Table 1. When a seed coat was remained, the correlation coefficients of plural whole seeds and a single seed were not good. Figure 11 shows the scattering plots between the standardized second derivative NIR spectral reading at 1708 nm vs. linoleic acid moiety ratio for whole plural seeds. The characteristics mentioned above were poorly appeared, and then no good correlation was obtained as described in Tables 1 and as shown in Fig.11.

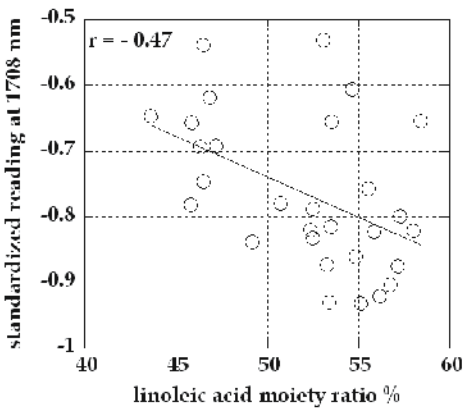

Fig. 11. The scattering plots between the standardized second derivative NIR spectral reading at 1708 nm vs. linoleic acid moiety ratio for whole plural seeds. There is no good correlation.

The correlation coefficients showed: the stronger the absorption band at 1708 nm downward, the higher the percentage of the linoleic acid moiety as mentioned above. The results showed that the rough estimation of the percentage of the linoleic acid moiety (C18:2) was possible. However, in the case of whole plural seeds in a rotating cup, the inclination was not good enough.

The variations of the samples harvested in 2000 were smaller than those in 1999: the smaller cultivated areas and the less varieties, and then their correlation coefficients were better for the powder analysis. Figure 12 shows the two year data combined. However, the regression lines were closely coincident.

This method can be used for the selection of varieties with higher or lower levels of linoleic (or oleic) acid moiety ratio in soybean. The above-mentioned method did not use empirical calibration equations but was instead based on spectroscopic pattern analysis. There is no need to develop a calibration equation in advance.

So far, the authors have successfully introduced the NIR method for single-seed, or very small amount, analysis of fatty acid composition, such as that in a sunflower seed (Sato et al., 1995b) , in sesame seed (Sato et al., 2003), and in a rapeseed (Sato et al., 1998). In this

samples	oleic acid moiety ratio	linoleic acid moiety ratio	linolenic acid moiety ratio	linoleic + linolenic acid moiety ratio
whole plural seeds in a rotating cup	0.48	-0.47	-0.38	-0.48
whole single seed in a mormal single grain cup	0.59	-0.53	-0.56	-0.58
whole single seed in a special single grain cup	0.48	-0.40	-0.57	-0.46
pealed single seed in a single grain cup	0.87	-0.83	-0.73	-0.86
standard amount of powder, obtained by a ultracentrifugal mill in a standard cup	0.97	-0.96	-0.71	-0.97
small amount of powder, obtained by a ultracentrifugal mill in a single grain cup	0.97	-0.96	-0.69	-0.98
crashed particles in a single grain cup	0.81	-0.79	-0.64	-0.82
small amount of powder, drilled from a seed in a modified single grain cup	0.86	-0.85	-0.63	-0.87
extracted oil in a British cup	0.98	-0.95	-0.76	-0.98

Table 1. The correlation coefficients between the standardized second derivative NIR spectral reading at 1708 nm vs. oleic acid moiety ratio, linoleic acid moiety ratio, linolenic acid moiety ratio, and linoleic + linolenic acid moiety ratio (soybean cultivated in 2000).

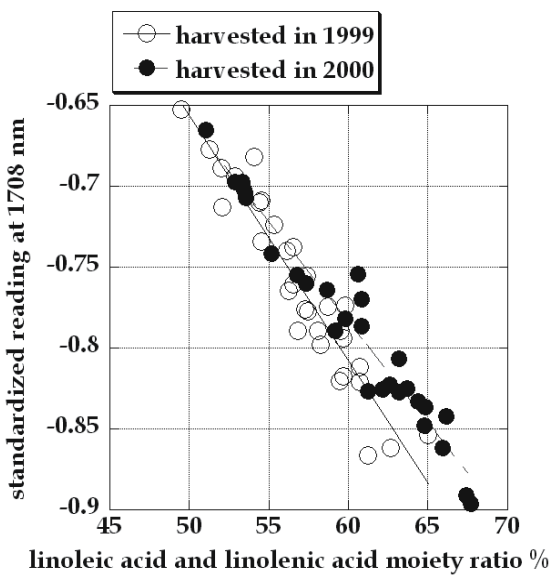

Fig. 12. The two year data combined for a small amount of powder in a modified single grain cup analysis case.

report, the authors used a very small amount (about 8 mg) of soy flour, and this amount can be sampled several times from a single soybean seed.
Using the nuclear magnetic resonance method, Yoshida *et al*. (1989) successfully carried out the fatty acid analysis of an intact single soybean seed. However, it took almost one hour for accumulations. On the other hand, it took only one minute in the authors' case. Further, when a Fourier transform type instrument is used instead of a scan type instrument, the measurement time becomes faster. Furthermore, if sampling of a small amount of soybean powder is possible using a mini-drill without destroying a hypocotyl in a seed, germination ability can be retained, and the method can be adopted for screening of the varieties not only with the required fatty acid composition but also with useful properties linked with the fatty acid composition based on the spectral assignments. The authors have successfully introduced a nearly nondestructive method.

4. Conclusions

The near infrared (NIR) spectroscopy has been recognized as a powerful analytical tool and has been widely used for estimating the contents of the constituents or the property of various agricultural products. However, the NIR method is thought to be an empirical method using multivariate statistical analyses. In this chapter, the author took another approach based on the NIR spectral pattern analysis to develop a simple and rapid method for the determination of the fatty acid composition in soybean oil. The NIR wavelength range from 1600 to 1724 nm includes the information concerning the unsaturation and the chain length of fatty acid moiety. First, the author examined the conditions of the mathematical treatment for the conversion of the original spectral data to the second derivative one in order to make this information clearer. Then, the NIR spectral data was standardized to compare easily by making the spectral value at 1600 nm as 0.0 and the spectral value at the minimum around 1724 nm as -1.0. The correlation coefficient between the corrected second derivative NIR readings at 1708 nm and the percentage of linoleic acid moiety in soybean oil was good enough for a rough estimation of it even with a very small amount of powder. Using this technique, the authors examine the determination of fatty acid composition of other oilseeds: (husked) sunflower, rapeseed and sesame without developing the calibration equations. These findings give theoretical bases to NIR technology. Further, if this nondestructive method will be used as a marker of the property linked with the fatty acid composition, it will develop its use in the new fields.

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Part 3

Physiology

29

Dietary Content and Gastrointestinal Function of Soybean Oligosaccharides in Monogastric Animals

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1. Introduction

Soybean is a major ingredient in non-ruminant animal diets throughout the world. There is an extensive body of information suggesting that soybean is an excellent source of high quality protein, whereas less attention has been paid to soybean oligosaccharides. Soybean oligosaccharides, also referred to as α-galacto-oligosaccharides, oligosaccharides of the raffinose family or simply α-galactosides, are water-soluble, low-molecular weight carbohydrates raffinose, stachyose and verbascose. In maturing seeds, oligosaccharides are formed by successive addition of galactosyl moieties to a sucrose primer. Alpha-galactosides are characterized by the presence of α(1→6) linkages between galactose moieties which are bonded via α(1→3) to terminal sucrose. Unlike other oligosaccharides, soybean α-galactosides can be extracted directly from the raw material and do not require enzymatic manufacturing processes.

Soybean oligosaccharides comprise approximately 4% of the soybean dry matter (DM) and during processing in the preparation of soybean meal (SBM) they are not removed or destroyed. Therefore, in SBM, α-galactosides represent approximately 5-6% but could be as high as 8% DM. Other processed soybean products, however, may contain significantly less oligosaccharides than SBM. The oligosaccharide content of soy protein concentrates (SPC) is as low as 3% DM while soy protein isolates (SPI) contain only trace amounts of oligosaccharides.

Soybean oligosaccharides appear to be indigestible in the upper intestinal tract of monogastric animals due to the absence of α-galactosidase enzyme. However, they are easily fermented by the lower gut microflora, resulting in the production of various gases and short-chain fatty acids. Studies have shown considerable microbial fermentation of α-galactosides in the small intestine with some authors referring to soybean oligosaccharides as bifidogenic factors which stimulate the growth of beneficial bacteria and others claiming that increased consumption of oligosaccharides may lead to negative effects in the large intestine of mammals, such as flatulence, diarrhea, and excessive dietary protein decay.

The content of α-galactosides in animal diets usually ranges from 0.5 to 3% and, since SBM is a rich source of oligosaccharides, an increase in the SBM content of the ration results in an increase in the concentrations of α-galactosides. A trend for increased inclusion of SBM in animal diets has been observed in recent decades due to the EU ban on the use of meat-and-bone meals and a decrease in fishmeal production. In animals whose nutrient requirements are high, such as young meat-type turkeys, the SBM content of the diet may be as high as 50%. Therefore, the objective of our research presented in this chapter is to determine whether excessive amounts of α-galactosides in turkey diets may increase the risk of diarrhea and result in reduced growth performance.

2. Chemical properties and occurrence of α-galactosides

Oligosaccharides, next to sucrose, are the most widely distributed water-soluble carbohydrates in the plant kingdom (Han & Baik, 2006). Oligosaccharides (the name is derived from the Greek word *oligos*, meaning a few) are compounds that yield only monosaccharide units upon complete hydrolysis (Kadlec et al., 2001). Depending on the number of monosaccharide residues, oligosaccharides are classified as trisaccharides, tetrasaccharides and so forth. The main group of oligosaccharides present in SBM are the raffinose family oligosaccharides (RFOs), so named after the first member of this homologous series of α-galactosides, which are characterized by the presence of α(1→6) links between the galactose moieties (Han & Baik, 2006). In addition to raffinose, this group comprises stachyose, verbascose and ajugose, which consist of 1, 2 and 3 α(1→6) linked units of galactose bonded through α(1→3) to terminal sucrose (Kadlec et al., 2001). The remaining α-galactosides are galactosyl cyclitos, mainly ciceritol (Barnabé et al., 1993) and unnamed longer-chain oligosaccharides up to nonasaccharides (Cerning-Beroard & Filiatre-Verel, 1976). According to the International Union of Pure and Applied Chemistry, raffinose is a trisaccharide (α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) composed of fructose, glucose and galactose. Stachyose (a tetramer) consists of two α-D-galactose units, one α-D-glucose unit, and one β-D-fructose unit sequentially linked as α- D-Galp-(1→6)-α- D-Galp-(1→6)- α- D-Glup(1→2)-β-Fru. Verbascose is a pentasaccharide with a longer chain of galactose units joined to sucrose as α-D-Galp-(1→6)-α- D-Galp-(1→6)-α- D-Galp-(1→6)-α- D-Glup(1→2)-β- D-Fru.

Chemically, α-galactosides are low molecular weight non-reducing carbohydrates that are soluble in water and aqueous alcohol solutions (Arentfot et al., 1993). They are associated with the onset of desiccation tolerance during seed development, and with seed storability (Blackman et al., 1992; Horbowicz & Obendorf, 1994; Obendorf et al., 1998). The synthesis of α-galactosides is also affected by growing conditions and the rate of seed maturation. Obendorf et al. (1998) found that the axes of seed matured at 25ºC accumulated higher concentration of sucrose and raffinose, whereas stachyose content remained unchanged.

Among the grain legume crops grown in Europe, faba been and lentil seeds are characterized by a low α-galactoside content, while pea seeds contain a moderate and lupin seeds contain a high level of α-galactosides. (Table 1). Although lupine seeds have a relatively high α-galactoside content they play a limited role in intensive animal farming. Pea, lentil and faba bean seeds are used in animal diets but not as extensively as soybean. Soybean α-galactosides comprise approximately 4% of the soybean dry matter (Karr-Lilienthal et al., 2005). During SBM processing, α-galactosides are not removed or destroyed, therefore, in toasted SBM α-galactosides may account for 5% (Seve et al., 1989;

	Lentil	Pea	Faba bean	White lupine	Narrow leaf lupine	Yellow lupine
Raffinose	0.3	0.8	0.2	0.7	1.4	2.2
Stachyose	1.7	2.5	0.9	6.6	5.5	6.9
Verbascose	0.4	1.7	1.4	0.5	2.2	2.8
Total	2.4	5.0	2.5	7.8	8.6	11.9

Table 1. The α-galactoside content of the seeds of legume crops (%) (Kozlowska et al., 2001)

Coon et al., 1990), 6-7% (van Kempen et al., 2006) or even 8% of DM (Grieshop et al., 2003). Some authors reported a lower, i.e., below 3% α-galactoside content of SBM, DM (Smirickey et al., 2002). Similar and considerably lower α-galactoside levels were observed in the seeds of selected and genetically modified soybean lines (Kerr et al. 1993; Parsons et al., 2000; Neus et al., 2005). Low-oligosaccharide SBM obtained from the seeds of the improved lines would contain only 0.2-0.5% α-galactosides (Parsons et al., 2000). In the future, such soybean lines could be grown on a large scale providing yields comparable to that of conventional varieties.

Soy products, mainly SBM, have superior nutritional characteristics in terms of a high protein content and amino acid profile, which is why SBM is a major protein source in swine (Cromwell, 2000) and poultry diets (Baker, 2000; Grieshop et al., 2000) . In addition to protein, SBM contains over 30% total carbohydrates (Grieshop et al., 2003). Approximately one third of which are non-structural low molecular weight carbohydrates, including oligosaccharides (Karr-Lilienthal et al., 2005). Diets for growing pigs with an average content of SBM of 16% contain less than 1% α-galactosides (Kozlowska et al., 2001). The α-galactoside content of poultry diets usually ranges from 0.5 to 3%, with the main sources, in decreasing order, being: soybean meal (6% DM), pea (5%), faba beans (4%), rapeseed meal (3%) and sunflower meal (2%) (Carré et al., 1984).

The seeds of soybean and other legume species intended for human consumption are processed by soaking, cooking, irradiation, fermentation and enzymatic treatment to decrease the α-galactoside content (Machaiah & Pednekar, 2002; Gote et al., 2004; Egounlety & Awort, 2003; Yoo & Hwang, 2008).

Table 2 shows changes in the levels of raffinose and stachyose in raw and cooked soybean seeds subjected to standard soaking, soaking under ultrasound and soaking under high hydrostatic pressure. After 3 h soaking, the raffinose and stachyose contents of uncooked seeds decreased from 6.01 to 4.01% and from 3.50 to 1.87%, respectively. A more significant decrease in the levels of raffinose and stachyose was observed in seeds soaked under ultrasound and under high hydrostatic pressure. The lowest content of raffinose (1.03%) and stachyose (1.30%) was noted in cooked seeds soaked under ultrasound for 3 h (Han & Baik, 2006). Cooked seeds soaked under high hydrostatic pressure contained the largest amounts of oligosaccharides.

The α-galactoside content of SBM used in poultry and swine diets may be decreased either by the extraction with ethanol (Seve et al., 1989; Coo et al., 1990; Leske et al., 1993; Irish et al., 1995; Leske et al., 1999a, b) or by the development of genetically modified soybean varieties (Frias et al., 1999; Parsons et al., 2000; Grieshop et al., 2003; van Kempen et al., 2006). The use of SPC and SPI represents another means of reducing dietary soybean α-galactosides.

Table 3 shows selected chemical components of various soybean products used in turkey diets (Jankowski et al., 2009). The content of soluble sugars, including α-galactosides, was

over two-fold lower in SPC than in SBM. Somewhat smaller differences between SBM and SPC were noted for dietary fiber fractions. Soy protein isolate had the lowest content of soluble sugars and structural carbohydrates while hulls were the richest source of fiber components resulting in limited use of these latter product in animal diets.

Sample	Treatment	Raffinose[1]	Stachyose[1]
Uncooked seeds	Soaked, 3 h	4.01	1.87
	Soaked, 12 h	2.63	1.53
	Soaked under ultrasound, 1.5 h	3.21	3.11
	Soaked under ultrasound, 3 h	2.66	2.50
	Soaked under high hydrostatic pressure, 0.5 h	4.10	3.48
	Soaked under high hydrostatic pressure, 1 h	3.97	3.24
Cooked seeds	Unsoaked	6.93	5.48
	Soaked, 3 h	3.62	2.97
	Soaked, 12 h	2.96	2.43
	Soaked under ultrasound, 1.5 h	3.35	3.10
	Soaked under ultrasound, 3 h	1.03	1.30
	Soaked under high hydrostatic pressure, 0.5 h	3.24	2.99
	Soaked under high hydrostatic pressure, 1 h	2.64	2.40

[1]The initial raffinose and stachyose content of seeds was 6.01% and 3.50%, respectively.

Table 2. The raffinose and stachyose content of soybean seeds processed by various methods (Hab & Baik, 2006)

Component	Soybean product			
	Soybean meal	Protein concentrate	Protein isolate	Hulls
Crude protein	47.0	64.9	85.9	12.7
Soluble sugars				
Monosaccharides	0.9	0.5	0.7	1.3
Sucrose	5.7	1.0	0.1	1.5
Oligosaccharides	5.3	2.5	0.1	0.8
Dietary fiber fractions				
Crude fiber	3.5	2.8	0.2	36.4
Acid detergent fiber	5.5	4.4	nd	42.3
Neutral detergent fiber	7.7	7.1	0.2	55.0
Non-starch polysaccharides (NSP)				
Total NSP	12.6	12.9	1.3	46.1
Water-soluble NSP	2.0	1.9	0.3	6.6
Water-insoluble NSP	10.5	11.0	1.0	39.5

Table 3. The content of crude protein and carbohydrate fractions (%) in different soybean products (Jankowski et al., 2009)

The utility of selected soybean products (including SPC and SPI) in the nutrition of baby pigs and chickens has been demonstrated (Coon et al., 1990; Sohn et al., 1994; Russett, 2002; Batal & Parsons, 2003). Swick (2007) indicated that the major advantage to the use of SPC and SPI is that diets higher in density and lower in water-soluble carbohydrates may be formulated and also the proteins are less allergenic.

3. Physiological properties of α-galactosides in the gastrointestinal tract and any potential antinutritive effects of their presence in the diet

Because of the lack of appropriate mucosal enzymes in the small intestine of monogastric animals, α-galactosides are considered as non-digestible carbohydrates. However, the oligosaccharides pass into the lower gut and are fermented by the intestinal microflora (Saini & Gladstone, 1986; Veldman et al., 1993; Price et al., 1988). Alpha-galactosides can also be fermented, to some extent, by bacterial populations in the distal ileum of non-ruminants (Liying et al., 2003). This fermentation pattern may result in both positive (bifidogenic) and negative (antinutritional) effects (Karr-Lilienthal et al., 2005).

For many years α-galactosides have been considered as antinutritional factors. Kuriyama and Mandel (1917) were the first to report that a meal containing 3 or 5 g raffinose resulted in severe diarrhea in rats. Such response can be explained by the fact that the intestinal mucosa of humans and monogastric animals lacks the enzyme α-galactosidase required to cleave α(1→6) linkages (Gitzelman & Auricchio, 1965). As a result, dietary raffinose and stachyose may produce diarrhea resulting in an increased digesta passage rate and decreased digestion and absorption of dietary nutrients (Wiggings, 1984).

For the past two decades increased attention has been paid to the antinutritional effects of α-galactosides in intensively fed animals, including fast-growing broiler chickens. It was found that that the concentration of metabolizable energy in SBM was low in comparison to the gross energy content (Pierson et al., 1980), and subsequently the presence of α-galactosides in SBM was implicated as the major reason for the low metabolizability of energy (Coon et al., 1990). The above finding was validated by the results of experiments in which chickens were fed ethanol-extracted SBM with no raffinose and stachyose (Coon et al., 1990; Leske et al., 1999a, b). Leske et al. (1995) found that when fed to chicks ethanol-extracted SBM contributed to better protein utilization and amino acid availability than did non-extracted SBM. The negative effects of dietary oligosaccharides on nutrient utilization as manifested in reduced energy digestibility (Leske et al. 1993) and reduced ileal digestibility (Bedford, 1995) have been related to a reduction of up to 50% in intestinal digesta passage rate and to elevated hygroscopic properties of excreta. Careé et al. (1995) found an apparent α-galactoside digestibility of 87% in broiler chickens and 99% in adult cockerels, which suggested extensive (increasing with age) microbial fermentation in the lower part of the gastrointestinal tract. In this regard, the efficiency of energy utilization from the products of microbial fermentation is lower compared with the utilization of energy from carbohydrates directly absorbed from the small intestine. It has also been demonstrated that the ethanol extraction of SBM decreased energy loss due to a reduced amount of hydrogen gas produced by the chicks fed ethanol-extracted SBM in comparison to control meal (Leske et al., 1999b).

In a study with cannulated piglets, it was found that 3 h after feeding 39% of α-galactosides disappeared from the stomach and small intestine (Gdala et al., 1997). The relatively high digestion of α-galactosides in the upper gut may be attributed to the presence of

endogenous plant and microbial α-galactosidase. Although the results have been inconclusive, attempts have been made to alleviate the adverse effect of α-galactosides by the addition of fungal α-galactosidase to diets. In one of the first studies on the use of fungal α-galactosidase in piglet diets it was demonstrated that the addition of this enzyme did not alleviate the adverse effects of α-galactosides in legume-based diets. It was concluded that an increase in fermentable substrates in the lower part of the digestive tract may disturb the microbial balance, thus increasing the risk of diarrhea (Veldman et al., 1993). In another experiment it was shown that the addition of pectinase and α-galactosidase to broiler diets tended to improve growth performance and increased ($P=0.06$) apparent metabolizable energy content, from 12.13 to 12.55 MJ/kg (Igbasen et al., 1997). In another study dietary supplementation with α-galactosidase significantly increased the cumulative feed intake in chickens, without any apparent effect on α-galactoside digestibility (Daveby et al., 1998). Addition of exogenous α-galactosidase has shown no beneficial effect on the ME content of SBM (Irish et al., 1995).

Relatively few studies on the physiological properties of α-galactosides have been conducted in the last decade. In one experiment, the average true metabolizable energy content of SBM with low total raffinose, stachyose and galactitol content (0.7% and 0.25%) was 9.8% higher ($P<0.05$) compared with conventional SBM (Parsons et al., 2000). Positive results were reported for an enzyme cocktail containing multi-activities, including α-galactosidase, α-amylase, β-glucanase, protease, xylanase, and cellulase. Broilers fed diets supplemented with the enzyme cocktail showed a better feed conversion ratio, although no effects on growth, immunity, or carcass attributes were noted (Kidd et al., 2001). In another experiment, application of α-galactosidase reduced the stachyose and raffinose content of enzyme-treated SBM by 69% and 54%, respectively, and decreased the concentrations of these oligosaccharides in the excreta (<0.1mg/g), but it did not influence the growth of chickens (Graham et al., 2002). In a similar study, although significant oligosaccharide hydrolysis in the chicken gut was achieved (57%), no improvement in growth performance was noted (Slominski et al., 2006).

In a study with cannulated young pigs, soy oligosaccharides reduced nutrient digestibility, but the reduction was small, ranging from 1.1 to 7.4 percentage units (Smiricky et al., 2002). The results of another experiment with growing pigs indicated that the ileal digestibility of α-galactosides added to a semi-purified diet was higher than 75% (Smiricky-Tjardes et al., 2003). According to some authors (Liying et al., 2003), oligosaccharides at the concentrations found naturally in a typical corn-SBM diet may have little effect on nutrient digestibility. However, it has been demonstrated that nutritionally relevant variability does exist in soy varieties and that a low stachyose content is important for maximizing energy utilization (van Kempen et al., 2006). In addition, soybean meal obtained from low-oligosaccharide varieties may have higher concentrations of most essential amino acids than the conventional SBM. The results of the above experiments and similar studies (Iji and Tivey, 1998) indicate that soybean oligosaccharides can be regarded as a factor capable of decreasing the health status and growth rate of animals.

4. Soybean oligosaccharides as potential prebiotics

Prebiotics are dietary ingredients, typically oligosaccharides and polysaccharides with defined properties, administered intentionally to improve and stimulate the growth and activity of intestinal microflora, and thereby reduce the risk of disease. To be considered as a prebiotic, a compound must conform to the following: (1) it must be resistant to digestion in

the upper gastrointestinal tract (remain unaltered through hydrolytic-enzymatic digestion), (2) it must selectively stimulate one or a limited number of beneficial microbiota and (3) it must benefit host health by improving colonic microbiota composition (Roberfroid et al., 1998). According to Gibson et al. (2004), a prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity of the gastrointestinal microflora, that confer benefits upon host well-being and health.

It is well known that the most important characteristic of non-digestible carbohydrates is their fermentability by bacteria in the large intestine of animals and humans. The main products of the fermentation processes are short-chain fatty acids (SCFAs) and the gases: H_2, CO_2, and CH_4 (Krause et al., 1994). The health status of the gastrointestinal tract is significantly affected by the amounts and proportions of SCFAs (acetate, propionate and butyrate), bacterial enzyme activity (e.g. pro- or anti-carcinogenic activity), the content of different bacterial metabolites in feces (e.g. phenols, cresols, products of bacterial breakdown of protein and urea) as well as by the amount and bulking of stool (Salminen et al., 1998, Loo et al., 1999). All SCFAs are rapidly absorbed from the hindgut where they stimulate salt and water absorption. The SCFAs are metabolized principally by the gut epithelium, the liver and muscles, with virtually none appearing in urine and only small amounts present in feces (Salminen et al., 1998). These compounds play a very important role in the function of the large bowel as an energy source for the colonic epithelium. Particularly important for large bowel health is butyrate, which regulates epithelial cell growth and differentiation (Miller & Wolin, 1996).

The name "prebiotics" may be given to the saccharides which selectively enhance the populations of beneficial microflora, primarily endogenous lactic acid bacteria and *Bifidobacteria* (Delzene and Roberfroid, 1994; Gibson and Roberfroid, 1995; Walker and Duffy, 1998). Such properties have been demonstrated for fructans (inulin and oligofructose) obtained from plant sources (mainly chicory root) and the products of biotechnological processing (enzymatic transglycosylation of sucrose). Fructans are among the most popular prebiotic supplements, available with 60% of the publications on the topic of prebiotic supplementation being devoted to fructans (Barry et al., 2009). Reports on the use of soybean α-galactosides as potential prebiotics are scarce, comprising only 9% of the prebiotic literature available. According to one of the first publication dealing with the production of commercial prebiotic preparations, the estimated production of soybean oligosaccharides (based on data obtained by surveying major manufactures of food-grade oligosaccharides) was 2000 tons (Crittenden and Playne, 1996). However, relevant data for subsequent years are not available. The above work is cited in a recent paper (Wang, 2009). Under laboratory and semi-technical conditions, α-galactosides are also extracted from the seeds of legume species other than soybean. The physiological properties of those products may vary. Depending on the plant source and the degree of purification, α-galactoside preparations contain different amounts of stachyose, raffinose, verbascose and sucrose (Table 4.)

In one of the very first experiments it was found that α-galactosides obtained from SBM were well utilized by beneficial bacteria, and also reduced the activity of enzymes specific to pathogenic bacteria (Masai et al., 1987). The ingestion of 10 g of soybean oligosaccharide extract (23% stachyose and 7% raffinose) significantly increased the counts of bifidobacteria in six healthy adult males (Hayakawa et al., 1990). A recent study on young female volunteers showed that a soy oligosaccharide intake of 3 g/day was enough to increase fecal bifidobacteria counts, short-chain fatty acids concentration, and fecal lipid output (Bang et al., 2007).

Component	Soybean		Pea		Lupine	
	Semi-pure[1]	Pure α-G[1]	Semi-pure[2]	Pure α-G[3]	Semi-pure[2]	Pure α-G[3]
Dry matter	97.0	95.0	95.6	95.3	94.0	97.0
Sucrose	44.0	2.0	24.5	20.3	35.0	9.1
Raffinose	7.0	20.0	12.3	4.0	3.1	30.2
Stachyose	23.0	71.0	40.9	51.5	32.7	47.0
Verbascose	-	-	-	19.5	-	10.7
Other	23.0	2.0	17.9	-	23.2	-

[1]Masai et al., 1987, [2]Juskiewicz et al., 2003, [3]Gulewicz et al. 2002

Table 4. Average content of α-galactosides and sucrose in α-galactoside (α-G) preparations obtained from soybean, pea and lupine seeds

In an *in vitro* experiment, the addition of soybean oligosaccharides to a growth medium increased the counts of bifidobacteria, measured as a proportion of total viable counts in the culture. Azoreductase, β-glucuronidase and β-glucosidase activities decreased, but only the activity of azoreductase was lowered significantly by soybean oligosaccharide treatment (Saito et al., 1992). In an experiment with young women who received 1.5 or 3 g/day of soy oligosaccharides for 30 days (LSO and HSO group, respectively), the counts of bifidobacteria in feces increased significantly in the HSO group. Significantly higher concentrations of fecal SCFAs, propionate and butyrate, were also noted in this group.

In an experiment with young pigs, dietary supplementation with 1% stachyose increased the counts of lactobacilli in the ileum as well as bifidobacteria in the cecum and colon. However, the weight gains of piglets were lower compared with the control group (Liying et al., 2003). A higher content of stachyose in the diet (2%) had a negative effect on body weight gain of piglets and lactobacilli and bifidobacteria counts in the cecum. The data indicate that at least a portion of the growth depression observed when soybean is included in the diet of weaning pigs can be attributed to the presence of α–galactosides (mainly stachyose). In a similar experiment, the dry matter content of cecal digesta was significantly lower when compared to control and the activities of bacterial β- and α-galactosidase, α-glucosidase and β-glucuronidase were significantly higher in rats fed diets containing oligosaccharides extracts from pea or lupine seeds (see Table 4) (Juśkiewicz et al., 2003). Compared with cellulose, the total production of SCFAs in the cecum was significantly higher when a diet contained 4.9% of α-galactosides and it tended to be lower when α-galactoside content decreased (3.9%). According to Lan et al. (2004), soybean oligosaccharides can increase the survival rates of lactic acid producing bacteria in broiler chickens infected with *E. tenella*. Furthermore, in a subsequent study Lan et al. (2007) revealed that soybean oligosaccharides can increase the population of a group of lactic acid bacteria (of the genera *Lactobacillus*, *Pedicoccus*, *Weissella* and *Leuconostoc*) in the cecal contents of young broiler chickens. The authors concluded that soybean oligosaccharides show promise for use as a product which may promote competitive exclusion of potential pathogens in young broiler chickens. However, they emphasized that the selective proliferation of *Lactobacillus* by soybean oligosaccharides could not be confirmed.

The results of experiments with chickens have indicated that the physiological effect of α-galactosides would depend on their concentration in the diet; with the level of 0.4% not affecting metabolizable energy and amino acid digestibilities, but level above 0.8% potentially depressing energy utilization (Biggs et al., 2007).

5. The physiological effects of α-galactosides in turkey diets

Little is known how turkeys respond to a different content of α-galactosides in their diets, particularly when SBM is added at a relatively high amount to replace meat and meat-and-bone meals. Meat-type turkeys have high protein and essential amino acid requirements. Starter diets from 1 to 4 weeks of age, should contain at least 27% of protein (NRC, 1994). In the absence of alternative high-protein components, the SBM content of turkey diets is often very high, approaching 50%, with the α-galactoside level exceeding 2.5%. As shown in Table 5, the α-galactoside content of diets for young growing turkeys may be decreased provided that SBM is partially or entirely replaced with other high-protein soy products, including SPC and SPI (Jankowski et al., 2009). In this study, the use of SPC as a replacer for SBM had no effect on the concentrations of non-starch polysaccharides (NSP). The content of water-insoluble NSP was considerably lower in a diet containing SPI, compared with the other groups (8% vs. 10%). The results of an experiment in which turkeys were fed diets from 0 to 8 weeks of age are presented in Table 6.

Ingredient, %	Starter diets from 1 to 4 weeks of age			
	SBM	SBM-SPC	SPC	SPI
Soybean meal (SBM)	44.3	28.3	3.9	-
Soybean protein concentrate (SPC)	-	10	25.3	-
Soybean protein isolate (SPI)	-	-	-	19.5
Soybean hulls	2.5	2.8	3.2	4.8
Soybean oil	5.5	3.6	0.7	-
Wheat	42.3	49.9	61.5	69.9
Minerals, amino acids, vitamins	5.4	5.4	5.4	5.8
Crude protein	27.0	27.0	27.0	27.0
Crude fiber	3.4	3.5	3.4	3.2
Oligosaccharides	2.6	2.0	0.9	0.2
Non-starch polysaccharides	10.2	10.2	10.1	8.0

Table 5. Composition of turkey diets containing different soybean products (Jankowski et al., 2009)

In the second phase of the experiment (4 to 8 wks) the diets contained lower amounts of soybean products, proportionally to the lower concentration of total protein (25.5%), and therefore the α-galactoside content decreased to 2.3, 1.7, 0.9 and 0.05%, respectively. When substituted for SBM, SPC and SPI increased in the weight and water content of cecal digesta in turkeys of 4 weeks of age. No increase in the activity of microbial β-glucosidase was noted, whereas the activity of β-glucuronidase produced by the potentially pathogenic bacteria increased in turkeys fed SPC and SPI. There were no differences in the quantities of SCFAs produced in the ceca. In turkeys of 8 wks of age, the concentration of SCFAs produced in the ceca was proportional to the dietary α-galactoside content. In comparison with other dietary groups, the average body weight of turkeys fed SPI was significantly lower. A reduction in the dietary α-galactoside content in the second phase of the experiment did not result in any beneficial effects. It was found that partial or almost complete replacement of SBM with SPC in turkey diets, which was associated with a decrease in dietary oligosaccharide content, suppressed the fermentation process in the

Item	Main protein source in the diet			
	SBM	SBM-SPC	SPC	SPI
Cecal parameters at 4 weeks of age				
Cecal digesta weight, g/kg of BW	1.91^{c}	2.19^{bc}	2.94^{a}	2.83^{ab}
Dry matter of digesta, %	14.7^{c}	14.6^{c}	19.4^{a}	18.8^{ab}
β-glucosidase activity, U/g	0.20	0.14	0.27	0.28
β-glucuronidase activity, U/g	0.34^{bc}	0.39^{c}	0.50^{a}	0.45^{ab}
SCFA pool, mol/kg of BW	208.6	208.2	232.1	204.5
Cecal parameters at 8 weeks of age				
Cecal digesta weight, g/kg of BW	2.98	2.71	2.48	2.52
Dry matter of digesta, %	15.8^{b}	17.9^{b}	17.3^{b}	21.6^{a}
β-glucosidase activity, U/g	0.47	0.43	0.43	0.42
β-glucuronidase activity, U/g	0.98	0.69	0.65	0.60
SCFA pool, mol/kg of BW	380.6^{a}	255.5^{b}	218.0^{b}	214.7^{b}
Body weight of turkeys, kg				
4 weeks of age	1.074^{a}	1.078^{a}	1.075^{a}	1.038^{b}
8 weeks of age	4.324^{b}	4.452^{a}	4.459^{a}	4.282^{b}
Feed conversion ratio, kg/kg				
0 to 4 weeks of age	1.416^{a}	1.469^{a}	1.416^{a}	1.368^{b}
0 to 8 weeks of age	1.760^{a}	1.787^{a}	1.742^{a}	1.667^{b}

Table 6. Selected results of an experiment in which different soybean products were fed to turkeys (Jankowski et al., 2009)

cecum but increased the body weight of 8-week-old turkeys. The concentrations and the pool of SCFAs in the cecum were positively correlated with the content of dietary oligosaccharides. An almost complete removal of oligosaccharides from the diet, due to the use of SPI as a substitute for SBM, improved the average feed conversion ratio, but decreased the average body weight of 4-week-old turkeys and had no effect on the overall growth rate of turkey up to 8 weeks of age (Jankowski et al., 2009).

