IntechOpen

Soybean

Genetics and Novel Techniques for Yield Enhancement

Edited by Dora Krezhova

SOYBEAN – GENETICS AND NOVEL TECHNIQUES FOR YIELD ENHANCEMENT

Edited by **Dora Krezhova**

Soybean - Genetics and Novel Techniques for Yield Enhancement
http://dx.doi.org/10.5772/727
Edited by Dora Krezhova

Contributors

Raffaella Tudisco, Serena Calabro', Monica Isabella Cutrignelli, Vincenzo Piccolo, Federico Infascelli, Arumugam Gnanamani, V Kavitha, Ganesan Sekaran, Asit Baran Mandal, Reda Helmy Sammour, Jongil Chung, Eduardo Antonio Gavioli, Xiangwen Pan, Changyi Cao, Yan-Hua Li, Guo-Dong Wang, Wei-Guang Du, Qiuying Zhang, Xiu-hong Wu, Robert Upchurch, Christine Foyer, Berhanu Fenta, Urte Schluter, Karl Kunert, Muhammad Zia, Sang Moo Kim, Xiao Ting Fu, Benjamin Matthews, Vincent P. Klink, Kyuya Harada, Satoshi Watanabe, Zhengjun Xia, Yasutaka Tsubokura, Toyoaki Anai, María Cristina Lurá, María Latorre Rapela, Mauro Colombini, Ana González, Stella Vaira, Roxana Maumary, Elena Carrera, John Wiersma, Shamaraz Firdous, Dora Krezhova

Notice

First published in Croatia, 2011 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

Additional hard and PDF copies can be obtained from orders@intechopen.com

Soybean - Genetics and Novel Techniques for Yield Enhancement
Edited by Dora Krezhova

p. cm.

ISBN 978-953-307-721-5

eBook (PDF) ISBN 978-953-51-5156-2

Meet the editor

Professor Dora Krezhova was born in Sofia and obtained master degree in telecommunication from the Technical University of Sofia, Bulgaria. Since graduating, after a six-year position of research fellow at the Institute of computing techniques she moved to the Solar-Terrestrial Research Institute. Presently, she is Associate Professor at the Space and Solar-Terrestrial Research Institute at the Bulgarian academy of sciences.

Krezhova published more than 187 research papers. She has contributed to the design of more than 10 scientific devices and systems and her PhD thesis and researches have made use of multichannel spectrometers for solving problems in remote sensing of the Earth and planets. For participation in the Bulgarian space programmes for remote sensing of the Earth by means of air and space-born equipment she was awarded with honorary badges, diplomas and medals. Her scientific contributions come from accurate determination of incident solar radiation and studies of spectral reflectance characteristics of natural formations, fluorescence of plants, and the impact of abiotic and biotic stress factors on plant physiology. She has a special interest in data processing and development of new mathematical models for description of the details of spectral characteristics and classification of natural formations and processes.

Contents

Preface

Soybean is one of the most important and valuable agricultural crops. Owing to its high nutritive value and versatility soybean offers resources to address world food issues through current and future utilization practices. Rapid increases of soybean demand in the last decade challenge the reliability of supply, stock levels, and reasonable pricing. Future soybean production is expected to increase steadily in proportion to increased demand. This book presents the importance of applying of novel genetics and breading technologies. The efficient genotype selections and gene transformations provide for generation of new and improved soybean cultivars, resistant to disease and environmental stresses. The book introduces also a few recent modern techniques and technologies for detection of plant stress and characterization of biomaterials as well as for processing of soybean food and oil products. The contributions are organized in two sections based on genetics researches and novel technical practices. Each of the sections covers a wide range of topics and the authors are from countries all over the world. This underlines the global significance of soybean research. I am certain that the book will provoke interest to many readers and researchers, who could find information useful for advancing their fields.

Prof. Dora Krezhova
Space and Solar-Terrestrial
Research Institute at the
Bulgarian Academy of Sciences
Bulgaria

Part 1

Genetics and Breeding

1

Genetic Diversity and Allele Mining in Soybean Germplasm

Reda Helmy Sammour
Tanta University, Faculty of Science, Botany Department
Egypt

1. Introduction

Soybean, *Glycine max* (L.) Merrill is recognized as the most important grain legume in the world in terms of total production and international trade (Golbitz, 1995), being an important source of protein and oil. There are developing thousands of breeding lines and hundreds of elite cultivars yearly in the soybean hybridization programmes over the world. The developing of these breeding lines increased genetic uniformity in the frame of species. Therefore, the genetic basis of these released cultivars is rather narrow. Generations of new and improved cultivars can be enhanced by new sources of genetic variation; therefore criteria for parental stock selection need to be considered not only by agronomic value, but also from the point of view of their genetic dissimilarity. That is why the evaluation of genetic variation is a very important task not only for population genetics but also for plant breeders. The study of genetic variation has fallen within population genetics which has focused on analyzing, measuring and partitioning genetic. The genetic diversity can be analyzed by agronomic and biochemical traits, and molecular marker polymorphisms, Analysis of gene marker data enables estimation of the mating system and monitoring of genetic changes caused by factors affecting the reproductive biology of a species. A key factor driving utilization of exotic germplasm is potential benefit. Benefit can be quite apparent for characteristics such as disease resistance or agronomic traits, but vague for yield or abiotic stress resistance.

2. Origin and diversification center of the soybean

Scholars generally agree that cultivated soybean (*Glycine max*) has originated in the eastern half of North China in the eleventh century B.C. or perhaps a bit earlier (Fukuda, 1933 and Singh, 2010). It is believed on world wide scale that soybean has been domesticated from the annual wild soybean *Glycine soja* Sieb.*et* Zucc. Many studies based on old Chinese literature, the geographic distribution of the wild ancestral species, the levels and types of genetic diversity of soybean varieties and the archeological evidence consistently indicated that China is the origin and diversification center of the cultivated soybean (Fukuda, 1933; Hymowitz, 1970; Zhuang, 1999). The evidences that China is the origin and main center of diversity of soybean are (1) the distribution of *G. soja* in China is the most extensive in terms of the numbers and diversity of types; (2) China has the earliest written records of soybean cultivation, about 4500 years ago; (3) soybean has been found in unearthed artifacts; (4)

soybeans cultivated in different countries in the world were introduced directly or indirectly from China; and (5) the pronunciation of the word of soybean in many countries is about the same as the Chinese 'Shu'; for instance, it is pronounced 'soya' in England, 'soy' in the USA, and in other languages.

Although, the origin of soybean cultivation may be China, scholars have different viewpoints on the original areas of soybean domestication. One of these views is the theory that soybean originated from northeast China (Fukuda, 1933). This theory based on the observations that semi-natural wild soybeans are extensively distributed in northeast China, that is, there are large numbers of soybean varieties that possess 'primitive' characteristics, such as small black soybean germplasm that extensively distributed in the lower and middle reaches of the yellow river North provinces. The second theory is that soybean cultivation originated in South China. In this theory, it has been thought that south China could be the origin of soybean (Wang, 1947). The evidences for that are the wide distribution of wild soybean in this area, extensive presences of primitive soybean varieties such as Nidou, Maliao Dou, Xiao Huangdou and others that have (1) the short-day character, which is considered to be the initial physiological state of soybean, and (2) the primitive agronomic characteristics related to yield and quality of soybean varieties. The other evidence supporting this theory is the close relatedness between cultivated soybeans in southern China, to wild soybeans in genetic terms based on isoenzymes, and RFLP (Restriction Fragment Length Polymorphism) markers of chloroplast and mitochondrial DNA, SSR data and botanical traits (Ding *et al*., 2008; Guo *et al*., 2010). In the third theory, it has been thought that the origin of soybean was the eastern part of northern China (i.e. the lower reaches the Yellow River) (Hymowitz, 1970). The evidences for his thought are the same blooming dates for both wild soybean and cultivated soybean at 35°N, confirming that cultivated soybean varieties may have been derived from local wild soybean at around 35°N. In addition, the protein content of cultivated soybean is close to that of wild soybean at 34–35°N. The fourth theory stated that the cultivated soybeans have multiple origins (Lü, 1978). The evidences for that postulation are (1) both South and North China have regions with early developed cultures, that is: the ancients in these regions used local wild soybean as food and did not domesticated wild soy-beans into cultivated ones; (2) the occurrence of wild soybean and cultivated soybean in the same regions and the similarities of both of them in morphological characters; (3) the successful cultivation of both wild and cultivated soybeans in different regions across China. In addition, the geographical distribution of the short-day character of wild soybean indicates the possibility of multiple origins of cultivated soybean.

3. Genetic diversity of soybean germplasm based on morphological traits

As we know, phenotypic traits are controlled by genes and affected by environment, but large numbers of accessions can adapt to environments. The phenotypic data has more polymorphism in genetic diversity and reveal genetic variation indirectly. On the contrary, the molecular data reveal genetic variation directly, but fewer markers have less polymorphism. It is very difficult to obtain molecular data for a large number of accessions that has enough polymorphism to show the genetic diversity of germplasm. So, the morphological traits are the suitable and practical tools for studying the genetic diversity on large numbers of accessions.

Variation in shape of plants has always been an important means of (1) distinguishing individuals; (2) controlling seed production; and (3) identifying the negative traits those

effects on yield, the genetic diversity centers of annual wild soybean and the soybean lines resistance to pod shatter, drought, pests or disease (Truong *et al.,* 2005; Malik *et al.,* 2006, 2007; Ngon *et a.,l* 2006). The studied soybean germplasm exhibited a wide range of phenotypic variation for pod number, seed number, and plant yield. It also showed that soybean developing stages had close association with agronomic traits as well as yield and yield components (Malik *et al.,* 2006, 2007; Ngon *et al.,* 2006).

Pod shape is one of the important descriptors for evaluating soybean genetic resources (IPGRI, 1998; USDA, 2001). Truong *et al.* (2005) tested the applicability of elliptic Fourier method for evaluating genetic diversity of pod shape in 20 soybean (*Glycine max* L. Merrill) genotypes. They concluded that principal component scores based on elliptic Fourier descriptors yield seemed to be useful in quantitative parameters not only for evaluating soybean pod shape in a soybean breeding program but also for describing pod shape for evaluating soybean germplasm.

The genetic diversity was evaluated for genotypes of soybean based on the yield-related traits (Rajanna *et al.,* 2000; Malik *et al.,* 2006, 2007; Ngon *et al*., 2006). It has been reported that differences among genotypes for all the characters were highly significant and the grain yield was positively and significantly correlated with number of pods per plant. The selection for the character had positive direct effect on yield. However, some traits had negative direct effects on yield, such as the leaf area, first pod height, days to 50% flowering, days to flowering completion, days to maturity, plant height, oil content and protein content.

The study of the genetic diversity of wild soybean is invaluable for efficient utilization, conservation and management of germplasm collections. Dong *et al*. (2001) statistically analyzed the agronomic traits of the data base from the National Germplasm Evaluation Program of China to study the geographical distribution of accessions, genetic diversity of characters and genetic diversity centers of annual wild soybean. The results showed that most annual wild soybeans are distributed in northeast China, and the number of accessions decreases from the northeast to other directions in China. They proposed three genetic diversity centers for annual soybean grown in China, the northeast, the Yellow River Valley and the Southeast Coasts of China. Based on these results and Vavilov's theory of crop origination, two opposing possible models for the formation of the three centers are proposed, either these centers are independent of each other and the annual wild soybeans in these centers originated separately, or the northeast center was the primary center for annual wild soybeans in China, while the Yellow River Valley center was derived from this primary center and served as the origin for the southeast Coast center.

The genetic variability in 131 accessions of edamame soybeans (the Japanese name for a type of vegetable soybean eaten at the immature R6 stage) was analyzed using phenotypic traits e. g. maturity information, testa color, and 100-seed weight for breeding new edamame lines resistance to pod shatter (Mimura, 2001). The 131 accessions include 108 Japanese edamame, 11 Chinese maodou, 8 WSU breeding lines, 2 US edamame and 2 US grain soybeans. The obtained results indicated that Edamame genetic diversity was generally clustered around maturity groups and testa color. It was also reported that the genetic diversity among the Japanese edamame cultivars was narrow, compared to Chinese maodou; Japanese edamame and Chinese maodou soybeans may have different genetic pools.

Soybean genotypes, which exhibit genetic diversity in root system developmental plasticity in response to water deficits in order to enable physiological and genetic analyses of the regulatory mechanisms involved, were identified (Young, 2008). These genotypes can

tolerate drought stress which is the major factor that limiting soybean yield. The results showed substantial genetic diversity in the capacity for increased lateral root development (number and total length of roots produced) and in the responses of overall root and shoot growth under water deficit conditions.

The extent of between- and within-species differences in the resistance of the four commonest species of Glycine (*G. canescens*, *G. clandestina* , *G. tabacina* and *G. tomentella*) to leaf rust caused by *Phakopsora pachyrhizi* was investigated by Burdon & Marshall (1981). The results of their study showed qualitative and quantitative resistance to leaf rust, and considerable variation in a number of disease characteristics both between and within populations of each species.

4. Genetic diversity in soybean germplasm based on karyological traits

Genetic diversity based on genome size among and within plant species has been well documented in the literature (Rayburn, 1990; Bennett and Leitch, 1995; Rayburn *et al*., 1997). The variation was pronounced in Chinese germplasm collected from diverse geographic locations. It was attributed to the environmental factors (Knight and Ackerly, 2002), cell size, minimum generation time, cell division rate and growth rate (Edwards and Endrizzi, 1975; Bennett *et al*., 1983) and polypoid species, in species with large seeds, and habits type (Bennett *et al*., 1998; Chung *et al*., 1998).

Reports of genome size variation in soybean [*Glycine max* (L.)] have ranged from 40 to 0% (Rayburn *et al.,* 2004). This wide range is highly reproducible and has resulted in doubts of the existence of intra-specific DNA variation in soybean. Rayburn *et al*. (2004) determined genome size of 18 soybean lines, selected on the basis of diversity of origin, by flow cytometry. They found that genome size variation between these lines was at approximately 4%. This amount of DNA variation is lower than was originally reported (Doerschug *et al*., 1978; Yamamota and Nagato, 1984; Hammatt *et al*., 1991; Graham *et al.,* 1994). Doerschug *et al*. (1978) is the first to determine genome size of soybean, upon examining 11 soybean lines, reporting over a 40% variation in nuclear DNA content. Graham *et al.* (1994) observed a 15% variation among soybean cultivars while Rayburn *et al*. (1997) reported a 12% variation among 90 Chinese soybean introductions. Chung *et al*. (1998) observed among 12 soybean strains a 4.6% DNA content variation. Yamamota and Nagato (1984) stated about 60% variation, while Hammatt *et al.* (1991) reported that the variation of genome size in 14 different *Glycine* species from different parts of the world was approximately 58%. These results indicated that the variability between DNA content was varied between the different scholars. The wide variation in genome size between soybean germplasm makes these accessions good candidates for crop improvement.

5. Evaluation of genetic diversity in soybean germplasm at the biochemical level

The genetic markers have made possible a more accurate evaluation of the genetic and environmental components of variation. The biochemical markers are ones of the interesting measures of genetic diversity. They include protein techniques and isozymes. The protein techniques are practical and reliable methods for cultivars and species identification because seed storage proteins are largely independent of environmental fluctuation (Sammour, 1992, 1999; Camps *et al*., 1994; Jha and Ohri, 1996). They are less expensive as compared to DNA

markers. SDS-PAGE is one of these techniques, widely used to describe seed protein diversity of crop germplasm (Sammour, 2007; Sammour *et al.,* 2007). Genetic diversity and the pattern of variation in soybean germplasm have been evaluated with seed proteins (Hirata *et al.,* 1999; Bushehri *et al*., 2000; Sihag *et al*., 2004; Malik *et al.,* 2009). SDS-PAGE (Bushehri *et al*., 2000) and discontinuous polyacrylamide slab gel electrophoresis (Sharma and Maloo, 2009) were used very successfully in evaluating the genetic diversity and identifying soybean (*Glycine max*) cultivars. Malik *et al*., (2009) evaluated the genetic variation in 92 accessions of soybean collected from five different geographical regions using the electrophoretic patterns of seed proteins. The accessions from various sources differed considerably, indicating that there is no definite relationship between genetic diversity and geographic diversity. Similar results were reported by (Ghafoor *et al.,* 2003). Based on the results of Ghafoor *et al.,* (2003) and Malik *et al*., (2009), SDS-PAGE cannot be used for identification of various genotypes of wild soybean at the intra-specific level, because some of the accessions that differed on the basis of characterization and evaluation exhibited similar banding patterns. However, it might be used successfully to study inter rather than intra-specific variation (Sammour, 1989; Sammour *et al*., 1993; Karam *et al*., 1999; Ghafoor *et al*., 2002). 2-D electrophoresis can be used to characterize the genotypes exhibited similar banding patterns (Sammour, 1985).

Allozyme markers have been used in soybean to evaluate genetic diversity in accessions from diverse geographic regions (Yeeh *et al*., 1996; Chung *et al*., 2006), wild soybean in natural populations from China, Japan and South Korea (Pei *et al*., 1996; Fujita *et al*., 1997), and Asian soybean populations (Hymowitz & Kaizuma, 1981; Hirata *et al*., 1999). From an analysis of the Kunitz trypsin inhibitor (*Ti*) and beta-amylase isozyme (*Sp1* = *Amy3*), Hymowitz & Kaizuma (1981) defined seven soybean germplasm pools in Asia: (1) northeast China and the USSR, (2) central and south China, (3) Korea, (4) Japan, (5) Taiwan and south Asia, (6) north India and Nepal and (7) central India. Hirata *et al*. (1999) compared the genetic variation at 16 isozyme of 781 Japanese accessions with the genetic variations of 158 Korean and 94 Chinese accessions, detecting a number of region-specific alleles that discriminated Japanese from Chinese accessions. The presence of alleles specific to the Japanese population suggested that the present Japanese soybean population was not solely a subset of the Chinese population.

6. Evaluation of genetic diversity in soybean germplasm using molecular markers

6.1 Introduction

The soybean genome is consisting of around 1115 Mbp, much smaller than the genomes of maize and barley, but larger than the genomes of rice and *Arabidopsis* (Arumuganathan & Earle, 1991). Soybean is a tetraploid plant, evolved from a diploid ancestor (n=11), went aneuploid loss (n=10), followed by polyploidization (n=20) and diploidization (chromosome pairing behavior) (Hymowitz, 2004). As a result of polyploidization soybean has a significant percentage of internal duplicated regions distributed among its chromosomes (Pagel *et al*., 2004). Sequence diversity in cultivated soybean is relatively low compared to other species leading to a major challenge in the improvement of this important crop. To efficiently broaden the genetic base of modern soybean cultivars, we have a detailed insight into genetic diversity of soybean germplasm. Such insight could be achieved through molecular characterization using DNA markers, which are more informative, stable and

reliable, compared to pedigree analysis and traditionally used morphological markers. The genetic markers include RFLP, RAPD, SSR and AFLP markers were used to probe the genetic differences between wild and cultivated soybeans or for the origin and dissemination of soybeans (Brown-Guedira *et al*., 2000; Tian *et al*., 2000; Li & Nelson, 2001; Xu & Zhao, 2002; Abe *et al*., 2003). These studies have revealed higher levels of genetic diversity in wild soybean.

6.2 RFLP (Restriction Fragment Length Polymorphism)

This analysis exploits variation in the occurrence of restriction sites in genomic sequences hybridizing to a cloned probe. Originally, RFLP analysis required Southern blotting and hybridization, making the method fairly slow and laborious. This technique is still used to generate ''anchor'' markers, used by many scholars to make consensus recombinational maps, though it is often implemented with the polymerase chain reaction (PCR) to generate the polymorphic fragments (Schulman, 2007).

Chung *et al*. (2006) evaluated levels of genetic diversity in USDA soybean germplasm (107 accessions), originated from six provinces in central China, using RFLP analysis. They detected significant genetic differentiation among the six provinces (mean *G*ST = 0.133). These results suggest that Chinese germplasm accessions from various regions or provinces in the USDA germplasm collection could be used to enhance the genetic diversity of US Cultivars.

6.3 AFLP (Amplified Fragment Length Polymorphism)

AFLP is an anonymous marker method, detects restriction sites by amplifying a subset of all the sites for a given enzyme pair in the genome by PCR between ligated adapters. To some extent, it like RFLP detects single nucleotide polymorphisms (SNPs) at restriction sites.

Ude *et al*. (2003) analyzed the genetic diversity within and between Asian and North American soybean cultivars by AFLP. They found that the average genetic distance between the North American soybean cultivars and the Chinese cultivars was 8.5% and between the North American soybean cultivars and the Japanese cultivars was 8.9%, but the Chinese soybean was not completely separated from the Japanese soybean. They also revealed that Japanese cultivars may constitute a genetically distinct source of useful genes for yield improvement.

6.4 RAPD (Random Amplified Polymorphic DNA)

RAPD analysis uses conserved or general primers that amplify from many anonymous sites throughout the genome. It is indeed rapid, and need only short primers of random sequence, but suffers from low polymorphism information content (PIC), poor correlation with other marker data, and problems in reproducibility due to the low annealing temperatures in the reactions.

The genetic diversity in the wild soybean populations from the Far East region of Russia was analyzed using RAPD markers (Seitova *et al*., 2004). The results obtained suggest that (1) genetically different groups of wild soybean have active development, (2) level of polymorphism was significantly higher than in the cultivated soybean and (3) geographically isolated subpopulations showed maximum distance from the main population of wild soybean. The high level of polymorphism between the wild and cultivated soybean accessions was also reported by Kanazawa *et al.* (1998) in their study on

soybean accessions from the Far East using RAPD profiles of mitochondrial and chloroplast DNA. Xu & Gai (2003), Pham Thi Be Tu *et al*. (2003), An *et al*. (2009) confirmed the results of Kanazawa *et al*. (1998) and Seitova *et al*. (2004) in terms of the high genetic variation between the wild and cultivated soybean accessions. They also found that the diversity of *G. soja* was higher than that of *G. max*; and environmental factors may play important roles in soybean evolution. Furthermore, they revealed that accessions within each species tend to form sub-clusters that are in agreement with their geographical origins, demonstrating that an extensive geographical genetic differentiation exists in both species. Consequently, it was indicated that geographical differentiation plays a key role in the genetic differentiation of both wild and cultivated soybeans. The relationship between geographical differentiation and genetic diversity appeared in the work of Chen & Nelson (2005) who identified significant genetic differences between soybean accessions collected from different provinces in China. Their data provided pronounced evidence that primitive cultivars of China were generally genetically isolated in relatively small geographical areas. Similar results were obtained by Li & Nelson (2001, 2002) in their study on soybean accessions from 8 provinces in China using a core set of RAPD primers with high polymorphism in soybean (Thompson *et al*., 1998). On the contrary, Brown-Guedira *et al*. (2000) did not find an association between origin and RAPD markers among soybean lines of more modern origin. It is likely that these genotypes have been dispersed by human intervention from the areas of actual origin.

The relationship between genetic differentiation and origin of 120 soybean accessions from Japan, South Korea and China was evaluated with RAPDs (Li & Nelson, 2001). They found that the Japanese and South Korean populations were more similar to each other, whereas both were genetically distinct from the Chinese population, suggesting that the S. Korean and Japanese gene pools might be probably derived from a relatively few introductions from China. Li *et al*. (2001) compared the genetic diversity of ancestral cultivars of the N. American (18) as well as the Chinese soybean germplasm pools (32) using RAPD markers, the N. American ancestors have a slightly lower level of genetic diversity. Cluster analyses generally separated the two gene pools. In particular, a great genetic variability was detected between the ancestors of northern U.S. and Canadian soybeans and the Chinese ancestors.

Chowdhury *et al*. (2002) examined the level of genetic similarity among forty-eight soybean cultivars imported out of their country Thailand using DNA (RAPD) markers. They found high level of genetic similarities between these cultivars. Cluster analysis of the obtained data classified the 48 cultivars into four groups at 0.57 similarity scale, even though the cultivars are morphologically or geographically very close. Comparing agronomic performance and RAPD analysis via dendrogram, a total of 11 cultivars can be useful to soybean breeders in Thailand who want to utilize genetically diverse introductions in soybean improvement. Baránek et *al*. (2002) evaluated the genetic diversity within 19 soybean genotypes included in the Czech National Collection of Soybean Genotypes by RAPD method. The polymorphism among the studied genotypes was 46%. Presented results enable the selection of genetically distinct individuals. Such information may be useful to breeders willing to use genetically diverse introductions in soybean improvement process.

6.5 SSRs (Simple sequence repeats)

SSRs molecular markers have been widely applied in the genetic diversity studies of the soybean germplasm (Abe *et al*., 2003; Wang *et al*., 2006; Fu *et al*., 2007; Li *et al*., 2008; Wang &

Takahata, 2007; Wang *et al*., 2008; Yoon *et al*., 2009). The advantages of SSR over other types of molecular markers are that they are abundant, have a high level of polymorphism, are codominant, can be easily detected with PCR and typically have a known position in the genome. High levels of polymorphism at SSR loci have been reported for both the number of alleles per locus and the gene diversity (Diwan & Cregan, 1997; Abe *et al*., 2003; Wang *et al*., 2006; Fu *et al*., 2007 ; Wang *et al*., 2010).

Wang *et al*. (2010) used 40 SSR primer pairs to study genetic variability in 40 soybean accessions of cultivars, landraces and wild soybeans collected from China. These results indicated that wild soybeans and landraces possessed greater allelic diversity than cultivars and might contain alleles not present in the cultivars which can strengthen further conservation and utilization. The UPGMA (Unweighted Pair Group Method with Arithmetic) results also exhibited that wild soybean was of more abundant genetic diversity than cultivars.

A total of 2,758 accessions of Korean soybean landraces were profiled and evaluated for genetic structure using six SSR loci (Yoon *et al*., 2009). The accessions within collections were classified based on their traditional uses such as sauce soybean (SA), sprouted soybean (SP), soybean for cooking with rice (SCR), and others-three different Korean *Glycine max* collections and for groups distinguished by their usage, such as SA, SP, and SCR. Nei's average genetic diversity ranged from 0.68 to 0.70 across three collections, and 0.64 to 0.69 across the usage groups. The average between-group differentiation (Gst) was 0.9 among collections, and 4.1 among the usage groups. The similar average diversity among three collections implies that the genetic background of the three collections was quite similar or that there were a large number of duplicate accessions in three collections (Yoon *et al*., 2009). The selection from the four groups classified based upon usage may be a useful way to select accessions for developing a Korean soybean landrace core collection at the RDA gene bank.

Hudcovicová *et al*. (2003) analyzed allelic profiles at 18 SSR loci of 67 soybean genotypes of various origins. Six only of SSR markers differentiated all 67 genotypes each from others successfully. Guan *et al*. (2010) investigated the genetic relationship between 205 Chinese soybean accessions that represent the seven different soybean ecotypes and 39 Japanese soybean accessions from various regions using 46 SSR loci. Cluster analysis with UPGMA separated the Chinese accessions from Japanese accessions, suggesting that soybean in these two countries form different gene pools. It also showed that (1) accessions from China have more genetic diversity than those from Japan, (2) studied germplasm was divided into three distinct groups, "corresponding to Japanese soybean, Northern China soybean, Southern China soybean and a mixed group in which most accessions were from central China", and (3) Japanese accessions had more close relationship with Chinese northeast spring and southern spring ecotypes. This study provides interesting insights into further utilization of Japanese soybean in Chinese soybean breeding.

Abe *et al*. (2003) analyzed allelic profiles at 20 SSR loci of 131 accessions introduced from 14 Asian countries. UPGMA-cluster analysis clearly separated the Japanese from the Chinese accessions, suggesting that the Japanese and Chinese populations formed different germplasm pools; showed that Korean accessions were distributed in both germplasm pools, whereas most of the accessions from south/central and southeast Asia were derived from the Chinese pool; indicated that genetic diversity in the southeast and south/central Asian populations was relatively high; and exhibited the absence of region-specific clusters in the southeast and south/central Asian populations. The relatively high genetic diversity

and the absence of region-specific clusters in the southeast and south/central Asian populations suggested that soybean in these areas has been introduced repeatedly and independently from the diverse Chinese germplasm pool. Therefore the two germplasm pools can be used as exotic genetic resources to enlarge the genetic bases of the respective Asian soybean populations.

Chotiyarnwong *et al.* (2007) evaluated the genetic diversity of 160 Thai indigenous and recommended soybean varieties by examining the length polymorphism of alleles found in 18 SSR loci from different linkage groups. UPGMA-Cluster analysis and principal component analysis (PCA) separated Thai indigenous varieties from recommended soybean varieties. However, the genetic differentiation between the indigenous and recommended soybean varieties was small.

Shi *et al*. (2010) performed genetic diversity and association analysis among 105 food-grade soybean genotypes using 65 simple sequence repeat (SSR) markers distributed on 20 soybean chromosomes. Based on the SSR marker data, the 105 soybean genotypes were divided into four clusters with six sub-groups. Thirteen SSR markers distributed on 11 chromosomes were identified to be significantly associated with oil content and 19 SSR markers distributed on 14 chromosomes with protein content. Twelve of the SSR markers were associated with both protein and oil QTL. A negative correlation was obtained between protein and oil content.

Mimura *et al*. (2007) investigated SSR diversity in 130 vegetable soybean accessions including 107 from Japan, 10 from China and 12 from the United States. Eighteen of the 130 accessions were outliers, and the rest of the accessions were grouped into nine clusters. The majority of food-grade soybean cultivars were released from Japan and South Korea because of the market availability and demands. However, the genetic diversity of South Korea food-grade soybean remains unreported (Mimura *et al*., 2007).

Nguyen *et al.* (2007) used 20 genomic SSR and 10 EST-SSR SSR to explore the genetic diversity in accessions of soybean from different regions of the world. The selection of the thirty SSR primer-pairs was based on their distribution on the 20 genetic linkage groups of soybean, on their trinucleotide repetition unit and on their polymorphism information content. All analyzed loci were polymorphic. A low correlation between SSR and EST-SSR data was observed, thus genomic SSR and EST-SSR markers are required for an appropriate analysis of genetic diversity in soybean. They observed high genetic diversity which allowed the formation of five groups and several subgroups. They also observed a moderate relationship between genetic divergence and geographic origin of accessions.

Xie *et al*. (2005) analyzed genetic diversity of 158 Chinese summer soybean germplasm, from the primary core collection of *G. max* using 67 SSR loci. The Huanghuai and Southern summer germplasm were different in the specific alleles, allelic-frequencies and pairwise genetic similarities. UPGMA cluster analysis based on the similarity data clearly separated the Huanghuai from Southern summer soybean accessions, suggesting that they were different gene pools. The data indicated that Chinese Huanghuai and Southern summer soybean germplasm can be used to enlarge genetic basis for developing elite summer soybean cultivars by exchanging their germplasm.

Most diversity studies on cultivated soybean published by now have focused on North American (Brown-Guedira *et al*., 2000; Narvel *et al*., 2000; Fu *et al*., 2007) Asian (Abe *et al*., 2003; Xie *et al*., 2005; Wang *et al*., 2006; Li *et al*., 2008; Wang *et al*., 2008; Yoon *et al*., 2009) as well as South American (Bonato *et al*., 2006) soybean germplasm. In several studies only a few genotypes of European origin have been represented among germplasm studied

(Brown-Guedira *et al*., 2000; Narvel *et al*., 2000; Fu *et al*., 2007; Hwang *et al*., 2008). Baranek *et al*. (2002) evaluated genetic diversity of 19 G*lycine max* accessions from the Czech National Collection using RAPD markers. Recently, Tavaud-Pirra *et al.* (2009) evaluated SSR diversity of 350 cultivated soybean genotypes including 185 accessions from INRA soybean collection originating from various European countries and 32 cultivars and recent breeding lines representing the genetic improvement of soybean in Western Europe from 1950 to 2000. They found the genetic diversity of European accessions to be comparable with those of the Asian accessions from the INRA collection, whereas the genetic diversity observed in European breeding lines was significantly lower. Breeding material and registered soybean cultivars in southeast European countries are strongly linked to Western breeding programs, primarily in the USA and Canada. There is little reliable information regarding the source of germplasm introduction, its pedigree and breeding schemes applied. Consequently, use of these genotypes in making crosses to develop further breeding cycles can result in an insufficient level of genetic variability. Assessing the genetic diversity of this germplasm at genomic DNA level would complement the knowledge on the European soybean gene pool (germplasm) and facilitate the utilization of the resources from southeastern Europe by soybean breeders. Ristova *et al*. (2010) therefore assess genetic diversity and relationships of 23 soybean genotypes representing several independent breeding sources from southeastern Europe and five plant introductions from Western Europe and Canada using 20 SSR markers. Cluster analysis clearly separated all genotypes from each other assigning them into three major clusters, which largely corresponded to their origin. Results of clustering were mainly in accordance with the known pedigrees.

6.6 EST (Expressed Sequence Tags)

The use of functional molecular markers, such as those developed from EST allows direct access to the population diversity in genes of agronomic interest that they represent coding sequences, facilitating the association between genotype and phenotype. Nelson and Shoemaker (2006) identified approximately 45,000 potential gene sequences (pHaps) from EST sequences of Williams/Williams 82, an inbred genotype of soybean (*Glycine max* L. Merr.) using a redundancy criterion to identify reproducible sequence differences between related genes within gene families. Analysis of these sequences revealed single base substitutions and single base indels are the most frequently observed form of sequence variation between genes within families in the dataset. Genomic sequencing of selected loci indicates that intron-like intervening sequences are numerous and are approximately 220 bp in length. Functional annotation of gene sequences indicates functional classifications are not randomly distributed among gene families containing few or many genes. The identification of potential gene sequences (pHaps) from soybean allows the scientist to get a picture of the genomic history of the organism as well as to observe the evolutionary fates of gene copies in this highly duplicated genome.

7. Allele mining in soybean germplasm

7.1 Concept

Exploitation of gene banks for efficient utilization depends on the knowledge of genetic diversity, in general, and allelic diversity at candidate gene(s) of interest, in particular. Hence, allele mining seems to be a promising in characterization of genetic diversity or allelic/genic diversity among the accessions of the collection in terms of its utility for

improving a target trait (Kaur *et al*., 2008). The availability of sequence and sequence variation that affects the plant phenotype is of utmost importance for the utilization of genetic resources in crop improvement (Graner, 2006).

The existing allelic diversity in any crop species is caused by mutations, the evolutionary driving force (Kumar *et al*., 2010). Mutations create new alleles or cause variations in the existing allele and allelic combinations. They take place in coding and non-coding regions of the genome either as single nucleotide polymorphism (SNP) or as insertion and deletion (InDel). As far it is known, there is no cited literatures on the effect of mutations on transcript synthesis and accumulation which in turn alter the trait expression in 5′ UTR including promoter, introns and 3′ UTR in the genome of soybean. In coding region, it may have tremendous effect on the phenotype by altering the encoded protein structure and/or function. For example, the AtAHASL protein encoded by *csr1-2* differs from the native AtAHASL protein by one amino acid substitution of a serine with an asparagine at residue 653 (S653N) which results in tolerance to imidazolinone containing herbicides. Besides the altered herbicide binding, the protein retains its biological function in the plant. Soybean line CV127 is tolerant to herbicides that contain imidazolinone. The another example is the mutations in soybean microsomal omega-3 fatty acid desaturase genes which resulted in reduce of linolenic acid concentration in soybean seeds (Bilyeu *et al*., 2005). Alternatively, several studies suggested that many diseases resistant alleles like soybean aphid [*Aphis glycines* Matsumura (Hemiptera: Aphididae)] resistance like *Rag1* from Germplasm collection (Kim *et al*., 2010), brown Stem Rot resistance like *Rbs1* and *Rbs3* from soybean lines L78-4049 and PI 437.833, and PI 84946-2 (Eathington *et al*., 1995; Klos *et al*., 2000), soybean cyst nematode (SCN) resistance genes like *rhg*1 and *Rhg*4 *from soybean* lines PI 88788, PI 437.654, Peking, PI90763 and PI209332, sudden death syndrome (SDS) resistance like Rfs1, rfs2, and rft from soybean lines PI 437654 (Meksem *et al*., 2001).

7.2 Approaches

Two major approaches are available for the identification of sequence polymorphisms for a given gene in the naturally occurring populations: (1) modified Targeting Induced Local Lesions in Genomes (TILLING) procedure and (2) sequencing based allele mining.

7.2.1 TILLING approach

In the TILLING approach, the polymorphisms (more specifically point mutations) resulting from induced mutations in a target gene can be identified by heteroduplex analysis (Till *et al*. 2003). This technique represents a means to determine the extent of variation in mutations artificially induced. EcoTilling represents a means to determine the extent of natural variation in selected genes in the primary and secondary crop gene pools (Comai and Henikoff, 2006 and Kumar *et al.* 2010). Like TILLING, it also relies on the enzymatic cleavage of heteroduplexed DNA, formed due to single nucleotide mismatch in sequence between reference and test genotype, with a single strand specific nuclease under specific conditions followed by detection through Li-Cor genotypers. At point mutations, there will be a cleavage by the nuclease to produce two cleaved products whose sizes will be equal to the size of full length product. The presence, type and location of point mutation or SNP will be confirmed by sequencing the amplicon from the test genotype that carry the mutation.

7.2.2 Sequencing-based allele mining

This technique involves amplification of alleles in diverse genotypes through PCR followed by identification of nucleotide variation by DNA sequencing. Sequencing-based allele

mining would help to analyze individuals for haplotype structure and diversity to infer genetic association studies in plants. Unlike EcoTilling, sequencing-based allele mining does not require much sophisticated equipment or involve tedious steps, but involves huge costs of sequencing. (Kumar *et al*., 2010)

7.3 Applications

Allele mining can be effectively and efficiently used for (1) discovery of superior alleles, through 'mining' the gene of interest from diverse genetic resources, (2) providing insight into molecular basis of novel trait variations and identifying the nucleotide sequence changes associated with superior alleles, (3) studying the rate of evolution of alleles; allelic similarity/dissimilarity at a candidate gene and allelic synteny with other members of the family, (4) paving way for molecular discrimination among related species through development of allele-specific molecular markers, and (5) facilitating introgression of novel alleles through Marker Assisted Selection (MAS) or deployment through Genetic Engineering (GE). Allele mining can also be potentially employed in the identification of nucleotide variation at a candidate gene associated with phenotypic variation for a trait. Through this, the frequency, type and the extent of occurrence of new haplotypes and the resulting phenotypic changes can be evaluated.

7.4 Challenges

The genetic resources collections, which are held collectively in various gene banks, harbour a wealth of undisclosed allelic variants. Now the challenge is how to efficiently identify and exploit the useful variation of these collections to exploit in crop improvement. The challenges stand as stampling block to make use of these collections are (1) selection of genotypes, (2) handling genomic resources, (3) demarcation of promoter region, (4) characterization of regulatory region, and (5) higher sequencing costs. The selection of germplasm to be 'mined' is one of the utmost challenges face the scholars because of the huge genetic resources collections. To overcome the aforementioned challenges, we must (1) narrow down the core collection to a manageable size while maintaining the variability, (2) refine phenotyping protocols to increase the efficiency of allele mining, (3) exploit the developments in allele mining, association genetics and comparative genomics by combining expertise from several disciplines, including molecular genetics, statistics and bioinformatics, (4) develop cheaper and faster sequencing platforms for high through put detection of allelic variations (5) develop flexible computational tools to manage genetic resources, select desirable alleles, analyze the functional nucleotide diversity to predict specific nucleotide changes responsible for altered function, accurately predict the core promoter region based on the representation/over-representation of consensus regulatory motifs, and get the snapshot of the regulatory elements which can be further examined through suitable experiments.

8. Conclusion

Soybean oil is used in many foods, industrial and fuel products. Whereas soybean meal is incorporated into animal feed. The variation in the quality and quantity of these products is basically dependent on the genetic diversity of soybean germplasm. The genetic diversity in

soybean germplasm was evolved from the dispersion of the cultivated soybean domesticated by the Chinese farmers. Many factors are affecting the dispersion of soybean including regional adaptation and selection. Morphological, cellular, biochemical (proteins and isozymes) and molecular markers have been used on the wide scale for the study of the genetic diversity of the cultivated and wild relative of soybean. These analyses were carried out to meet wide rang of objectives from simply testing the usefulness of a particular marker system to identifying exotic germplasm accessions to expand the genetic diversity of the elite germplasm pool in order to permit genetic improvement for increased soybean yield. Exploitation of soybean germplasm for efficient utilization depends on the knowledge of genetic diversity, in general, and allelic diversity at candidate gene(s) of interest, in particular. The beneficial alleles from vast soybean genetic resources existing worldwide were derived from cultivated germplasm. However, a significant portion of these beneficial alleles were still resided in the wild soybean germplasm. Nowadays, considerable attention has focused on allele mining (gene polymorphisms) and their potential use to alter protein function in ways that might prove biologically important. But increasing numbers of polymorphisms are also being identified in the regulatory and non coden regions of genes. Therefore, allele mining is a promising approach to dissect naturally occurring allelic variation at candidate genes controlling key agronomic traits which has potential applications in crop improvement programs. Allele mining can be effectively used for discovery of superior alleles, through 'mining' the gene of interest from soybean germplasm. It can also provide insight into molecular basis of novel trait variations and identify the nucleotide sequence changes associated with superior alleles. In addition, the rate of evolution of alleles; allelic similarity/dissimilarity at a candidate gene and allelic synteny with other members of the family can also be studied. Allele mining may also pave way for molecular discrimination among related species within the genus *Glycine*, development of allele-specific molecular markers, facilitating introgression of novel alleles through Marker Assisted Selection or deployment through genetic engineering. The alleles mining approaches and the challenges associated with it are also discussed.

9. References

Abe, J., Xu D. H., Suzuki, Y., Kanazawa, A. & Shimamoto, Y. (2003). Soybean germplasm pools in Asia revealed by nuclear SSRs. *Theor. Appl. Genet.,* 106, 445-453.

An, W., Zhao, H., Dong, Y., Wang, Y., Li, Q., Zhuang, B., Gong, L. & Liu, B. (2009). Genetic diversity in annual wild soybean (*Glycine Soja* SIEB. ET ZUCC.) and cultivated soybean (*G. Max*. MERR.). from different latitudes in China. *Pak. J. Bot*., 41. 2229-2242.

Arumuganathan, I., & Earle, E. D. (1991). Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep*., 9, 208-219.

Baranek, M., Kadlec, M., Raddova, J., Vachun, M. & Pidra, M. (2002). Evaluation of genetic diversity in 19 *Glycine max* (L.) Merr. accessions included in the Czech national collection of soybean genotypes. Czech J. Genet. Plant Breed, 38 , 69–74.

Bennett, M. D., Heslop-Harrison, J. S., Smith, J. B. & Ward, J. P. (1983). DNA density in mitotic and meiotic metaphase chromosomes of plants and animals. *J. Cell Sci*., 63, 173-179.

Bennett, M. D. & Leitch, I. J. (1995). Nuclear DNA amounts in angiosperms. *Ann. Bot.*, 76, 113-176.

Bennett, M. D., Leitch, I. J. & Hanson, L. (1998). DNA amounts in two samples of angiosperm weeds. *Ann. Bot.*, 82, 121-134.

Bilyeu, K., Palavalli, L., Sleper, D. & Beuselinck, P. (2005). Mutations in soybean microsomal omega-3 fatty acid desaturase genes reduce linolenic acid concentration in soybean seeds. Crop Sci., 45,1830–1836

Bonato, A. L. V., Calvo E. S., Geraldi, I. O., Arias, C. A. A. (2006). Genetic similarity among soybean (Glycine max (L). Merrill) cultivars released in Brazil using AFLP markers. *Gen Mol Biol*., 29, 692-704.

Brown-Guedira, G. L, Thompson, J. A, Nelson, R. L, Warburton, M. L (2000). Evaluation of genetic diversity of soybean introductions using RAPD and SSR markers. *Crop Sci*., 40, 815–823.

Burdon, J. J. & Marshall, D. R. (1981). Inter- and intra-specific diversity in the disease-response of Glycine species to the leaf-rust fungus. *Phakopsora pachyrhizi. Journal of Ecology*, 69, 381-390.

Bushehri, S. A. A., Abd-Mishani, C., Yazdi-Samadi, B., Sayed-Tabatabaei, B. E. (2000). Variety specific electrophoretic profiles of soybean cultivars. *Iranian J. Agric. Sci.,* 31, 55-61.

Camps, G., Vernetti Fde, J., Augustin, E. & Irigon, D. (1994). Morphological and electrophoretical characterization of twenty soybean cultivars. *Pesqui. Agropecu. Bras*., 29: 1779-1787.

Chen, Y. & Nelson, R. L. (2005). Relationship between Origin and Genetic Diversity in Chinese Soybean Germplasm. *Crop Sci*., 45, 1645–1652.

Chowdhurya, A.K, Srinivesb, P., Tongpamnakc, P., Saksoongd, P. & Chatwachirawong, P. (2002). Genetic Relationship among exotic soybean introductions in Thailand: consequence for varietal registration. *ScienceAsia*, 28, 227-239.

Chotiyarnwong, O., Chatwachirawong, P., Chanprame, S. & Srinives, P. (2007). Evaluation of genetic diversity in thai indigenous and recommended soybean varieties by SSR markers. *Thai Journal of Agricultural Science,* 40, 119-126.

Chung, J., Lee J. H., Arumuganathan K., Graef G. L., & Specht J. E. (1998). Relationship between nuclear DNA content and seed and leaf size inn soybean. *Theor. Appl. Genet.,* 96, 1064–1068.

Chung, M. G., Chung, M. Y., Johnson, C. & Palmer, R. G. (2006). Restriction fragment length polymorphisms in the USDA soybean germplasm from central China. *Botanical Studies*, 47, 13-21.

Comai, L. & Henikoff, S. (2006). TILLING: practical single-nucleotide mutation discovery. Plant J., 45,684–94."

Ding, Y. L., Zhao, T. J. & Gai, J. Y. (2008). Genetic diversity and ecological diversity of soybean cultivars from China, Japan, North America, and North American Ancestral Lines determined by amplified fragment length polymorphism. *Crop Sci,* 43, 1858–1867.

Diwan, N., Cregan, P. B. (1997). Automated sizing of fluorescent labeled simple sequence repeat (SSR) markers to assay genetic variation in soybean. *Theor Appl Genet.*, 95,723– 733.

Doerschug, E. B., Miksche, J. P., Palmer, R. G. (1978). DNA content, ribosomal-RNA gene number, and protein in soybeans. *Canadian Journal of Genetics and Cytology,* 20, 5331-5338.

Dong, Y. S., Zhuang, B. C., Zhao, L.M., Sun H. & He, M.Y. (2001). The genetic diversity of annual wild soybeans grown in China. *Theor Appl Genet.*, 103, 98–103.

Earthington, S. R., Nickell, C. D. & Gray, L. E. (1995). Inheritance of brown stem rot resistance in soybean cultivar BSR 101. *J. Hered*, 86:55-60.

Edwards, G. A. & Endrizzi, J. L. (1975). Cell size nuclear size and DNA content relationships in Gossypium. *Can. J. Genet. Cytol.*, 17, 181-186.

Fu, Y. B., Peterson, G. W., Morrison, M. J. (2007). Genetic diversity of Canadian soybean cultivars and exotic germplasm revealed by simple sequence repeat markers. *Crop Science*, 47, 1947-1954.

Fujita, R., Ohara, M., Okazaki, K., Shimamoto, Y. (1997). The extent of natural cross-pollination wild soybean (*Glycine soja*). *J Hered.*, 88,124–128.

Fukuda, Y. (1933). Cytogenetical studies on the wild and cultivated Manchurian soybeans (*Glycine*L.). *Japanese Journal of Botany* 6, 489–506.

Ghafoor, A., Gulbaaz, F. N., Afzal, M., Ashraf, M., Arshad, M. (2003). Inter-relationship between SDS-PAGE markers and agronomic traits in Chickpea (*Cicer Arietinum*). *Pak. J. Bot.*, 35, 613-624.

Ghafoor, A., Zahoor, A., Qureshi, A. S., Bashir, M. (2002). Genetic relationship in *Vigna mungo* (L.). Hepper and *V. radiata* (L.) R. Wilczek based on morphological traits and SDS-PAGE. *Euphytica*, 123, 367-2002.

Golbitz, P. (2007). *Soya and Oilseed Bluebook*, Soyatech, Inc., Bar Harbor, ME., Graham, MJ.

Graham, M.J., Nickell, C.D.& Rayburn, A.L. (1994). Relationship between genome size and maturity group in soybean. *Theoretical andApplied Genetics*, 88, 429-432.

Graner, A. (2006). Barley research at IPK, In: *Molecular markers for allele mining*, de Vicente, M.C. & Glaszmann, J.C., pp. (25-25). IPGRI, Italy.

Guan, R., Chang, R., Li, Y., Wang, L., Liu, Z., Qiu, L. (2010). Genetic diversity comparison between Chinese and Japanese soybeans (Glycine max (L.) Merr.) revealed by nuclear SSRs. *Genet Resour Crop Evol.* 57, 229–242.

Guo J., Wang, Y., Song, C., Zhou, J., Qiu, L., Huang, H., & Wang, Y. (2010). A single origin and moderate bottleneck during domestication of soybean (*Glycine max*): implications from microsatellites and nucleotide sequences. *Annals of Botany*, 106, 505-514.

Hammatt, N., Blackwell, N. W. & Davey, M. R. (1991). Variation in the DNA content of *Glycine* species. *Journal of Experimental Botany,* 42, 659-665.

Hirata, T., Abe, J. & Shimamoto, Y. (1999). Genetic structure of the Japanese soybean population. Genet. Resour. *Crop Evol.*, 46, 441–453.

http://www.ipgri.cgiar.org/system, 1998.

Hudcovicova, M. & Kraic, J. (2003). Utilisation of SSRs for characterisation of the soybean (*Glycine max* (L.). Merr.). genetic resources. *Czech J. Genet. Plant Breed.*, 39, 120–126.

Hwang, T.Y., Nakamoto, Y., Kono, I., Enoki, H., Funatsuki, H., Kitamura, K., Ishimoto, M. (2008). Genetic diversity of cultivated and wild soybeans including Japanese elite cultivars as revealed by length polymorphism of SSR markers. *Breeding Sci*., 58,315-323.

Hymowitz, T. (1995). Evaluation of wild perennial *Glycine* species and crosses for resistance to *Phakopsora*, In: *Proceedings of the Soybean Rust Workshop*, J.B. Sinclair & G.L.Hartman, 33–37, Urbana, IL, USA: National Soybean Rsearch Laboratory.

Hymowitz, T. (1970). On the domestication of the soybeans. *Economic Botany* 23, 408–421.

Hymowitz, T. (2004). Speciation and Cytogenetics, In Soybeans: improvement, production, and uses, H. R. Boerma & J. E. Specht, 97-136, American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, Wis.

Hymowitz, T., & N. Kaizuma. (1981). Soybean protein electrophoresis profiles from 15 Asian countries or regions: Hypotheses on paths plasm for soybean yield improvement. *Crop Sci.,* 38, 1362–1368.

Jha, S. S., Ohri, D. (1996). Phylogenetic relationships of *Cajanus cajan* (L.) Millsp. (pigeonpea). and its wild relatives based on seed protein profiles. *Genet. Resour. Crop Evol*., 43, 275–281.

Kanazawa, A., Tozuka, A., & Akimoto, S. (1998). Phylogenetic relationships of the mitochondrial genomes in the genus *Glycine* subgenus soja. Genes Genet. Syst., 73, 255–261.

Karam, M. A., Sammour, R. H., Ahmed, M. F., Ashour, F. M. & El-Sadek, L.M. (1999). Relationships among some *Medicago* species as revealed by isozyme electrophoresis. J. *Union Arab Biol*., 9 ,269-279.

Kaur, N., Street, K., Mackay, M., Yahiaoui, N., Keller, B. (2008). Molecular approaches for characterization and use of natural disease in wheat. . *Eur J Plant Patho.,* 121,387–97.

Kim, K-S., Bellendir S., Hudson, K. A., Hill, C. B., Hartman, G. L., Hyten, D. L., Hudson, M. E & Diers, B. W. (2010). Fine mapping the soybean aphid resistance gene *Rag*1 in soybean. *Theor. Appl. Genet.,* 120,1063 – 1071.

Klos, K. L. E., Paz, M. M., Fredrick, L., Marek, Cregan, P. B. & Shoemaker, R. C. (2000). Molecular markers useful for detecting resistance to brown stem rot in soybean. Crop Sci., 40,1445–1452.

Knight, C. A. & Ackerly, D. D. (2002).Variation in nuclear DNA content across environmental gradients: a quantile regression analysis. *Ecology Letters*, 5, 66-76.

Kumar, G. R., Sakthivel, K., Sundaram, R.M., Neeraja, C.N., Balachandran, S.M., Shobha Rani, N., Viraktamath, B.C., Madhav, M.S. (2010). Allele mining in crops: Prospects and potentials. Biotechnology Advances, 28, 451–461.

Li, C., Fatokun, C. A., Ubi, B., Singh, B. B. & Scoles, G. J. (2001). Determining genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. *Crop Sci.,* 4, 189-97.

Li, W., Han, Y., Zhang, D., Yang, M., Teng, W., Jiang, Z., Qiu, L., Sun, G. (2008). Genetic diversity in soybean genotypes from north-eastern China and identifi cation of

candidate markers associated with maturity rating. *Plant Breeding*, 127,494-500.

Li, Z. L & Nelson, R. L (2001). Genetic diversity among soybean accessions from three countries measured by RAPDs. *Crop Sci*., 41,1337-1347.

Li, Z. & Nelson, R. L. (2002). RAPD marker diversity among cultivated and wild soybean accessions from four Chinese provinces. *Crop Sci.,* 42, 1737-1744.

Lü, S.L. (1978). Discussion on the original region of cultivated soybean in China. *Scientia Agricultura Sinica,* 4, 90–94.

Malik, M.F.A., Qureshi, A.S., Ashraf, M. & Ghafoor, A. (2006). Genetic variability of the main yield related characters in soybean. *Inter. J. Agri. & Biol.,* 8, 815-619.

Malik, M. F. A., Ashraf, M., Qureshi, A. S., & Gjafoor, A. (2007). Assessment of genetic variability, correlation and path analyses for yield and its components in soybean. *Pak. J. Bot*., 39, 405-413.

Malik, M.F.A., Qureshi, A.S., Ashraf, M., Khan, M.R., & Javed, A. (2009). Evaluation of genetic diversity in soybean (*Glycine max*). lines using seed protein. Electrophoresis. *Australian Journal of Crop Science,* 3,107-112.

Meksem, K., Pantazopoulos, P., Njiti, V.N., Hyten, L.D., Arelli, P.R. & Lightfoot, D.A. (2001). 'Forrest' resistance to the soybean cyst nematode is bigenic: saturation mapping of the *Rhg1* and *Rhg4* loci. *Theor Appl Genet*., 103,710–717.

Mimura, M. (2001). *Genetic Diversity of Edamame Soybean [Glycine max (L.) Merr.]*, M.S. thesis, Washington State University.

Mimura, M., Coyne, C. J., Bambuck, M. W. & Lumpkin, T. A. (2007). SSR Diversity of vegetable soybean [*Glycine max* (L.) Merr.]. *Genet. Resour. Crop Evol.,* 54, 497-508.

Narvel, J. M., Fehr, W. R., Chu, W. C., Grant, D., Shoemaker, R. C. (2000). Simple sequence repeat diversity among soybean plant introductions and elite genotypes. *Crop Sci*., 40,1452–1458.

Nelson, R. L. & Shoemaker, R. C. (2006). Identification and analysis of gene families from the duplicated genome of soybean using EST sequences. *BMC Genomics,* 7,204 -215.

Ngon, T. T., Van, K., Kim, M. Y., & Lee, S. H. (2006). Genetic variation in flowering time and maturity and its relationship among agronomic characters in soybean. *Korean Crop Science,* 51, 163-168.

Nguyen, H. T., Wu, X. L., Vuong, T. D, Sleper, D. A, Shannon, G. J. & Stacey, G. (2007). Genome maps, genetic diversity and marker-assisted selection for soybean improvement. *Molecular Plant Breeding*, 2007, 5, 196-198.

Pagel, J., Walling, J.G., Young, N.D., Shoemaker, R.C. & Jackson, S.A. (2004). Segmental duplications within the Glycine max genome revealed by fluorescence in situ hybridization of bacterial artificial chromosomes. *Genome*, 47, 764-768.

Pei, Y. L., Wang, L., Ge S., Wang, L. Z. (1996). Study on genetic diversity of *Glycine soja*-isozyme variation in four populations (China). *Soybean Sci*., 15, 302-309.

Pham Thi Be Tu., Nguyen Thi Lang & Bui Chi Buu. (2003). Soybean genetic diversity analysis *Omonrice,* 11, 138-142.

Rayburn, A. L. (1990). Genome size variation in southwestern Indian maize adapted to various altitudes. *Evolutionary Trends in Plants,* 4, 53-57.

Rayburn, A. L., Birdar, D. P., Bullock, D. G., Nelson, R. L., Gourmet, C. & Wetzel J. B. (1997). Nuclear DNA content diversity in chinese soybean introductions *Annals of Botany,* 80, 321-325.

Rayburn, A. L., Biradar, D. P., Nelson, R. L., McCloskey, R., & Yeater, K. M. (2004). Documenting intraspecfic genome size variation in soybean. *Crop Sci*., 44, 261–264.

Rajanna, M. P., Viswanatha, S. R., Kulkarni, R. S. & Ramesh, S. (2000). Correlation and path analysis in soybean (*Glycine max* L. Merrill).. *Crop Res. Hisar*., 20, 244-2447.

Ristova, D., Šarcevic, H., Šimon, S., Mihajlov, L. & Pejic, I. (2010). Genetic diversity in southeast European soybean germplasm revealed by SSR markers. *Agriculturae Conspectus Scientificus,* 75 , 21-26.

Sacks, F. M., Lichtenstein, A., Van Horn, L., Harris, W., Kris-Etherton, P., Winston, M. (2006). "Soy protein, isoflavones, and cardiovascular health: an American Heart Association Science Advisory for professionals from the Nutrition Committee". *Circulation* 113, 1034–44.

Sammour, R. H. (1985). *Use of proteins characters in the taxonomy of peas and beans*. Thesis (Ph.D.), Ph D thesis, Tanta University, Tanta, Egypt.

Sammour, R. H. (1989). Electrophoresis of the seed proteins of *Vicia faba L.* and their immediate progenitors, -a reappraisal. *Plant Breeding,* 104,196-201.

Sammour, R. H. (1991). Using electrophoretic techniques in varietal identification, biosystematics analysis, phylogenetic relations. *Journal of Islamic Academy of Sciences*, 4, 221-226, 1991.

Sammour, R. H. (1992). An electrophoretic analysis of the variation in the seed storage proteins of *Vicia faba L.* Cultivars. *Fedds Repertorium,* 103, 555-557.

Sammour, R. H., Hamoud, M. A., Haidar, A. S. & Badr, A. (1993). Electrophoretic analysis of the seed proteins of some species in genus *Lotus*. *Feddes Repertorium,* 104, 251-257.

Sammour, R. H. (1999). SDS-PAGE analysis of the seed protein of some *Trifolium* taxa. *Plant Varieties and Seeds*, 12, 11-210.

Sammour, R. H. (2007). Electrophoretic and immunochemical techniques for the molecular reassessment of relationships within *Vicieae*. *Acta Agronomica Hungarica* 55, 131-147, 2007.

Sammour, R. H., Radwans, S. A & El-Koly, A. (2007). Genetic Variability in *Phaseolus* spp. as Revealed by SDS-PAGE Markers. *Seed Technology*, 29, 50-59.

Schulman, A. H. (2007). Molecular markers to assess genetic diversity. *Euphytica*, 158, 313–321.

Sharma, S. C. & Maloo, S. R. (2009). Genetic diversity and phylogenetic relationship among soybean [*Glycine max* (L.) Merrill] varieties based on protein, evolutionary and morphological markers. *Indian Journal of Plant Genetic Resources*, 22.

Shi, A., Chen, P., Zhang, B. & Hou, A. (2010). Genetic diversity and association analysis of protein and oil content in food-grade soybeans from Asia and the United States. *Plant Breeding*, 129, 250–256.

Sihag, R., Hooda, J. S., Vashishtha, R. D., Malik, B. P. S. (2004). Genetic divergence in soybean [*Glycine max* (L.). Merrill]. *Annals Biol,*. 20, 17-21.

Singh, G. (2010). *The soybean: botany, production and uses: The origin and history of soybean*, L. J. Qui & R. Z. Chang, 1-23, CAB International.

Seitova, A. M., Ignatov, A. N., Suprunova, T. P., Tsvetkov, I. L., Deineko, E. V., Dorokhov, D. B., Shumnyi, V. K., & Skryabin, K. G. (2004). Genetic Variation of Wild Soybean Glycine soja Sieb. et Zucc. in the Far East Region of the Russian Federation. *Russian Journal of Genetics*, 40, 165–171.

Tavaud-Pirra, M., Sartre, P., Nelson, R. L., Santoni, S., Texier, N., Roumet, P. (2009). Genetic diversity in a soybean collection. *Crop Sci*., 49, 895-902.

Thompson, J. A., Nelson, R. L., & Vodkin, L. O. (1998). Identification of diverse soybeangermplasm using RAPD markers. *Crop Sci*., 38, 1348-1355.

Tian, Q. Z, Gai, J. Y, Yu, D. Y, Jia, J. Z (2000). A study of amplified fragment length polymorphism (AFLP). in soybean (Chin.).. *Soybean Sci.,* 19, 210-217.

Till, B.J., Reynolds, S.H., Green, E.A., Codomo, C.A., Enns, L.C.& Johnson, J.E., Burtner, C., Odden, A. R., Young, K., Taylor, N. E., Henikoff, J. G., Comai, I. & Henikoff, S. (2003). Large-scale discovery of induced point mutations with high-throughput TILLING. Genome Res., 13, 524–30.

Truong, N. T. , Gwag, J. G., Park, Y. J, & Lee, S. H. *(2005).* Genetic Diversity of Soybean Pod Shape Based on Elliptic Fourier Descriptors. *Korean J. Crop Sci., 50,* 60-66.

Ude, G. N., Kenworthy, W. J., Costa, J. M., Cregan, P. B., & Alvernaz, J. (2003). American Ancestral Lines Determined by Amplified Fragment Length Polymorphism. *Crop Sci.*, 43,1858–1867.

USDA, (2001). Soybean Germplasm Collection Descriptors.

Wang, J.L. (1947). Evaluation of soybean traits. Agricultural Journal, 5, 6–11.

Wang, K. J.& Takahata, Y. (2007). A preliminary comparative evaluation of genetic diversity between Chinese and Japanese wild soybean (*Glycine soja*). germplasm pools using SSR markers. Genet Resour Crop Evol., 54, 157-165.

Wang, L., Guan, R., Zhangxiong, L., Chang, R. & Qiu, L. (2006). Genetic Diversity of Chinese Cultivated Soybean Revealed by SSR Markers. Crop Sci, 46,1032-1038.

Wang, M., Li, R., Yang, W. & Du, W. (2010). Assessing the genetic diversity of cultivars and wild soybeans using SSR markers. African Journal of Biotechnology, 9, 4857-4866.

Xie, H., Guan, R., Chang, R. & Qiu, L. (2005). Genetic diversity of Chinese summer soybean germplasm revealed by SSR markers. *Chinese Science Bulletin,* 50, 526-535.

Xu, D.H. & Gai, J.Y. (2003). Genetic diversity of wild and cultivated soybeans growing in China revealed by RAPD analysis. *Plant Breeding*, 122, 503-506.

Xu, L. & Zhao, G. F. (2002). Microsatellite DNA marker and its application in genetic diversity research. *Acta Botanica Boreali Occidentalia Sinica*, 22, 714-722.

Yeeh Y., Kang, S.S. & Chung, M.G. (1996). Evaluations of the natural monument 1 populations of *Camellia japonica* (Theaceae). in Korea based on allozyme studies. *Bot. Bull. Acad. Sin.*, 37, 141-146.

Yamamoto, K. & Nagato, Y. (1984). Variation of DNA content in the genus *Glycine. Japanese Journal of Breeding*, 34, 163-170.

Yoon, M., Lee, J., Kim, C., Kang, J., Cho, E., Baek, H. (2009). DNA profi ling and genetic diversity of Korean soybean (*Glycine max* (L.). Merrill). landraces by SSR markers. *Euphytica*, 165,69-77.

Young, H. (2008). *Plasticity of soybean* (*Glycine Max* (L.). Merrill) *root development under mild water deficits*. Thesis (M.S.), University of Missouri-Columbia.

Zhuang, B. C. (1999). *Biological Studies of Wild Soybeans in China*, Science Press, Beijing (in Chinese).

2

Importance of Seed [Fe] for Improved Agronomic Performance and Efficient Genotype Selection

John V. Wiersma
University of Minnesota/Northwest Research and Outreach Center
United States

1. Introduction

Plants require a continuous supply of iron (Fe) to maintain proper growth. Although the most abundant micronutrient in surface soils (Fageria et al., 2002), Fe is the most limiting to agricultural production throughout the world (Kochian, 2000) and to soybean production in the North Central United States (Hansen et al., 2003). Iron deficiency is a complex disorder and occurs in response to multiple soil, environmental, and genetic factors. Iron deficiency chlorosis (IDC) is symptomatic of the disorder and commonly observed on high pH, highly calcareous soils. Planting Fe deficiency- resistant soybean [*Glycine max.* (L.) Merr.] varieties has been promoted as the best strategy to alleviate or avoid Fe deficiency where soybean is grown on high pH, highly-calcareous soils (Fairbanks et al., 1987; Goos and Johnson, 2000; Naeve and Rehm, 2006). However, screening nurseries used to identify more resistant varieties based on visual chlorosis scores (VCS) do not always provide consistent, reliable results. A major obstacle to breeding for Fe chlorosis resistance in soybean has been that Fe deficiency symptoms and resistance scores cannot be consistently replicated among experiments. Inconsistent results preclude precise recommendations. Naeve and Rehm (2006), using nine highly tolerant and one moderately tolerant genotype, concluded that variety evaluation for IDC must be done at multiple IDC prone locations with varying soil chemical factors. One hypothesis is that this lack of consistency is probably due to the complex chemical and physical criteria in both the plant and soil that must be met for chlorosis to occur (Fairbanks, 2000; Naeve and Rehm, 2006). A more accurate and precise estimate of resistance to Fe deficiency may be expressed by a different plant character.

Ideally, plant traits measured to characterize resistance to Fe deficiency would be accurate, precise, simple, rapid, and inexpensive. Few plant traits or measures satisfy all of these requirements. For resistance to Fe deficiency, the "measure of choice" for decades (Weiss, 1943; Cianzio et al., 1979; Froehlich and Fehr, 1981; Fairbanks et al., 1987; Penas, et al., 1990; Goos and Johnson, 2000; Helms et al., 2010) has been a subjective, discontinuous, visual estimate of the degree of chlorosis, i.e. VCS, of the most recently fully-expanded middle leaflet of the third or developmentally younger trifoliolate. Cianzio et al. (1979) concluded that evaluation of foliar chlorosis, rather than measurement of chlorophyll concentration, is the most efficient procedure for comparison of cultivars because it requires relatively less labor. However, visual estimates of chlorosis when only the first trifoliolate leaf is fully

developed (Cianzio et al. 1979) may be more a reflection of planting seed [Fe][1] than resistance to Fe deficiency (Ambler and Brown, 1974; Tiffin and Chaney, 1973; Chaney et al., 1992). Furthermore, Naeve and Rehm (2006) concluded that varietal screening based on VCS likely requires that evaluation is conducted at multiple locations to be predictive. This suggests that using VCS to identify more resistant cultivars may not be the most efficient or least expensive procedure. It has been suggested that the plant character (plant height, seed number, grain yield, seed [Fe], VCS, relative chlorophyll [SPAD] reading) used to measure Fe deficiency is of primary importance in the classification of genotypes for resistance to Fe deficiency (Wiersma, 2007). Many of the characters mentioned are known to vary markedly in screening nurseries as well as in management studies (Helms et al., 2010; Naeve and Rehm, 2006; Wiersma, 2005, 2007, and 2010).

In measuring the indirect effects of recurrent selection for Fe efficiency in soybean, Beeghly and Fehr (1989) reported that Fe efficiency was not associated closely with grain yield, time of maturity, plant height, seed protein or oil, leaflet traits, and most micronutrients, except seed [Fe]. Seed weight declined 12%; seed [Fe] increased 13%; whereas, seed Fe content did not change over seven cycles of selection (Beeghly and Fehr, 1989). For soils known to have yield-limiting availabilities of specific micronutrients, increasing the concentration of that micronutrient in seed used for planting has reduced Mo deficiency in corn (*Zea mays* L.) (Weir and Hudson, 1966), Zn deficiency in several species (Rashid and Fox, 1992), Fe and Zn deficiency in rice (*Oryza sativa* L.) (Gregorio et al., 2000), B deficiency in soybean (Rerkasem, et al., 1997), and Fe deficiency in dry bean (*Phaseolus vulgaris* L.) (Beebe et al., 2000) and wheat (*Triticum aestivum* L.) (Shen et al., 2002). Since seed [Fe] can be regarded as an integrated measure of resistance to Fe deficiency that is manifest at maturity, perhaps seed [Fe] should be considered the "measure of choice" in determining susceptibility or resistance to IDC (Bouis et al., 2003; Nestle et al., 2006).

This chapter presents evidence that supports the use of seed [Fe] as an accurate and consistent measure of genotypic differences in Fe efficiency and agronomic performance. This 'evidence' has been garnered from recent soybean Fe deficiency trials conducted on high pH, highly calcareous soils in the North Central region of the USA (Wiersma, 2005, 2007, and 2010), from variety evaluation trials of the Univ. of Minn. Soybean Plant Breeding and Genetics Project, from IDC nurseries managed by R.J. Goos (http://www.soilsci.ndsu.nodak.edu/yellowsoybeans/) and from varietal trials conducted on partially limed and fully limed, acid soils in Brazil (Spehar, 1994).

2. Agronomic performance

Average visual chlorosis scores (VCS) in chlorosis screening nurseries and in management trials involving various treatments are commonly accepted as reasonable estimates of the severity of Fe deficiency. Minor, although statistically significant, differences in VCS observed in the near absence of chlorosis, or in another trial, the near death of many cultivars, have little meaning. In the research discussed here, the severity of Fe deficiency ranged from almost no chlorosis (VCS= 1.2) to mild chlorosis (VCS=2.3) to moderate chlorosis (VCS=3.0) to severe chlorosis (VCS=4.2), nonetheless, consistent genotypic differences usually were observed when genotypes were first grouped into classes based on published VCS, field VCS observed at V3, or planting seed Fe concentration or content.

[1][Fe] is iron concentration.

Class variances were calculated and tested for homogeneity (Snedecor and Cochran, 1980; Gomez and Gomez, 1984) and when class variances were homogeneous, regression equations were developed using class means consisting of both independent and dependent variables. Management studies involving increasing rates of seeding, increasing rates of Fe-EDDHA application, and increasing rates of N application were conducted using resistant, moderately resistant, and susceptible cultivars, without first categorizing the smaller number of genotypes into classes.

2.1 Increasing seeding rates with low Fe-EDDHA rates

It is generally reported that increasing seeding rates will reduce visual chlorosis ratings (early and/or mid-season) and often will increase grain yield when soybean is grown where Fe deficiency is moderate to severe (Uvalle-Bueno and Romero, 1988; Penas et al., 1990; Goos and Johnson, 2001; Lingenfelser et al., 2005; Wiersma, 2007). Increasing seeding density (seeds unit^{-1} of row), and, presumably, increasing the volume of soil occupied by roots unit^{-1} of row, can lead to higher yields and higher seed [Fe]s, but may have little influence on early-season VCS (Fig. 1 A, C, E). When averaged across 3 years, 4 replications, 3 cultivars, and 5 rates of Fe-EDDHA, increasing seeding density almost 3-fold reduced visual chlorosis about 12% (Fig. 1 A). On the other hand, increasing Fe-EDDHA rates (in accordance with the severity of IDC) will markedly reduce early season VCSs, but may have little influence on grain yield (Fig. 1 B, D, F). Averaged across 3 years, 4 replications, 3 cultivars, and 5 seeding densities, increasing the Fe-EDDHA rate 4-fold reduced early season visual chlorosis about 70% (Fig. 1 B). Fe acquisition, measured as seed [Fe], appears to be regulated primarily by genotype, yet Fe acquisition by less Fe-efficient cultivars can be increased by increasing SD or reducing the severity of Fe deficiency. It is possible to slightly increase seed [Fe] of both susceptible and resistant cultivars grown under severe chlorosis if high rates (>4.48 kg ha^{-1}) of Fe-EDDHA are used (Table 1; Fig. 1 F). Rates of Fe-EDDHA used in these studies (Fig. 1) were much lower (1.12 to 4.48 kg ha^{-1}) than those evaluated in other studies (2.24 to 11.2 kg ha^{-1}) and may have been responsible for the moderate responses to increasing rates of Fe-EDDHA.

2.2 High Fe-EDDHA rates

Research to reduce or alleviate IDC in soybean by applying various seed, soil, or foliar Fe chelates or fertilizers has been conducted for decades. Although the results have been mixed (Mortvedt, 1986), and are seldom directly comparable, positive responses to foliar (Randall, 1981), seed (Karkosh et al., 1988), and soil (Penas et al., 1990; Wiersma, 2005) application have been reported. Other researchers have observed only small, if any, response to similar treatments (Goos and Johnson, 2000; Goos and Johnson, 2001; Heitholt et al., 2003). Lack of consistent results may be related to differing levels of chlorosis severity among experiments; soil, environmental, or genetic differences; and/or the low rates of Fe often applied to ensure economic feasibility. Low rates of Fe probably do not satisfy the requirement of a continuous supply of Fe as plant development progresses (Goos and Johnson, 2001).

Responses to higher (beyond economic feasibility) rates of Fe-EDDHA appear variety specific and occur over an extended period, manifest at maturity (Fig. 2). As plant development progresses, there are earlier, limited responses to low rates of Fe-EDDHA, whereas higher rates appear to provide Fe continuously and to promote later, larger responses.

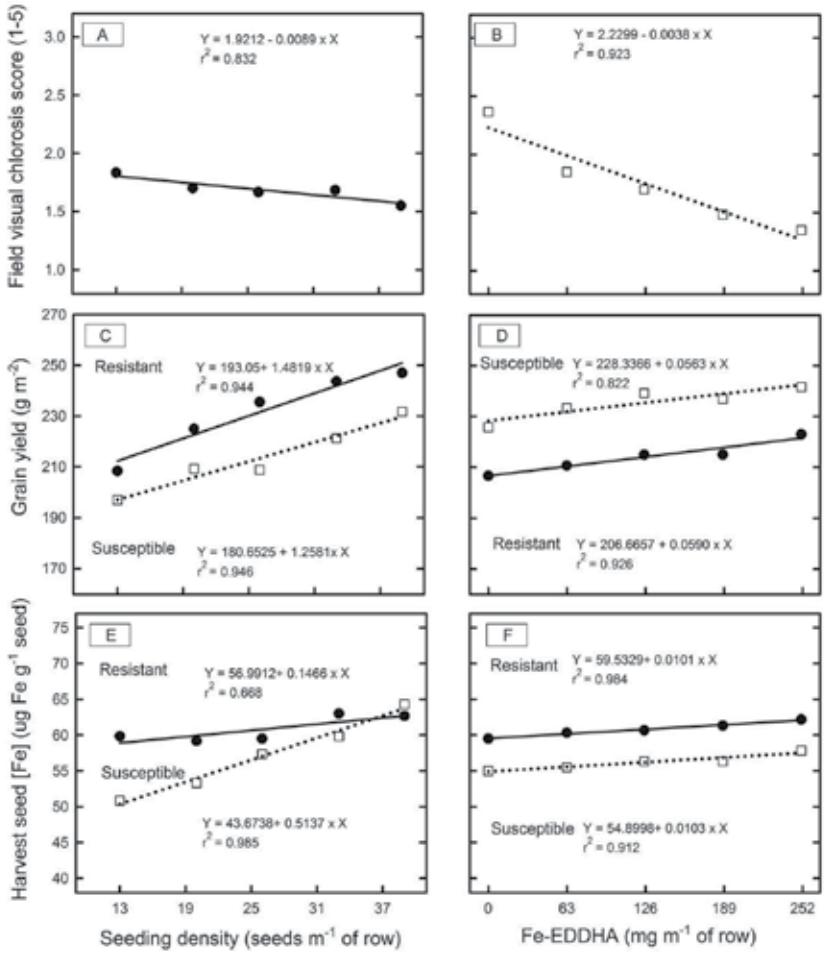


Fig. 1. Visual chlorosis, grain yield, and harvest seed [Fe] of resistant and susceptible cultivars in response to seeding density and Fe-EDDHA rate.

However, it should be noted that the severity of Fe deficiency and the plant characters used to measure treatment response are both crucial when deciding the suitability of various treatments for improving Fe acquisition. For example, measures of field visual chlorosis at low rates of Fe-EDDHA discriminate nicely between resistant and susceptible cultivars, but at higher Fe rates, almost all cultivars have similar scores (Table 1A). In contrast, measures of harvest seed [Fe] provide nearly identical discrimination among cultivars at each rate of Fe-EDDHA, and are nearly the same as the [Fe] of the seed used for planting (Table 1B).

At lower rates of Fe-EDDHA, resistant cultivars often exceed susceptible cultivars in plant height, seed number, and grain yield, whereas at higher rates, susceptible cultivars approach values similar to resistant cultivars (Fig. 2). With only slight chlorosis (Fig. 3, Fisher, MN 2003) seed [Fe] changed very little in response to added Fe for either resistant or susceptible cultivars. With severe chlorosis (Fig. 3, Crookston, MN, 2003), resistant cultivars increased harvest seed [Fe] about 15% in each portion of the canopy, whereas susceptible cultivars changed harvest seed [Fe] little in any portion of the canopy. Harvest seed [Fe]s of

A.

Variety	Initial seed Fe conc.	Fe-EDDHA (kg Fe ha^{-1})						Published chlorosis rating
		0.00	2.24	4.48	6.72	8.96	11.20	
	(µg g^{-1})	field visual chlorosis rating (1-5)						(1-5)
MN0302	88.6	2.1 B†	1.2 A	1.1 A	1.0 A	1.0 A	1.0 A	1.6
GC3104	73.1	3.4 A	1.9 A	1.2 A	1.0 A	1.0 A	1.0 A	2.5
Norpro	91.3	2.6 B	1.4 A	1.2 A	1.0 A	1.0 A	1.0 A	3.3
S2000 2020	57.6	3.8 A	1.5 A	1.1 A	1.0 A	1.0 A	1.0 A	3.7
LSD (0.05)		0.7	NS	NS	.	.	.	
CV		14.9	26	15.7	.	.	.	

B.

Variety	Published chlorosis rating	Fe-EDDHA (kg Fe ha^{-1})						Initial seed Fe conc.
		0.00	2.24	4.48	6.72	8.96	11.20	
	(1-5)	harvest seed [Fe] (µg g^{-1} seed)						(µg g^{-1})
MN0302	2.1 B	64.1 B	64.7 B	66.3 B	66.3 B	77.7 A	74.3 A	88.6
GC3104	3.4 A	42.4 C	49.5 C	47.8 C	51.3 C	53.2 B	52.7 B	73.1
Norpro	2.6 B	79.3 A	77.1 A	78.6 A	76.4 A	76.9 A	80.0 A	91.3
S2000 2020	3.8 A	41.3 C	46.4 C	45.5 C	45.4 C	45.4 B	49.8 B	57.6
LSD (0.05)	0.7	8.3	6.0	5.8	6.3	12.2	9.7	
CV	14.9	9.2	6.3	6.2	6.5	12.0	9.4	

† Means followed by the same letter within a column are not statistically significant at the 5 % level of probability.

Table 1. Field visual chlorosis rating recorded at V3 and seed [Fe] at harvest of four cultivars grown at six rates of Fe-EDDHA applied at planting.

resistant cultivars were consistently higher than that of susceptible cultivars whether chlorosis was nil or severe. This observation is similar to that of Beebe et al. (2000) and Blair et al. (2009) who, from work done with dry beans (*Phaseolus vulgaris* L.), concluded that seed micronutrient densities of Fe (and Zn) were consistent, reliable estimates of resistance to Fe deficiency. Genotypically superior and inferior cultivars could be identified consistently across years and locations (Bouis et al., 2003; Nestle et al., 2006; Ghandilyan et al., 2006). Other research (Wiersma, 2005) has shown that plotting relative grain yield vs seed [Fe] for several environments exhibits a narrow range of seed [Fe] associated with wide ranges in relative yield and that there are consistent seed [Fe] differences between resistant and susceptible cultivars regardless of relative yield. These conclusions have led to the concept that individual genotypes have a seed [Fe] "threshold" that is presumably, genetically

predetermined, yet seldom exceeded, and that seed [Fe] could supplement or replace VCS as a measure of resistance to IDC.

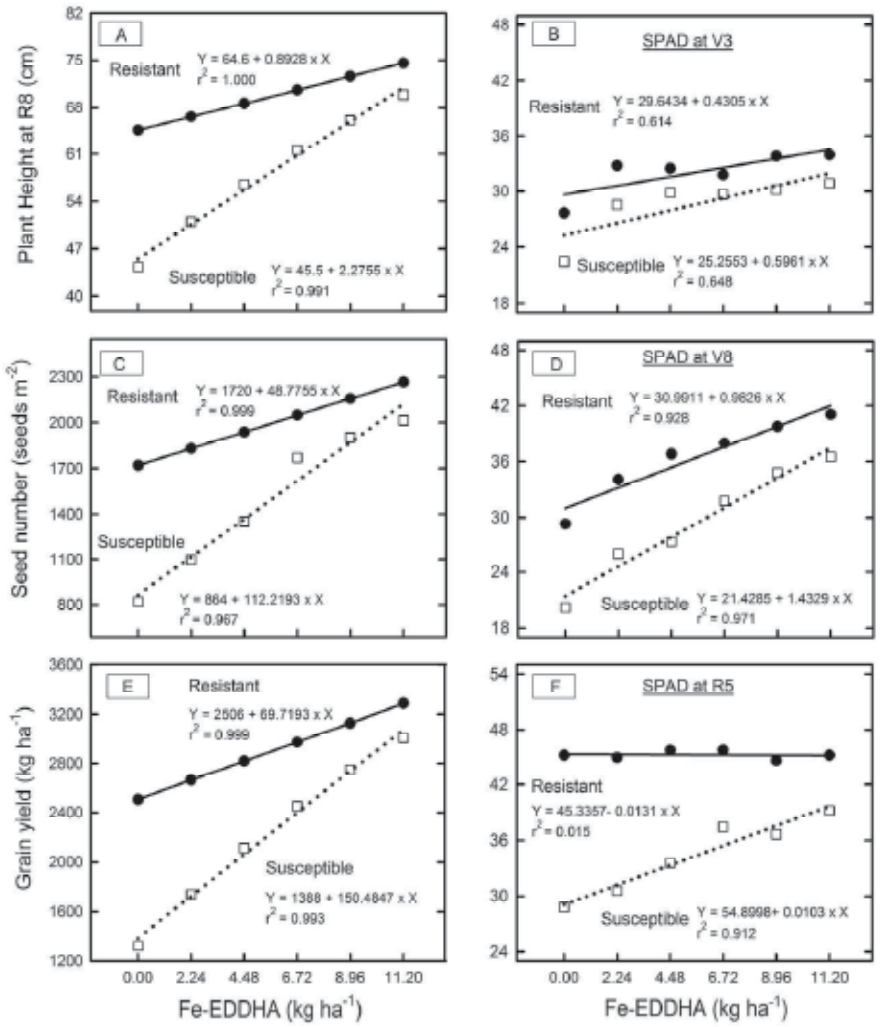


Fig. 2. Agronomic measures of resistance to Fe deficiency in soybean in response to increasing rates of applied Fe-EDDHA averaged over three environments (panels A, C, and E). SPAD measures were recorded at three stages of development in one environment (panels B, D, and F).

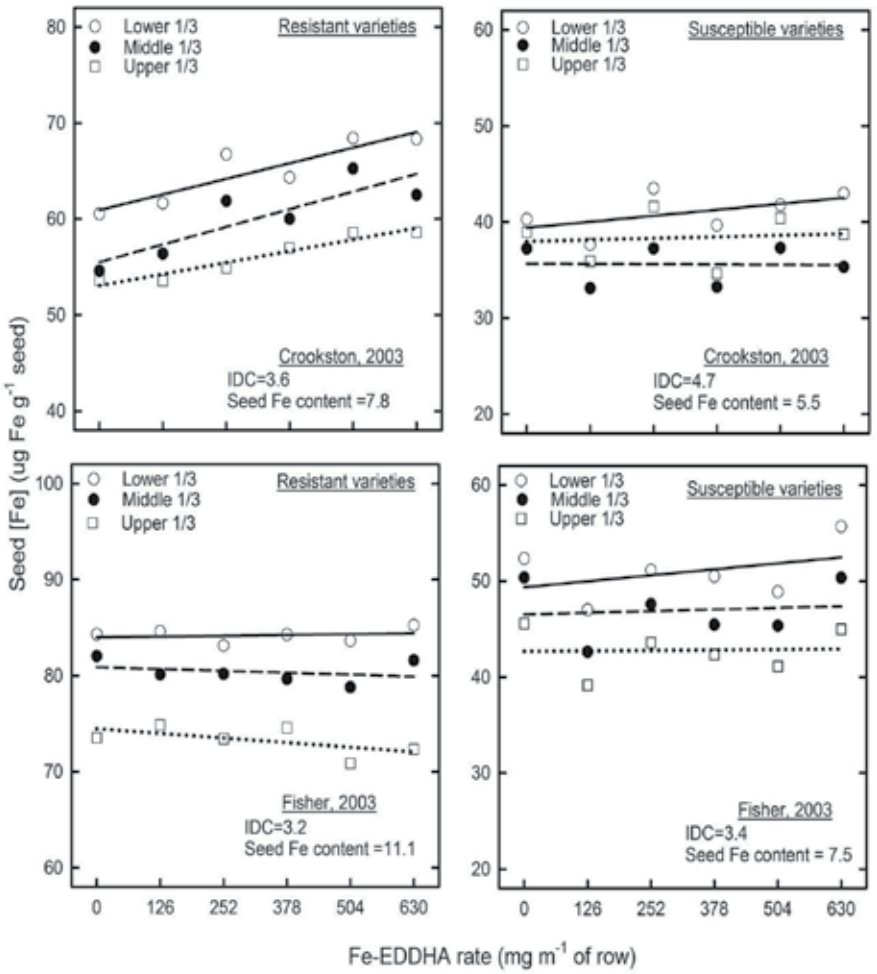


Fig. 3. Seed [Fe] at harvest for different canopy positions of resistant and susceptible cultivars grown under mild to severe Fe deficiency (Crookston, MN, 2003) and nil to mild Fe deficiency (Fisher, MN, 2003).

2.3 Fe-EDDHA rates - canopy position

Ten consecutive plants within row two of each plot in the Fe-EDDHA trials mentioned above were harvested at R7-R8, the total number of main stem nodes was counted, averaged, and used to separate plants into the upper, middle, and lower thirds of the plant. Sections were combined and the number of seeds, total seed weight, and seed [Fe] of the three sections of the canopy were determined. Averaged across cultivars, seed [Fe] decreased from approx. 50 µg g^{-1} at the lower canopy position, to 45 µg g^{-1} in the middle

one-third, to 40 μg g^{-1} in the top one-third (Fig. 3). This decrease occurred under both nil and severe Fe chlorosis and suggests that developmentally younger and older seeds respond similarly to increasing Fe-EDDHA rates. Increases in seed [Fe] occur primarily in resistant cultivars grown under harsh Fe deficiency. Susceptible cultivars show little response to added Fe-EDDHA whether Fe deficiency is nil or severe.
With limited Fe deficiency (Fisher, MN, 2003), both resistant and susceptible cultivars attain their genetically predetermined seed [Fe] (Fig. 3). Taken together, these results suggest that developmentally younger, intermediate, and older seed accumulate Fe at similar rates, but for different lengths of time and that cultivars and canopy positions have very similar regression slopes, but different intercepts or "thresholds" (Fig. 4).

2.4 Nitrogen rates

The response of soybean cultivar resistance to IDC to differing N rates was evaluated in a field study. Six rates of fertilizer N (0, 34, 68, 102, 136, and 170 kg ha^{-1}) were applied to six cultivars differing in resistance to IDC (2 Fe efficient, 2 moderately Fe efficient, and 2 Fe inefficient) over a three year period. Nodulation decreased linearly in response to added N for all cultivars, regardless of their Fe efficiency characterization or yearly growing conditions. In contrast, relative foliar chlorophyll concentrations (SPAD readings) differed markedly among cultivars, but showed little consequential response to increasing nitrogen rates (NR) (Fig. 5). Plant height, seed number, grain yield, and seed [Fe] decreased linearly in response to increasing NRs for Fe-inefficient cultivars, whereas these responses in Fe-efficient and moderately efficient cultivars changed little as NR increased (Figs. 5 and 6). Despite these differences, the ranking of cultivars based on seed [Fe] was only slightly affected by increasing NRs.

3. Genotype selection

When the results of variety evaluations conducted in large-scale chlorosis nurseries are considered, there is little evidence of consequential decreases in VCS among cultivars during the last decade (Fig. 7). However, selecting more resistant genotypes in large screening nurseries is complicated by the large genotype x nursery (environment) interaction, especially where VCSs are used to estimate 'resistance'. Inconsistent variety responses have been attributed to environments, soil heterogeneity, and large variations in soil chemistry (Jolley et al., 1996; Fairbanks, 2000). Ferric chelate reductases and quantitative determination of iron reduction have been suggested as reliable indicators of the genetic potential for chlorosis resistance (Jolley et al., 1996; Fairbanks, 2000). Factors controlling absorption and transport of Fe are known to be located in the root and to be genetically determined (Brown et al., 1958; Brown et al., 1972). Although these measures appear to be reliable, they require specialized equipment and knowledge, limiting the number of potential genotypes that can be evaluated in a reasonable amount of time. Within years, genotypic rank correlations of VCSs (Table 2) are often highly significant across locations, suggesting reasonable reliability. This can be deceiving, however, because large-scale nurseries often have 'normal' distributions with nearly all VCSs being between 2.5 and 3.5 at each location (Fig. 8). In a field study conducted during 2007, 2008, and 2009 rank correlations were calculated among 14 genotypes that had been included each year (Table 5). These results suggest that VCSs may not be the most appropriate measures of Fe efficiency. During the same decade, micronutrient densities (primarily Fe and Zn) in both

grasses and legumes have been found to be reliable and consistent across both years and locations. Research conducted on dry bean (*Phaseolus vulgaris* L.), wheat (*Triticum aestivum* L.), and rice (*Oryza sativa* L.) cultivars demonstrated that genotypes with high micronutrient densities of Fe and Zn during one year at one location will also be among the highest at another location in another year (Gregorio, 2002; Shen et al., 2002; Bouis et al., 2003; Nestle et al., 2006; Blair et al., 2009; Blair et al., 2010). Perhaps, measures of resistance to Fe deficiency in soybean should involve integrated estimates of uptake, transport, and accumulation of Fe that are manifest at maturity, such as Fe content 1000^{-1} seeds, seed [Fe], and/or iron removal with seed (μg Fe m^{-2}).

3.1 Roundup ready vs conventional cultivars

As Roundup Ready™ (RR) cultivars were first being released there was local concern among growers and crop consultants that resistance to Fe deficiency may not have been incorporated during development of earlier releases. During 2002, ten RR™ and ten 'conventional' cultivars were grown at two rates of Fe-EDDHA (0 and 8.96 kg ha^{-1}) at the University of Minnesota Northwest Research and Outreach Center (NWROC) on soils with a known history of mild to severe Fe deficiency. A relatively high rate of application of Fe-EDDHA increased relative chlorophyll readings at V3 about 13% (4.6 SPAD units) and increased grain yield nearly 18% (434 kg ha^{-1}). Roundup Ready cultivars out-yielded conventional cultivars by approximately the same amount, 19% (453 kg ha^{-1}). Nonetheless, seed [Fe] at harvest did not differ between Fe-EDDHA rates, nor between RR and conventional cultivars (Table 3). Seed [Fe] at harvest was moderately related to both published visual chlorosis score (r^2=0.452) and Fe concentration of the seed used for planting (r^2=0.458). Classifying cultivars on the basis of their published VCS and then their planting seed [Fe] resulted in the same cultivars being in each class and, consequently, having the same r^2 values. This research involved a relatively small sample of cultivars grown under harsh conditions and may not have fairly represented the importance of Fe 1000^{-1} seeds, seed [Fe], and/or Fe removal. Similarly, it is important to remember that these results cannot be extended to all RR and conventional soybean cultivars.

3.2 Variety x Fe-EDDHA rate

Four cultivars (two Fe deficiency resistant, two Fe deficiency susceptible) and six rates of Fe-EDDHA (0, 2.24, 4.48, 6.72, 8.96, and 11.2 kg ha^{-1}) were evaluated at one location in 2002 and six cultivars (two Fe deficiency resistant, two moderately resistant, and two susceptible) were evaluated at two locations in 2003. Visual chlorosis scores recorded at V3 could distinguish resistant from susceptible cultivars only when no Fe-EDDHA was applied, whereas harvest seed [Fe] could discriminate among resistant and susceptible cultivars at all six rates of Fe-EDDHA and in exactly the same order at each level of added Fe chelate (Table 1). Although grain yield increased markedly with added Fe chelate (Fig. 1. C), seed [Fe] changed very little (approx. 11%) (Fig. 1. F). The rank order of cultivars for harvest seed [Fe] was also the same as that of the cultivars' initial or planting seed [Fe], providing some evidence that seed [Fe] reflects varietal differences in resistance to Fe deficiency. Similar results recorded for the two 2003 trials, where two additional cultivars were evaluated under different severities of Fe deficiency, extend the applicability of this concept to evaluations conducted on similar soils.

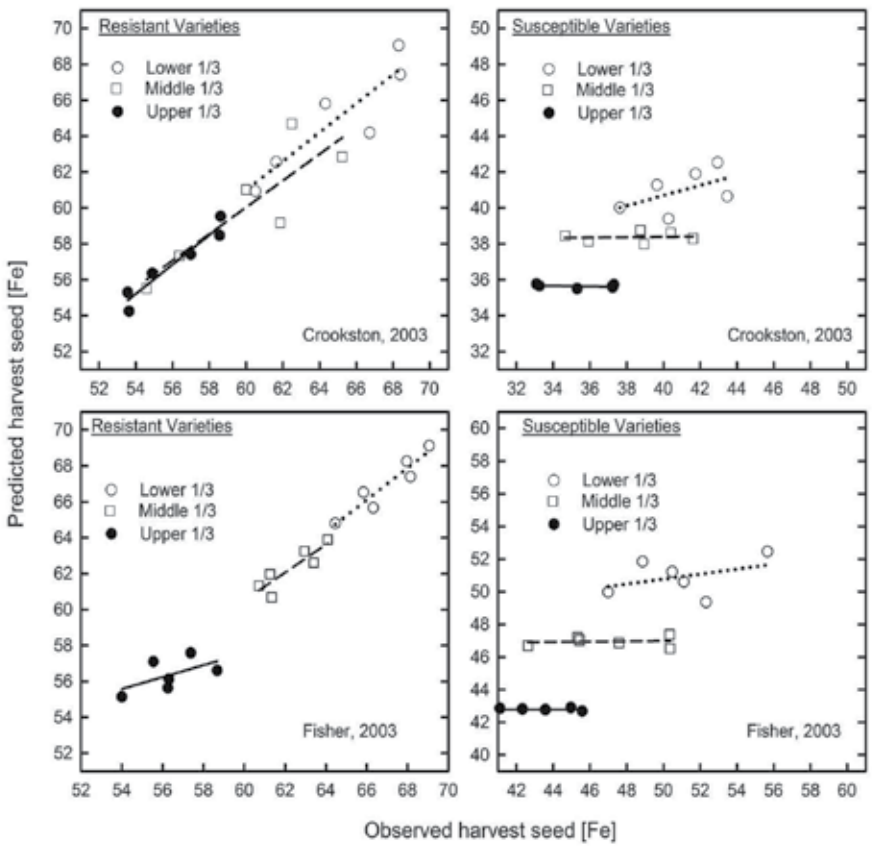


Fig. 4. Results (observed and predicted values) from linear regression equations of harvest seed [Fe] on applied Fe-EDDHA were significant for each one-third of the canopy for resistant cultivars, but for none of the canopy thirds for susceptible cultivars, Crookston, MN and Fisher, MN, 2003. Rates of applied Fe-EDDHA were: 0, 125, 250, 375, 500, 625 mg Fe-EDDHA per m of a 0.56 m wide row.

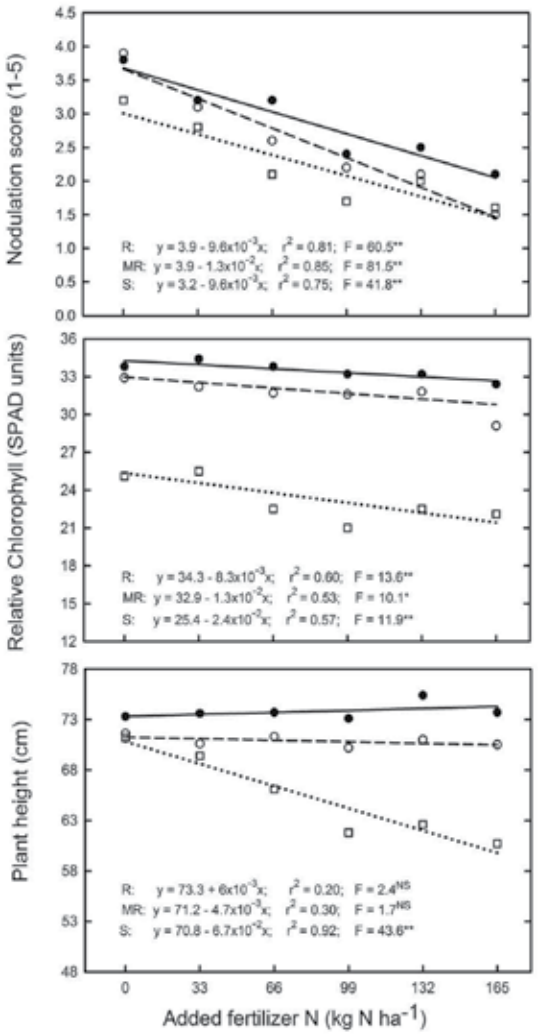


Fig. 5. Varietal differences in linear response to added N, averaged across three years, for plant height, relative chlorophyll, and nodulation score.

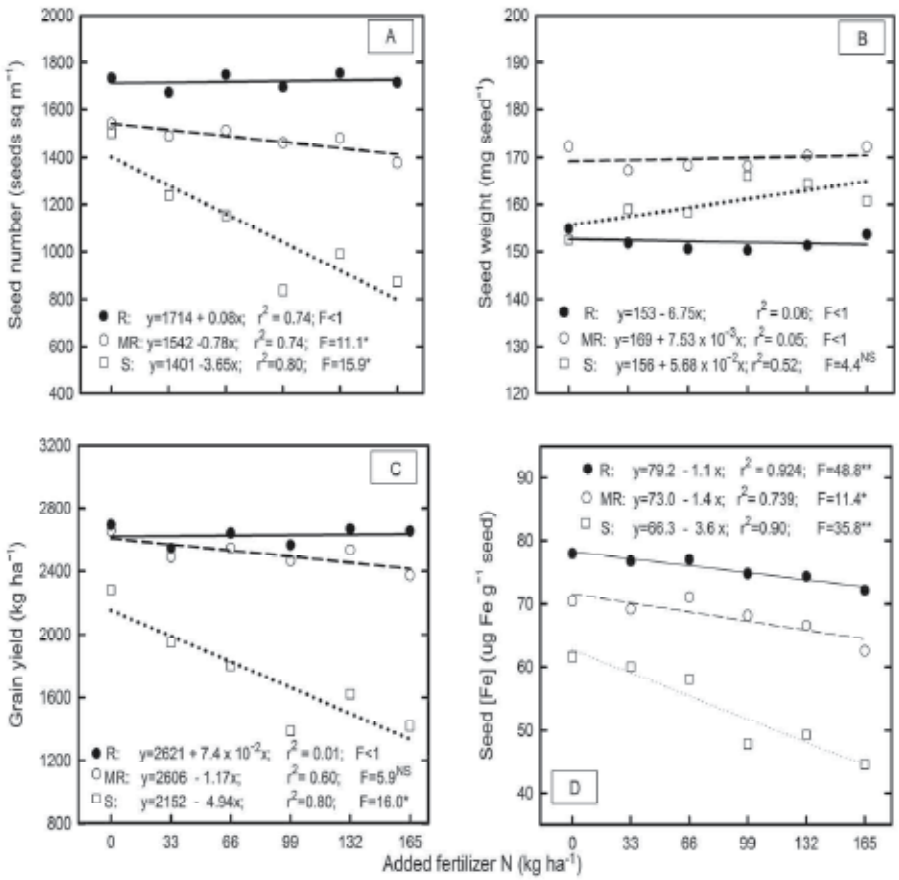


Fig. 6. Varietal differences in linear response to added N, averaged across three years for seed number, seed weight, grain yield, and seed [Fe].

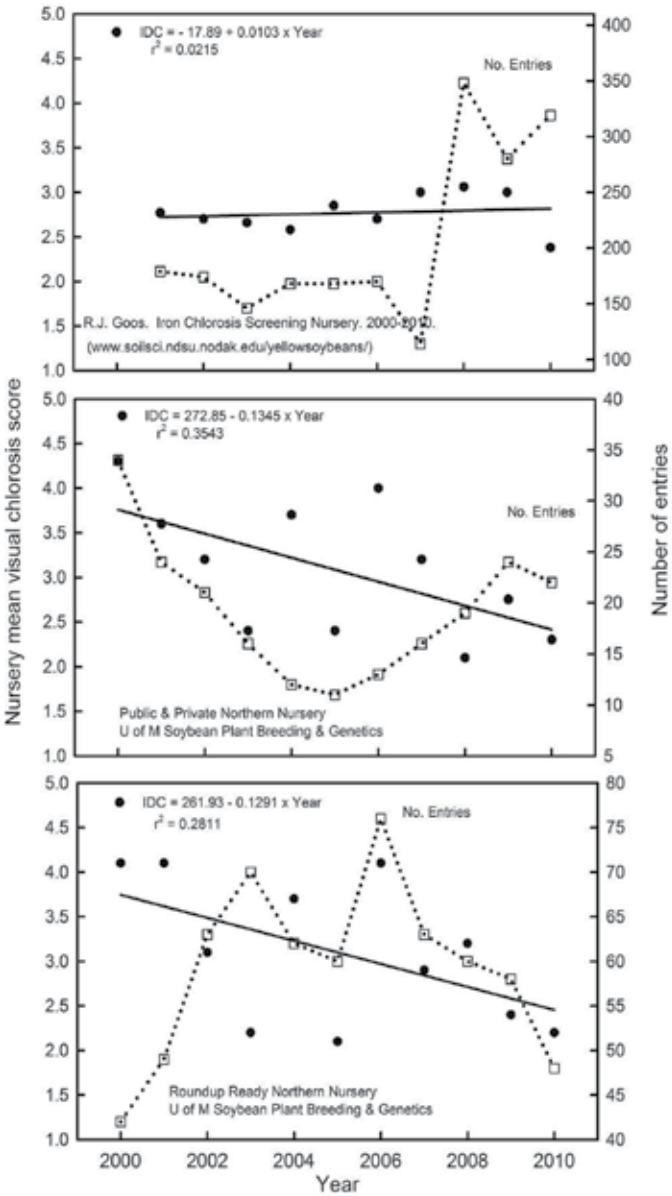


Fig. 7. Changes in means of visual chlorosis score and number of entries during the last decade for three large-scale chlorosis nurseries.

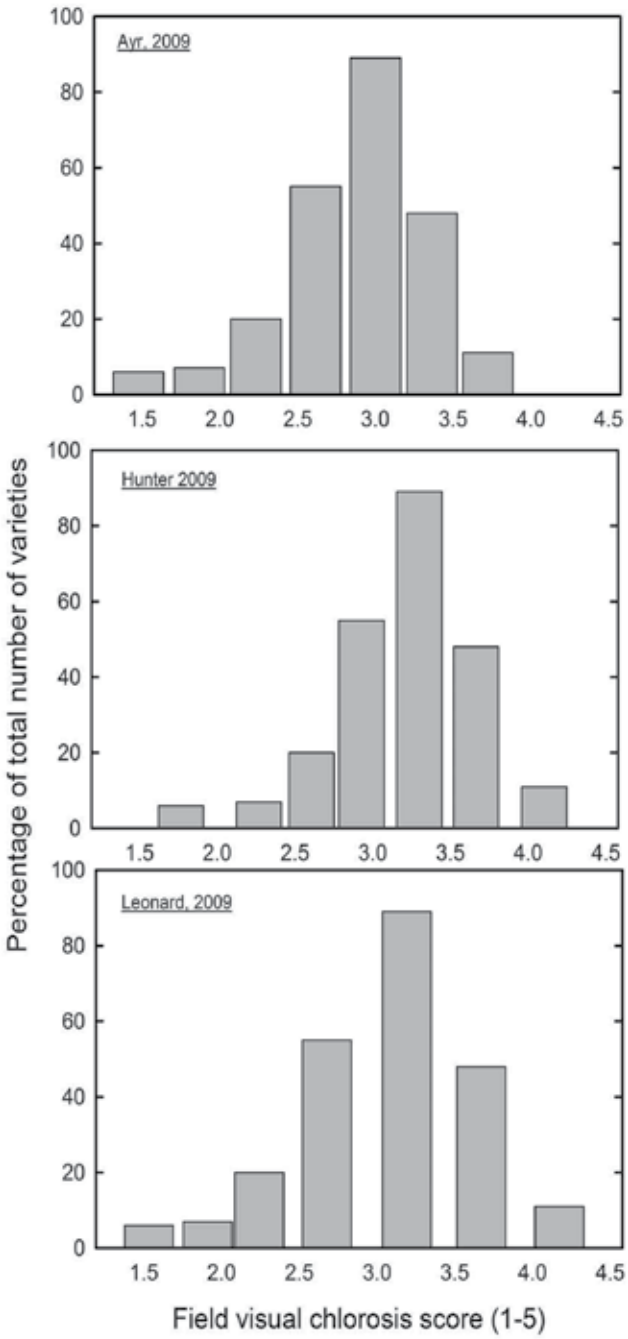


Fig. 8. Frequency (as a percentage of the total) of cultivars in increasing, field visual chlorosis categories at three North Dakota locations in 2009.