In another experiment, turkeys were fed four diets containing SBM or SPI and different levels of soybean hulls, to increase the crude fiber content of the ration (Juskiewicz et al., 2009). The results of this study are presented in Tables 7 and 8.

Dietary treatment	Fecal DM		LMI	
	4 weeks	8 weeks	4 weeks	8 weeks
α-galactoside content of the diet				
High (2.44%)	21.4^{b}	19.4^{b}	1.96^{a}	2.39^{a}
Low (0.15%)	25.9^{a}	21.4^{a}	1.25^{b}	1.57^{b}
Crude fiber content of the diet				
Low (3.5%)	23.6	19.6	1.54	2.00
High (5.3%)	23. 7	21.2	1.68	1.96

Values within each row with the same superscript letter are not different at $P<0.05$

Table 7. Fecal dry matter content (DM, %) and litter moistness index (LMI, points) (Juskiewicz et al., 2009)

The use of SPI as a substitute for SBM reduced the α-galactoside content of diets from 2.44 to 0.15%, thus significantly increasing the dry matter content of excreta (Table 7) with greater differences observed for turkeys at 4 wks of age. The water content of excreta was well correlated with the moisture content of a litter. It would appear that high content of dietary α-galactosides may affect excreta moisture content, with no obvious symptoms of diarrhea. There were no differences when turkeys were fed diets with a different crude fiber content (3.5 and 5.3%). There was no interaction between the levels of α-galactosides and crude fiber.

The differences in the α-galactosides and crude fiber contents affected some duodenal mucosal structures in turkeys (Table 8).

Parameter	4 weeks				8 weeks			
	α-galactosides		Crude fiber		α-galactosides		Crude fiber	
	High	Low	Low	High	High	Low	Low	High
Villus height, mm	2.01	2.03	2.00	2.04	2.41	2.25	2.51[a]	2.16[b]
Villus width, mm	0.14	0.15	0.15	0.13	0.14	0.14	0.13	0.15
GL thickness[1], mm	0.30	0.27	0.28	0.29	0.30	0.33	0.28[b]	0.35[a]
Crypt depth, mm	0.19[a]	0.17[b]	0.19	0.18	0.19[b]	0.21[a]	0.18[b]	0.22[a]
VCR[1]	10.6	11.9	10.5	11.3	12.7[a]	10.7[b]	13.9[a]	9.82[b]
Number of GC[2]	6.28	5.94	6.37	5.89	5.90	6.45	6.75[a]	5.60[b]

[1]Glandular layer, [2]Villus height/crypt depth ratio, [3]Number of goblet cells per 150 μm of the villus area

Table 8. Duodenal mucosal structures in turkeys fed diets with different levels of α-galactosides and crude fiber at 4 and 8 weeks of age (Juskiewicz et al., 2009)

The effect of dietary oligosaccharides on the duodenal epithelial surface and nutrient utilization depended on the age of turkeys. In contrast to older turkeys (8 weeks of age), younger 4 wks old birds responded differently to dietary treatments with lower α-galactoside levels. Different concentrations of dietary crude fiber affected the turkeys' response to α-galactosides, as reflected in changes in the duodenal crypt depth (interaction P=0.093) and in the number of duodenal goblet cells (interaction P<0.05) in birds at the age of 4 and 8 weeks, respectively. A high dietary content of α-galactosides decreased crypt depth and increased the villus height/crypt depth ratio in 8-week-old turkeys. However, the results of the above studies indicate that the physiological effect of α-galactosides on mucosal structures in the small intestine was not significantly influenced by different levels of crude fiber. The presence of α-galactosides in diets for young turkeys should be considered as a factor positively affecting the development of the duodenal mucosa. It could be concluded that a high content of α-galactosides in the diet increased the hydration of the intestinal contents, but had no significant effect on DM digestibility and nitrogen, calcium and phosphorus utilization (Juśkiewicz et al., 2009).

The third experiment was conducted to investigate the effect of dietary α-galactoside and crude fiber levels on gastrointestinal functions and the growth performance of young turkeys fed diets with a different content of SBM, SPC, SPI and soybean hulls. Table 9 shows the chemical composition of experimental diets at the second stage of growth (5 - 8 weeks). SBM-based diets contained approximately 2.3% α-galactosides, and a partial replacement of SBM with SPC decreased α-galactoside levels to 1.7%. α-Galactoside content was low

(0.94%) in diets where the predominant high-protein component was SPC. Trace amounts of α-galactosides (below 0.1%) were noted in diets in which SBM was completely replaced with SPI. The initial crude fiber content of approximately 3.5% was increased to over 5% by the addition of soybean hulls. The effects of both experimental factors are summarized in Table 10.

Diet composition, %	Main protein source and low (LF) and high (HF) crude fiber content of the diet							
	SBM_{LF}	SBM_{HF}	SBM-PC_{LF}	SBM-PC_{HF}	SPC_{LF}	SPC_{HF}	SPI_{LF}	SPI_{HF}
Soybean meal (SBM)	39.4	40.5	30.5	29.9	6.2	5.7	-	-
Soy protein concentrate (SPC)	-	-	5.6	6.6	20.8	21.8	-	-
Soy protein isolate (SPI)	-	-	-	-	-	-	17.4	17.9
Soybean hulls	2.6	9.0	2.7	9.1	3.1	9.6	4.6	11.1
Soybean oil	4.6	7.1	3.6	5.8	0.8	3.0	-	2.1
Wheat	48.8	39.0	53.0	43.9	64.4	55.3	72.9	64.1
Minerals, amino acids, vitamins	4.6	4.4	4.6	4.7	4.7	4.6	5.1	4.8
Crude protein	25.4	25.4	25.5	25.4	26.0	25.9	25.6	25.3
Crude fiber	3.68	5.23	3.61	5.38	3.69	5.45	3.69	5.17
α-galactosides	2.27	2.36	1.70	1.68	0.93	0.94	0.05	0.07

Table 9. Composition of experimental diets for turkeys at 5-8 weeks of age (Zdunczyk et al., 2010)

Item	Soybean product				CF level	
	SBM	SBM-PC	SPC	SPI	Low	High
Intestinal tissue mass, g/kg BW	40.5^{a}	40.2^{a}	37.2^{b}	32.2^{c}	35.8	39.3
Viscosity of intestinal digesta, MPas	1.91^{b}	2.08^{ab}	2.44^{a}	2.53^{a}	2.28	2.20
Cecal digesta, g/kg BW	2.90	2.57	2.54	2.54	2.47	2.55
Dry matter of cecal digesta, %	17.4^{bc}	16.4^{c}	18.4^{b}	20.8^{a}	18.3	18.2
SCFA concentrations, µmol/g	127.8^{a}	100.7^{b}	89.3^{b}	88.2^{b}	102.3	100.8
SCFA pool, µmol/kg BW	367.5^{a}	243.8^{b}	223.5^{b}	223.6^{b}	267.2	262.0
Body weight at 8 weeks, kg	4.32^{bc}	4.36^{b}	4.82^{a}	4.27^{c}	4.38	4.35
FCR for 0-8 weeks, kg/kg	1.75^{b}	1.76^{b}	1.75^{b}	1.65^{a}	1.74	1.74

Table 10. Selected parameters of the gastrointestinal tract function and growth performance of turkeys fed diets containing various soybean products and different crude fiber levels for 8 weeks (Zdunczyk et al., 2010)

The use of SPC and SPI, which reduced the α-galactoside content of diets, decreased the weight of small intestinal wall and digesta in turkeys. This may have resulted from increased digesta viscosity and a slower rate of transit through the gastrointestinal tract. The weight of cecal digesta was comparable in all groups. Diets containing SPC and SPI

increased the dry matter content of cecal digesta. A decrease in the α-galactoside content of diets resulted in a reduction in the production of cecal SCFAs. Statistically significant differences were found between the group fed the SBM-based diet and the groups fed diets with SBM substitutes with decreased α-galactoside contents. Different crude fiber concentrations in experimental diets had no effect on any parameter investigated.
After 8 weeks of experiment, the highest body weight was observed for the group fed the SPC-based diet containing approximately 1% α-galactosides, while the lowest body weight was noted in the group receiving the SPI-based diet with the α-galactoside content of 0.1%. The latter group had the best feed conversion ratio, which suggests that feeding diets with a reduced α-galactoside content may improve growth performance.

6. Conclusions

Proportionally to the SBM content of diets for monogastric animals, α-galactoside concentrations vary within a broad range of 0.5% to over 2.5%. The results of experiments with chickens and piglets indicate that the physiological effects of α-galactosides are determined by the concentrations of these carbohydrates in the diet. According to some studies, high α-galactoside levels may produce antinutritional effects, e.g. disturb the intestinal passage of digesta and the digestibility of some nutrients. Based on many studies, however, there is little evidence that the oligosaccharides at a normal dietary level pose a nutritional concern and may be even considered as potential prebiotics, although the mechanism of this effect requires further research.
Neither too high (2.3-2.6%) nor too low (0.05-0.1%) α-galactoside content of diets is recommended for young growing turkeys. The best production results have been reported in turkeys fed SPC which allowed for the reduction of dietary α-galactoside content of diets to 1%. A high content of α-galactosides in the diet enhances fermentation processes within the intestines (increased production of SCFAs) and increases the hydration of the intestinal contents, thus increasing the risk of diarrhea. A decrease in α-galactoside levels below 0.1% significantly increases the viscosity of the intestinal contents and has a negative influence on the development of duodenal structures. The physiological effects of α-galactosides, administered at high or low concentrations, are not influenced by different levels of crude fiber in turkey diets.
In view of the development and physiology of the gastrointestinal tract as well as the growth of birds, it may be concluded that a total withdrawal of soybean α-galactosides from turkey diets does not seem to be advisable from a nutritional viewpoint. Thus, SPC, in contrast to SPI, could be considered as an effective SBM substitute.

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Physiological Mechanisms Regulating Flower Abortion in Soybean

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1. Introduction

Soybean yield is determined by the number of pods (seeds) produced per unit area and individual seed weight. The seed number depends upon the number of floral buds that initiate pods and attain maturity. Generally, soybean plants produce an abundance of floral buds, but a large proportion of them abort during development. Indeed, rates of flower and pod abscission/abortion were estimated to reach 80% (Shibles et al., 1975). Alleviation of this abortion rate should increase pod and seed number, and thereby increase yield.
In a series of studies conducted in the 1950s and 1960s, Kato and his group examined abscised/aborted buds, flowers, and pods, and classified them according to their developmental stage. The greatest rate of abscission/abortion took place after fertilization, particularly during early stages of embryo development (Table 1). Based on this observation, they concluded that most critical stage causing flower abortion was at proembryo development after fertilization (Kato & Sakaguchi, 1954; Kato et al., 1955; Kato, 1964). This important finding provided a basis for further anatomical and physiological analyses of the pod abortion process.

Stage reached at failure	Days after fertilization	Frequency of abortion (%)	
		1952	1953
Flower differentiation		5.2	1.4
Reproductive cell division		16.3	13.2
Flowering (fertilization)	0	4.3	3.6
Initial phase of proembryo	3-7	43.3	↕
Later phase of proembryo	10-15	12.8	81.8
Cotyledon stage	20-25	17.9	↕

Table 1. Frequencies of developmental stages of bud, flower and pod abortion at each developmental stage. Adapted from Kato & Sakaguchi (1954) and Kato et al. (1955). Results from two years (1952, 1953) are shown.

Subsequent studies conducted in the 1970s and 1980s suggested that soybean abortion is primarily caused by deficiency in or competition for photoassimilates and nutrients among growing organs (Brevedan et al., 1978; Brun & Betts, 1984; Antos & Wiebold, 1984). Shading treatment imposed at different reproductive growth stages clearly showed that reduced solar radiation significantly decreased the number of pods and seeds, and thereby grain yield (Kokubun, 1988). The yield reduction was particularly marked when the shading treatment was imposed during the period from flowering to early grain development, due to a decrease in dry matter production and/or dry matter partitioning to reproductive organs (Fig. 1).

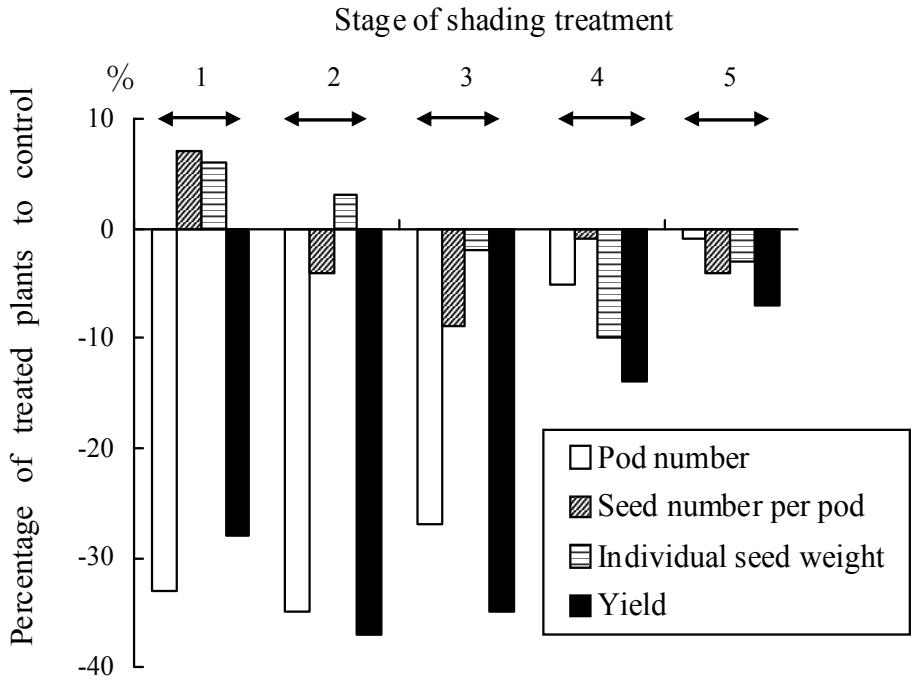

Fig. 1. Effects of 10-day shading treatment imposed at different reproductive growth stages. Stages of shading treatment: 1 Flowering, 2 Pod elongation, 3 Early pod-filling stage, 4 Middle pod-filling stage, 5 Late pod-filling stage. Cultivar: Raiden. Adapted from Kokubun (1988).

Water deficit during reproductive development was shown to be a dominant environmental factor accelerating the rate of abortion (Kato, 1964; Westgate & Peterson, 1993). Based on these findings, Raper & Kramer (1987) concluded that water stress imposed during flower development reduces photosynthesis and the amount of photoassimilates allocated to reproductive tissues, thereby accelerating the rate of abortion.

Another possible physiological factor affecting flower abortion in soybean is the availability of plant hormones (Huff & Dybing, 1980; Heindl et al., 1982; Carlson et al., 1987). There are a number of reports showing that the application of 6-benzylaminopurine (BA) to racemes reduced the rate of abortion and thereby increased pod set. Analysis of endogenous cytokinins revealed that cytokinins detected in root pressure exudates showed a maximum

concentration during a period from 0 to 9 days after initial flowering, when most flowers are destined to either initiate pods or abort (Heindle et al., 1982; Carlson et al., 1987).
Based on these previous findings, detailed mechanisms regulating the abortion have been under investigation since 2000. This chapter highlights major findings of our recent studies on these mechanisms, with an emphasis on 1) involvement of plant hormones in the occurrence of abortion, and 2) water stress as a factor affecting the abortion. A clarification of the mechanisms involved will promote the development of genetic or agricultural methods to alleviate pod abortion, leading to increased pod set and grain yield.

2. Role of phytohormones in flower development in soybean

2.1 Evidence indicating the involvement of phytohormones in the regulation of flower development

The critical role of phytohormones in the formation and abortion of reproductive organs in soybean was clearly recognized when Huff & Dybing (1980) observed that extracts from flowers and young pods applied to growing flowers accelerated flower abortion. They then applied a lanolin paste containing either indoleacetic acid (IAA), giberellin (GA) or 6-benzylaminopurine (BA) to the growing raceme, and found that IAA enhanced the abortion rate, as did the extract, whereas GA and BA did not. These results indicated that IAA plays a crucial role in increasing the abortion rate, although there was a conflicting report indicating that IAA delays the abortion (Oberholster et al., 1991).

2.2 Cytokinin as a key hormone regulating pod development

Among phytohormones, cytokinins are considered to play a vital role in floral development in soybean. There have been numerous reports showing that the application of BA to racemes reduced seed abortion and increased pod number (Crosby et al., 1981; Carlson et al., 1987; Dyer et al., 1987; Peterson et al., 1990; Mosjidis et al., 1993; Reese et al., 1995; Nagel et al., 2001). A limited number of reports support the notion that cytokinins are produced by the root system and transported to the shoot, where they are involved in the regulation of shoot development (Sitton et al., 1967; Wareing et al., 1968). To examine the hypothesis that cytokinins produced in the root system are transported to the shoot, Heindle et al. (1982) collected root pressure exudates from detopped roots, and analyzed the forms and quantities of cytokinins in these exudates. Using high-performance liquid chromatography, they isolated and quantitated several forms of cytokinins: zeatin, zeatin riboside, and their dihydro derivatives, dihydrozeatin and dihydrozeatin riboside. Their results indicated that cytokinin fluxes were independent of exudate flux, and that the ribosides accounted for the majority of the observed transport. In a later study, they also found a peak in cytokinin concentration during the period from the beginning of anthesis until 9 days after initial anthesis (Heindl et al., 1982; Carlson et al., 1987). This period corresponds to a stage in which most flowers are destined to either initiate or abort floral structure. Based on these findings, they concluded that cytokinins exported from the root may function in the regulation of reproductive growth in soybean.

2.3 Relationship between intra-raceme variation in pod-set probability and cytokinin concentrations

The magnitude of the rate of flower abortion was observed to vary with the position on the plant. For example, it was higher in the branches, in the lower part of the main stem and in

the top nodes of the main stem (Hansen & Shibles, 1978; Wiebold et al., 1981; Heindl & Brun, 1983, 1984; Gai et al., 1984). Within individual racemes, flowers on the distal portions on the rachis exhibited a higher probability of abortion than did those on the basal portions (Huff & Dybing, 1980; Spollen et al., 1986a, 1986b; Dybing et al., 1986; Carlson et al., 1987; Wiebold, 1990; Wiebold & Paciera, 1990).

Fig. 2. Development of pod after flowering. Genotype: IX93-100. DAA, Days after anthesis of first flower in raceme. Photo by Nonokawa.

As described above, the role of cytokinins in promoting development of floral structures in soybean had been clarified by several studies (Huff & Dybing, 1980; Heindl et al., 1982; Carlson et al., 1987). However, the nature of ontogenetic changes in the cytokinin content of racemes, and the relationship between the location of cytokinin on the plant and pod abortion remained unknown until 1990s. For precise analyses of intra-raceme variation in pod set and cytokinin contents, soybean genotype IX93-100, which has long racemes (approximately 10 cm, depending on the environmental conditions), was used as plant material. The developmental stages of this genotype are shown in Fig. 2.

We examined the ontogenetic changes in cytokinins detected in different portions in racemes to determine whether a relationship exists between cytokinin concentration and pod-set probability at a specific floral position on a raceme (Kokubun & Honda, 2000). In an experiment using genotype IX93-100 grown in an environmentally controlled chamber (30/20°C day/night temperature, 15 h day length, 600 μmol mol^{-2} s^{-1} photosynthetic photon flux density (PPFD)), we found that the total amount of cytokinin in racemes peaked one to two weeks after the first flowering event on a raceme, when pod development initiated. Within individual racemes, the total amount of cytokinin was greater at more proximal floral positions, as was the probability of pod set (Fig. 3). Removal of proximal flowers increased both cytokinin content and pod-set probability at middle positions on the raceme. Thus, cytokinin content in racemes was closely associated with pod-set probability within individual

racemes. Each flower on a raceme initiated a pod 4 to 7 days after anthesis. During this period, a fertilized ovule develops embryo and endosperm, and cells are dividing actively (Carlson & Lersten, 1987). Microscopic observation showed that most of the abscised flowers are in the proembryo stage, which occurs several days after anthesis (Kato, 1964; Abernethy et al., 1977). Abernethy et al. (1977) speculated that the frequent occurrence of abscission at this stage was due to the reduced level of a cell division mediating factor.

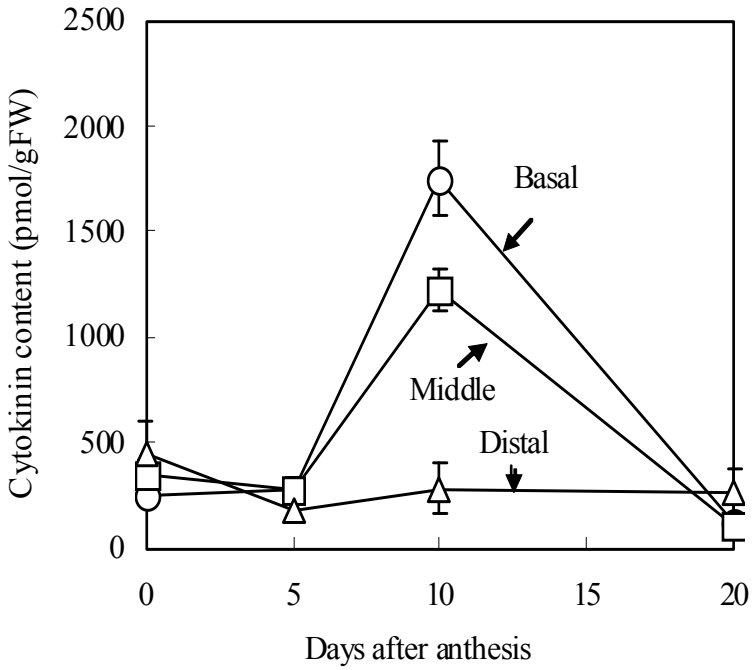

Fig. 3. Changes in the amount of cytokininis in various portions (basal, middle and distal) of racemes during reproductive development in soybean. Portions: 1-3 (basal), 4-6 (Middle), and 7 and above (Distal), numbered from the most basal portion on the rachis. Adapted from Kokubun & Honda (2000).

The biosynthetic pathway for cytokinins has not been fully elucidated in soybean. In our study, the dominant forms of cytokinins were identified to be *cis*-zeatin riboside (*c*-ZR) and isopentenyladenosine (iPA), which differed from the forms detected in exudates previously reported by Heindl et al. (1982) and Carlson et al. (1987). In chickpeas, *cis*-isomers were found to be predominant in seeds (Emery et al., 1998), while *trans*-isomers are the more commonly reported forms in higher plants (McGaw & Burch, 1995; Prinsen et al., 1997). Further studies are currently in progress to identify the forms of cytokinins detected both in exudates and racemes in our laboratory.

2.4 Effects of cytokinin application in plants with varying source-sink ratios

The clear evidence that cytokinin plays a prompting role in flower development raised the following question. Does cytokinin accelerate flower development independently or synergistically with photoassimilate supply? To address this question, we examined the combined effects of these two physiological circumstances by manipulating source-sink

ratios at specific nodes in soybean plants (Yashima et al., 2005). As the source-sink ratio increased, the number of pods per node on manipulated plants increased curvilinearly, reaching a plateau at high source/sink ratios. By contrast, when cytokinin was also applied to the nodes, the number of pods per node increased with increasing source/sink ratio with no plateau, either through an increase in the number of flowers or through an increase in the pod-set probability, depending on the year (Fig. 4). These results indicate that cytokinin plays an augmenting role in pod number increase for plants with high levels of assimilate availability. In another experiment in which synthetic cytokinin (6-benzylaminopurine) was introduced into the xylem stream through a cotton wick during anthesis, Nagel et al. (2001) found that the effect of cytokinin application on pod number and seed yield was consistently significant in the greenhouse, but it was variable in the field. These findings suggest that increased supplies of cytokinins produced in roots and translocated to racemes are able to accelerate pod development in plants with high source/sink ratios, but that the positive effect of cytokinins on flower development can vary with environmental factors.

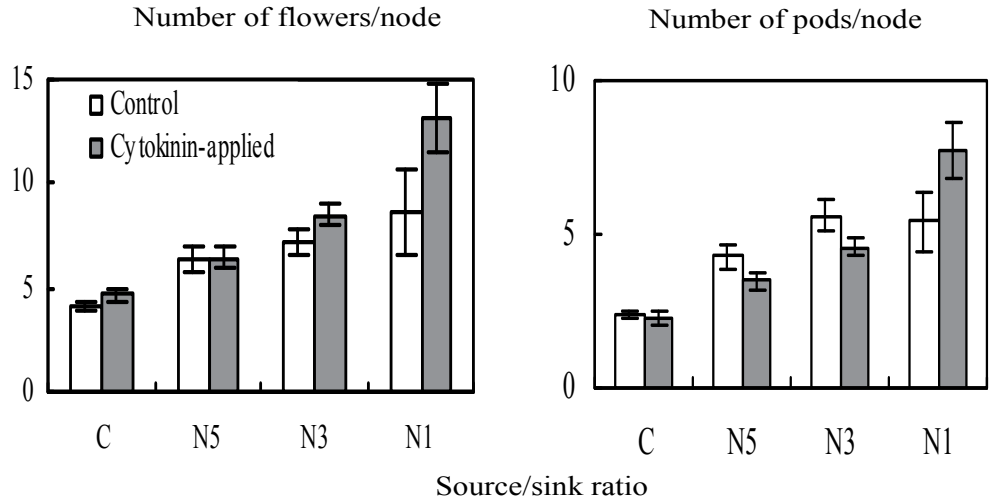

Fig. 4. Effects of source/sink ratio and cytokinin application on the number of flowers per node and the number of pods per node. Values represent the mean ± SE of six plants. C: Control. N5, N3 and N1: Nodes five, three and one node, respectively were allowed to bear racemes. Adapted from Yashima et al. (2005).

As indicated above, the significant effects of cytokinin and IAA application were observed only in pot-grown plants, but their effects were obscured in field-grown plants (Cho et al., 2002; Nonokawa et al., 2007). This instability in the effects of hormones application to field-grown plants may be ascribed to climatic variation across years. Therefore, the specific climatic conditions in which hormones, particularly cytokinins, enhance pod set should be examined to optimize the agricultural use of cytokinins in the field.

2.5 Combined effects of multiple phytohormones on the regulation of flower development

Plant hormones often do not act alone, but in conjunction with or in opposition to each other in such a manner that the final state of plant development reflects the net effect of the

interplay of two or more hormones. Regarding the effects of hormones other than cytokinins on soybean floral development, there have not been many studies (Huff & Dybing, 1980; Oberholster et al., 1991). Views on the role of IAA were conflicting, which could be a reflection of genotypic or cultural differences in those experiments.

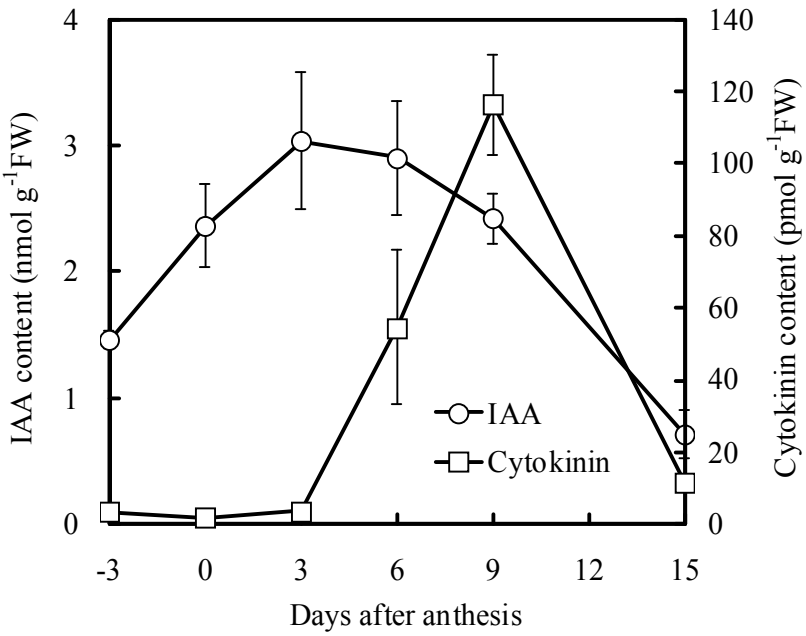

Fig. 5. Changes in the endogenus concentration of IAA and cytokinin (*t*-ZR equivalent) in racemes during reproductive development of soybean plant. Racemes were sampled for analysis at intervals before and after anthesis. Values represent the mean ± SE (n = 6). Adapted from Nonokawa et al. (2007).

Using the same plant material (IX93-100) grown in pots and in the field, we examined changes in the concentrations of endogenous auxin and cytokinin within racemes and the effects of application of the two hormones on pod set. The auxin (IAA) concentration in racemes was high for a long period from pre-anthesis to ca. 10 days following the anthesis (DAA) of the first flower on a raceme, but the cytokinin concentration remained elevated for a shorter period, with a peak at 9 DAA (Nonokawa et al. (2007) (Fig. 5). The two phytohormones are located primarily at different positions within a raceme; the IAA concentration was higher in distal portion of racemes, whereas the cytokinin concentration was higher in basal portions of racemes. IAA application to racemes reduced the number of flowers and pods throughout the reproductive stage. In contrast, the effect of cytokinin (BA) application varied depending on the growth stage: application of BA at around 7 DAA significantly increased the pod-set percentage, while at other stages BA application reduced pod set. Thus, the concentrations of the two endogenous hormones changed in a different manner, with cytokinins exerting a positive effect, and auxin exerting a negative effect on pod set, depending on the growth stage.

3. Difference in susceptibility to water stress between male and female structures

3.1 Water stress as a factor increasing the abortion

Deficient water supply during reproductive development is a major environmental factor that increases the rate of pod abortion in soybean (Kato, 1964; Westgate & Peterson, 1993). While the effect on yield of increased pod abortion at one stage can be offset an increase in pod set at another stage of development, or by an increase in seed mass, frequent or long-term water deficit during reproductive development reduces the yield of soybean (Shaw & Laing, 1966; Sionit & Kramer, 1977). In fact, the rate of abortion substantially increases when soybean plants are subjected to water deficient conditions during reproductive development, particularly during later flowering stages. Water stress imposed during flowering and early pod development reduces photosynthesis and the amount of photosynthetic assimilates allocated to floral structures, which is likely to increase the rate of abortion (Raper & Kramer, 1987). These findings raise the following question. Does water deficit prior to flowering impair the development of male or female structures?

3.2 Differences between pollen and ovule in susceptibility to water stress

To address the above question, we conducted experiments to clarify a) whether water deficit imposed on a soybean plant prior to flowering caused abortion of the basal flowers on the rachis, which are very likely to develop seed-bearing pods under favorable conditions, and b) whether the abortion, if occurred, was due to the impairment of stamen (pollen) or pistil (ovule) function (Kokubun et al., 2001; Kokubun, 2004). IX93-100 plants were grown for this experiment in controlled environment chambers (30/20ºC day/night temperature, 60 ± 10% relative humidity, 15-h photoperiod at 600 μmol m^{-2} s^{-1} PPFD on the plant canopy). Plants were irrigated frequently to maintain soil moisture at 70% of the water-holding capacity of the soil, except during periods of experimental restriction of the water supply. When plants began to flower, the water supply was restricted to a level at which the water potential of the leaves fell below -1.5 MPa. This treatment was imposed on half of the plants and lasted for three days, then plants were rewatered to the original moisture level. After the water restriction treatment was initiated, well-watered (WW) and water-deficient (WD) plants were reciprocally hand pollinated (WW×WD and WD×WW) daily for 10 days. Water stress caused by restriction of watering for three days during the pre-anthesis stage significantly increased the abortion of the basal flowers, which are destined to develop into pods under optimal irrigation (Fig. 6). The experiment also revealed that the pistils of well-watered plants, whether pollinated with water-stressed or unstressed pollen, produced pods at a considerable rate, whereas only a small percentage of water-stressed pistils developed into pods, even when crossed with unstressed pollen (Fig. 7).

In soybean, water deficiency decreased leaf water potential (ϕ_w), resulting in decreased photosynthesis when the leaf ϕ_w was below -1.1 MPa (Boyer, 1970). In our experiment, the leaf ϕ_w of water-deficient plants fell below -1.1 MPa for several days, and photosynthetic rates of the stressed leaves were substantially lower than those of well-watered plants. A decrease in photosynthetic rate may have reduced photoassimilate allocation to reproductive organs, which could have increased the rates of flower abortion in water-deficient plants, particularly during the period from one to four days after the initiation of the water restriction treatment. However, after the leaf ϕ_w of the water-deficient plants recovered to the control level after rewatering, the photosynthetic rates also recovered.

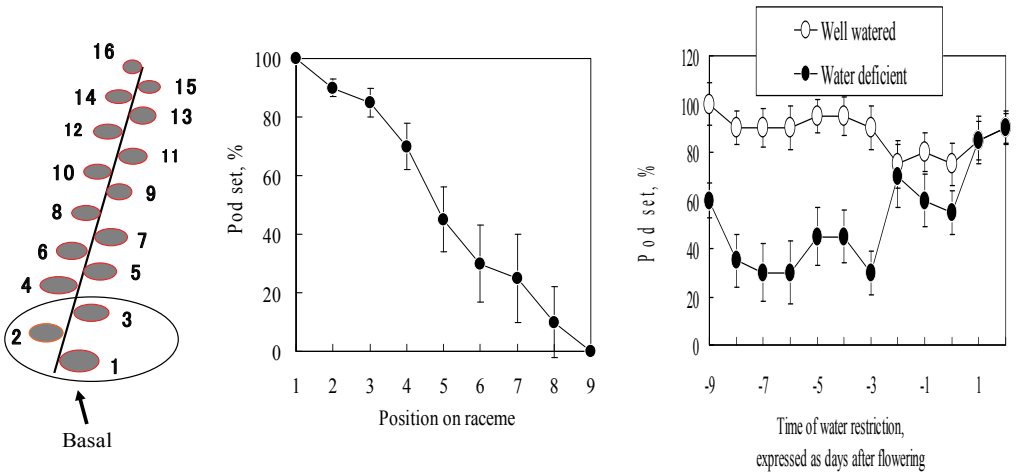

Fig. 6. Pod-set percentage of flowers at different positions in a raceme, and the effect of water restriction on pod-set percentage of flowers located on the basal portion of the raceme. Left: flowers numbered from basal portion on rachis. Center: Pod-set percentage of flowers at different positions on a raceme of well-watered plants. Right: pod-set percentage of well-watered plants and water-deficient plants which were subjected to water restriction treatment for three days imposed for three days prior to flowering. Adapted from Kokubun et al. (2001).

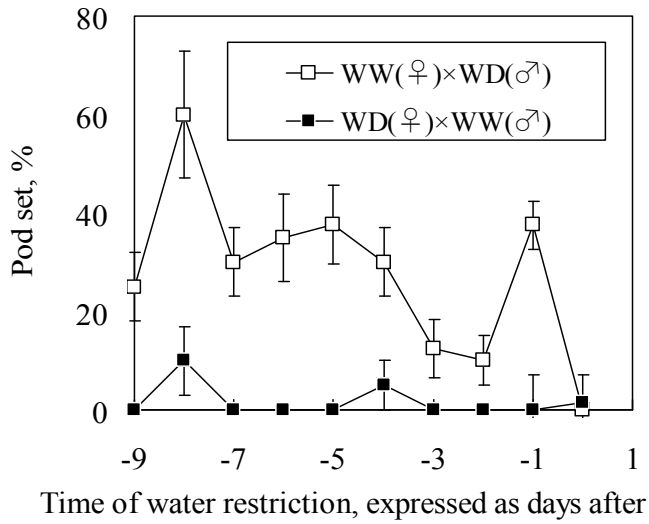

Fig. 7. Pod-set percentage of hand pollinated flowers that were reciprocally crossed between well-watered (WW) and water-deficient (WD) plants. From Kokubun et al. (2001).

Despite the recovery of leaf ϕ_w and photosynthetic rates within a few days following rewatering, the water-deficient plants exhibited significantly lower pod-set percentage than the well-watered plants. This evidence suggests that the decreased pod-set percentage might be caused by factor(s) other than a reduction of photosynthetic assimilation.

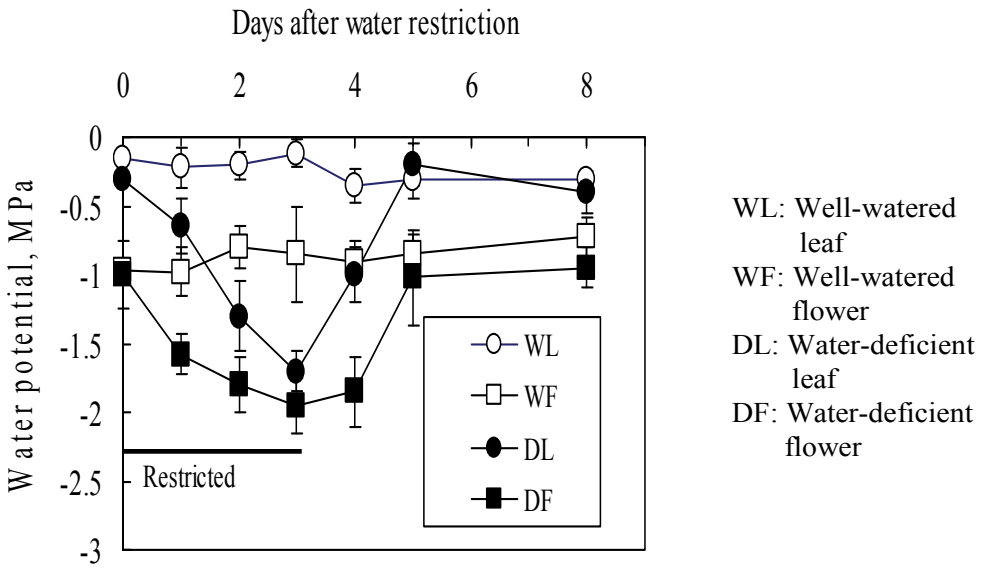

Fig. 8. Effect of water restriction on water potentials of leaves and flowers. From Kokubun et al. (2001).

No data describing the ϕ_w of pollen and pistils had been available, likely due to the technical difficulty of making these measurements. We measured the ϕ_w of whole flowers, and found that the flower ϕ_w was always lower than the leaf ϕ_w, by a difference of nearly 0.5 MPa. After a three-day water restriction, the ϕ_w fell to -1.7 MPa in leaves and -2.0 MPa in flowers, respectively (Fig. 8). In maize, pollen ϕ_w was generally found to be substantially lower than silk ϕ_w, and silk ϕ_w was found to vary with the water status of a plant, while pollen ϕ_w did not (Westgate & Boyer, 1986a). Because maize and soybean have different physiological characteristics and reproductive structures, evidence from maize may not hold true for soybean. However, it is noteworthy that pollen desiccation may not be a factor limiting kernel set in maize, because pollen does not lose viability at ϕ_w as low as -12.5 MPa (Westgate & Boyer, 1986b). In our experiment, pollen which received severe water stress was still able to contribute to the development of a considerable number of pods, suggesting that pollen was not a limiting factor in pod set under water-stressed conditions.

It is difficult to identify directly whether pollen and/or pistils are relatively viable under water-stressed conditions. We indirectly evaluated their viability by measuring the pod-set percentages of flowers that were reciprocally crossed between well-watered and deficient plants (Kokubun et al., 2001). The pistils of well-watered plants, even when pollinated with pollen from water-deficient plants, were capable of initiating pods at a considerable rate, whereas water-deficient pistils developed few pods even when crossed with unstressed pollen. These results suggest that soybean flower abortion caused by a water deficit prior to anthesis is not attributable to an impairment of pollen function, but is more likely due to impairment of pistil function.

There have been few reports on the involvement of phytohormones in regulation of abortion under water-stressed conditions. Lie et al. (2003) found that reduced pod-set percentages induced by water deficit were associated with an increase in the ABA content of

reproductive structures during a period from 3 to 5 days after anthesis. Because cytokinins play such a pivotal role in flower development, clarifying the interacting roles of cytokinins and other phytohormones, including ABA, will be the aim of future research.

4. Conclusion and future research needs

A large proportion of soybean flowers abort during development, a problem which is exacerbated by environmental stresses, particularly by water scarcity. This paper summarized the results of our recent studies, with an emphasis on 1) the involvement of phytohormones in the regulation of abortion (pod set), and 2) the differences in susceptibility of male and female organs to water stress during floral development.

Analyses of the concentrations of two endogenous hormones (cytokinin and auxin) and examination of the effects of their application on pod set have revealed that cytokinins have a positive, and auxin a negative effect, on pod set. Because the effects of application of synthetic cytokinins were unstable in field tests, the use of synthetic cytokinins as chemical enhancer of pod set in the field may be impractical. Genetic improvement of synthesis and transport of endogenous cytokinins from the root system, via conventional breeding or molecular approaches, may strengthen pod-set capacity of agriculturally significant genotypes. Clarification of the physical and chemical properties of the rhizosphere optimizing synthesis of endogenous cytokinins in roots should improve pod set.

Recent molecular and genomic analyses have facilitated the discovery of genes involved in regulating abiotic stresses, enabling genetic engineering using functional or regulatory genes to activate pathways involved in stress tolerance (Shinozaki-Yamaguchi et al., 2006). Of the genes that have been studied so far, *DREB* transgenes are known to impart tolerance to multiple stresses including chilling, heat, dehydration and salinity. Attempts to incorporate the *Arabidopsis DREB* genes, or homologs from other species, into crop species are currently underway. For example, transgenic rice plants overexpressing *DREB1* have exhibited enhanced tolerance to drought. Similar attempts to incorporate these genes into soybean are being undertaken. Sequencing and analysis of a large collection of cDNA clones and subsequent clarification of genome sequence will build the molecular basis to accelerate the creation of novel soybean genotypes (Umezawa et al., 2008; Schmutz et al., 2010). Such recent advances have been magnificent, and we may soon be able to design ideal crops with multiple environmental stress tolerances.

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31

Soybean and Obesity

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The human body is a beautiful specialized engine and there is not any other machine like it. Your body will always do everything possible to stay healthy; in its interior it will sacrifice some things for others in order to conserve the equilibrium.

1. Introduction

Obesity is an alteration of body composition characterized by excess adipose tissue, that involves an imbalance between energy intake and output, which can be produced by a series of genetic, biochemical, dietary and behavioral alterations; therefore, it could be viewed as a multifactorial disease, in which inside the body it can find various regulatory elements of the system of feeling hunger-satiety signals commanded by chemicals that regulate food intake. The environment plays an important role in the development of cultural elements of each individual, determining the amount, type, and frequency of consumption of foods; this also determines their nutritional status. Eastern societies have based their feeding in legumes such as soybeans. These groups have low rates of obesity, which has drawn wide attention towards these foods and especially soybeans. Soy is a valuable food from a nutritional standpoint, because its protein is among the highest biological value, its lipids are mostly polyunsaturated, contains fiber and carbohydrates, is rich in vitamins and minerals; additionally containing various phytochemicals with antioxidant and anti-obesity activity. Several studies in experimental animals and humans have shown that a diet whose protein content is based on soy protein, have a beneficial effect in obese individuals as measured by the decrease in body weight associated with the body calorie intake, liver triglycerides, cholesterol, hepatic synthesis of fatty acids, enzymes responsible for mRNA synthesis of fatty acids and hyperinsulinemia. Moreover, the soy protein has been shown to contribute to the increase in LDL receptors, the increase in insulin sensitivity, and also in the activity of enzymes responsible for fatty acid metabolism, especially those involved in the β-oxidation. Considering the above, in this chapter shall be reviewed how the consumption of soybean and soybean sprouts may reduce the incidence of obesity and diseases closely related to it as the Diabetes Mellitus and Cardiovascular Disease.

2. Obesity

Through the years, the world has lived terrible wars, in which many people survived in extreme conditions. Under these circumstances, the body created defense mechanisms that would allow it survives on similar conditions relying on fat. This is particularly important in any energy deprivation state such as prolonged fasting, skipping meals, performing to much exercise and not feeding correctly. However, nowadays there is a general tendency to cumulate fat; the excess of this component is associated with obesity.

When a disease is produced by multiple factors, a lot of different definitions and etiological explanations arise, such is the case of obesity, in which the conceptual analysis can be as diverse depending on the focus from which it is signaled. The definition varies from the clearest and simplest concept***: "the alteration of the body composition characterized for an excess of adipose tissue"***, through the most complex concept: ***"Unbalance between food intake and energy expenditure produced by series of genetic, biochemical, dietary and behavior alterations."*** Besides, the problem gets worse when it involves different population factors as ethnicity, dietary habits and the decrease of the vulnerability to diseases that previously limited life expectancy and conferred the opportunity to gain weight.

In many affected subjects it is clear that overfeeding and low physical activity causes an accumulation of excessive body fat. Currently there are many individual differences in the energy processing and tendency to calorie storing. Below we will provide a brief description of the great complex interrelated factors. Exposing different causes, we can constitute a group of syndromes based on diverse origins. Unfortunately it is a combination of these factors the ones that affect the majority of the population (**Figure 1**).

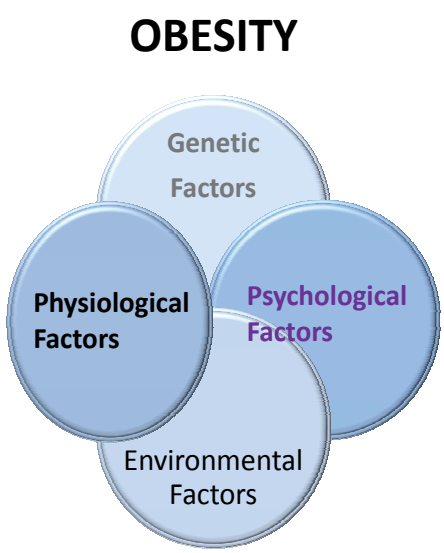

Fig. 1. Obesity Etiology. The obesity has origin in four main factors: genetic, physiological, environmental and psychosocial.

Genetic Factors

Genetics seems to establish the obesity scenario; nevertheless, diet, exercise and life style will be the ones determining the magnitude of the problem, for that reason it is convenient to analyze the physiological pathology of obesity from a wider focus.

Some studies based on comparing the behavior of identical twins exposed to different environmental conditions established that the impact of genetics, as an obesity causal factor, was approximately 30-40%, while environment was ascribed 60-70% (Bouchard, et al. 1993). Although other researches report an interval between 20 to 80%, depending on some particular characteristics of obesity or age of appearance (Groop & Orho-Melander, 2001).

In the research of the genetic factors that modulate satiety and body mass, several studies have been realized in animal models. In transgenic animal models has been studied the role of genes involved in the body fat increase as the effect related to the suppression of melanocortin-4 receptor, the reduction of glucocorticoid receptor in brain, the over expression of the corticotrophin releasing hormone, suppression of uncoupling protein in brown adipose tissue, the over expression of agouti protein, the suppression of β-3 adrenergic receptor and dysfunction of GLUT-4 in fat and intracellular adhesion molecule-1.

In humans, there are clearly identified genetic syndromes in which obesity is a characteristic, such as Prader-Willi and Bardet-Biedl syndrome. However, obesity-related genetic alterations have been identified only in very few individuals. These alterations may be mutations in leptin and its receptor, melanocortin-4 receptor, propiomelanocortin, endopeptidase, prohormone convertase-1, in β-3 adrenergic receptor, peroxisome proliferator-activated receptor-γ2, among others (**Figure 2**). Despite the discovery of these single-gene disorders, the genetic model in the most cases of obesity in humans is non-Mendelian polygenic. In the genomics of obesity in humans, it has been determined that there are at least 15 genes that were significantly associated with body fat or the percentage of body fat, and 5 genes are associated with abdominal visceral fat (Sims, 2001), although surveys of large populations have identified over 250 genes, markers and chromosomal

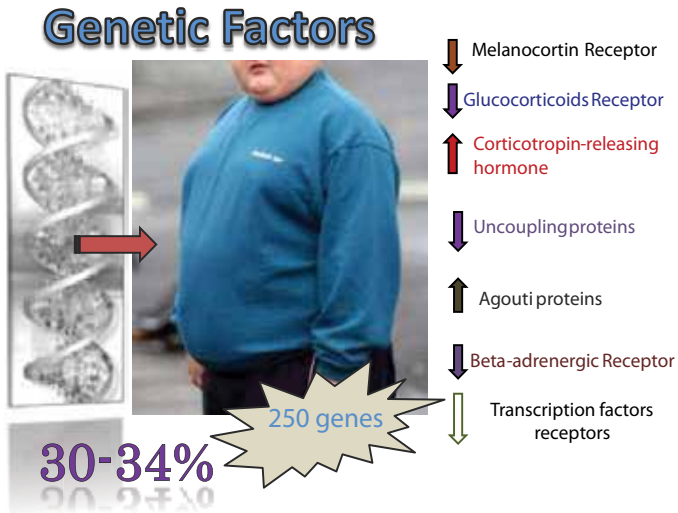

Fig. 2. Genetic factors of obesity. Two hundred and fifty genes have been related with obesity. Melanocortin and glucocorticoid receptors, corticotrophin-releasing hormone, uncoupling proteins, Agouti protein and transcription factors

regions associated with obesity (Perusse et al., 2000). Therefore, in humans, the potential interactions among multiple genes and their interaction with the environment, lead to the phenotypic expression of obesity.

Physiological factors

The accumulation of body fat requires an increment on the relation between intake and energy expenditure during a long period. However the simplicity of this premise vanishes when the modulator effect of other physiological variables as intrauterine development influences, hormonal functions (growth hormone and reproductive hormone) and the fine regulation of the feedback systems that try to keep a constant energy balance are included.

In a study of obese and non-obese subjects with periods of caloric restriction and excess of calorie consumption, it was observed a decrease in the total and resting energy expenditure when they lost 10-20% body weight, possibly due to the adaptation of caloric deprivation. With the increase of weight it was observed increment on the energy expenditure, which delayed weight gain. These findings suggest the existence of a compensator mechanism that tends to maintain body weight (Leibel et al., 1995).

Physiologically, there are many hormones and peptides that act in a feedback system composed of gastrointestinal system, adipocytes, hypothalamus and the hypothalamic-pituitary-adrenal axis. The main appetite suppressants, at gastrointestinal level, are the glucagon-like peptide-1, the 6-29 amino acid segment of glucagon, cholecystokinin, enterostatin, the peptide YY 3-36 and the ghrelin. In addition, gastric distension and contractions produce signals of satiety and decreased appetite. This highly accurate system is also influenced by the serum glucose concentrations. When glycemia is reduced about 10% it causes an increase of appetite (Campfield et al., 1985).

The discovering of leptin and its receptor interactions has established new paths for investigation on obesity physiopathology. Although it have been established that leptin is a fundamental protein in the energy equilibrium in rodents, the physiological role and the regulatory mechanisms of its secretions in humans have been object of great interest.

This protein hormone is secreted by adipocytes in response to activation of insulin receptors, adipogenic hormones, adrenergic receptor, and also when detecting fat repletion. That secretion has a periodicity of 7 min and diurnal variation. When the hormone is liberated, it stimulates to its receptor located in the paraventricular nucleus of the hypothalamus which induces the release of the neurpeptide, whose main functions are appetite suppression and the stimulation of the thyroid function, the sympathetic nervous system and, of the thermogenesis. All these effects tend to limit weight gain. Therefore, the adipocyte and the hypothalamus form a classic feedback mechanism in which the adipogenesis and lypolisis are revealed as highly regulated processes (Figure 3).

Aside of this path there are many afferent signals that affects the intake and energy expenditure. The adipocyte also receives signals from the gastrointestinal tract, the peripheral nervous system and the endocrine system. The integration of these systems involves the adequate adaptation to food deprivation periods, but it also leads to a poor adaptation to overfeeding.

Several studies have confirmed direct interaction between hyperleptinemia and the percentage of body fat that suggests a leptin resistance (Rosenbaum et al., 1997). This resistance can occur at different levels: in the transport across the hematoencephalic barrier, in its hypothalamic receptor and/or other neural circuits in which this hormone influences. Recent studies have shown hypertrigliceridemia-mediated alterations in the transport of leptin through the hematoencephalic barrier (Banks et al., 2004).

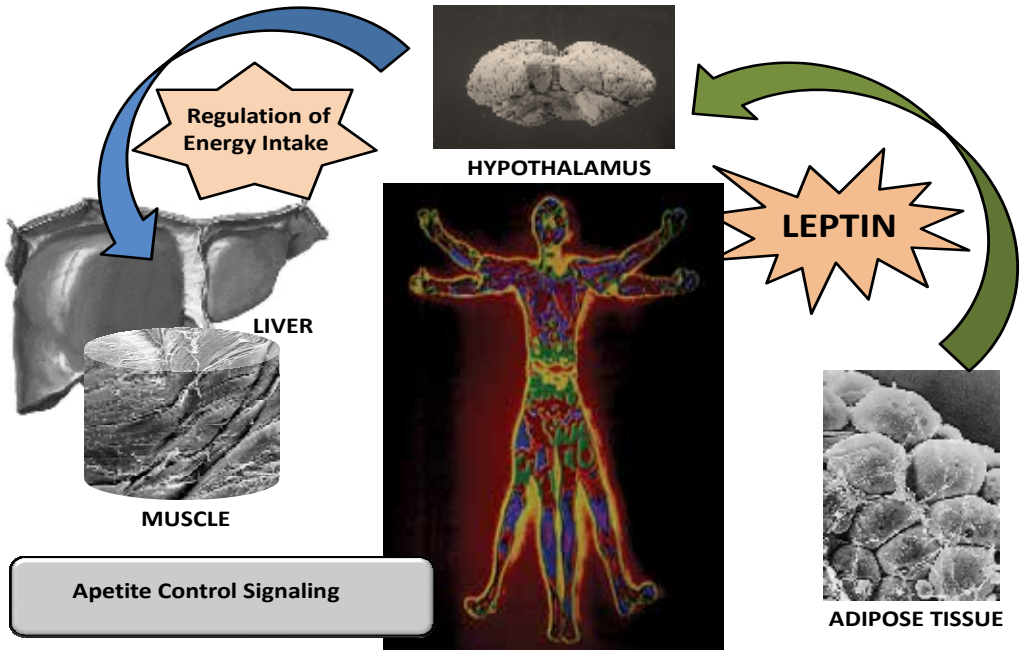

Fig. 3. Role of leptin on energy intake. Leptin is a 16 kDa protein hormone that plays a key role in regulating energy intake and energy expenditure, including appetite and metabolism.

Besides the role of leptin in the origin of obesity, have emerged reports about the deleterious effect of hyperleptinemia on the complications of obesity. It has been reported that leptin causes insulin resistance in hepatocytes (effect mediated by the dephosphorylation of insulin receptor substrate-1) and has fibrosis-inducing effects in various chronic liver diseases of metabolic or toxic etiology (Cohen et al., 1996; Crespo et al., 2002; Leclercq et al, 2002).

The hypothalamus exerts control over appetite, satiety and thermogenesis. To carry out this function, the hypothalamus requires mediators such as afferent hormonal signals (leptin, glucose), and regulation by the autonomic nervous system, through vagal afferents from gastrointestinal system and even from oropharyngeal stimulus. The main sites involved in this regulation are the nucleus of the solitary tract, the arcuate and paraventricular nucleus, as well as the ventromedial and lateral regions of the hypothalamus and amigdala. Leptin acts on the control of satiety in the arcuate and ventromedial nucleus. When there is destruction of the ventromedial hypothalamus, leptin is unable to suppress food intake at this level. In this process is also involved a large number of monoamines (such as norepinephrine and serotonin) and other neurotransmitters or neuromodulaters (Campfield, 2000) (Figure 4).

Other metabolic abnormalities related to obesity pathogeny are the defects on the lypolisis regulation (Sheehan & Jensen, 2000), actions in adipose tissue of rennin-angiotensin system (Goossen et al., 2003), tumor necrosis factor (TNF) (Bulló et al., 1999), and several neuropeptide systems (Cummin & Schwartz, 2003; PI-Sunyer, 2002). In this final sections, it

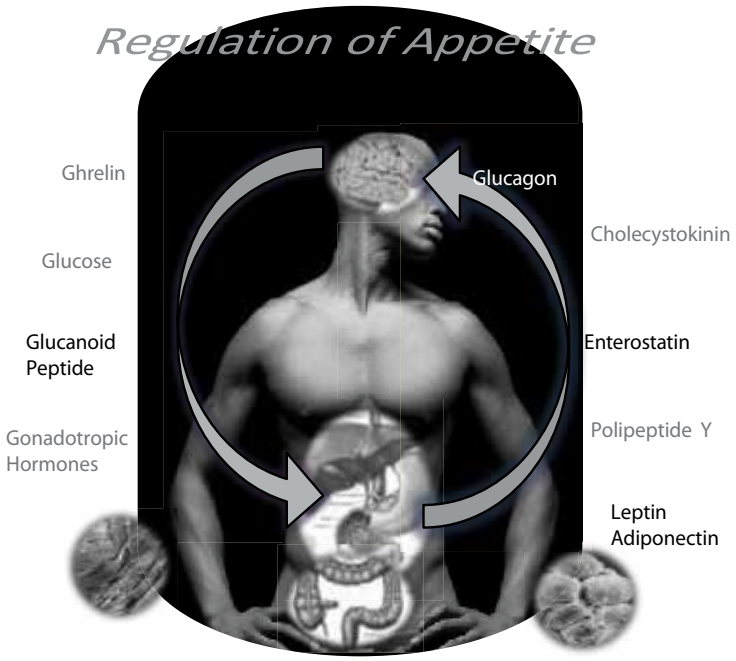

Fig. 4. Appetite control. The fluctuation of some molecules and hormone levels results in the motivation of an organism to consume food.

has been implicated the autonomic nervous system imbalance with obesity and metabolic syndrome. In animal models with beta-adrenergic receptors suppressed, there is a severe obesity due to failure in diet-induced thermogenesis (Bachman et al., 2002). Pima population studies have linked the low adrenal sympathetic activity to weight gain (Tatarann, 1997).

Another etiological factor of supreme importance is the aging process, in which, there are various elements that determine the weight gain and the changes in body fat distribution, such as the decreased physical activity and metabolic responses to dietary or environmental modifications; hormonal changes, for example, the decline in estrogens and progesterone that alters the adipocyte biology; the emergence of comorbidities, as well as behavioral disturbances and depression among others.

As we move on obesity knowledge, new routes and pathophysiological interactions are discovered and it will be increasingly difficult to attribute them a greater pathogenic impact.

Environmental factors

The exaggerated increase in the prevalence of obesity in the last 20 years has been favored by changes in the environment that determine the increasing energy input and the reduction of the physical activity, included individuals without genetic predisposition.

The environment influence can initiate since gestation. Diverse studies have related obesity with prenatal exposure to an excess of caloric intake, diabetes, smoking and the lack of breastfeeding (Dabelea, et al., 2000; Power & Jefferis, 2002; Silverman et al., 1998).

The weight gain is very common in people that quit smoking. This fact has been attributed to the suppression of nicotine exposure. The average weight gain is about 4 - 5 Kg in 4 to 6 months. It has estimated that suppression of smoking increase to 2.4 times the risk of obesity in comparison to non smoking people (Flegal, 1995).

The increasingly sedentary lifestyle is an important determinant of obesity. Some authors suggest that the decrease in energy expenditure may have more impact than the increase in the caloric intake (Prentice & Jebb, 1995). A health study reported that watching television for 2 hours a day is associated with an increment of about 23 and 14% of obesity and diabetes risk respectively (Hu et al., 2003). The reduction in the number of hours watching television has been demonstrated to decrease the appearance of obesity (Robinson, 1999) Obesity is more prevalent in adults with physical or sensorial incapacity, or with mental diseases (Levine et al., 2000).

The relationship between environment and physiology is an important factor on the obesity epidemic in industrialized countries. It has emerged an abundant availability of food; the food intake prevails at the end of day and the physical activity has been reduced. This supposed "environment mutation" causes that the susceptible central nervous system (CNS) loses its capability to detect the internal and external rhythm. Since CNS employs the autonomous nervous system (ANS) to regulate internal rhythm, it has been proposed that unbalance and loss of rhythm could be the most important mechanisms in the origin of the metabolic syndrome. The metabolic syndrome is a clinic concept that is characterized by the association of Diabetes Mellitus, glucose intolerance, hypertension, central obesity, dyslipidemia, micro albuminury and atherosclerosis (Kreier et al., 2003).

Psychosocial factors

There are descriptions about some psychiatric disorders related to obesity. The "night eating" syndrome is defined as the consumption of at least 25% (generally more than 50%) of the energy between dinner and the next morning breakfast. It is an eating disorder of the obese that is accompanied by sleeping disorders and it has been considered as a component of the sleep apnea. It occurs in 10-64% of the obese subjects. Binge eating disorder is a psychiatric disease characterized by ingesting large amounts of food in a relatively short time period, with the subjective feeling of control loss and without a compensatory behavior. Its prevalence is 7.6 to 30% in different groups of obese (Stunkard et al., 1996). Progressive hyperphagic obesity starts from childhood, and affected individuals which generally exceed 140 Kg of weight at age 30 (Bray, 1976). Obesity is more prevalent in subjects of low socioeconomic level, but it has not determined the precise reason of this finding.

Although increasingly new evidence about genetic influence and neuroendocrine unbalance of obesity are emerging, is necessary to consider a holistic model in which the biological and psychological factors interact in a complex way. Thus we can expect better results in the comprehension, prevention and treatment for this important health problem.

There have been many attempts to find an adequate treatment that contributes to diminish the amount of body fat of individuals that for any of the previously mentioned reasons have acquired a *Body Mass Index (BMI)* above to the standard established for health care (**Figure 5**). Due to the multifactorial etiology of this disease, it has been complicated to find a unique or standard therapeutic, since each individual has a different development of the disease.

Looking for options that can contribute to the decrease of the exceeding body fat in the population that have obesity, it has been observed that some populations tend to develop in a lesser frequency this disease, such is the case of the eastern populations which have an obesity incidence about 5% in subjects over 18 (WHO, 2002), compared to the highest incidence in countries as USA, where 1 of every 3 habitants are obese. It has been observed that the eastern populations have healthier life styles and also a diet where soybeans have long been a major component.

BODY MASS INDEX (BMI)

IS ONE OF THE MOST IMPORTANT CRITERIA TO KNOW THE NUTRITIONAL STATUS OF ADULTS, CONSIDERING FACTORS AS CURRENT WEIGHT, HEIGHT AND SEX OF THE PERSON

PARAMETERS ARE RANGING FROM 18 TO 24.9

BMI

OBESITY: 27

OVERWEIGHT : **Over 25 and under 27**

Malnutrition : **EQUAL TO OR LESS THAN 18**

http://www.issste.gob.mx/aconseja/índice.html

Fig. 5. BMI, over 80 developing countries have adopted BMI as part of their national policy to improve child health (WHO, 2002).

3. Soybean

Chemical composition

Soy (*Glycine max*, family Leguminosae, subfamily Papilionoidae) is a species of legume native to East Asia. The height of the plant varies from 0.50 m to 1.50 m. It has large trifoliate and pubescent leaves and the fruit is a hairy pod that grows in clusters of 3–5, and usually contains 2–4 spherycal or elongate seeds (**Figure 6**). This legume has important feeding characteristics. Japan was the first country to consider the nutritional benefits of the soybean. The protein concentration of the soybean is the largest of all legumes. The soybean contains in sufficient amount the indispensable amino acids to satisfy the healthy adult requirements (Ridner, 2006). This legume also has lipids that are rich in poly-unsaturated fat acids, standing out for the high content of linoleic acid (51%). Approximately 1.5 to 2.5% of the lipids present in the soybean are found as lecithin, this has an emulsifier function. We can found also in considerable proportions tocopherols and vitamin E, both of them can act as antioxidants (FAO, 1992)

Fig. 6. Soybeans. Soy its a legume containing between one and four yellow or black seeds

This is the legume with the highest content of galactooligosaccharides, which constitute an important prebiotic (Ridner, 2006). The soybean also has significant amounts of minerals like: calcium, iron, copper, phosphorus and zinc; however, the availability seems diminished by the presence of phytate; this disadvantage decreases with cooking, fermentation or germination process (FAO, 1992).
In addition, soybean contains a series of compounds which are known to have specific functions in both the plant and the organisms that consume it; such as the isoflavones genistein, daidzein and glycitein (**Figure 7**), as well as flavonoids, lignin, saponins and sterols. Some of them have showed to have antioxidant capacity (Dixon, 2004; Mikstacka et al., 2010).

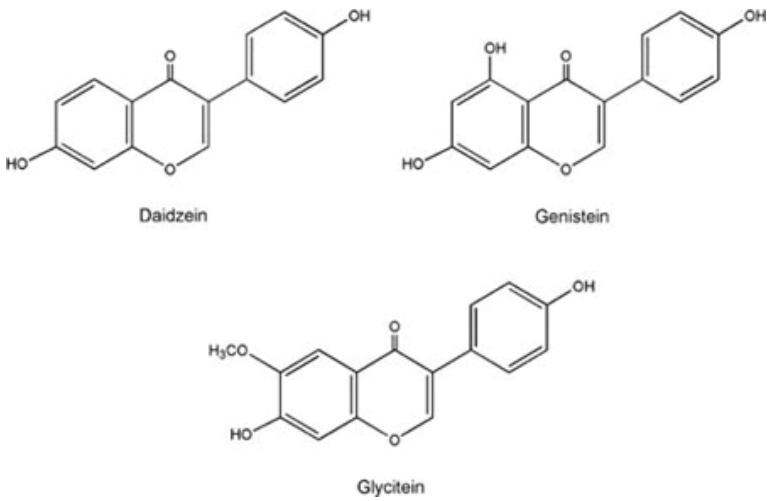

Fig. 7. Soy Isoflavones. Isoflavones comprise a class of organic compound, often naturallity occurring, related to the isoflavonoids. Many act as phytoestrogens and antioxidants.

Soybean and obesity

Metabolic syndrome is a combination of medical disorders that increase the risk for cardiovascular disease and Type II diabetes. Obesity may lead to metabolic syndrome because it increases the prevalence of visceral obesity, insulin resistance, increased very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol, decreased high-density lipoprotein (HDL) cholesterol, elevated triglycerides, hypertension (high blood pressure), and fatty liver, which are important factors of metabolic syndrome.
Visceral obesity, a hallmark for the male obese phenotype, which is characterized by excess fat storage around the abdomen, is the prime cause of metabolic abnormalities; therefore, men are usually at higher risk of cardiovascular disease than women. Along with the realization of many studies it has been observed that animals and humans fed with seeds of soybean tend to lose more weight than those who were fed animal protein such as casein (Hurley et al., 1998; Iritani et al., 1997).

The mechanisms of action by which the soy protein isolates have beneficial effects on obesity are not completely clear yet. However, there are many studies confirming that different components included in the soybean have specific functions in the human body such as the absorption of the lipids, the insulin resistance, fat acid metabolism and other hormonal, cellular and molecular changes related with adipose tissue (Wang & González, 2005).

Soybean, obesity and Diabetes Mellitus II

Murine Models

Some researches performed on animal models have demonstrated that the consumption of soy has effects on the diminution of glucose levels and in insulin resistance, and also the increase in insulin receptors. Hurley et al. (1998) examined the metabolic effects of different types of protein in male Sprague-Dowley rats. Plasma glucose and insulin concentrations, in addition to total and metabolizable energy intake and body weight gain, were lower in rats fed soy protein isolate- cornstarch diet compared with casein-cornstarch diet. In the study of Lavign et al. (2000), it was evaluated the effect of feeding different types of proteins (cod, soy and casein) in Wistar rats finding that cod and soy protein improved fasting glucose tolerance and peripheral insulin sensitivity when compared with casein.

Subsequently, based on these findings, further studies were done in animal models with specific features of obesity and various chronic degenerative diseases.

Iritani et al. (1997) investigated the effects of different dietary fatty acids and proteins on glucose tolerance and insulin receptor gene expression, in Wistar fatty rats (genetically obese, noninsulin-dependent diabetes mellitus). After three weeks, dietary soybean protein could help to reduce the insulin resistance, but only when a diet low in polyunsaturated fatty acids was consumed. In addition, dietary soybean protein stimulated insulin receptor gene expression in comparison with casein.

Another possible mechanism of action of soy protein is by stimulation of adiponectin, a cytokine produced by fat cells that has an important role in regulating differentiation and secretory function of adypocytes by increasing insulin sensitivity (Anderson et al, 2004). High blood levels of this hormone reduce obesity (Arita et al, 1999; Weyer et al,. 2001). There is a report indicating that the consumption of soy isolate reduces the adiponectin concentration in Wistar rats genetically engineered to develop obesity (Dietze et al., 2005).

In another study it was compared the effects with an energy-restricted diet, low fat intake (5%) and high protein content (35%) from soy and casein in male genetically obese yellow KK mice. The plasma total cholesterol and glucose levels as well as the body fat and body weight were lower in mice fed soy protein isolate (SPI) compared to group fed whey protein isolate (Aoyama et al., 2000).

Nagasawa et al. (2002) studied the effects of energy-restricted diet containing soy protein isolate (SPI) on body composition, blood glucose, lipid and adiponectin levels and the expression of genes involved in the metabolism of glucose and fatty acid in male obese mice KK-A. Body weight and brown adipose and mesenteric tissues were lower in animals fed SPI compared with animals fed casein diet. There no was significant difference in gene expression between both diets. It was concluded that SPI diminishes body fat quantity and glucose levels more efficiently than hypocaloric diets based in casein in obese mice.

The content of isoflavones of the soy protein is important to the antiobesity effect, because has been shown to decrease fat accumulation in animal models of obesity (Manzoni et al., 2005; Banz et al., 2004).