Year		Amenia	Ayr	Colfax	Galesburg	Leonard
2008						
	Amenia	1	0.7902**	0.8386**	0.8118**	0.6696**
	Ayr		1	0.7676**	0.7559**	0.6368**
	Colfax			1	0.7958**	0.6832**
	Galesburg				1	0.6372**
	Leonard					1
2009						
		Ayr	Hunter	Leonard		
	Ayr	1	0.7989**	0.7817**		
	Hunter		1	0.7964**		
	Leonard			1		
2010						
		Ayr	Colfax	Galesburg	Leonard	Prosper
	Ayr	1	0.6932**	0.7227**	0.5606**	0.6674**
	Colfax		1	0.5829**	0.5109**	0.6168**
	Galesburg			1	0.5151**	0.5800**
	Leonard				1	0.5270**
	Prosper					1

** Significant at 1% level of probability.

Table 2. Genotypic rank correlations across several North Dakota locations during 2008, 2009, and 2010.

3.3 Northwest Minnesota - seventy-two cultivars, 4 environments

Results from earlier research (Table 1; Figs.1, 2, and 3) provided preliminary, but convincing, evidence that grain yield and harvest seed [Fe] were closely related to planting seed [Fe] across several genotypes when conditions favorable for Fe deficiency prevailed. These studies, however, involved only 4 to 29 cultivars. To confirm this observation we evaluated 72 cultivars expressing a wide range of seed Fe concentrations. Our primary objective was to compare plant traits thought to represent measures of resistance to Fe deficiency. This collection of Maturity Group 00 (MG 00) genotypes had a range of planting seed Fe concentrations from 64 to 106 ug Fe g^{-1} seed. We assumed that the seed we obtained had been grown with adequate Fe availability. Plants were grown in nutrient solution as well as field nurseries. Nutrient solution culture procedures described by Chaney et al. (1992) were used to evaluate genotypes grown with moderate to severe Fe deficiency under controlled conditions. Other researchers have concluded that similar quantitative trait loci (QTL) are identified in nutrient solution and field tests and, therefore, both systems identify similar genetic mechanisms of iron uptake and/or utilization (Lin et al., 2000).

These seventy-two genotypes also were grown on high pH, highly calcareous soils at three locations in 2003 and one location in 2004. Measures of resistance to Fe deficiency were: harvest seed [Fe] (µg g^{-1} seed); harvest seed Fe content (µg 1000^{-1} seeds); and Fe removal (µg Fe m^{-2}). Classification variables were: published visual chlorosis (VC); in-field visual chlorosis at V3 (V3); planting seed [Fe] (µg Fe g^{-1} seed) (FC); planting seed Fe content (µg Fe 1000^{-1} seeds) (FS); and relative chlorophyll concentration (SPAD values) (GC).

Type 3 Tests of Fixed Effects

Effect	Numerator df	Denominator df	F value	Pr > F
Fe Rate	1	6	20.83	0.0038
Entry	19	114	4.49	<.0001
Fe Rate * Entry	19	114	0.67	0.8369

Estimates

Label	Estimates	Standard Error	df	t Value	Pr > F
Mean High Rate	35.0325	0.7198	6	48.67	<.0001
Mean Low Rate	30.3862	0.7198	6	42.22	<.0001
HI vs Low Fe	4.6463	1.0179	6	4.56	0.0038
RR	33.8887	0.5781	9.94	58.62	<.0001
Conv.	31.5300	0.5781	9.94	54.54	<.0001
RR vs Conv.	2.3588	0.5482	114	4.30	<.0001
Rate 1 – RR	31.8850	0.8175	9.94	39.00	<.0001
Rate 2 – RR	35.8925	0.8175	9.94	43.90	<.0001
R1 vs R2 – RR	-4.0075	1.1562	9.94	-3.47	0.0061
Rate 1 – Conv.	28.8875	0.8175	9.94	35.34	<.0001
Rate 2 – Conv.	34.1725	0.8175	9.94	41.80	<.0001
R1 vs R2 – Conv.	-5.2850	1.1562	9.94	-4.57	0.0010

Contrasts

Label	Numerator df	Denominator df	F value	Pr > F
Hi vs Low Fe (C1)	1	6	20.83	0.0038
RR vs Conv. (C2)	1	114	18.51	<.0001
C1 x C2	1	114	1.36	0.2464

† SPAD values are described as consistent and accurate measures of leaf chlorophyll content (umoles m^{-2} of leaf area) (Markwell, et al., 1995; Markwell and Blevins, 1999).

Table 3. Analysis of variance of SPAD† at V3 for 10 RR and 10 conventional soybean cultivars grown at two rates of Fe-EDDHA under severe Fe deficiency.

Type 3 Tests of Fixed Effects

Effect	Numerator df	Denominator df	F value	Pr > F
Fe Rate	1	6	1.54	0.2614
Entry	19	114	2.37	0.0026
Fe Rate*Entry	19	114	0.61	0.8950

Estimates

Label	Estimates	Standard error	df	t value	Pr. [t]
Mean High Rate	2797.27	247.64	6	11.30	<.0001
Mean Low Rate	2363.11	247.64	6	9.54	<.0001
HI vs LOW Fe	434.16	350.21	6	1.24	0.2614
RR	2806.51	181.92	6.99	15.43	<.0001
Conv.	2353.87	181.92	6.99	12.94	<.0001
RR vs Conv.	452.64	98.65	114	4.59	<.0001
Rate 1 – RR	2568.48	257.27	6.99	9.98	<.0001
Rate 2 – RR	3044.55	257.27	6.99	11.83	<.0001
R1 vs R2 – RR	-476.07	363.84	6.99	-1.31	0.2321
Rate 1 – Conv.	2157.75	257.27	6.99	8.39	<.0001
Rate 2 – Conv.	2550.00	257.27	6.99	9.91	<.0001
R1 vs R2 – Conv.	-392.25	363.84	6.99	-1.08	0.3168

Contrasts

Label	Numerator df	Denominator df	F value	Pr > F
Hi vs Low Fe (C1)	1	6	1.54	0.2614
RR vs Conv. (C2)	1	114	21.05	<.0001
C1 x C2	1	114		0.6717

Table 4. Analysis of variance of grain yield for 10 RR and 10 conventional soybean cultivars grown at two rates of Fe-EDDHA under severe Fe deficiency.

We acknowledge that in our studies genotype and seed [Fe] are confounded and that Fe availability, as well as genotype, likely will influence final seed [Fe]. Although seed [Fe] and genotype are confounded, this is not unlike visual chlorosis score and genotype. A better approach would have been to use several genotypes each having a range of seed [Fe] from 50 to 120 μg g^{-1} seed. We were unable to identify or create these treatments. Nonetheless, we know from earlier research (Wiersma, 2005, 2007, and 2010) that large (25-50%) differences in agronomic performance, within the same genotype, often are associated with rather small (5-10%) differences in harvest seed [Fe]. In this case, correlations among agronomic characters and seed [Fe] are relatively small ($r < 0.4$). We also know from field experiments that differences between cultivars of <10 μg Fe g^{-1} seed are not likely to be statistically significant (Wiersma, 2005, 2007,and 2010). Similarly, Shen et al. (2002) concluded that in wheat "In fact, it is impossible to distinguish completely the role of the genotype vs seed Fe content in the early response to Fe deficiency because the difference in seed Fe content can be an aspect of genotypic difference to Fe deficiency". It is reasonable to think that seed [Fe], seed Fe content, or Fe removal can also be considered aspects of genotypic differences in response to Fe deficiency.

	Year		
Year	2007	2008	2009
2007	1	0.6176*	0.5472*
2008		1	0.3714^{NS}
2009			1

$^{*, NS}$ Significant at 5% level of probability, and not significant at 5% level of probability

Table 5. Genotypic rank correlations of 14 genotypes across several North Dakota locations during 2007, 2008, and 2009.

In-field visual chlorosis is better predicted using at planting seed [Fe] than the recorded visual chlorosis score, although the relationship is far from perfect. The complexity of using individual genotype means, without first classifying them into groups, is illustrated in Fig. 9. The extent of yellowing (VCS) among plots within a nursery often approaches a continuous distribution from green to yellow. Historically, this range of expression has been sub-divided into classes prior to analysis (Fehr, 1982). Thus, to evaluate relationships among characters, the 72 genotypes in each environment were first divided into 5 classes on the basis of several characters: recorded visual chlorosis (VC); visual chlorosis at V3 (V3); seed Fe concentration (FC); seed Fe content (FS); and growth chamber SPAD (GC). Then, Levene's F test (Littell et al., 2006) was used to assess homogeneity of error variances across classes for each measure used in classifying genotypes. Welch's F test was used to test the equality of means across the levels of the single class terms (Littell et al., 2006). Ideally, plant traits used to classify genotypes would have homogeneous error variances (non-significant Levene's F) and significant differences among class means (significant Welch's F).

Classifying genotypes on the basis of visual observations, either VC or V3, rarely (16%) yielded homogeneous class variances, although differences among class means were almost always (83%) significant (Table 6). The heterogeneous class variances indicate that VCSs may not be the most appropriate measures of Fe efficiency and suggest that the slow

improvement in genotypic resistance mentioned earlier may be related to the plant trait used to measure resistance.

Classifying genotypes on the basis of relative chlorophyll concentration (GC) yielded homogeneous class variances; however, differences among class means were not statistically significant (Table 6). The severity of Fe chlorosis in nutrient solution culture was especially harsh and may have limited genotypic expression of resistance to those genotypes having high Fe-efficiency (Jessen, et al., 1988). Although other researchers have concluded that similar QTL for visual chlorosis are identified in nutrient solution and field tests (Lin et al., 2000), other QTL may be identified when integrated measures of resistance, manifest at maturity, are evaluated.

Classifying genotypes on the basis of planting seed Fe content (ng Fe $seed^{-1}$ or ug Fe 1000^{-1} seeds) resulted in homogeneous class variances for each measure of resistance, whereas, differences among class means were significant only for harvest seed Fe content (Table 6; Fig. 10). Measures of planting seed Fe content should provide reliable measures of resistance to Fe deficiency defined as Fe accumulation. Using planting seed [Fe] to classify genotypes resulted in homogeneous class variances for Fe accumulation and harvest seed [Fe], but not for Fe removal. Differences among class means were significant for each measure of resistance. The heterogeneous class variances for Fe removal is a warning that planting seed [Fe] may not provide consistent, reliable estimates of resistance to Fe deficiency defined as Fe removal at harvest. Another interpretation is that Fe removal at harvest may not be an acceptable measure of resistance to Fe deficiency because it involves the primary yield component, seeds m^{-2}. Grain yield is not necessarily a suitable measure of resistance to Fe deficiency, especially where Fe is not yield-limiting, such as on non-IDC prone sites (Helms, et al., 2010). Helms, et al. (2010) also noted that visual chlorosis scores could not identify the highest-yielding genotype even where Fe was yield-limiting.

The severity of Fe deficiency among environments ranged from almost no chlorosis (Fisher, MN, 2003; VCS=1.2) to mild chlorosis (Crookston, MN, 2003; VCS=2.3) to moderate chlorosis (Ada, MN, 2003; VCS=3.0) to severe chlorosis (Crookston, MN, 2004; VCS=4.2). Across this wide range of IDC severity, our results emphasize the difficulty of identifying superior genotypes when visual observations, either VC or V3, are used to classify genotypes, whereas, the importance of seed [Fe] for efficient genotype selection (consistency and reliability) is underscored. Genotypes were also ranked in each environment for three putative measures of resistance to Fe deficiency and for four classification variables thought to represent potential measures of resistance to Fe deficiency. These values were then correlated to determine genotypic rank correlations among environments (Table 7).

When genotypes were ranked using published visual chlorosis scores or in-field visual chlorosis scores, there was little association with measures of resistance to Fe deficiency. In contrast, when genotypes were ranked using planting seed [Fe] or planting seed Fe content, there often was a close association with measures of resistance to Fe deficiency. These generalizations, however, do not include Crookston, 2004 where IDC was especially severe and some genotypes barely survived. Nonetheless, planting seed [Fe] and content are substantially superior to measures of visual chlorosis for identifying consistent, reliable estimates of resistance to Fe deficiency (Fig. 10). The consistency and reliability of using seed [Fe] as a measure of resistance to Fe deficiency in soybean is further illustrated in the article written by Spehar (1994).

Using results from this study of 45 cultivars of soybean grown on partly- and fully-limed acid soils in Brazil, it is possible to calculate a genotypic rank correlation coefficient

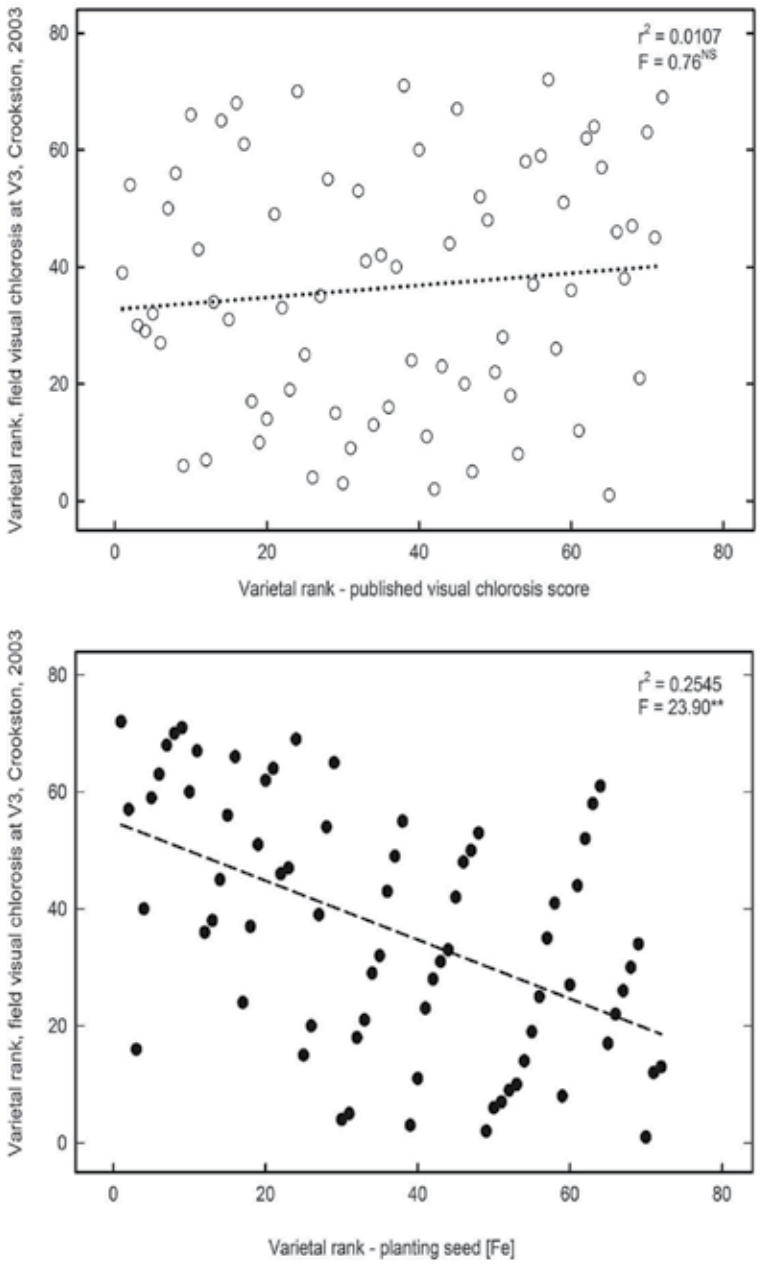


Fig. 9. Regression of varietal rank for visual chlorosis at V3 on varietal rank for published visual chlorosis score (VC) and planting seed [Fe].

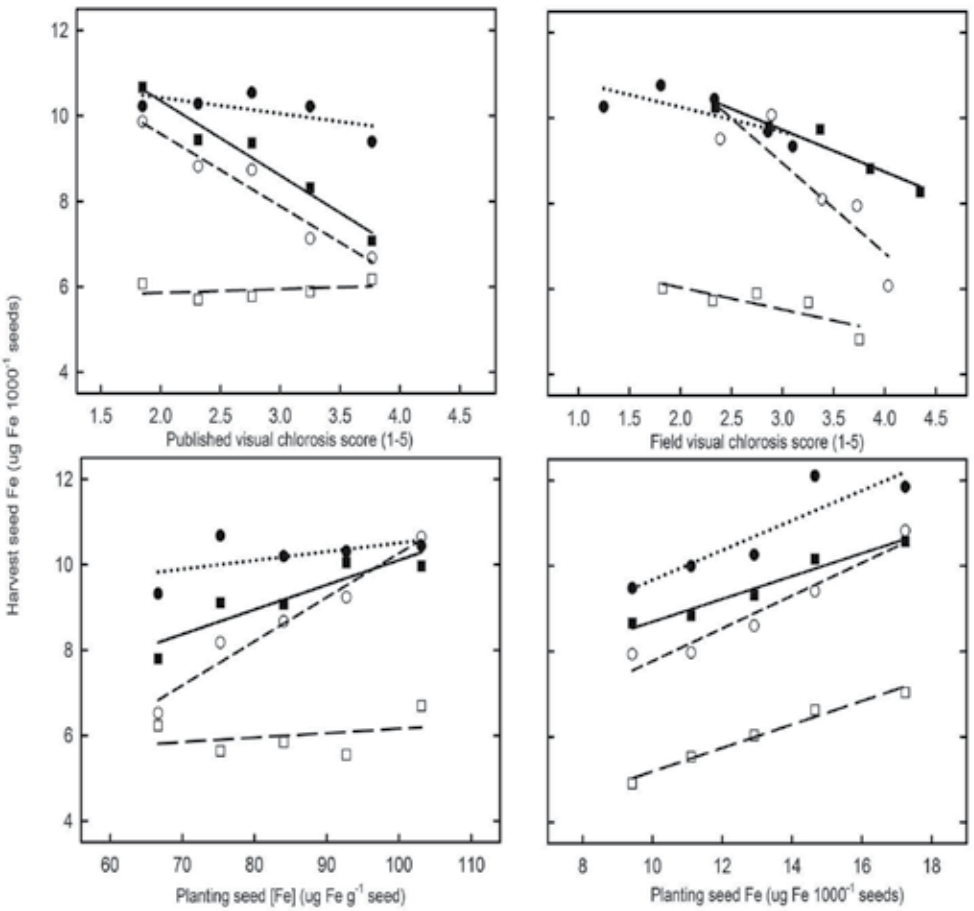


Fig. 10. Relationships between seed Fe content at harvest and four classification variables. Each regression equation is represented by the same symbol and line type for each environment in each panel: filled circle, dotted line is Fisher, MN, 2003; filled square, solid line is Ada, MN, 2003; open circle, short dash line is Crookston, MN, 2003; open square, long dash line is Crookston, MN, 2004.

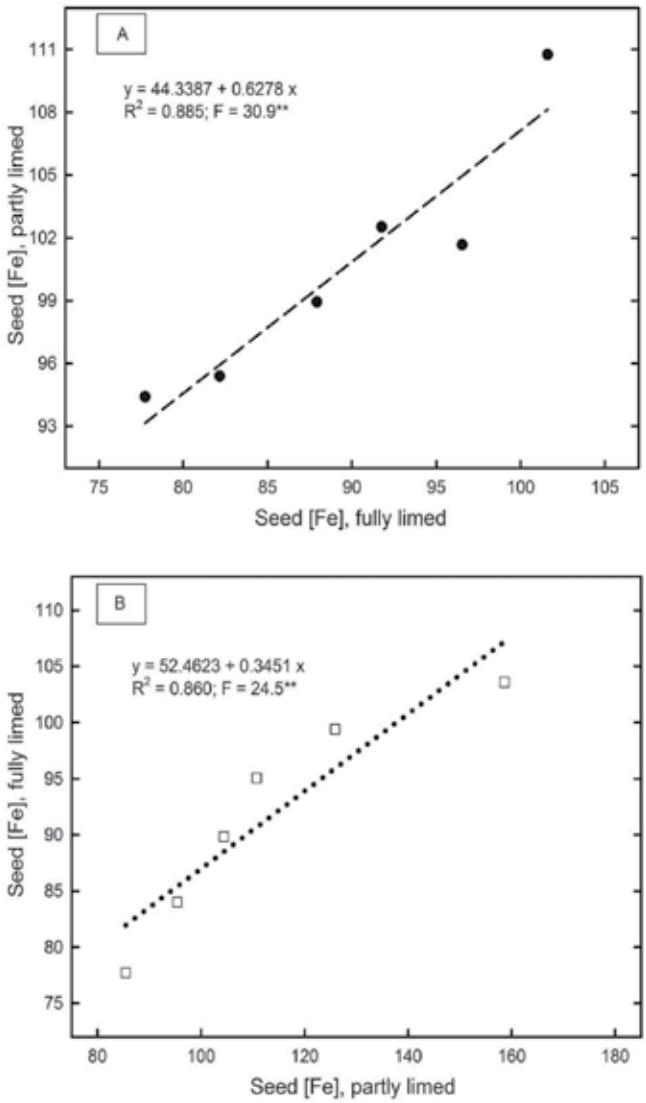


Fig. 11. Seed [Fe] determined from genotypes grown on fully-limed acid soils can be used to predict seed [Fe] of those same genotypes when grown on partly-limed soils (A); similarly, seed [Fe] determined from genotypes grown on partly-limed soils can be used to predict seed [Fe] of those same genotypes when grown on fully-limed soils (B).

Classification variable	Measure of resistance	Mean†	Standard deviation‡	Levene's F§	Welch's F¶
Published visual chlorosis score (VC)	ug Fe 1000^{-1} seeds	VC1=9.2 VC2=8.5 VC3=8.6 VC4=7.9 VC5=7.3	VC1=9.2 VC2=8.5 VC3=8.6 VC4=7.9 VC5=7.3	<1.0^{NS}	2.36^{NS}
	ug Fe g^{-1} seed	VC1=74.7 VC2=70.3 VC3=64.7 VC4=62.0 VC5=59.8	VC1=19.3 VC2=20.2 VC3=17.5 VC4=14.9 VC5=13.2	5.01**	3.66*
	Fe removal (ug Fe m^{-2})	VC1=10.6 VC2= 9.5 VC3= 8.2 VC4= 6.6 VC5= 6.0	VC1=3.7 VC2=3.7 VC3=2.8 VC4=2.0 VC5=1.4	9.02**	12.68**
Field visual chlorosis (V3)	ug Fe 1000^{-1} seeds	V31=9.9 V32=8.0 V33=8.5 V34=8.2 V35=7.9	V31=1.8 V32=2.5 V33=2.4 V34=1.8 V35=2.3	3.16*	7.50**
	ug Fe g^{-1} seed	V31=82.8 V32=62.7 V33=66.6 V34=63.9 V35=63.6	V31=13.4 V32=20.5 V33=20.1 V34=14.3 V35=13.6	18.42**	14.71**
	Fe removal (ug Fe m^{-2})	V31=5.6 V32=7.6 V33=9.8 V34=8.4 V35=7.2	V31=0.4 V32=3.3 V33=3.7 V34=2.6 V35=3.3	3.42**	21.87**
Planting seed [Fe] (FC)	ug Fe 1000^{-1} seeds	FC1=7.5 FC2=8.4 FC3=8.4 FC4=8.8 FC5=9.4	FC1=2.2 FC2=2.3 FC3=2.1 FC4=2.4 FC5=2.0	<1.0^{NS}	2.89*
	ug Fe g^{-1} seed	FC1=55.6 FC2=64.5 FC3=69.0 FC4=72.2 FC5=75.8	FC1=14.6 FC2=17.4 FC3=18.2 FC4=19.7 FC5=18.6	1.88^{NS}	7.16**
	Fe removal (ug Fe m^{-2})	FC1=7.0 FC2=7.9 FC3=8.6 FC4=9.5 FC5=10.6	FC1=2.4 FC2=2.8 FC3=3.5 FC4=4.0 FC5=3.5	5.01**	4.24**

Classification variable	Measure of resistance	Mean†	Standard deviation‡	Levene's F§	Welch's F¶
Planting seed Fe content (FS)	ug Fe 1000^{-1} seeds	FS1=7.7 FS2=8.1 FS3=8.6 FS4=9.6 FS5=10.1	FS1=2.0 FS2=2.4 FS3=2.1 FS4=2.6 FS5=2.1	1.59^{NS}	5.63^{**}
	ug Fe g^{-1} seed	FS1=67.6 FS2=64.9 FS3=68.0 FS4=66.3 FS5=71.8	FS1=18.4 FS2=18.4 FS3=19.2 FS4=17.1 FS5=18.8	$<1.0^{NS}$	$<1.0^{NS}$
	Fe removal (ug Fe m^{-2})	FS1=8.4 FS2=8.0 FS3=8.7 FS4=8.3 FS5=10.4	FS1=3.2 FS2=3.2 FS3=3.5 FS4=3.2 FS5=3.3	$<1.0^{NS}$	1.49^{NS}
Relative chlorophyll concentration SPAD (GC)	ug Fe 1000^{-1} seeds	GC1=8.2 GC2=8.0 GC3=9.0 GC4=8.7 GC5=9.9	GC1=2.2 GC2=2.3 GC3=2.5 GC4=2.2 GC5=2.6	$<1.0^{NS}$	$<1.0^{NS}$
	ug Fe g^{-1} seed	GC1=66.3 GC2=64.6 GC3=69.3 GC4=80.6 GC5=80.0	GC1=17.7 GC2=18.3 GC3=17.8 GC4=20.6 GC5=24.8	$<1.0^{NS}$	$<2.45^{NS}$
	Fe removal (ug Fe m^{-2})	GC1=8.4 GC2=8.0 GC3=9.1 GC4=10.9 GC5=11.4	GC1=3.1 GC2=3.1 GC3=3.8 GC4=4.3 GC5=4.7	1.77^{NS}	1.65^{NS}

† Mean of resistance measure for each level of classification.

‡ Standard deviation of resistance measure for each level of classification.

§ Test to assess homogeneity of error variances across levels of classification

¶ Test to assess the equality of classification means.

^{NS}, *, ** Not significant at 5% level of probability, significant at 5% level of probability, and significant at 1% level of probability.

Table 6. Classification of genotypes into 5 levels of selected plant traits thought to represent measures of resistance to Fe deficiency, defined as Fe accumulation (ug Fe 1000^{-1} seeds), harvest seed [Fe] (ug Fe g^{-1} seed), and Fe removal (ug Fe m^{-2}).

between partially- and fully-limed conditions of r=0 .686 ($P<0.001$) using nearly all genotypes. However, some genotypes were missing seed [Fe]s under one treatment or another and 7 cultivars were not included in later calculations. Using 38 genotypes and 6 seed [Fe] classes (5 ppm), seed [Fe] on partly-limed soil could be predicted from seed [Fe] on fully-limed soil: linear, $r^2=0.883$, $F=30.2^{**}$ (Fig. 11, A). Using 38 genotypes and 6 seed [Fe] classes (10 ppm), seed [Fe] on fully-limed soil could be predicted from seed [Fe] on partly-

limed soil: linear, r^2=0.860, F=24.5** (Fig. 11, B). Differences in class sizes (5 vs 10 ppm) are related to the range of seed [Fe] values observed for the independent variable. Nonetheless, the same genotypes usually were included in the same classes whether based on 5 or 10 ppm, which led to nearly identical results.

Presuming that seed [Fe] can be regarded as an integrated measure of resistance to Fe deficiency that is manifest at maturity, then Spehar's data provides additional evidence that individual genotypes have a seed [Fe] "threshold" that is genetically predetermined, yet seldom exceeded, and that seed [Fe] should supplement or replace VCS as a measure of resistance to Fe deficiency.

		Measures of resistance to Fe deficiency		
Environment	Classification variable	Harvest seed [Fe] (ug Fe g^{-1} seed)	Harvest Fe content (ug Fe 1000^{-1} seeds)	Fe removal (ug Fe m^{-2})
Ada, 2003	VC†	-0.2464*	-0.2007^{NS}	-0.0254^{NS}
	V3	-0.0984^{NS}	-0.0137^{NS}	0.1932^{NS}
	FE	0.4382**	-0.2505*	0.3687**
	FS	0.3454**	0.1730^{NS}	0.3646**
Crookston, 2003	VC	-0.2214^{NS}	-0.0554^{NS}	0.0186^{NS}
	V3	-0.3616**	-0.3838**	-0.1832^{NS}
	FE	0.5698**	0.4548**	0.4402**
	FC	0.1701^{NS}	0.4038**	0.2148^{NS}
Fisher, 2003	VC	-0.1722^{NS}	-0.0659^{NS}	.‡
	V3	-0.0999^{NS}	0.0722^{NS}	.
	FE	0.3568**	0.0975^{NS}	.
	FC	0.2468*	-0.0057^{NS}	.
Crookston, 2004	VC	-0.1282^{NS}	0.0320^{NS}	-0.0099^{NS}
	V3	-0.1158^{NS}	-0.0887^{NS}	-0.1048^{NS}
	FE	0.2211 ^{NS}	-0.0298^{NS}	0.0482^{NS}
	FC	0.0044^{NS}	-0.0950^{NS}	-0.0365^{NS}

† VC, V3, FE, and FC are published visual chlorosis score, in-field visual chlorosis score, planting seed [Fe], and planting seed Fe content.

‡ Missing data.

Table 7. Correlations among genotypes, across four environments, ranked on the basis of three measures of resistance to Fe deficiency and genotypes ranked on the basis of four classification variables though to represent potential measures of resistance to Fe deficiency.

4. Conclusions

Conclusions from the research discussed in this chapter are: (1) soybean seed [Fe] and/or seed Fe content provide reliable and consistent measures of genetic differences in resistance to Fe deficiency; (2) seed [Fe] is tightly controlled genetically; (3) it is not likely that

susceptible genotypes will accumulate high seed [Fe] even when excess Fe is available; (4) seed [Fe] or content at harvest, more so than VCS, can also reflect responses to management practices designed to reduce or alleviate Fe deficiency; (5) when soybean is grown on chlorosis-prone soils, increasing seeding density can markedly increase grain yield and seed [Fe] of both susceptible and resistant cultivars, whereas applying higher rates of Fe-EDDHA especially benefits susceptible cultivars; (6) increasing rates of added fertilizer nitrate have little influence on Fe deficiency of resistant cultivars, but severely depress plant height, grain yield and harvest seed [Fe] of susceptible cultivars; (7) genotypic rank correlations of visual chlorosis scores across locations within a year are reasonably consistent and reliable; however, rank correlations across years are not; (8) classifying genotypes using VCS can result in heterogeneous class variances indicating that VCSs may not be appropriate (consistent, reliable) measures of Fe efficiency; (9) measures of genetic resistance to Fe deficiency should include measures of seed [Fe] or content; and (10) the slow improvement in genotypic resistance to Fe deficiency may be related to the plant trait used to measure resistance.

Seed [Fe] is very useful for identifying superior genotypes in management and agronomic performance trials; it also provides a consistent, reliable estimate of resistance to Fe deficiency, thereby enhancing genotype selection.

5. References

Aktas, M., and F. van Egmond. 1979. Effect of nitrate nutrition on iron utilization by an Fe-efficient and an Fe-inefficient soybean cultivar. Plant Soil 51:257-274.

Ambler, J. E. and J. C. Brown. 1974. Iron supply in soybean seedlings. Agron. J. 66:476-478.

Beebe S.A., V. Gonzalez and J. Rengifo. 2000. Research on trace minerals in the common bean. Food Nutr. Bul. 21:387-391.

Beeghly, H.H., and W.R. Fehr. 1989. Indirect effects of recurrent selection for Fe efficiency in soybean. Crop Sci. 29:640-643.

Blair, M.W., C. Astudillo, M.A. Grusak, R. Graham, and S.E. Beebe. 2009. Inheritance of seed iron and zinc concentrations in common bean (*Phaseolus vulgaris* L.). Mol. Breeding 23:197-207.

Blair, M.W., J.I. Medina, C. Astudillo, J. Rengifo, S.E. Beebe, G. Machado, and R. Graham. 2010. QTL for seed iron and zinc concentration and content in a Mesoamerican common bean (*Phaseolus vulgaris* L.) population. Theor. Appl. Genet. 121:1059-1070.

Bouis, H.E., B.M. Chassy, and J.O. Ochanda. 2003. Genetically modified food crops and their contribution to human nutrition and food quality. Trends Food. Sci. Tech. 14:191-209.

Brown, J.C., J.E. Ambler, R.L. Chaney, and C.D. Foy, 1972. Differential responses of plant genotypes to micronutrients. *In* J.J. Mortvedt, P.M. Giordano, and W.L. Lindsay (ed.) Micronutrients in agriculture. Soil Sd. Soc. Am., Madison, WI.

Brown, J.C., R.S. Holmes, and L.0. Tiffin. 1958. Iron chlorosis in soybeans as related to the genotype of rootstaIk. Soil Sci. 86:75-82.

Chaney, R.L., B.A. Coulombe, P.F. Bell, and J. Scott Angle. 1992. Detailed method to screen dicot cultivars for resistance to Fe-chlorosis using FeDTPA and bicarbonate in nutrient solutions. J. Plant Nutr. 15:2063-2083.

Cianzio, S. Rodriguez de, W.R. Fehr, and I.C. Anderson. 1979. Genotypic evaluation for iron deficiency chlorosis in soybeans by visual scores and chlorophyll concentration. Crop Sci. 19:644-646.

Ellsworth, J.W., V.D. Jolley, D.S. Nuland, and A.D. Blaylock. 1998. Use of hydrogen release or a combination of hydrogen release and iron reduction for selecting iron-efficient dry bean and soybean cultivars. J.Plant Nutr. 21:2639-2651.

Fageria, N.K., V.C. Baligar, and R.B. Clark. 2002. Micronutrients in crop production. Adv. Agron. 77:185-268.

Fairbanks, D.J., J.H. Orf, W.P. Inskeep, and P.R. Bloom. 1987. Evaluation of soybean genotypes for iron-deficiency chlorosis in potted calcareous soil. Crop Sci. 27:953-957.

Fairbanks, D.J. 2000. Development of genetic resistance to iron-deficiency chlorosis in soybean. J. Plant Nutr. 23:1903-1913.

Fehr, W.R. 1982. Control of iron-deficiency chlorosis in soybeans by plant breeding. J. Plant Nutr. 5:611-621.

Froehlich, D.M. and W.R. Fehr. 1981. Agronomic performance of soybeans with differing levels of iron deficiency chlorosis on calcareous soil. Crop Sci. 21:438-441.

Ghandilyan, A., D. Vreugdenhil, and M.G.M. Aarts. 2006. Progress in the genetic understanding of plant iron and zinc nutrition. Physiol. Plant. 126:407-417.

Gomez, K. A., and A. A. Gomez. 1984. Statistical procedures for agricultural research. 2nd ed. John Wiley & Sons, New York.

Goos, R.J., and B. Johnson. 2000. A comparison of three methods for reducing iron-deficiencey chlorosis in soybean. Agron. J. 92:1135-1139.

Goos, R.J., and B. Johnson. 2001. Seed treatment, seeding rate, and cultivar effects on iron deficiency chlorosis of soybean. J. Plant Nutr. 24:1255-1268.

Gregorio, G.B., D. Senadhira, H. Htut, and R.D. Graham. 2000. Breeding for trace mineral density in rice. Food Nutr.21:382-386.

Gregorio, G.B. 2002. Progress in breeding for trace minerals in staple crops. J. Nutr. 132:500S-502S

Hansen, N.C., M.A. Schmitt, J.E. Anderson, and J.S. Strock. 2003. Iron deficiency of soybean in the Upper Midwest and associated soil properties. Agron. J. 95:1595-1601.

Heitholt, J.J., J.J. Sloan, C.T. MacKown, and R.I. Cabrera. 2003. Soybean growth on calcareous soil as affected by three iron sources. J. Plant Nutr. 26:935-948.

Helms, T.C., R.A. Scott, W.T. Schapaugh, R.J. Goos, D.W. Franzen, and A.J. Schlegel. 2010. Soybean iron-deficiency chlorosis tolerance and yield decrease on calcareous soils. Agron. J. 102:492-498.

Imsande, J. 1998. Iron, sulfur, and chlorophyll deficiencies: A need for an integrative approach in plant physiology. Physiol. Plant. 103:139-144.

Jessen, J., M.B. Dragonuk, R.W. Hintz, and W.R. Fehr. 1988. Alternative breeding strategies for the improvement of iron efficiency in soybean. J. Plant Nutr. 11:717-726.

Jolley, V.D., K.A. Cook, N.C. Hansen, and W.B. Stevens. 1996. Plant physiological responses for genotypic evaluation of iron efficiency in strategy I and strategy II plants – a review. J. Plant Nutr. 19:1241-1255.

Karkosh, A.E., A.K. Walker, and J.J. Simmons. 1988. Seed treatment for control of iron-deficiency chlorosis of soybean. Crop Sci. 28:369-370.

Kochian, L.V. 2000. Molecular physiology of mineral nutrient acquisition, transport, and utilization. p.1204-1249. *In* B.B. Buchanan, W. Gruissem, R.L. Jones (ed.) Biochemistry and molecular biology of plants. Am. Soc. of Plant Biol., Rockville, MD.

Lin, S.F., J. Baumer, D. Ivers, S. Cianzio, and R. Shoemaker. 2000. Nutrient solution screening of Fe chlorosis resistance in soybean evaluated by molecular characterization. J. Plant Nutr. 23:1915-1928.

Lingenfelser, J.E., W.T. Schapaugh, Jr., J.P. Schmidt, and J J. Higgins. 2005. Comparison of genotype and cultural practices to control iron deficiency chiorosis in soybean. Comm. Soil Sci. Plant Anal. 36:1047-1062.

Littell R.C., G.A. Milliken, W.W. Stroup, R.D. Wolfinger, and O. Schabenberger. 2006. SAS® for Mixed Models, 2nd ed. Cary, NC: SAS Institute Inc.

Markwell, J., and D. Blevins. 1999. The Minolta SPAD-502 leaf chlorophyll meter: An exciting new tool for education in the plant sciences. Am. Biol. Teach. 61:672-676.

Markwell, J., J.C. Osterman, and J.L. Mitchell. 1995. Calibration of the Minolta SPAD-502 leaf chlorophyll meter. Photosynthesis Res. 46:467-472.

Mortvedt, J.J. 1986. Iron sources and management practices for correcting iron chlorosis problems. J. Plant Nutr. 9:961-974.

Naeve, S.L. and G.W. Rehm. 2006. Genotype x environment interactions within iron deficiency chlorosis-tolerant soybean genotypes. Agron. J. 98:808-814.

Nestle, P., H.E. Bouis, J.V. Meenakshi, and W. Pfeiffer. 2006. Biofortification of staple food crops. J. Nutr. 136:1064-1067.

Penas E.J., R.A. Wiese, R.W. Elmore, G.W. Hergert, and R.S. Moomaw. 1990. Soybean chlorosis studies on high pH bottomland soils. Bull. 312. Univ. Nebraska Inst. Agric. Nat. Resour., Lincoln.

Randall, G.W. 1981. Correcting iron chlorosis in soybeans. Soils Fact Sheet 27(revised). Minnesota Agric. Ext. Serv., St. Paul.

Rashid, A., and R.L. Fox. 1992. Evaluating internal zinc requirements of grain crops by seed analysis. Agron. J. 84:469-474.

Rerkasem, B., R.W. Bell, S. Lodkaew, and J.F. Loneragan. 1997. Relationship of seed boron concentration to germination and growth of soybean *(Glycine max).* Nutrient Cycling in Agroecosystems 48:217-223.

Shen, J.F. Zhang, Q. Chen, Z. Rengel, C. Tang, and C. Song. 2002. Genotypic difference in seed iron content and early responses to iron deficiency in wheat. J. Plant Nutr. 25:1631-1643

Snedecor G.W., and W.G. Cochran. 1980. Statistical Methods. 7th ed. Iowa State Univ. Press, Ames.

Spehar, C.R. 1994. Seed quality of soya bean based on mineral composition of seeds of 45 varieties grown in a Brazilian Savanna acid soil. Euphytica 76:127-132.

Tiffin, L.O., and R.L. Chaney. 1973. Translocation of iron from soybean cotyledons. Plant Physiol. 52:393-396.

Uvalle-Bueno, J.X., and M.A. Romero B. 1988. Chlorosis of soybean [*Glycine max* (L.) Merr.] and its relation with the content of chlorophyll, phosphorus, phosphorus/iron and pH vegetative tissues. J. Plant Nutr. 11:797-807.

Weir, R.G., and A. Hudson. 1966. Molybdenum deficiency in maize in relation to seed reserves. Aust. J. Exp. Agric. Anim. Husb. 6:35-41.

Weiss, M.C. 1943. Inheritance and physiology of efficiency in iron utilization in soybeans. Genetics 28:253-268.

WHO. World Health Report 2002. Geneva, Switzerland:World Health Organization; 2002. Reducing Risks, Promoting Healthy Life.

Wiersma, J.V. 2005. High rates of Fe-EDDHA and seed iron concentration suggest partial solutions to iron deficiency in soybean. Agron. J. 97:924-934.

Wiersma, J.V. 2007. Iron acquisition of three soybean varieties grown at five seeding densities and five rates of Fe-EDDHA. Agron. J. 99:1018-1028.

Wiersma, J.V. 2010. Nitrate-induced iron deficiency in soybean varieties with varying iron-stress responses. Agron. J. 102:1-7.

3

Positional Cloning of the Responsible Genes for Maturity Loci *E1, E2* and *E3* in Soybean

Kyuya Harada[1], Satoshi Watanabe[1], Xia Zhengjun[2],
Yasutaka Tsubokura[1], Naoki Yamanaka[3] and Toyoaki Anai[4]
[1]*National Institute of Agrobiological Sciences, Tsukuba,*
[2]*Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin,*
[3]*Japan International Research Center for Agricultural Sciences, Tsukuba,*
[4]*Faculty of Agriculture, Saga University, Saga*
[1,3,4]*Japan*
[2]*China*

1. Introduction

The change from vegetative to reproductive growth is a critical developmental transition in the life of plants. Various external cues, such as photoperiod and temperature, are known to initiate plant flowering under the appropriate seasonal conditions. Endogenous cues include a system of juvenile to adult transition that affects competence to flower. To understand the molecular mechanism of flowering, extensive studies have been performed using model plants, *Arabidopsis thaliana* and rice (*Oryza sativa*), and these have revealed the numerous regulatory network components associated with flowering (Jung & Muller, 2009; Amasino, 2010). The general concept of the photoperiodic induction of flowering (photoperiodism) and the range of response types among plant species was established by Garner and Allard (1920). Among the external cues, light is the most important, being received by several photoreceptors including phytochromes, cryptochromes and phototropins. The role of phytochromes, that is the R-light- and FR-light- absorbing photoreceptors, in flowering has been investigated in several plant species. In *Arabidopsis*, a quantitative long-day (LD) plant, a phyA mutant flowered later in either long-day or short-day (SD) conditions with a night break (Johnson et al., 1994; Reed et al., 1994). In rice, a SD plant, the phyA monogenic mutant exhibited the same flowering time as the wild type under LD conditions, while, in the phyB and phyC mutant backgrounds, the flowering was greatly accelerated relative to phyB and phyC monogenic mutants (Takano et al., 2005). In pea, a LD plant, loss- or gain-of-function phyA mutants displayed late or early flowering phenotypes, respectively (Weller et al., 1997, 2001). Day length is found to be perceived by leaves by Knott (1934). Because flowering occurs in the shoot apical meristem (SAM), the leaves must transmit a signal to the SAM and this signal is referred to as florigen (Chailakhyan, 1936). In *Arabidopsis*, three genes, *CONSTANS* (*CO*), *GIGANTEA* (*GI*) and *FLOWERING LOCUS T* (*FT*) were found to be involved in the production of a flowering promoter in LD conditions (Koornneef et al., 1991; Kardailsky et al., 1999). FT protein is now known to be florigen, and CO and GI are key players in the activation of FT expression. CO is a zinc-finger protein that

functions as a transcription factor (Putterill et al., 1995), and GI is a large protein involved in circadian clock function (Fowler et al., 1999; Park et al., 1999). FT is a small protein with some resemblance to RAF kinase inhibitors (Kardailsky et al., 1999; Kobayashi et al., 1999) that is produced in leaves and moves to the SAM (Corbesier et al., 2007; Jaeger & Wigge, 2007; Mathieu et al., 2007; Tamaki et al., 2007; Notaguchi et al., 2008). The rice orthologs of *Arabidopsis CO* and *FT* genes, *Heading date 1* (*HD1*) and *Heading date 3a* (*Hd3a*), respectively, have been identified (Yano et al., 2000; Kojima et al., 2002; Hayama et al., 2003). The promotion of flowering in *Arabidopsis* in LD conditions results from activation of *FT* by *CO*, while the delay in flowering in rice in LD conditions results from repression of *Hd3a* by *Hd1* (Izawa et al., 2000; Kojima et al., 2002; Roden et al., 2002; Hayama et al., 2003). A *CO*/*FT* module is likely to be conserved throughout the plant kingdom. CYCLING DOF FACTORS (CDFs) exhibit circadian cycling and bind to *CO* promoter and repress *CO* expression. The abundance of CDFs is controlled by FLAVIN-BINDING, KELCH REPEAT, F-BOX PROTEIN1 (FKF1) that appears to be involved in the ubiquitin-mediated degradation of CDFs. GI protein physically interacts with FKF1 and stabilizes it promoting CDF degradation and subsequent *CO* expression (Imaizumi et al., 2005.; Sawa et al., 2007; Fornara and Coupland, 2009; Imaizumi, 2009). Despite the conserved functions of *FT* orthologs, their expression may be controlled by different systems in different species. Non-*CO*/*FT* pathways have been proposed for several plants, such as morning glory (*Pharbitis nil*) (Hayama et al., 2007) and tomato (Ben-Naim et al., 2006; Lifschitz et al., 2006). In rice, *Early heading date 1* (*Ehd1*) has been found to promote flowering by inducing *FT*-like gene expression only under SD conditions independently of *Hd1* (Doi et al., 2004). There is no *Ehd1* ortholog in *Arabidopsis*.

Soybean is a typical SD plant whose photoperiodic sensitivity was discovered by Garner and Allard in 1920. Compared to the model plants, photoperiodic control of flowering in soybean is far less understood. The eight loci, *E1* to *E8*, conditioning flowering has been genetically identified (Bernard, 1971; Buzzel, 1971; Buzzel and Voldeng, 1980; McBlain and Bernard, 1987; Bonato and Vello, 1999; Cober and Voldeng, 2001; Cober et al., 2010). At each of these loci, two alleles have been identified, and except for *E6*, the recessive alleles at the *E* loci condition early flowering under both LD and SD conditions. The partially dominant alleles at the *E* loci delay flowering under LD conditions. Near-isogenic lines (NILs) for *E* loci have been developed and used for studies to elucidate the flowering in soybean (Saidon et al., 1989a,b; Upadhyay et al., 1994a,b; Cober et al., 1996a). Among these *E* loci, *E1*, *E3*, *E4* and *E7* are known to be involved in the response to the phtoperiod (Buzzell, 1971; Buzzell and Voldeng, 1980; McBlain et al., 1987; Cober et al., 1996b; Cober and Voldeng, 2001; Abe et al., 2003). The *E3* locus was first identified with the use of fluorescent lamps to extend day length. The *e3e3* recessive homozygote can initiate flowering under LD conditions where the day length was extended to 20 hr using fluorescent lamps (FLD) with a high red to far-red (R: FR) ratio (Buzzell, 1971). The *E4* locus was identified by extending the natural day length to 20 hr with incandescent lamps with a low R: FR ratio (Buzzell and Voldeng, 1980). The insensitivity of *e4e4* genotype to LD conditions with a low R: FR ratio is necessary of *e3e3* background (Buzzell and Voldeng, 1980; Saindon et al., 1989b; Cober et al. 1996b). The *E1* and *E7* loci are involved in the control of insensitivity to artificially induced LD conditions in the *e3* and *e4* backgrounds (Cober et al., 1996b; Cober and Voldeng 2001). Of the known *E* loci, the *E1* locus is considered to have the largest effect on time to flowering under field conditions (Stewart et al., 2003).

Flowering time is a very important trait which is related to productivity, adaptability and domestication. Soybean breeders have attempted to modify flowering and maturity to expand growing areas for soybean. Molecular identification of *E* loci and flowering network of soybean is useful for efficient breeding to control adaptability and increase yield of soybean. We have identified flowering-time quantitative loci (QTL), *FT1*, *FT2* and *FT3*, and found to correspond to *E1*, *E2* and *E3*, respectively (Yamanaka et al., 2001). We successfully identified the responsible genes for the *E1* (Xia et al., unpublished), *E2* (Watanabe et al., 2009) and *E3* (Watanabe et al., in press) by positional cloning strategy. In this chapter, we will describe the process of identification of responsible genes for the *E1*, *E2* and *E3* loci with variation of alleles and propose a tentative major flowering time pathway in soybean.

2. Strategy for fine mapping and positional cloning

As flowering time is a quantitative trait, we employed QTL analysis (Tanksley, 1993) to dissect the genetic factors for flowering time into individual components by using recombinant inbred lines (RIL) derived from Misuzudaizu, a Japanese variety, and Moshidou Gong 503, a weedy line from China. To identify the underlying molecular basis for each QTL, map-based cloning method was performed because molecular or biochemical information for soybean flowering was very few or totally not available. Although NILs are usually used for fine mapping of each QTL, developing NILs is time-consuming and laborious process especially in soybean. Alternatively, we have proposed fine mapping using residual heterozygous lines (RHLs) (Yamanaka et al., 2005). An RHL selected from an RIL population harbors a heterozygous region where the target QTL is located but contains a homozygous background for most other regions of the genome. The progenies of the RHL are expected to show a simple phenotypic segregation based on the effects of the target QTL at the heterozygous region (Fig. 1). A similar term, heterogeneous inbred family (HIF), was used by Tuinstra et al. (1997) to identify the QTL associated with seed weight in sorghum. The RHL strategy has already been used to identify loci underlying pathogen resistance in soybean (Njiti et al., 1998; Meksem et al., 1999; Triwitayakorn et al., 2005). Genotypes of a trait in recombinants identified in the progenies of RHL, could be determined in the next generation.

The probability of discovering RHLs for a target QTL depends on the heterozygosity ratio in a population and the size of the population. If p is the ratio of hetrozygosity of any population with size n, then the probability of detecting k individuals with a heterozygous genotype is supposed as ${}_nC_k\ p^k\ (1-p)^{n-k}$ based on a binomial distribution. In the case of an F_7 generation of RILs, the ratio of heterozygosity (p) is 0.0156 and with a population size of 200 (n), the probability of detecting at least one RHL is more than 0.95. We propose that QTL analysis using the F_6-F_8 RIL population in combination with the RHL strategy is useful for dissecting genetic factors for an agronomic trait into each QTL where the homozygous ratio is sufficiently high to evaluate traits with replication and the heterozygosity ratio is not so low and will allow the identification of a sufficient number of RHLs.

In progenies of an RHL, we can identify NILs for the target QTL. New DNA markers in the heterozygous region were developed using NILs, bulked segregant analysis (BSA) in progenies of the RHL, and sequences of bacterial artificial chromosome (BAC) clones covering the target QTL. We usually developed amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and sequence characterized amplified region (SCAR) markers. Genetic analyses of flowering phenotypes and DNA markers were performed in the

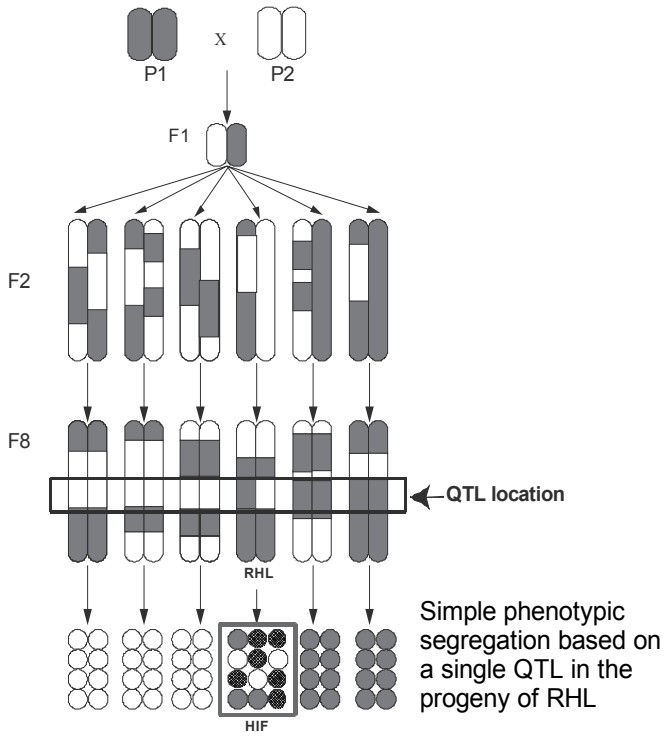


Fig. 1. A schematic representation of RHL. An RHL harbors a heterozygous region where the target QTL is located but contains a homozygous background for most other regions of the genome. Meshed circles show heterozygous individuals.