Human research

Positive results obtained with murine models supported researches to evaluate the effect of SPI diets in human subjets.

Mikkelsen et al. (2000) compared the effect of feeding low-fat diets containing pork, soy protein or carbohydrates in 12 young people with overweight and grade II obesity and a body mass index (BMI) between 26 and 32. Energy expenditure was measured in a respiratory chamber and was significantly higher (by >3%) in subjects consuming protein-rich diets. This indicates that protein has a thermogenic and satiety effect greater than carbohydrates, a fact that may be relevant in the prevention and treatment of obesity.

Allison et al. (2003) evaluated the efficiency and the safety of a low-calorie diet based on soy in the treatment of obesity in obese individuals. Soy diet induced a higher weight loss than animal protein diet. Anderson et al. (2004) demonstrated, in human studies, that SPI consumption had an effect on reduction of appetite compared to egg protein.

In another study (Deibert et al, 2004) it was compared diets from two different lifestyles (balanced nutrient reduction diet, and soy protein substitution diet with and without physical activity program). It was shown that a high-soy-protein low-fat diet induced a higher weight loss through fat but not muscle mass in overweight and obese people.

Soybean, obesity and cardiovascular diseases

Murine models

There are *in vivo* evidences showing that soy protein influences the lipogenesis on the liver. It was demonstrated that triglycerides (TGC) in the blood and especially in the liver were decreased by the consumption of a diet with a protein input based on soybean. These effects were associated with the activity reduction of lipogenic enzymes, particularly dehydrogenase 6-phosphate, malic enzyme, synthetase fatty acid, as well as acetyl CoA carboxylase (ACC) meaning that soy protein decreases the liver TGC inhibiting the synthesis in the same (Xiao et al., 2006)

Recently in a study with obese Zucker rats that were fed isoflavones-rich SPI, it was showed a decrease on fatty liver and reduced alanine and aspartate transaminases levels in plasma. These effects were accompanied by an increase in mitochondrial and peroxisomal β-oxidation activity and acetyl CoA carboxilase activity, among others. The ACC is the rate limiting enzyme in catalyzing the carboxylation of acetyl CoA to form malonyl CoA and is the main enzyme in the biosynthesis of long chain fatty acids. Aoki et al. (2006) reported that SPI feeding decreased the hepatic contents of ACC alpha mRNA mainly by regulating PI promoter in rats.

In addition, it was shown that soy protein decreased levels of TGC in rat liver also reducing the adipose tissue weight. These changes were associated with increased gene expression of skeletal muscle enzymes which produce the fatty acid oxidation, including carnintin palmitoyl-transferase (CTP1), β-hydroxyacyl-CoA dehydrogenase (HAD), acyl CoA-oxidase and the medium-chain acyl CoA-dehydrogenase activities.

It has been reported that soybean saponins can also reduce serum cholesterol (Oakenful et al, 1984), but their role in lipid metabolism is not completely clear. A study on hamsters reported that a diet containing soy saponins without isoflavones induced a reduction in cholesterol and TGC levels as well as in total cholesterol/HDL ratio. The phospholipids may have an antilipidemic effect, since they reduced the hepatic synthesis of fatty acids along

with the malic enzyme, glucose-6-phosphate dehydrogenase and pyruvate kinase activities in a study in rats (Rouyer et al, 1999).

Human studies

The consumption of soy protein in a group of patients with hyperlipoproteinemia, reduced the low density lipoproteins (LDL) cholesterol and TGC levels by 16.4% and 15.9%, respectively, in blood and liver, besides to reduce intestinal absorption of endogenous and exogenous cholesterol (Wright & Salter, 1998). Soy protein has also been shown to directly affect LDL hepatic metabolism and the activity of LDL receptors. Lovati et al. (1987) found that SPI diet dramatically affected the degradation of LDL by mononuclear cells.

In another research in humans, it was found that soybean can reduce the insulin/glucagon ratio which increases its antihypercholesterolemic effect (Gudbrandsen et al., 2006; Hubbard et al., 1989). In a similar work it was evaluated the effect of a hypocaloric diet containing casein or soy protein in a long and short term assays. The measured parameters were body weight reduction in subjects with 50% over ideal weight and lipoproteins levels. All participants lost weight (in a similar way in both diets), but the high density lipoproteins (HDL) level was lower in individuals consuming casein. Authors concluded that soy protein could have a greater benefit than casein in patients who need hypocaloric diet for long periods (Bosello et al., 1998).

In a twelve-week trial with obese subjects, it was compared the soy protein effects against milk protein in hypocaloric diets of 1200 kcal/day. People who consumed soy lost more weight than those who consumed milk (9% vs 7.9%) but the difference was not statistically significant. However, the reduction of LDL cholesterol and TGC levels were significant (Anderson et al., 2005).

Soy and its relation with PPAR and SREBP-1

The type of foods that are ingested has effect in the phenotype of an individual by modulating transcription factors that modifies the expression of genes and determines individual characteristics.

The transcriptional control of these genes is mediated by a family of transcription factors designated as sterol regulatory element binding proteins (SREBPs) (Torres, 2006). The isoflavones acts through multiple mechanisms that include inhibition of cholesterol synthesis and esterification of fat (Orgaard & Jensen, 2008). Isoflavones regulate the activity or the expression of SREBP-1. In addition, SREBP-1 regulates the expression of stearoyl–CoA desaturase 1 (SCD-1), D5 desaturase and D6 desaturase involved in fatty acid desaturation to form monounsaturated and polyunsaturated fatty acids. The balance between saturated, monounsaturated and polyunsaturated fatty acids is essential for the formation of triglycerides and phospholipids in the liver. Hepatic SCD-1 activity determines the metabolic fate of endogenous lipids, driving newly synthesized fatty acids preferentially to triglyceride esterification and very LDL (VLDL) assembly and secretion rather than mitochondrial influx and C-H oxidation (Torres, 2006). Soy protein causes an increase in bile acid secretion and inhibits intestinal cholesterol absorption.

The mechanisms by which soy protein prevents triglyceride accumulation are by reducing hepatic fatty acid and triglyceride biosyntheses and by increasing fatty acid oxidation through the activation of the transcription factor peroxisome proliferator-activated receptor α (PPARα). PPARα is a ligand-dependent transcription factor of the nuclear receptor superfamily. A soy protein diet up-regulates PPARα gene expression (Tovar, 1998). PPARγ

is highly expressed in adipose tissue and is involved in critical physiological functions such as adipogenesis and glucose and cholesterol metabolism. This transcription factor induces a preadipocyte differentiation program leading to mature functional adipocytes. PPARγ stimulates fatty acid uptake and triglyceride esterification in a concerted action with SREBP-1 that regulates lipogenesis to fill the lipid droplet. The differentiation of adipose tissue protect to other organs of fat accumulation called lipotoxicity. Many studies have demonstrated that the soy protein associated isoflavone genistein is able to activate PPARγ, resulting in an up-regulation of adipogenesis and probably fatty acid uptake from plasma (Mezei, 2003).

Another molecule related in the energy homeostasis is AMP-activated protein kinase (AMPK). AMPK induces a cascade of events within cells in response to the ever changing energy charge of the cell. The role of AMPK in regulating cellular energy charge places this enzyme at a central control point in maintaining energy homeostasis. In a recent research the consumption or administration of soy peptides increased AMPK level, and promoted the fat mass loss (Jang, 2008).

Soybean germination, obesity and cardiovascular diseases

Germination of soybean and changes in chemical composition and aminoacid profile

Germination is a simple, low-cost process that produces a natural product, eliminates or inactivates certain antinutritional factors, and increases the digestibility of proteins and starches in legumes. Germination process has been developed to overcome the disadvantage of soybean seeds used in food products. Germination causes changes in secondary metabolite distribution, mobilizes the reserve proteins stored in the cotyledon protein bodies, changes amino acids composition (Davila et al., 2003) and produces intermediate molecular weight peptides (Mora-Escobedo et al., 2009). Germination could improve the nutritional and nutraceutical properties of legumes by modifying metabolites content and generating peptides and amino acids with possible biological activity. Paucar-Menacho et al., (2009) found that germination of soybean for 42 h at 25 °C resulted in an increase of 61.7% of lunasin, decrease of 58.7% in lectin and 70.0% in lipoxygenase activity. Optimal increases in the concentrations of isoflavone aglycones were observed in combination of 63 h of germination and 30°C. A significant increase of 32.2% in the concentration of soy saponins was observed in combination of 42 h of germination at 25 °C.

Our group conducted an investigation in which soybean seeds (BM2 donated by the Forestry Research Institute of Agricultural and Livestock, INIFAP, México) were germinated according Mora-Escobedo et al. (2009). The germinated seeds were harvested at different times (0, 48, and 72 h), lyophilized and ground. In order to evaluate the effect of germination on the main nutrients provided by the soybeans, were characterized the flours obtained at different times of germination. Table 1 shows the results. The protein content of the soybean was of 34.85 ± 0.65 to 35.63 ±0.87, with no significant difference ($p > 0.05$) between different times of germination. In germinated flour was an increase in the lipid content of 11.14% ($p \leq 0.05$) for 48 h and 16.18% ($p < 0.05$) for 72 h compared to ungerminated flour. The increase in lipid content observed in this study probably was due to the decrease of other seed components, as some carbohydrates. Paredes-López and Mora-Escobedo (1989) reported a decrease in starch content of 25% in germinated amaranth flour by 72 h.

Soybean flour	Carbohydrates	Protein	Fat	Ashes
Ungerminated	37.52	34.85 ± 0.65	22.43 ± 1.04	5.20 ± 0.03
48h of germination	34.66	35.63 ± 0.87	24.93 ± 0.8*	4.78 ± 0.02
72h of germination	33.62	35.18 ± 0.64	26.06 ± 0.8*	5.14 ± 0.10

*p<0.05 regarding non-germinated flour

Table 1. Composition of soybean flour with different times of germination (g/100 g meal dry basis)

Amino acid content of germinated soybean proteins

The method proposed by Rickert et al., (2004) was used with slight modifications in order to obtain soybean proteins. The protein yield was 59.7%. The total protein content and amino acid analysis of germinated soy proteins are shown in Table 2. The protein content in the sample obtained at 72 h of germination decreased. The amino acid composition was altered significantly compared to the different times of germination. Analyzing the essential amino acids, it was observed in the isolate of soy germinated for 48 h, a significant increase ($p \leq 0.05$), between 10 and 21% for phenylalanine, leucine, threonine, and isoleucine. These results were consistent with those reported by Dasinova (1994), who observed an increase of 5 to 23% in soybean, lentils, barley and wheat sprouts, for the essential amino acids leucine, phenylalanine and tryptophan. By extending the germination time to 72 h, this group of amino acids showed a more significant increase of 16 to 25%. Valine was the amino acid with the greatest increase and it was seen in the germination of 48 h (54.06% at $p \leq 0.05$).

Lysine is an amino acid important for human consumption, since it intervenes in the metabolism of carbohydrates and fats and is needed for protein synthesis. It is the main limiting amino acid in cereals, especially wheat. The isolate obtained from soybean germinated for 72 h, was able to increase the contribution of lysine by 26% ($p \leq 0.05$). Contrary to expectations, the methionine content increased with germination time up to 23.98% at 72 h ($p \leq 0.05$). This is the limiting amino acid in soy and the germination was able to increase their concentration; a significant event when viewed from the nutritional point of view. However, methionine is the precursor of homocysteine, a compound that at high levels in the blood can be an independent risk factor for cardiovascular disease (Steed & Tyagi, 2010).

The amino acid of interest for this study was arginine. This amino acid was link with the decrease in the progression of atherosclerotic plaque and protection against damage produced by ischemia-reperfusion (Piñeiro et. al., 2010). In this research after germination for 72 h, there was an increase of 8.5% ($p \leq 0.05$). Considering these results it was decided to continue the study using the isolated protein obtained from soybeans germinated for 72 h, free of isoflavones, because the objective was to study only the effect of proteins, since Orgaard and Jensen (2008) studied the effect of soy isoflavones on obesity in humans and they found that this effect may depend on whether the isoflavones are consumed in combination with soy protein.

Germination induced degradation of the α and α´ fractions of β-conglycinin, after third day of germination generating low molecular weight peptides (Figure 7). At least six polypeptides, ranging from 25 to 37 kDa molecular weight, appeared as apparent degradation products of β-conglycinin (Mora-Escobedo et al. 2009). Analyzing the electrophoretic profile, it is demonstrated that there was a turnover of proteins and

	Ungerminated	48 h of germination	72 h of germination
Proteins	84.6%	81.39%	76.90%
Essential amino acids (g/100 g of protein)			
Valine	3.64	5.60*	4.12
Isoleucine	3.33	4.05*	4.17*
Treonine	3.48	4.09*	4.07*
Fenilalanine	4.96	5.48*	5.83*
Leucine	7.46	8.42*	8.67*
Lisina	5.61	5.91	7.06*
Metionine	0.98	1.19*	1.22*
Cisteíne	1.37	1.64*	1.68*
Non essential amino acids (g/100 g of protein)			
Histidine	2.43	2.69	2.69
Aspartic Acid	11.47	11.95*	12.29*
Serine	7.69	8.3*	8.32*
Glutamic Acid	18.58	18.36	17.93*
Proline	5.18	5.32	5.28
Glicine	4.32	4.76*	5.67*
Alanine	4.27	5.07*	5.12*
Tirosine	3.68	4.41*	4.51*
Arginine	4.90	5.07	5.31*

*p<0.05 regarding non-germinated flour

Table 2. Protein and aminoacid content of soybean proteins obtained at different times of germination of BM2 variety.

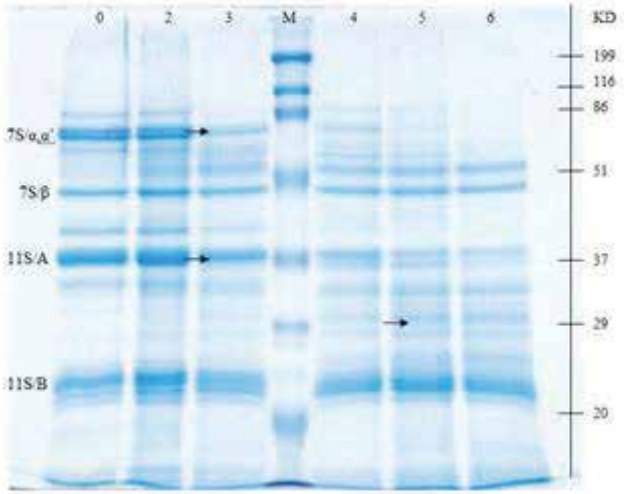

Fig. 7. Electrophoretic profile of the soy protein isolates germinated at different times (0-6 days) (Mora-Escobedo, 2009).

nonprotein nitrogen; equilibrium resulting of the degradation and synthesis processes during germination.

Biological experiments

Rat experimental design: 27 female rats were randomly distributed in 3 groups with 9 rats each. Group 1 (control): hypercholesterolemic diet (HCD); Group 2 (soybean): 0.43 g of germinated soybean protein/Kg of weight and Group 3 (blank): milled Rodent chow 5008. The weight of the rats was registered in order to adjusting the doses. HDC was: Cholesterol 1% (C8503, Sigma), Sodium Cholate 0.5% (C1254, Sigma), butter without salt 5%, glass sugar 30%, casein 10% (Teckland, MA) and Rodent food 5008 53.5% (Matsuda, 1986). The soybean protein/ treatment were administered orally for a period of 40 days. On day 40, myocardial infarction was provoked in all animals, following the procedure reported by Piñeiro et al., 2010.

Table 3 shows weight increase of different groups at the end of the treatment. Analyzing body weight in all groups it was observed a significant decrease in Group 2 ($p \leq 0.05$). It was an important finding since it indicates that consumption of soy protein may help control weight. Bau et al., (2000) found that a diet rich in germinated soybean seeds may possibly have beneficial effects in preventing obesity (Bau et al., 2000). On the other hand the results found in this work showed protein soy tendency to diminish the problems generated by ischemic reperfusion and then it is possible to say that the changes in aminoacid profile could be responsible for the protector effect.

	Group 1 (Control)	Group 2 (Soybean)	Group 3 (Blank)
Weight increase (%)	41.5 ± 6.24	37.67 ± 5.03*	34.5 ±4.45*
Heart (relative weight)	0.47 ± 0.05	0.43 ± 0.03	0.44 ± 0.07
HAA/TA	0.75	0.59	0.58

*Significant difference ($p<0.05$) respecting to control. Relative weight: heart weight/100 g of rat weight. HAA/TA=Heart attack area/Total area.

Table 3. Weight increase; heart relative weight and damaged area in dyslipidemic rats

Studies *in vitro* and *in vivo* suggest that consumption of soy protein have favorable effects on obesity and lipid metabolism. Cell culture has been used as a model for the study of obesity, supporting the study of phenomena such as disorders in the metabolism of carbohydrates and lipids. It is useful to elucidate the possible mechanisms by which soy protein has beneficial effects on diabetes, cardiovascular disease and obesity (Jang et al, 2009; Gonzalez, et al. 2009; Tsou, et al. 2010).

Taking into account the results obtained with studying the germinated soy protein it was realized an *in vitro* study of antiobesity effect of germinated soy proteins using 3T3-L1 adiposities. This research was done to determine if germination improves the antiobesity properties of soybean protein through generation of amino acids or bioactive peptides. Soybean was germinated during 1 to 6 days and proteins were isolated from germinated samples. The protein isolates were hydrolizated by sequential *in vitro* digestion using pepsin and pancreatin, the protein profile was observed by SDS-PAGE.

These hydrolysates were tested in 3T3-L1 cells (mice fibroblast) differentiated into adiposities. The amount of accumulated lipid was measured by red oil technique. Degrees

of hydrolysis ranged from 60-63%. The 2 days germinated soy protein hydrolysate with isoflavones had the best effect antiadipogenic. These results indicate that the consumption of germinated soybean could have an impact on reducing body fat and thereby mitigate the effect of obesity, promoting the use of germinated soybean for the elaboration of functional foods. The development of products from germinated soybean could further increase the versatility and utilization of soybean.

4. References

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ABA Increased Soybean Yield by Enhancing Production of Carbohydrates and Their Allocation in Seed

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1. Introduction

Soybean is the most expanding crop worldwide, and in the last 30 years it has doubled the cultivated area (FAO, 2002). Argentina is one of the main producers, where soybean production increased from 11 millions of tons in the campaign 1990-1991 to 55 millions of tons in the campaign 2009- 2010, with a planted area that reached 18.7 million hectares (GEA, 2010). Soybean expansion in Argentina has been so significant that the crop is now located in areas that were previously considered "marginal" or "not suitable for the crop" due to environmental conditions (Qaim & Traxler, 2005; Monti, 2008; Zak et al., 2008). In the last five years, this crop has moved approximately 4.6 millions of hectares of other crops and pasture lands (Pengue, 2009). The same as in other countries in Latin America such as Brazil, Bolivia and Paraguay, soybean culture has been the main cause of deforestation during the last years (Kaimowitz & Smith 2001; Steininger et al., 2001; Pengue, 2009).
Several technological advances have allowed the development of a new agricultural model that having less input is able to increase yields therefore generating an intensive system that in turn increases the financial profit (Monti, 2008). The use of practices such as direct sowing, fertilization, and genetically modified materials resistant to glyphosate that allow easy weed control and with high yield potential, have permitted yield increases and consolidated the new agricultural model. This agricultural model, based in monoculture, has made soybean production very economic to crop growers allowing an interesting income in a short period with low investment of resources. Argentina's economy is greatly dependent on the currency generated by exportation of primary products and it is one of the top three producers and exporters of vegetable oils, and the biggest worldwide exporter of soybean oil (FAO, 2008). Due to the country's size and geographical diversity, soybean has a high potential to satisfy the increasing international demand of bio-fuels (Tomei & Upham, 2009). Therefore it is possible to think in a future of increasing soybean production.

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However, although this leguminous is rich in proteins and oils, its monoculture presents risks from the point of view of the system's sustainability on the long term because of the probable negative environmental impact (Steward, 2007; Gudynas, 2008; Holland et al., 2008; Aizen et al., 2009; Altieri, 2009; Pengue, 2009). Especially regarding the organic matter soil content (the main indicator used to assess its quality and agronomical sustainability in the long term; Reeves, 1997), since in the agricultural nucleus area for the soybean crop the organic matter has been reduced from 4-5 % (original soil) to 2.5% (Cordone et al., 2005). Moreover this reduction has also impaired the soil nutrient content, the porous structure, and the soil biological activity. Nowadays, it is necessary to manage the soil appropriately in order to maintain a high grain production along the time. A system based in soybean monoculture will provoke great soil damage due to the low contribution of rubbish by the crop; in this scenario nitrogen is quickly discomposed remaining a scarce portion for the production of humus, thus leaving the soil without protection (Franzluebbers et al., 1995; Wright & Hons, 2005). This agro-ecological imbalance leads towards loss of the soils productive capacity.

A good alternative to soybean monoculture is rotation with cereals that contribute with a big mass of stubble. In the Argentinean Pampas the rotation of soybean is being performed mainly with wheat since the cycles of both crops are adapted for a continuous sequence (Andrade & Calviño, 2004). This agricultural practice however has high yield potential only in conditions of enough water availability given that the relatively high transpiratory demand of the two cultures. Also the sequence wheat/soybean/maize is used, which involves the sowing of the two gramineous in two consecutive years with the participation of soybean as the intermediate crop. This alternative provides a bigger contribution of stubbles, not only in the surface, but also in the soil volume because the mass of roots. In experiments done in soils degraded by agriculture, it has been observed that when soybean was seeded in sequences that included gramineous, yield increases by 10% as compared with those of soybean-soybean (Bacigaluppo et al., 2009).

The productive capacity of soils submitted to continuous agriculture will not be maintained by direct sowing and nitrogen fertilization since both do not provide enough carbon to repair the natural losses. Only the combination of all of these practices, i.e. direct sowing, nitrogen fertilization and rotation with cereals, will make possible to maintain the soil content of organic matter and thus to benefit the soil water balance through the presence of the harvest's rubbish on the surface that improve the soil capacity to store water (Cordone et al., 2005).

2. Yield components

In any productive system the main objective is to increase the performance; that is to say, to maximize the production of seeds by surface unit according to the availability of light, water and nutrients on each portion of land. Although the yield of soybean crops is the result of processes and changes that occur in the plants from the moment of the sowing to the harvest, it results from two main components, the number of seeds established by unit area and the seed weight (Kantolic et al., 2003). Albeit there are compensations between these two components, there is certain independence between them, which allows thinking that an increase in any of them can produce an increase in the yield (Kantolic et al., 2003). The number of seeds is the most difficult component to estimate since it depends on different factors. In soybean the flowers are arranged in open bunches that develop on the nodes. The

number of pods per node will depend on the number of fecundated flowers that last in the plant until the fruit matures, which in turn will depend on the number of nodes the plant develop, not only on its main stem but also in the lateral branches that may also vary in quantity. The mature pods can present two or three seeds, but the number of them is a character genetically stable and it does not affect the crop yield (Herbert & Litchfield, 1982; Liu et al., 2006).

Nevertheless, there is a period where the number of legumes and seeds are determined, which begins around flowering and extends through pod set, including the beginning of the seed-filling period (R1-R6). This period is called "critical period" since crop yield is established at this time (Egli, 1998). A close relationship has been found between the number of seeds per area unit and the plant growth rate during a critical period, especially between R3-R6, independently of the changes in growth for rest of the plant cycle (Jiang & Egli, 1995; Board & Tan, 1995). During the critical period both the production of reproductive organs and its survival are defined.

The final weight of the seed depends on the growth rate and the duration of grain filling, and both factors are genetically determined and vary according to the environmental conditions. The duration of the reproductive period can be changed by manipulating the plant responses to the environmental factors that control its development, particularly temperature and photoperiod; these factors act simultaneously in the plants and there are evidences of their interaction (Sinclair et al., 1991; Kantolic et al., 2003). Though temperature has influence during the whole crop cycle, the photoperiod starts its regulation when the juvenile part ends. The main effect of the photoperiod is induction of flowering; the soybean is a short day plant because the beginning of the process of flowering is induced under days getting shorter (Hicks, 1978). The photoperiod influences and regulates the major part of the reproductive events by conditioning the beginning and the ending of the different phases and the rate of these changes inside the plant (Kantolic et al., 2003). Since a long time ago many authors demonstrated the existence of a genetic variability in the sensibility of the soybean crop to the photoperiod during the period of post-flowering (Thomas & Raper, 1976, Guiamet & Nakayama, 1984; Ellis et al., 2000; Kantolic & Slafer, 2001). Recently, it has been established for soybean cultivars of indeterminate growth that an increase in the photoperiod length could extend the time of the critical period and so the time that determines the seeds; such response was observed in a wide range of photoperiods longer to those required for each cultivar studied (Kantolic & Slafer, 2005).

To increase the length of the critic period through the modification of the plant responsiveness to the photoperiod would be a useful strategy to achieve a balance between photoassimilates production and sink demand. Thus, the number of legumes per node at harvest is the result of the equilibrium between production and mortality of flowers and legumes. The production of pods in turn includes the appearance of floral buds and their development in matured flowers, but then the abortion of young and matured flowers and pods are sensible to changes in photoassimilates production (Jiang & Egli, 1993; Bruening & Egli, 2000). The extension of the critical period may also have a negative effect on seeds production if during this period light or temperatures are reduced. The best diurnal temperatures for photosynthesis in soybean range between 30º and 35º C, and a decrease in pods set have been noticed below 22º C, indicating that pods number is sensible to low temperatures (Thomas & Raper, 1976; Hume & Jackson, 1981). Also, the seed growth is generally limited by the supply of assimilates, which is reduced by low temperatures (Egli, 1999). Studies under controlled conditions demonstrated that the exposition to long

photoperiods caused reduction in seeds growth rate, thus affecting negatively the seeds weight (Raper & Thomas, 1978; Morandi et al., 1988). Nowadays, several scientific and technological efforts are oriented to obtain cultivars sensible to photoperiod that by flowering earlier will further develop under light and temperature appropriate to achieve a good yield (Kantolic & Slafer, 2001). In other words, to achieve optimal environmental conditions during the critical period can be a good strategy to improve the performance due to the critical role that they have on the control of the plant development.

The weather conditions also define when and how long the yield components are determined and how much of the production arrives at end of the crop (Kantolic et al., 2003). However, water deficiencies or any stress that affects the photoassimilates production and transport during the critical period reduces the number of pods per node and the number of seeds at harvest. During dry farming, and even under irrigation, plants may suffer daily variations of water supply (an unbalance between what roots absorbed and leaves transpired) that generate stressful situations. If such stress is mitigated during the crop's critical period, its performance may be improved. This objective becomes really important when soybean varieties of short cycle are used, since they have a shorter critical period than those of long cycle. In fact, the major part of soybean in South America is cultivate in rain fed, and variability in crops performance is strongly related with differences in rain's total amount and distribution along the plant's cycle and soil water availability. Since water availability is an important limitation for the application of the strategy before mentioned, the success of any crop not only depends on water supply but also on the plant's ability to use it (Andriani, 1997). Thus, crops with long cycle cultivars are more flexible and balanced than those with short cycle cultivars, like those used to rotate with wheat. The later have a high yield potential when there is good water availability, particularly during the period where the number of seeds is defined. Therefore, a way of improving the performance of short cycle soybean crops is to optimize water usage. To achieve this will let to obtain a more profitable soybean harvest, and to maintain an appropriate rotation of the crops. Such benefit could be achieved through induction of partial stomata closure in a way that the concentration of intracellular CO_2 would be enough for photosynthesis, and at the same time water looses may be minimized (Kang et al., 1998).

3. Abscisic acid functions

The phytohormone abscisic acid (ABA) regulates a variety of physiological plant processes, including seed maturation, seed and bud dormancy, root growth, foliar senescence and the transition between the vegetative to reproductive development, among others (Leung & Giraudat, 1998).

When plants are confronted to abiotic stresses, ABA is the main candidate in the plant's responses signaling, since it is well known that the phytohormone concentration varies according to the stressful situation (Zhu, 2002). In fact, ABA acts as signal in the plant responses to environmental stresses imposed by either cold, drought and/or high levels of salt, through the modulation of genes sensible to stress (Christmann et al., 2006). It has been proven that during periods of stress synthesis and concentration of ABA may drastically vary in specific tissues (Zeevaart & Creelman, 1988). For example, under conditions of water stress the concentration of ABA in leaves can increase up to 50 times in a period of 4 to 8 hours; after rehydration ABA levels are reduced to normal in a similar period of time (Taiz & Zeiger, 2006). This relationship strongly suggests that ABA is one of the mediators of the

plant responses to the stressful conditions (Galau et al., 1986; Zeevaart & Creelman, 1988; Bray, 1991; Christmann et al., 2006; Hirayama & Shinozaki, 2007).

It has been demonstrated that ABA is able to alleviate the water stress not only in daily variations but also in long term draught by provoking a stomatal closure, since stomata are responsible for the major proportion of plant water losses (Leung & Giraudat, 1998; Zhang & Outlaw, 2001; Taiz & Zeiger, 2006). In fact, the signal transduction in guard cells in response to ABA has been well documented (Luan 2002; Levchenko et al., 2005; Pei & Kuchitsu 2005; Vahisalu et al., 2008).

During seed development, ABA has a main role in regulating synthesis of proteins and lipids and in promoting the seed's tolerance to dryness. This hormone has also a protective role for the seed by inducing embryonic dormancy not allowing its early germination and inhibiting it in conditions of osmotic stress. As well, ABA has been identified as one of the main chemical signal that regulates genetic expression during stress (Seo & Koshiba 2002). Different evidences suggest that the hormone perception may be produced both extra and intracellular (Bray, 1997; Finkelstein et al., 2002). Although it has not been identified yet a primary receptor of the hormone, there are a wide variety of second messengers involved that contribute to the signaling pathway (Moreno, 2009). In different species, the application of this hormone increased the plant resistance by provoking the expression of ABA-dependent genes.

For the expression of specific genes in response to water and saline stresses, the existence of two types of signal chains have been postulated, one that depends on ABA and another independent of the hormone (Chandler & Robertson, 1994; Shinozaki & Yamaguchi-Shinozaki, 1997). Within the ABA-dependent signal chain there is production of proteins with protective function, enzymes responsible for osmolite synthesis, antioxidant enzymes, transcription factors and other proteins involved in the responses to water stress (Bray, 1997; Xiong et al., 2002). Amongst the main proteins are the dehydrins, which are highly hydrophilic and replace water in its function of maintaining the structure of other proteins and membrane phospholipids protecting the cell cytoplasm of dehydration (Hare et al., 1999). There are also the LEA (late embryogenesis abundant proteins) proteins, that form many families of proteins accumulating at high levels during the matured period of embryogenesis, just before the moment of the seed drying (Moreno, 2009). Some of them are accumulated in vegetative tissues in response to the osmotic stress generated by different factors such as dehydration, salinity, cold and freezing (Baker et al., 1988; Bray, 1993). The information available about different LEA proteins indicates that they have an important role in the protection against the cell's dehydration (Battaglia et al., 2008).

As well, the synthesis of osmoregulators like proline, glycine-betaine, sugars and sugar alcohols are related with the cells osmotic status since these components facilitate the water uptake by the plant (Cushman, 2001). Over-expression of some of the genes that lead the synthesis of osmoregulators have been used to protect plants to the osmotic stress in many species (Abebe et al., 2003; Tamura et al., 2003; Waditee et al., 2005; Hmida-Sayari et al., 2005; Ashraf & Foolad, 2007). On the other hand, antioxidant enzymes together with non protein components protect plants from free radicals that are generated due to an increase in the rate of O_2 photoreduction in chloroplasts (Danon et al., 2004; Robinson & Bunce, 2000). Among the main antioxidant enzymes are superoxide dismutase, catalase, ascorbate peroxidase, peroxidase, glutathione reductase and monodehidroascorbate reductase (Apel & Hirt, 2004).

During the water stress the expression of different transcription factors that mediate the gene response to the stress is also induced; some of these transcription factors are related to specific sequences in the region that promotes the genes (Guiltinan et al., 1990; Busk et al., 1997).
ABA is also able to activate metabolization of carbohydrates temporally stored in the stem of rice (Yang et al., 2003) and wheat (Travaglia et al., 2007 and 2010) plants. As well, ABA applications lead to the accumulation of carbohydrates in grains consequently accelerating the filling process. The application of fluridone, inhibitor of carotenes and (hence) ABA synthesis, leads to reduction in the activity of enzymes related with starch synthesis (Grappin et al., 2000).

4. ABA promotes yield in field-cultured soybean

The roles of ABA in basic physiology have been extensively studied, but information regarding participation of this hormone in field crops eco-physiology is rather limited. The evaluation of stress effects under artificially controlled conditions is very useful to recognize the physiological plant response and allows determining the presence of mechanisms of resistance. Such mechanisms could be useful traits of cultivar selection in programs of genetic performance. However, the plant responses under these conditions may not be representative of what happens in field conditions. Growth and development of a crop under field conditions is the product of the interaction among a series of genetic and environmental factors, so the formulation and application of agronomic practices for the crop's improvement will depend on the understanding of the eco-physiological basis for their development.
Taking into account all the antecedents cited, we worked with the following hypothesis: "a plant that grows in a field and receive the stress signal (i.e., it is treated with ABA) before a situation of stress is produced, it is then more prepared to face the stress and the effect is minimized".
From here on we inform the results obtained by our research group in evaluating the effects of ABA applications on the performance of a short cycle cultivar (GM 3.4, Dekalb, Argentina) of *Glycine max* L. under field conditions. The experiments were performed during three consecutive years through direct sowing in rain fed conditions at the experimental field of the Universidad Nacional de Río Cuarto Campus, Río Cuarto, Provincia de Córdoba, Argentina (33° 07' S, 64° 14' W). Sowing density was 32 seeds m^2, and at harvest there were 23 plants m^2. The crops had a water supply during the crop seasons of 611, 466 and 571 mm of rainfall that satisfied the calculated crop's evapo-transpiration demand. The results of the field experiments were also complemented with an experiment in greenhouse. ABA (Lomon Biotech, Beijing, China, 90 % purity) was sprayed in a dose of 300 mg L^{-1} at the phenological stages V7 and R2, which are previous and close to the critical periods; the solutions contained 0.1% Triton for emulsification, and the spraying was done at sunset to avoid ABA photo-destruction. Further details of the experimental conditions can be found in Travaglia et al. (2009).
The results showed that ABA application improved physiological variables related with the photosynthetic capacity of the plants. Control and ABA-treated plants at R5 phenological stage (beginning of seed filling), did not show differences in the length of the main stalk. However, ABA-treated plants had higher shoot's dry weight than controls (Figure 1).
Bigger stem diameter (data not shown) and increase in the foliar area of the leaves 2 and 3 (counting from the apex) as compared with the control plants were observed (Figure 2).
The leaves of these plants were greener and presented a bigger proportion of chlorophyll than the control ones (Figure 3), however they did not present main differences in the levels of carotene (data not shown).