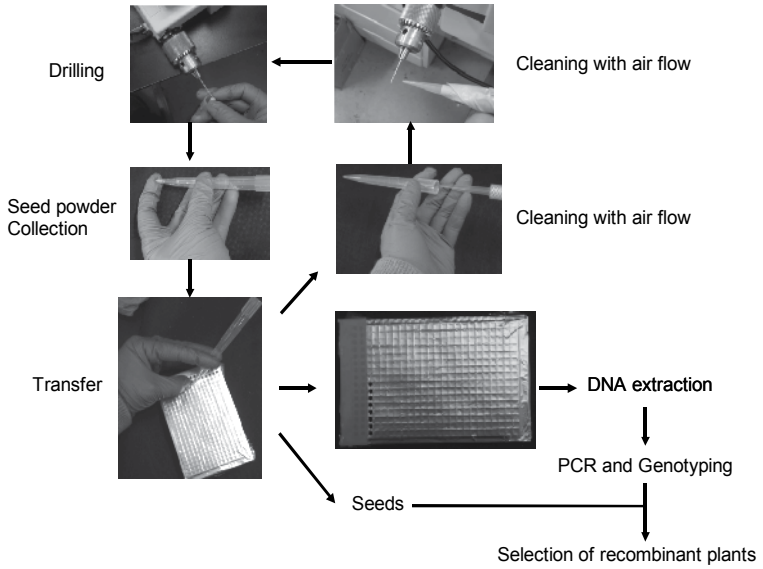


Fig. 2. A procedure for seed genotyping.

progenies of RHLs with a large population. Recombinants of DNA markers were identified in the population and the genotypes of flowering time of recombinants were confirmed by progeny test. The cosegragated region of DNA markers with genotypes of flowering time, and BAC contig covering the region were identified. Sequencing of BAC clones covering the target region and annotation of sequences were performed. Confirmation of a candidate gene was carried out by association of phenotypes and sequence polymorphism of several alleles and gene disruption by induced mutation.

Population size of progenies of RHL for fine mapping depends on recombination frequency, that is, the position of a QTL. We usually used about 1,000 individuals but more than 10,000 plants are necessary when the target locus is located in the peri-centromeric or centromeric region. For high throughput genotyping, the cotyledon flour was obtained by drilling a hole on the surface of seed without any damage to the embryonic axis (Fig. 2). The initially drilled material was discarded to eliminate any possible contamination from the seed coat. Collected materials were transferred into wells in 384-well plate. The drill and tube were cleaned by air flow.

3. Positional cloning of the responsible genes for the *E1, E2* and *E3* loci

A population of 156 RILs ($F_{8:10}$) derived from a cross between Misuzudaizu and Moshidou Gong 503 was used for QTL analysis of flowering. Three QTLs for flowering time, *FT1*, *FT2* and *FT3* were identified at LG C2 (Chr. 6), LG O (Chr. 10) and LG L (Chr. 19), respectively (Fig. 3). The *FT1*, *FT2* and *FT3* were considered to correspond to *E1*, *E2*, *E3*, respectively, based on their map positions (Yamanaka et al., 2001; Watanabe et al., 2004). The late-flowering alleles *FT1*, *FT2* and *FT3* are partially dominant over the early-flowering alleles, *ft1*, *ft2* and *ft3*, respectively. Misuzudaizu harbored the late-flowering allele of the *FT1* and *FT3* loci, whereas Moshidou Gong 503 carried the late-flowering alleles of the *FT2* locus.

3.1 Positional cloning of the responsible gene for the *E2* locus

The line RIL6-8 was found to be heterozygous for the *FT2* locus and was designated as RHL6-8 (Fig. 4). DNA marker analysis showed that RHL6-8 harbored a heterozygous region covering approximately 10 cM including the *FT2* locus. The RHL6-8 generated NILs6-8-*FT2* and –*ft2* among its progenies. Using BSA, a polymorphic AFLP marker, E7M19, was detected between the early-flowering bulk and late-flowering bulk derived from the progeny of RHL6-8. This marker was located close to the LOD peak position of the QTL assigned *FT2* (Fig. 5). We developed additional DNA markers tightly linked to the *FT2* locus using NILs6-8. Among the products amplified from all possible 4,096 primer pair combinations, only five polymorphic bands showed constant polymorphism between the contrasting genotypes of *FT2*/*FT2* and *ft2*/*ft2* in NILs6-8. These polymorphic bands were excised from the gel, sequenced and converted to SCAR markers. Three SCAR markers, originating from five AFLP bands, were developed and used for screening of 10 BAC clones from two independent BAC libraries. A contig covering the *FT2* region was constructed based on the results of PCR analysis using the BAC end sequences. Five of the 10 BAC clones were then subjected to shotgun sequence analysis. Each BAC clone was separately analyzed and assembled, and the sequence information then combined using overlapping sequences. The total length covered by the five clones was approximately 430 Kb. A total of three DNA markers, including one AFLP-derived marker (marker 2) and two PCR-based markers developed from BAC sequences (markers 1 and 3), were used in the fine mapping to minutely restrict the *FT2* locus (Table 1). The positions of these markers are shown in Fig. 6.

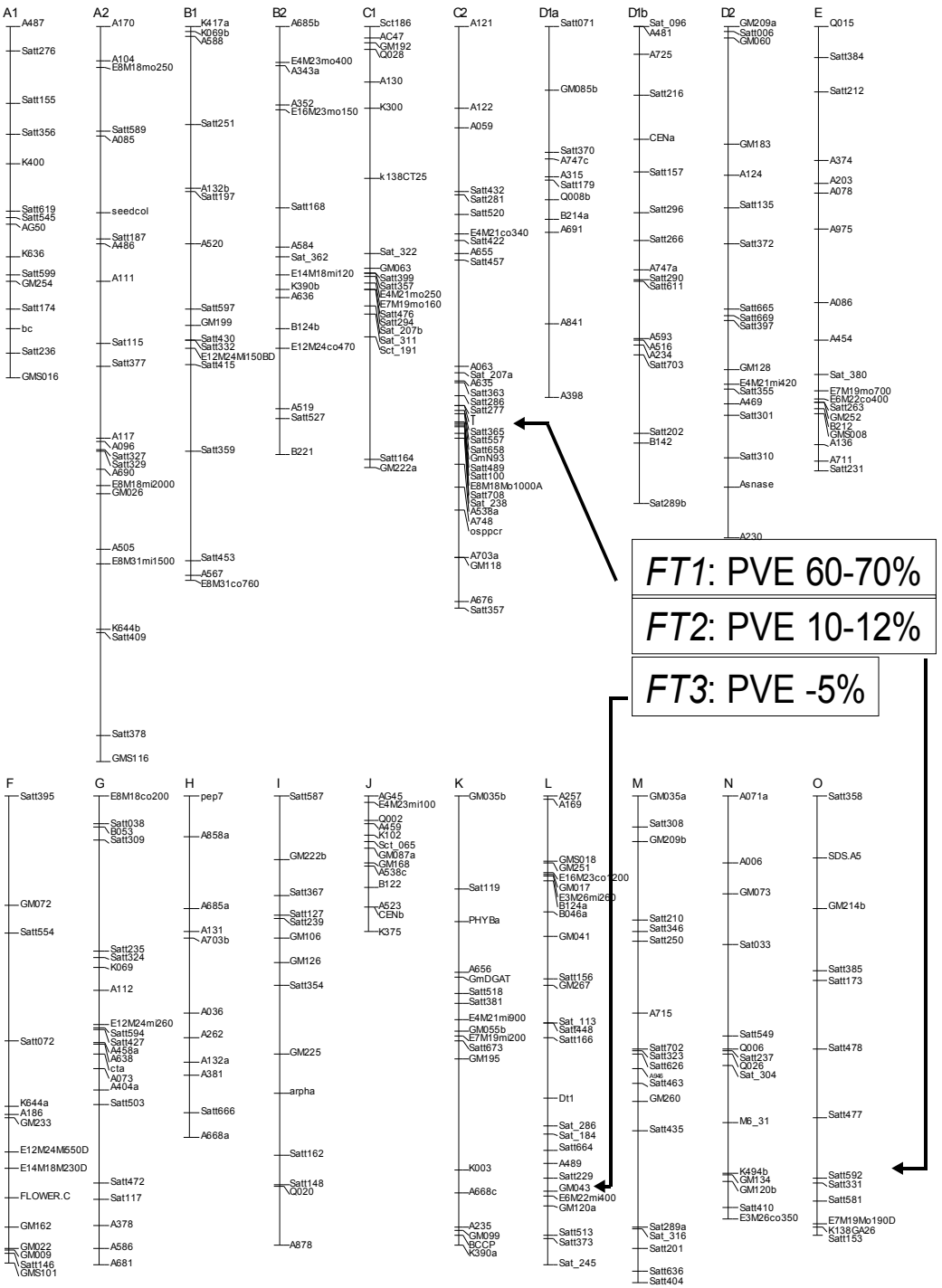


Fig. 3. QTLs for flowering time identified in the RIL population. PVE: phenotypic variance explained by each QTL.

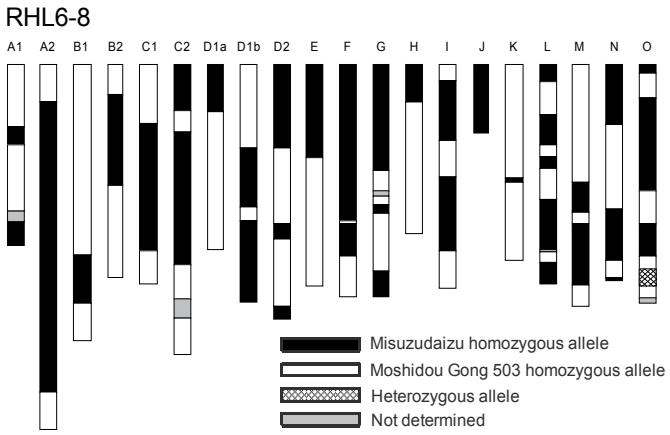


Fig. 4. Graphical genotype of RHL6-8. Solid bars, open bars and meshed bar indicate Misuzudaizu homozygous, Moshidou Gong homozygous genotypes and heterozygous genotype, respectively.

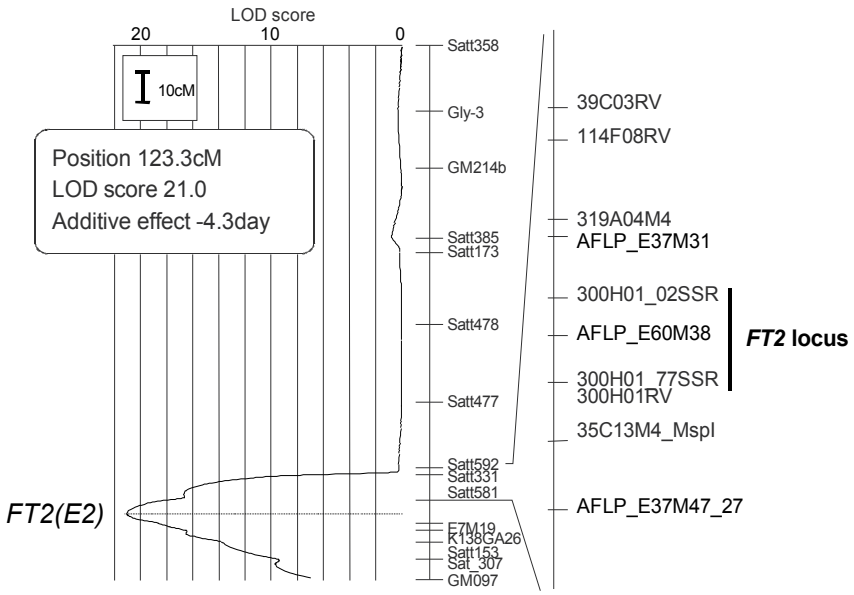


Fig. 5. QTL analysis for the *FT2* locus in the RIL population. The LOD scores for the *FT2* locus calculated by composite interval mapping and displayed in the left panel. DNA markers closely linked to the *FT2* locus are shown in the right panel.

A population consisting of 888 plants, derived from several RHL6-8 plants, was used for fine mapping of the *FT2* locus. Recombination between in this region was found in 21 plants among 843 plants. The remaining 45 individuals were omitted from the analysis because of missing data for phenotypes or genotypes. The number of *FT2* homozygous late-flowering genotypes (n=213), heterozygous (n=420), and *ft2* homozygous early-flowering genotypes (n=210) fitted well with a 1: 2: 1 segregation ratio. The additive effect and dominant effect of

this QTL were estimated to be -5.17 days and 0.57 days, respectively. The ratio of genetic variance explained by the *FT2* locus accounted for 87.9 % of the total variance, indicating that the variation observed in this population was largely controlled by the single QTL effect. The genotypes of the selected 3 markers and flowering genotypes confirmed by progeny test are shown in Fig. 6. The genotypes of marker 2 cosegregated with flowering genotypes indicating that the QTL was close to this marker. Among the recombinants, line 6-8_501 rec had a recombination point between marker 1 and marker 2. Another lines, 6-8_452rec_A, 528rec_B and 6-8_120 rec, generated a recombination between marker 2 and marker 3. Marker 1 and 3 originated from the end sequences of a BAC clone MiB300H01. Considering the recombination points in each line and their flowering genotypes, this indicated that the *FT2* locus was restricted to the single BAC clone, MiB300H01. To identify the responsible gene for this QTL, the nucleotide sequence of this BAC clone was determined.

Marker name	Type of marker	Clone name	Direction	Sequence (5'-3')	Glyma1.0 (Gm10) [b]
Marker 1	BAC end	GMJMiB300H01RV	Fw	CATAGCCGACCTTCTCCAAA	44,787,669
			Rv	AGCCCAATATGGCAGCATAC	44,787,287
Marker 2 [a]	AFLP(SCAR)	E60M38	Fw	CAGTGTTCGCCAGGCTTAGT	44,726,500
			Rv	GCTTGGGTAAACATCCCAAA	44,726,011
Marker 3	BAC end	GMJMiB300H01fw	Fw	GAGAGCAGGGTTATTGGATGA	44,696,157
			Rv	GCCACTGTGCCACATTACAC	44,696,810

a) The digestion with the restriction enzyme *Eco*RI was needed to detect polymorphism.

b) Physical position at Gm10 in Glyma1.0 (http://www.phytozome.net/).

Table 1. List of DNA markers used for fine mapping of the *FT2* locus.

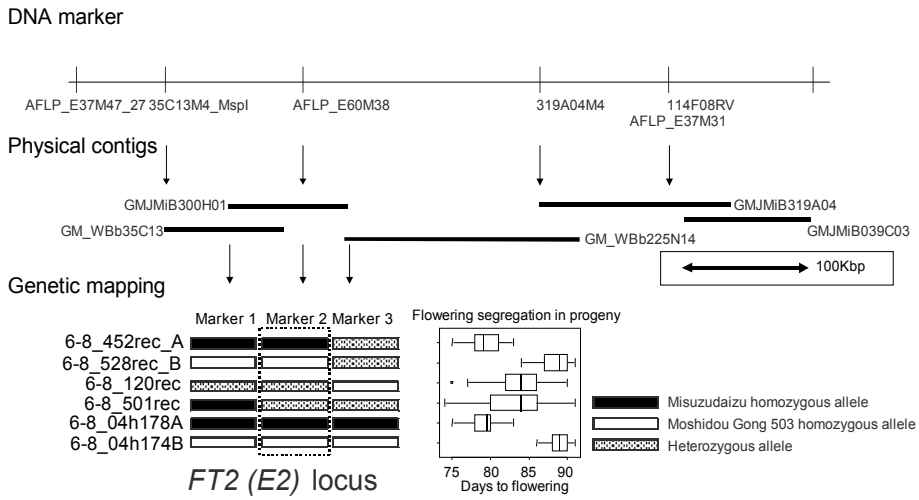


Fig. 6. Fine mapping of the *FT2* locus. The genotypes of each DNA marker of recombinants are shown in the left panel and segregation of flowering in the progenies is displayed in the right panel at the bottom of the figure. The interquatile region, median, and range are indicated by a box, vertical line, and horizontal line, respectively.

In the 94 Kb sequence of MiB300H01, nine annotated genes were predicted. One of these genes, Glyma 10g36600 (assigned in phytozome ver. Glyma 1.0 http://www.phytozome.net/), with a high level of similarity to *GIGANTEA* (*GI*) gene, was

considered a strong candidate for the *FT2* locus. We isolated the complete predicted coding region using an RNA sample extracted from leaves of NILs6-8-*FT2*. We refer to this gene as *GmGIa*, since another *GI* gene, *GmGIb*, was also obtained from the same RNA sample. The coding sequence of *GmGIa*-Mo from Moshidou Gong 503 was extended to a 20Kb genomic region and contained 14 exons (Fig. 7A). Marker 2, which cosegragated with the *FT2* genotypes and originated from the AFLP marker, E60M38, was located in the 5th intron (Fig. 7). Compared to *GmGIa*-Mo, the Misuzudaizu early flowering allele, *GmGIa*-Mi, showed four single nucleotide polymorphisms (SNPs) in its coding sequence. One of these SNPs, detected in the 10th exon, introduced a premature stop codon mutation that led to a truncated 521 amino acids GI protein in the *GmGIa*-Mi allele (Fig. 7B). This stop codon mutation was considered a candidate for a functional nucleotide polymorphism in *GmGIa*. A derived amplified polymorphic sequence (dCAPs) marker was developed to examine the identity of this stop codon mutation in other NILs originating from Harosoy (*e2*/*e2*). The genotypes of all NILs tested coincided well with the genotype of this diagnostic dCAPs marker. This result indicated that the responsible gene for the *FT2* and *E2* loci was identical to each other, and that a conserved mutation might have caused the early flowering phenotype in the recessive alleles. To validate the significance of the mutation in the *GmGIa*, we screened a mutant line from X-ray irradiated and ethyl methanesulfonate (EMS) treated libraries by targeting-induced local lesions in genomes (TILLING) (McCallum et al., 2000). The sequence of *GmGIa* in the wild type Bay cultivar was completely identical to that of the *E2* allele. One mutant line harboring a deletion in the 10th exon that caused a truncated protein (735 amino acids) (Fig. 7B) showed a significant earlier (8days) flowering phenotype than the wild type under natural day-length conditions. These results indicate that *GmGIa* is the gene responsible for the *E2* locus.

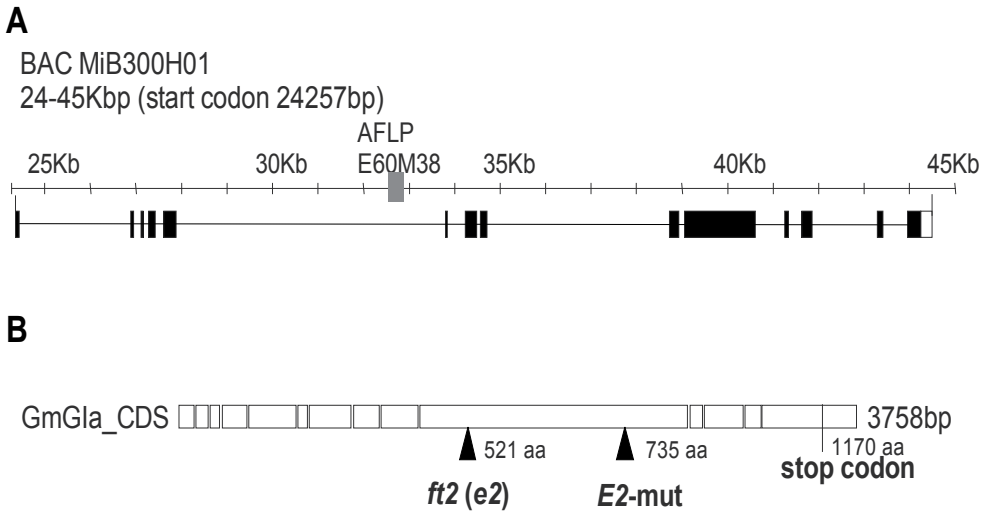


Fig. 7. Variation of gene structure of *GmGIa*. A: Exons, a part of the 3'UTR, and introns of the *GmGIa* gene in the 24-45 Kb region of MiB3300H01 are indicated by bold boxes, open boxes and lines, respectively. The location of marker 2, originating from AFLP marker E60M38, is presented in the 5th intron by the gray box. B: The truncated sites of amino acid sequences in *ft2* (*e2*) and the mutant allele (*E2*-mut) are indicated by the solid triangles.

3.2 Identification and variation analysis of the responsible gene for the *E3* locus

The line RIL1-146 was found to be heterozygous for the *FT3* locus. One other line, RIL6-22, showed segregation for growth habit. This trait is controlled by the *Dt1* locus and is linked to the *FT3* locus at a distance of about 25 cM. The segregating region of RIL6-22 included both the *Dt1* and the *FT3* loci. A single plant with a genotype of *dt1dt1 FT1FT1 ft2ft2 FT3ft3* was selected from RIL1-146, and 5plants with a genotype of *Dt1dt1 ft1ft1 ft2ft2 FT3ft3* were selected from RIL6-22 and designated as RHL1-146 and RHL6-22, respectively. From both progenies of these RHLs, two NILs, 1-146-*FT3* and –*ft3*, and 6-22-*FT3* and –*ft3* were selected. Using BSA analysis, a polymorphic AFLP marker, E6M22, was detected between the early-flowering bulk and the late-flowering bulk derived from the progeny of RIL1-146. This marker was located at the LOD peak position of the *FT3* (Fig. 8).

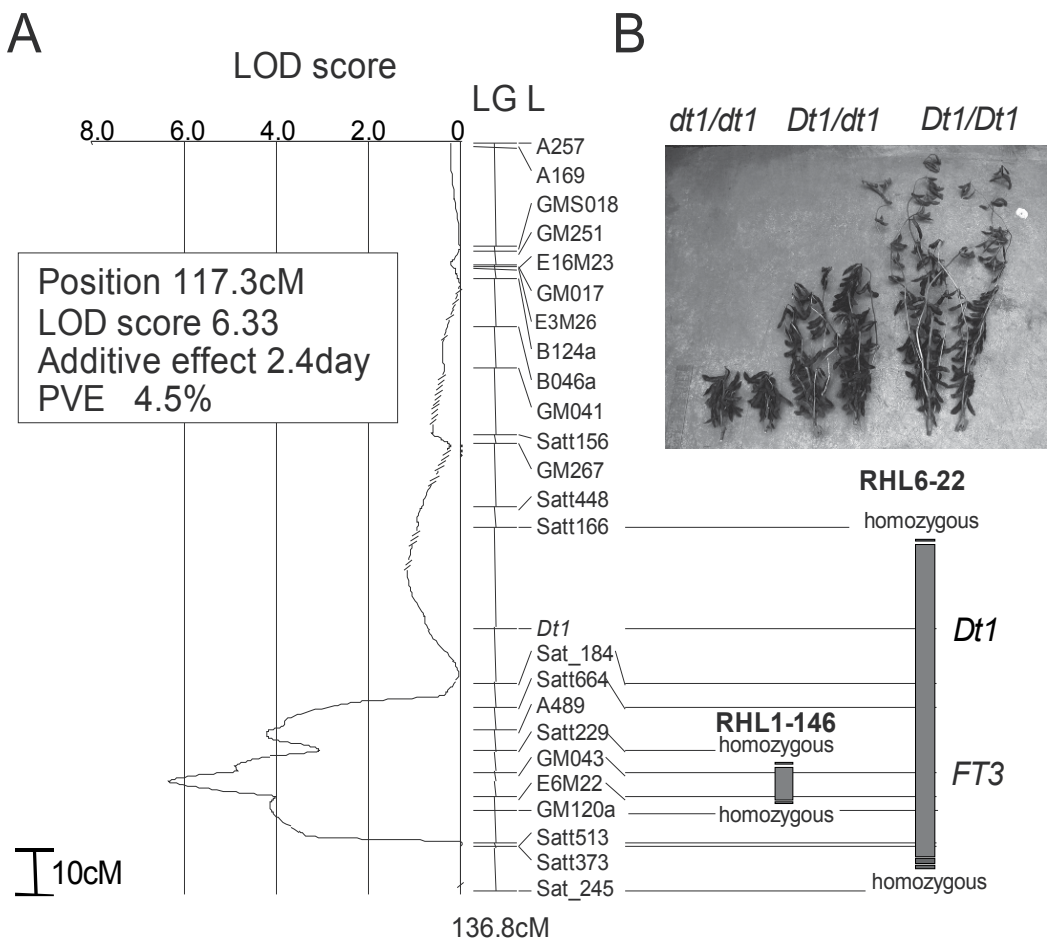


Fig. 8. LOD scores for the *FT3* locus and heterozygous regions of RHLs. The location of the *FT3* locus and the segregating regions of two RHLs, 6-22 and 1-146 are shown. Solid line indicates the LOD scores calculated by composite interval mapping for the QTL (A). Shaded bars indicate the heterozygous regions of two RHLs (B).

As a result of marker analysis, the heterozygous region in RHL1-146 extended for about 5 cM including the *FT3* locus. In contrast, the heterozygous region in RHL6-22 extended for about 40 cM including the *FT3* and *Dt1* loci. Two groups of NILs, NILs1-146 and NILs6-22, were used to develop the AFLP markers tightly linked to the *FT3* locus. Of all possible 4096 primer pairs, only six fragments showed constant polymorphism between the genotypes of *FT3*/*FT3* and *ft3*/*ft3* in NILs1-146 and NILs6-22. These polymorphic bands were excised from the gel, then sequenced, and converted to codominant SCAR markers. Several BAC and transformation-competent bacterial artificial chromosome (TAC) clones were screened using the SCAR markers. The nucleotide sequences of a BAC clone, GMJMiB242F01, and a TAC clone, GM_TMiH_H17D12, were determined. These BAC/TAC sequences were used to develop new PCR-based markers. A total of six DNA markers, including three AFLP-derived markers (markers 1, 3, and 6) and three PCR-based markers developed from the BAC/TAC sequences (markers 2, 4, and 5) were used for fine mapping of the *FT3* locus (Table 2).

A population of 897 plants derived from seven RHL1-146 plants was used for precise mapping of the *FT3* locus. No recombination between these markers was found in 883 plants. The numbers of *FT3* homozygous late-flowering genotype (n=208) and heterozygous (n=441) and *ft3* homozygous early flowering genotypes (n=234) fitted a 1: 2: 1 segregation ratio. These results suggested the presence of a single QTL for flowering time within a small heterozygous region in RHL1-146. The additive effect and the dominant effect of this QTL were estimated to be 3.0 and 0.98 days, respectively. The ratio of genetic variance explained by the *FT3* locus accounted for 70.7 % of the total variance. On the other hand, 14 plants showed recombination between these markers (Fig. 9) and the recombination points were determined by the genotype of markers 2-5. The *FT3* genotypes in each recombinant completely coincided with the genotypes of marker 3 that originated from the closest AFLP marker E6M22 to the LOD peak position (Fig. 8). Moreover, recombination points occurred on both sides of marker 3 and corresponded to both sides of the TAC clone, GM_TMiH_H17D12. These results suggested that the gene responsible for the *FT3* locus was restricted to the physical region covered by GM_TMiH_H17D12 (Fig. 9).

Name of marker	Origin of marker [a]	Direction	Primer sequence (5'-3')	Glyma1.0 (Gm19) [e]
Marker1	AFLP	FW	GAATGTGCTTGTGTTTGTGTC	47,172,164
	(SCAR_E54/56M59)	RV	TAAAGAACCCAGATACAGTCC	47,171,779
Marker2 [b]	TAC_end sequence	FW	CACACAGAAAGCCACAGCAT	47,441,801
	(GM_TMiH_H17D12-RV)	RV	GCTGATCCTTGTGCTGATGA	47,442,133
Marker3 [c]	AFLP	FW	TGGGTCTTCAGTTCAGTTGG	47,516,419
	(SCAR_E6M22)	RV1	TGCTTCCTTTCACTTTCTGATG	47,519,757
		RV2	AGGAAAGGTGGAAGGCGTAT	47,518,322
Marker4 [d]	BAC internal sequence	FW	AATTGAGCTCAGGGAACAGC	47,536,043
	(GMJMiB242F01)	RV	GGATAGAGATGGCGAAGCAA	47,536,374
Marker5	BAC_end sequence	FW	GCCATGGAAGAGAGGAAGATT	47,549,930
	(GMJMiB 242F01-M4)	RV	CACCGTCGTTTTCCTTCTTC	Not found with BLAST search
Marker6	AFLP	FW	CAGTAAAATTGATGGGTGCC	47,672,769
	(SCAR_E30M47)	RV	CTGATACACCCAAAGGAAAC	47,672,997

a) Code of AFLP markers and the clone name are indicated in parentheses.

b) Digestion using the restriction enzyme *Mse*I was applied to detect polymorphism.

c) The two combinations of PCR (FW-RV1 and FW-RV2) enabled to distinguish the each allele as a co-dominant marker.

d) The amplified region by marker 4 was included in GM_TMiH_H17D12 and was located 1.5 Kbp from the end of this clone.

e) Physical position at Gm19 in Glyma1.0 (http://www.phytozome.net/).

Table 2. List of DNA markers used for fine mapping of the *FT3* locus.

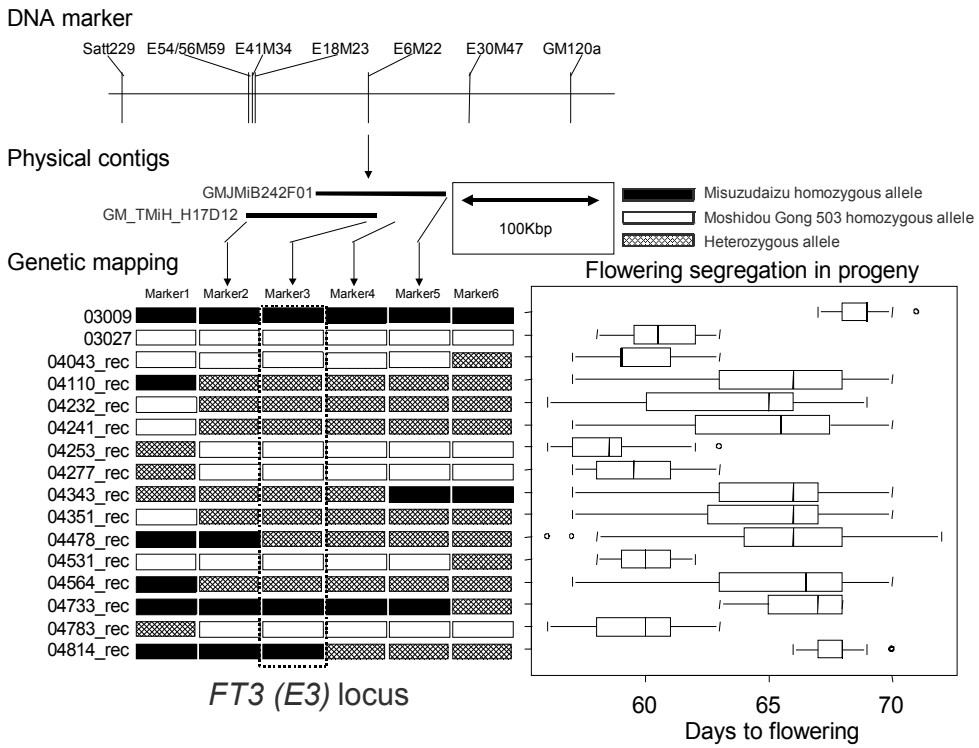


Fig. 9. Fine mapping of the *FT3* locus. The genotypes of each recombinant are shown in the left panel. Misuzudaizu homozygous, Moshidou Gong 503 homozygous and heterozygous genotypes are indicated by solid, open and meshed boxes, respectively. The phenotypic segregation in the progenies of each recombinant was shown in the right panel. The interquartile region, median, and range are indicated by a box, bold vertical line, and horizontal line, respectively.

A total of 11 genes were predicted in the sequence of GM_TMiH_H17D12. Previous studies had suggested that the *FT3* locus may be identical to the maturity locus *E3* (Yamanaka et al., 2001) and that the *E3* gene which showed a large effect on flowering time under FLD conditions had some association with a photoreceptor (Cober et al., 1996b). Considering these findings, one gene highly similar to that encoding phytochrome A was considered to be the gene responsible for the *FT3* locus. To confirm this assumption, differences in this gene between the parental lines were investigated. This phytochorome gene was referred to as *GmPhyA3*, since two other phytochrome A genes had been previously designated as *GmPhyA1* and *GmPhyA2* by Liu et al. (2008). *GmPhyA3* obtained from Misuzudaizu (*GmPhyA3*-Mi) was found to encode a protein composed of 1130 amino acids. A BLAST search found that *GmPhyA3*-Mi displayed normal features of phytochrome A, including a chromophre-attached domain, two PAS domains, and a histidine kinase domain as conserved domains. Compared to *GmPhyA3*-Mi, the *GmPhyA3* gene of Moshidou Gong 503 (*GmPhyA3*-Mo) showed a large insertion in the fourth intron and one SNP for a nonsynonymous amino acid substitution (glycine to arginine) in the third exon (Fig. 10). This SNP corresponded to the polymorphism detected by the AFLP marker E6M22. The inserted sequence was 2.5 Kb in length and a part of this sequence was found to be highly

similar to that of the non-long-terminal-repeat (LTR) retrotransposon reverse transcriptase element, but did not resemble the Ty1/copia or Ty1/gypsy sequences in the *e4* allele (Liu et al., 2008). Moreover, this inserted sequence showed a similar short sequence on both sides of the inserted position. To collect allelic information about *GmPhyA3*, the genes from Harosoy and Harosoy-*e3* were also isolated and designated as *GmPhyA3-E3* and *GmPhyA3-e3*, respectively. While a large retrotransposon-like insertion sequence was observed in *GmPhyA3-E3*, similar to that in *GmPhyA3*-Mo, the amino acid sequences encoded by *GmPhyA3*-Mi and –*E3* were identical (Fig. 10). On the other hand, a large deletion of 13.33 Kb at a position after the third exon was detected in *GmPhyA3-e3* (Fig. 10). Additionally, one mutant (*GmPhyA3*-mut), with a 40-bp deletion in the middle of the first exon of the *GmPhyA3* gene was screened from the mutant libraries of Bay by TILLING (Fig. 10). The sequence of *GmPhyA3* from Bay was identical to that of *GmPhyA3-E3*.

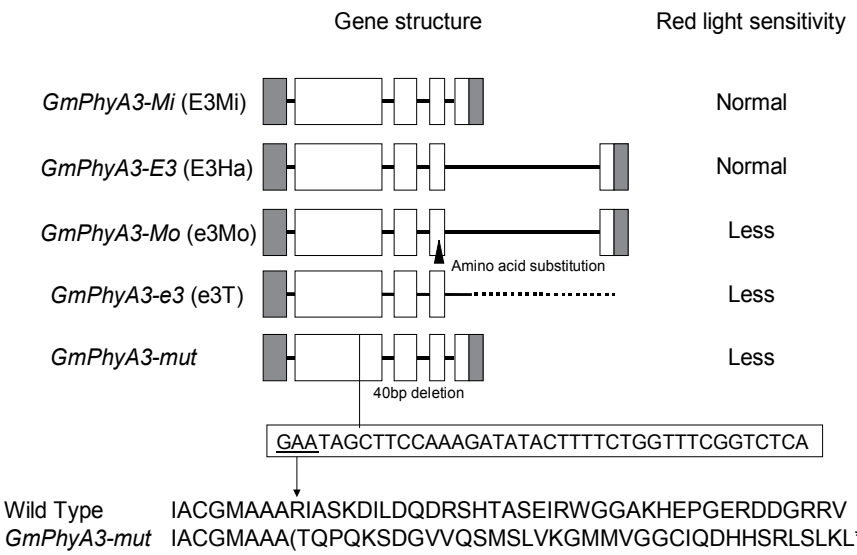


Fig. 10. Variation of gene structure of *GmPhyA3* and red light sensitivity. Open boxes, shaded boxes, and horizontal lines indicate exons, UTRs, and introns, respectively. The deleted region detected in Harosoy-*e3* is denoted by a dotted line. The deleted region in the middle part of the first exon of the mutant is shown at the bottom of the figure. The sequence of 40-bp deletion and the corresponding translated amino acid sequence in the wild-type plant are displayed. As a result of the deletion, a stop codon following the 36 amino acids at the deletion site appears in the mutant.

For allelism test among the *E3*, *FT3*, and *ft3* alleles, two population from crosses between Harosoy (*Dt1Dt1 e1e1 e2e2 E3E3*) and 6-22-*FT3* (*Dt1Dt1 ft1ft1 ft2ft2 FT3FT3*) and 6-22-*ft3* (*Dt1Dt1 ft1ft1 ft2ft2 ft3ft3*) were developed. Genetic analysis revealed that only the crossing population of Harosoy and 6-22-*ft3* showed a significant difference in genetic effect on flowering time. This indicated that the *E3* and *FT3* alleles had the same effect. The large insertion-like retrotransposon observed in *GmPhyA3-E3* and –Mo therefore might have no effect on the phenotype, whereas the one-amino-acid substitution observed in the *GmPhyA*-Mo might have weakened the effect of the *FT3* allele.

Since Cober's study (1966b) indicated that the *E3* allele exerted a large effect under FLD, the sensitivity to FLD conditions between the three NILs (Harosoy and *–e3*, 6-22-*FT3* and *–ft3*, 1-146-*FT3* and *–ft3*) and the mutant line for the *GmPhyA3* gene were evaluated. While the flowering days of each line varied because of their different genetic backgrounds, the effect of the *E3*/*FT3* allele was enhanced under FLD conditions in all the NILs. The mutant line with *GmPhyA3*-mut flowered 15 days earlier than the original variety Bay under extended mercury-vapor lamp with high red/far-red (R/FR) conditions like FLD.
These results strongly suggest that *GmPhyA3* is the gene responsible for the locus *E3*/*FT3*.
We designated the type of gene structure of *GmPhyA3*-Mi, *GmPhyA3*-*E3*, *GmPhyA3*-Mo and *GmPhyA3*-*e3* as E3Mi, E3Ha, e3Mo and e3T, respectively, hereafter. Distribution of these alleles was investigated using several cultivars and lines covering all the maturity groups in Japan. Three primer pairs were designed for discrimination among E3Mi, E3Ha/e3Mo and e3T. The sequences of these primers are shown in Table 3 and the positions of these primers are indicated in Fig. 11. The e3Mo type was distinguished from E3Ha type by *Mse1* digestion of a PCR product using specific primers, E3_07666FW and E3_08417RV. PCR products or digested fragments were separated by 1% agarose gel electrophoresis. Among the 80 accessions randomly selected from Genebank of the National Institute of Agrobiological Sciences (NIAS) in Japan, the E3Mi and e3T types were equally abundant, while the E3Ha and e3Mo types seldom occurred.

Marker type	Primer name	5'-sequence
STS	E3_08557FW	TGGAGGGTATTGGATGATGC
	E3_09908RV	CTAAGTCCGCCTCTGGTTTCAG
	E3Ha_1000RV	CGGTCAAGAGCCAACATGAG
	e3T_0716RV	GTCCTATACAATTCTTTACGACG
CAPS_*Msel*	E3_07666FW	CTCGGATCTTGACAGCATCA
	E3_08417RV	CAACTGAACTGAAGACCCACAA

Table 3. The DNA markers for genotype analysis of the *E3* locus.

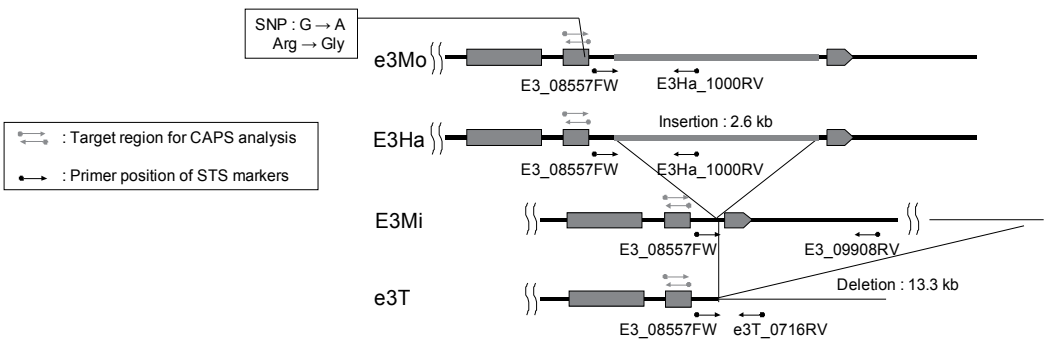


Fig. 11. Variation of *GmPhyA3* gene structure with the position and orientation of PCR primers.

The *E3* region were amplified with four pairs using the total DNA of 30 varieties, and four PCR products were designated as E3f1 to E3f4 (Table 4). The positions of these primers and PCR fragments are indicated in Fig. 12. Sequencing primers were constructed at intervals of

approximately 500 bases on the fragments. The PCR products were sequenced and alignment of the sequences was carried out. Days from sowing to the first flowering and alleles at the *E3* locus of 30 accessions are listed in Table 5. The results showed that E3Mi and e3T types were abundant, followed by the E3Ha type, while the e3Mo seldom occurred. No other type has been detected so far. The E3Ha type was detected in the accessions from China and North America. The latest flowering group harbored the E3Mi type, while the earliest flowering group, the e3T type. There was no clear relationship between the flowering time and the alleles at the *E3* locus in the other groups, because the flowering time depends on the combination of alleles at many loci.

Fragment	Primer name	5'-Sequence
E3f1	E3_00527FW	TCATGTCCAGCAACGCGTAGCATATT
	E3_03552RV	GAGACACTGCCATGCCATAA
E3f2	E3_03384FW	GGCTGCAATCATCATCACAT
	E3_06355RV	TCACTGCATCCAGTTCTTGC
E3f3	E3_05879FW	AACAAGGTGTGGCGATTAGG
	E3_08417RV	CAACTGAACTGAAGACCCACAA
E3f4(E3Mi/E3Ha/e3Mo)	E3_08115FW	TTGCATGAAGTTTTGGTTGC
	E3_09908RV	CTAAGTCCGCCTCTGGTTTCAG
E3f4(e3T)	E3_08115FW	TTGCATGAAGTTTTGGTTGC
	e3T_3544RV	AACTGGCCAAATCAAAGTGC

Table 4. Anchor primers for sequence analysis at the *E3* locus.

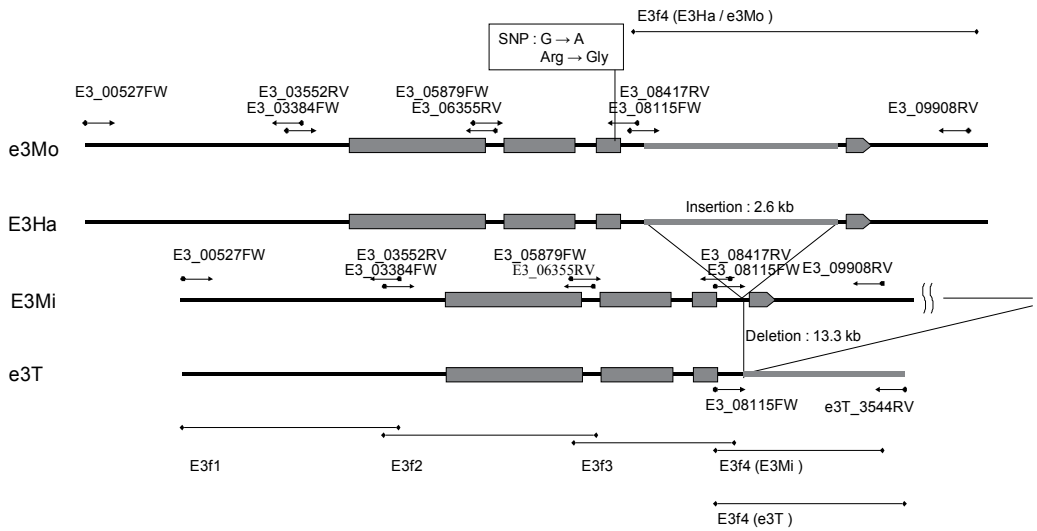


Fig. 12. Variation of *GmPhyA3* gene structure with the position and orientation of primers for PCR walking. The PCR products (E3fi, E3f2, E3f3 and E3f4) are shown at the bottom of the figure. As the e3T type lacked a portion of the third intron and the downstream region, the reverse primer for E3f4 was different from that for other alleles.

Accsession	Days of flowering	Genotype	Origin
Akisengoku	69.6	E3Mi	Japan
Tamanishiki	64.4	E3Mi	Japan
Oushokuakidaizu	62.2	E3Mi	Japan
Fukuyutaka	58.8	E3Mi	Japan
Hyoukeikuro 3	58.6	E3Mi	Japan
Akishirome	55.6	E3Mi	Japan
Misuzudaizu	52.6	E3Mi	Japan
Peking	52.2	E3Ha	China
akasaya(Nagano)	50.8	E3Mi	Japan
Ani	48.0	e3T	Japan
Enrei	46.0	e3T	Japan
Tamatsukuri	45.0	e3T	Japan
Norin 2	44.6	e3T	Japan
Harosoy_E1	44.4	E3Ha	Canada
Aohigu	43.3	e3T	Japan
Kin	42.2	e3T	Japan
Matsuura	42.0	E3Mi	Japan
Wasekin	41.2	e3T	Japan
NIL-13-E1	38.4	e3T	Japan
Harosoy_E2	37.8	E3Ha	Canada
Tokachinagaha	37.6	e3T	Japan
Moshidou Gong 503	36.8	e3Mo	China
Kingen 1	36.4	E3Ha	China
Toyomusume	36.2	E3Mi	Japan
Harosoy	34.8	E3Ha	Canada
NIL-11-e4	33.6	e3T	Japan
Harosoy_e4	33.6	E3Ha	Canada
NIL-13-e2	33.4	e3T	Japan
Harosoy_e3	31.2	e3T	Canada
Sakamotowase	28.6	e3T	Japan

Table 5. Days from sowing to the first flowering and alleles at the *E3* locus. These accessions were sown on June 10, 2008 at the NIAS

3.3 Toward the positional cloning of the *E1* gene

Among the the 156 RILs, a single line was identified as being heterozygous around the *FT1* locus (approximately 17 cM) based on the genotypes of the DNA markers, and was named RHL1-156 (Fig. 13). A population of 1,006 plants derived from RHL1-156 was used for fine mapping of the *FT1* locus. The *FT1* locus mapped between tightly linked DNA markers, Satt365 and GM169 (Fig. 14).

As it was difficult to find AFLP markers around this region in this population, we used mapping populations derived from a cross between Harosoy-*E1* (*E1E1 e2e2 E3E3*) and Harosoy (*e1e1 e2e2 E3E3*). The *E1* locus was mapped proximate to Satt557 between Satt365 and Satt289 using the F_2 population (117 plants). In a $F_{2:4}$ population (mixed progenies from F2 heterozygotes at Satt557 locus) with 1,442 individuals, seven recombinants were identified between Satt365 and Satt289. The flowering genotypes for each recombinant are confirmed by the progeny segregation pattern. With these recombinants, we were able to delimit the *E1* region to approximately 289 kb between markers A and E5 (Fig. 15). No recombination was found between markers S8 and Satt557, despite a physical distance of 133 kb. Because more than 40 genes were identified in the 289 kb region, more intense fine

mapping was conducted by using more than 13,000 plants with a protocol for large-scale genotyping of soybean seeds (Fig. 2) and a candidate gene was identified (Xia et al., unpublished).

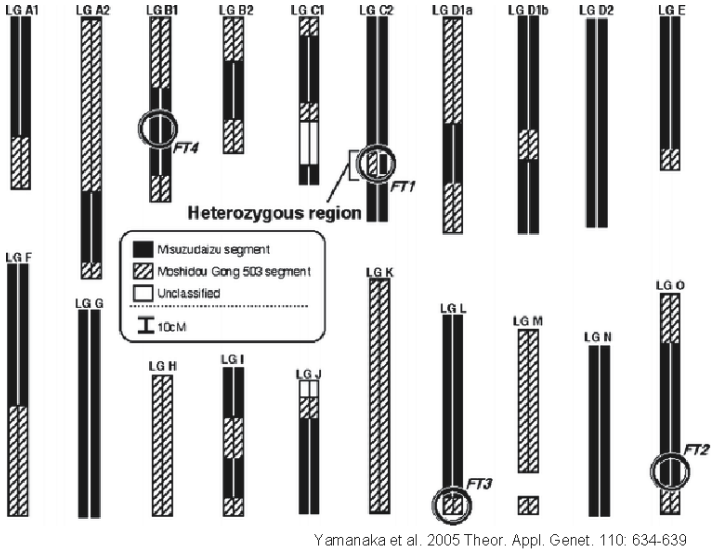


Fig. 13. Graphical genotype of RHL1-156. Solid bars and bars with slanted lines represent Misuzudaizu and Moshidou Gong 503 homozygous segments, respectively. Open bars represent unclassified segments. Putative location of each QTL is circled.

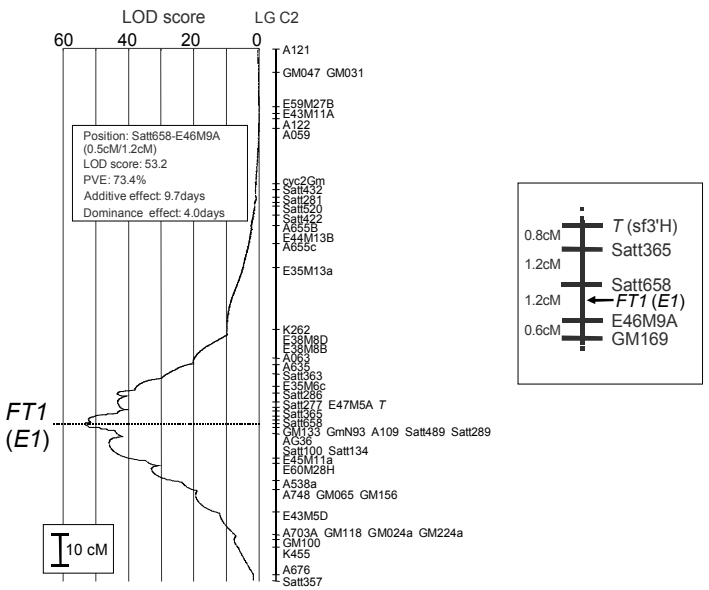


Fig. 14. QTL analysis for the *FT1* locus. LOD scores calculated by interval mapping are shown in the left panel. Close-up of the *FT1* region is highlighted in the right panel.

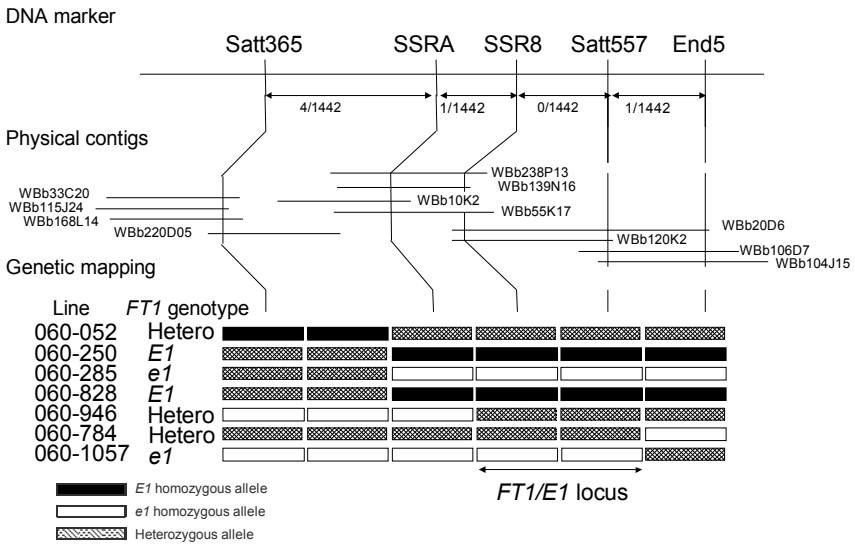


Fig. 15. Fine mapping of the *FT1/E1* locus. *E1* homozygous, *e1* homozygous and heterozygous genotypes are shown by solid, open and meshed boxes, respectively. The *FT1* genotype of each recombinant was identified by progeny test.

The *FT1* locus was genetically mapped into the semi-central domain of linkage group C2 (Fig. 3) and was included in the pericentromeric region of chromosome 06 (http://www.phytosome.net/). In the heterochromatic regions, the ratio of physical to genetic distance is 3.5Mb/cM in comparison of 197 Kb/cM in euchromatic regions (Schmutz et al., 2010). The responsible gene for *FT1/E1* locus is characterized by relatively lower mRNA abundance. In fact, no EST data of the *FT1/E1* gene could be retrieved from public databases. The gene encodes a novel small protein and is unique in the sense of no apparent orthologs in model plants *Arabidopsis* or rice. We are analyzing the ligands of this protein and the interaction with DNA sequences.

4. Putative pathway of flowering time in soybean

The responsible gene for the *E4* locus was identified as *GmPhyA2* through the candidate gene approach (Liu et al., 2008). At the *e4* allele, a Ty1/copia-like retrotransposon was inserted in exon 1 of the gene, which resulted in dysfunction of the gene and photoperiod insensitivity. Similarly, natural and artificial mutations of *GmPhyA3* resulted in weak or complete loss of photoperiod sensitivity (Watanabe et al., 2009). The *FT* homologs in soybean have been identified (Kong et al., 2010) and two of them, *GmFT2a* and *GmFT5a*, were highly upregulated under SD conditions and showed diurnal expression patterns with the highest expression 4h after dawn. Under LD conditions, expression of *GmFT2a* and *GmFT5a* was downregulated and did not follow a diurnal pattern. Ectopic expression analysis in *Arabidopsis* confirmed that both *GmFT2a* and *GmFT5a* had the same function as *Arabidopsis FT*. A double-mutant (*e3e3 e4e4*) for *GmPhyA2* and *GmPhyA3* expressed high levels of *GmFT2a* and *GmFT5a* under LD conditions (18-h light) with an R: FR ratio of 1.2, and it flowered slightly earlier under LD than the wild type (*E3E3 E4E4*) grown under SD. The expression levels of *GmFT2a* and *GmFT5a* were regulated by PHYA-mediated

photoperiodic regulation system, and the *GmFT5a* expression was also possibly regulated by photoperiod-independent system in LD.

GI have the conserved function of controlling the expression of the *FT* gene in *Arabidopsis*, rice and pea (Hayama et al., 2003; Mizoguchi et al., 2005; Hecht et al., 2007). We analyzed the expression of *GmFT2a* and *GmFT5a* at 9:00 a.m. 4 weeks after sowing under natural day-length conditions using *E2* (*FT2*) NILs in which photoperiod changed from LD to SD. A clear association between the *GmFT2a* expression level and early flowering phenotype was observed in both NILs. However, there was no significant difference in the *GmFT5a* expression levels between these NILs. These results suggested that *GmGIa* probably controlled flowering time through the regulation of *GmFT2a*. The recessive alleles of the *E2* (*FT2*) locus were perhaps unable to suppress *GmFT2a* expression and resulted in the early flowering phenotype.

There are strong interaction among the effects of *E1* (*FT1*) and *E2* (*FT2*), *E1* (*FT1*) and *E3* (*FT3*) (Yamanaka et al. 2000; Watanabe et al. 2004). The *e3e3* recessive homozygote can initiate flowering under R-enriched LD, but the *e3e3* genotype is necessary for plants with *e4* mutant allele to flower under FR-enriched LD. In the mapping population with *e3* background, photoperiodic insensitivity could occur in either genotypes of *e1E4*, *E1e4* or *e1e4* (Abe et al., 2003). These results suggest that *E1*, *E2*, *E3* and *E4* might concurrently mediate photoperiodic flowering in a shared pathway. The expression of the candidate gene for the *E1* locus was found to be repressed under SD. Under SD conditions, *E3*/*E4*-mediated photoperiodic regulation system up-regulates the expression of *GmFT2a* and *GmFT5a* possibly through the repression of the *E1* gene (Fig. 16). The *E2* locus also might control the *GmFT2a* expression through the *E1* gene.

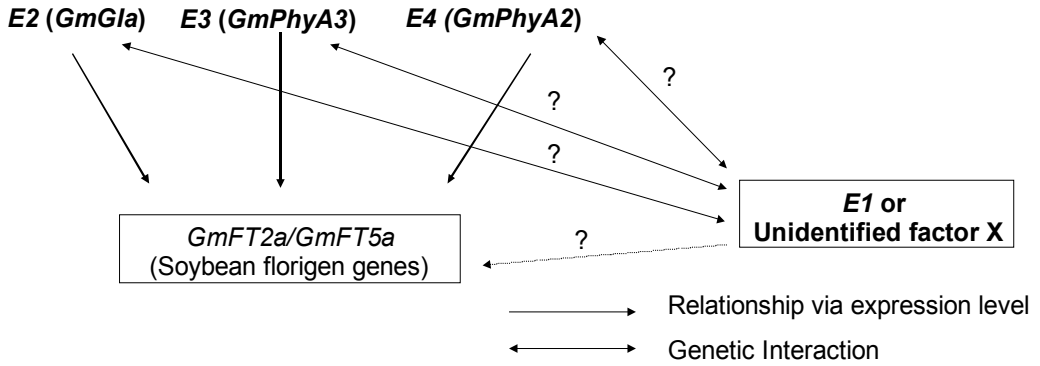


Fig. 16. A putative network of flowering time genes in soybean.

5. Conclusion

We successfully identified the responsible genes for the *E1*, *E2* and *E3* by positional cloning strategy and proposed a tentative flowering time gene network in soybean based on interaction of these genes. We used RHLs derived from RIL for fine mapping a single QTL effectively. An RHL harbors a heterozygous region where the target QTL is located and a homozygous background in most other regions of the genome. Novel DNA markers tightly linked to the locus were developed based on AFLP between the NILs of the locus derived from an RHL. A large-scale population derived from RHLs was used to locate the target

locus precisely. We developed manual large-scale genotyping of seeds, in which powdered cotyledon was obtained by drilling a hole on the surface of seed without any damage to the embryonic axis. Recombinants carrying crossovers in the target region were selected based on genotypes of DNA markers around the region. Genotypes of the flowering time locus of recombinants were determined by progeny test and identified the cosegregated region based on these genotypes. Physical contigs were constructed with BAC/TAC clones screened by SCAR markers converted from these AFLP fragments. By sequencing the BAC contig covering the cosegregated region, we identified the candidate genes. Confirmation of the responsible gene was performed by investigation of association between natural and induced variation of the candidate gene structures and flowering time. Mutant screening was carried out with TILLING using X-ray irradiated or EMS treated mutant libraries. The interactions between the identified genes were analyzed using several NILs and segregating population for the *E* loci. A tentative flowering time network in soybean was proposed taking into consideration the possible functions of responsible genes for *E1*, *E2*, *E3* and *E4* loci and *GmFT*s. Further characterization of other *E* loci is necessary to reveal the molecular mechanism of flowering in soybean.

Recently, soybean genome sequence has been reported (Shumutz et al., 2010) and a large number of SSR (Song et al., 2010) and SNP (Hyten et al., 2010a; Lam et al., 2010) markers has been developed. New high-throughput sequencing technologies, and multiplex assays for genotyping a huge number of SNPs have become available. These technologies and information will accelerate the identification of responsible genes for agriculturally important loci. But methods and materials to precisely locate the target loci in the genome are still important. Moreover, variation of regional genome structure and gene content (Kim et al., 2010 ; William et al., 2010; Xia et al., unpublished) will need the sequencing of genome clones covering the target region.

6. Acknowledgment

We thank Dr. S. Sato and Dr. S. Tabata at the Kazusa DNA Research Institute, for nucleotide sequencing of BAC and TAC clones, Dr. H. Kanamori at the Institute of the Society for Techno-innovation of Agriculture and Dr. Y. Katayose at the National Institute of Agrobiological Sciences for nucleotide sequencing of the *E3* genes, and Dr. R. L. Nelson at the U. S. Department of Agriculture, University of Illinois, for supplying seeds of the Harosoy, Harosoy-*E1,* -*E2* and -*e3*. This study was partly supported by the Programs for the Promotion of Basic Research Activities for Innovative Biosciences, Research Fellowships from the Japan Society for the Promotion of Science for Young Scientists (17-2554), a grant from Ministry of Agriculture, Forestry and Fisheries of Japan (Genomics for Agricultural Innovation, DD-2040), from Grant-in Aid for Scientific Research A (18208001).

7. References

Abe, L., Xu, D., Miyano, A., Komatsu, K. & Kanazawa, A. (2003). Photoperiod-insensitive Japanese soybean landraces differ at two maturity loci. *Crop Science*, Vol.43, pp. 1300-1304

Amasino, R. (2010). Seasonal and developmental timing of flowering. *The Plant Journal*, Vol.61, pp. 1001-1013

Ben-Naim, O., Eshed, R., Parnis, A., Teper-Bamnolker, P., Shalit, A., Coupland, G., Samach, A. & Lifschitz, E. (2006). The CCAAT binding factor can mediate interaction between CONSTANS-like proteins and DNA. *The Plant Journal*, Vol. 46, pp. 462-476

Bernard, R.L. (1971). 2 Major genes for time to flowering and maturity in soybeans. *Crop Science*, Vol.11, pp. 242-247

Bonato, E.R. & Vello, N.A. (1999). *E-6*, a dominant gene conditioning early flowering and maturity in soybeans. *Genetics and Molecular Biology*, Vol.22, pp.229-232

Buzzel, R.I. (1971). Inheritance of a soybean flowering response to fluorescent-daylength conditions. *Canadian Journal of Genetics and Cytology*, Vol.13, pp. 703-707

Buzzel, R.I. & Voldeng, H.D. (1980). Inheritance of insensitivity to long daylength. *Soybean Genetics Newsletter*, Vol.7, pp. 26-29

Chailakhyan, M.K. (1936). New facts in support of the hormonal theory of plant development. *Doklady Akademii Nauk SSSR*, Vol. 13, pp. 79-83

Cober, E.R., Molnar, S.J., Charette, M. & Voldeng, H.D. (2010). A new locus for early maturity in soybean. *Crop Science*, Vol.50, pp. 524-527

Cober, E.R., Tanner, J.W. & Voldeng, H.D. (1996a). Genetic control of photoperiod response in early-maturing, near-isogenic soybean lines. *Crop Science*, Vol.36, pp.601-605

Cober, E.R., Tanner, J.W. & Voldeng H.D. (1996b) Soybean photoperiod-sensitivity loci respond differentially to light quality. *Crop Science*, Vol. 41, pp. 698-701

Cober, E.R. & Voldeng, H.D. (2001). Low R:FR light quality delays flowering of *E7E7* soybean lines. *Crop Science*, Vol.41, pp. 1823-1926

Corbesier, L., Vincent, C., Jang S., Fornata, F., Fan, O., Searle, I, Gialountis, A., Farrona, S., Gissort, L., Turnbull, C. & Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of *Arabidopsis*. *Science*, Vol.316, pp. 1030-1033

Doi, K., Izawa, T., Fuse, T., Yamanouchi, U., Kubo, T., Shimatani, Z., Yano, M. & Yoshimura, A. (2004). *Ehd1*, a B-type response regulator in rice, confers short-day promotion of flowering and controls *FT*-like gene expression independently of *Hd1*. *Genes & Development*, Vol.18, pp. 926-936

Fornara, F. & Coupland, G. (2009). Plants phase transitions make a SPLash. *Cell*, Vol.138, pp. 625-627

Fowler, S., Lee, K., Onouchi, H., Samach, A., Richardson, K., Morris, B., Coupland, G. & Putterill, J. (1999). *GIGANTEA*: a circadian clock-controlled gene that regulates photoperiodic flowering in *Arabidopsis* and encodes a protein with several possible membrane-spanning domains. *The EMBO Journal*, Vol. 18, pp. 4679-4688

Garner, W.W. & Allard, H.A. (1920). Effect of the relative length of day and night and other factors of the environment on growth and reproduction in plants. *Journal of Agricultural Research*, Vol.18, pp553-606

Hayama, R., Agashe, B., Luley, E., King, R. & Coupland, G. (2007). A circadian rhythm set by dusk determines the expression of *FT* homologs and short-day photoperiodic flowering response in *Pharbitis*. *The Plant Cell*, Vol.19, pp. 2988-3000

Hayama, R., Yokoi, S., Tamaki, S., Yano, M. & Shimamoto, K. (2003). Adaptation of photoperiodic control pathways produces short-day flowering in rice. *Nature*, Vol.422, pp. 719-722

Hecht, V., Knowles, C.L., Vander Schoor, J.K., Liew, L.C., Jones, S.E., Mistry, J., Lambert, M. & Weller, J.L. (2007). Pea *LATE BLOOMER1* is a *GIGANTEAE* ortholog with roles in photoperiodic flowering, deetiolation, and transcriptional regulation of circadian clock gene homologs. *Plant Physiology*, Vol.144, pp. 648-661

Hyten, L.H., Cannon, S.B., Song, Q., Weeks, N., Ficks, E.D., Shoemaker, R.C., Spect, J.E., Farmer, A.D., May. G.D. & Cregan, P.B. (2010) High-throughput SNP discovery through deep resequencing of a reduced representation library to anchor and orient scaffolds in the soybean whole genome sequence. *BMC Genomics*, Vol. 11, 38

Imaizumi, T. (2009). *Arabidopsis* circadian clock and photoperiodism: time to think about location. *Current Opinion in Plant Biology*, Vol.13, pp. 83-89

Imaizumi, T., Schultz, T.F., Harmon, F.G., Ho, L.A. & Kay, S. A. (2005). FKF1 F-box protein mediates cyclic degradation of a repressor of CONSTANS in *Arabidopsis*. *Science*, Vol.309, pp. 293-297

Izawa, T., Oikawa, T., Sugiyama, N., Tanisaka, T., Yano, M. & Shimamoto, K. (2002). Phytochrome mediates the external light signal to repress *FT* orthologs in photoperiodic flowering of rice. *Genes & Development*, Vol.16, pp.2006-2020

Jaeger, K.E. & Wigge, P.A. (2007). FT protein acts as a long-range signal in *Arabidopsis*. *Current Biology*, Vol.17, pp. 1050-1054

Johnson, E., Bradley, J.M., Harberd, N.P. & Whitelam, G.C. (1994). Photoresponces of light-grown *phyA* mutants of *Arabidopsis*: phytochrome A is required for perception of daylength extensions. *Plant Physiology*, Vol.105, pp. 141-149

Jung, C. & Muller, A.E. (2009) Flowering time control and application in plant breeding. *Trends in Plant Science*, Vol.14, pp. 563-573

Kardailsky, L., Shukla, V.K., Ahn, J.H., Dagenais, N. Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J. & Weigel, D. (1999) Activation tagging of the floral inducer *FT*. *Science*, Vol.286, pp. 1962-1965

Kim. M.Y., Lee, S., Van, K., Kim, T.H., Jeong, S.C., Choi, I.Y., Kim, D.S., Lee, Y.S., Park, D., Ma, J., Kim, W.Y., Kim, B.C., Park, S., Lee, K.A., Kim, D.H., Kim, K.H., Shin, J.H., Jang, Y.E., Kim, K.D., Liu, W.X., Chaisan, T., Kang, Y.J., Lee, Y.H., Kim, K.H., Moon, J.K., Schmutz, J., Jackson, S.A., Bhak, J. & Lee, S.H. (2010). Whole-genome sequencing and intensive analysis of the undomesticated soybean (*Glycine soja* Sieb. and Zucc.) genome. *Proceedings of the National Academy of Sciences of the United States of America,* Vol.107, pp. 22032-22037

Knott, J.E. (1934). Effect of a localized photoperiod on spinach. *Proceedings of the American Society for Horticultural Science*, Vol. 31, pp. 152-154

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. & Araki, T. (1999). A pairs of related genes with antagonistic roles in mediating flowering signals. *Science*, Vol.286, pp. 1960-1962

Kojima, S., Takahashi, Y., Kobayashi, Y., Monna, L., Sasaki, T., Araki, T, & Yano, M. (2002). *Hd3a*, a rice ortholog of the *Arabidopsis FT* gene, promotes transition to flowering downstream of *Hd1* under short-day conditions. *Plant & Cell Physiology*, Vol.43, pp. 1096-1105

Kong, F., Liu, B., Xia, Z., Sato, S., Kim, B., Watanabe, S., Yamada, T., Tabata, S., Kanazawa, A., Harada, K. & Abe, J. (2010). Two coordinately regulated homologs of

FLOWERING LOCUS T are involved in the control of photoperiodic flowering in soybean. *Plant Physiology*, Vol.154, pp. 1220-1231

Koornneef, M., Hanhart, C.J. & van der Veen, J.H. (1991). A genetic and physiological analysis of late flowering mutations in *Arabidopsis thaliana*. *Molecular and General Genetics*, Vol.229, pp. 57-66

Lam, H.-M., Xu, X., Liu, X., Chen, W., Yang, G., Wong, F.-L., Li, M.-W., He, W., Qin, N., Wang, B., Li, J., Jian, M., Shao, G., Wang, J., Sun, S.S.-M. & Zhang, G. (2010). Resequencing of 31 wild and cultivated soybean genomes identifies patterns of genetic diversity and selection. *Nature Genetics*, Vol.42, pp. 1053-1059

Lifschitz, E., Eviatar, T., Rozman, A., Shalit, A., Goldshmidt, A., Amsellem, Z., Alvarez, J.P. & Eshed, Y. (2006). The tomato *FT* ortholog triggers systemic signals that regulate growth and flowering and substitute for diverse environmental stimuli. *Proceedings of the National Academy of Sciences of the United States of America*, Vol.103, pp. 6398-6403

Liu, B., Kanazawa, A., Matsumura, H., Takahashi, R., Harada, K. & Abe, J. (2008). Genetic redundancy in soybean photoresponses associated with duplication of the phytochrome A gene. *Genetics*, Vol.180. pp. 995-1007

Mathieu, J., Warthmann, N., Kuttner, F., & Schmid, M. (2007). Export of FT protein from phloem companion cells is sufficient for floral induction in *Arabidopsis*. *Current Biology*, Vol.17, pp. 1055-1060

McBlain, B.A. & Bernard R.L. (1987). A new gene affecting the time of flowering and maturity in soybean. *The Journal of Heredity*, Vol.178, pp. 68-70

McBlain, B.A., Bernard, R.L., Cremeens, C.R. & Korczak, J.F. (1987). A procedure to identify genes affecting maturity using soybean isoline testers. *Crop Science*, Vol.27, pp. 1127-1132

McCallum, C.M., Comai, L., Greene, E.A. & Heinkoff, S. (2000). Targeting induced local lesions in genomes (TILLING) for plant functional genomics. *Plant Physiology*, Vol.123, pp. 436-442

Meksem, K., Doubler, T.W., Chancharoenchai, K., Nijti, V.N., Chang S.J.C., Rao Arelli, A.P., Cregan, P.E., Gray, L.E., Gibson, P.T. & Lightfoot, D.A. (1999). Clustering among loci underlying soybean resistance to *Fusarium solani*, SDS and SCN in near-isogenic lines. *Theoretical and Applied Genetics*, Vol.99, pp. 1131-1142

Mizoguchi, T., Wright, L., Fujiwara, S., Cremer, F., Lee, K., Onouchi, H., Mouradov, A., Fowler, S., Kamada, H., Putterill, J. & Coupland, G. (2005). Distinct roles of *GIGANTEA* in promoting flowering and regulating circadian rhythms in Arabidopsis. *The Plant Cell*, Vol.17, pp. 2255-2270

Njiti, V.N., Doubler, T.W., Suttner,R.J., Gray, L.E., Gibson, P.T. & Lightfoot, D.A.(1998). Resistance to soybean sudden death syndrome and root colonization by *Fusarium solani* f. sp. *glycine* in near-isogenic lines. *Crop Science*, Vol.38, pp.472-477

Notoguchi, M., Abe, M., Kimura, T., Daumon, Y., Kobayashi, T., Yamaguchi A., Tomita, Y., Dohi, K., Mori, M. & Araki, T. (2008). Long-distance, graft-transmissible action of Arabidopsis FLOWERING LOCUS T protein to promote flowering. *Plant & Cell Physiology*, Vol. 49, pp. 1645-1658

Park, D.H., Somers, D.E., Kim, Y.S., Choy, Y.H., Lim, H.K., Soh, M.S. Kim, H.J., Kay, S.A. & Nam, H.G. (1999) .Control of circadian rhythms and photoperiodic flowering by the *Arabidopsis GIGATEA* gene. *Science*, Vol.285, pp. 1579-1582

Putterill, J., Robson, F., Lee, K., Simson, R., & Coupland, G. (1995).The *CONSTANS* gene of Arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell*, Vol. 80, pp. 847-857

Reed, J., Nagatani, A., Elich, T.D., Fangan, M. & Chory, J. (1994). Phytochrome A and phytochrome B have overlapping but distinct functions in Arabidopsis development. *Plant Physiology*, Vol.104, pp1139-1149

Roden, L.C., Song, H.R., Jackson, S., Morris, K. & Carre, I.A. (2002). Floral responses to photoperiod are correlated with the timing of rhythmic expression relative to dawn and dusk in *Arabidopsis*. *Proceedings of National Academy of Sciences of the United States of America*, Vol.99, pp. 13313-13318

Saindon, G., Beversdorf, W.D. & Voldeng, H.D. (1989a). Adjustment of the soybean phenology using the *E4* loci. *Crop Science*, Vol.29, pp. 1361-1365

Saindon, G., Voldeng, H.D., Beversdorf, W.D. & Buzzel, R.I. (1989b). Genetic control of long daylength response in soybean. *Crop Science*, Vol.29, pp. 1436-1439

Sawa, M., Nusinow, D.A., Kay, S.A. & Imaizumi, T. (2007). FKF1 and GIGANTEA complex formation is required for day-length measurement in *Arabidopsis*. *Science*, Vol. 318, pp. 261-265

Schumutz, J., Cannon, S.B., Schlueter, J., Ma, J., Mitros, T., Nelson, W., Hyten, D.L., Song, Q., Thelen, J.J., Cheng, J., Xu, D., Hellsten, U., May, G.D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M., Sandhu, D., Valliyodan, B., Lindquist, E., Peto, M., Grant, D., Shu, S., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J., Tian, Z., Zhu, L., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X.-C., Shinozaki, K., Nguyen, H., Wing, R.A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R.C. & Jackson, S.A. (2010). Genome sequence of the palaeopolyploid soybean. *Nature*, Vol.463, pp.178-183

Stewart, D.W., Cober, E.R. & Bernard R.L. (2003). Modeling genetic effects on the photothermal response of soybean phenological development. *Agronomy Journal*, Vol.95. pp. 65-70

Song, Q., Jia, G., Zhu, Y., Grant, D., Nelson, R.T., Hwang, E.-Y., Hyten, D.L. & Cregan, P.B. (2010). Abundance of SSR motifs and development of candidate polymorphic SSR markers. *Crop Science*, Vol.50, pp. 1728-1734

Takano, M., Inagaki, N., Xie, X., Yuzurihara, N., Hihara, F., Ishizuka, T., Yano, M., Nishimura, M., Miyao, A., Hirochika, H. & Shinomura, T. (2005). Distinct and cooperative functions of phytochrome A, B, and C in the control of deetiolation and flowering in rice. *The Plant Cell*, Vol.17, pp. 3311-3325

Tamaki, S., Matsuo, S., Wong, H.L., Yokoi, S. & Shimamoto, K (2007). Hd3a protein is a mobile flowering signal in rice. *Science*, Vol.316, pp. 1033-1036

Tanksley, S.D. (1993) Mapping polygenes. *Annual Review of Genetics*, Vol.27, pp. 205-233

Triwitayakorn, K., Njiti, V.N., Iqbal, M.J., Yaegashi, S., Town, C., & Lightfoot, D.A. (2005). Genomic analysis of a region encompassing *QRfs1* and *QRfs2*: genes that underlie

soybean resistance to sudden death syndrome. *Genome*, Vol.48, pp. 125-138

Tuinstra, M.R., Ejeta, G. & Goldsbrough, P.B. (1997). Heterogeneous inbred family (HIF) analysis: a method for developing near-isogenic lines that differ at quantitative traits loci. *Theoretical and Applied Genetics*, Vol.95, pp. 1005-1011

Upadhyay, A.P., Asumadu, H., Ellis, R.H. & Qi, A (1994a). Characterization of photothermal flowering responses in maturity isolines of soybean [*Glycine-max* (L.) Merrill] Cv Clark. *Annals of Botany*, Vol.74, pp. 87-96

Upadhyay, A.P., Ellis, R.H., Summerfield, R.J., Roberts E.H. & Qi, A. (1994b). Variation in the duration of the photoperiod-sensitive and photoperiod-insensitive phase of development to flowering among 8 maturity isoline of soybean [*Glycine-max* (L.) Merrill]. *Annals of Botany*, Vol.74, pp.97-101

Watanabe, S., Hideshima, R., Xia, Z., Tsubokura, Y., Sato, S., Nakamoto, Y., Yamanaka, N., Takahashi, R., Ishimoto, M., Anai, T., Tabata, S. & Harada, K. (2009). Map-based cloning of the gene associated with the soybean maturity locus *E3*. *Genetics*, Vol.182, pp. 1251-1262

Watanabe, S., Tajuddin, T., Yamanaka, N., Hayashi, M. & Harada, K. (2004). Analysis of QTLs for reproductive development and seed quality traits in soybean using recombinant inbred lines. *Breeding Science*, Vol.54, pp. 399-407

Watanabe, S., Xia, Z., Hideshima, R., Tsubokura, Y., Sato, S., Yamanaka, N., Takahashi, R., Anai T., Tabata, S., Kitamura, K. & Harada, K. (2011). A map-based cloning strategy employing a residual heterozygous line reveals that the *GIGANTEAE* gene is involved in soybean maturity and flowering. *Genetics*, in press

Weller, J.L., Beauchamp, N., Kerckhoffs, L.H, Platten, J.D. & Reid, J.B. (2001). Interaction of phytochromes A and B in the control of de-etiolation and flowering in pea. *The Plant Journal*, Vol.26, pp. 283-294

Weller, J.L., Murfet, I.C. & Reid, J.B. (1997). Pea mutants with reduced sensitivity to far-red light define an important role for phytochrome A in day-length detection. *Plant Physiology*, Vol.114, pp. 1225-1236

Williams, J., Hyten, D.L., Xu, W.W., Gerhardt, D.J., Albert, T.J., Richmond, T., Jeddeloh, J.A., Jia, G., Springer, M., Vance, C.P. & Stupar, M. (2010). The composition and origins of genomic variation among individuals of the soybean reference cultivar Williams 82. *Plant Physiology*, Vol.155, pp.645-655

Yamanaka, N., Nagamura, Y., Tsubokura, Y., Yamamoto, K., Takahashi, R., Kouchi, H., Yano, M., Sasaki, T. & Harada, K. (2000). Quantitative trait locus analysis of flowering time in soybean using a RFLP linkage map. *Breeding Science*, Vol.50, pp. 109-115

Yamanaka, N., Ninomiya, S., Hoshi, M., Tsubokura, Y., Yano, M., Nagamura, Y., Sasaki, T. & Harada, K. (2001). An informative linkage map of soybean reveals QTLs for flowering time, leaflet morphology and regions of segregation distortion. *DNA Research*, Vol.8, pp. 61-72

Yamanaka, N., Watanabe, S., Toda, K., Hayashi, M., Fuchigami, H., Takahashi R. & Harada, K. (2005) Fine mapping of the *FT1* locus for soybean flowering time using a residual heterozygous line derived from a recombinant inbred line. *Theoretical and Applied Genetics*, Vol.110, pp. 634-639

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Taguchi, F., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y. & Sasaki, T. (2000). *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene *CONSTANS*. *The Plant Cell*, Vol. 12, pp. 2473-2483

4

Changes in the Expression of Genes in Soybean Roots Infected by Nematodes

Benjamin F. Matthews[1], Heba M.M. Ibrahim[1,2] and Vincent P. Klink[3]
[1]*United States Department of Agriculture, Agricultural Research Service, Soybean Genomics and Improvement Laboratory, Beltsville,*
[1,2]*Genetics Department, Cairo University, Cairo,*
[3]*Department of Biological Sciences, Mississippi State University, Mississippi State, MS*
[1,3]*USA*
[2]*Egypt*

1. Introduction

1.1 Plant nematodes

Plant parasitic nematodes cause severe damage to plants and are responsible for billions of dollars of losses worldwide (Koenning et al. 2007). Soybean cyst nematode (SCN; *Hederodera glycines*; Fig. 1a) and root-knot nematode (RKN; *Meloidogyne* spp.; Fig. 1b) are sedentary obligate parasites of plants. SCN is the major pest of soybean and causes an estimated one billion dollars in losses annually in the US (Wrather & Koenning 2006). RKN is a major pest of vegetables and can become a serious problem on soybean, especially on edible soybean planted in areas used to grow vegetables (Adegbite & Adesiyan 2005). The genera *Meloidogyne* is widespread and is considered, economically and agriculturally, as a very important group of plant pathogens. The host range of *Meloidogyne* is very wide as it attacks almost all plant species (Sasser 1980). Both SCN and RKN are sedentary endoparasites and they cause dramatic morphological and physiological changes in plant cells while inflicting severe decreases on yield. Chemical methods of nematode control are costly and can damage the environment, especially with contamination of ground water. Therefore, the preferred method of nematode control is the use of resistant or tolerant varieties, when available. Unfortunately, a plant with resistance to one population of nematode is often susceptible to a different population due to the wide genetic variation of nematode populations.

When a plant parasitic nematode infects a plant root, the nematode and the plant enters an intricate interactive relationship with the host that is attempting to inhibit nematode development, while the nematode's goal is to develop and reproduce. The life cycle of SCN and cellular responses of soybean to SCN infection have been documented and reviewed extensively (Bird & Koltai 2000; Endo 1964; Endo, 1965; Endo, 1992; Goverse *et al*. 2000; Lilley *et al*. 2005; Mitchum & Baum 2008; Niblack *et al*. 2006; Williamson & Gleason 2003; Abad & Williamson 2010; Klink *et al.* 2011a). The SCN egg can be found in soil and within the mature female. The second stage juvenile (J2) hatches from the egg, searches for a root of a plant host, penetrates the root epidermis, and migrates intracellularly, using its stylet and enzyme secretions to disrupt cells and force its way toward the vascular tissue.

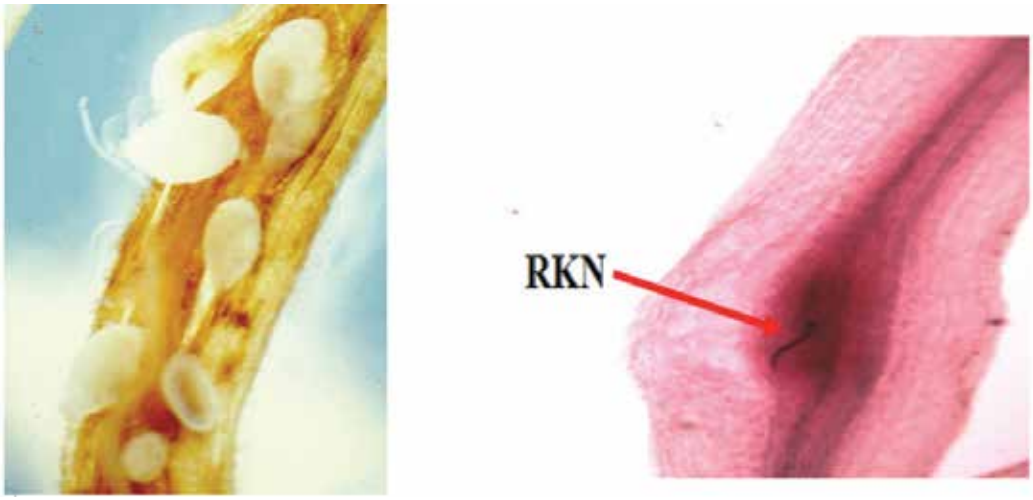


Fig. 1. (*Left*) Female cyst of the soybean cyst nematode at 21 days after infection(dai). (*Right*) Gall formed by the root-knot nematode at 14 dai. The RKN appears red after staining with acid fuchsin.

There nematode selects a feeding cell adjacent to the vascular tissue, pierces the cell wall and injects material from its esophageal gland. The proteins injected by SCN alter the physiology and metabolism of the plant cell and surrounding cells so a syncytium is formed by dissolution of the walls of surrounding cells and the fusion of those adjacent cells The nematode becomes sedentary, feeds and molt three times to reach maturity. The anterior portion of the female SCN remains inside the root while the posterior portion breaks through the epidermis of the root at approximately 12 to 14 dai. At maturity, the outer integument of the mature female SCN hardens to protect eggs within its body, while some eggs are extruded in a gelatinous mass. SCN can complete its life cycle in three to four weeks with one female producing 200 to 600 eggs (Young, 1992). Thus, SCN can complete numerous life cycles during the soybean growing season and infest a field rapidly.

The RKN follows a similar pattern of development to that of SCN. The RKN also goes through five different developmental stages starting with the J1 which molts once inside the egg. After hatching, the motile J2 immediately searches for a plant host and infects immediately behind the root tip and migrates between the plant cells. RKN does not feed during this stage; instead it uses its lipid reserves in the gut (Eisenback & Triantaphyllou, 1991). When the RKN J2 reaches the vascular cylinder, it becomes sedentary and establishes its permanent feeding site by injection of proteins into selected parenchymal adjacent to the vascular system to form giant cells (Caillaud *et al.*, 2001). The giant cells expand and undergo multiple rounds of mitosis without cell division. After feeding for only 24 hours, the RKN molts three times to reach the adult stage (Eisenbach & Triantaphyllou, 1991). The entire body of the RKN remains within the root and infection of roots by RKN can be easily recognized by the "knots" or "galls" formed where they feed and develop (Caillaud *et al.*, 2001). The mature adult female deposits its eggs in a gelatinous mass, which remain attached to the end of the female's body and can be observed on the gall surface. One adult female can lay hundreds to thousands of eggs in three months.

It is important to reiterate that the SCN and RKN puncture the plant cell wall with its stylet to inject secretions from its esophageal glands. These secretions are important to altering the

plant cell morphology and metabolism to form a feeding structure, called the syncytium in the case of SCN or giant cell in the case of RKN. More than 60 genes have been identified that are expressed in the esophageal glands of SCN, many of which have no known function (Gao *et al*. 2001, 2003; Williamson & Gleason 2003; Davis *et al.,* 2004; Davis & Mitchum, 2005). Some of the genes encoding these proteins are similar to microbial genes or genes of animal-parasitic nematodes. Knowledge about these secreted proteins from the nematode and their interactions with targets within the plant cell during infection provides a better understanding of the interaction between the host cells and the parasite.

During the establishment of their feeding sites, nematodes secrete into the plant cell several different proteins and enzymes made in the esophageal gland (Davis *et al*. 2004; Gao *et al*. 2001, 2003). The SCN esophageal glands produce β-1,4-endoglucanase and pectate lyase to degrade the plant cell wall (Smant *et al*., 1998; Hang *et al*., 2003). Some enzymatic reactions of these nematode proteins on the cell wall may produce compounds that interact with signal transduction receptors on the plant host cells (Davis *et al*. 2004; Davis & Mitchum 2005: Mitchum & Baum, 2008). A model of a potential secretomes from plant parasitic nematode has been proposed by Davis et al. (2004) and shows involvement of cell wall remodeling proteins, such as endoglucanases, and expansions. Plant parasitic nematodes also produce proteins that may mimic plant proteins, such as chorismate mutase (Doyle & Lambert, 2003; Bekal *et al*. 2003; Lambert *et al.* 1999) and CLAVATA (Wang *et al*. 2005; Wang *et al.,* 2010; Replogle *et al*. 2010). Some of the secreted proteins contain a peptide sequence that targets the protein to the nucleus, while other proteins remain in the cytoplasm of the plant cell (Elling *et al*., 2007).

2. Gene expression in soybean

Gene expression has been examined in both compatible and incompatible interactions of SCN with soybean roots using Affimetrix microarrays containing approximately 37,000 set of probes (Klink et al. 2007 a; 2009a, 2010, 2011b) (Ithal et al. 2007a,b). The identification of gene expression occurring specifically within the syncytium was first reported by Klink et al. (2005). The experiments provided a means for examining expression at the genomic scale. Also, changes in gene expression in the cells at the feeding site of the nematode have been examined using microarrays (Klink et al. 2007b, 2009a, 2010a, 2011b; Ibrahim et al. 2011). In all of these studies approximately two to ten per cent of the genes represented on the microarray changed in expression by over 1.5-fold. Through microarray studies, many genes were identified that are involved in metabolism, energy, defense and other areas, which provided new insights into plant-pathogen interactions. At the first phase of parasitism, which is prior to feeding or at 12 h after infection (dai), gene expression patterns in the root were found to be similar in both the susceptible and resistant reaction, when the nematode first attempts to establish itself in the host. Gene expression during the second phase depends on the defense response of the host plant (Klink *et al*., 2007a). If the host is resistant or displays an incompatible interaction to the nematode, then gene expression patterns are different than if the host is susceptible or if the host displays a compatible reaction with the nematode, although there are some commonalities (Klink et al. 2007b, 2009a, 2010b). In either case a syncytium is formed. However, in the incompatible interaction, the syncytium degrades, whereas the syncytium is maintained and expands in the compatible interaction. During the formation of the nematode feeding sites, many pathways are involved in the induction of syncytia. For example, solidifying and lignifying

the cell wall of the syncytium, down-regulation of the plant defense system, such as the pathway leading to jasmonic acid, occur in the plant selected feeding cells during the nematode parasitism process (Ithal *et al.,* 2007a; Klink et al., 2007b). Meanwhile other genes and pathways are utilized by the plant exhibiting an incompatible reaction (Klink et al., 2007b, 2009a, 2010b), wherein the syncytium degrades.

Gene expression during only the compatible interaction has been studied between RKN and soybean using soybean Affymetrix microarrays roots (Ibrahim et al., 2010). The nematode not only triggers the defense response of the root and forms a feeding site or giant cell, but also redesigns the morphology of root cells to form a gall. The giant cell is interesting in that it undergoes karyokinesis, but not cytokinesis. Furthermore, genes encoding enzymes in important biochemical pathways were found to be either highly induced or highly suppressed during the infection of the soybean roots with RKN (Ibrahim *et al*. 2010).

Analysis of microarray data can be complex and requires a great deal of time and effort. Commonly, microarray data sets are very large and take a long time to analyze, identify and understand changes in metabolic pathways. Most of the time, only genes already known to be involved in resistance are focused in on with the rest of the data never analyzed to its full potential. PAICE (Pathway Analysis and Integrated Coloring of Experiments) (PAICE (Paice_v2_90.jar) http://sourceforge.net/projects/paice/ (Hosseini *et al*. unpublished) software has been used to analyze microarray data and connect gene expression results between microarrays and illustrations of biochemical pathways found in the Kyoto Encyclopedia of Genes and Genomes (Ibrahim *et al.,* 2011; Klink *et al*., 2009a, 2010b, 2011b; Tremblay *et al.,* 2010). This program provides visualization of microarray gene expression data relevant to known biochemical pathways with a color scheme coding up-regulated genes in various shades of green and down-regulated genes in various shades of red depending on gene expression level. This tool makes key changes in gene expression in biochemical pathways stand out and makes comparison of pathway changes among treatments and across time points easier. This tool will be used in this chapter to display some of the gene expression data from various relevant publications.

2.1 Carbohydrate and energy

The female nematode requires large amounts of energy from its host so it can develop and produce large quantities of eggs. In sycnytia formed during both a compatible interaction at 5 and 10 dai and the incompatible interaction at 6 dai of soybean roots with SCN (Ithal *et al*. 2007a,b; Klink et al. 2007b, 2009a, 2010a); Fig 2) and in galls formed by RNK at 12 dai in a compatible interaction (Ibrahim *et al*. 2010), genes involved in glycolysis are up-regulated. Genes that are in common and up-regulated between the compatible and incompatible interactions of SCN with roots include genes encoding enzymes encompassing the entire pathway between α-D-glucose-6-phosphate and pyruvate. Also, transcripts of genes encoding enzymes between β-D-Fructose-6-phosphate and α-D-glucose and β-D-glucose are elevated in both cases. There are two differences in gene expression levels in the glycolosis/gluconeogenesis pathway that are striking. First the amount transcript of the gene encoding aldose 1-epimerase (EC 5.1.3.3), catalyzing the first step in galactose metabolism that converts -D-glucose into α-D-glucose, is moderately lower at 10 dai in syncytia formed by SCN, but is elevated in the SCN incompatible reaction at 6 dai and in root galls formed by RKN at 12. An increase in this enzyme is associated with a decrease in

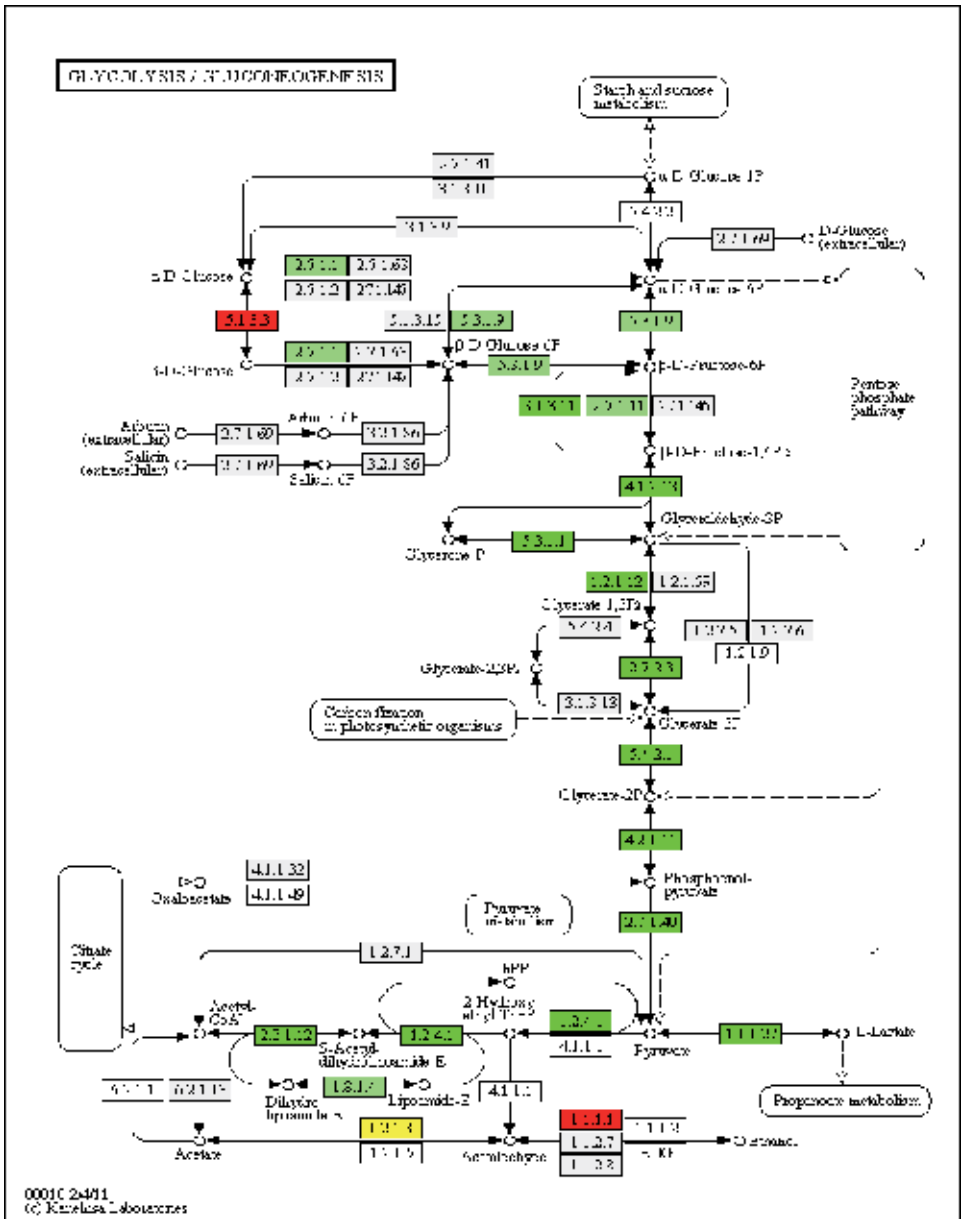


Fig. 2. Expression profiles at 10 dai in a susceptible reaction of Williams 82 with SCN are displayed for the genes encoding enzymes in glycolysis/gluconeogenesis on the KEGG pathway diagram. Enzyme commission numbers in the rectangles are provided by KEGG. Rectangles are colored light green for genes up-regulated in the first 50%, medium green for genes up-regulated in the 50 to 75 quartile and dark green for genes up-regulated in the top 25 %. Enzymes colored in red are encoded by down-regulated genes using a similar scheme. Enzymes colored in yellow are encoded by more than one gene and different copies of that gene are up- and down-regulated, respectively. Rectangles colored light gray indicate that the genes encoding those enzymes are not annotated in our soybean microarray database.

the production of cellulose (Fekete *et al*. 2008). The second pronounced difference is that the gene encoding fructose-bisphosphatase (EC 3.1.3.11) is not elevated in galls, whereas it is one of the genes with the most highly elevated abundance of transcripts in syncytia during the compatible interaction at 5 and 10 dai in syncytia. It is not elevated at 9 dai in the incompatible interaction of SCN with soybean. The reaction of fructose-bisphosphatase is in the direction of starch formation. This supports metabolite studies of the interaction of *Arabidopsis* with the sugar beet nematode, *Heterodera schachtii*, indicate that syncytia accumulate starch during this interaction (Hofmann & Grundler 2008a,b, 2010).

2.2 Cell wall modification and remodeling in soybean

Syncytial cells formed by SCN may encompass 200 to 400 cells, while giant cells formed by RKN sometimes reach more than 400-times the size of a normal cell and may contain more than one hundred nuclei (Caillaud *et al*., 2008). The expansion of the syncytium and the giant cell are accompanied by extensive cell wall modification. Microarray data indicate that the expression of many genes involved in cell wall extension and remodeling is altered (Klink *et al.* 2007b, 2009a,b; Ithal *et al.* 2007; Ibrahim *et al.* 2011). For example more pectinases are expressed in the syncytium during a compatible interaction at 10 dai than in an incompatible reaction at 9 dai (Fig 3). One gene represented by

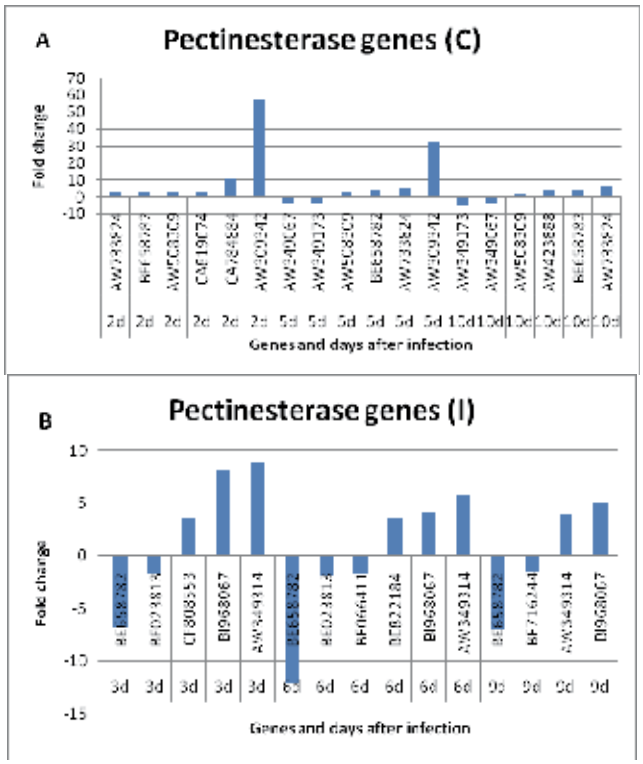


Fig. 3. (A)Fold change in expression of pectinesterases in syncytia in a compatible interaction (C) at 2, 5 and 10 dai. Data from Ithal. *et al. (*2007b) and (B) an incompatible interaction (I) at 3, 6 and 9 dai Data from Klink et al.(2009a). Genes are represented by GenBank numbers.

GenBank number AW309342 experiences more than a 50-fold increase in expression at 2 dai and over 30-fold increase in expression at 5 dai in syncytia in the susceptible reaction. Only three genes encoding pectinesterase are overexpressed in syncytia of the incompatible reaction at 3 and 6 dai and one gene represented by BE658782 is over 5-fold decreased in expression.

Nine genes encoding xyloglucanases are up-regulated in syncytia at 2 and 5 dai during the susceptible reaction. At 5 dai three genes, represented by GenBank numbers BU764179, AW707175, and BQ298739 are more than 15-fold increased in transcript abundance (Fig. 4a). Only four genes encoding xyloglucanases are up-regulated in syncytia during the incompatible reaction at 3 and 6 dai, while one gene represented by AW310549 is down-regulated approximately 30-fold (Fig. 4b). The lack of sustained upregulation and in some cases the actual downregulation of cell remodeling genes in the incompatible reaction is indicative of the fact that the syncytium is not sustained in the incompatible reaction for more than two or three days before it degrades.

Numerous cellulases, endo-1,4-β–glucanases, are altered in regulation in soybean roots upon SCN infection. Two genes encoding cellulases are increased in expression over 60-fold at 3 dai in the incompatible reaction, BI969418 and BI785739. The first, BI969418, decreases to 10-fold over expression at 6 and 9 dai, while the second, BI785739, returns to control levels, while CF806812 increases over 50-fold in expression at 6 and 9 dai in the incompatible interaction (Klink *et al*., 2009a). In contrast in the compatible reaction, two genes, represented by CD394414 and BI971040, encoding cellulases are increased at 2 dai 5- and 10-fold, respectively, while genes represented by BM091956 and BI968056 are increased approximately 28- and 46-fold at 5 dai. At 10dai two genes are increased over 36- and 78--fold, MI968056 and BN091956, respectively (Ithal. *et al.* 2007b).

Expansion of giant cells formed by RKN also requires extensive cell wall remodeling and modification. After infection with RKN (12 dai and 10 wai (weeks after infection)) soybean genes encoding cell-wall modifying xyloglucan endotransglycosylase/hydrolase and endoxyloglucan transferase A2 are differentially expressed (Ibrahim *et al*. 2011). These enzymes are known to have an important role in cell wall softening and degradation (Nishitani, 1998). In addition, some β-endo-1,4-glucanases family members, involved in cell wall remodeling and expansion, were shown to be up-regulated at both 12 dai and 10 wai. Many genes encoding endo-1,4-β–glucanases family members were up-regulated at both time points, 12 dai and 10 wai (Ibrahim *et al.* 2011). This enzyme is also involved in cell wall remodeling and expansion. Some, members of the endo-1,4-β-glucanase gene family are expressed in feeding cells formed by RKN and cyst nematode in tobacco plants (Goellner *et al*., 2001). The promoter of one of these genes is strongly activated in feeding cells formed by *Meloidogyne incognita* as indicated by strong GUS expression (Mitchum *et al.;* 2004). Also, there is an increase in expression of the gene encoding expansin A, which is consistent with other investigations, wherein the expansin (LeEXPA5) genes in *A. thaliana* and tomato were shown to be up-regulated in developing giant cells after infection of roots with *Meloidogyne* (Jammes *et al.,* 2005; Gal *et al*., 2006). Moreover, down-regulation of cellulose synthase and over-expression of pectinesterase that degrades pectin to pectate coincide with a breakdown of the cell wall during the early time points of infection with RKN. These results are consistent with those of Jammes *et al.* (2005), wherein genes encoding pectin esterases and pectate lyases were activated in *Arabidopsis thaliana* (roots after infection with *Meloidogyne incognita* and the cell walls loosening process occurred during the development of the giant cell as well.

2.3 Plant defense system

When a nematode invades a plant root, it must repress or control the plant defense response, so it can successfully establish its permanent feeding site (Caillaud *et al*., 2001). These defense responses may include the production of jasmonic acid and salicylic acid, the hypersensitive response, cell wall strengthening, the production of pathogenesis related (PR) proteins, and other cellular defense responses. There are changes in the expression of genes involved in many of these defense responses in both compatible and incompatible interactions of SCN with soybean and with RKN and soybean in the compatible interaction. Many of the same genes are altered in expression in both the compatible and incompatible interaction. However, the amount of change in transcript abundance may be very important and in some cases a gene is up regulated in one interaction and down regulated in another interaction

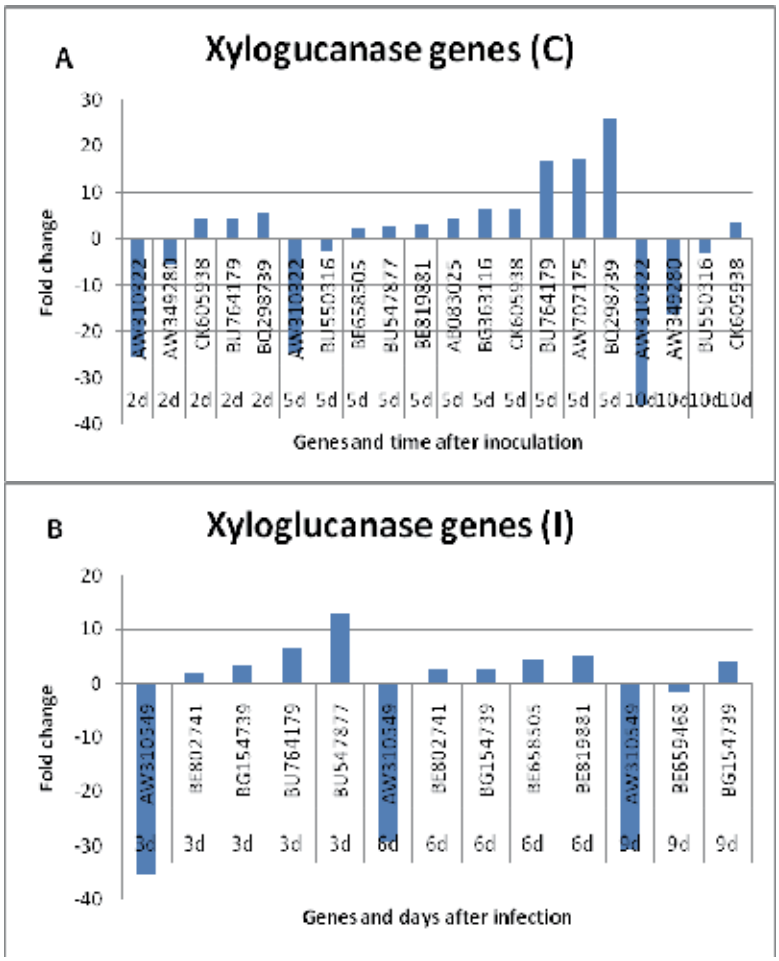


Fig. 4. (A) Fold change in expression of genes encoding xyloglucanases in syncytia in a compatible interaction (S) at 2, 5 and 10 dai. Data from Ithal. *et al. (*2007b) and (B) an incompatible interaction at 3, 6 and 9 dai Data from Klink *et al*.(2009a). Genes are represented by GenBank numbers.