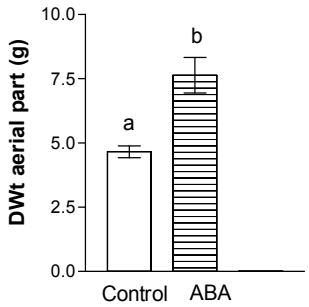

Fig. 1. Average dry weight (D Wt) in three years of experiments with field-grown soybean plants at R5. Treatments: control, ABA treated plants. Bars indicate SEM of 30 plants. Different letters show significant differences at $p<0.05$ with the Fisher alpha Test.

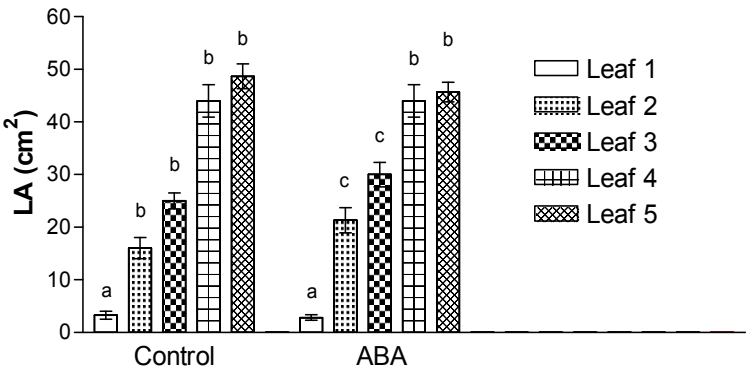

Fig. 2. Average leaf area (LA) at different plant levels in three years of experiments with field-grown soybean plants at R5. Treatments: control, ABA treated plants. Bars indicate SEM of 30 plants. Different letters show significant differences at $p<0.05$ with the Fisher alpha Test. Figure adapted from Travaglia et al. (2009).

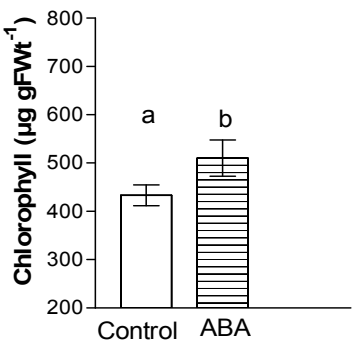

Fig. 3. Average of total chlorophyll content in three years of experiments with field-grown soybean plants at R5. Treatments: control, ABA treated plants. Bars indicate SEM of 15 plants. Different letters show significant differences at $p<0.05$ with the Fisher alpha Test.

Generally, the action of ABA has been related with processes of inhibition, although there is recent evidence of its presence in developing tissues where it may have a promoting action (Finkelstein & Rock 2002; Sansberro et al., 2004; Peng et al., 2006). Our results demonstrate that ABA sprayed to the leaves benefits dry matter accumulation favoring vegetative growth of soybean plants grown in field conditions (Travaglia et al., 2009). For the cultivar and the dose used, no restrain of shoot growth was observed as had been previously reported (Sloger & Caldwell, 1970). These results coincide with those found in field-grown wheat plants under water stress that had been treated with ABA, in which these plants showed higher shoot biomass accumulation (Travaglia et al., 2007 and 2010). This effect of ABA has also been seen in studies carried out under controlled conditions with different levels of water deficit, both in *Arabidopsis* (Finkelstein & Rock 2002) and *Ilex paraguariensis* (Sansberro et al., 2004), where the plants sprayed with ABA had greater growth than those with lower (endogenous) levels of ABA. In agreement with our previous findings (Travaglia et al., 2007 and 2010), these new results suggest that ABA is an important regulator of cell and whole-plant water content, likely due to an increased turgor that allows optimal cellular expansion (Acevedo et al., 1971).

ABA-treated soybean plants also improved leaf area, which can be considered useful since it benefits the interception of light, particularly in short cycle varieties that sometimes do not cover the soil surface until late in the cycle (Andrade & Calviño, 2004). Another interesting aspect to take into account is the photosynthetic capacity of the plant. It has been reported in literature that ABA stops the photosynthesis of different species under controlled conditions (Daie & Campbell, 1981; Xu et al., 1995; Gong et al., 1998; Wilkinson & Davies, 2002; Reddy et al., 2004; Liu et al., 2005); but our results showed an increase in the content of dry matter that increases in correlation with chlorophyll levels (Travaglia et al., 2009). These results are similar to those we observed during three years of essays with field-grown wheat treated with ABA; the treated plants showed higher levels of chlorophyll and maintained green leaves longer (5 to 10 days) than control plants (Travaglia et al., 2010). Longer photosynthetic activity should benefit higher accumulation of dry matter in harvest products (Thomas & Howarth, 2000). According to Ivanov et al. (1995) ABA had a protective effect on the photosystem II complex (PSII) in barley plants since it avoided the deleterious effect of high intensity light. ABA would act protecting the membranes, especially those of chloroplasts, when they are under stress (Travaglia, 2008). These authors also reported higher carotene levels in ABA-treated plants, an effect that was also observed in wheat (Travaglia et al., 2007 and 2010), but not in soybean. Although in this investigation the soybean plants were not under a long water deficit, they would suffer temporary water stress during hours of high light intensity where there is an unbalance between transpiration and water absorption; the ABA-treated plants showed higher chlorophyll levels and maintained green leaves longer. These results indicate that ABA protect the photosynthetic system and delayed foliar senescence under conditions of temporary water stress allowing the plant to generate and accumulate more dry matter and also produce more seeds.

The root density (number of lateral roots) was higher in ABA-treated plants as compared to controls (Figure 4). Such higher production of lateral roots is well expressed by roots dry weight improvement in ABA-treated plants as compared with controls (Figure 5). There were no significant differences in number, weight and viability of nodules in plants treated respect to the control (data not shown).

Fig. 4. Picture showing soybean plants grown in plastic pots (24 L) during 80 days; ABA-treated (left), control (right) plants. Bars = 10 cm.

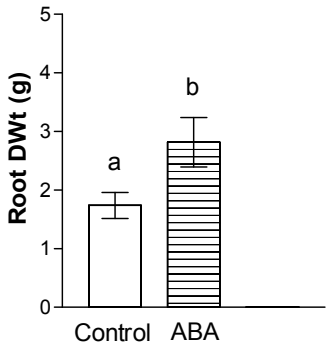

Fig. 5. Dry weight (D Wt) of roots of soybean plants grown in plastic pots (24 L) during 80 days. Treatments: control, ABA treated plants. Bars indicate SEM of 30 plants. Different letters show significant differences at $p<0.05$ with the Fisher alpha Test.

Vysotskaya et al. (2008) suggested that ABA promotes radical growth instead of any specific growth of the aerial part. Our results indicate however that ABA stimulated an increase of the total biomass. It is possible that at the beginning the application of the phytohormone

may induce the distribution of biomass towards the roots, but our results indicated an increase in the quantity of dry matter for both, aerial part and roots.
Enhancement in the number of lateral roots, therefore augmenting the density of the root system, by ABA has been previously informed for other species by Trewavas & Jones (1991). According to Lian & Harris (2005) it is an effect that ABA has on roots of leguminous species, but in non leguminous species ABA inhibits lateral root development. The increasing in the density of lateral roots provoked by ABA can be a good effect to obtain a higher number of nodules by plant.
As well, Zhang et al. (2004) found more biomass and more nodules in well-irrigated soybean plants treated with ABA or inhibitors of GA biosynthesis. In our study ABA did not affect the nodules but increased plants' root density, which is highly beneficial considering that soybean has a high requirement of N that is accumulated in seed proteins. The amount of N used for this crop is the sum of the inorganic N present in the soil and the atmospheric N that is symbiotically fixed by *Bradyhizobium japonicum* (Madrzak et al., 1995). The crop gets *ca.* 50 to 60 % of its N at the beginning of the symbiosis (Salvagiotti et al., 2008). The performance of soybean is related in a positive way with the absorption of N by plant. This is supported by the findings of Salvagiotti et al. (2008), who analyzed 637 essays in USA between 1966 and 2006, observing an increase in grain yield of 13 kg per kg of N accumulated in aerial biomass. When soil aeration is minimal, such as in direct sowing, the rate of mineralization is reduced, and N immobilized and consumed by denitrificating microorganisms increases. This fact augments the necessity of N, stimulating N biological fixation (NBF) from the early stages of the crop (Bonel et al., 2005). The soybean N requirements changes according to the stage of development, increasing between R3-R6. In the Argentinean Pampas region, the contribution of NBF is ca. 40%. Any environmental situation of stress, like drought or flood, has negative repercussions in the NBF compromising yield (González, 1997). Drought also affects NBF because the nodule must have more than 80% of relative water content (RWC), otherwise the capacity of NBF is seriously reduced when RWC is below 60%. The critical period of the grain filling is compromised by any situation that affects NBF hence reducing the yield potential of the crop (Bonel et al., 2005).
As well, ABA-treated plants showed reduction in stomatal conductance during 24 h after the hormone has been applied as compared to controls, but then the conductance values kept more stable and become equal to the controls 11 days later (Figure 6). The ABA-treated plants also showed a lower foliar temperature (data not shown).
Closing of stomata immediately after ABA treatment is an expected response, but the partial (and stable) closing observed later could be accountable of regulating enough CO_2 entrance in the chlorenchyma cells to support photosynthesis and to minimize water losses, though maintaining evaporation in a way that foliar temperature is lower than in control plants. At the interface between atmosphere and plant, leaf stomata provide the entryway for CO_2 for photosynthetic carbon fixation, while preventing excessive water loss. Through their role in transpiration, stomata also help to control leaf temperature. The net stomatal conductance depends on both, plant-specific traits and signals received from the environment, which is the result of stomata functioning. It has been demonstrated that exogenous ABA promotes the closure of stomata (Zhang & Outlaw, 2001; Davies et al., 2005; Pei & Kuchitsu, 2005). So the observed low conductance immediately post-treatment was a desired answer, but the later increase of the conductance was maintained in the time. In papers related with field-grown wheat under water deficit, it is observed a similar behavior. The ABA treated plants

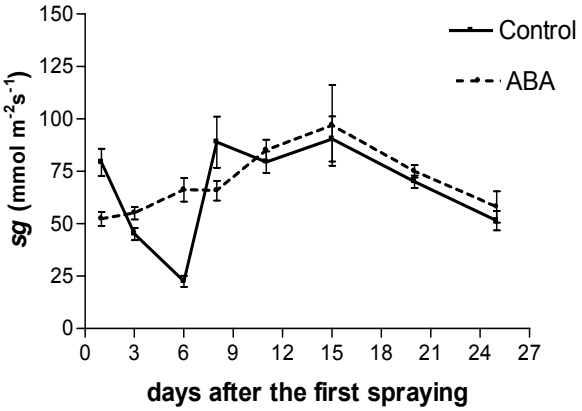

Fig. 6. Average values of leaf conductance (sg) in the 3rd fully expanded leaf (one leaf per plant, 15 plants per treatment) at 01:00 PM in three years of experiments with control and ABA-treated field-grown soybean plants since 24 h until 25 days after the first spraying. Bars indicate SEM of the 15 plants. Different letters show significant differences at $p<0.05$ with the Fisher alpha Test. Figure taken from Travaglia et al. (2009).

closed their stomata immediately after the hormonal application, so decreasing the conductance and the rate of transpiration, but 21 days after the ABA treatment leaf conductance and transpiration rate increased more than in control plants (Travaglia et al., 2010). As well, in front of a water deficiency, the treatment with ABA had a long time effect on to the stomata behavior, maintaining some ostiolar aperture in the hours of high light while stomata in control plants are closed. This ostiolar aperture in the ABA-treated plants, although limited, was enough to maintain a leaf conductance higher than in the control plants. Although in our experiments with soybean the grade of stomata aperture was not recorded, the similarity of the results suggest that exogenous ABA promoted the immediate stomata closure, even though the plant is not under water stress, but later it benefits since stomata are maintained semi-opened with high irradiation. This may be the reason why leaf conductance is more stable along the time doing the balance between water looses and CO_2 gain more efficient. The partial stomata closure allows that the concentration of intracellular CO_2 may be sufficient so that the photosynthesis would be maximal and the water loses minimum (Kang et al., 1998). After evaluation of two leguminosae, *Phaseolus vulgaris* and *Trifolium pratense*, in front of the combined stress (drought + high temperatures), Reynolds-Henne et al. (2010) proposed that the stomata behavior is complex. However, these authors consider that in all the cases the plants achieve a balance that allows them to loose water and to avoid overheating. If stress is moderated, an important stomata closure is produced and leaf conductance is impeded giving priority to water relations, while if the stress is severe stomata conductance increases maintaining leaf temperature to avoid metabolic damages and to protect the photosynthetic apparatus. In our soybean experiments, where stress was of short time, the application of ABA immediately reduced transpiration, but in the mid term sustained water evaporation more constant so leaf temperature may be lower than in non-treated plants. Given that photosynthetic activity by crop canopy declines gradually during the effective grain filling period and current photosynthesis (rather than remobilization of stored carbohydrate) is considered to be main source for seed growth in soybean (Liu et al., 2006), supporting a regular photosynthesis could be the cause of the

higher carbohydrate amounts found at the flowering period in shoots of the ABA-treated plants as compared to controls; the difference in shoots disappears at harvest because of an increased carbohydrate remobilization (21%) to the seed in the ABA-treated plants (Travaglia et al., 2009). These results confirm the participation of ABA in promoting source to sink transport of assimilates during the stage of seed filling. This effect has been also reported in rice (Zhang et al., 1998; Yang et al., 2001; 2004), and by our group in wheat (Travaglia et al., 2007; 2010). Also in grapes, Moreno (2009) found that ABA stimulates carbon translocation from source to sink.

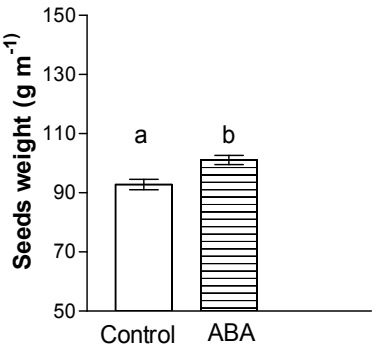

Fig. 7. Seeds weight per meter in three years of experiments with field-grown soybean plants. Treatments: control, ABA treated plants. Bars indicate SEM of 15 plants. Different letters show significant differences at $p<0.05$ with the Fisher alpha Test.

The number of ripped pods in the ABA-treated soybean plants was similar to the controls; however it was observed that during the first periods of development the pods in ABA treated plants were bigger, difference that disappeared at maturity (data not shown). The weight of seeds per m^2 was significantly higher in the ABA-treated plants (Figure 7); the seeds maintain the same protein content and higher oil concentration as compared with the controls (Figure 8 a and b). Thus, the treatment with ABA did not affect the quality of seeds, an important characteristic since the seed quality is one of the key aspects for agriculture success.

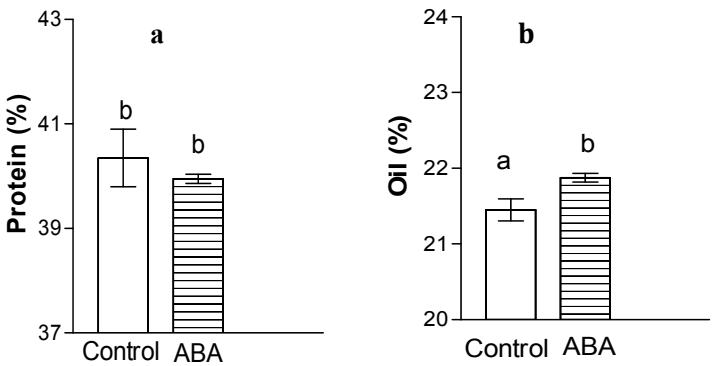

Fig. 8. Protein (a) and oil (b) percentage in grains in three years of experiments with field-grown soybean plants. Treatments: control, ABA treated plants. Bars indicate SEM of 15 plants. Different letters show significant differences at $p<0.05$ with the Fisher alpha Test.

The final chemical composition of the seeds is the result of accumulation of reserve substances in the cotyledons during the filling period. The contents of oils and proteins are influenced by genetic and environmental factors (Ojo et al., 2002; Wilson, 2004; Minuzzi et al., 2007). In the last years a better understanding of the genetic regulation in the proportions of the different components that define the quality of seeds has been achieved, and cultivars known with "enhanced value" have been obtained by traditional methods or genetic manipulation. Regarding the environment, the influence of the conditions during the seeds filling period is direct, since soybean seeds accumulate oils during this period, with a maximum rate occurring around 30 days after flowering (Wilson, 2004). The short-cycle cultivars, i.e. the short term maturity group (MG), usually have their seed filling period exposed to higher temperatures than the long-cycle MG genotypes. Yaklich and Vinyard (2004) also found higher oil concentration under high temperatures. These authors suggest that by monitoring the minimum temperatures and the daily increase during the seed filling period might have predictive value for seeds oil concentration.

It has been informed that not only the high temperatures but also the presence of water stress during the filling of seeds produces alterations in the contents of proteins and oils (Boydak et al., 2002). It is also frequently found a negative relationship between seed protein content and crop yield (Shannon et al., 1972; Yin & Vyn, 2005); moreover, there are evidences of a negative correlation between oils and proteins concentration. None of these relations were seen in our experiments since ABA enhanced both yield and seed proteins, because ABA not only increased yield but also improved the seed quality; that is, ABA did not affect protein levels but enhanced oil yield. We have also found benefits with the application of exogenous ABA in the performance of wheat grown under water restrictions. When stress was very strict, the application of ABA increased the number of grains per spike. When water stress was moderated, ABA not only increased the weight but also the number of grains. Such effect was reflected in a yield increase that ranged between 11 and 34 % (Travaglia et al., 2010). Since the food value of grain wheat relays on the protein content, it is not only necessary to achieve higher yields but also to maintain a high percentage of protein in the grain. It has been mentioned that this is difficult to achieve considering that there is an inverse relationship between yield and protein content (Cuniberti, 2001). In our study however, the grain protein content under severe drought increased in ABA-treated plants, whereas in moderate drought there was no difference in grain protein between ABA-treated and control plants (Travaglia et al., 2007 and 2010).

5. Conclusion

Recent biotechnological advances have opened great possibilities for the agricultural production and have permitted a big expansion of soybean in the Americas in general and in Argentina in particular. Having in mind that currency generation by exports is of outmost importance in the economy of the countries, everything indicates that the high production soybean system will continue.

Soybean monoculture generates a selective decreasing of the soil nutrients and can generate their exhaustion and the necessity of adding more fertilizers. According to this point of view rotation with other crops, especially cereals, seems to be an appropriate solution. However, the profits for soybean production have been differentially favorable as compared to other crops so many producers will continue with the monoculture. A profitable alternative is through practices that take into consideration the rotation of the crops; for example, short

cycle soybean-wheat. To achieve this purpose unfavorable environmental conditions during the critical period of the crops must be minimized, especially the most frequent factor that is water deficit.

The results obtained in the experiments with soybean grown in field conditions support the idea that ABA enhances yield by a combination of factors. Therefore, foliar application of ABA may be an alternative tool for enhancing yield of short-cycle soybean, since it gives relief to temporary situations of water stress, such as the stress that happens in the hours of maximum irradiance, where an imbalance between water transpiration and absorption it is frequently produced. ABA seems to improve a combination of factors that contribute to increase the number of lateral roots and the density of the radical system, to protect the photosynthetic apparatus, to keep the stomata conductance more stable over the time, and to enhance carbon allocation and partitioning to the seeds. The results presented here are also related to those obtained for wheat and other species, and open the possibility for the future use of this hormone in commercial products. Although nowadays its relative cost is high, it has decreased remarkable in the last years and some commercial products are now registered around the world; besides, its application will not represent an environmental threat since ABA is a natural compound produced by plants, fungus and bacteria.

6. References

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Soybean Cultivars Affecting Performance of *Helicoverpa armigera* (Lepidoptera: Noctuidae)

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1. Introduction

Helicoverpa armigera (Hübner) is one of the major devastating and highly polyphagous insect pests in many parts of the world (Liu et al., 2004; Naseri et al., 2009 a, b; Naseri et al., 2011). This species has a high potential for population increase and outbreak on different host plants including soybean (*Glycine max* (L.) Merrill) throughout the world. *Helicoverpa armigera* is a major pest of more than 60 cultivated and uncultivated plant species, distributed in 47 families (Zalucki et al., 1994).

To determine the potential of different soybean cultivars to help manage *H. armigera* populations, data on the effects of various cultivars on the pest's digestive enzymes, survival, development time, reproduction, population growth and nutritional indices are necessary. Such experiments essentially measure the potential for antibiosis resistance. Host plant resistance has been used effectively in sustainable integrated management programs for several crop pests. Plants with antibiosis machanism may reduce directly insect survival, size or weight, longevity, and fecundity in new generation adults, or they may have an indirect effect by increasing the exposure of the insect to its natural enemies due to prolonged developmental time (Sarfraz et al., 2006).

In terms of production and trade, soybean is the most important leguminous crop in the world due to its high protein (35-40 %) and oil content (15-22 %). In recent years (especially after 1950), soybean production has been seriously affected by *H. armigera*. In spite of high level of natural mortality, *H. armigera* needs to be controlled by synthetic pesticides (Fitt, 1994). Despite extensive use of synthetic insecticides to control *H. armigera*, it has developed/evolved resistance to these insecticides, extremely reducing the number of effective approaches to its control (Armes et al., 1992). Consequently, there is considerable interest in alternative management tactics, which might be applied in area-wide or more restricted basis. Environmentally safe techniques are not widely used in rural areas, probably because these products are too expensive or their effectiveness is highly variable (Sharma, 2001). Therefore, the study of potential resistance of soybean cultivars by comparing the performance of *H. armigera* on these cultivars can play an important role in identifying the anti-digestive or anti-feedant compounds and their further use in the pest management programs.

In this chapter we would like to emphasize the strong effect of the selected soybean cultivars (as representatives of the gene pool of soybean) on life table, nutritional indices and physiology of digestive enzymes of *H. armigera* and discover the crucial importance of the cultivar selection and breeding in control program of the pest.

2. Soybean cultivars pods affecting the life table parameters of *H. armigera*

It is known that the quality and quantity of food ingested by an insect can influence directly its survival, development and reproduction (Razmjou et al., 2006). So, the fitness of plant-feeding insects depends upon the nutritious substances in the host plant (Du et al., 2004). To study the dynamics of animal populations, especially arthropods, life table parameters are appropriate tools because these tools provide very important demographic parameters (Maia et al., 2000). Demographic information may also be useful in constructing population models (Carey, 1993). The life table gives the most extensive description of the survivorship, development and reproduction of a population which are basic factors in both theoretical and applied population ecology (Taghizadeh et al., 2008). Effects of various host plants, apart from soybean cultivars, were studied on life table parameters of *H. armigera* (Patal & Koshyia, 1997; Liu et al., 2004; Reddy et al., 2004).

2.1 Case study

2.1.1 Experimental conditions

Helicoverpa armigera tested on different soybean cultivars (356 (Delsoy4210), M4, M7, M9, Clark, Sahar, JK, BP, Williams, L17, Zane, Gorgan3 and DPX), had already been reared for two generations on the same cultivars. All experimental insects were kept inside a growth chamber at 25 ± 1°C, relative humidity of 65 ± 5% and a photoperiod of 16:8 (L:D) h (Naseri et al., 2009 a, b). In order to study the life table parameters of *H. armigera* on different soybean cultivars, the adult moths emerged from the larvae reared on different soybean cultivars were used in the experiments. Life table parameters of *H. armigera* were studied using the same aged eggs laid within 24 h by females reared as larvae on the related cultivars. Each cohort of eggs (fifty eggs) was used to start experiment on each cultivar. Upon egg hatching, the newly emerged larvae were transferred individually into plastic Petri dishes (8 cm in diameter by 2 cm in height) with a hole covered with a fine mesh net for ventilation. These Petri dishes contained the fresh detached leaves of different test plants for feeding of the 1st instar larvae. The petioles of the detached leaves were inserted in water-soaked cotton to maintain their freshness. The 2nd to 5th instars were fed on the pods of different soybean cultivars until pre-pupation. The larva in each Petri dish was observed daily for the mortality or ecdysis. The fifth instar larvae were kept in plastic containers (3 cm in diameter by 5 cm in height) for pre-pupation and pupation (Naseri et al., 2009 a, b). Duration of pre-pupal and pupal stages and their mortality were recorded daily.

A pair of female and male adults emerged from the pupae were introduced into each plastic oviposition container (14 cm in diameter by 19 cm in height), which was closed at the top with a fine mesh net for ventilation. To provide a source of carbohydrate for the adult feeding, a small cotton wick soaked in 10% honey solution was placed in the oviposition containers. Daily number of eggs laid per female, the longevity and gender of each adult were recorded.

The intrinsic rate of natural increase (r_m) for *H. armigera* on different cultivars was estimated (Birch, 1948). The net reproductive rate (R_0), finite rate of increase (λ), mean generation time

(*T*) and doubling time (*DT*) were also estimated (Birch, 1948; Southwood & Henderson, 2000). The life table parameters of *H. armigera* on different soybean cultivars were analyzed using one-way ANOVA. The means associated with soybean cultivars for each parameter were separated using least significant differences (LSD) test when significantly different values were obtained.

2.1.2 Results

According to the results of the study conducted (Naseri et al., 2009 a), among different life history parameters of *H. armigera* reared on different soybean cultivars during the larval stage, development time of the immature stages, life span and fecundity are affected by the pods of soybean cultivars. Both the larval period and entire development time were longest on L17 and shortest on M7 (Table 1). Taking longer time required to complete immature stages of *H. armigera* on L17 may enhance the effectiveness of its management techniques by using natural enemies and insecticides, so the use of cultivar L17 can be a part of an IPM strategy (Du et al., 2004). It is also indicated that because of shortest larval period and development time of *H. armigera* on the cultivar M7, it may be more suitable host plant, perhaps because of higher nutritional quality compared with the other cultivars tested. Variations in the development time of *H. armigera* on different soybean cultivars can be attributed to either differences in nutrients or primary and secondary compounds among the soybean cultivars pods, or physiological differences depending on the host plant.

As can be seen in Table 2, the lowest development index (the ratio between the percentage of individuals completing development and the average period required to do) of the immature stages of *H. armigera* is observed on L17 and BP. It may be due to the presence of some phytochemicals in these cultivars as antibiotic agent, or the absence of some primary nutritious substances essential for development of *H. armigera*, leading to higher percentage of mortality on these cultivars. Since the lowest level of mortality of immature stages is recorded on M7, the development index of *H. armigera* is higher on this cultivar. Differences in mortality and the development index of the pest on different soybean cultivars might be the result of antibiotic effects, poor nutritional quality of the food ingested, and/or secondary phytochemicals (Naseri et al., 2009 a).

This study shows that the fecundity (daily and total number of oviposited eggs per female) of *H. armigera* is affected by different soybean cultivars consumed by the larvae (Table 3). The females reared as larvae on DPX produced the highest total number of eggs (582.70 eggs) whereas the lowest total number of eggs was observed on 356 (177.10 eggs), suggesting that the quantity and/or the quality of nutrients in cultivar 356 are less suitable for larval feeding compared with other soybean cultivars (Naseri et al., 2009 a).

We have demonstrated the significant difference of life table parameters of *H. armigera* on different soybean cultivars (Naseri et al., 2009 b). The net reproductive rate (R_0) is a key parameter, summarizing the physiological ability of an animal related to its reproductive capacity. Comparison of the net reproductive rate often provides important perception beyond that available from the independent analysis of individual life cycle parameters. The net reproductive rate of *H. armigera* was the highest on M7, whereas the values of R_0 varied from 89.35 on 356 to 354.92 females/female on M7 (Table 4). Liu et al. (2004) showed that the R_0 values of *H. armigera* differed depending on host plant, which ranged from 5.1 on hot pepper to 117.6 females/female on cotton. According to the literature, the net reproductive rate of *H. armigera* was 143.77 on sunflower (Reddy et al., 2004) and 374.01 females/female on pearl millet (Patal & Koshyia, 1997).

						Longevity (days)		Whole life span (days)	
Cultivar	Incubation period (days)	Larval period (days)	Pre-pupal period (days)	Pupal period (days)	Development time (days)	Male	Female	Male	Female
M7	3.00 a (0.00)	17.30 f (0.60)	2.23 a (0.15)	11.63 a (0.17)	34.21 f (0.77)	12.71 a (0.94)	9.69 a (0.36)	46.57 de (1.49)	44.00 d (1.12)
JK	3.00 a (0.00)	20.86 cd (0.66)	2.62 a (0.18)	12.05 a (0.34)	38.42 bcde (0.89)	9.25 a (0.81)	9.33 a (0.62)	48.08 cde (0.88)	44.92 cd (0.94)
Clark	3.00 a (0.00)	18.50 f (1.22)	2.79 a (0.22)	12.41 a (0.36)	36.82 def (1.49)	8.89 a (0.35)	10.14 a (0.52)	45.11 e (0.45)	46.00 bcd (1.22)
M4	3.00 a (0.00)	20.21 cde (0.96)	2.68 a (0.15)	12.44 a (0.23)	38.39 bcde (0.92)	9.69 a (0.65)	9.70 a (0.86)	47.54 cde (1.04)	48.30 ab (0.82)
M9	3.00 a (0.00)	18.17 ef (0.64)	2.48 a (0.21)	12.12 a (0.33)	36.06 ef (1.03)	10.75 a (1.12)	9.92 a (0.47)	48.00 cde (1.48)	44.77 cd (1.32)
L17	3.00 a (0.00)	26.20 a (1.62)	2.39 a (0.18)	1.06 a (0.27)	42.71 a (1.41)	9.40 a (1.06)	9.08 a (0.67)	53.40 a (1.70)	49.82 a (0.95)
356	3.00 a (0.00)	18.80 def (0.48)	2.65 a (0.20)	11.86 a (0.31)	36.71 cde (0.69)	11.25 a (0.97)	10.63 a (0.99)	47.00 de (0.53)	47.00 abcd (0.99)
DPX	3.00 a (0.00)	21.28 bc (0.74)	2.55 a (0.23)	12.00 a (0.39)	38.59 bcd (0.95)	12.37 a (0.86)	10.92 a (0.53)	50.37 abc (1.13)	47.92 abc (0.90)
BP	3.00 a (0.00)	23.33 ab (1.01)	2.59 a (0.17)	12.29 a (0.32)	40.29 ab (1.28)	10.12 a (1.49)	9.50 a (0.80)	51.86 ab (1.07)	46.54 bcd (1.55)
Zane	3.00 a (0.00)	21.77 bc (1.05)	2.73 a (0.17)	11.35 a (0.39)	37.95 bcde (0.91)	10.89 a (0.90)	10.85 a (1.02)	49.22 bcd (1.07)	48.31 ab (1.52)
Sahar	3.00 a (0.00)	21.56 bc (0.74)	2.65 a (0.28)	11.60 a (0.21)	39.20 abc (0.75)	9.44 a (1.09)	9.54 a (0.68)	48.14 cde (1.53)	48.00 abc (1.14)
Gorgan3	3.00 a (0.00)	22.09 bc (0.79)	2.30 a (0.17)	11.44 a (0.21)	38.28 bcde (0.64)	9.44 a (0.72)	9.31 a (0.62)	47.00 de (0.97)	48.00 abc (0.77)
Williams	3.00 a (0.00)	18.41 ef (0.73)	3.00 a (0.23)	12.30 a (0.17)	36.60 def (0.76)	9.69 a (0.67)	8.42 a (0.55)	48.08 cde (1.18)	43.82 d (0.73)

*The means followed by different letters in the same columns are significantly different ($P<0.01$, LSD)

Table 1. Mean development time and longevity of *Helicoverpa armigera* on different soybean cultivars

	Larvae		Pre-pupae		Pupae		Overall immature	
Cultivar	Mortality (%)	D.I.	Mortality (%)	D.I.	Mortality (%)	D.I.	Mortality (%)	D.I.
M7	0.00 (46)*	5.78	4.35 (46)	42.89	13.64 (44)	7.42	17.39 (46)	2.41
JK	15.38 (52)	4.06	4.45 (44)	36.43	9.52 (42)	7.51	26.92 (52)	1.90
Clark	23.08 (52)	4.16	5.26 (40)	33.96	10.53 (38)	7.21	34.61 (52)	1.77
M4	24.00 (50)	3.76	0.00 (38)	37.31	5.26 (38)	7.61	28.00 (50)	1.87
M9	8.00 (50)	5.06	0.00 (46)	40.32	26.09 (46)	6.10	32.00 (50)	1.88
L17	29.63 (54)	2.68	20.00 (40)	33.47	5.55 (36)	7.83	40.74 (54)	1.39
356	20.00 (50)	4.25	0.00 (40)	37.73	30.00 (40)	5.90	44.00 (50)	1.52
DPX	28.00 (50)	3.38	0.00 (36)	39.21	5.55 (36)	7.87	32.00 (50)	1.76
BP	28.00 (50)	3.09	0.00 (36)	38.61	22.22 (36)	6.33	44.00 (50)	1.39
Zane	18.52 (54)	3.74	0.00 (44)	36.63	9.09 (44)	8.01	25.92 (54)	1.95
Sahar	28.00 (50)	3.34	5.55 (36)	35.64	11.76 (34)	7.61	40.00 (50)	1.53
Gorgan3	8.33 (48)	4.15	4.76 (42)	41.41	10.00 (40)	7.87	20.83 (48)	2.07
Williams	15.38 (52)	4.60	9.09 (44)	30.30	0.00 (40)	8.13	23.08 (52)	2.10

*Numerals in parentheses are the number of samples tested

Table 2. Percentage of mortality and development index (D.I.) of *Helicoverpa armigera* on different soybean cultivars

The life table parameters, particularly, the intrinsic rate of natural increase (r_m), are the most important parameters that can be used to assess plant resistance level to insects (Razmjou et al., 2006). The r_m value of *H. armigera* ranged from 0.1324 to 0.1848 female/female/day, which was minimum on 356 and maximum on M9 (Table 4). The higher r_m value of *H. armigera* on M9 is due to the greater fecundity, lower mortality and shorter development time of the pest fed on this cultivar. Therefore, *H. armigera* fed on M9 has greater potential to population increase and outbreaks in the next generation. However, lower r_m value on 356 was mainly as a result of the poor fecundity and survivorship as well as longer development time of *H. armigera* on this cultivar (Naseri et al., 2009 b). The intrinsic rate of natural increase for *H. armigera* was estimated 0.1135 on sunflower (Reddy et al., 2004) and 0.1423 on pearl millet (Patal & Koshyia, 1997). Some possible reasons for disagreement are due to physiological differences of the host plant tested, genetic differences as a result of laboratory rearing or variation in geographic populations of the pest.

The higher value of r_m indicates the susceptibility of a host plant to insect feeding, while the lower value indicates that the host plant species is resistant to the pest. Therefore, these data show the considerable population growth capacity of *H. armigera* under desirable conditions. Furthermore, since some soybean cultivars such as M9, M7, Clark and Zane were susceptible hosts, *H. armigera* had the greatest opportunity to increase its population on these cultivars. However, some cultivars including L17, 356, BP, Sahar and Gorgan3 were pretty unsuitable host plants, suggesting that they are partially resistant to *H. armigera* compared with others.

Cultivar	Pre-oviposition period (days)	Oviposition period (days)	Post-oviposition period (days)	Daily fecundity	Total fecundity
M7	3.58 abcde* (0.25)	4.33 a (0.44)	1.83 bc (0.23)	118.40 a (14.54)	569.30 a (104.76)
JK	3.73 abcd (0.27)	4.64 a (0.50)	1.45 cd (0.20)	55.39 efg (13.25)	280.6 cde (62.41)
Clark	3.00 de (0.27)	5.54 a (0.52)	1.61 bcd (0.21)	98.45 ab (11.76)	518.00 ab (92.19)
M4	3.20 bcde (0.19)	4.90 a (0.72)	1.60 bcd (0.30)	83.35 h (13.77)	419.30 abcd (113.49)
M9	3.00 de (0.27)	5.50 a (0.54)	1.42 cd (0.43)	85.90 abc (10.99)	511.70 ab (78.73)
L17	3.44 abcde (0.29)	5.00 a (0.47)	1.22 cd (0.22)	81.03 bcde (10.42)	450.50 abc (85.70)
356	2.90 e (0.17)	5.10 a (0.74)	3.00 a (0.78)	37.88 g (5.09)	177.10 e (41.48)
DPX	3.90 ab (0.37)	4.40 a (0.59)	2.40 ab (0.33)	118.92 a (14.61)	582.70 a (95.72)
BP	3.40 abcde (0.26)	4.70 a (0.74)	1.40 cd (0.21)	76.48 bcd (10.40)	393.90 abcd (40.20)
Zane	4.00 a (0.32)	6.50 a (0.94)	0.75 d (0.21)	63.06 defg (8.32)	423.70 abcd (89.98)
Sahar	3.54 abcde (0.15)	5.18 a (0.51)	1.36 cd (0.24)	63.86 defg (12.38)	302.30 bcde (58.51)
Gorgan3	3.83 abc (0.23)	4.25 a (0.47)	1.25 cd (0.12)	47.81 fg (7.60)	262.00 cde (39.98)
Williams	3.11 cde (0.20)	3.67 a (0.40)	1.33 cd (0.28)	66.45 cdef (14.52)	225.50 de (35.45)

The means followed by different letters in the same columns are significantly different ($P<0.01$ and $P<0.05$*, LSD)

Table 3. The mean (SE) pre- and post-oviposition and oviposition periods and fecundity of *Helicoverpa armigera* emerging from larvae reared on different soybean cultivars

3. Soybean cultivars pods affecting the nutritional indices of *H. armigera*

Food consumption and utilization link plant attributes with insect performance (Slansky, 1990). For polyphagous insects, the accessibility of various host plants plays an important function triggering population increase and outbreaks (Singh & Parihar, 1988). Growth, development and reproduction of insects are strongly dependent upon the quality and quantity of ingested nourishment (Scriber & Slansky, 1981). The factors determining nutrient availability for growth and maintenance over a given time of development are the quantity and type of food consumed and the efficiency with which is utilized (Barton Browne & Raubenheimer, 2003). Feeding and foraging behaviour of *H. armigera* on mung bean, *Vigna radiata* (L.) R. Wilczek were determined by Johnson & Zalucki (2007). The effect of morpho-physical factors on consumption and coefficient of utilization of *H. armigera* has been already demonstrated (Ashfaq et al., 2003).

3.1 Case study

3.1.1 Experimental conditions

The neonate larvae, collected from the stock culture which reared on cowpea-based artificial diet, were divided into four replicates (10 larvae in each) and transferred into plastic

container (diameter 16.5 cm, depth 7.5 cm). The first instar larvae were reared in groups until developing to the third instar, then they were separated and transferred to plastic tubes (diameter 3 cm, depth 5 cm) individualy to prevent cannibalism. The fifth instar larvae were kept in the above-described tubes for pre-pupation and pupation.

			Parameter (mean±SE)		
Cultivar	Net reproductive rate (R_0)	Intrinsic rate of natural increase (r_m) (day^{-1})	Finite rate of increase (λ) (day^{-1})	Mean generation time (T) (day)	Doubling time (DT) (day)
M7	354.92±52.34 a	0.1820±0.0063 a	1.20±0.007 a	31.92±0.410 d	3.80±0.013 e
JK	133.47±28.85 def	0.1476±0.0070 cde	1.16±0.008 cde	33.30±0.291 c	4.68±0.211 bc
Clark	210.58±26.57 bc	0.1759±0.0046 a	1.19±0.005 a	30.45±0.192 ef	3.94±0.104 e
M4	170.09±20.25 cde	0.1577±0.0051 bc	1.17±0.006 bc	31.99±0.381 d	4.39±0.145 cd
M9	274.32±31.70 ab	0.1848±0.0050 a	1.20±0.005 a	30.00±0.359 f	3.75±0.104 e
L17	127.76±22.33 def	0.1329±0.0050 f	1.14±0.006 f	36.61±0.391 a	5.21±0.200 a
356	89.35±18.71 f	0.1324±0.0052 ef	1.14±0.006 f	34.12±0.552 c	5.23±0.211 a
DPX	226.56±37.22 bc	0.1549±0.0044 bcd	1.17±0.005 cd	35.09±0.240 b	4.47±0.127 cd
BP	142.11±15.76 cdef	0.1402±0.0024 ef	1.15±0.003 def	35.40±0.408 b	4.94±0.086 ab
Zane	194.23±29.61 bcd	0.1697±0.0067 ab	1.19±0.007 ab	28.85±2.509 de	4.08±0.165 de
Sahar	114.99±22.87 ef	0.1413±0.0065 def	1.15±0.007 def	33.70±0.369 c	4.89±0.232 ab
Gorgan3	119.85±18.29 ef	0.1367±0.0047 ef	1.14±0.004 ef	35.10±0.380 b	5.06±0.177 ab
Williams	107.48±16.28 ef	0.1572±0.0043 bc	1.17±0.005 bc	29.82±0.391 f	4.40±0.113 cd

The means within columns followed by different letters are significantly different ($P < 0.01$, LSD)

Table 4. Life table parameters of *Helicoverpa armigera* on different soybean cultivars

To determine the nutritional indices of *H. armigera*, the consumption of the fourth instar, fifth instar and second to fifth instar larvae on the soybean cultivars were measured by using the gravimetric technique (Waldbauer, 1968). The nutritional indices were measured based on dry weight for weight gain, food consumption and feces produced by *H. armigera* larvae. After measuring the weight of the second instar larvae, they were introduced onto the pods of different soybean cultivars and the weight of the larvae were recorded daily before and after feeding until they reached the pre-pupal stage. The pre-pupa, pupa and adults from the larvae reared on each cultivar were weighed as well. The initial fresh pods and the pods and feces remaining at the end of each experiment were weighed daily. The quantity of food ingested was determined by subtracting the diet remaining at the end of each experiment from the total weight of diet provided. The weight of feces produced by the larvae fed on each soybean cultivar was recorded daily. All of the calculations to determine the nutritional parameters were based on dry-weight determinations made after the extra

specimens including the pods, feces and larval to adult stages (20 specimens for each) had been oven dried (48 hours at 60°C) to a constant weight (Naseri et al., 2010 a).
The following formulae can be used to calculate *CI* (consumption index), *AD* (approximate digestibility), *ECI* (efficiency of conversion of ingested food) and *ECD* (efficiency of conversion of digested food) (Waldbauer, 1968):

$$CI = E/A$$

$$AD = E\text{-}F/E$$

$$ECI = P/E$$

$$ECD = P/E\text{-}F$$

where, *A* = mean dry weight of insect over unit time, *E* = dry weight of food consumed, *F* = dry weight of feces produced and *P* = dry weight gain of insect.

3.1.2 Results

According to the results (Naseri et al., 2010 a) a significant difference was found within the nutritional indices especially *ECI* and *ECD* values of whole larval instars (second to fifth instars) of *H. armigera* reared on different soybean cultivars (Table 4), suggesting that the host plants can change their nutritional values. *ECI* is an overall variable of an insect's ability to utilize the food that it ingests for growth and *ECD* is a variable of the efficiency of conversion of digested food into growth (Nathan et al., 2005).
The nutritional indices of the fourth instar larvae of *H. armigera* were significantly different depending on the type of soybean cultivar (Table 5). However, no significant difference was observed on the nutritional indices of the fifth instar on soybean cultivars except for the larval weight and *ECI*. Therefore, the data obtained for the fourth and fifth instars are not coherent with each other. This may be due to this fact that the nutritional requirements of an insect change during development and such changes are typically reflected in changes of food consumption and feeding behavior (Barton Browne & Raubenheimer, 2003). In a larva, the nutritional requirements over different developmental periods are positively correlated with growth over the period, since growth is directly funded by nutrients.
According to Barton Browne & Raubenheimer (2003), total consumption in the fifth instar of *H. armigera* fed on a navy bean-based diet was about 3.5 times greater than in the fourth, mainly due to the greater rate of ingestion. Another possible reason for this variation could be due to the larval age in a particular stadium at the time of weighing. For instance, the weights of either fourth or fifth stadia are expected to be lower when the larvae are near to enter the next stadium (where the larva stops feeding while entering to next stadium) or recently entered to next stadia (where it losses some water and exuviae) as compared to the larvae growing in the middle age of any stadia. Additionally, differences in physiological changes during penultimate and ultimate instar larvae are probably responsible for the differences in data generated for these two larval instars on soybean cultivars.
Physiological changes in the nervous system of ultimate instar (fifth instar) cessation of feeding, cause wandering behavior, and metabolic changes happen in the fat body. Because of such physiological and behavioral changes, feeding period of the larvae fed on soybean cultivars was shorter in fifth instar than fourth instar, and subsequently nutritional responses of these two larval instars were different (Naseri et al., 2010 a).

The highest *ECI* value of *H. armigera* was on the cultivars Zane and M7, indicating that they were more efficient at the conversion of ingested food to biomass. As can be seen in Table 3, the larvae fed on the cultivar Sahar had the lowest value of *ECD*, which suggests that these larvae were apparently not as efficient in turning digested food into biomass. It is well known that the degree of food utilization depends upon the digestibility of food and the efficiency, which digested food is converted into biomass (Batista Pereira et al., 2002). The reduction in dietary utilization suggests that reduction in nutritional values may be resulted from both behavioral and physiological effects (Nathan et al., 2005).

Cultivar				Parameter (mean±SE)			
	FC (mg)	*FP* (mg)	*DW* (mg)	*CI*	*AD*	*ECI*	*ECD*
M7	78.89 ± 3.06b	26.67 ± 6.11a	30.725 ± 3.453a	7.351 ± 0.958a*	0.610 ± 0.042c	0.524 ± 0.040a	0.820 ± 0.046a
JK	119.15 ± 17.43a	17.55 ± 4.44a	21.580 ± 3.549a	6.906 ± 1.197ab	0.848 ± 0.070ab	0.287 ± 0.075b	0.357 ± 0.123c
Clark	84.60 ± 3.98b	21.44 ± 1.89a	16.037 ± 4.606a	4.457 ± 0.352c	0.699 ± 0.041bc	0.495 ± 0.022a	0.625 ± 0.056ab
M4	120.80 ± 18.33a	18.44 ± 3.79a	16.310 ± 4.306a	4.693 ± 1.566bc	0.857 ± 0.069a	0.281 ± 0.073b	0.357 ± 0.123c
M9	95.54 ± 9.46ab	23.89 ± 6.50a	16.037 ± 4.606a	6.939 ± 1.214ab	0.858 ± 0.064a	0.489 ± 0.052a	0.581 ± 0.077abc
L17	81.66 ± 4.59b	23.96 ± 0.66a	15.497 ± 0.911a	5.302 ± 0.331abc	0.704 ± 0.016abc	0.482 ± 0.017a	0.687 ± 0.039ab
356	88.17 ± 0.735c	23.66 ± 4.13a	24.788 ± 4.548a	4.022 ± 0.870c	0.733 ± 0.044abc	0.495 ± 0.054a	0.693 ± 0.108ab
DPX	84.60 ± 3.99b	31.67 ± 2.80a	19.034 ± 2.624a	4.236 ± 0.313c	0.841 ± 0.090ab	0.502 ± 0.041a	0.705 ± 0.117ab
BP	79.76 ± 3.07b	28.67 ± 2.74a	23.197 ± 1.494a	3.462 ± 0.152c	0.643 ± 0.020c	0.502 ± 0.033a	0.786 ± 0.065a
Zane	81.05 ± 2.81b	21.15 ± 2.01a	26.809 ± 3.221a	5.488 ± 0.922abc	0.597 ± 0.039c	0.499 ± 0.035a	0.787 ± 0.071a
Sahar	118.28 ± 15.12a	28.91 ± 2.73a	23.197 ± 1.494a	5.302 ± 0.331abc	0.843 ± 0.067ab	0.279 ± 0.068b	0.353 ± 0.119c
Gorgan3	98.77 ± 5.95ab	17.55 ± 4.26a	18.244 ± 3.975a	3.594 ± 0.222c	0.852 ± 0.060ab	0.467 ± 0.058a	0.505 ± 0.058bc
Williams	84.60 ± 3.99b	22.40 ± 1.94a	19.579 ± 2.583a	4.457 ± 0.352c	0.736 ± 0.019abc	0.456 ± 0.036a	0.621 ± 0.054ab

The means followed by different letters in the same columns are significantly different ($P < 0.01$, $P < 0.05^*$, LSD)

FC = dry weight of food consumed, *FP* = dry weight of feces produced, *DW* = mean dry weight of larvae, *CI* = consumption index, *AD* = approximate digestibility, *ECI* = efficiency of conversion of ingested food and *ECD* = efficiency of conversion of digested food

Table 4. Nutritional indices of whole larval instars (second to fifth instars) of *Helicoverpa armigera* on different soybean cultivars

Cultivar				Parameter (mean±SE)			
	FC (mg)	*FP* (mg)	*DW* (mg)	*CI*	*AD*	*ECI*	*ECD*
M7	80.62 ± 5.73a*	25.95 ± 3.60ab*	18.102 ± 2.563a	5.309 ± 0.466a	0.779 ± 0.043abc	0.149 ± 0.016a	0.244 ± 0.044abc
JK	69.73 ± 5.12abc	23.35 ± 5.26ab	18.891 ± 2.109a	3.357 ± 0.415cdef	0.610 ± 0.055def	0.147 ± 0.020a	0.147 ± 0.028e
Clark	76.43 ± 5.19ab	19.95 ± 3.99abc	20.767 ± 2.565a	3.496 ± 0.405cde	0.699 ± 0.047bcde	0.125 ± 0.021a	0.251 ± 0.043ab
M4	55.37 ± 4.56c	11.28 ± 0.15c	16.330 ± 1.570a	3.390 ± 0.279cdef	0.783 ± 0.022abc	0.126 ± 0.012a	0.147 ± 0.006e
M9	75.85 ± 8.78ab	17.66 ± 4.13bc	17.860 ± 2.066a	4.247 ± 0.492bc	0.754 ± 0.050abc	0.133 ± 0.017a	0.191 ± 0.032bcde
L17	62.12 ± 4.93bc	26.55 ± 7.05ab	17.653 ± 2.357a	3.377 ± 0.313cdef	0.603 ± 0.083def	0.138 ± 0.036a	0.211 ± 0.038abcde
356	53.82 ± 4.83c	11.46 ± 2.36c	20.150 ± 1.714a	2.671 ± 0.239ef	0.807 ± 0.029ab	0.098 ± 0.015a	0.133 ± 0.023e
DPX	64.02 ± 8.63abc	21.42 ± 3.85abc	17.824 ± 2.240a	3.724 ± 0.459bcd	0.532 ± 0.071f	0.124 ± 0.013a	0.155 ± 0.024de
BP	81.72 ± 7.62a	22.55 ± 3.92ab	17.335 ± 1.973a	4.716 ± 0.440ab	0.722 ± 0.039bcd	0.106 ± 0.011a	0.164 ± 0.023bcde
Zane	69.75 ± 7.17abc	27.04 ± 4.52ab	19.342 ± 2.240a	2.431 ± 0.216f	0.867 ± 0.019a	0.150 ± 0.019a	0.299 ± 0.049a
Sahar	69.40 ± 6.20abc	22.68 ± 3.24ab	19.012 ± 2.359a	4.609 ± 0.477ab	0.659 ± 0.061cdef	0.113 ± 0.016a	0.158 ± 0.022cde
Gorgan3	64.49 ± 8.20abc	17.98 ± 4.37bc	22.869 ± 2.894a	3.039 ± 0.303def	0.713 ± 0.045bcde	0.127 ± 0.017a	0.211 ± 0.029abcde
Williams	64.87 ± 4.83abc	30.38 ± 4.44a	16.958 ± 2.229a	3.825 ± 0.284bcd	0.585 ± 0.062ef	0.152 ± 0.021a	0.241 ± 0.042abcd

The means followed by different letters in the same columns are significantly different ($P < 0.01$, $P < 0.05^*$, LSD) *FC* = dry weight of food consumed, *FP* = dry weight of feces produced, *DW* = mean dry weight of larvae, *CI* = consumption index, *AD* = approximate digestibility, *ECI* = efficiency of conversion of ingested food and *ECD* = efficiency of conversion of digested food

Table 5. Nutritional indices of fourth instar larvae of *Helicoverpa armigera* on different soybean cultivars

The body weight is an important indicator of fitness of an insect, which can be measured easily (Liu et al., 2004). The pupae produced by the larvae reared on Sahar and M4 were lighter than those produced by the larvae reared on the other cultivars (Table 7). This reinforces the suggestion that Sahar and M4 are more unsuitable host plants for *H. armigera* larvae in comparison with the others. Liu et al. (2004) showed that the pupal weight of *H. armigera* was affected by different host plants, which was ranged from 167.1 ± 3.9 mg on tomato to 285.2 ± 4.2 mg on corn. Furthermore, the heaviest pupal weight of *H. armigera* was on cultivar Clark.

Despite significant difference between the pupal weight of *H. armigera* on 13 soybean cultivars, no significant differenc was observed for adult weight on these cultivars. Pupal and adult phenotypic characteristics may be affected by the quality of larval food. Apparent influences of larval diets are body distortions in the pupa and wing malformations in the adult (Rosenthal & Dahlman, 1975). The fecundity (number of eggs laid per female),

longevity and fore-wing area of lepidopteran adults are the most commonly used parameters to determine the larval diet effect on adult stage. Probably, because of no significant effect of the soybean cultivar as larval food on the adult size (fore-wing area) of the pest, this effect has disappeared in the adult. In addition, ability of an insect to store energy (e.g., pupal weight and lipids and glycogen levels) is varied depending on host plant of its larvae (Liu et al., 2007). The results of this study suggest that M7 and Zane are more nutritive and M4, Sahar and JK are less nutritive for *H. armigera* larvae compared to the others.

Cultivar				Parameter (mean±SE)			
	FC (mg)	*FP* (mg)	*DW* (mg)	*CI*	*AD*	*ECI*	*ECD*
M7	124.69 ± 9.57a	93.27 ± 9.53a	48.55 ± 3.19bcd	2.119 ± 0.167a	0.393 ± 0.054a	0.198 ± 0.018abc	0.675 ± 0.132a
JK	137.79 ± 7.99a	90.08 ± 11.35a	58.08 ± 5.31abcd	2.139 ± 0.218a	0.420 ± 0.051a	0.222 ± 0.021a	0.500 ± 0.041a
Clark	118.04 ± 8.33a	75.38 ± 10.29a	59.83 ± 3.83ab	2.051 ± 0.104a	0.401 ± 0.053a	0.163 ± 0.019bc	0.437 ± 0.070a
M4	111.68 ± 8.24a	63.09 ± 10.93a	48.32 ± 4.18cd	2.311 ± 0.170a	0.460 ± 0.075a	0.156 ± 0.017c	0.379 ± 0.056a
M9	123.54 ± 8.95a	91.36 ± 10.64a	56.98 ± 4.79abcd	2.168 ± 0.157a	0.262 ± 0.052a	0.187 ± 0.027abc	0.483 ± 0.080a
L17	107.62 ± 7.25a	73.99 ± 10.04a	52.34 ± 3.92bcd	2.056 ± 0.138a	0.429 ± 0.050a	0.193 ± 0.010abc	0.476 ± 0.060a
356	114.43 ± 6.47a	65.67 ± 8.68a	59.25 ± 3.65abc	1.931 ± 0.109a	0.458 ± 0.050a	0.231 ± 0.014a	0.513 ± 0.064a
DPX	138.60 ± 8.38a	77.33 ± 10.16a	48.19 ± 4.32cd	2.147 ± 0.192a	0.424 ± 0.062a	0.225 ± 0.018a	0.618 ± 0.116a
BP	116.33 ± 9.38a	84.62 ± 11.07a	57.65 ± 4.18abcd	2.018 ± 0.163a	0.387 ± 0.051a	0.212 ± 0.015ab	0.544 ± 0.067a
Zane	136.48 ± 9.10a	63.72 ± 7.10a	51.79 ± 5.47bcd	1.788 ± 0.220a	0.378 ± 0.047a	0.235 ± 0.018a	0.437 ± 0.070a
Sahar	115.32 ± 6.24a	60.95 ± 10.50a	47.42 ± 4.18d	2.146 ± 0.124a	0.445 ± 0.052a	0.203 ± 0.027abc	0.463 ± 0.049a
Gorgan3	120.04 ± 8.78a	51.38 ± 6.32a	54.52 ± 4.46bcd	1.788 ± 0.220a	0.461 ± 0.038a	0.203 ± 0.019abc	0.431 ± 0.053a
Williams	119.19 ± 5.85a	80.71 ± 9.31a	66.79 ± 2.97a	1.940 ± 0.095a	0.400 ± 0.050a	0.168 ± 0.017bc	0.498 ± 0.054a

The means followed by different letters in the same columns are significantly different ($P < 0.05$, LSD)
FC = dry weight of food consumed, *FP* = dry weight of feces produced, *DW* = mean dry weight of larvae, *CI* = consumption index, *AD* = approximate digestibility, *ECI* = efficiency of conversion of ingested food and *ECD* = efficiency of conversion of digested food

Table 6. Nutritional indices of fifth instar larvae of *Helicoverpa armigera* on different soybean cultivars

Cultivar	Pre-pupal weight (mg)		Pupal weight (mg)		Adult weight (mg)		Fore-wing area (cm^2)
	Wet	Dry	Wet	Dry	Wet	Dry	
M7	278.88 ± 14.57bcde	71.67 ± 3.75bcde	242.61 ± 8.99bc	84.91 ± 3.15bc	163.00 ± 7.13a	63.57 ± 2.78a	1.124 ± 0.030a
JK	299.86 ± 18.08abcd	77.06 ± 4.65abcd	245.47 ± 9.30abc	85.91 ± 3.26abc	156.80 ± 9.12a	61.00 ± 3.55a	1.081 ± 0.045a
Clark	317.73 ± 13.07a	81.66 ± 3.36a	269.50 ± 9.35a	94.33 ± 3.27a	155.50 ± 6.24a	60.65 ± 0.65a	1.144 ± 0.043a
M4	254.00 ± 9.62e	65.28 ± 2.47e	203.75 ± 7.87e	71.31 ± 2.75e	143.58 ± 6.78a	56 ± 2.64a	1.154 ± 0.041a
M9	264.00 ± 23.01de	67.85 ± 5.92de	241.79 ± 6.56bc	84.63 ± 2.30bc	163.36 ± 7.81a	63.71 ± 3.05a	1.234 ± 0.057a
L17	271.73 ± 15.24cde	69.83 ± 3.92cde	237.62 ± 5.97bcd	83.17 ± 2.09bcd	153.55 ± 6.95a	59.88 ± 2.71a	1.106 ± 0.048a
356	316.82 ± 12.18a	81.42 ± 3.13a	268.61 ± 10.35a	94.01 ± 2.62a	159.82 ± 12.65a	62.01 ± 4.91a	1.173 ± 0.055a
DPX	286.73 ± 13.34abcde	73.69 ± 3.43abcde	237.89 ± 8.24bcd	83.26 ± 2.88bcd	159.00 ± 7.71a	61.53 ± 2.98a	1.125 ± 0.033a
BP	298.18 ± 10.96abcd	76.63 ± 2.82abcd	261.89 ± 8.93ab	91.66 ± 3.13ab	160.29 ± 6.28a	62.35 ± 2.44a	1.170 ± 0.032a
Zane	313.07 ± 10.59ab	80.46 ± 2.72ab	256.87 ± 12.38ab	89.90 ± 4.33ab	157.31 ± 8.68a	61.35 ± 3.38a	1.132 ± 0.036a
Sahar	249.00 ± 9.21e	63.99 ± 2.37e	216.68 ± 8.81de	75.84 ± 3.08de	155.09 ± 8.35a	60.49 ± 3.25a	1.181 ± 0.031a
Gorgan3	307.65 ± 13.03abc	79.07 ± 3.35abc	228.72 ± 8.31cd	80.05 ± 2.91cd	148.00 ± 7.08a	57.42 ± 2.75a	1.176 ± 0.044a
Williams	293.19 ± 13.42abcd	75.35 ± 3.45abcd	255.68 ± 6.40ab	89.49 ± 2.24ab	148.77 ± 6.41a	57.87 ± 2.52a	1.100 ± 0.062a

Table 7. The mean (±SE) body weights of pre-pupa, pupa and adult stages and fore-wing area of *Helicoverpa armigera* on different soybean cultivars. The means followed by different letters in the same columns are significantly different ($P < 0.01$, LSD)

4. Soybean cultivars seeds affecting the life table parameters of *H. armigera*

Determining the effect of different diets on the life table parameters of insects is of particular importance in understanding host suitability of plant infesting species and determining magnitude of injury to the crops attacked by them (Greenberg et al., 2001). Population parameters are important in measurement of population growth capacity of species under specified conditions. These parameters are also used as indices of population growth rates responding to selected conditions and as bioclimatic indices in assessing the potential of a pest population growth in a new area (Southwood & Henderson, 2000).

4.1 Case study

4.1.1 Experimental conditions

To study the effect of soybean cultivars seeds on the life table parameters of *H. armigera*, the artificial diet based on the seeds of various soybean cultivars including Clark, Gorgan3, L17, M7, M4, M9, Sahar, Sari, Tellar and Zane was used. The artificial diet contained: soybean

seed powder (250 g), wheat germ (30 g), yeast (35 g), sorbic acid (1.1 g), ascorbic acid (3.5 g), sunflower oil (5 ml), agar (14 g), methyl-p-hydroxy benzoate (2.2 g), formaldehyde 37% (2.5 ml) and distilled water (650 ml) (Teakle, 1991). The prepared artificial diets were kept refrigerated for no longer than two weeks before use (Soleimannejad et al., 2010). The experimental conditions to determine the life table parameters of *H. armigera* were the same as the previously described conditions on the pods of soybean cultivars (see the section 2.2).

4.1.2 Results

According to the results (Soleimannejad et al., 2010), there was strong effect of the seeds of different soybean cultivars on development time of *H. armigera* when incorporated into artificial diet (Table 8). The larvae reared on Clark and Sari had comparatively shorter development time of immature stages which was more than the value (29.7 days on cotton) previously reported on different hosts by Liu et al. (2004). However, the development time of the immature stages was lower on our artificial diets than published by Shanower et al. (1997) for *Cajanus scarabaeoides* (53 days). Slower development time on a particular host means a longer life cycle, usually a lower reproductive ability and slower population growth (Singh & Parihar, 1988). In this study the larvae which had eaten an artificial diet based on Sahar, L17, Gorgan3 and M4 completed their larval period in five instars, as reported already by Saour & Causse (1996) and Naseri et al. (2009 a). On the other examined cultivars (Clark, M7, M9, Sari, Tellar, Zane) the larvae completed development in six instars. Six instars of *H. armigera* have been reported by Goyal & Rathore (1988) and Borah & Dutta (2002).

Using Sahar, L17 and Gorgan3 diets resulted in very poor survival. Survival rate on soybean was low (12 %) in comparision with chickpea and maize as artificial diets for *H. armigera* (Singh & Rembold, 1992). The pupae produced by the larvae reared on Sahar and L17 were much lighter than others Table 8). These findings support the suggestion that Sahar and L17 are less suitable host plants for *H. armigera* larvae than other cultivars.

Adult life span was significantly different depending upon the soybean seed which the larvae had been feeding on. The adults reared as larvae on some soybean cultivars have lower longevity (Table 8) compared with values reported by Borah & Dutta (2002) (6.38: 8.66 days, male: female on pigeon pea) and Liu et al. (2004) (12.1: 14 days male: female on hot pepper). Such effects might be due to the presence of some secondary allelochemicals in seeds of these cultivars. It seems that the life span of *H. armigera* tended to be sensitive to different artificial diets.

Fecundity in heliothines is influenced by temperature, humidity, and larval and adult nutrition (Adjei-Maafo & Wilson, 1983, Liu et al., 2004). Females laid the highest number of eggs when reared on Sari compared with the other diets, and females reared on Sahar oviposited least number of eggs during their oviposition period (Table 9).

As can be seen in Table 10, the lower value of r_m is on Sahar and L17, which might be attributed to considerably lower fecundity and survivorship. Similarly a close association was found between the effects of pods and seeds of soybean cultivars on life table parameters as minimum r_m was 0.132 on L17 (Naseri et al., 2009 b). A skewed female-biased sex ratio was not observed in *H. armigera* reared on pods of soybean (Naseri et al., 2009 b) nor has this phenomenon been reported on other host plants. The main reasons for male-killing agents are less clear but clues have been provided by the timing of male death (Hurst & Majerus, 1992).

Cultivar	Egg period	Larval stages						Pre pupal period	Pupal period	Duration of immature stages	Pupal weight	Immature survival (%)
		First	Second	Third	Fourth	Fifth	Sixth					
Clark	2.50 ± 0.12 f	4.3 ± 0.02 e	2.40 ± 0.24 e	2.50 ± 0.66 cd	2.10 ± 0.28 d	3.03 ± 0.05 h	2.00 ± 0.24 e	3.50 ± 0.02 c	10.81 ± 0.28 f	32.00 ± 0.57 f	271.30 ± 1.13 a	58
Gorgan3	4.50 ± 0.57 c	5.6 ± 0.12 ab	4.60 ± 0.24 bc	3.50 ± 0.24 a	3.00 ± 0.25 ac	5.70 ± 0.03 c	-	5.00 ± 0.00 b	16.39 ± 0.28 b	48.77 ± 1.20 bc	237.78 ± 0.94 cd	35.55
L17	6.00 ± 0.47 a	5.5 ± 0.33 bc	7.27 ± 0.30 a	3.40 ± 0.25 ac	3.54 ± 0.15 a	7.62 ± 0.02 a	-	4.55 ± 0.28 b	18.76 ± 0.22 a	56.66 ± 0.84 a	233.20 ± 1.44 cd	49.05
M4	4.75 ± 0.24 bc	5.5 ± 0.09 ab	5.33 ± 0.66 b	3.50 ± 0.24 a	3.25 ± 0.20 ab	6.75 ± 0.037 b	-	4.69 ± 0.20 b	16.50 ± 0.06 b	50.66 ± 0.88 b	240.07 ± 0.76 bcd	54
M7	4.25 ± 0.24 cd	4.7 ± 0.06 d	3.65 ± 0.28 de	2.60 ± 0.28 cd	2.80 ±0.25 bd	4.77 ± 0.06 e	4.20 ± 0.24 b	3.50 ± 0.28 c	13.50 ± 0.07 d	44.00 ± 0.95 c	258.5 ± 1.10 abcd	67.24
M9	4.25 ± 0.24 cd	4.0 ± 0.03 e	4.00 ± 0.00 cd	2.40 ± 0.28 d	2.80 ± 0.28 bd	5.36 ± 0.06 d	4.50 ± 0.14 a	2.50 ± 0.28 d	14.50 ± 0.28 c	45.25 ± 1.10 c	250.76 ± 1.31 bcd	47.5
Sahar	5.60 ± 0.25 ab	5.8 ± 0.040 a	5.50 ± 0.28 b	3.81 ± 0.22 a	3.40 ± 0.24 ab	7.49 ± 0.04 a	-	5.80 ± 0.15 a	16.79 ± 0.20 b	54.16 ± 0.70 ab	229.50 ± 0.75 d	45.09
Sari	3.18 ± 0.44 ef	4.7 ± 0.05 e	3.00 ± 0.40 de	3.06 ± 0.28 acd	2.50 ± 0.03cd	3.68 ± 0.05 g	2.50 ± 0.28 d	3.63 ± 0.06 c	12.42 ± 0.02 e	38.00 ± 0.57 e	274.05 ± 0.97 a	55.31
Tellar	4.00 ± 0.28 cde	4.2 ± 0.52 e	3.25 ± 0.25 de	2.50 ± 0.24 cd	2.75 ± 0.00bd	4.60 ± 0.03 f	3.60 ± 0.37 bc	3.40 ± 0.24 c	13.50 ± 0.28 d	41.75 ± 1.31 d	262.08 ± 0.98 abcd	54.16
Zane	3.49 ± 0.28 def	5.4 ± 0.28 c	3.00 ± 0.00 de	3.25 ± 0.28 acd	2.50 ± 0.10 cd	4.59 ± 0.07 f	3.27 ± 0.02 c	3.75 ± 0.25 c	13.20 ± 0.04 d	42.00 ± 0.96 d	265.00 ± 0.95 ab	47.22

Values followed by the same letter in each column are not significantly different (P <0.01 ,Duncan)

Table 8. The mean (± SE) duration of different development stages, survivorship and pupal weight of *Helicoverpa armigera* on different soybean cultivars

Cultivar	Longevity		Whole life span		Fecundity		Pre-oviposition period	Oviposition period	Post-oviposition period	Adult weight
	Female	Male	Female	Male	Total	Daily				
Clark	17.30 ± 0.18 j	7.30 ± 0.10 j	50.92 ± 0.08 i	39.65 ± 0.18 h	1779.78 ± 51.91b	102.66 ± 2.73 b	1.10 ± 0.03 e	9.75 ± 0.13 a	6.38 ± 0.04 d	178.50 ± 3.25 b
Gorgan3	22.30 ± 0.14 d	15.36 ± 0.18 d	70.75 ± 0.14 c	64.51 ± 0.57 d	741.21 ± 32.19 ef	33.68 ± 2.29 ef	10.22 ± 0.08b	6.30 ± 0.11e	6.48 ± 0.04 d	145.50 ± 0.63 bc
L17	26.10 ± 0.089 a	20.60 ± 0.17 a	81.68 ± 0.29 a	76.91 ± 0.23 a	916.61 ± 66.47 e	35.23 ± 3.52 e	10.74 ± 0.06b	3.91 ± 0.32 f	11.07 ± 0.09 a	143.00 ± 0.74 bc
M4	23.57 ± 0.12 c	16.30 ± 0.15 c	74.06 ± 0.25 b	67.16 ± 0.16 c	1157.61 ± 15.16 d	48.20 ± 1.01 d	7.75 ± 0.03 c	5.24 ± 0.05 e	10.10 ± 0.05 b	147.7 ± 0.56 abc
M7	21.25 ± 0.13 e	12.22 ± 0.13 f	65.75 ± 0.32 d	56.16 ± 0.18 e	1253.89 ± 59.26 d	59.71± 2.61 d	6.81 ± 0.06 d	6.85 ± 0.12 d	7.59 ± 0.03 c	155.90 ± 0.7 abc
M9	18.65 ± 0.04 g	14.43 ± 0.23 e	63.25 ± 0.32 f	59.87 ± 0.17g	1451.29 ± 65.6 c	76.36 ± 2.83 c	3.28 ± 0.06 f	8.35 ± 0.10 b	7.07 ± 0.03cd	153.2 ± 0.63 abc
Sahar	25.37 ± 0.12 b	18.30 ± 0.10 b	79.25 ± 0.16 ab	72.77 ± 0.18 b	589.67 ± 43.58 f	23.30 ± 6.81 f	10.96 ± 0.08 a	3.78 ± 0.13 f	10.10 ± 0.08 b	139.83 ± 0.91 c
Sari	17.30 ± 0.05 i	9.42 ± 0.11 i	54.92 ± 0.14 h	47.47 ± 0.18 i	2558.40 ± 86.83 a	149.87± 4.82 a	1.10 ± 0.05 g	10.00 ± 0.05 a	6.10 ± 0.07 de	183.67 ±1. 46 a
Tellar	19.56 ± 0.04 f	11.20 ± 0.10 g	61.75 ± 0.32 e	52.36 ± 0.32 f	1679.19 ± 25.91 b	83.10 ± 1.29 c	4.58 ± 0.05 e	7.73 ± 0.06 c	6.47 ± 0.04 d	162. 2± 1.29 abc
Zane	17.30 ± 0.05 h	10.45 ± 0.15 h	59.70 ± 0.66 g	52.75 ± 0.02 h	1432.74 ± 58.05c	84.29 ± 3.22 c	1.75 ± 0.04 g	8.16 ± 0.10 b	6.75 ± 0.05 cd	162.60 ± 1.35 ab

Values followed by the same letter in each column are not significantly different (P <0.01; Duncan).