2.3.1 Alpha-linolenic acid and jasmonic acid biosynthesis

The pathway leading to jasmonic acid biosynthesis is one of the pathways associated with pathogen resistance that was significantly affected by both SCN and RKN infection. In soybean there are several lipoxygenase gene family members. Several members of this gene family are expressed higher in the compatible reaction of SCN with soybean at 2, 5 and 10 dai, specifically CF808603, CD409280 and BM092012, which are elevated 2.4- to 6.3-fold (Data from Ithal *et al.* 2007b). In contrast, in the incompatible reaction of SCN with soybean, several members of the gene family are down-regulated, while others are up- regulated, ranging between approximately -22- to 22-fold (Klink *et al.* 2007a). Genes encoding allene oxide synthase (AOS) and allene oxide cyclase (AOC) are not greatly changed in the compatible interaction at 2, 5 and 10 dai fold (data from Ithal *et al.* 2007b). However, three members of the AOS gene family are down regulated in the incompatible interaction at 3 dai, while syncytia are forming. Then expression of one gene family member is increased at 6 and 9 dai as the syncytia collapse and become non-functional (Fig. 5 A; Klink *et al.* 2007b). Expression of genes encoding AOC is increased in syncytia during the incompatible reaction, especially at 3 dai, then decreases in expression at 6 and 9 dai (Fig. 5B; data from Klink *et al.* 2007a). A genes encoding 12-oxyphytodienoate reductase 1 (OPR1), represented by BI968944, is strongly down-regulated in the compatible interaction of SCN with soybean roots (Ithal *et al.* 2007b), while a gene encoding OPR3, represented by BU765938, is up regulated 14-fold at 6 dai in the incompatible reaction (Fig 5C; Klink *et al.* 2007b). Thus, there is an increase in transcripts for specific gene members encoding enzymes through the pathway leading to JA biosynthesis in the incompatible reaction of SCN with soybean roots, while there is either no effect on genes encoding AOS and AOC or a decrease in transcript levels in the case of the gene encoding OPR1 in the compatible reaction. JA biosynthesis is one of the pathways affected in soybean roots by infection with RKN at 12 dai and 10 wai (Ibrahim *et al.*, 2011). At 12 dai, most of the genes encoding enzymes encoding lipoxygenase family members were up-regulated. Lipoxygenase is important in the biosynthesis of oxylipins and it is important in the response of plants during wounding and attack by pathogens (Gobel *et al*, 2001). Reduction of the expression of the gene encoding this lipoxygenase resulted in an increase in susceptibility of transgenic potato plants to insect attack (Gobel *et al*., 2001). Over-expression of the gene encoding lipoxygenase could mean a high accumulation of 9-HPOTrE, as it is one of the major products of lipoxygenase (Fig. 6). Interestingly, 9-HPOTrE is involved in the activation of the plant defense response directly or through its metabolites. In potato plants, 9-HPOTrE is produced in response to injury or infection. The role of 9-HPOTrE in the plant defense response suggests that there may be a new pathway leading to LOX-mediated defense responses (Reddy *et al.*, 2000). The same results have been observed in pigeon pea seedlings after infection with *Fusarium udum* (Reddy *et al*., 2000).

Transcript abundance of genes encoding lipoxygenase was much lower at 10 wai (weeks after infection) than at 12 dai in roots infected by RKN (Ibrahim *et al.* 2011). Three of seven gene family members encoding lipoxygenase were down-regulated. Also, all of the allene oxide synthase gene family members were greatly down-regulated at 10 wai This suggests that at 12 dai the plant defense system is still struggling to fight the infection, but after prolonged infection (10 wai) most of the genes that encode enzymes responsible for the production of jasmonic acid were turned off in the compatible interaction. Genes in this pathway could be a target for testing to determine if resistance to nematode infection can be increased in transformed plants by over-expression of these genes.

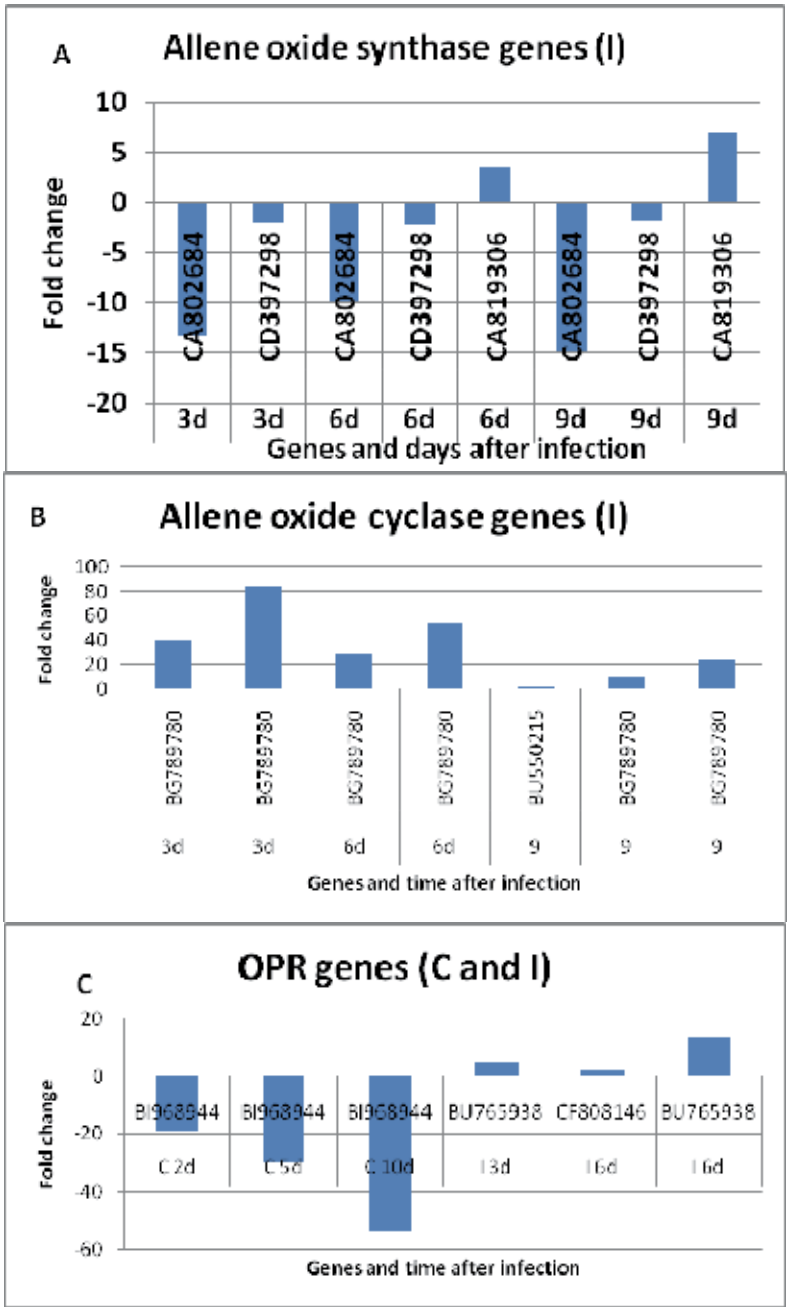


Fig. 5. A) Fold change in expression of genes encoding allene oxide synthase (AOS); (B) fold change in expression of genes encoding allene oxide cyclase (AOC) in sysncytia of an incompatible reaction of SCN with soybean; and C) fold change in expression of genes encoding 12-Oxyphytodienoate reductase in syncytia in a compatible interaction (C) at 2, 5 and 10 dai (data from Ithal. *et al.* 2007b) and incompatible interaction (I) at 3, 6 and 9 dai (data from Klink *et al.* 2009b). Genes are represented by GenBank numbers.

2.3.2 Pathogen related protein (PR) and transcription factors:

Pathogen related (PR) proteins are induced systemically by the interaction of a pathogen with its host (Van Loon & Van Strien, 1999). PR-1 and PR-2 are induced by SA (Ohishima *et al.,* 1990, Hennig *et al*., 1993), while basic PR genes are induced by JA (Niki *et al.* 1998). Genes encoding enzymes involved in JA synthesis were discussed above. Unfortunately, genes important to salicylic acid biosynthesis were either not represented on the microarray chip or were not annotated. However, genes encoding proteins of the PR-1, PR-2 and PR-5 families were up-regulated at 3, 6 and 9 dai in the incompatible interaction of soybean with SCN, suggesting that salicylic acid or its derivatives may be synthesized at these time points.

The PR1 gene, represented by CF806816, was increased 900, 2100 and 1600-fold at 3, 6, and 9 dai, respectively in the incompatible interaction of SCN with soybean, while the PR1 gene, represented by BQ628525, was over expressed 70, 240, 160-fold at 3, 6, and 9 dai (Klink *et al.* 2009b). During the compatible interaction, few PR1 genes were increased in expression and only one gene, represented by BU548404, was increased over 10-fold (Ithal. *et al.,* 2007b) and this was at 2 dai, when the nematode first initiates feeding. At 5 dai only two genes were increased in expression and this was at 5.6-fold and 2.8-fold, respectively. Only one PR-1 gene was increased in expression at 10 dai in the compatible interaction and that was only 5-fold increased in expression. Transcript levels of genes encoding PAL are also more strongly up-regulated in tomato roots displaying an incompatible interaction with the potato cyst nematode (*Globodera rostochiensis*), than in the compatible interaction (Uehara *et al.,* 2010). Arabidopsis roots infected with beet-cyst nematode (*Heterodera schachtii*), transcript levels of PR-1, PR-2, and PR-5 were increased, while PR-3 and PR-4 remained at similar levels to control plants (Hamamouch *et al.* 2010). Transcript levels of genes encoding PR-1 and PR5 were also increased in the incompatible interaction of Arabidopsis with the RKN, *M. incognita*, while transcript levels of PR-3 were elevated to a lesser extent. PR-3 and PR-4 are different types of chitinase. Seven chitinase genes are increased in expression at 3 dai in the incompatible reaction of soybean with SCN; three are approximately 20-fold over-expressed. At six dai, three genes encoding chitinase are expressed; one is 74-fold; A second gene is 33-fold increased in expression. No genes encoding chitinase are over-expressed in the incompatible reaction at 2 dai, and only one gene is over expressed at 5 and 10 dai, 6- and 15-fold, respectively (Fig 6). PR10 genes, represented by X60043, CF921432 and CF805736, are increased in expression 200-fold or more at all time points in both the compatible and incompatible interactions of SCN with soybean roots.

During the interaction of soybean roots with RKN, many genes encoding several PR proteinas were altered in expression (Ibrahim *et al.*, 2011). Transcripts of the gene encoding PR-1 were increased 78-fold at 12 dai in the compatible interaction of soybean roots with RKN. After prolonged infection by RKN at 10 wai, transcript levels of two genes encoding PR-1 were 17- and 350-fold increased. Genes encoding chitinase (PR-3 and PR-4) were down-regulated 4.6-fold at 12 dai in the compatible interaction of soybean roots with RKN, however, by 10 wai transcripts of two chitinase genes were up-regulated 15- to 26-fold, respectively. Transcripts of genes encoding PR-10 (SAM22) were increased 5- to 10-fold at 12 dai and remained at a similar level at 10 wai.

The increase in PR-1 protein suggests that there may be an increase in the level of salicylic acid. Interestingly, there are two different possible routes to salicylic acid production (Chen *et al*. 2009). Salicylic acid is known as a signal molecule for defense against nematodes (Branch *et al.,* 2004).

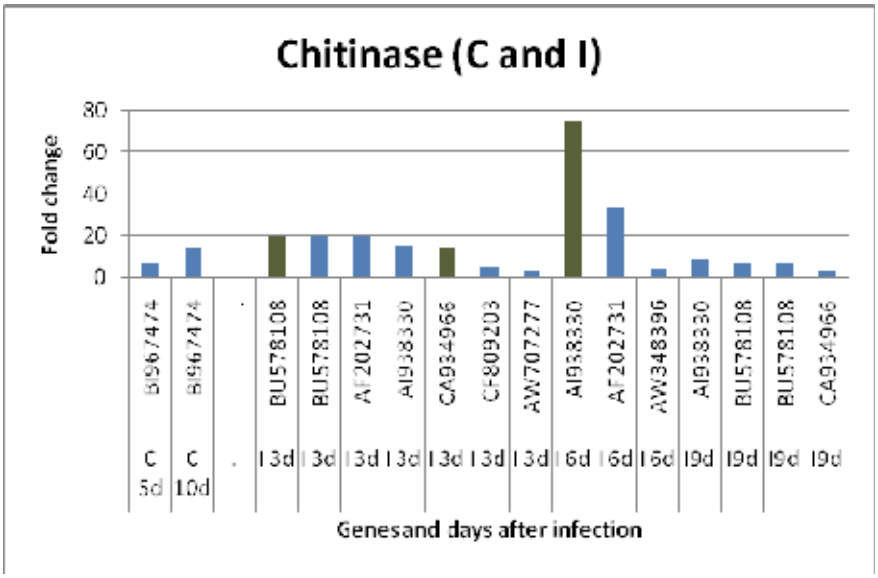


Fig. 6. Fold increase in expression of chitinase genes in the compatible (C) interaction (data from Ithal. *et al.* 2007b) and incompatible interaction (I) at 3, 6 and 9 dai (data from Klink *et al.* 2009b). Genes are represented by GenBank numbers.

The pathway that has the most scientific support involves isochorismate synthase (Wildermuth *et al*. 2001) and is not represented or is not annotated on the microarray. The other pathway involves phenylalanine. In the latter pathway, we found a high increase in the tyrosine aminotransferase enzyme (EC:2.6.1.5) which would lead to high level of phenylalanine. Genes encoding phenylalanine ammonia-lyase (EC:4.3.1.24) and salicylate 1-monooxygenase (1.14.13.-) were over-expressed at 6.9 and 2.9 F.C, respectively. Loon *et al.* (2006) reported that the PR-1- type proteins and also proteinase inhibitors were induced in abscission zones, which suggest the involvement of these proteins in cell wall loosening and degradation of the scarified cells as a defense response against fungal and bacterial pathogens. Also, transgenic tobacco over-expressing PR-1 was more resistant to blue mold and black shank caused by *Peronospora tabacina* and *Phytophthora parasitica f. sp. nicotianae*, respectively (Loon *et al.*, 2006). In addition, PR-3 and PR-4 showed chitinase activity that is required for embryogenesis during the globular stage in carrot (Loon *et al.,* 2006). Genes encoding PR-3 and PR-4 family proteins are reported to be up-regulated by jasmonic acid and ethylene (Niki *et al.,* 1998). Also, PR-4 showed ribonuclease activity against fungal protein in wheat (Loon *et al*., 2006).

2.3.3 Phenylpropanoid biosynthesis

The phenylpropanoid pathways leads to the synthesis of coumarins, flavonoids, phytoalexins, lignins, and lignans, all which can play roles in plant defense. Several genes encoding enzymes in this complex pathway are up regulated in the incompatible interaction at 6 dai (Fig . 7; data from Klink et al. 2007b). And there are notable differences in the expression of genes encoding enzymes in this pathway between the compatible interaction at 2, 5 and 10 dai and the incompatible reaction at 6 and 9 dai. One major difference is in the expression of the genes encoding enzymes involved in the production of phenylpropanoids.

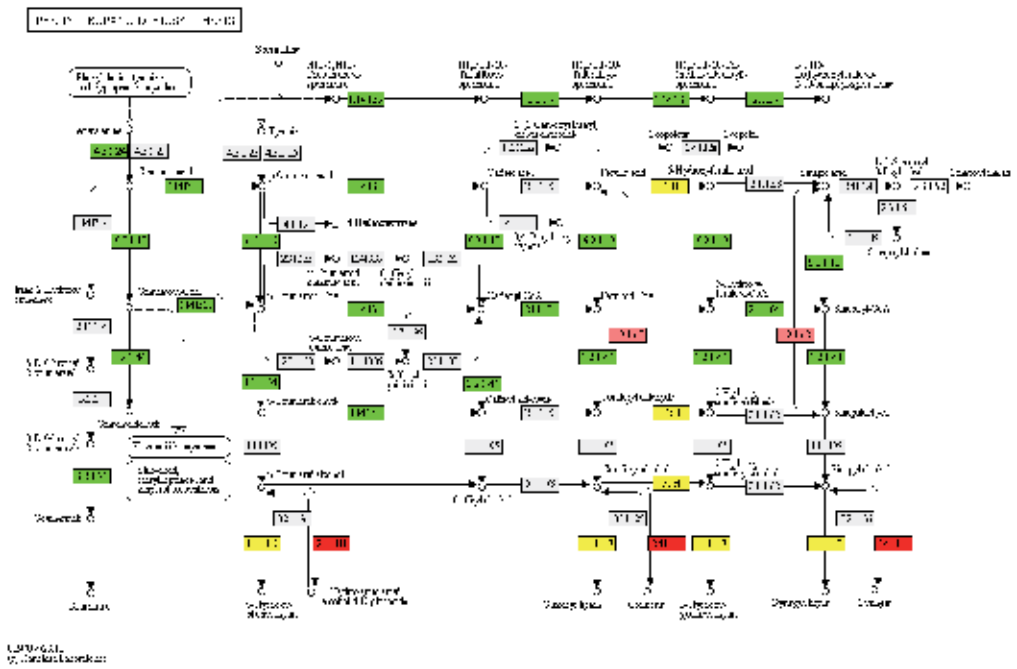


Fig. 7. Expression of genes encoding enzymes involved in phenylpropanoid biosynthesis (A) at 10 dai in a compatible reaction between Williams 82 with SCN and (B) at 6 dai in an incompatible reaction between cv. Peking and SCN are displayed on the KEGG pathway diagram. Enzyme commission numbers in the rectangles are provided by KEGG. Rectangles are colored light green for genes up-regulated in the first 50%, medium green for genes up-regulated in the 50 to 75 quartile and dark green for genes up-regulated in the top 25 %s. Enzymes colored in red are encoded by down-regulated genes using a similar scheme. Enzymes colored in yellow are encoded by more than one gene and different copies of that gene are up- and down-regulated, respectively. Rectangles colored light gray indicate that the genes encoding those enzymes are not annotated in our soybean microarray database.

Phenylalanine ammonia-lyase (EC 4.3.1.34; PAL) can be considered a control point for entry into the phenylpropanoid pathway. There is no major change in expression of genes encoding PAL in the compatible interaction, however at 3, 6 and 9 dai in the incompatible interaction genes encoding PAL are increased in expression, thus suggesting an increased metabolic flow into the pathway. Genes encoding PAL and represented by BI701520, CK606172, and AW351172 are 20- to more than 40-fold increase in expression over that time course (Klink *et al.* 2007a). Increased PAL enzyme activity has been noted in resistant tomato roots infected with RKN, while PAL activity was depressed in susceptible tomato roots (Brueske, 1980). Similarly, in potato PAL activity is higher in resistant plants (Giebel, 1973) . Certainly certain genes involved in isoflavonoid production are increased in expression in the incompatible reaction. For example, the gene encoding chalcone synthase (EC 2.3.1.74),represented by BQ081473, is more than 40-fold increased in expression at 3 and 9 dai in the incompatible interaction, but there is no change in the compatible interaction, while one gene encoding chalcone isomerase is eleveated 4-, 6- and 17-fold in the incompatible interaction (Klink *et al.* 2007b).

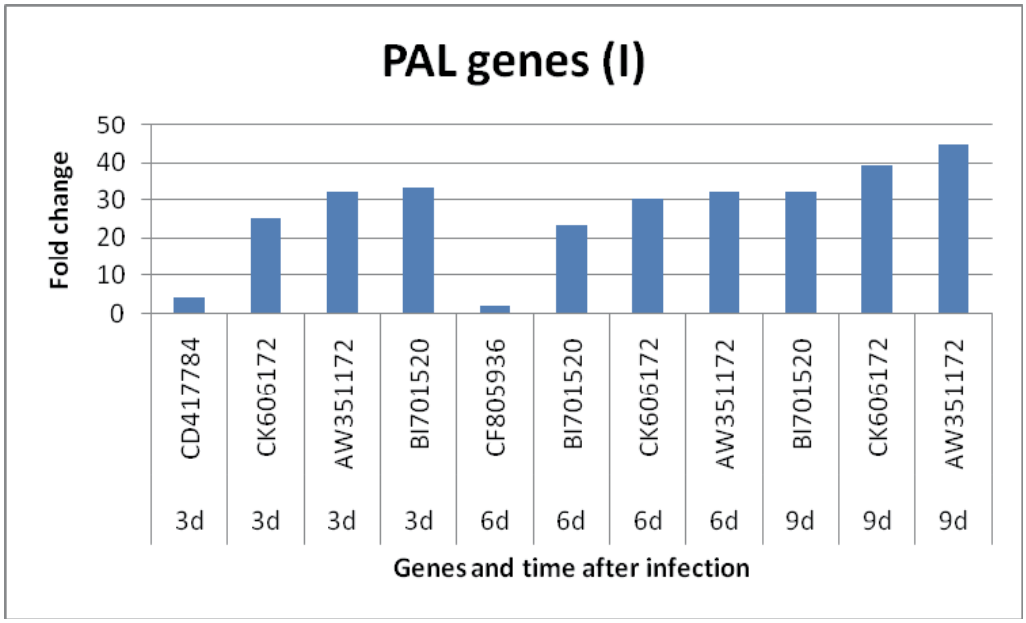


Fig. 8. Expression of genes encoding phenylalanine ammonia-lyase (EC 4.3.1.24) at 3, 6 and 9 dai after infection with SCN in an incompatible interaction with soybean roots (Data from Klink *et al.* 2007b) phenylalanine ammonia-lyase (PAL; EC 4.3.1.24; Fig. 7; Data from Klink *et al.* 2007b).

While microarray studies of genes expressed in the incompatible reaction of soybean plants against SCN revealed an increase in transcript levels of certain genes encoding enzymes involved in glycolysis/gluconeogenesis, jasmonic acid biosynthesis, phenylpropanoid biosynthesis, pathogenesis related proteins, flavonoid biosynthesis, and the methionine salvage pathway (Klink *et al.*, 2010; Alkharouf *et al.*, 2006), the expression of many genes encoding proteins having regulatory and signaling functions, such as cyclins, phosphokinases and transcription factors, were also affected. Genes encoding enzymes belonging to pathways depicted in KEGG and that were highly preferentially expressed were related to those KEGG pathways using PAICE software (Hosseini *et al.*, in preparation) to make interpretation of the data easier. Thus, relationships among genes and pathways were recognized with less difficulty.

3. Conclusions

Soybean genes involved in glycolysis/gluconeogenesis are up-regulated during nematode feeding and several lines of evidence indicate that the gluconeogenesis is occurring. This would allow soybean cells to provide carbohydrates as an energy source to the nematode. Genes encoding enzymes involved in cell wall molding are up-regulated, including cellulases, pectinesterases and xyloglucanases. These increases in gene expression allow the development and expansion of the syncytium for nematode feeding. Genes encoding important enzymes involved in the synthesis of jasmonic acid are down-regulated in the compatible interaction. This would quench the defense response controlled by jasmonic acid and related compounds and allow the nematode to grow and develop in a compatible

reaction. In general, genes encoding pathogenesis-related proteins are more highly expressed in the incompatible interaction and a gene encoding phenylalanine ammonia lyase is much more highly expressed in the incompatible interaction of soybean roots with SCN. Phenylalanine ammonia lyase is major gateway to phenylpropanoid metabolism and to the synthesis of numerous secondary compounds involved in plant defense, All of these data indicate that there is a stronger production of transcripts of genes encoding proteins involved in the plant defense response in the incompatible interaction, while transcripts of many of these genes are lower or the genes are down-regulated leading to a weaker defense response in the compatible reaction of soybean roots to SCN. Gene expression studies performed in soybean has resulted in the understanding gene expression during infection by SCN. The challenge to scientists now is in testing the function genes to understand the molecular circuitry occurring between plants and their parasitic nematodes so new methods of nematode control can be developed.

4. Acknowledgments

The authors gratefully acknowledge support from United Soybean Board project number Y9254, the US-Egypt Science and Technology project number BIO8-001-002 and from the BioGreen 21 Program (no. PJ007031), Rural Development Administration, Republic of Korea and Mississippi Soybean Promotion Board.

5. References

Abad, P. & Williamson V.M. (2010). Plant Nematode Interaction: A sophisticated dialogue. *Advances in Botanical Research* 53:147-192.

Adegbite, A. & Adesiyan, S. (2005). Root extracts of plants to control root-knot nematode on edible soybean. *World Journal of Agricultural Sciences* 1: 18-21.

Alkharouf, N.W., V. Klink, I.B. Chouikha, H.S. Beard, M.H. MacDonald, S. Meyer, *et al.* (2006). Microarray analyses reveal global changes in gene expression of susceptible *Glycine max* (soybean) roots during infection by *Heterodera glycines* (soybean cyst nematode). *Planta* 224: 838-852.

Bekal, S., Niblack, T.L. & Lambert K.N. (2003). A chorismate mutase from the soybean cyst nematode *Heterodera glycines* shows polymorphisms that correlate with virulence. *Molecular Plant-Microbe Interactions* 16:439-446

Bird, D.McK. & Koltai, H. (2000). Plant parasitic nematodes: Habitats, hormones, and horizontally-acquired genes. *Journal of Plant Growth Regulation* 19:183-194.

Branch, C., Hwang, C.F., Navarre, D.A. & Williamson, V.M. (2004). Salicylic acid is part of the *Mi-1*-mediated defense response to root-knot nematode in tomato. *Molecular Plant-Microbe Interactions* 17:351-358/

Bruska, C.H. (1980). Phenylalanine ammonia lyase acitivity in tomato roots infected and resistant to the root-knot nematode, *Meloidogyne incognita. Physiological Plant Pathology* 16:409-414.

Caillaud, M.; Dubreuil, G.; Quentin, M.; Perfus-Barbeoch, L.; Lecomte, P.; Engler, J.; Abad, P.; Rosso, M.& Favery, B. (2001). Root-knot nematodes manipulate plant cell functions during a compatible interaction. *Moleculaar Plant Microbe Interactions* 13:288-299

Caillaud, M.C., Dubreuil, G., Quentin, M., Barbeoch, L.P., Lecomte, P., Engler, *et al.* (2008). Root-knot nematodes manipulate plant cell functions during a compatible interaction. *Journal of Plant Physiology* 165: 104—113.

Chen, Z., Zhen, Z., Huang, J., Lai, Z. & Fan, B. (2009). Biosynthesis of salicylic acid in plants. *Plant Signaling & Behavior* 2009, 4(6):493-496.

Davis, E.L., Hussey, R.S & Baum, T.J. (2004). Getting to the root s of parasitism by nematodes. *TRENDS in Parasitology* 20:134-141.

Davis, E.L. & Mitchum, M.G. (2005). Nematodes: Sophisticated parasites of Legumes. *Plant Physiology* 137:1182-1188.

Doyle, E.A. & Lambert, K.N. (2003). *Meloidogyne javanica* chorismate mutase 1 alters plant cell development. Molecular Plant Microbe Interactions 16:123-131.

Elling, A.A., Davis, E.L., Hussey, R.S. (2007). Active uptake of cyst nematode proteins into the plant nucleus. *International Journal of Parasitology* 37:1269-1279.

Eisenback and Triantaphyllou, (1991) Root-knot nematodes: *Meloidogyne* species and races. *In* Nickle, W.R. (ed), *Manual of Agricultural Nematology*. Marcel Dekker Inc, New York USA, pp. 191-274.

Endo, B.Y. (1964). Penetration and development of *Heterodera glycines* in soybean roots and related anatomical changes. *Phytopathology* 54:79-88.

Endo, B.Y. (1965). Histological responses of resistant and susceptible soybean varieties, and backcross progeny to entry and development of *Heterodera glycines. Phytopathology* 55:375-81.

Endo BY. Cellular responses to infection. In *Biology and Management of the Soybean Cyst Nematode.* Ed Riggs RD, Wrather JA, St. Paul, MN. APS Press 1992:37-49.

Fekete, E., Seiboth B., Kubicek C., Szentirmai A. & Karaffa, L. (2008). Lack of aldose 1-epimerase in *Hypocrea jecorina* (anamorph *Trichoderma reesei*): a key to cellulose gene expression on lactose. *Proceedings National Academy of Science* 105 (20):7141-6

Giebel, J. (1973). Phenylalanine and tyrosine ammonia-lyase activities in potato roots and their significance in potato resistance to *Heterodera rostochiensis. Nematologica* 19:3-6.

Gobel, C., Feussner, I. Schmidt, A., Scheel, D., Sanchez-Serrano, J., Hamberg, M. *et al*. (2001). Oxylipin profiling reveals the preferential stimulation of the 9-lipoxygenase pathway in elicitor-treated potato cells. *The Journal of Biological Chemistry* 276:6267-6273.

Goellner, M., Wang, X. & Davis, E.L. (2001). Endo-β-1,4-glucanase expression in compatible plant-nematode interactions. *The Plant Cell* 13:2241-2255.

Goverse, A., Engler, J.D.A., Verhees, J., Krol, S.V.D., Helder, J. & Gheysen, G. (2000). Cell cycle activation by plant parasitic nematodes. *Plant Molecular Biology* 43:747-761.

Hamamouch, N., Li, C., Seo, P.J., Park, C.M. & E.L. Davis. 2010. Expression of Arabidopsis pathogenesis-related genes during nematode infection. *Molecular Plant Pathology* DOI: 10.1111/J.1364-3703.2010.00675.x/pdf

Hennig, J., Dewey, R.E., Cutt, J.R. & Klessig, D.F. (1993). Pathogen, salicylic acid and developmental dependent expression of β-1,3-glucanase/GUS gene fusion in transgenic tobacco plants. *Plant Journal* 4:481-493.

Hofmann, J.& Grundler, F.M.W. (2008a). Starch as a sugar reservoir for nematode-induced syncytia. *Plant Signaling & Behavior* 3:961-962

Hofmann, J., El Ashry, A.E.N., Anwar, S., Erban, A., Kopka, J.& Grudler, F. (2010). Metabolic profiling reveals local and systemic responses of host plants to nematode parasitism. *The Plant journal* 62:1058-1071

Hofmann, J., Szakasits, D., Blochl, A., Sobczak, M., Daxboc-Horvath, S., Golinowski, W., Bohlmann, W.,& Grundler, F.M.W.. (2008b). Starch serves as carbohydrate storage in nematode-induced syncytia. *Plant Physiology* 146:228-235

Huang, G.Z., Gao, B., Maier, T., Allen, R. , Davis, E.L., Baum, T.J. & Hussey, R.S. 2003. *Molectular Plant-Microbe Interactions* 19:376-381.

Ibrahim, H.M.M., Alkharouf, N.W. , Meyer, S.L.F., Aly, M.A.M., Gamal El-Din, A.E.K., Hussein, E.H.A.& Matthews, B.F. (2010). Post-transcriptional gene silencing of root knot-nematode in transformed soybean roots. *Experimental Parasitology* 127 (1): 90-99.

Ibrahim, H.M.M., Hosseini, P., Alkharouf, N.W., Aly, M.A.M., Gamal El-Din A.E.K.Y., Hussein, E.H.A., & Matthews, B.F. (2011). Analysis of gene expression in soybean roots in response to root-knot nematode using microarray and KEGG pathways. *BMC Genomics*. In press.

Ithal, N., Recknor, J., Nettleston, D., Nettleton, D., Maier, T., Baum, T.J. & Mitchum, M.G. (2007a). Parallel genome-wide expression profiling of host and pthaogen during soybean cyst nematode infection of soybean.. *Molecular Plant-Microbe Interactions* 20: 510-525

Ithal, N., Recknor, J., Nettleston, D., Hearne, L., Maier, T., Baum, T.J. & Mitchum, M.G. (2007b). Developmental transcript profiling of cyst nematode feeding cells in soybean roots. *Molecular Plant-Microbe Interactions* 20: 293-305

Jammes, F., Lecomte, P., de Almeida-Engler, J., Bitton, F., Martin-Magniette, M.L., Renou, J.P. *et al.* (2005) Genome-wide expression profiling of the host response to root-knot nematode infection in Arabidopsis. *The Plant Journal* 44:447-458.

Klink, V.P., Alkharouf, N., MacDonald, M. & Matthews, B.F. (2005). Laser capture microdissection (LCM) and analysis of Glycine max (soybean) syncytial cells formed by the soybean cyst nematode *Heterodera glycines*. *Plant Molecular Biology* 59: 969-983.

Klink, V.P., Hosseini, P., Matsye, P., Alkharouf, N.W. & Matthews, B.F.(2009a). A gene expression analysis of syncytia laser microdissected from the roots of the *Glycine mzx* (soybean) genotype PI 548402 undergoing a resistant reaction after infection by *Heterodera glycines* (soybean cyst nematode). *Plant Molecular Biology* 71:525-567

Klink, V.P., Hosseini, P., MacDonald, M.H., Alkharouf, N.W.& Matthews, B. F. (2009b). Population-specific gene expression in the plant pathogenic nematode *Heterodera glycines* exists prior to infection and during the onset of a resistant or susceptible reaction in the roots of the *Glycine max* genotype Peking. *BMC Genomics,* 10. http://www.biomedcentral.com/1471-2164/10/111

Klink, V.P., Hosseini, P., Matsye, P., Alkharouf, N.W. & Matthews, B.F (2010a). Syncytium gene expression in *Glycine max* [PI88788] roots undergoing a resistant reaction to

the parasitic nematode *Heterodera glycines*. *Plant Physiology and Biochemistry*. 48:176-193

Klink, V.P., Kim, K.H., Martins, V., MacDonald, M.H., Beard, H.S., Alkharouf, N.W., Lee, S.K., Park, S.C.& Matthews, B. F. (2009b). A correlation between host-mediated expression of parasite genes as tandem inverted repeats and abrogation of development of female *Heterodera glycines* cyst formation during infection of *Glycine max*. *Planta* 230, 53–71.

Klink, V.P., Matsye PD, Lawrence GW. 2011a. Developmental Genomics of the Resistant Reaction of Soybean to the Soybean Cyst nematode, pp. 249-270, In Plant Tissue Culture and Applied Biotechnology. Eds. Kumar A., Roy S. Aavishkar Publishers, Distributors, India (In Press)

Klink, V.P., Hosseini, P., Matsye, P.D., Alkharouf, N., Matthews, B.F. 2011b. Differences in gene expression amplitude overlie a conserved transcriptomic program occurring between the rapid and potent localized resistant reaction at the syncytium of the *Glycine max* genotype Peking (PI 548402) as compared to the prolonged and potent resistant reaction of PI 88788. *Plant Molecular Biology* 75: 141-165.

Klink, V.P., Overall, C.C., Alkharouf, N., MacDonald, M.H., Matthews, B.F. (2007a). A time-course comparative microarray analysis of an incompatible and compatible response by *Glycine max* (Soybean) to *Heterodera glycines* (soybean cyst nematode). *Planta* 226: 1423-1447

Klink, V.P., Overall, C.C., Alkharouf, N., MacDonald, M.H., Matthews, B.F. (2007b). Laser capture microdissection (LCM) and comparative microarray expression analysis of syncytial cells isolated from incompatible and compatible soybean roots infected by soybean cyst nematode (*Heterodera glycines*). *Planta* 226: 1389-1409

Klink, V.P., Overall, C.C., Alkharouf, N., MacDonald, M.H. & Matthews, B.F. (2010b). Microarray detection calls as a means to compare transcripts expressed within syncytial cells isolated from incompatible and compatible soybean (*Glycine max*) roots infected by the soybean cyst nematode (*Heterodera glycines*). *Journal of Biomedicine and Biotechnology* 1-30

Koenning, S. R., Overstreet, C., Noling, J. W., Donald, P. A., Becker, J. O. & Fortnum, B. A. (1999). Survey of crop losses in response to phytoparasitic nematodes in the United States for 1994. *Journal of Nematology* 31(4S): 587–618.

Lambert, K.N., Allen, K.D., & Sussex, I.M. (1999). Cloning and characterizationof an esophageal-gland specific chorismate mutase from the phytopatogenic nematode *Meloidogyne javanica.* Molecular Plant Microbe Interactions 12:328-336

Lilley, C.H., Atkinson, H.J., Urwin, P.E. (2005). Molecular aspects of cyst nematodes. *Molecular Plant Pathology* 6:577-588.

Mitchum, M.G., Sukno, Wand, X., Shani, z., Txabary, G. Shoseyov, O. & Davis E.L. (2004). The promoter of the *Arabidopsis thaliana Cel1* endo-1,4-β glucanase gene is differentially expressed in plant feeding cells induced by root-knot and cyst nematodes. *Molecular Plant Pathology* 5:175-181.

Mitchum, M.G. & Baum, T.J. (2008). Genomics of the soybean cyst nematode-soybean interaction. *In* Stacy, G. (ed.), *Genetics and Genomics of Soybean*, Springer Science & Business Media pp. 321-341.

Niblack, T.L., Lambert, K.N. & Tylka, G.L. (2006). A model plant pathogen from the kindom Animalia: *Heterodera glycines*, the soybean cyst nematode. In Annual Reveiw of Phytopathology 44:283-303.

Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N., & Ohashi, Y. (1998). Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant Cell Physiology* 39:500-507.

Nishitani, K. (1997). The role of endoxyloglucan transferase in organization of plant cell walls *International Review of Cytology* 173:157-206.

Oshima, M., Itoh, H., Matsuoka, M. Murakami, T. & Pjasjo. U. (1990). Analysis of stress-induced or salicylic acid-induced expression of the pathogenesis-related 1a protein gene in transgenic tobacco. *Plant Cell* 2:95-106.

Reddy PS, Kumar T C, Reddy,MN, Reddanna SP. Differential formation of octadecadienoic acid and octadecatrienoic acid products in control and injured/infected potato tubers. *Biochimica et Biophysica Acta* 2000, 1483:294-300.

Replogle, A., Wang, J., Bleckmann, A., Hussey, R.S., Baum, T.J., Sawa, S., *et al.* (2011). Nematode CLE signaling in Arabidopsis requires CLAVATA2 and CORYNE. *The Plant Journal* 65:430-440.

Sasser, J.N. (1980) Root-knot nematodes: A global menace to crop production. *Plant Disease* 64:36-41.

Smant, G., Stokkermans, J.P.W.G., Yan, Y. DeBoer, J.M., Baum, T.J.Wang, X. *et al.* (1998). Endogenous cellulases in animals: isolation of beta-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes. *Procedings National Academy of Science USA* 95:4906-4911.

Tremblay, A., Hosseini, P., Alkharouf, N.W., Li, S. & Matthews, B.F. (2010). Transcriptome analysis of a compatible response by *Glycine max* to *Phakopsora pachyrhiza* infection. Plant Science. 2010: Article ID 491217, 30 pages

Uehara, T., Sugiyama, S., Matsuura, H., Arie, T. & Masuta, C. (2010). Resistant and susceptible response in tomato to cyst nematode are differentially regulated by salicylic acid.*Plant & Cell Physiology* 51:1524-1536.

Van Loon, L.C., Rep, M. & Pieterse, C.M.J. (2006). Significance of Inducible Defense-related Proteins in Infected Plants. *Annual Review of Phytopathology* 44:135–162.

Van Loon, L.C. & Van Strien, E.A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology.* 55:85-97.

Wang, X., Mitchum, M.G., Gao, B., Li, C., Diab, Hl, Baum, TlJl, Hussey, R.S., & Davis, E.L. (2005). A parasitism gene from a plant-parasitic nematode with function similar to *CLAVATA3/ESR (CLE)* of *Arabidopsis thaliana. Molecular Plant Microbe Interactions* 14:536-544.

Wang, J., Replogle, A., Hussey, R., Baum,T., Wang, X., Davis, E.L. & Mitchum, M.G. (2010). Identification of potential host plant micis of CLV3/ESR (CLE)-like peptides from the plant-parasitic nematode *Heterodera schachtii. Molecular Plant Pathology.* DOI: 10.1111/J.1364-3703.2010.00660.X.

Wildermuth, M.C., Dewdney, J., Wu, G. & Ausubel, F.M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. *Nature* 414:562-565.

Williamson, V.M. & Gleason, G.A. (2004). Plant-nematode interactions. *Current Opinion in Plant Biology* 6:327-333.

Wrather J.A. & S.R. Koenning. (2006). Estimates of disease effects on soybean yields in the United States 2003 to 2005. *Journal of Nematology* 38:173-180.

Young, L.D. (1992). Epiphytology and life cycle, *in* Riggs, R.D. & Wrather, A.W. (ed.), *Biology and management of the Soybean Cyst Nematode.* APS Press, St. Paul, pp 27-36.

5

Phenotypic and Genotypic Variability in *Cercospora kikuchii* Isolates from Santa Fe Province, Argentina

María G. Latorre Rapela, Mauro A. Colombini, Ana M. González, Stella M. Vaira, Roxana Maumary, Mónica C. Mattio, Elena Carrera and María C. Lurá
Universidad Nacional del Litoral
Argentina

1. Introduction

More than 100 fungal diseases affecting soybean (*Glycine max* (L.) Merr.) culture have been found worldwide, though only 35 of them are of great economic concern since they cause a 10 to 15% yield decrease (Ivancovich & Botta, 2003; Yeh & Sinclair, 1980).

In Argentina, "Late-cycle diseases" (LCD) constitute a complex of infections affecting the culture of this leguminous, particularly in the reproductive stages. The first symptoms appear from the onset of carpel formation, thus producing premature plant maturation, a reduction in yield (5 to 8%) and seed quality loss. Due to the climate conditions of the region, leaf blight and purple seed stain, is one of the LCD prevailing in the central-northern region of Santa Fe Province (Formento, 2005; Ivancovich & Botta, 2003).

The causal agent of the disease is the fungus *Cercospora kikuchii* (T. Matsumoto & Tomoyasu) M.W. Gardner, which produces irregular injuries on the leaves, forming reddish-purple necrotic areas, pale pink to dark purple stains on the seeds, together with cracks on the outer coat (Formento, 2005). *C. kikuchii* belongs to the *Cercospora* taxonomic complex, which is assumed to be host-specific (Crous & Braun, 2003), and therefore its species are normally identified from the phenotypic characteristics they show when grown on their natural substrate, being much more difficult to be characterized from artificial media (Almeida et al., 2005; Gams et al., 2007).

One of the most important factors determining the pathogenicity of this fungus is the production of cercosporin, a red exotoxin (Kuyama & Tamura, 1957; Upchurch et al.,1991). Another aspect to consider is the genetic variability found in some fungi, which has determined the description of different races; that is why the use of some methodology to detect inter- and intraspecific variations between isolates is recommended (Kuyama & Tamura, 1957). In this sense, the Random Amplified Polymorphic DNA (RAPD) technique allows the differentiation between strains since amplification focuses only on the whole genome (Tigano et al., 2003; Williams et al., 1990).

Although some Brazilian research groups have been studying aspects related with this phytopathogen (Almeida et al., 2003) only a few reports have been found in Argentina and, in particular in Santa Fe Province, about epidemiology and population structure of *C. kikuchii.*

The aim of the present work was to determine the occurrence of phenotypic and genotypic variability between isolates of *C. kikuchii* in various regions of Santa Fe Province.

2. Methods

2.1 Fungal isolation

Nineteen samples with visible symptoms of leaf blight obtained from soybean lots not less than 0.5 ha in size, were processed. Soybean samples were collected from several places like Emilia, Margarita, Esperanza, Gobernador Crespo and San Justo, in Santa Fe Province. Sampling was carried out between January and May (2005-2006 campaign) under similar climatic conditions (Table 1).

Meteorological Station	Average			
	Tmax °C	Tmin °C	Tmed °C	RH %
1	26.8	14.4	22.8	67
2	28.8	15.0	22.5	73
3	28.1	15.0	22.9	70

Table 1. Climatic conditions corresponding to different meteorological stations in Santa Fe Province, Argentina. 2005-2006 Campaign. 1: Esperanza; 2: Emilia, Gobernador Crespo and San Justo; 3: Margarita; Tmax: maximum temperature; Tmin: minimum temperature; Tmed: medium temperature; RH: relative humidity.

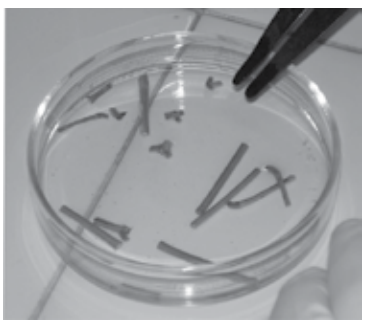


Fig. 1. Tissue pieces being disinfected by immersion.

Fig. 2. Tissue pieces in moist chambers.

Tissue pieces were disinfected by immersion in 3% w/v sodium hypochlorite for 3 min, rinsed with sterile distilled water (Fig.1) and incubated in moist chambers at 26 ± 0.5 ºC, under alternate light cycles (16 h cold light and 8 h in the dark) (Salvador & Garrido, 1990). (Fig. 2).

Observation with stereoscopic magnifying lens (BOECO Germany, BTB 3-C) was performed from the 3rd day of incubation onwards. Once the conidial structure growth was verified, conidia were taken with a sterile needle and suspended in 1 mL sterile water so as to obtain a homogeneous suspension. To produce monosporic cultures, an aliquot (100 µL) was spread over Potato Dextrose Agar (PDA) in a Petri dish and incubated as described above (Dunkle & Levy, 2000). Isolates were labeled with letter C and correlative numbers (Table 2), stored and subsequently included in the strain collection.

Isolate	Location	Lot	Cultivar/varietal
C14	Emilia	A01	A5409
C15	Emilia	A01	A5409
C16	Emilia	A01	A5409
C17	Emilia	A01	A5409
C18	Emilia	A01	A5409
C19	Emilia	A01	A5409
C20	Margarita	A04	TJ2070
C21	Margarita	A04	TJ2070
C22	Margarita	A04	TJ2070
C23	San Justo	A02	A4910
C24	Margarita	A04	TJ2070
C25	G. Crespo	A04	A8000
C26	Emilia	B01	A5409
C27	G. Crespo	A04	A8000
C28	Esperanza	Lote Rural	A7636
C29	Esperanza	Lote Rural	A7636
C30	Esperanza	Lote Rural	A7636
C31	Esperanza	Lote Rural	A7636
C32	Esperanza	Lote Rural	A7636

Table 2. Origin and designation of *Cercospora kikuchii* regional isolates. G. Crespo: Gobernador Crespo.

2.2 Phenotypic characterization

Each fungus was inoculated with a single touch in the center of a Petri dish containing PDA and incubated under the conditions previously described. Macro and microscopic observations were conducted after 15 days, taking into account color and diameter of the colony, red pigment production (cercosporin) and the presence or absence of typical elements of reproduction. For the latter case, plates were discarded only after 25 days of incubation.

Cercosporin production was confirmed following Jenns et al. methodology (Jenns et al, 1989) with slight modifications as described by González et al. (González et al., 2008).

2.3 Genotypic characterization

Every isolate was streak-plated over PDA and incubated for 4 days under the conditions described above. Following incubation, 5 mL sterile water was added and the surface of the colony was rubbed with a wire loop in order to free the fungal elements. The resulting suspension was added to 100 mL of culture medium for *Colletotrichum* (Martinez Culebras et al., 2000), and incubated 48 h in the dark at 28-30 ºC in orbital motion (Orbital Shaker, Forma Scientific, Inc.) at 180 rpm. The mycelium was placed on a nylon filter (200 µm pore diameter) and dried with absorbent paper until all moisture was removed. Once dry, it was extended forming a layer as thin as possible and was dried in stove at 37 $\pm$ 0.2 ºC until constant weight. Then it was ground to thin powder in a mortar.

The same procedure was applied to two strains belonging to the NITE Biological Resource Center (Japan) collection: *C. kikuchii* NBRC 6711 and *C. sojina* NBRC 6715.

In order to extract total DNA, Di Conza et al. (2007) protocol was followed. DNA was quantified through absorbance reading in a spectrophotometer, and its quality determined by means of electrophoresis in 0.8% w/v agarose gel (Sambrook et al., 1989). RAPDs were carried out following Williams et al. (1990), using 20 oligonucleotides (FAGOS/Ruralex, Argentina) (Table 3).

The reaction mixture was prepared for a 50 µL total volume with these components: 2.5 mM magnesium chloride, 125 µM of each dNTPs (INBIOHIGHWAY), 1 µM oligonucleotide, 5 U *taq* DNA polymerase (INBIOHIGHWAY) and 20 ng DNA per reaction. Amplification was carried out using a MJ Research Thermal Cycler under the following conditions: 1 cycle of 5 min at 95ºC, 40 cycles comprising 75 s at 94ºC, 90 s at 36ºC and 150 s at 72ºC, and 1 final cycle of 10 min at 72ºC. Amplification products were separated by electrophoresis in 1.5% w/v agarose gel with 0.5X TBE 0.089M Tris-borate, 0.002 M EDTA. The run time was 180 min, with a 100V constant voltage. The gel was stained with ethidium bromide, and the molecular weight marker was 100-bp DNA Ladder (Promega). Band profiles obtained were photographed and analyzed with Gel Doc XR System (BIORAD-Life Science Cat. # 170-8170) using the Quantity One Software.

All RAPD reactions were done in duplicate.

A matrix with 0 and 1 corresponding to absence and presence of band, respectively, for each one of the images resultant from each oligonucleotide used, was constructed, giving a total of 18 binary matrices with order mx21, where 18 represents the amount of nucleotides considered, m the bands obtained and 21 the total fungi analyzed. From these matrices, distance matrices (21x21) were obtained with the Jaccard coefficient, designed for asymmetric binary variables (Everitt & Hothorn, 2009; Johnson & Wichern, 1998) (Table 4).

Then, an average distance matrix (21x21), essential to apply the cluster technique, was calculated with the 18 distance matrices (Abonyi & Feil, 2007; Bolshakova & Azuaje, 2003; Peña, 2002).

The choice of clustering algorithm depended on the type of data and the purpose of using the technique. It was considered convenient to apply more than one clustering algorithm for a database and then compare the agreement between the results.

Therefore, a partition (FANNY) and a hierarchical (AGNES) algorithm were selected. FANNY computes "diffuse" clusters, giving each item a degree of belonging to the cluster. The algorithm combines the optimum choice of the amount of clusters (k) and the parameter r, linked to the degree of diffusivity or *membership exponent*.

AGNES makes agglomerative hierarchical clusters, the distance between clusters (k) being analyzed by Ward method.
Silhouette Coefficient (SC) and Agglomeration Coefficient (AC) were used for FANNY and AGNES, respectively, to evaluate cluster quality (Kaufman & Rousseeuw, 1990).
Data were processed with R version 2.10.1 Software (Torgo, 2003).

Oligonucleotide	Sequence
OPA-01	5′-CAGGCCCTTC-3′
OPA-02	5′-TGCCGAGCTG-3′
OPA-03	5′-AGTCAGCCAC-3′
OPA-04	5′-AATCGGGCTG-3′
OPA-05	5′-AGGGGTCTTG-3′
OPA-06	5′-GGTCCCTGAC-3′
OPA-07	5′-GAAACGGGTG-3′
OPA-08	5′-GTGACGTAGG-3′
OPA-09	5′-GGGTAACGCC-3′
OPA-10	5′-GTGATCGCAG-3′
OPA-11	5′-CAATCGCCGT-3′
OPA-12	5′-TCGGCGATAG-3′
OPA-13	5′-CAGCACCCAC-3′
OPA-14	5′-TCTGTGCTGG-3′
OPA-15	5′-TTCCGAACCC-3′
OPA-16	5′-AGCCAGCGAA-3′
OPA-17	5′-GACCGCTTGT-3′
OPA-18	5′-AGGTGACCGT-3′
OPA-19	5′-CAAACGTCGG-3′
OPA-20	5′-GTTGCGATCC-3′

Table 3. Oligonucleotides used in RAPD reaction

		Fungi j		Total
		1	**0**	
Fungi i	**1**	a	b	a+b
	0	c	d	c+d
Total		a+c	b+d	a+b+c+d

Table 4. Contingency table summarizing the coincidences in the presence/absence of bands between pairs of fungi. Letters i and j: correspond to the same fungus or to different fungi; 1: presence of bands; 0: absence of bands; a, b, c and d are the frequencies[1].

[1]coefficient of distance is given by:

$J = \frac{b+c}{a+b+c}$. No 0-0 matches in numerator or denominator.

3. Results

3.1 Phenotypic characterization

When considering PDA cultures, the colonies, with sizes between 26-50 mm diameter, appeared as white, green-greyish and pink colored, depending on the isolate. The reverse side showed green, brown or red-brown color. All of them presented a reddish touch on their edge and abundant colorless exudate which became amber to ochre with the passing of time. The pigment diffused to the medium in some of them. Fructification was observed after 15 days of incubation, though sporulation was scarce and unevenly distributed over the colony surface.