Table 9. The mean (± SE) adult longevity, life span, fecundity, pre- and post- oviposition and adult weight of *Helicoverpa armigera* on different soybean cultivars

Cultivar	R_0	r_m	λ	T	DT
Clark	270 ± 4.2 a	0.113 ± 0.003 ab	1.121 ± 0.004 a	40.32 ± 0.61 bc	6.08 ± 0.18 e
Gorgan3	34 ± 1.1 f	0.093 ± 0.001 bc	1.078 ± 0.002 e	42.98 ± 0.57 ab	7.43 ± 0.05 ab
L17	16 ± 1.56 g	0.090 ± 0.008 c	1.086 ± 0.003 de	42.21 ± 0.95 abc	8.10 ± 0.29 a
M4	20 ± 0.90g	0.092 ± 0.005 bc	1.097 ± 0.006 cd	42.46±1.18 abc	7.69 ± 0.18 a
M7	17 ± 0.87 g	0.099 ± 0.001 abc	1.105 ± 0.001 de	45.28 ± 0.75 a	7.98 ± 0.11 a
M9	94 ± 1.01 cc	0.100 ± 0.004 abc	1.100 ± 0.003 cd	36.72 ± 1.30 d	7.34 ± 0.14 abc
Sahar	17.43 ± 0.87 cg	0.084 ± 0.001 c	1.087 ± 0.001 de	45.28 ± 0.75 a	7.98 ± 0.10 a
Sari	162.27 ± 2.49 cb	0.114 ± 0.005 a	1.116 ± 0.005 ab	40.68 ± 0.56 bc	6.18 ± 0.22 cde
Tellar	83.91 ± 0.13 d	0.100 ± 0.001 abc	1.111± 0.004 abc	39.54 ± 0.32 cd	6.70 ± 0.08 ab
Zane	153.78 ± 0.48 b	0.110 ± 0.007 ab	1.107 ± 0.007 abc	41.48 ± 0.73 bc	6.65 ± 0.35 cde

Values followed by the same letter in each column are not significantly different (P <0.01; Duncan).

Table 10. Life table parameters of *Helicoverpa armigera* on different soybean cultivars

5. Soybean cultivars seeds affecting the nutritional indices of *H. armigera*

5.1 Case study

5.1.1 Experimental conditions

A total of 50 larvae of 3rd and 4th instars in five replicates (10 larvae in each) were weighed to an accuracy of 0.001 g and provisioned on adequate and weighed amount of the rearing food. In each daily observation, all the larvae, food remains and feces were weighed and a fresh weighed amount of food provided for each larva. In the old larvae (5th instars to end of larval stage), each larva and provided food were weighted individually. Pre-pupae, pupae and adults were weighted and compared in each cohort. The nutritional indices as described by Waldbauer (1968) were calculated. All indices were calculated using dry weights. To estimate initial dry weights, three fresh larvae and three blocks of the diet were measured separately. The larvae and food were each oven-dried at 60ºC for 72 h and then weighed to determine dry weights. The measured nutritional indices were relative growth rate (*RGR*), relative consumption rate (RCR), efficiency of conversion of ingested food (*ECI*), efficiency of conversion of digested food (*ECD*), approximate digestibility (*AD*) and consumption index (*CI*) and calculated as follows:

$$RGR = B/Tb$$

$$RCR = F/Tb$$

$$ECI = RGR/RCR\ (\%)$$

$$ECD = [B/\ (F\text{-}M_F)] \times 100$$

$$AD = (F\text{-}M_F)/F \times 100$$

$$CI = F/b\ (mg/mg/d)$$

where B= weight gained in feeding period (weight of live insects on the last day - weight of insects at first day (mg)), b = mean dry weight of larva during the feeding period (mg), T= feeding period (day), F= weight of food ingested (mg), M_F = weight of feces (mg). The weights (mg) of pupae and adults were recorded on the second day after pupation and adult emergence.

Effects of the main factor (host plant) on the life table parameters and nutritional indices of *H. armigera* were analyzed using ANOVA multiple comparisons by Duncan test. A t-test was used to compare nutritional index values between young and old larvae.

5.1.2 Results

Different soybean seed diets had significant effects on nutritional indices of *H. armigera*. The lowest *AD* on Clark (48.1%) and the highest ECD on Sari (38.3%) indicated that these seeds had a large effect on the physiology of *H. armigera.* The highest value of *AD* in young larvae was noticed on Sahar (9.57 %), however the lowest *AD* in old larvae (48.09% on Clark) was five times higher, and this significant difference may be related to better feeding ability of *H. armigera* in these stages than diet effects.

Significant differences of *ECD* and *ECI* were observed in both young and old larvae reared on seeds of various soybean cultivars (Table 11). The lowest value of *AD* in old larvae was 48.1% higher than that reported by Wang et al. (2006) on wheat based artificial diet. This could be in related to higher performance of soybean compared with wheat as an artificial diet.

Among soybean cultivars, Sahar has presented as the most resistant cultivar to *Tetranychus*

Cultivar	*RGR* (mg/mg/d)	*RCR* (mg/mg/d)	*ECI* %	*ECD* %	*CI* (mg/mg/d)	*AD* %
Young Larvae (3th and 4th instars)						
Clark	0.08 ± 0.002 a	2.62 ± 0.59 a	3.11 ± 0.19 a	3.17 ± 0.12 a	2.44 ± 1.02 a	9.15 ± 0.67 b
Gorgan3	0.01 ± 0.003 e	1.64 ± 0.27 cd	0.91 ± 0.27 de	0.90 ± 0.079 c	1.53 ± 2.66 c	9.47 ± 0.65 a
L17	0.01 ± 0.003 e	1.60± 0.76 d	0.84 ± 0.06 e	0.88 ± 0.06 c	1.40 ± 2.73 c	9.50 ± 0.55 a
M4	0.02 ± 0.002 d	1.82 ± 0.83 cd	0.98 ± 0.12 de	1.32 ± 0.18 bc	1.61 ± 1.44 bc	9.47 ± 0.68 a
M7	0.03 ± 0.011 c	1.92 ± 0.25 cd	1.49 ± 0.15 cd	1.74 ± 0.24 b	1.88 ± 1.15 bc	9.40 ± 1.45 ab
M9	0.02 ± 0.003 c	1.83 ± 0.87 cd	1.28 ± 0.09 cde	1.56 ± 0.16 b	1.74 ± 4.76 bc	9.45 ± 0.71 ab
Sahar	0.01 ± 0.000 e	1.47 ± 0.88 d	0.79 ± 0.14 e	0.85 ± 0.17 c	1.26 ± 0.99 c	9.57 ± 0.57 a
Sari	0.05 ± 0.003 b	2.20 ± 0.35 ab	2.51±0.76 b	2.71 ± 0.87 a	2.22 ± 3.22 a	9.16 ± 0.42 b
Tellar	0.04 ± 0.002 bc	2.05 ± 0.16 bc	1.83 ± 0.22 c	1.91 ± 0.23 b	2.08 ± 2.90 a	9.37 ± 1.35 ab
Zane	0.04 ± 0.002 bc	1.94 ± 0.39 cd	1.65 ± 0.10 c	1.80 ± 0.11 b	2.02 ± 2.62 a	9.35±1.34 ab
Old Larvae (5th instars to end of larval stage)						
Clark	0.59 ± 0.01 a	3.36 ± 0.28 a	17.24 ± 0.77 a	36.40 ± 2.46 a	3.02 ± 0.10 a	48.09 ± 2.42 c
Gorgan3	0.32 ± 0.02 de	2.62 ± 0.07 bcd	10.12 ± 1.03 d	15.01 ± 2.73 c	1.14 ± 0.11 cd	63.52 ± 2.34 a
L17	0.23 ± 0.01 d	2.33 ± 0.11 cd	12.28 ± 0.99 bcd	21.43 ± 1.98 bc	1.08 ± 0.16 d	64.62 ± 2.184 a
M4	0.27 ± 0.01 de	2.57 ± 0.12 bcd	10.54 ± 1.40 cd	18.39 ± 1.41 bc	1.21 ± 0.10 cd	61.99 ± 3.65 a
M7	0.36 ± 0.01 c	2.65 ± 0.10 bc	13.80 ± 1.39 b	24.98 ± 1.60 b	1.58 ± 0.12 bcd	61.08 ± 3.22 ab
M9	0.35 ± 0.01 c	2.64 ± 0.16 bc	13.24 ± 0.41 bc	22.20 ± 1.73 b	1.38 ± 0.15 bcd	61.28 ± 5.112 ab
Sahar	0.20 ± 0.03 e	2.13 ± 0.12 d	9.81 ± 0.63 d	14.81 ± 1.38 c	0.94 ± 0.28 d	66.97 ± 2.48 a
Sari	0.45 ± 0.01 b	3.02 ± 0.23 ab	15.23 ± 1.04 ab	38.30 ± 2.94 a	1.93 ± 0.31 b	50.58 ± 3.04 c
Tellar	0.39 ± 0.01 bc	2.68 ± 0.15 bc	14.76 ± 0.83 ab	33.37 ± 2.34 a	1.60 ± 0.07 bcd	53.05 ± 2.91 bc
Zane	0.40 ± 0.02 bc	2.71 ± 0.21 bc	15.05 ± 0.64 ab	32.53 ± 2.50 a	1.78 ± 0.21 bc	50.91 ± 2.66 c

Values followed by the same letter in each column of larvae stages groups are not significantly different (Duncan).

*values of each nutritional index on each cultivar between larval stage groups (young and old larvae) are significantly different, ($P < 0.01$, t- test).

Table 11. Nutritional indices of Young (3th and 4th instars) and old larvae (5th instars to end of larval stage) of *Helicoverpa armigera* on different soybean cultivars

*urtica*e (Koch) and L17 was classified as susceptible (Sedaratian et al., 2009), however L17 was resistant cultivar for *H. armigera*. Overall we suggest that Sahar could serve as a key tool in integrated pest management in soybean fields due to its resistance to *H. armigera* and *T. urtica*e. Our observations provided evidence that seeds from different soybean cultivars as a diet for immatures affected life history and nutrition of *H. armigera*.

6. Soybean cultivars pods affecting digestive proteolytic and amylolytic activities of *H. armigera*

Insect digestive proteases catalyze the release of peptides and amino acids from dietary proteins in the insect digestive system to meet its nutritional requirements (Terra & Ferreira, 1994). The larval midgut in Lepidoptera harbors complex digestive proteolytic activities including trypsins, chymotrypsins, elastases, cathepsin-B like proteases, aminopeptidases and carboxypeptidases. Works on the protease digestive enzymes of lepidopteran insects showed that they prevalently (95% of total digestive activity) depending on serine proteases for protein digestion (Bown et al., 1997). In addition to complexity of multiple protease specificities, there usually exists a set of diverse protease isoforms. The gut of *H. armigera* has been known to contain around 20 different types of active serine protease isoforms at any given moment (Purcell et al., 1992). The α-amylases (α-1, 4-glucan-4-glucanohydrolases; EC 3.2.1.1) are hydrolytic enzymes that are found in microorganisms, plants and animals, which catalyze the hydrolysis of α-D-(1, 4)-glucan linkage in starch and related carbohydrates (Stroble et al., 1998). In insects, the activity of digestive enzymes such as proteases and α-amylases depends upon the nature of food sources or chemical substances consumed (Slansky, 1982). Protease and α-amylase activities in crude extracts of larval guts of *H. armigera* have been described by some researchers (e.g. Patankar et al., 2001; Chougule et al., 2005; Kotkar et al., 2009).

6.1 Case study

6.1.1 Experimental conditions

In order to preparation of crude midgut enzyme extracts of *H. armigera* larvae, fifth-instar larvae reared on either cowpea-based artificial diet or different soybean cultivars for 24 h are cold-immobilized, rapidly dissected under a stereomicroscope. The haemolymph is washed away with precooled distilled water, and the midguts are then cleaned by removal of extraneous tissues. The midguts, including contents, are collected into a known volume of distilled water, homogenized with a hand-held glass grinder on ice and the homogenates centrifuged at 16,000 × g for 10 min at 4°C. The resulting supernatant is collected, frozen in aliquots and stored at -20°C until required for protease and amylase assays (Naseri et al., 2010 b).

General proteolytic activity present in the midgut of *H. armigera* larvae fed either on artificial diet or different soybean cultivars can be determined using azocasein as a substrate at the pH optimum. The universal buffer system (50 mM sodium phosphate-borate) is used to determine the pH optimum of proteolytic activity over a pH range of 7 to 12. To determine the azocaseinolytic activity, the reaction mixture containing 80 µl of 1.5% azocasein solution in 50 mM universal buffer pH 12 and 50 µl of crude enzyme is incubated at 37°C for 50 min. Proteolysis is stopped by the addition of 100 µl of 30% trichloroacetic acid (TCA), followed by cooling at 4°C for 30 min and centrifugation at 16,000 × g for 10 min. An equal volume of 2 M NaOH is added to the supernatant and the absorbance is measured at 440 nm.

Appropriate blanks in which TCA had been added prior to the substrate are prepared for each assay. Unit activity is expressed as an increase in optical density per milligram protein of the tissue min^{-1} due to ***azocasein*** proteolysis (Naseri et al., 2010 b)

Digestive trypsin-, chymotrypsin- and elastase-like activities of the larvae fed either on artificial diet or soybean cultivars using final concentrations of 1 mM BAρNA, 1 mM SAAPFρNA and 1 mM SAAAρNA as substrates, respectively were estimated. A reaction mixture consisted of 20 µl enzyme extract for trypsin- and elastase-like activities and 10 µl enzyme extract for chymotrpsin-like activity, 75 µl universal buffer at the appropriate pH optimum (pH 10.5 for trypsin- and chymotrpsin-like enzymes and pH 11 for elastase-like enzyme), and 5 µl of the above-mentioned substrate. Absorbance was then measured at 405 nm for 40 min (at 2, 1 and 4 min time intervals, respectively). All assays were carried out in triplicate against appropriate blanks (Naseri et al., 2010 b).

The dinitrosalicylic acid (DNSA) method (Bernfeld, 1955), with 1% soluble starch as the substrate can be used to assay digestive amylolytic activity of *H. armigera* larvae fed either on artificial diet or the different soybean cultivars. According to this method, fifty microliters of the enzyme are incubated with 250 µl universal buffer pH 10 and 20 µl soluble starch for 30 min at 37°C. The reaction is stopped by addition of 50 µl DNSA and heating in boiling water for 10 min. The absorbance is then read at 540 nm after cooling on ice. One unit of amylase activity is defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 37°C under the given assay conditions. All assays are performed in triplicate (Highley, 1997; Naseri et al., 2010 b).

We determined the effect of different protease inhibitors on proteolytic activities of midgut extract of *H. armigera* larvae. Chemical and plant inhibitors are used at final concentrations of: 1 mM PMSF (serine protease inhibitor), 0.5 mM TLCK (trypsin inhibitor) and 0.05 mM chymostatin (chymotrypsin inhibitor), 0.002 mM SKTI (trypsin inhibitor), 0.002 mM STI (trypsin inhibitor) and 0.002 mM SBBI (trypsin-chymotrypsin inhibitor). All inhibition assays are conducted as described in the enzyme assay section except that the enzyme extract and inhibitor are pre-incubated in the buffer (pH optimum) at room temperature for 15 min prior to addition of the substrate (Naseri et al., 2010 b).

To determine the effect of different combinations of protease inhibitors on endogenous proteolytic activity of *H. armigera*, fifth instar larvae (20 larvae) are fed for 24 hours on artificial diet containing either 0.5% (w/v) SKTI, STI or SBBI or control diet (without any inhibitor). The larvae are placed in 250 ml plastic container with holes pierced in the lid, and lined with tissue paper to take out moisture. After larval feeding for 24 hours, the midguts are dissected and the midgut enzymes are prepared as above. Relative contributions of different proteolytic activities are assessed using combinations of inhibitors in assays of general and specific proteolytic activities using appropriate substrates. The percentage inhibition of general and specific proteolytic activities by individual inhibitors, or mixture of inhibitors, is measured (Naseri et al., 2010 b).

The visualization of protease activity present in homogenates of larval midguts fed on different soybean cultivars is carried out after non-denaturing SDS-polyacrylamide gel electrophoresis (PAGE) using the procedure of Garcia-Carreno et al. (1993) with minor modification (Naseri et al., 2010 b). Electrophoresis is performed in a 7.5% (w/v) separating gel and a 4% stacking gel. The sample buffer contained 25% glycerol, Tris-Hcl 0.2 M (pH 6.8), 5% SDS and 2.5% bromophenol blue, but no mercaptoethanol, and was not boiled. Electrophoresis is conducted at room temperature at a constant voltage of 110 V until the blue dye reached the bottom of the slab gel. For visualization of protease activity, gels are

washed by shaking gently in 0.1 M phosphate buffer (pH 7.5) containing 2.5% triton X-100 thrice for 10 min, followed by 0.1 M borate buffer (pH 8) for 30 min. Gels are then incubated in 0.5% casein for 120 min, and gel strips stained with commassie blue to detect protease activity bands as clear zones against a dark blue background.
Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard (2, 1.5, 1, 0.5, 0.25, 0.125 and 0.063 mg ml^{-1}).

6.1.2 Results

Helicoverpa armigera larvae show complicated and diverse forms of proteolytic digestion that is influenced by the host plant on which they are feeding (Patankar et al., 2001; Chougule et al., 2005). We investigated how diet affected gut proteolytic activity and subsequent sensitivity to inhibition by plant-derived or chemical protease inhibitors (PIs) (Naseri et al., 2010 b). The highest level of general proteolytic activity was in the artificial diet-fed larvae (Figure 1), suggesting its nutritionally balanced composition. Artificial diets are usually complete nourishments formed for high insect performance and commonly considered to be better than natural diets (Hari et al., 2007). According to Kotkar et al. (2009) *H. armigera* fed on artificial diet completed its life cycle somewhat early compared with natural diets. Among different soybean cultivars, the highest general proteolytic activity was in the larvae reared on L17, M4 and Sahar, indicating the presence of some PIs on these cultivars, leading to hyperproduction of proteases by midgut cells of *H. armigera* in response to protease inhibition by PIs. The larvae of *H. armigera* fed on chickpea show more than 2.5 to 3- fold protease activity compared with those reared on the other host plants (Patankar et al., 2001). Higher protease activities in the chickpea or artificial diet-fed larvae may be due to either high protein content of the diet or response of the insect to the dietary PIs which partly inhibit the activity of midgut proteases (Patankar et al., 2001). Furthermore, hyperproduction of proteases in response to consumed PIs leads to an additional load on the insect for energy and essential amino acids resulting in postponement of the insect growth and development (Broadway & Duffy, 1986).

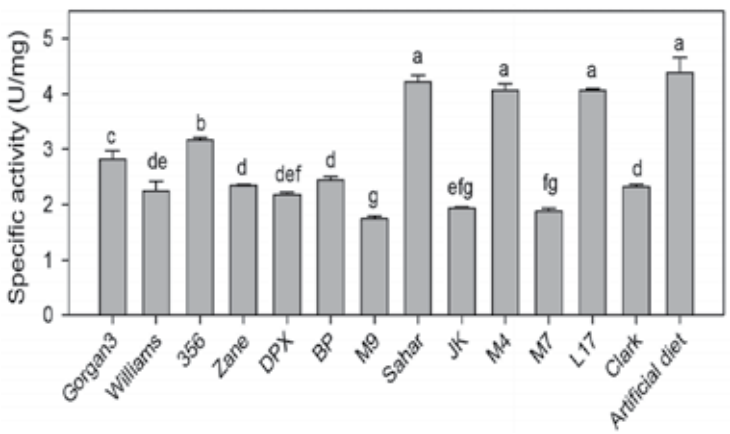

Fig. 1. General proteolytic activity of midgut extracts from *Helicoverpa armigera* larvae reared on either artificial diet or different soybean cultivars using azocasein as substrate, pH 12. Bars represent means of three independent estimations associated with standard error (P = 0).

The highest trypsin- and elastase-like activities were also in artificial diet-fed larvae compared with the soybean cultivars (Figure 2). Among soybean cultivars, the activity of trypsin-like enzymes in the midgut extract of larvae reared on L17 and Sahar was the lowest. It could be suggested that the inhibition of tryptic activity by PIs present in these two cultivars happened probably to decrease activity of trypsin-like enzymes in midgut extracts of the larvae fed on these cultivars. However, the larvae reared on L17 and Sahar had the highest chymotryptic activity compared with the other cultivars may be because of overexpression of chymotrypsin-like enzymes in response to the trypsin inhibitors on these cultivars.

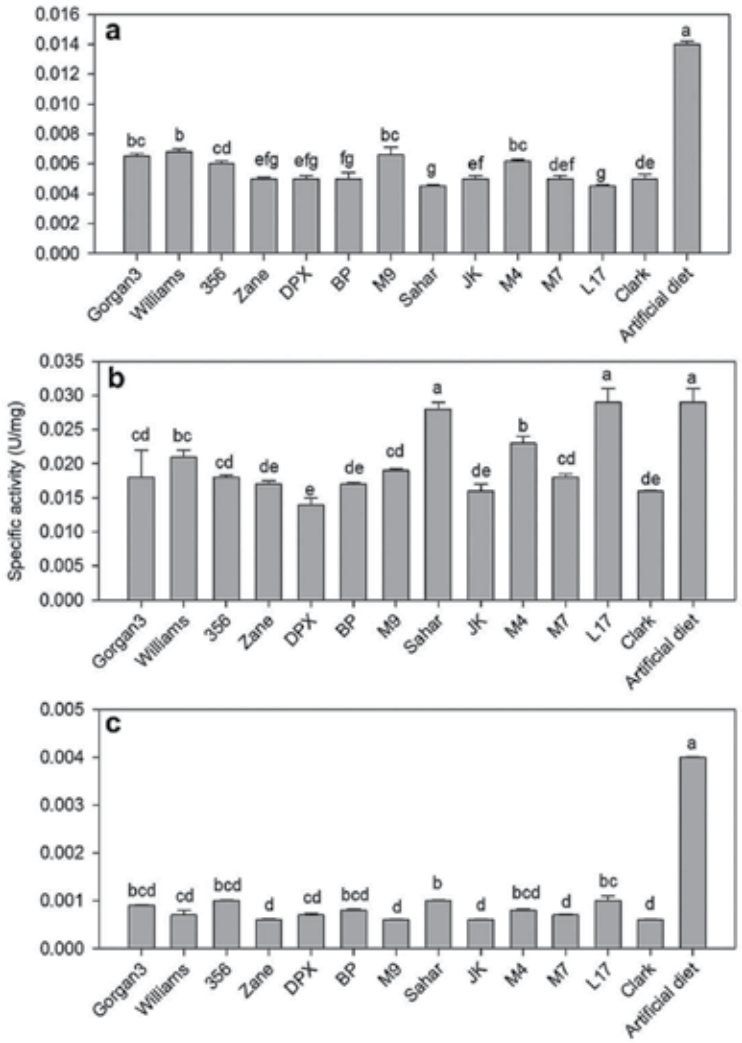

Fig. 2. Trypsin- (a), chymotrypsin- (b) and elastase-like (c) activities of midgut extracts from *Helicoverpa armigera* larvae reared on either artificial diet or different soybean cultivars using BAρNA, pH 10.5; SAAPFρNA, pH 10.5 and SAAAρNA, pH 11 as substrates, respectively. Bars represent means of three independent estimations associated with standard error (P = 0).

The same result on elastase-like activity was detected in Sahar fed larvae in response to the presence of trypsin inhibitors of this cultivar. Previous studies pointed out that *H. armigera* fed on L17 and Sahar had a weakly potential to population increase and these cultivars were less suitable host plants for the growth and development of *H. armigera* than the other cultivars tested (Naseri et al., 2009 a,b). By composing the results of our earlier studies on demographic parameters of *H. armigera* on L17 and Sahar and results of proteolytic activity of the larvae fed on these two cultivars, it would be deduced that, perhaps, the presence of some PIs in these cultivars, which acting as antibiosis agents were responsible for a weakly performance of *H. armigera* reared on these cultivars. Generalized overexpression of some trypsin-like and chymotrypsin-like proteases was reported in *H. armigera* fed on various non-host plant protease inhibitors (Chougule et al., 2005).

Digestive amylolytic activity of *H. armigera* is affected by either artificial diet or different soybean cultivars (Naseri et al., 2010 b). Artificial diet-fed larvae of *H. armigera* showed nearly two times higher midgut amylase activity than those fed on soybean cultivars (Figure 3). Such inconsistency in enzyme activities of the artificial and natural diet-fed insects has been reported by Chougule et al. (2005). Artificial diet-fed larvae of *H. armigera* are healthier and they can complete their life history earlier compared with natural diet, indicating that the artificial diet does not exert pressure for a metabolic adjustment. There were little significant differences in amylolytic activity of midgut extracts from *H. armigera* larvae reared on the most soybean cultivars. It could be suggested that since the total carbohydrate substance in soybean cultivars was probably equal with each other, thus any high significant differences in amylase activity of the larvae fed on different soybean cultivars were not detected. However, the amylolytic activity was the highest on M4 and lowest on Williams and DPX. Kotkar et al. (2009) have indicated that natural diet-fed *H. armigera* had three times lower gut amylase activities compared with those fed on artificial diet. It was also reported by above-mentioned researchers that the larvae reared on legume and vegetable crops showed double gut amylolytic activity than those fed on ornamental and cereal crops.

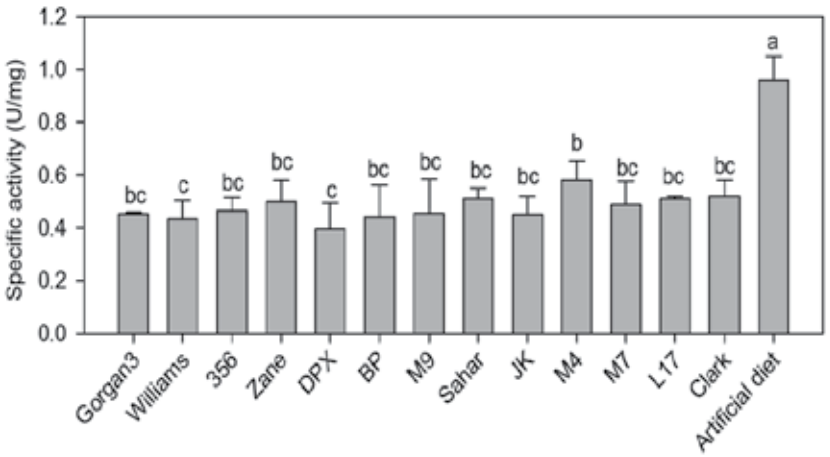

Fig. 3. Amylolytic activity of midgut extracts from *Helicoverpa armigera* larvae reared on either artificial diet or different soybean cultivars using 1% starch as substrate, pH 10. Bars represent means of three independent estimations associated with standard error (P = 0).

According to Figure 4, since the azocaseinolytic activity of STI-fed larvae was lower than SBBI- or SKTI-fed larvae, it could be suggested that the recompense of proteolytic inhibition by means of hyperproduction of enzymes in response to STI had no effect on general proteolytic activity.

The percentage inhibition of serine proteases by PMSF in artificial diet and artificial diet containing protease inhibitors indicates that higher inhibition of enzyme in SBBI- and SKTI-fed larvae by PMSF may be due to the induction of hyperproduction of serine proteases by these two plant protease inhibitors (Figure 5a). According to Bown et al. (1997), PMSF could inhibit 28% of the proteolytic activity of *H. armigera* larvae fed on an artificial diet.

The percentage inhibition of tryptic activity by STI in inhibitor-free diet was more than that of STI-fed larvae, may be attributed to the overexpression of trypsin-like enzymes to compensate for inhibitory effect of STI. Inhibitory activity of STI in inhibitor-free diet was more 2-fold than that of TLCK. The comparison of inhibitory effect of *in vitro* use of STI in STI-fed larvae with the inhibitory effect of *in vitro* use of SBBI and SKTI in the larvae reared on SBBI and SKTI indicates that the STI diet-fed insects compensate the enzyme inhibition by hyperproduction of proteolytic enzymes (Figure 5b) (Naseri et al., 2010 b).

Although the inhibitory effect of STI *in vivo* and *in vitro* was more than SBBI and SKTI, it could not completely inhibit tryptic activity, probably because of the high sensitivity to STI, leading to the overexpression of trypsin-like proteases to compensate enzyme inhibition (Naseri et al., 2010 b). Assays with *Spodoptera exigua* (Hübner) larvae fed on PIs-containing diets have showed increases (Broadway and Duffy, 1986) or reductions (Lara et al., 2000) of tryptic activity. Johnston et al. (1993) have reported that STI was more effective on the inhibition of *H. armigera* larval growth and larval midgut protease activities than SBBI.

According to the reports of Broadway and Duffy (1986) the potato protease trypsin inhibitor (PPTI) and soybean trypsin inhibitor (STI) had no effects on the *in vivo* digestion of protein, and the trypsin activity was significantly elevated. Thus, they concluded that the mode of action of protease inhibitors was to cause the hyperproduction of trypsin. For *H. armigera*, Johnston et al. (1993) reported that SKTI caused continued stoppage in the *in vivo* trypsin-like enzyme activity.

Chymotryptic activity of the larvae fed on artificial diet and artificial diet containing plant protease inhibitors was powerfully inhibited by chymostatin, indicating the presence of high levels of chymotrypsin-like enzyme in these larvae. STI-fed larvae did not show the inhibition of chymotrypsin, suggesting the lack of inhibitory effect by STI on chymotrypsin-like activity (Figure 5c) (Naseri et al., 2010 b).

SBBI inhibited trypsin-like activity more than chymotrypsin-like, in contrast to *Heliothis virescens* (Fabricius) and *lacanobia oleracea* L. larval gut proteases, where it inhibited chymotrypsin-like activity more than trypsin-like (Johnston et al., 1995; Gatehouse et al., 1999). Chougule et al. (2005) noted that SBBI inhibited chymotrypsin-like and trypsin-like activity of *Mamestra brassicae* L. gut proteases almost equally.

High inhibitory effect of chymostatin in PIs-fed larvae demonstrated that the insect's chymotrypsin-like enzymes had not any sensitivity to the diets including STI and SBBI. Jongsma et al. (1996) have reported that the chymostatin inhibited 88% of proteolytic activity of *S. exigua* larvae. The gut protease activity of *H. armigera* larvae reared on cotton, okra and pigeonpea was inhibited 39, 45 and 78%, respectively by chymostatin (Patankar et al., 2001).

Visualization of the protease activity of midgut extracts from *H. armigera* larvae reared on different soybean cultivars using substrate SDS-PAGE electrophoresis revealed the presence of at least seven bands (Naseri et al., 2010 b). Although the majority showed similar profiles,

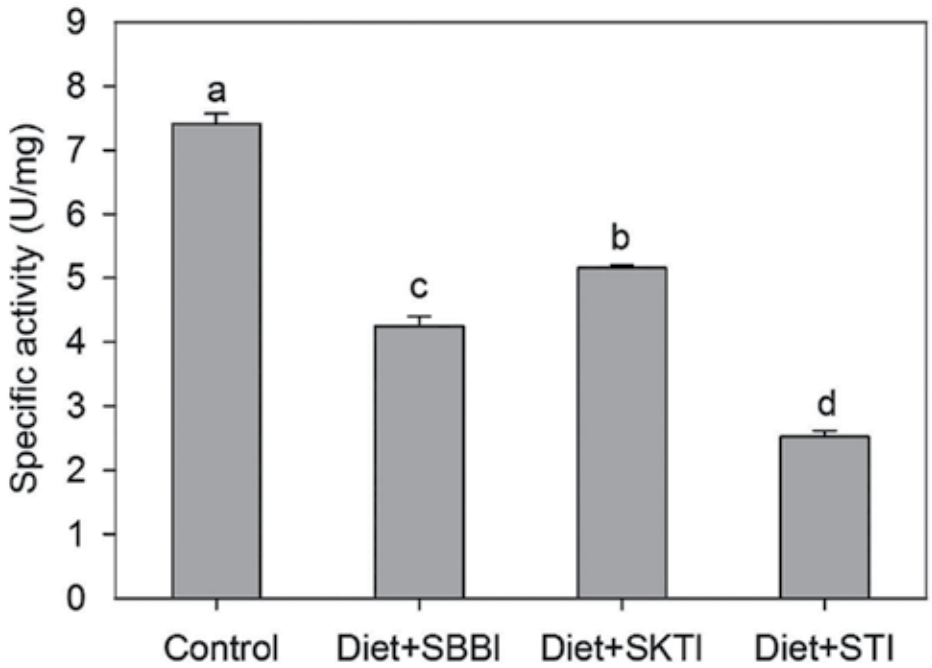

Fig. 4. The effects of three protease inhibitors, when incorporated into artificial diet at a single concentration (0.5% W/V) on general proteolytic activity of *Helicoverpa armigera* larvae using azocasein as substrate, pH 12. Bars represent means of three independent estimations associated with standard error (P = 0). STI: soybean trypsin inhibitor; SKTI: soybean Kunitz trypsin inhibitor; SBBI: soybean Bowman-Birk inhibitor.