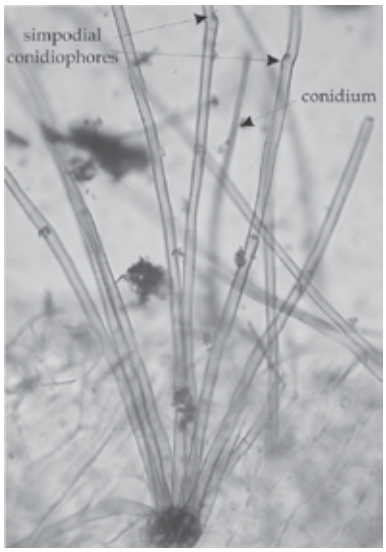


Fig. 3. Growth of *Cercospora kikuchii* on natural substratum (400x)

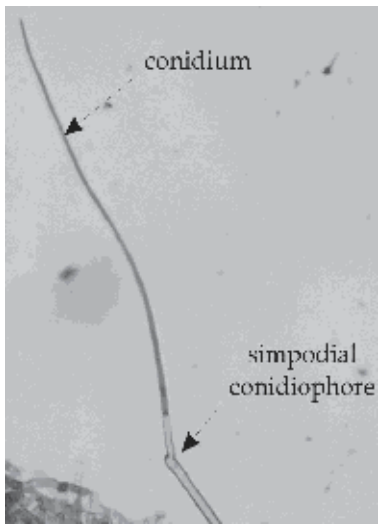


Fig. 4. *Cercospora kikuchii.* Single conidium (400x)

Similar morphologic structures were observed in the 19 isolates. The direct examination of the injury showed brown pigmented, fasciculated and septate conidiophores (200-300 µm long per 4-5 µm wide), with simpodial growth and conidiogenous cells integrated, terminal or intercalary, with thickened and darkened conidiogenous loci (Figure 3). Single, long, acicular, hyaline, pluriseptate (12 to 26 septa) conidia with truncate bases and subacute apices (170 to 190 µm long per 3 µm wide at the base) were observed (Figure 4) as described by Ellis (1971), Crous & Braun (2003) and Solheim (1929).
Conidia formation process involves internal and external walls of the conidiogenous cells, so when the conidio arises, a scar appears in its origin, on the conidiogenous cell (Cai, 2004; Fernández et al., 1991).

3.2 Genotypic characterization

A total of 90 bands were obtained, 100% of them were polymorphic. Amplification size ranged between 107 bp and 2750 bp, an average of 51 amplified fragments/oligonucleotide being produced. OPA-01 was the oligonucleotide which produced the least amount of bands (1) and OPA 12 the most (23) amplified fragments (Table 5). Amplifications with OPA-02 and OPA-06 were not satisfactory.
Figure 5 shows band profiles obtained with oligonucleotide OPA-14.

Oligonucleotide	Maximum number of bands	Minimum number of bands	Total bands	Number of polymorphic bands	Pair of bases (bp)
OPA-01	19	1	49	49	1846-168
OPA-03	17	9	49	49	1437-202
OPA-04	16	6	46	46	1451-156
OPA-05	19	7	48	48	1485-107
OPA-07	14	5	49	49	1599-197
OPA-08	16	3	58	58	2009-253
OPA-09	14	7	53	53	1561-134
OPA-10	18	4	56	56	1797-114
OPA-11	13	5	45	45	1422-212
OPA-12	23	7	55	55	1883-200
OPA-13	17	8	51	51	1495-156
OPA-14	15	6	44	44	1999-309
OPA-15	16	7	53	53	2730-241
OPA-16	18	8	50	50	2252-215
OPA-17	16	4	60	60	2673-213
OPA-18	18	9	51	51	1655-229
OPA-19	18	9	58	58	2750-208
OPA-20	16	7	51	51	1572-242
Total			926	926	

Table 5. Total number of bands and polymorphic bands obtained with the oligonucleotides selected for RAPD

A FANNY cluster analysis with k= 6 and r =1.2 (optimum combination) was carried out. Few distances near 0.2 were observed (Fig 6). C21 and C22 (isolated from Margarita and

same cultivar) were strongly related, as well as C30 and C31, both of them isolated from Esperanza (same cultivar). Great genetic distances for the remaining fungi were detected (Fig.7).

Given the optimum combination obtained with Fuzzy analysis (FANNY), the AGNES algorithm was applied considering the number of clusters found (k=6).

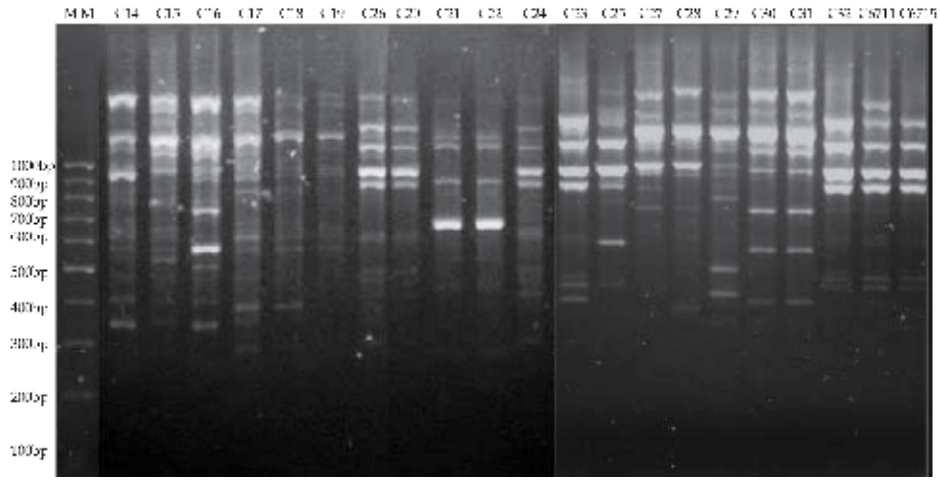


Fig. 5. Sample gel with patterns produced by *Cercospora kikuchii* isolates and NBRC strains using OPA-14. MM: molecular marker 100 bp; C14, C15, C16, C17, C18, C19, C26, C20, C21, C22, C24, C23, C25, C27, C28, C29, C30, C31, C32: regional isolates; C6711: *Cercospora kikuchii* NBRC 6711; C6715: *Cercospora sojina* NBRC 6715.

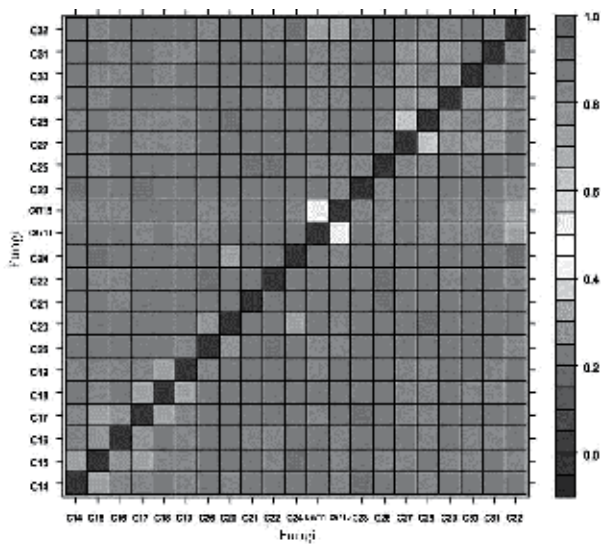


Fig. 6. Average Distance Matrix. C32, C31, C30, C29, C28, C27, C25, C23, C24, C22, C21, C20, C26, C19, C18, C17, C16, C15, C14: regional isolates; C6711: *Cercospora kikuchii* NBRC 6711; C6715: *Cercospora sojina* NBRC 6715.

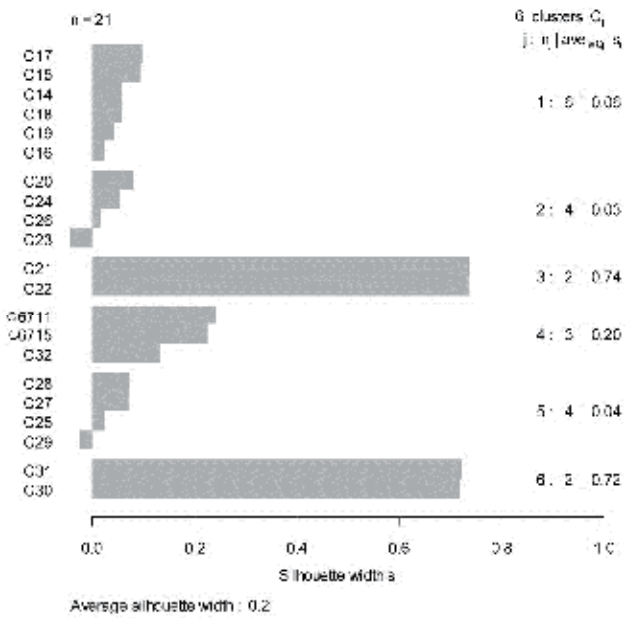


Fig. 7. Silhoutte plot of FANNY (x= average distance; k=6; membership exponent (r) = 1.2). C17, C15, C14, C18, C19, C16, C20, C24, C26, C23, C21, C22, C32, C28, C27, C25, C29, C31, C30: regional isolates; C6711: *Cercospora kikuchii* NBRC 6711; C6715: *Cercospora sojina* NBRC 6715.

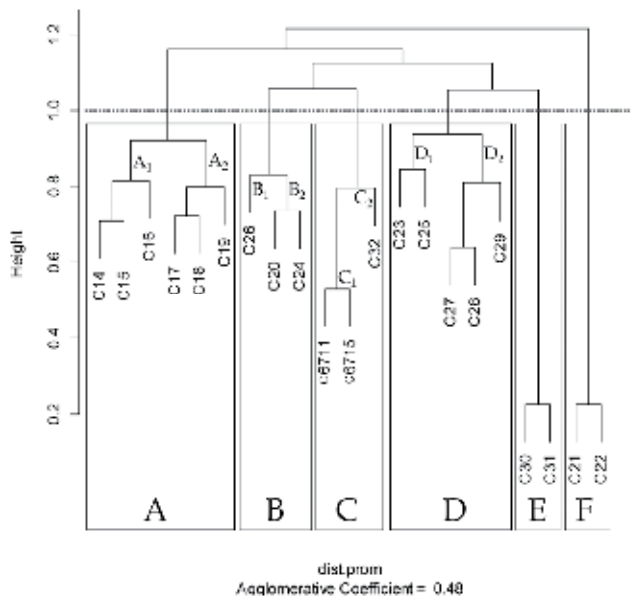


Fig. 8. Dendrogram of AGNES (x= average distance, method = Ward). C14, C15, C16, C17, C18, C19, C26, C20, C24, C32, C23, C25, C27, C28, C29, C30, C31, C21, C22: regional isolates; C6711: *Cercospora kikuchii* NBRC 6711; C6715: *Cercospora sojina* NBRC 6715.

The AGNES cluster analysis resulted in a dendrogram that showed great genetic distances between different fungi (Figure 8). Cluster A included six isolates, C14, C15 and C16 in cluster A_1 and C17, C18 and C19 in A_2, all of them from the same origin (Emilia, Table 2). Cluster B included three fungi, which were grouped in two subclusters. B_1 included C26 which, although isolated from Emilia, belonged to another lot, and B_2 included C20 and C24, both isolated from the same region (Margarita) and cultivar (Table 2).
Cluster C included both strains NBRC (C6711 and C6715) (subcluster C_1) and C32 isolated from Esperanza (subcluster C_2). Cluster D included 5 fungi, C23 and C25 from different origin and cultivar (subcluster D_1) and C27 (same origin than C25), C28 and C29 isolated from Esperanza (subcluster D_2) (Table 2). Finally, clusters E and F included C30 and C31, (isolated from Esperanza) and C21 and C22 (from Margarita), respectively.

4. Discussion

Soybean is one of the most important crops in Argentina, and it has been characterized by an incredible rate of adoption and growth. Twenty-one percent of the cultivated lands of Argentina are in Santa Fe Province, whose main crop, soybean, makes it the main national producer (Aizen et al., 2009; Penna & Lema, 2002). However, soybean crops are affected by several diseases which decrease the total production.
This study aims at contributing to the knowledge of one of the most frequent soybean phytopathogens. Phenotypic and genotypic variations among isolates of *C. kikuchii* from infected soybean corresponding to the centre-northern region in Santa Fe Province, geographically separated but with similar climatic conditions, were studied.
When grown on PDA, and considering micromorphology, the isolated fungi showed colonies with different macroscopic aspects. These results would be in agreement with those reported by Almeida et al. (2005) on Brazilian isolates of *C. kikuchii*. The red pigment observed around some of the colonies was caused by the presence of cercosporin, a pathogenicity factor of *C. kikuchii,* as described by many authors (Assante et al., 1997; Fajola, 1978; Upchurch et al., 1991).
In general, fungi belonging to genus *Cercospora*, as well as other similar genera, exhibit great difficulty for sporulating on artificial culture media (Avila de la Calle et al., 2004; Cadwell, 1994; Yeh & Sinclair, 1980). This limitation was also proved in this study.
Information about which is the optimum medium for studying *Cercospora* sporulation is diverse and confuse, there being no uniform criteria between the different authors. This particular fact evidences the great "inter" and "intra" variability among species belonging to this genus (Brunelli, 2004; Cai, 2004; Chen et al., 1979; Jenns et al., 1989; Salvador & Garrido, 1990).
Given that genus *Cercospora* belongs to the group of Dematiaceous (dark-colored) fungi, it shows septated and olive-brown pigmented hyphae. It is worth mentioning that the number of conidia, its size and number of septa are affected both by the environment and the culture media used (Cai, 2004). Yeh & Sinclair (1980) reported that conidiophore and conidia size differed between isolates even when incubated under the same conditions.
Since typing is a necessary first step in knowing pathogens (Redondo et al., 2009), techniques based on DNA polymorphisms are especially valuable to enhance epidemiological studies.

Genetic variability could be found both between isolates from the same region and between those from different regions, thus confirming previous results (González et al., 2008; Lurá et al., 2007), as reported by Almeida et al. and Cai for *C. kikuchii* and other species (Almeida et al., 2003, 2005; Cai, 2004). No isolates turned out to be 100 % similar. These findings are not easy to be explained. However, it is essential to consider that this fungus is transmitted through the soybean seeds and, being necrotrophic, it can survive in the stubble. As a great increase in the soybean producing area has occurred in Argentina in the last decade, the turnover of seeds from traditional to new production areas has increased accordingly (Secretaría de Agricultura, Ganadería, Pesca & Alimentos [SAGPyA], 2003). The survival of the fungus in the stubble, on the other hand, makes it the main source of primary inoculum for the re-infection in the next campaign (Sillón, 2007).

According to Pujol Vieira dos Santos et al. (2002) and Stenglein & Ballati (2006), many factors could have been affecting polymorphism analysis, e.g. the intraspecific variants of a pathogen, the number of samples selected for analysis, genetic flow between populations, environmental adaptation and selective pressure and migration.

Other factors to be considered, which could explain the genetic variability detected in the study, would be the changes in DNA within the populations of filamentous fungi. As reported by MacDonald (1997), these changes are the consequence of mutations, deletions, pairing systems or gene migration or flow, and population selection, since sexual reproduction is not known in *C. kikuchii* (Almeida et al., 2005). Daboussi & Capy (2003) and Kempken & Kück (1998), on the other hand, state that changes mediated by transposable elements, together with transposition and recombination, provide a wide range of genetic variation, which is useful for the natural self-adaptation of the population to the changing environment and the interaction with another organisms.

With reference to the strains *C. kikuchii* NBRC 6711 and *C. sojina* NBRC 6715, from the Culture Collection of the National Institute of Technology and Evaluation (NITE), Japan, no data were found concerning either their isolation source, location or country of origin. The low similarity they showed, as compared to the regional isolates, could be attributed to the fact that they come from regions naturally different from those selected for this work.

As regards the two oligonucleotides that gave unsatisfactory results to amplification, very few bands were detected with OPA-06, which could be accounted for considering that efficiently amplified DNA regions must be located between two sites complementary to the primers and separated by a distance of a few kb (Williams et al., 1990). In the *C. kikuchii* under study, the sites for this primer could be less frequent, which made the amplification of fragments technically impossible. OPA-02, on the other hand, showed a pattern with numerous bands, its reading and interpretation becoming thus very difficult.

The 6 groups identified by the clustering techniques allowed to distinguish both genetic variability among them and similarity among the fungi belonging to the same group. After comparing the results of both clustering methods, a high genetic homogeneity could be observed in two out of the six ones (third and sixth FANNY groups with the corresponding F and E AGNES groups) since Silhouette coefficient associated to them turned out to be higher than 0.70 with FANNY algorithm and agglomerative coefficient in AGNES technique was close to zero for the same two clusters. Besides, clusters A and C (AGNES) corresponded with the 1st and 4th FANNY clusters, respectively. As regards the rest of the

fungi, little homogeneity was confirmed between the isolates comprising clusters B and D generated by AGNES and clusters 2 and 5 obtained with FANNY.

It was shown that *C. kikuchii* isolates from the same geographic region appeared in different groups. Molecular analyses showed intraspecific variability within *C. kikuchii* isolates from soybean collected in different regions, so it was difficult to establish a relationship between this variability and that of the soybean cultivars from which *C. kikuchii* isolates were obtained. Similar results had been previously reported by González et al. (2008) and Almeida et al. (2005). According to the results here obtained, Argentinian populations of *C. kikuchii* are phenotypically, genotypically and geographically variable. In agreement with Almeida et al. (2005), who consider that this pathogen is easily transmitted by seeds, it is not surprising to find the same haplotypes in different regions.

In Argentina, and in this region in particular, there has been a rapid increase in the soybean producing area since 1970 (Aizen et al., 2009); therefore, the traffic of seeds from traditional to new areas could be responsible for the geographical variability since *C. kikuchii* is a seed borne pathogen. Unfortunately, an insufficient number of isolates was obtained from each area to permit the evaluation of gene flow among populations more accurately.

For countries like Argentina, with large soybean areas it is very important to know the variability of the pathogen in advance in order to prevent resistant cultivars when sown in different areas.

Few studies have been reported concerning the molecular characterization of genus *Cercospora*. Therefore, widening the scope of knowledge about this pathogen biology, and developing strategies to control the cultures intended for human and/or animal use in this region, therefore, would be a great contribution of this work.

5. Conclusions

Results revealed a considerable degree of phenotypic and genotypic variation in the population of *C. kikuchii* infecting soybean crops from the centre-northern region of Santa Fe Province, Argentina.

From the phenotypic- in vitro- viewpoint, isolates were differentiated by color and size of the colonies, as well as by sporulation capacity. As far as genotypic aspect is concerned, differences in the genome of fungi from the different regions were detected, aside from differences among isolates from the same population.

The two statistical techniques applied proved to be adequate since not only genetic variants could be detected among the isolates under study but also similar clusters were obtained in both of them, thereby giving validity to the results. The detection of genetically similar isolates, on the other hand, would make decision-making easier so as to intervene in health issues such as the prevention of diseases produced by these phytopathogenic fungi.

6. Acknowledgements

This work was carried out within CAI+D 2009 Program (Universidad Nacional del Litoral, Argentina). The authors are grateful to engineers participating in RiiA Program.

7. References

Abonyi, J. & Feil, B. (2007). *Cluster analysis for data mining and system identification*. Birkhäuser Verlag AG, ISBN 987-3-7643-7987-2, Germany

Aizen, M., Garibaldi, L. & Dondo, M. (2009). Expansión de la soja y diversidad de la agricultura argentina. *Ecología Austral*, Vol.19, No. 1, pp. 45-54, ISSN 1667-782X

Almeida, A., Abdelnoor, R., Arrabal Arias, C., Carvalho, V., Jacoud Filho, D., Marin, S., Benato, L., Pinto, M. & Carvalho, C. (2003). Genotypic diversity among Brazilian isolates of *Macrophomina phaseolina* revealed by RAPD. *Fitopatologia Brasileira,* Vol.28, No.3, pp. 279-285, ISSN 0100-4158

Almeida, A., Piuga, F., Marin, S., Binneck, E., Sartori, F., Costamilan, L., Teixeira, M. & Lopes, M. (2005). Pathogenicity, molecular characterization, and cercosporin content of Brazilian isolates of *Cercospora kikuchii. Fitopatologia Brasileira,* Vol.30, No.6, pp. 594-602, ISSN 0100-4158

Assante, G., Locci, L., Camarda, L., Merlini, L. & Nasini, G. (1977). Screening of the genus *Cercospora* for secondary metabolites. *Phytochemistry*, Vol.16, No.2, pp. 243-247

Ávila de la Calle, A., Benali, A. & Trapero Casas, A. (2004). Variabilidad morfológica y cultural de *Pseudocercospora cladosporioides*, agente del emplomado del olivo. *Boletín de Sanidad Vegetal-Plagas*, Vol.30, No.2, pp. 369-384, ISSN 0213-6910

Bolshakova, N. & Azuaje, F. (2003). Cluster validation techniques for genome expression data. *Signal Processing,* Vol.83, No.4, pp. 825–833, ISSN 0165-1684

Brunelli, K. (2004). *Cercospora zeae-maydis*: *Esporulaçao, diversidade morfogenética e reaçao de linhagens de milho*. Doctoral Thesis, Universidade de Sao Paulo, Brasil

Cadwell, V. (1994). *Cercosporioid fungi on Australian native plants*. Doctoral Thesis, University of Melbourne, Australia

Cai, G. (2004). *Cercospora Leaf Blight of soybean: Pathogen vegetative compatibility groups, population structure, and host resistance*. Doctoral Thesis, Universidad de Lousiana, USA, Available from http://etd.lsu.edu/docs/available/etd-12112003-143624/

Chen, M., Lyda, S. & Halliwell, R. (1979). Environmental factors influencing growth and sporulation of *Cercospora kikuchii*. *Mycologia*, Vol.71, No.6, pp. 1150-1157, ISSN 0027-5514

Crous, P. & Braun, U. (2003). *Mycosphaerella and its anamorphs: 1. Names published in Cercospora and Passalora*. Centraalbureau voor Schimmelcultures, ISBN 90-70351-49-8, The Netherlands

Daboussi, M. & Capy, P. (2003). Transposable elements in filamentous fungi. *Annual Review of Microbiology*, Vol.57, pp. 275-299, ISSN 0066-4227

Di Conza, J., Nepote, A., Gonzalez, A. & Lurá, M. (2007). $(GTG)_5$ Microsatellite regions in citrinin-producing *Penicillium. Revista Iberoamericana de Micología*, Vol.24, No.1, pp. 34-37, ISSN 1130-1406

Dunkle, L. & Levy, M. (2000). Genetic relatedness of African and United State populations of *Cercospora zeae-maydis. Phytopathology*, Vol.90, No.5, pp. 486-490, ISSN 0031-949X

Ellis, M. (1971). *Dematiaceous Hyphomycetes.* Commonwealth Mycological Institute, ISBN 85198-027-9, England

Everitt, B. & Hothorn, T. (2009). *A handbook of statistical analyses using R* (2nd Edition), Chapman & Hall/CRC, ISBN 978-1420079333, England

Fajola, A. (1978). Cercosporin, a phytotoxin from *Cercospora* species. *Physiological Plant Pathology*, Vol.13, No.2, pp. 157-164

Fernández, F., Glawe, D. & Sinclair, J. (1991). Microcycle conidiation and nuclear behavior during conidiogenesis in *Cercospora kikuchii*. *Mycologia*, Vol.83, No.6, pp. 752-757, ISSN 0027-5514

Formento, N. (December 2005). Enfermedades de fin de ciclo de la soja en Entre Ríos, In: *Instituto Nacional de Tecnología Agropecuaria*, 10.11.2010, Available from http://www.inta.gov.ar/parana/info/documentos/produccion_vegetal/soja/enfermedades/20320_051207_enfe.htm

Gams, W., Verkleij, G. & Crous, P. (2007). *CBS Course of Mycology* (5th edition), Centraalbureau voor Schimmelcultures, The Netherlands

González, A., Turino, L., Latorre Rapela, M. & Lurá, M. (2008). *Cercospora kikuchii* aislada en la provincia de Santa Fe (Argentina): variabilidad genética y producción de cercosporina *in vitro*. *Revista Iberoamericana de Micología*, Vol.25, pp. 237-241, ISSN 1130-1406

Ivancovich, A. & Botta, G. (October 2003). Enfermedades de final de ciclo de la soja, In: *Instituto Nacional de Tecnología Agropecuaria*, 08.10.10, Available from http://www.inta.gov.ar/rafaela/info/documentos/misc100/misc100_15.pdf

Jenns, A., Daub, M. & Upchurch, R. (1989). Regulation of cercosporin accumulation in culture by medium and temperature manipulation. *Phytopathology*, Vol.79, No.2, pp. 213-219, ISSN 0031-949X

Johnson, R. & Wichern, D. (1998). *Applied multivariate statistical analysis* (4th edition), Prentice Hall, ISBN 9780138341947, England

Kaufman, L. & Rousseeuw, P. (1990). *Finding groups in data. An introduction to cluster analysis* (9th edition), John Wiley & Sons, ISBN 978-0471878766, USA

Kempken, F. & Kück, U. (1998). Transposons in filamentous fungi: facts and perspectives. *BioEssays*. Vol.20, No.8, pp. 652–659, ISSN 0265-9247

Kuyama, S. & Tamura, T. (1957). Cercosporin. A pigment of *Cercospora kikuchii* Matsumoto et Tomoyasu. I. Cultivation of fungus, isolation and purification of pigment. *Journal of the American Chemical Society*, Vol.79, No.21, pp. 5725-5726, ISSN 0002-7863

Lurá, M., Di Conza, J., González. A., Latorre Rapela, M., Turino, L., Ibáñez, M. & Iacona, V. (2007). Detección de variabilidad genética en aislamientos de *Cercospora kikuchii* contaminantes de un mismo sembradío de soja. *Revista Argentina de Microbiología*, Vol.39, No.1, pp. 11-14, ISSN 1851-7617

MacDonald, B. (1997). The population genetics of fungi: Tools and techniques. *Phytopathology*, Vol.87, No.4, pp. 448-453, ISSN 0031-949X

Martinez Culebras P., Barrio, E., García M. & Querol A. (2000). Identification of *Colletotrichum* species responsible for anthracnose of strawberry based on the

internal transcribed spacers of the ribosomal region. *FEMS Microbiology Letters*, Vol. 189, No.1, pp 97-101, ISSN 0378-1097

Penna, J. & Lema, D. (2002). Adoption of herbicide resistant soybeans in Argentina: an economic analysis, In: *Economic and Environmental impacts of Agbiotech*, Kalaitzandonakes N, Dordrecht: Kluwer Academic Publishers, Available from http://www.inta.gov.ar/ies/docs/doctrab/adoption_dt_18.PDF

Peña, D. (2002). *Análisis de datos multivariantes*, McGraw-Hill S.A./Interamericana, ISBN 8448136109, España

Pujol Vieira dos Santos, A., Santos Matsumura, A. & Van der Sand, S. (2002). Intraspecific genetic diversity of *Drechslera triciti-repentis* as detected by a ramdom amplified polymorphic DNA analysis. *Genetic and Molecular Biology*, Vol.25, No.2, pp. 243-250, ISSN 1415-4757

Redondo, C., Cubero, J. & Melgarejo P. (2009). Characterization of *Penicillium* species by ribosomal DNA sequencing and BOX, ERIC and REP-PCR analysis. *Mycopathologia*, Vol.168, No.1, pp. 11-22, ISSN 0301-486X

Secretaría de Agricultura, Ganadería, Pesca & Alimentos (SAGPyA). (December 2003). Mercado de granos, In: *Informe diario*, (05.12.2003), Available from http://www.minagri.gob.ar/new/0-0/programas/dma/Informe_diario/2003/infogra_2003-12-05.pdf

Salvador, D. & Garrido, M. (1990). Características culturales y patogenicidad del hongo causante de la mancha en cadena del sorgo. *Fitopatologia Venezolana*, Vol.3, pp. 11-15, ISSN 0798-0035

Sambrook, J., Fritsch, E. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual* (2nd edition), Cold Spring Harbor, ISBN 0-87969-309-6, New York

Sillón, M. (April 2007). Proyecto de asintomáticas: ¿Qué esconden nuestros cultivos de soja?, In: *El Litoral*, (07.04.2007), Available from http://www.ellitoral.com/ index.php/diarios/2007/04/07/laregion/REG-11.html

Solheim, W. (1929). Morphological studies of genus *Cercospora*. *Illinois Biological Monographs*. Vol.12, pp. 1-84, ISSN 0073-4748

Stenglein, S. & Ballatti, P. (2006). Genetic diversity of *Phaeoisariopsis griseola* in Argentina as revealed by pathogenic and molecular markers. *Physiological and Molecular Plant Pathology*, Vol.68, No.4-6, pp. 158-167, ISSN 0885-5765

Tigano, M., Aljanabi, S. & Marques de Mello, S. (2003). Genetic variability of Brazilian *Alternaria* spp. isolates as revealed by RAPD analysis. *Brazilian Journal of Microbiology*, Vol.34, No.2, pp. 117-119, ISSN 1517-8382

Torgo, L. (2003). *Data Mining with R: learning with case studies*. CRC Press, ISBN 9781439810187. 05.05.2010, Available from http://www.math.iupui.edu /~samiran/STAT521f09_files/DataMiningWithR.pdf

Upchurch, R., Walker, J., Rollins, J., Ehrenshaft, M. & Daub, M. (1991). Mutants of *Cercospora kikuchii* altered in cercosporin synthesis and pathogenicity. *Applied of Environmental Microbiology*, Vol.57, No.10, pp. 2940-2945, ISSN 0099-2240

Williams, J., Kubelik, A., Livak, K., Rafalski, J. & Tingey, S. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, Vol.18, No.22, pp. 6531-6535, ISSN 0305-1048

Yeh, C. & Sinclair, J. (1980). Sporulation and variation in size of conidia and conidiophores among five isolates of *Cercospora kikuchii*. *Plant Disease*, Vol.64, No.4, pp. 373-374, ISSN 0191-2917

6

Soybean Fatty Acid Desaturation Pathway: Responses to Temperature Changes and Pathogen Infection

Robert G. Upchurch
Agricultural Research Service-US Department of Agriculture, Raleigh, NC USA

1. Introduction

Soybean [Glycine max (L.) Merr] is the largest oilseed crop produced and consumed worldwide, accounting for 58% of the world oilseed production (SoyStats, 2011), yet the oil produced from most available cultivars is still lacking in several quality characteristics. For example, the oil is too low in oleate and/or too high in linolenate content with resulting negative impacts on oil stability and human nutrition. Three fatty acid metabolism enzymes, the stearoyl-acyl carrier protein-desaturases (encoded by the *GmSACPD* genes), the omega-6 desaturases (*GmFAD2s*), and the omega-3 (*GmFAD3s*) desaturases largely determine the relative degree of unsaturated fatty acids and the content of the C_{18} fatty acids stearate (18:0), oleate (18:1), linoleate (18:2), and linolenate (18:3) in vegetative and seed lipids. In vitro studies have shown that it is possible to redesign soluble fatty acid desaturases from plants for altered fatty acid substrate and double bond position (Cahoon et al., 1997, Whittle et al., 2005) and in that way potentially alter the fatty acid content of plant lipids. Since the fatty acid composition of seed lipid is such an important determinate of oil quality, intensive efforts have also been mounted to select advantageous desaturase alleles (Wilson et al., 2001, Rajcan et al., 2005) and to manipulate molecularly desaturase expression and activity (Buhr et al., 2002), the goal being to produce elite soybean varieties with enhanced oil traits for the needs of industry and for improved human nutrition.

Both field and growth chamber experiments have shown that the fatty acid composition in soybean tissues is responsive to environmental temperature. In field studies, temperatures during the growing season affected seed linolenic content most clearly (Hou et al., 2006). Experiments to model climate change by increasing temperatures and [CO_2] in controlled environment chambers (Thomas et al., 2003) showed that exposure to increasing [CO_2] had no measurable effect, but higher temperatures (greater than 32/22^{o}C day/night) reduced total seed oil concentration while oleate increased and linolenate decreased with increasing temperature. Transcripts of β-glucosidase, a gene expressed during seed development, was detected in seeds grown at 28/18^{o}C but not detected in seeds grown at 40/30^{o}C. This observation suggested that one mechanism by which climate change may affect soybean seed development is through the regulation of gene transcription. The ability to adjust membrane lipid fluidity by changing the levels of unsaturated fatty acids is provided mainly by the regulated activity of fatty acid desaturases (Iba 2002). Through this

mechanism, the modification of membrane fluidity in response to temperature stress results in the maintenance of a membrane environment suitable for the function of critical integral proteins, such as the photosynthetic machinery in chloroplasts (Nishiuchi et al., 1998). The fatty acid composition in soybean tissues is, in addition, responsive to biotic (pathogen) attack (Iba, 2002, Upchurch, 2008) and fatty acids and fatty acid-derived compounds act as signals of plant defense gene expression (Kachroo et al., 2001, Weber, 2002). Evidence suggests that the levels of 18:0 and 18:1 are critical for defense against pathogens in soybean as they have been shown to be in *Arabidopsis thaliana* (Kachroo & Kachroo, 2009). Moreover, the oleate and linoleate content of soybean seeds appears to influence the course of seed colonization by a fungal pathogen (Xue et al., 2008).

Plants often encounter temperatures that are stressing, as well as pathogens and insects in the environment, sometimes simultaneously. Thus, the current worldwide situation of diminishing farm land and the heightened effects of global climate change on the productivity of agriculture (Garrett et al., 2006) increase the need to understand stress responses in crop plants such as soybean. More complete knowledge of fatty acid metabolism and its regulation in this and other important oilseed crops may significantly aid the development of effective strategies for managing abiotic and biotic stresses in the agricultural environment. This chapter focuses on a concise description of three fatty acid desaturase gene families and their contributions to the acclimation of soybean and other plants to high and low temperature and pathogen infection. Investigations of the regulation of desaturase expression and activity by temperature and pathogens are relatively recent in soybean and current results suggest complexity, yet a basic understanding of these phenomena are required if varieties are to be developed that possess stable and durable expression of desirable stress-acclimation traits.

2. Soybean delta-9 stearoyl-acyl carrier protein-desaturases

The Δ-^{9} stearoyl-acyl carrier protein-desaturases are soluble enzymes localized to the stroma fraction of plastids that introduce the first double bond into stearoyl-ACP (18:0-ACP) to produce oleoyl (18:1$^{\Delta 9}$)-ACP. Delta 9-stearoyl-ACP-desaturases thus occupy a key position in C_{18} fatty acid biosynthesis since perturbation of SACPD gene expression and/or enzyme activity may modulate the relative cellular content of both stearate and oleate. Three alleles of *SACPD* have been identified and characterized from soybean (Table 1).

Enzyme function	Gene name	GenBank accession	Chromosome, Linkage Group	Transcript expression	References
Δ^{9}-Stearoyl-ACP-Desaturase	*GmSACPD-A*	AY885234	7, M	Vegetative and seeds	Byfield et al., 2006 Zhang et al., 2008 Ha et al., 2010
	GmSACPD-B	AY885233	2, D1b	Vegetative and seeds	
	GmSACPD-C	EF113911	14, B2	Highly in seeds	

Table 1. Soybean (*Glycine max* L.) Δ^{9}-stearoyl-acyl carrier protein-desaturase genes including putative chromosome and linkage group assignment and tissue transcript expression.

Transcripts of the *GmSACPD-A* and *-B* were detected in developing seeds and other tissues, but differences in transcript abundance between *-A* and *-B* were not dramatic (Byfield et al., 2006). Translation of the 1158-bp transcript of *SACPD-A* or *-B* predicts a protein of 411 amino acids with a molecular mass of 47.2 kDa. The enzyme is a homodimer with each mature subunit containing an independent binuclear iron cluster. Soybean *SACPD*s contain two characteristic histidine box motifs. High transcript levels of a unique third allele, *GmSACPD-C*, is expressed only in developing seeds (Zhang et al., 2008). Structurally, *SACPD-C* is composed of two exons, not three as for *SACPD-A* and *-B*, separated by an 846-bp intron. Thus, *SACPD-C* differs from the *SACPD-A* and *-B* alleles in that it lacks their large intron located immediately after the putative transit peptide-encoding region. Mutations at *SACPD-C* in two soybean germplasm sources, the mutants A6 (30% 18:0) and FAM94-41 (9% 18:0) (Pantalone et al., 2002), have decreased *SACPD-C* expression and elevated seed stearic acid levels. This finding suggests, conversely, that germplasm with high SACPD-C gene expression and/or enzyme activity would produce elevated 18:1 levels. Polymerase chain reaction-based CAPS (Cleaved Amplified Polymorphism) gene probes (Zhang et al., 2008) were developed to screen soybean germplasm for mutations at *SACPD-C*, since varieties with elevated stearate are desirable for certain industrial uses such as food shortening and soap making.

The effect of increasing temperatures (from 22/18^{o}C to 30/26^{o}C) during seed development on 18:0 accumulation and *SACPD-A* and *-B* transcript accumulation has been measured in growth chamber environments (Byfield and Upchurch, 2007A). At the cool temperature, transcript accumulation of both *SACPD-A* and *-B* was significantly elevated and significantly decreased at the warmer temperature. Decreased *SACPD-A* and *-B* transcript accumulation at the warmer temperature was positively associated with a significant increase in the level of seed 18:0, but only in the high stearate mutant A6. It was suggested that temperature modulation of 18:0 content in wild type soybeans may be more complex, potentially involving in addition to the *SACPDs*, plastid thioesterase *FAT* genes, or warm-temperature post-translational modulation of SACPD enzyme activity.

The role of fatty acid desaturation pathways in mediating pathogen defense signaling has been, until recently, examined mainly in *Arabidopsis*. The *SSI2* gene cloned from *Arabidopsis* was shown to encode an (*At*) Δ^9-stearoyl-ACP-desaturase. Plants with the recessive mutation *ssi2* had a 10-fold reduction in SACPD enzyme content resulting in elevated 18:0 and reduced 18:1 content. Reduced SACPD activity in the *ssi2* mutant lead to induction of a salicylic acid-signaled defense response to the oomycete *Peronospora parasitica,* plant dwarfing and spontaneous leaf lesion formation, but also to inhibition of the jasmonic acid-signaled defense response to the fungus *Botrytis cinerea* (Kachroo et al., 2001, Nandi et al., 2003, Kachroo et al., 2005, Kachroo et al., 2007). In a situation similar to that of *Arabidopsis*, suppression of the rice fatty-acid desaturase gene *OsSSI2* (a rice Δ^9-stearoyl-ACP-desaturase) by transposon insertion or RNAi-mediated knockdown increased 18:0 and reduced 18:1 in plants and markedly enhanced resistance to the blast fungus *Magnaporthe grisea* and the leaf blight bacterium *Xanthomonas oryzae* pv. *oryzae* (Jiang et al., 2009). On the other hand, multiple stresses imposed on avocado fruits including inoculation with the fungal pathogen *Colletotrichum gloeosporioides*, exposure to ethylene, $C0_2$, fruit wounding, and low temperature exposure increased transcript abundance of avocado (*Av*) Δ^9-stearoyl-ACP-desaturase. The up-regulation of *AvSACPD* was accompanied by increases in the concentration of 18:2 (presumably from increased 18:1), increase in an antifungal diene volatile and enhanced resistance to fungal infection (Madi et al., 2003). In soybean as in

Arabidopsis, silencing of the *SACPD* genes (*-A*,*-B*, and *-C*) by a *Bean pod mottle virus*-based vector resulted in plants with reduced 18:1, elevated 18:0, the formation of spontaneous lesions, increased salicylic acid accumulation, and constitutively expressed pathogenesis-related genes. These plants also exhibited enhanced resistance to bacterial and oomycete pathogens (Kachroo et al., 2008, Kachroo & Kachroo, 2009).

3. Soybean omega-6 oleate fatty acid desaturases

The soybean ω-6 oleate fatty acid desaturases (FAD2s) are microsomal enzymes that initiate the primary route of polyunsaturated lipid biosynthesis by catalyzing the first extra-plastidal desaturation to convert 18:1 esterified to phosphatidylcholine to α-18:2 (Heppard et al., 1996). Omega-6 desaturase enzymes are typical of other microsomal desaturases in that they contain three histidine box motifs, possess a C-terminal signal for endoplasmic reticulum retention (Li et al. 2007) and have four predicted transmembrane spanning domains (Tang et al., 2005). Four different soybean ω-6 desaturase genes comprise the soybean *FAD2* gene family (Schlueter et. al., 2007) including *GmFAD2-1* and *GmFAD2-2* and their alleles (Heppard et al., 1996, Tang et al., 2005, Bachlava et. al., 2009), *GmFAD2-3* (Li et al. 2007), and *GmFAD6* (Heppard et al., 1996, Bachlava et al., 2009) (Table 2).

Enzyme function	Gene name	GenBank accession	Chromosome, linkage group	Transcript expression	References
Omega (ω)-6 Fatty Acid Desaturase	*GmFAD2-1A*	AB188250	20, I	Highly in seeds	Heppard et al., 1996, Tang et al. 2005, Bachlava et al. 2009, Li et al., 2007, Ha et al., 2010
	GmFAD2-1B	AB188251	10, O	Highly in seeds	
	GmFAD2-2A	AB188252	19, L	Vegetative and seeds	
	GmFAD2-2B	AB188253	19, L	Vegetative and seeds	
	GmFAD2-2C	AC166742.25	15, E	Vegetative and seeds	
	GmFAD2-2D	AC166091.3	3, N	Vegetative and seeds	
	GmFAD2-3	DQ53237	3 ,N	Vegetative and seeds	
	GmFAD6	L29215	2, D1b	chloroplasts	

Table 2. Soybean (*Glycine max* L.) omega-6 fatty acid desaturase genes including putative chromosome assignment and tissue transcript expression.

GmFAD2-1 genes have a short intron immediately after the start ATG which is spliced out and their mature transcripts encode proteins of approximately 387 amino acids (Tang et. al., 2005). *GmFAD2-1s* are highly expressed during lipid synthesis in developing seeds and not in vegetative tissues, while *GmFAD2*-2s are constitutively expressed in both vegetative tissue and developing seeds. Although the FAD2-2s contribute to the production of 18:1 in all tissues, transcript expression analysis suggests that the FAD2-1s play the major role in

the conversion of 18:1 to 18:2 in developing seeds. Two seed specific isoforms of FAD2-1, FAD2-1A and FAD2-1B, have been described that differ in stability at elevated temperature (Tang et al., 2005). Recent soybean genomic analysis has shown that *FAD2-2* exists as four alleles, *GmFAD2-2A, 2-2B*, *2-2C*, and *2-2D* (Schlueter et al., 2007, Bachlava et al., 2009, Ha et al., 2010). The expression level of *GmFAD2*-2C has been shown to increase eightfold in developing pods grown at 18/12°C in comparison to those grown at 32/28°C. The third gene, *GmFAD2-3*, is also constitutively expressed in both vegetative and developing seed tissues but shows no significant changes in transcript abundance in cold stressed leaves (Li et al., 2007). The fourth gene, *GmFAD6,* encodes an omega-6 desaturase that localizes to the plastid membrane. The expression pattern of the FAD6 gene does not suggest changes in transcript abundance in response to different temperatures (Heppard et al., 1996).

Significant efforts have been expended to select soybean varieties that produce higher seed oil 18:1 content, for example, mid-oleic soybean line N98-4445A which produces 50-60% 18:1 as a percent of total seed lipid fatty acids (Burton et al., 2005). Our understanding of the phenomena of elevated seed oleate and efforts to develop soybeans with this phenotype have been facilitated by the isolation and characterization of the X-ray induced mutant M23 and others with similar oleate phenotypes (Takagi, Rahman, 1996, Anai et al. 2008) and the earlier molecular characterizations of *FAD2-1* in high-oleate producing peanut mutants (Martinez-Rivas et al., 2001, Lopez et al., 2002). M23 was found to contain a large genomic lesion that completely deleted *GmFAD2-1A* (Alt et al., 2005, Sandhu et al., 2007) and mutant KK21 has a deletion of 232-bp downstream of the *FAD2-1A* ATG initiation codon (Anai et al., 2008). Both mutants produce 50-60% 18:1 in their seed lipid compared to approximately 20% 18:1 for conventional soybean cultivars. Many of the higher oleate soybean lines under development are progeny of crosses with the M23 mutant. Field trials have uncovered environmental instability in the expression of this trait in the M23-derived lines (Oliva et al., 2006, Scherder et al., 2008), as well as reductions in seed yield, protein, and oil (Scherder & Fehr, 2008). Possibly, the large genomic deletion in M23 (which extends outside of *FAD2-1A*) or additional X-ray induced mutations in M23 may be responsible for some or all of these additional phenotypic alterations. To develop soybean lines with more stable expression of elevated 18:1 without yield penalty, additional approaches involving reverse genetics have been applied. Ribozyme termination cassettes were employed with the aim of producing transgenic soybean with down-regulated GmFAD2-1 gene expression. Soybean transformants were recovered that stably displayed 18:1 levels in seed lipids of over 75% (Buhr et al., 2002). An intron sense suppression construct of *GmFAD2-1A* was employed with the aim of specifically reducing *FAD2-1* transcripts in developing seeds (Mroczka et al., 2010). Single copy transformants were recovered in which both *FAD2-1* alleles were suppressed that produced seeds with 18:1 levels elevated to 65 to 70% and corresponding reduction of 18:2. Targeting Induced Local Lesions In Genomes (TILLING) was employed with the aim of producing mutations in *GmFAD2-1A*. A missense amino acid mutation was recovered that resulted in an increase in seed 18:1 and a decrease in 18:2 compared to the wild type Williams 82 cultivar (Dierking & Bilyeu, 2009). Recently, soybean lines were identified that contain a single missense mutation in *GmFAD2-1A* or in *GmFAD2-1B* as a result of unique single nucleotide polymorphisms (SNPs) that were predicted to alter seed 18:1 content. Crosses were made to combine the two mutant FAD2-1 alleles from these otherwise conventional lines (Pham et al. 2010). Progeny homozygous for both mutant alleles consistently produced 80% seed 18:1 at different geographic locations, two in Missouri in the US and one in Costa Rica.

In both soybean seed and leaf tissues, the levels of 18:2 and 18:3 gradually increase as temperature decreases to 18/12°C, but the levels of *GmFAD2-1*, *GmFAD2-2*, and *GmFAD6* transcripts were found not to increase at low temperature. This suggests that the elevated 18:2 and 18:3 in developing seeds grown at low temperature are not due to enhanced expression (transcriptional control) of these ω-6 genes (Heppard et al., 1996). On the other hand, in developing soybean seed, the levels of 18:2 and 18:3 decreases as temperature increases to 30/26°C and higher, and the levels of *GmFAD2-1A* and *2-1B* transcripts were found to decrease. This suggests transcriptional down-regulation of the *GmFAD2-1* genes does occur as growth temperatures increase (Byfield & Upchurch, 2007A). Substantial evidence suggests that post-translational regulatory mechanisms likely play an important role in modulating FAD2-1 enzyme activities. The FAD2-1A isoform was found to be more unstable than FAD2-1B, especially at elevated growth temperatures. In addition, the FAD2-1s were phosphorylated during seed development. Evidence suggests that phosphorylation may down regulate FAD2-1 enzyme activity. Thus, growth at elevated temperature results in increased 18:1 and decreased 18:2 and 18:3 because the FAD2-1 oleate desaturase enzymes are substantially inactivated (Tang et al. 2005).

Evidence for the participation of microsomal ω-6 fatty acid desaturases in the responses of plants to pathogen infection is not plentiful. Treatment of cultured parsley cells with the Pep25 peptide elicitor derived from the soybean oomycete pathogen *Phytophthora sojae* resulted in a strong local resistance response. Omega-6 fatty acid desaturase transcripts accumulated rapidly and transiently in elicitor-treated cells, protoplasts, and leaves, suggesting that 18:1 desaturation is an early component of the response of parsley to pathogen infection (Kirsch et al. 1997). Growth chamber experiments (Thomas et al., 2003, Xue et al., 2008) have shown that elevated growth temperatures (34/26 versus 22/18°C) during seed development results in higher 18:1 and reduced 18:2 content in seed lipid. Mature soybean seeds with higher ratios of 18:1 to 18:2 that were inoculated with the fungal pathogen *Cercospora kikuchii* were colonized more heavily by the fungus than inoculated seeds with lower 18:1 to 18:2 ratios (Xue et. al., 2008).

4. Soybean omega-3 linoleate fatty acid desaturases

The membrane lipids of higher plants including soybean are characterized by a high proportion of polyunsaturated fatty acids, in particular, fatty acids in the plastidic galactolipids in most plant species are made up of about 70-80% of the trienoic fatty acids, hexadecatrienoic and α-linolenic acids (16:3 and 18:3) (Harwood 1980). In soybean phosphatidylglycerol (PG) is the only lipid synthesized by the prokaryotic type pathway, one of the two glycerolipid synthetic pathways in plants. The other leaf glycerolipids, monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), digalactosyldiacylglycerol (DGDG), and sulphoquinovosyldiacylglycerol (SQDG) are synthesized through the eukaryotic lipid pathway. Soybean lacks hexatrienoic acid (16:3) and contains α-linolenic (18:3) as the only trienoic fatty acid (Browse, Somerville, 1991). Omega-3 fatty acid desaturases are microsomal enzymes that catalyze the insertion of a third double bond into α-linoleic acid ($18:2^{\Delta 9,\ 12}$) to produce α-linolenic acid ($18:3^{\Delta 9,\ 12,\ 15}$). They, like the microsomal ω-6 desaturases, are characterized by the presence of a diiron cofactor that interacts with three conserved histidine motifs (Byfield & Upchurch, 2007B). Three soybean microsomal ω-6 desaturase genes have been isolated: *GmFAD3A*, *GmFAD3B*, and *GmFAD3C* (Bilyeu et al., 2003, Anai et al., 2005). *GmFAD3A* was found to be highly

expressed in seeds and *FAD3B* and *FAD3C* in both vegetative tissues and seeds. *GmFAD3A*, *B*, and *C* encode proteins that lack N-terminal chloroplast signal peptides. Soybean lines have been identified that produce low (2.8% compared to 8% for wild type) levels of 18:3 in their seed lipid. Low 18:3 in soybean seed lipid is a desired trait since 18:3 contributes to oil instability and rancidity. Molecular characterization of the low 18:3 line showed that a missplice mutation was present in *FAD3A* and also a single SNP altering a codon glycine to glutamic acid was present in *FAD3C (*Bilyeu et al., 2005). Molecular identity probes (CAPS markers, SNPs) were developed for all three soybean FAD3 genes and deployment of these probes for screening combinations of *FAD3* mutant alleles have allowed the development of new soybean lines with 1% 18:3 (Bilyeu et al., 2006, Beuselinck et al. 2006). Chloroplast localized soybean ω-6 fatty acid desaturase genes, designated *GmFAD7* and *GmFAD8* (after *Arabidopsis* chloroplast ω-6 desaturase functional nomenclature) have been partially characterized (Collados et al., 2006) and they do possess N-terminal chloroplast signal peptides (Table 3).

Enzyme function	Gene name	GenBank accession	Chromosome, linkage group	Transcript expression	References
Omega (ω)-3 Fatty Acid Desaturase	*GmFAD3A*	AY204710	14, B2	Seeds highly, vegetative	Bilyeu et al., 2003, Collados et al., 2006, Ha et al., 2010, Upchurch & Ramirez, 2011
	GmFAD3B	AY204711	2, D1b	Vegetative and seeds	
	GmFAD3C	AY204712	18, G	Vegetative and seeds	
	GmFAD7	HM769340	18, G and 7, M	chloroplasts	
	GmFAD8	HM769341	3, N and 1, D1a	chloroplasts	

Table 3. Soybean (*Glycine max* L.) omega-3 fatty acid desaturase genes including putative chromosome and linkage group assignment, and tissue transcript expression.

The discussion that follows focuses mainly on regulation of ω-3 FAD activity at the level of transcription control. A recent report has provided compelling evidence for a temperature-sensitive post-translational regulation of FAD3 protein abundance that involves a combination of cis-acting degradation signals and the ubiquitin-protease pathway that modulates FAD3 protein amounts in response to temperature (O'Quin et al., 2010). The half-life of FAD3 protein is greater at cooler temperatures and protein degradation required specific components of the endoplasmic reticulum protease pathway.