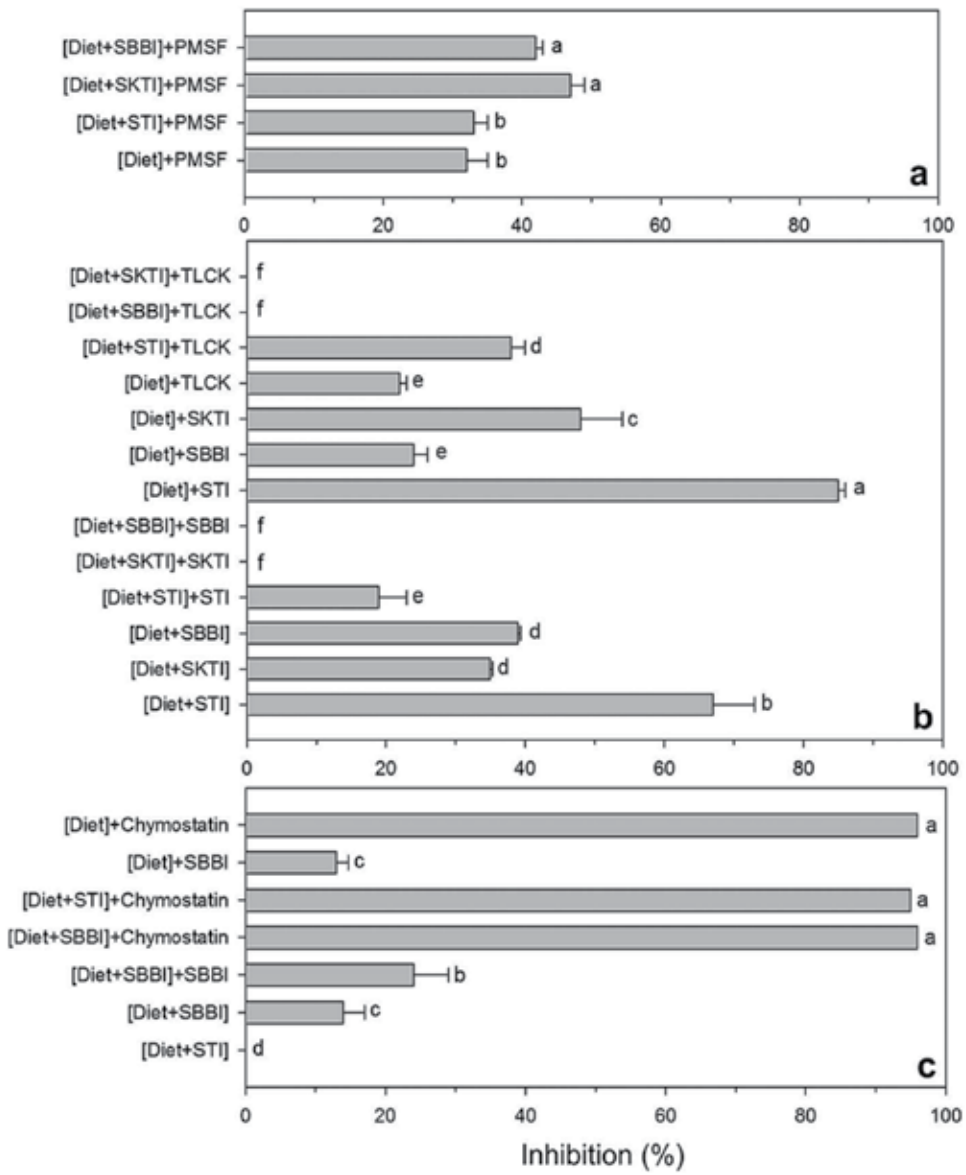

Fig. 5. Effect of different combinations of protease inhibitors *in vitro* and *in vivo* on enzyme inhibition (%) of gut protease activity (a), trypsin inhibition (%) (b) and chymotrypsin inhibition (%) (c) of gut enzyme extracts from *Helicoverpa armigera* larvae using azocasein, pH 12; BAρNA, pH 10.5 and SAAPFρNA, pH 10.5 as substrates, respectively. Bars represent means of three independent estimations associated with standard error (P = 0). The assay treatments were presented as "[*in vivo*] + *in vitro*". STI: soybean trypsin inhibitor; SKTI: soybean Kunitz trypsin inhibitor; SBBI: soybean Bowman-Birk inhibitor; TLCK: Nα-ρ-tosyl-$_L$-lysine chloromethyl ketone; PMSF: phenylmethylsulfonyl fluoride.

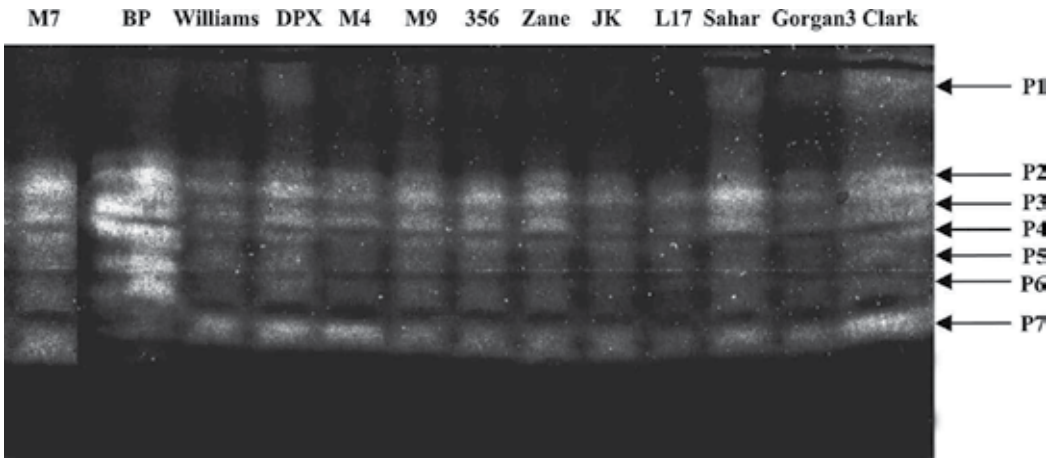

Fig. 6. Zymogram analysis of casein hydrolytic activity of midgut extracts from *Helicoverpa armigera* larvae reared on different soybean cultivars. Protease activity bands are indicated by arrows (P1-P7).

both qualitatively and quantitatively, four appeared to exhibit lower levels of activity (Williams, JK, L17 and Gorgan3). Three cultivars (Clark, Sahar and BP) appeared to exhibit different expression patterns especially due to high molecular weight proteases. However, perhaps of greater importance was the fact that M7, and in particular BP and Clark, exhibited different expression patterns (Figure 6). Visualized general proteolytic patterns in zymogram did not match completely with this issue except for the cultivar Sahar. This may be related to inactivation of the over-expressed proteases during gel electrophoresis**.**

7. Conclusion

All of the case studies substantiated in this chapter are to prove this fact that host plant cultivars (e.g. soybean cultivars) can significantly affect the life table parameters, nutritional indices and digestive enzymes activity of *H. armigera*, and can be used as a tool to control this devastating pest in integrated pest management programs. In this chapter, to demonstrate such influence of different crop cultivars on the performance of *H. armigera*, we reconsidered the works conducted on crop cultivars (including soybean cultivars and other host crops) effect on the life table parameters and nutritional indices of *H. armigera*. A complementary case study was also considered on digestive proteolytic and amylolytic activities of the larvae fed on different soybean cultivars, and response of the larvae to feeding on some soybean-based protease inhibitors. We have also emphasized on the influence of the seeds of different soybean cultivars on the life table parameters and nutritional indices of *H. armigera* when incorporated into artificial diets. It would be concluded that the leaves and green pods of the different soybean cultivars and the seeds of the examined soybean cultivars differed greatly in suitability as diets for *H. armigera* when measured in terms of the life table parameters (e.g., life history, fecundity and population growth parameters) and nutritional indices. By combining the data resulted from the studies on digestive enzymes activity, the life table parameters and nutritional indices of *H. armigera* reared on the leaves and pods of different soybean cultivars and findings of the life table parameters and nutritional indices of this pest on different soybean cultivars seeds, it could be concluded that *H. armigera* did not perform well on some

cultivars such as Sahar and L17, and therefore these cultivars were partially resistant to *H. armigera*. Among soybean cultivars, Sahar was the most resistant cultivar to *Tetranychus urtica*e (Koch) and L17 was reported as susceptible (Sedaratian et al., 2009). Our study, however, indicated that L17 was resistant cultivar to *H. armigera*. In general we conclude that Sahar could serve as a key tool in integrated pest management in soybean fields because of its resistance to *H. armigera* and *T. urtica*e.

The information obtained from these researches will be important in the management of *H. armigera* by providing a better understanding of its life history and its ability to survive on different host plants. Such information and further field and laboratory experiments are needed in developing integrated pest management (IPM) program of this pest and other economically important pests like spider mites (e.g. Sedaratian et al., 2009; Sedaratian et al., 2010).

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Soybean Performance under Salinity Stress

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1. Introduction

Soybean seed is a major source of high-quality protein and oil for human consumption (Katerji *et al.* 2001). The unique chemical composition of soybean has made it one of the most valuable agronomic crops worldwide (Thomas *et al.* 2003). Its protein has great potential as a major source of dietary protein. The oil produced from soybean is highly digestible and contains no cholesterol (Essa and Al-ani 2001). Growth, development and yield of soybean are the result of genetic potential interacting with environment. Soybean seed production may be limited by environmental stresses such as soil salinity (Ghassemi-Golezani *et al.* 2009). Minimizing environmental stress will optimize seed yield (Mc Williams *et al.* 2004).

Soil salinity, resulting from natural processes or from crop irrigation with saline water, occurs in many arid and semi-arid regions of the world (Meloni *et al.* 2004). The UNEP (United Nations Environment Program) estimates that 20% of the agricultural land and 50% of the cropland in the world is salt-stressed (Yan 2008). Most of the salt stresses in nature are due to Na^+ salts, particularly NaCl (Demirel 2005). High salinity lowers water potential and induces ionic stress, and results in secondary oxidative stress. It severely limits growth and development of plants by affecting different metabolic processes such as CO_2 assimilation, oil and protein synthesis (Nasir khan *et al.* 2007).

Plants vary tremendously in their ability to tolerate salinity (Bischoff and Warner 1999). The term halophyte means "salt tolerant plant" but is used specifically for plants that can grow in the presence of high concentration of Na^+. Plants that can not grow in presence of high concentration of Na^+ salts are called glycophytes (Brevedan and Egli 2003). Soybean is classified as moderately salt sensitive instead of moderately salt tolerant (Katerji *et al.*, 2000). Salt tolerance of plants may be dependent on growth stage, varieties, nutrition and environment (Bischoff and Warner 1999).

Netondo *et al.* (2004) reported that photosynthetic activity decreases when plants are grown under saline conditions leading to reduced growth and productivity. The reduction in photosynthesis under salinity can be attributed to a decrease in chlorophyll content (Jamil *et al.* 2007) and activity of photo-system *II* (Ganivea *et al.* 1998). Salinity can affect chlorophyll content through inhibition of chlorophyll synthesis or an acceleration of its degradation (Reddy and Vora 1986). Fluorescence of chlorophyll reflected the photochemical activities of photo-system *II* (Ganivea *et al.* 1998). Photochemical efficiency of photo-system *II* (fv/fm) could be reduced by salinity stress (Jamil *et al.* 2007; Netondo *et al.* 2004).

Plants have evolved complex mechanisms that contribute to the adaptation to osmotic stress caused by high salinity (Meloni *et al.* 2004). Osmotic adjustment has undoubtedly gained considerable recognition as a significant and effective mechanism of salinity tolerance in crop plants (Pakniyat and Armion 2007). In salt stressed plants, osmotic potential of vacuole decreased by proline accumulation (Yoshiba *et al.* 1997). Several possible roles have been attributed to supra-optimal level of proline including osmoregulation under salinity, stabilization of proteins and prevention of heat denaturation of enzymes and conservation of nitrogen and energy for a post-stress period (Aloni and Rosenshtein 1984).
Final seed weight is the result of seed filling rate during the linear phase and the duration of this period. Seed filling rate was described as the accumulation of seed dry matter per unit time, which varied among varieties and had positive correlation with final seed weight (Guffy *et al.* 1991). Researchers showed that environmental stresses may hasten the seed filling rate and decrease grain filling duration (Yazdi-Samadi *et al*. 1977). This can influence final yield of all grain crops such as soybean. Seed filling period is under genetic control and it is sensitive to salt stress (Brevedan and Egli 2003). Soybean seed protein and oil contents may be also influenced by environmental factors such as salinity (Nakasathien *et al*. 2000). Oil and protein syntheses occur during seed filling (Yazdi-Samadi *et al.* 1977). Approximately 18% to 21% of soybean seed dry weight is oil in the form of triacylglycerol. From 24 to 40 days after flowering, oil percentage increases rapidly and by the end of this period accounts for approximately 30% of the total oil of the mature seed. The remaining 70% is synthesized during 40 to 64 days after flowering, also a period of seed desiccation (Hajduch *et al.* 2005). The objective of this study is to evaluate the performance of soybean cultivars in response to different levels of NaCl salinity.

2. Materials and methods

Seeds of three soybean cultivars (Williams, Zan and L_{17}) were obtained from Agricultural Research Institute, Moghan, Iran. Two experiments with factorial arrangements on the bases of randomized complete block (RCB) with three replications were conducted in 2007 (Tabriz, Iran) and 2008 (Uremia, Iran) to investigate changes in chlorophyll content index (7 weeks) and fluorescence of chlorophyll (4 times) in leaves and to determine proline content and grain yield of three soybean cultivars under a non-saline (control) and three saline (3, 6 and 9 dS/m NaCl) conditions. Six seeds were sown 3 cm deep in each pot, filled with 900 g perlite, using 144 pots in each experiment. Pots were then placed in the greenhouse. The temperature variation in the greenhouse was 17-34^{o}C and 13-28^{o}C during the first and second experiments, respectively. Tap water and saline solutions were added to the pots in accordance with the treatments to achieve 100% FC.
After emergence, seedlings were thinned to keep 4 plants in each pot. During the growth period, the pots were weighed and the losses were made up with Hoagland solution (EC = 1.3 dS/m). Perlites within the pots were washed every 25 days and non-saline and salinity treatments were reapplied in order to prevent further increase in electrical conductivity (EC), due to adding the Hoagland solution.
Leaf chlorophyll content index (CCI) was measured by a chlorophyll meter (CCM-200, Opti-Science, USA) in weekly intervals for seven weeks. After seedling establishment, a plant was marked in each pot and CCI of upper, middle and lower leaves was measured at each stage. Subsequently, mean CCI for each treatment and replicate at each developmental stage was calculated.

The chlorophyll fluorescence induction parameters were measured in leaves by a chlorophyll fluorometer (OS-30, OPTI-SCIENCES, USA) every 10 days from 30 to 60 days after sowing. Fluorescence emission was monitored from the upper surface of the leaves. Dark-adapted leaves (30 min.) were initially exposed to the weak modulate measuring beam, followed by exposure to saturated white light to estimate the initial (F0) and maximum (Fm) fluorescence values, respectively. Variable fluorescence (Fv) was calculated by subtracting F0 from Fm. The Fv/Fm ratio measures the efficiency of excitation energy capture by open PSII reaction centers, representing the maximum capacity of light-dependent charge separation in PSII (Rizza *et al.* 2001).

The proline content was determined spectrophotometrically according to Bates *et al*. (1973). 200 mg leaf samples were powdered in liquid nitrogen and were homogenized in 5 ml sulphosalcylic acid. 2 ml acid ninhydrine and 2 ml glacial acetic acid were added to the extract. The samples were heated at 100 °C. The mixture was extracted with toluene and the free toluene was quantified spectrophotometrically at 520 nm.

During grain filling, four harvests were made at 10 days intervals, beginning 75 days after sowing. Grain yields of two experiments for the same replicates and treatments were mixed, in order to provide enough grains for the measurement of protein and oil. Percentages of oil and protein for each sample were measured, using a seed analyzer (model: Zeltex ZX-50). Subsequently, Protein and oil yields per grain and per plant were calculated. A regression model was used to describe the seeds oil and protein accumulation. The following equation was applied to calculate the rates of protein and oil accumulation in soybean grains under different treatments:

$$\textit{Accumulation rate (mg d}^{-1}\textit{)} = \textit{Maximum weight (mg)/Filling duration (day)}$$

At maturity, plants of each pot were separately harvested and grains were detached from the pods. Finally, grains were weighed and grain yield per plant for each treatment at each replicate was determined.

MSTATC software was used to analyze the data for CCI and chlorophyll fluorescence as factorial split plot and those for proline and grain yield as factorial. Means of the traits were compared at $p \leq 0.05$. SAS software was used for regression analysis of the grain filling data and Excel software was applied to draw figures.

3. Results

The results of analysis of variance showed highly significant ($P \leq 0.01$) effects of year, cultivar, salinity and time on both chlorophyll content index (*CCI*) and fluorescence of chlorophyll. Means of *CCI* and fv/fm in 2007 were higher than those in 2008. The CCI and fluorescence of chlorophyll in soybean leaves decreased with increasing salinity. L17 and Zan had the highest and the lowest *CCI* and fv/fm, respectively (Table 1).

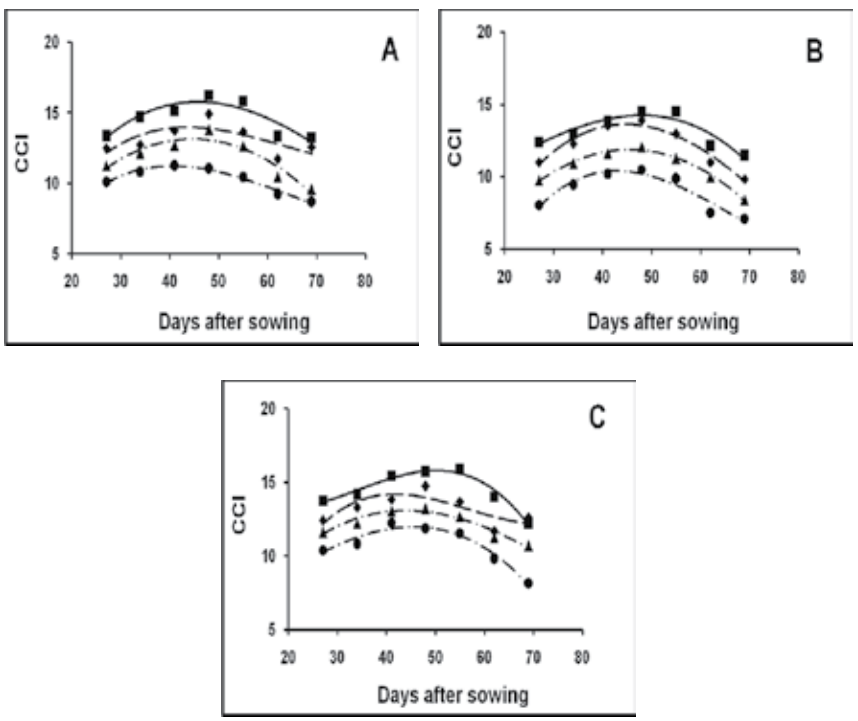
Means of *CCI* and chlorophyll fluorescence of soybean cultivars increased with progressing plant growth up to the points where maximum values were achieved under non-saline and saline conditions (Figures 1 and 2). Maximum CCI of all cultivars under salinity treatments was obtained earlier than that under non-saline treatment (Figure 1), but maximum chlorophyll fluorescence under all treatments was achieved at almost similar stage (Figure 2). Thereafter, due to senescing of leaves, CCI and chlorophyll fluorescence started to decrease. Means of CCI and chlorophyll fluorescence at all developmental stages decreased

as the salinity increased. In general, L17 and Williams had more *CCI* and chlorophyll fluorescence at different stages of growth and development, compared with Zan (Figures 1 and 2).

Treatment		*CCI*	fv/fm
year	1	13.66 a	0.779 a
	2	10.50 b	0.728 b
Salinity (dS m^{-1})	0	14.06 a	0.792 a
	3	12.81 b	0.768 b
	6	11.50 c	0.742 c
	9	9.97 d	0.713 d
Cultivar	L_{17}	12.63 a	0.764 a
	Zan	11.19 b	0.739 b
	Williams	12.43 a	0.758 a

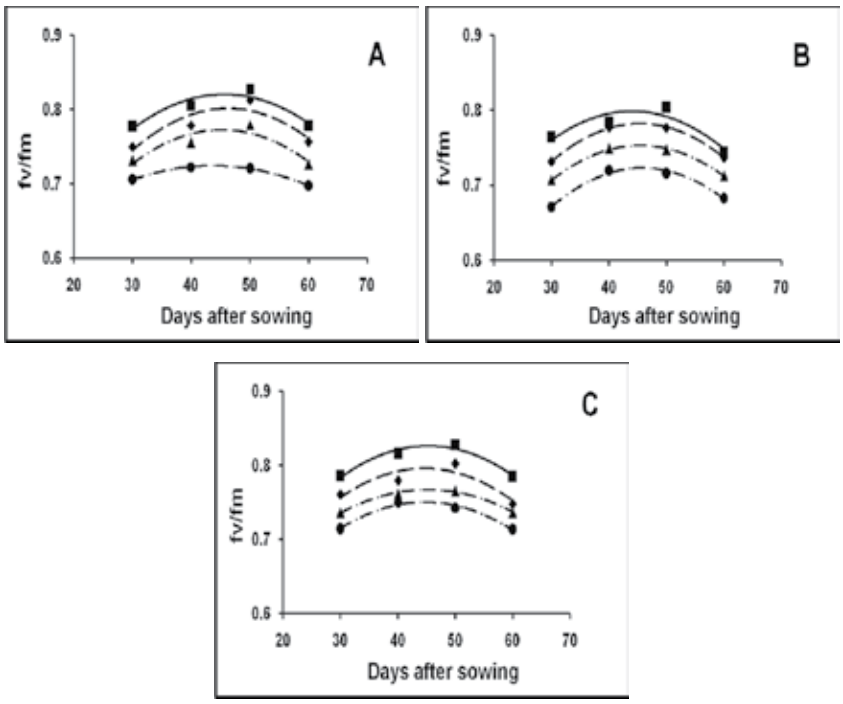
Different letters in each column indicate significant difference at p≤0.05.

Table 1. Comparison of means chlorophyll content index (*CCI*) and fluorescence of chlorophyll (fv/fm) of three cultivars of soybean under salinity stress



A: Williams B: Zan C: L17, — 0 dS/m – – 3 dS/m - - - 6 dS/m - - — - - 9 dS/m

Fig. 1. Changes in chlorophyll content index (*CCI*) of soybean cultivars under non-saline (control) and Saline conditions (means of two years).



A: Williams B: Zan C: L17, — 0 dS/m — — 3 dS/m - - - 6 dS/m - - — - - 9 dS/m

Fig. 2. Changes in chlorophyll fluorescence (fv/fm) of soybean cultivars under non-saline (control) and Saline conditions (means of two years).

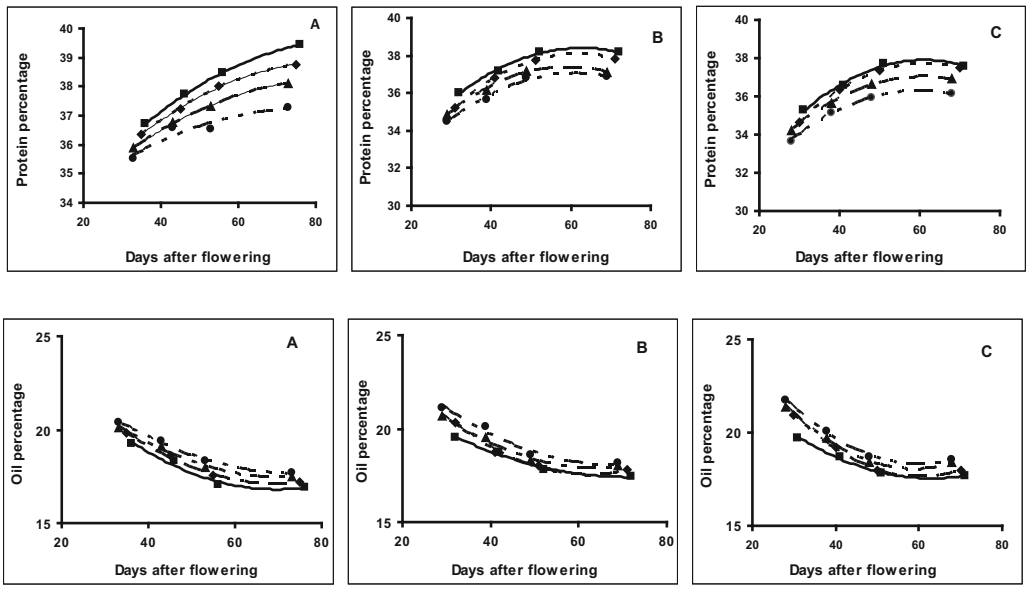
Leaf proline content and grain yield per plant were significantly ($P \leq 0.01$) affected by cultivar and salinity, but cultivar × salinity interaction was not significant for these traits ($P \square 0.05$). Leaf proline content of soybean increased with increasing salinity. Proline content of Zan was significantly higher than that of Williams and L_{17}. However, proline content of the latter cultivars was similar (Table 2). Grain yield per plant significantly decreased as salinity increased. Zan had the lowest grain yield per plant, but there was no significant difference in grain yield of L_{17} and Williams (Table 2).

Treatment		Proline content (Mm/g)	Grain yield per plant(g)
Salinity (dS m^{-1})	0	19.40 d	1.250 a
	3	26.12 c	0.892 b
	6	39.28 b	0.516 c
	9	45.89 a	0.274 d
Cultivar	L_{17}	31.71 b	0.782 a
	Zan	35.36 a	0.651 b
	Williams	30.96 b	0.766 a

Different letters in each column indicate significant difference at p≤0.05.

Table 2. Comparison of means of proline content and grain yield per plant of three cultivars of soybean under salinity stress

Protein percentage of soybean cultivars under all saline and non-saline conditions increased with increasing grain filling period up to a point where maximum value was achieved. Maximum protein percentage for Zan and L_{17} was attained about 10 days earlier than Williams (Figure 3). Protein percentage of all soybean cultivars at different stages of seed development decreased, as the salinity increased. However, this reduction was higher for Williams, compared with Zan and L_{17}. In contrast, oil percentage of all cultivars increased with increasing salinity. Oil percentage decreased as protein percentage increased with proceeding of grain filling (Figure 3).



A: Williams B: Zan C: L17, 0 dS/m 3 dS/m 6 dS/m 9 dS/m

Fig. 3. Changes in seed protein and oil percentage of soybean cultivars under non-saline (control) and Saline conditions

Protein and oil contents per grain of soybean cultivars under non-saline and all saline conditions increased with progressing seed development up to 50-65 days after flowering, depending on cultivar and salinity level (Figure 4). Maximum protein and oil contents per seed under salinity stress were achieved earlier than those under non-saline conditions. Although both protein and oil per seed decreased with increasing salinity, protein content per grain at different stages of seed development was much higher than oil content under all treatments (Figure 4).

Effects of salinity on rate of protein accumulation, duration of protein and oil accumulation, grain yield per plant and protein and oil yields per plant were significant ($P \leq 0.01$), but its effect on rate of oil accumulation was not significant (P□0.05). All these traits, except rate of protein accumulation and the duration of oil accumulation, were also significantly affected by cultivar (Table 3).

Means of all the traits, except rate of oil accumulation, decreased with increasing salinity. Despite this reduction, Rate of protein accumulation for control and 3 and 6 dS/m NaCl

salinity was statistically similar (Table 3). Williams had the highest rate and duration of protein accumulation and rate of oil accumulation, but L_{17} had the highest grain yield per plant. The lowest grain, protein and oil yields were obtained for Zan, while differences in protein and oil yields between Williams and L_{17} were not significant (Table 3).

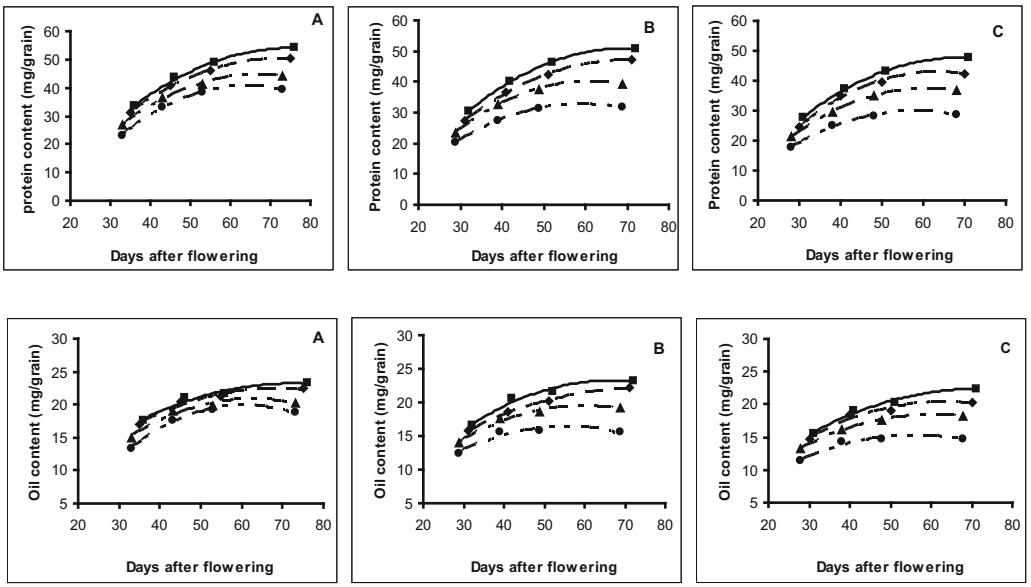


Fig. 4. Changes in seed protein and oil content of soybean cultivars under non-saline (control) and saline conditions

Treatment		Rate of protein accumulation (mg/day)	Duration of protein accumulation (day)	Rate of oil accumulation (mg/day)	Duration of oil accumulation (day)	Grain Yield per plant (g)	Protein yield per plant (mg)	Oil yield per plant (mg)
Salinity (dS m^{-1})	0	0.851 a	59.8 a	0.390 a	59.2 a	1.250 a	478.41 a	216.18 a
	3	0.846 a	55.6 b	0.398 a	55.4 b	0.892 b	338.70 b	157.27 b
	6	0.806 a	50.7 c	0.409 a	48.2 c	0.516 c	192.54 c	92.38 c
	9	0.712 b	47.2 d	0.388 a	44.6 d	0.275 d	101.12 d	49.84 d
Cultivar	Williams	0.841 a	55.3 a	0.415 a	52.8 a	0.766 a	296.65 a	131.65 a
	ZAN	0.791 a	53.8 a	0.389 ab	52.4 a	0.651 b	245.12 b	114.72 b
	L_{17}	0.779 a	50.7 b	0.384 b	50.3 a	0.782 a	291.31 a	140.38 a

Different letters in each column indicating significant difference at $p \leq 0.05$.

Table 3. Comparison of means of rate and duration of protein and oil accumulation in grains of three cultivars of soybean under salinity stress

4. Discussion

Decreasing chlorophyll content index (CCI) of soybean leaves with increasing salinity (Table 1, Figure 1) could be related to increasing the activity of chlorophyll degrading enzyme: chlorophyllase (Jamil *et al*. 2007), and the destruction of the chloroplast structure and the instability of pigment protein complexes (Singh and Dubey 1995). Similar results were reported for tomato (Lapina and Popov 1970), pea (Hamada and El-Enany 1994), alfalfa (Winicov and Seemann 1990), sunflower (Ashraf 1999), sorghum (Netondo *et al.* 2004), and wheat (El-Hendawy *et al.* 2005). Differences in CCI among cultivars (Table 1, Figure 1) indicate that this trait can be also influenced by genetic constitution.

Reduction in fv/fm due to salinity stress (Table 1, Figure 2) is possibly related to the damage of chlorophyll under saline conditions (Ganieva *et al.* 1998). Ashraf (2004) found that ionic imbalance can also cause the reduction in fv/fm under high salinity conditions. Nasir Khan *et al* (2007) reported that the decrease in chlorophyll content and PS *II* activity have adverse effect on growth and grain yield of treated plants.

Increasing leaf proline content under salinity stress (Table 2) might be caused by the induction or activation of proline syntheses from glutamate or decrease in its utilization in protein syntheses or enhancement in protein turnover. Thus, proline may be the major source of energy and nitrogen during immediate post stress metabolism and accumulated proline apparently supplies energy for growth and survival, thereby inducing salinity tolerance (Gad 2005). Zan had the highest proline content (Table 2) and the lowest CCI and fv/fm (Table 1). Gad (2005) also reported that proline content was much higher in sensitive cultivar of tomato than in salt-tolerant.

Large reductions in grain yield per plant clearly show that soybean is a salt sensitive crop, but the extent of this sensitivity varies among cultivars (Table 2). Salinity can severely limit crop production because high salinity lowers water potential and induces ionic stress and results in a secondary oxidative stress (Shanon 1998). Reductions in grain yield as a result of salt stress have also been reported for some other crop species (Ashraf 2004; Katerji *et al.* 1992; Sohrabi *et al.* 2008). These reductions are closely related with low *CCI* and PS *II* activity (Table 1) and high leaf proline content (Table 2) in soybean cultivars.

Oil and protein are the most important constituents of soybean grain. These are synthesized and deposited in the grain during pod filling (Yazdi-Samadi *et al.* 1977). Decreasing protein percentage and content with increasing salinity (Figures 3 and 4) could be attributed to the disturbance in nitrogen metabolism or to inhibition of nitrate absorption. It has been stated that the reduction in nitrogen under saline conditions might be due to the reduction of absorbed water and a decrease in root permeability (Strogonov *et al.* 1970). Medhat (2002) reported that salinity stress induce changes in the ion content of plant cell which intern induce changes in the activity of certain metabolic systems that might have serious consequences for protein.

The effect of salinity on oil percentage of soybean cultivars was opposite to that on protein percentage (Figure 4), suggesting that oil percentage increases as protein percentage decreases in response to salinity stress. Hobbs and Muendel (1983) reported similar results for soybean seeds under moisture stress. However, protein and oil contents of individual grains produced under non-saline conditions were higher than those produced under saline

conditions (Figure 4). This was associated with production of larger grains under non-saline conditions (Ghassemi-Golezani *et al*. 2009).

Salinity had little effect on rate of protein and oil accumulation in soybean grains. Therefore, decreasing oil and protein yields per plant with increasing salinity mainly resulted from the large reductions in durations of protein and oil accumulation and grain yield per plant under saline conditions (Table 3). Although, duration of protein accumulation and rate of oil accumulation for L_{17} were slightly lower than those for other cultivars, the lowest protein and oil yields of Zan were strongly associated with the lowest grain yield per plant of this cultivar (Table 3). The greater grain, protein and oil yields per plant of L_{17} and Williams were due to production of comparatively more grains per plant by the former and larger grains by the latter cultivars as previously reported by Ghassemi-Golezani *et al* (2009).

5. Conclusion

Salinity stress can considerably reduce chlorophyll content index and PS *II* activity and consequently grain yield per plant in soybean cultivars. These reductions enhance with increasing salinity. In contrast, leaf proline content increases due to NaCl salinity. Oil percentage of soybean grains increases as protein percentage decreases under salinity stress. However, both protein and oil contents of individual grains under non-saline conditions are higher than those under saline conditions. Oil and protein yields per plant of soybean cultivars decrease with increasing salinity as a result of reductions in durations of protein and oil accumulation and grain yield per plant in response to salinity stress. In general, soybean is a sensitive crop to salinity stress, but the extent of this sensitivity varies among cultivars.

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Soybean is an agricultural crop of tremendous economic importance. Soybean and food items derived from it form dietary components of numerous people, especially those living in the Orient. The health benefits of soybean have attracted the attention of nutritionists as well as common people.

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