Most of our understanding of ω-3 FAD activity and stress acclamation in plants, including temperature change and pathogen infection, comes from research with *Arabidopsis* and other plants. Characterization of *AtFAD7* gene sequence revealed an open reading frame of 1338 bp comprised of 8 exons that encoded a deduced 446 amino acid peptide of 51.1 kDa. Growth temperature had no apparent effect on the steady-state levels of *FAD7* transcripts in wild-type plants (Nishiuchi & Iba, 1998). The *AtFAD8* sequence was found to code for a 435 amino acid peptide of 50.1 kDa that also contained a consensus chloroplast transit peptide. The coding region of *AtFAD8* shared 75% nucleotide identity with *AtFAD7*. Transcript

abundance of *AtFAD8* strongly increases in plants grown at low temperatures suggesting that the role of *FAD8* in *Arabidopsis* is to provide increased chloroplast membrane 18:3+16:3 in plants that are exposed to low growth temperature (Nishiuchi & Iba, 1998). The temperature dependent regulation of *AtFAD8* expression is not due to the *FAD8* 5′ flanking region (promoter and untranslated region), but to the exon/intron structure that is inherent in the *AtFAD8* gene (Iba, 2002). Examination of *GmFAD7* and *GmFAD8* at NCBI GenBank accession numbers HM769340 and HM769341 revealed that both soybean genes have a similar structure containing 8 exons ranging in size from 67 to 521 nucleotides and 7 introns ranging in size from 90 to 393 nucleotides. The soybean *FAD7* and *FAD8* intron/exon structure is similar to *FAD7* and *FAD8* structures of other higher plants except that the rice *OsFAD8* contains 7 exons. For both soybean genes, an exonic sequence of 1362 base pairs encodes a predicted protein of 453 amino acid residues with molecular masses of 51.3 and 51.4 kDa, respectively, for *GmFAD7* and *GmFAD8*. Using *GmFAD7* and *GmFAD8* genomic sequences as queries to interrogate the Williams 82 genome database (Schmutz et al., 2010) revealed that each gene was present in the Williams 82 genome as two complete copies located on different chromosomes, one *GmFAD7* copy located on chromosome 18 and a second on chromosome 7. A recent report has shown that the second *GmFAD7* gene, designated *GmFAD7-2*, and located on chromosome 7 is paralogous to *GmFAD7-1* located on chromosome 18 (Andreu et al., 2010). The paralogous nature of *GmFAD7-1* and *GmFAD7-2* is supported by the finding of specific gene-related FAD7 protein conformations in soybean seeds. The FAD7 protein conformations were differentially affected by *in vitro* changes in redox conditions and iron availability suggesting the existence of tissue-specific post-translational mechanisms that affect the distribution and activity of the FAD7 enzymes. Two complete copies of *GmFAD8* were also present in the Williams 82 genome sequence and they may, as well, be paralogous. One is located on chromosome 3 and the second on chromosome 1. Recent studies have characterized soybean FAD7 chloroplast localization and the transcript expression patterns in response to light of both microsomal and plastidal ω-3 soybean desaturases. In situ analysis using confocal microscopy with FAD7 antibody and chlorophyll auto fluorescence has shown that the soybean FAD7 protein is preferentially localized to the chloroplast thylakoid membranes suggesting that not only the chloroplast envelope, but also the thylakoid membranes could be sites of lipid desaturation in higher plants (Andreu et al., 2007). *GmFAD3, GmFAD7*, and *GmFAD8* transcription and transcript stability have been found to be differentially regulated by light (Collados et al., 2006). In soybean cell suspension, darkness leads to an overall decrease in 18:3 levels and *GmFAD3* and *GmFAD8* transcripts are undetectable, but after reillumination *FAD3* and *FAD8* transcript abundance increased concomitant with an increase in 18:3 accumulation. *GmFAD7* transcript levels were remarkably similar under dark or light conditions and *GmFAD7* mRNA stability dramatically increased in the dark as well. FAD7 protein levels were also very stable in either light or dark conditions, suggesting that an additional post-translational regulatory mechanism may control the activity of FAD7 in response to light.

Numerous studies have shown that temperature regulates the transcript expression of plastid ω-3 and microsomal ω-3 desaturases and leaf trienoic fatty acid levels in plants. In *Brassica napus* leaf 16:3/18:3 levels increase in MGDG during low temperature acclimation (Williams et al. 1996). In birch (*Betula pendula*) seedlings exposed to low temperatures (+ 4 to -24^{o}C) increased 18:3 in the chloroplast membrane lipids (MGDG, DGDG and PG) were found in the leaves at colder temperatures. The higher 18:3 levels were associated with

upregulated expression of the birch ω-3 desaturases, *BpFAD3*, *BpFAD7* and *BpFAD8* (Martz et al. 2006). Transgenic tomato plants in which the microsomal omega-3 desaturases have been silenced have greatly reduced *LeFAD3* transcripts, contain low levels of 18:3, higher levels of 18:2, and exhibit long-term heat tolerance at 36°C (Wang et al. 2010). Heat stressed *LeFAD3*-suppressed plants produced greater fresh weight of aerial plant parts and had a more intact chloroplast membrane structure than did heat stressed wild-type plants. Growth of soybean plants at a cool temperature (22/18°C, D/N) during seed development resulted in elevated seed 18:3 and elevated Gm*FAD3A*, *FAD3B* and *FAD3C* transcript expression, and conversely, decreased 18:3 and transcript expression of these microsomal omega-3 desaturase genes at a warm temperature (30/26°C, D/N) during seed development (Byfield and Upchurch 2007B). A general conclusion to be drawn from experiments with *Arabidopsis* and other plants is that transcript expression of *FAD8* and *FAD3* change in response to changes in ambient temperature, and *FAD8* is cold-inducible whereas expression of *FAD7* is not affected by changes in temperature (McConn et al., 1994, Berberich et al., 1998, Iba, 2002, Upchurch & Byfield, 2007, Nair et al., 2009, Wang et al., 2010). Another conclusion is that the increased 18:3 level in chloroplast membranes due to upregulated *FAD8* expression is associated with low temperature tolerance in *Arabidopsis* and other plants. Presumably, temperature regulation of soybean *GmFAD 7* and *FAD8* follows a similar pattern.

Upregulation of *FAD7* and increased 18:3 levels in chloroplasts have physiological roles in modulating plant defense responses to pathogens in several plant-pathogen systems. For instance, *FAD7* has been shown to be required to provide 18:3 for the synthesis of a long-distance signal (not jasmonic acid) that is required for the induction of systemic acquired resistance (SAR) in *Arabidopsis* and tomato (Chaturvedi et al. 2008). The *A. thaliana FAD7 and FAD8* double mutation prevents the synthesis of trienoic acids in chloroplast lipids, causing a reduction in the production and accumulation of reactive oxygen intermediates in leaves, reduced levels of programmed cell death, and compromised resistance to several avirulent *Pseudomonas syringae* strains (Yaeno et al. 2004). On the other hand and in contrast, disease resistance to compatible and incompatible races of the rice blast fungus *Magnaporthe grisea* is enhanced in 18:2 accumulating and 18:3-deficient transgenic rice (F78Ri) in which *OsFAD7* and *OsFAD8* were suppressed. The 18:3 Jasmonate-mediated wound responses were suppressed, but the expression of jasmonate-responsive PR genes, PBZ1 and PR1b were induced after inoculation. In rice F78Ri mutant plants, the 18:2-derived hydroperoxides and hydroxides (HPODEs and HODEs) increased significantly and these molecules inhibited the growth of *M. grisea* more strongly than their 18:3-derived counterparts (Yara et al., 2007, 2008). In *Arabidopsis*, local mechanical wounding and pathogen attack causes a rapid rise of *AtFAD7* transcripts in the basal rosette leaves and induces *AtFAD7* expression in the roots. Inhibitors of the oxylipin octadecanoid pathway strongly suppress wound activation of the *FAD7* promoter in roots but not in leaves and stems (Nishiuchi et al., 1997). A specific region of the *AtFAD7* promoter is required for wound-activated expression of this gene in leaves and stems, while another region is necessary for wound-activated, jasmonic acid-responsive expression of the gene in roots (Nishiuchi et al., 1999) suggesting that a jasmonate-independent wound signal may induce the activation of the *FAD7* gene in leaves and stems. In tomato (*Lycopersicon esculentum*) containing a mutation in *Spr2* (which encodes the chloroplast ω-3 FAD gene, *LeFAD7*), the 18:3 content of the leaves was less than 10% of wild-type levels. The accumulation of hexadecatrienoic acid was also abolished and both wound-induced jasmonic acid biosynthesis and the production of a long-distance signal for expression of defensive genes were reduced such that *Spr2* plants were compromised in

defense against attack by tobacco hornworm (Li et al. 2003). Recently it was reported that silencing of the three soybean GmFAD3 genes enhanced the accumulation of *Bean Pod mottle virus* (BPMV) in plant tissues and enhanced susceptibility to virulent *Pseudomonas syringae* bacteria (Singh et al. 2011). Silenced plants exhibited increased levels of jasmonic acid and slightly reduced levels of 18:3 indicating that loss of microsomal ω-3 activity enhances jasmonate accumulation and thereby susceptibility to *BPMV* in soybean.

5. Conclusions

Stearoyl-ACP-desaturase, omega-6, and omega-3 desaturases are diiron cofactor, histidine box motif enzymes that introduce, respectively, the first, second or third double bond into the specific C_{18} fatty acid substrate to yield oleate (18:1), linoleate (18:2), or linolenate (18:3). The expression and activity of these enzymes significantly determines the fatty acid composition and overall quality of soybean oil, and also contributes to the physiological adaptation to environmental temperature and the induction of defense responses to pathogens. Investigations of the regulation of desaturase expression and activity by temperature and pathogens in soybean are relatively recent, but initial findings suggest similarities with *Arabidopsis* and other plants. Down regulation of the *SACPD* gene expression results in plants with reduced 18:1, elevated 18:0, the formation of spontaneous lesions, increased salicylic acid accumulation, and constitutively expressed pathogenesis-related genes (Kachroo & Kachroo 2009). These plants exhibit enhanced resistance to bacterial and oomycete pathogens. In both soybean seed and leaf tissues, the levels of 18:2 and 18:3 gradually increase as temperature decreases, but the transcript levels of the omega-6 desaturases do not increase at low temperature, suggesting that post-translational regulatory mechanisms likely play an important role in modulating the omega-6 (FAD2-1) enzyme activities. Transcript expression of the omega-3 desaturases *FAD8* and *FAD3* do change in response to changes in ambient temperature. *FAD8* is cold-inducible and the increased 18:3 level in chloroplast membranes due to upregulated *FAD8* expression is associated with low temperature tolerance. Upregulation of *FAD7* and increased 18:3 levels in chloroplasts modulate plant defense responses to pathogens through increased production of oxylipin antimicrobial and signaling molecules. SACPD, ω-6, and ω-3 fatty acid desaturase genes are present as multiple copies in the soybean genome as expected given the evidence (Schmutz et al. 2010, Ha et al., 2010) from cytogenetics, genetic mapping, and genomic sequencing that soybean is a paleopolyploid species that underwent at least two major genome duplications. The soybean genome possesses tissue-specific alleles for all three of C_{18} desaturase enzymes involved in the biosynthesis of triacylglycerols. The occurrence of seed-specific alleles of these genes provides for the accommodation of the great increase in lipid biosynthesis that occurs as the developing soybean seeds produce storage lipid reserves (Tang et al., 2005). Genomic (Schmutz et al., 2010) and gene expression analysis (Upchurch & Ramirez, 2010) using the Williams 82 soybean genome database is expected to expand knowledge of soybean gene regulatory sequences and their interaction with transcription complexes. Development of soybean SNP markers (Ha et al., 2010), mapping and dissection of Quantitative Trait Loci (Bachlava et al., 2008, Bachlava et al., 2009A, Bachlava et al., 2009B) and gene silencing analyses (Singh et al., 2011) may lead to the discovery of new genes for fatty acid biosynthesis and stress adaptation, and the potential epigenetic interactions between them. Since the capacity to induce host pathogen defenses is associated with specific desaturase-mediated changes in the levels of unsaturated C_{18} fatty

acids in plant lipid, global climate change (Garrett et al. 2006) may potentially negatively impact plant defenses.

6. Acknowledgements

I thank Dr. Ralph E. Dewey, Crop Science Department, North Carolina State University, Raleigh for helpful discussions on the soybean omega-6 fatty acid desaturase alleles and Dr. Martha E. Ramirez, ARS Soybean & Nitrogen Fixation Unit, Raleigh for a critical reading of the manuscript.

7. References

Alt, J.L., Fehr, W.R., Welke, G.A., & Sandhu, D. (2005). Phenotype and molecular analysis of oleate content in the mutant soybean line M23, *Crop Sci*ence Vol. 45: 1997-2000.

Anai, T., Yamada, T., Kinoshita, T., Rahman, S.M., Takagi, T. (2005). Identification of corresponding genes for three low-α-linolenic acid mutants and elucidation of their contribution to fatty acid biosynthesis in soybean seed, *Plant Sci*ence Vol. 168: 1615-1623.

Anai, T., Yamada, T., Hideshima, R., Kinoshita, T., Rahman, S.M., & Takagi, Y. (2008). Two high-oleic-acid soybean mutants, M23 and KK21, have disrupted microsomal omega-6 fatty acid desaturase, encoded by *GmFAD2-1a*, *Breeding Science* Vol. 58: 447-452.

Andreu, V., Collados, R., Testillano, P.S., Risueno, M.dC, Picorel, R., & Alfonso, M. (2007). In situ molecular identification of the plastid Ω-3 fatty acid desaturase FAD7 from soybean: evidence of thylakoid membrane localization, *Plant Physiology* Vol. 145: 1336-1344.

Andreu, V., Lagunas, B., Collados, R., Picorel, R. & Alfonso, M. (2010). The *GmFAD7* gene family from soybean: identification of novel genes and tissue-specific conformations of the FAD7 enzyme involved in desaturase activity, *Journal of Experimental Botany* Vol. 61(No. 12): 3371-3384.

Bachlava, E., Dewey, R.E., Auclair, J., Wang, S., Burton, J.W. and Cardinal, A.J. (2008). Mapping genes encoding enzymes and their cosegregation with QTL affecting oleate content in soybean, *Crop Science* Vol. 48: 640-650.

Bachlava, E., Dewey, R.E., Burton, J.W. and Cardinal, A.J. (2009A). Mapping and comparison of quantitative trait loci for oleic acid seed content in two segregating soybean populations. *Crop Science* Vol. 49: 433-442.

Bachlava, E., Dewey, R.E., Burton, J.W., Cardinal, A.J. (2009B). Mapping candidate genes for oleate biosynthesis and their association with unsaturated fatty acid seed content in soybean, *Molecular Breeding*. Vol. 23: 337-347.

Berberich, T., Harada, M., Sugawara, K., Kodama, H., Iba, K & Kusano, T. (1998). Two maize genes encoding ω-3 fatty acid desaturase and their differential expression to temperature, *Plant Molecular Biology* Vol. 36: 297-306.

Beuselinck, P.R., Sleper, D.A. & Bilyeu, K.D. (2006). An assessment of phenotype selection for linolenic acid using genetic markers, *Crop Science* Vol. 46: 747-750.

Bilyeu, K.D., Palavalli, L., Sleper, D.A., & Beuselinck, P.R. (2003). Three microsomal omega-3 fatty-acid desaturase genes contribute to soybean linolenic acid levels, *Crop Science* Vol. 43: 1833-1838.

Bilyeu, K., Palavalli, L., Sleper, D., & Beuselinck, P. (2005). Mutations in soybean microsomal omega-3 fatty acid desaturase genes reduce linolenic acid concentration in soybean seeds, *Crop Sci*ence Vol. 45: 1830-1836.

Bilyeu, K., Palavalli, L., Sleper, D.A., & Beuselinck, P. (2006). Molecular genetic resources for development of 1% linolenic acid soybeans, *Crop Sci*ence Vol. 46: 1913-1918.

Browse, J., Somerville, C. (1991). Glycerolipid synthesis: biochemistry and regulation, *Annual Review of Plant Physiology and Plant Molecular Biology* Vol. 42: 467-506.

Buhr, T., Sato, S., Ebrahim, F., Xing, A., Zhou, Y., Mathiesen, M., Schweiger, B., Kinney, A., Staswick, P. & Clemente, T. (2002). Ribozyme termination of RNA transcripts down-regulate seed fatty acid genes in transgenic soybean, *The Plant Journal* Vol. 30 (No. 2): 155-163.

Burton, J.W., Wilson, R.F., Novitzky, W., Rebetzke, G.J., and Pantalone, V.R. (2005). Registration of N98-4445A, a mid-oleic soybean germplasm line, *Crop Science* Vol. 46: 1010-1011.

Byfield, G.E., Xue, H., & Upchurch, R.G. (2006). Two genes from soybean encoding soluble Δ9 stearoyl-ACP desaturase, *Crop Sci*ence Vol. 46: 840-846.

Byfield, G.E. & Upchurch, R.G. (2007A). Effect of temperature on delta-9 stearoyl-ACP and microsomal omega-6 desaturase gene expression and fatty acid content in developing soybean seeds, *Crop Science* Vol. 47: 1698-1704.

Byfield, G. & Upchurch, R.G. (2007B). Effect of temperature on microsomal omega-3 linoleate desaturase gene expression and linolenic acid content in developing soybean seeds, *Crop Science* Vol. 47: 2445-2452.

Cahoon, E.B., Lindqvist, Y., Schneider, G., & Shanklin, J. (1997). Redesign of soluble fatty acid desaturases from plants for altered substrate specificity and double bond position, *Proceedings of the National Academy of Science, USA* Vol. 94: 4872-4877.

Chaturvedi, R., Krothapalli, K., Makandar, R., Nandi, A., Sparks, A.A, Roth, M.R., Welti, R. & Shah, J. (2008). Plastid Ω 3-fatty acid desaturase-dependent accumulation of a systemic acquired resistance inducing activity in petiole exudates of *Arabidopsis thaliana* is independent of jasmonic acid, *The Plant J*ournal Vol. 54: 106-117.

Collados, R., Andreu, V., Picorel, R., Alfonso, M. (2006). A light-sensitive mechanism differentially regulates transcription and transcript stability of Ω-3 fatty acid desaturases (FAD3, FAD7 and FAD8) in soybean photosynthetic cell suspensions, *Federation of European Biochemical Societies Letters* Vol. 580: 4934-4940.

Dierking, E.C. & Bilyeu, K.D. (2009). New sources of soybean seed meal and oil composition traits identified through Tilling, *BioMed Central Plant Biology* Vol. 9: 89-99.

Garrett, K., Dendy, S., Frank, E., Rouse, M. & Travers, S. (2006). Climate change effects on plant disease: genomes to ecosystems, *Annual Review of Phytopathology* Vol. 44: 489-509.

Ha, B-K, Monteros, M.J., Boerma, H.R. (2010). Development of SNP assays associated with oleic acid QTLs in N00-3350 soybean, *Euphytica* Vol. 176: 403-415.

Harwood, J.L. (1980). Plant acyl lipids: structure, distribution, and analysis *in* P.K. Stumpf and E.E. Conn (eds.), *The Biochemistry of Plants*, Vol. 4, Academic Press, New York.

Heppard, E.P., Kinney, A.J., Stecca, K.L., & Miao, G-H. (1996). Developmental and growth temperature regulation of two different microsomal ω-6 desaturase genes in soybeans, *Plant Physiology* Vol. 110: 311-319.

Hou, G., Ablett, G.R., Pauls, K.P., & Rajcan, I. (2006). Environmental effects on fatty acid levels in soybean seed oil, *Journal of the American Oil Chemists' Society* Vol. 83 (No. 9): 759-763.

Iba, K. (2002). Acclimative response to temperature stress in higher plants: approaches of gene engineering for temperature tolerance, *Annual Review of Plant Biology* Vol. 53 (No.): 225-245.

Jiang, C-J, Shimono, M., Maeda, S., Inoue, H., Mori, M., Hasegawa, M., Sugano, S., & Takatsuji, H. (2009). Suppression of the rice fatty-acid desaturase gene *OsSSI2* enhances resistance to blast and leaf blight diseases in rice, *Molecular Plant-Microbe Interactions* Vol. 22 (No. 7): 820-829.

Kachroo, P., Shanklin, J., Shah, J., Whittle E.J., & Klessig, D.F. (2001). A fatty acid desaturase modulates the activation of defense signaling pathways in plants, *Proceedings of the National Academy of Science, USA* Vol. 98: 9448-9453.

Kachroo, P., Venugopal, S.C., Navarre, D.A., Lapchyk, L., & Kachroo, A. (2005). Role of salicyclic acid and fatty acid desaturation pathways in *ssi2*-mediated signaling, *Plant Physiology* Vol. 139: 1717-1735.

Kachroo, A., Shanklin, J., Whittle E., Lapchyk, L., Hildebrand, D., Kachroo, P. (2007). The *Arabidopsis* stearoyl-acyl carrier protein-desaturase family and the contribution of leaf isoforms to oleic acid synthesis, *Plant Molecular Biology* Vol. 63: 257-271.

Kachroo, A., Fu, D.Q., Havens, W., Navarre, D., Kachroo, P., Ghabrial, S.A. (2008). An oleic acid-mediated pathway induces constitutive defense signaling and enhanced resistance to multiple pathogens in soybean, *Molecular Plant-Microbe Interactions* Vol. 21(No. 5): 564-575.

Kachroo, A. & Kachroo, P. (2009). Fatty acid-derived signals in plant defense, *Annual Review of Phytopathology* Vol. 47: 153-176.

Kirsch, C., Hahlbrock, K., & Somssich, I. (1997). Rapid and transient induction of a parsley microsomal Δ12 fatty acid desaturase mRNA by fungal elicitor, *Plant Physiology* Vol. 115: 283-289.

Li, C., Liu, G., Xu, C., Lee, G.I., Bauer, P., Ling, H-Q, Ganal, M.W., & Howe, G.A. (2003). The tomato *suppressor of prosystemin-mediated responses2* gene encodes a fatty acid desaturase required for the biosynthesis of jasmonic acid and the production of a systemic wound signal for defense gene expression, *The Plant Cell* Vol. 15: 1646-1661.

Li, L., Wang, X., Gai, J., Yu, D. (2007). Molecular cloning and characterization of a novel microsomal oleate desaturase gene from soybean, *Journal of Plant Physiology* Vol. 164: 1516-1526.

Lopez, Y., Nadaf, H.L., Smith, O.D., Simpson, C.E., & Fritz, A.K. (2002). Expressed variants of Δ^{12}-fatty acid desaturase for high oleate trait in spanish market-type peanut lines, *Molecular Breeding* Vol. 9 (No. 9): 183-190.

Madi, L., Wang, X., Kobiler, I., Lichter, A., Prusky, D. (2003). Stress on avocado fruits Δ^{9}-stearoyl ACP desaturase expression, fatty acid composition, antifungal diene level and resistance to *Colletotrichum gloeosporioides* attack, *Physiological and Molecular Plant Pathology* Vol. 62 : 277-283.

Martinez-Rivas, J.M., Sperling, P., Luhs, W. & Heinz, E. (2001). Spatial and temporal regulation of three different microsomal oleate desaturase genes (*FAD2*) normal-

type and high-oleic varieties of sunflower (*Helianthus annuus* L.), *Molecular Breeding* Vol. 8 (No. 8): 159-168.

Martz, F., Kiviniemi, S., Palva, T.E. & Sutinen, M-L. (2006). Contribution of omega-3 fatty acid desaturase and 3-ketoacyl-ACP synthase II (*KASII*) genes in the modulation of glycerolipid fatty acid composition during cold acclimation in birch leaves, *Journal of Experimental Botany* Vol. 57 (No. 4): 897-909.

McConn, M., Hugly, S., Browse, J., & Somerville, C. (1994). A mutation at the *fad8* locus of *Arabidopsis* identifies second chloroplast ω-3 desaturase, *Plant Physiology* Vol. 106: 1609-1614.

Mroczka, A., Roberts, P.D., Fillatti, J.J., Wiggins, B.E., Ulmasov, T., & Voelker, T. (2010). An intron sense suppression construct targeting soybean *FAD2-1* requires a double-stranded RNA-producing inverted repeat T-DNA insert, *Plant Physiology* Vol. 153: 8829-891.

Nair, P.M.G., Kang, I-S, Moon, B-Y, Lee, C-H. (2009). Effects of low temperature stress on rice (*Oryza sativa* L.) plastid Ω-3 desaturase gene, *OsFAD8* and its functional analysis using T-DNA mutants, *Plant Cell and Tissue Culture* Vol. 98: 87-96.

Nandi, A., Krothapalli, K., Buseman, C.M., Li, M., Welti, R., Enyedi, A., & Shah, J. (2003). Arabidopsis *sfd* mutants affect plastidic lipid composition and suppress dwarfing, cell death, and the enhanced disease resistance phenotypes resulting from the deficiency of a fatty acid desaturase, *The Plant Cell* Vol. 15: 2383-2398.

Nishiuchi, T., Hamada, T., Kodama, H., & Iba, K. (1997). Wounding changes the spatial expression pattern of the Arabidopsis plastid ω-3 fatty acid desaturase gene (*FAD7*) through differential signal transduction pathways, *The Plant Cell* Vol. 9: 1701-1712.

Nishiuchi, T. & Iba, K. (1998). Roles of plastid Ω-3 fatty acid desaturases in defense response of higher plants, *Journal of Plant Research* Vol. 111: 481-486.

Nishiuchi, T., Kodama, H., Yanagisawa, S., & Iba, K. (1999). Wound-induced expression of the *FAD7* gene is mediated by different regulatory domains of its promoter in leaves/stems and roots, *Plant Physiology* Vol. 121: 1239-1246.

Oliva, M.L., Shannon, J.G., Sleper, D.A., Ellersieck, M.R., Cardinal, A.J., Paris, R.L., & Lee, J.D. (2006). Stability of fatty acid profile in soybean genotypes with modified seed oil composition, *Crop Science* Vol. 46: 2069-2075.

O'Quin, J.B., Bourassa, L., Zhang, D., Shockey, J.M., Gidda, S.K., Fosnot, SS., Chapman, K.D., Mullen, R.T., & Dyer, J.M. (2010). Temperature-sensitive post-translational regulation of plant omega-3 fatty acid desaturases is mediated by the endoplasmic reticulum-associated degradation pathway, Journal of Biological Chemistry Vol. 285 (No. 28): 21781-21796.

Pantalone, V.R., Wilson, R.F., Novitzky, W.P., & Burton, J.W. (2002). Genetic regulation of elevated stearic acid concentration in soybean oil, *Journal of the American Oil Chemists' Society* Vol. 79 (No. 6): 549-553.

Pham, A.T., Lee, J.D., Shannon, J.G., Bilyeu, K.D. (2010). Mutant alleles of FAD2-1A and FAD2-1B combine to produce soybeans with the high oleic acid seed oil trait, BioMed Central Plant Biology Vol. 10: 195-207.

Rajcan, I., Hou, G., Weir, A.D. (2005). Advances in breeding of seed-quality traits in soybean, *Journal of Crop Improvement* Vol. 14: 145-174.

Sandhu, D., Alt, J.L., Scherder, C.W., Fehr, W.R., Bhattacharyya, M.K. (2007). Enhanced oleic acid content in soybean mutant M23 is associated with a deletion in the Fad2-1a

gene encoding a fatty acid desaturase, *Journal of the American Oil Chemists' Society* Vol. 84: 229-235.

Scherder, C.W., Fehr, W.R., & Shannon, J.G. (2008). Stability of oleate content in soybean lines derived from M23, *Crop Science* Vol. 48: 1749-1752.

Scherder, C.W., Fehr, W.R. (2008). Agronomic and seed characteristics of soybean lines with increased oleate content, *Crop Science* Vol. 48: 1755-1758.

Schlueter, J.A., Vasylenko-Sanders, I.F., Deshpande, S., Yi, J., Siegfried, M., Roe, B.A., Schlueter, S.D., Scheffler, B.E., Shoemaker, R.C. (2007). The FAD2 gene family of soybean: insights into the structural and functional divergence of a paleopolyploid genome, *Crop Science* Vol. 47(SI):S14- S26.

Schmutz, J., Cannon, S.B., Schlueter, J., Ma, J., Mitos, T., Nelson, W., Hyten, D.L., Song, Q., Thelen, J.J., Cheng, J., Xu, D., Hellsten, U., May, G.D., Yu, Y., Sakurai, T., Umezawa, T., Bhattacharyya, M.K., Sandhu, D., Valliyodan, B., Lindquist, E., Peto, M., Grant, DD., Shu, S., Goodstein, D., Barry, K., Futrell-Griggs, M., Abernathy, B., Du, J., Tian, Z., Zhu, L., Gill, N., Joshi, T., Libault, M., Sethuraman, A., Zhang, X-C, Shinozaki, K., Nguyen, H.T., Wing, R.A., Cregan, P., Specht, J., Grimwood, J., Rokhsar, D., Stacey, G., Shoemaker, R.C., Jackson, S.A. (2010). Genome sequence of the palaeopolyploid soybean, *Nature* Vol. 463: 178-183.

Singh, A.K., Fu, D-Q, El-Habbak, M., Navarre, D., Ghabrial, S., & Kachroo, A. (2011). Silencing genes encoding omega-3 fatty acid desaturase alters seed size and accumulation of *bean pod mottle virus* in soybean, *Molecular Plant-Microbe Interactions* Vol. 24 (No. 4): 506-515.

SoyStats (2011). American Soybean Association, Available at http://soystats.com , St. Louis, USA

Takagi, Y., Rahman, S.M. (1996). Inheritance of high oleic acid content in the seed oil of soybean mutant M23, *Theoretical and Applied Genetics* Vol. 92: 179-182.

Tang, G-Q, Novitzky, W.P., Griffin, H.C., Huber, S.C. and Dewey, R.E. (2005). Oleate desaturase enzymes of soybean: evidence of regulation through differential stability and phosphorylation, *Plant Journal* Vol. 44: 433-446.

Thomas, J.M.G., Boote, K.J., Allen, Jr., L.H., Gallo-Meagher, M. & Davis, J.M. (2003). Elevated temperature and carbon dioxide effects on soybean seed composition and transcript abundance, *Crop Science* Vol. 43: 1548-1557.

Upchurch, R.G. (2008). Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress, *Biotechnology Letters* Vol. 30: 967-977.

Upchurch, R.G., Ramirez, M.E. (2010). Gene expression profiles of soybeans with mid-oleic acid seed phenotype. *Journal of the American Oil Chemists' Society* Vol. 87: 857-864.

Upchurch, R.G., Ramirez, M.E. (2011). Soybean plastidal omega-3 fatty acid desaturase genes *GmFAD7* and *GmFAD8*: structure and expression, *Crop Science* Vol. 51: 1673-1682.

Wang, J., Ming, F., Pittman, J., Han, Y., Hu, J., Guo, B., Shen, D. (2006). Characterization of a rice (*Oryza sativa* L.) gene encoding a temperature-dependent chloroplast Ω-3 fatty acid desaturase, *Biochemical and Biophysical Research Communication* Vol. 340: 1209-1216.

Wang, H-S, Yu, C., Tang, X-F, Wang, L-Y, Dong, X-C & Meng, Q-W. (2010). Antisense-mediated depletion of tomato endoplasmic reticulum omega-3 fatty acid

desaturase enhances thermal tolerance, *Journal of Integrative Plant Biology* Vol. 52 (No. 6): 568-577.

Weber, H. (2002). Fatty acid-derived signals in plants, *Trends in Plant Science* Vol. 7 (No. 5): 217-224.

Whittle, E., Cahoon, E.B., Subrahmanyam, S., and Shanklin, J. (2005). A multifunctional acyl-acyl carrier protein desaturase from *Hedera helix* L. (English Ivy) can synthesize 16- and 18-carbon monoene and diene products, *Journal of Biological Chemistry* Vol. 280 (No. 31): 28169-28176.

Williams, J.P., Khan, M.U. and Wong, D. (1996). Fatty acid desaturation in monogalactosyldiacylglycerol of Brassica napus leaves during low temperature acclimation. *Physiologia Plantarum* Vol.96: 258-262.

Wilson, R.F., Burton, J.W., Novitzky, W.P., and Dewey, R.E. (2001). Current and Future Innovations in soybean (*Glycine max* L. Merr.) oil composition, *Journal of Oleo Science* Vol. 50 (No. 5): 353-358.

Xue, H.Q., Upchurch, R.G. & Kwanyuen, P. (2008). Relationships between oleic and linoleic acid content and seed colonization by *Cercospora kikuchii* and *Diaporthe phaseolorum,* Plant Disease Vol. 92 (No. 7): 1038-1042.

Yaeno, T., Matsuda, O. & Iba, K. (2004). Role of chloroplast trienoic fatty acids in plant disease defense responses, *The Plant Journal* Vol. 40: 931-941.

Yara, A., Yaeno, T., Hasegawa, M., Seto, H., Montillet, J-L, Kusumi, K., Seo, S. & Iba, K. (2007). Disease resistance against *Magnaporthe grisea* is enhanced in transgenic rice with suppression of Ω-3 fatty acid desaturases, *Plant Cell Physiology* Vol. 48 (No. 9): 1263-1274.

Yara, A., Yaeno, T., Montillet, J-L, Hasegawa, M., Seo, S.S., Kusumi, K., Iba, K. (2008). Enhancement of disease resistance to *Magnaporthe grisea* on rice by accumulation of hydroxyl linoleic acid, *Biochemical and Biophysical Research Communications* Vol. 370: 344-347.

Zhang, P., Burton, J.W., Upchurch, R.G., Whittle, E., Shanklin, J., & Dewey, R.E. (2008). Mutations in a Δ^9-stearoly-ACP-desaturase gene are associated with enhanced stearic are levels in soybean seeds, *Crop Sci*ence Vol. 48: 2305-2313.

7

Genetically Modified Soybean in Animal Nutrition

Tudisco Raffaella, Calabrò Serena, Cutrignelli Monica Isabella,
Piccolo Vincenzo and Infascelli Federico
Department of Animal Science and Food Control; University of Naples; Naples Italy

1. Introduction

In recent years, genetically modified (GM) plants, whose DNA has been changed using genetic engineering techniques, are mainly used as foods for human and feeds and foods for farm animals. To date, a number of GM products have been approved for human consumption but concerns over safety persist, mainly as regards either the detection of transgenic plant genes and proteins in animal systems or allergenicity and toxicity of GM plants.

Since their commercial release in 1996, the global cultivation area dedicated to the production of GM plants has increased significantly (ISAAA, 2010). The majority of GM crops currently produced, like soybean, corn, cotton and canola, have been engineered to enhance agronomic performance by transformation with genes encoding herbicide tolerance and pest resistance. GM soybean has been rendered tolerant to the glyphosate family of herbicides through expression of transgenic DNA from the CP4 strain of *Agrobacterium tumefaciens* that encodes 5-enolpyruvylshikamate-3-phosphate synthase (CP4 EPSPS). Roundup Ready (RR) soybean have been grown commercially from 1996 and continued to be the principal biotech crop in 2010. Farm animals are currently fed soybean and soybean meal developed from genetic transformation as well as corn and corn products. The European Union imports soybean from USA, Brazil, and Argentina, the main users of biotech crops globally. About 90% of the compound feed produced in the EU contains GM soybean.

Although regulations with regard to GM plants have been developed primarily from the perspective of human consumption of GM food, it is generally assumed that these criteria are suitable for a risk assessment of the consumption of GM feed by livestock. The protocol for establishing "substantial equivalence" of GM plant compared to isogenic parental lines does not complete a nutritional safety assessment of a GM plant, rather, it provides a starting point for the overall assessment (FAO/WHO, 2000). Based on the European novel food and feed regulation, all foods and feeds containing or derived from approved GM products in amounts greater than a 0.9% threshold are subject to labelling rules (European Commission, 2003). Labelling of feeds containing GM ingredients gives farmers the choice of using such feed for their livestock. However, products such as milk, meat, and eggs, that are derived from livestock fed transgenic feeds are exempt from EU-labelling laws. Several studies have been conducted to evaluate the safety of GM crops, but there is still a debate on the risk of GM consumption and their potential passage into tissues.

Current researches suggest that the passage of plant DNA fragments across the intestinal barrier is a natural event, as demonstrated by the detection of endogenous, high copy number chloroplast genes from plants in several animal tissues and products. Low copy endogenous and transgenic DNA in animal tissues have been detected but to a lesser extent than high copy genes.

For several years, no direct evidence that GM food may represent a possible danger for health has been reported and the scientific literature in this field is still quite poor, especially as to the possible effect of a diet involving a significant amount of GM plants. More recently, a number of papers have been published and controversial results have been obtained. However, some have found significant modifications in some nuclear features in mice fed GM soybean and, more recently, it has been reported that the activity of some enzymes was altered in rabbit and goats fed GM soybean, as confirmed also by histochemistry which showed a widespread distribution of enzyme activity in myocytes, myocardiocytes, epithelial cells of renal tubules and hepatocytes. These observations suggest that the risk of genetically modified crops cannot be ignored and requires further investigations in order to identify possible long-term effects of GM plants on both livestock and human consumption.

The main focus of this chapter concerns the genetically engineered soybean, its effects on human and animal health, the productivity of this GM crop and the outcome for environment.

2. Agronomic impact of genetically modified soybean

Genetic engineering has been widely applied to agriculture to obtain specific plant characteristics which can lead to an improvement in both food quality and yield. Compared with traditional plant breeding methods, such as artificial crossing or hybridization, biotechnology now allows for the introduction of DNA from outside the plant kingdom. Selective inclusion of single or multiple traits can be performed to change the quality of agricultural crops. According to statistics released by the International Service for the Acquisition of Agri-Biotech Applications (ISAAA, 2010), the area of planted transgenic crops was 148 million hectares in 2010, a approximately 87-fold increase from the 1996 level (1.7 million hectares of biotech crops). The number of countries adopting biotech crop cultivation has increased crops consistently from 6 in 1996 to 29 in 2010. The Unites States (US), followed by Brazil, Argentina, India, Canada, and China continued to be the principal adopters of biotech crops globally, with 66.8 million hectares planted in the US.

The majority of genetically modified (GM) crops currently produced have been engineered to enhance agronomic performance by transformation with genes encoding herbicide tolerance or pest resistance. From the first commercialization of biotech crops in 1996, to 2010 herbicide tolerance has consistently been the dominant trait. In 2010, herbicide tolerance deployed in soybean, corn, canola, cotton, sugarbeet and alfalfa, occupied 61% or 89.3 million hectares of the global biotech area. In 2010, the stacked double and triple traits occupied a larger area (22% or 32.3 million hectares) than insect resistant varieties (26.3 million hectares) at 17%. The insect resistance trait products were the fastest growing trait group between 2009 and 2010 at 21% growth, compared with 13% for stacked traits and 7% for herbicide tolerance.

Biotech herbicide tolerant soybean continued to be the principal biotech crop in 2010, occupying 73.3 million hectares or 50% of global biotech area, followed by biotech corn (46.8 million hectares at 31%), biotech cotton (21.0 million hectares at 14%) and biotech canola (7.0 million hectares at 5%) of the global biotech crop area.

Farm animals are currently fed soybean and soybean meal developed from genetic transformation as well as corn and corn products such as corn gluten feed and meal. Europe is strongly dependent upon the American continent for its protein requirements amounting up to 90 to 95% for soybean, 40 to 60% for corn derivatives and partly for canola grain or meal (Aumaitre, 2004).

3. Genetic modification of soybean

Traditionally, plants with desirable characteristics were chosen for food of the next generation. The desirable characteristics arose from naturally occurring variations in the genetic make-up of individual plants. Unlike conventional genetic modification that is carried out through time-tested conventional breeding of plants as combining genes from different organisms is known as recombinant DNA technology and the resulting organism is said to be genetically modified, or genetically engineered or transgenic (Pandey et al., 2010).

Transgenic plant is one that has received a segment of DNA or genes from another organism (known as heterologous or foreign DNA) using recombinant DNA techniques. The foreign DNA is integrated through natural systems present in plant cells into the plant's genome. The newly introduced genes are subsequently inherited in a normal Mendelian manner through pollen and egg cells. The mainly process of introducing DNA into plants (called transformation) uses the *Agrobacterium* mediated method and it can be achieved both in monocotyledonous plants such as wheat, barley and rice and in dicotyledonous plants such as soyabean, potato and tomato.

The soil bacterium *Agrobacterium tumefaciens* causes crown gall disease on some plants, in particular in dicotyledonous species. In causing crown gall disease *A. tumefaciens* transfers DNA (the transferred DNA or T-DNA) from the bacterium to the plant. In nature the transferred bacterial DNA cause the symptoms associated with crown gall disease. In the early 1980s scientists removed the disease causing genes from this bacterium and the T-DNA is now routinely used to transport foreign genes into plants. *Agrobacterium* cells carrying the foreign genes of interest are incubated with cultured cells of the recipient crop plant and transgenic plants are regenerated from them. Not all cells subjected to this process are successfully modified so it may be necessary to identify the modified cells using marker genes which are closely linked to the genetic material that is transferred. These selectable marker genes usually confer resistance to an antibiotic such as kanamycin or resistance to an herbicide.

The genetically modified soybean (named RoundUp Ready, RR) has been rendered tolerant to the glyphosate family of herbicides through expression of transgenic DNA from *Agrobacterium tumefaciens* sp. strain CP4 that encodes 5-enolpyruvylshikamate-3-phosphate synthase (CP4 EPSPS). The CP4 EPSPS protein expressed in GE glyphosate tolerant plants is functionally equivalent to endogenous plant EPSPS enzymes with the exception that CP4 EPSPS displays reduced affinity for glyphosate (Franz et al. 1997). This soybean is, also, composed of a 35S promoter from cauliflower mosaic virus (CMV) and a NOS-terminator, a terminator of nopaline synthase gene.

According to the Center for Environmental Risk Assessment (2010), the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS: EC 2.5.1.19) family of enzymes is ubiquitous in plants and microorganisms. EPSPS enzymes have been isolated from both sources, and their properties have been extensively studied. EPSPS proteins catalyze the

transfer of the enolpyruvyl group from phosphoenol pyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P), thereby yielding inorganic phosphate and 5-enolpyruvylshikimate-3-phosphate (Alibhai and Stallings, 2001). Shikimic acid obtained is a substrate for the biosynthesis of aromatic amino acids (phenylalanine, tryptophan and tyrosine) as well as many secondary metabolites, such as tetrahydrofolate, ubiquinone, and vitamin K. Importantly, the shikimate pathway and, hence, EPSPS proteins, are absent in mammals, fish, birds, reptiles and insects (Alibhai and Stallings, 2001). In contrast, it has been estimated that aromatic molecules, all of which are derived from shikimic acid, represent 35% or more of the dry weight of a plant (Franz et al. 1997).

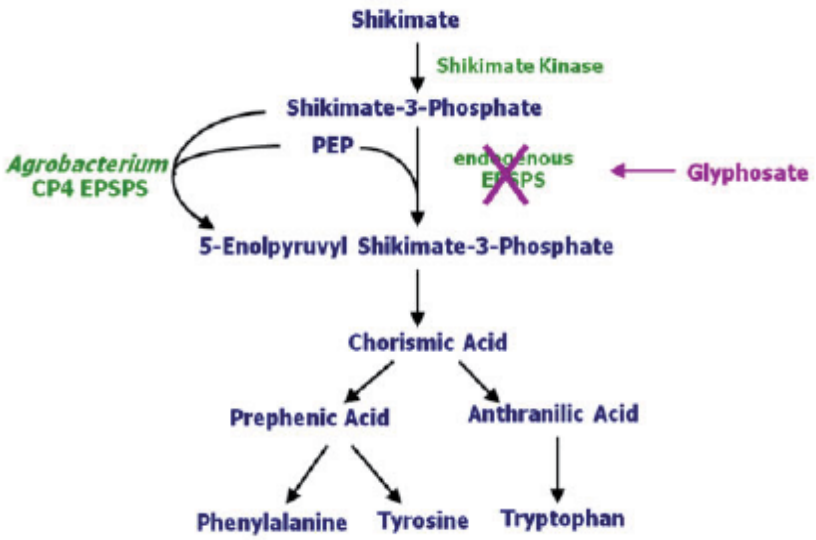


Fig. 1. Schematic representation of glyphosate mode of action and mechanism of CP4 EPSPS mediated tolerance (Center for Environmental Risk Assessment, 2010).

4. Nutritional assessment of genetically modified soybean

In animal nutrition, many studies with GM plants were carried out in target species using the substantial equivalence method. The application of this method to animal studies led to the development of the concept of nutritional equivalence which implies specific measurements regarding animal production. The European Commission has a combined safety approach that requires an assessment of risks for humans, animals, and the environment prior to approval of importation or cultivation of a novel crop (European Commission, 2001). An integral part of the safety evaluation of GM plants is to test for "substantial equivalence". The concept of substantial equivalence is the starting point and guiding concept for safety assessment (Food and Agriculture Organization/World Health Organization, 2000). It is not the conclusion, but it is part of the safety assessment (Konig et al., 2004; Kuiper & Kleter, 2003). The aim of such a test is to determine whether a transgenic plant is substantially equivalent to its conventional counterpart at a chemical and nutritional level. While the parameters to be measured have not been formally defined, minimal analyses performed should determine whether the major nutritional components (i.e., lipids, carbohydrates, proteins, vitamins, minerals, trace elements) and known antinutrients and

toxins of transgenic plants are equivalent to those in conventional varieties that have a history of safe use. Guidelines have been established by several organizations regarding assessment of the allergenic risk of each novel protein expressed in a GM plant, prior to market approval (FAO/WHO, 2000; Konig et al., 2004; Martens, 2000). These typically include comparison of amino acid sequence homology of the novel protein to known allergens and digestion of the protein in simulated gastric environments. While allergic reactions are primarily a concern for human consumption of GM foods, certain proteins in soybean have been shown to elicit allergenic reactions in calves and piglets. The assessment of the safety of GM organisms addresses both intentional and unintentional effects that may result as a consequence of genetic engineering of the food source. Future transgenic crops are expected to contain fewer or no marker genes in the final products since marker free insertion techniques or methods to eliminate marker genes from transgenic plants are being improved. The assessment of safety measures are a lengthy and tedious process (Figure 2). The nutritional aspects, risk characterization and exposure assessment are preliminary steps being taken. Before hitting the market, all GM products have to pass all the allergic tests and provide the details. Only those products find as possessing no harmful or allergic effects are only recommended.

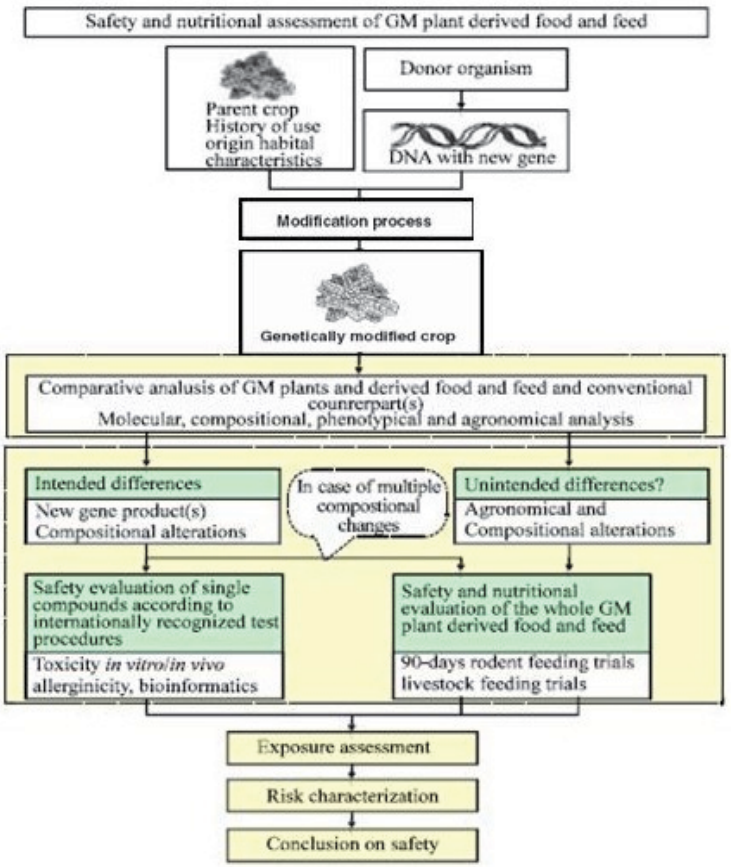


Fig. 2. Pre-market safety and nutritional testing of genetically modified plant derived food and fed (EFSA, 2008).

In most countries authorities and agencies involved in feed/food safety assessment have based their safety assessment strategies and guidelines on this approach. To provide consumers the opportunity for choice, in 2004 the European Union (EU) has extended regulations concerning GM foods to include animal feeds and feed additives. According to Regulation (EC) No. 1829/2003 (European Commission, 2003), all foods and feeds containing or derived from approved GM products in amounts greater than a 0.9% threshold are subject to labelling rules. Labelling of feeds containing GM ingredients informs farmers and gives them the choice of using such feed for their livestock. However, products such as milk, meat, and eggs, that are derived from livestock fed transgenic feeds are exempt from EU-labelling laws. One of the controversies, important for safety aspects of GM feeds, is a potential possibility of transfer of the transgenic DNA to animal tissues, and in consequence its negative effect on consumers of such products originating from animals fed diets containing GM plants. Detailed studies of the feeding qualities of GM plants for livestock and their nutritional evaluation have been reviewed previously (Aumaitre et al., 2002; Flachowsky et al., 2005a; Flachowsky & Aulrich, 2001). A lot of animal studies with GM plants aimed to evaluate the compositional and nutritional equivalency of transgenic feeds and their conventional counterparts.

Accordingly to the substantial equivalence theory, feed chemical analyses were performed to determine whether the macronutrients, vitamins, minerals and even trace elements were found at the same level as in the conventional or isogenic plants. The composition studies confirmed the substantial equivalence of genetically modified (GM) soybean to conventional counterpart (Table 1) (Cromwell et al., 2001; Padgette et al., 1996).

	Conventional soybean	GM soybean
Dry Matter	90.30	91.00
Crude Protein	51.50	51.20
NDF	4.95	4.85
Lysine	3.16	3.09
Methionine+Cysteine	1.47	1.51

Table 1. Chemical composition (% dry matter) of genetically modified soyabean (RoundUp Ready, RR) and its conventional counterpart (Adapted from Cromwell et al., 2001). NDF: neutral detergent fiber.

The concerns that have been raised with respect to the potential risk associated with the use of GM plant products in animal feed are related to the possible unintended effects of inserting novel DNA into the plant by biotechnology. The possible "side effects" of the genetically modification is often termed unintended effects and may result from the random integration in the genome of the novel DNA which may result in an over-expression in the plant of inherently toxic substances such as anti-nutritional factors (ANFs), silencing of endogenous plant genes (e.g essential nutrients), or alterations in host metabolic pathways (Novak & Hasleberger 2000; Saxena & Stotzky, 2001). The possible combination of an unexpected increase in expression of endogenous ANFs and the presence of new exogenous toxicants have been of particular concern as these could compromise the quality of feedstuffs and may affect animal health and nutrition (Francis et al., 2001). Studies also include any known anti-nutritional factors (ANFs), such as trypsin inhibitors in soybean,

interfering with nutrient absorption or natural toxicants typically present in the genus (Padgette et al., 1996). Trypsin inhibitors are similarly destroyed during heating associated with oil extraction and preparation of soybean meal. In addition, the activity of urease measured by the variation in pH is efficiently reduced by heat treatment whatever the genotype of the soybean kernel (Table 2).

	Parental soybean		RR	
	Raw	Meal	Raw	Meal
Lectins (HU/mg sample)	1.2	-	1.0	-
Trypsin inibithor (TIU/mg sample)	22.6	3.4	23.7	3.3
Urease (pH)	2.18	0.03	2.17	0.04

Table 2. Main anti-nutritional factors in raw soybean and soybean meal (parental vs. RR): absence of effect of genetic modification for glyphosate resistance (Adapted from Padgette et al., 1996).

Additional nutritional data can be of importance in the case of oil seeds or oil-rich cereals such as corn because they can markedly affect the composition of fatty tissues when fed to farm animals. Data obtained from the analysis of corn kernels demonstrated similar proportions of fatty acids in oil of kernels of insect resistant and herbicide-resistant corn. Similarly, and in the majority of cases, it has also been observed that insect resistant and herbicide-tolerant corn kernels contain similar proportions of amino acids. The introduction of Bt and herbicide tolerance genes in corn has never been found to create starch modifications expressed as the proportion of amylose and amylopectin. Thus similar proportions of 21.5 and 21.0% of amylose have been found in Bt and herbicide-tolerant modified corn, respectively, compared to 22.4 and 22.7% of amylose in starch of the isogenic varieties, respectively (Benetrix, 2000). Data from the literature have many times corroborated the substantial equivalence in major nutrients and minerals and trace elements in corn and kernels of GM compared to isogenic control corn (Brake & Vlachos, 1998; Sidhu et al., 2000). The whole modified corn plant (Clarke & Ipharraguerre, 2001; Faust, 2000) has also been found to be substantially equivalent in composition to isogenic plants. All these data suggest a similar nutritional value for the feed material derived from the modified plants (Aumaitre et al., 2002).

In order to evaluate the nutritive value of feeds for ruminants, nowadays the in vitro gas production technique (Theodorou et al. 1994) (IVGPT) is commonly used. IVGPT is based on the assumption that the accumulated gas production by a substrate, incubated in with rumen liquor, is proportional to the amount of digestible carbohydrates, and thus highly correlated to the energy value of feeds. In addition, IVGPT allows to study also the fermentation kinetics of feeds. Tudisco et al. (2004) in a research, aimed to compare the fermentation kinetics of Roundup Ready defatted soybean to its conventional counterpart by the IVGPT, found that the genetic modification, although did not affect the chemical composition, led to a significantly lower cumulative gas production and volume per gram of incubated organic matter. It could be hypothesized that the genetic modification may lead to pleiotropic effects (effect of a single gene on multiple phenotypic traits) that may alter the starch and/or protein structure. Alternatively, the results could be explained with a plant DNA transfer to ruminal bacteria which may modify their fermentation activity.

In any case, the results of this research arouse concerns in term of food safety, because other unpredictable metabolic effects, such as metabolic interferences, or direct or indirect insertional mutagenesis cannot be excluded. With this regards Seralini et al. (2011) report that by insertion of the transgene in varieties producing Cry1Ab toxin caused a complex recombination event, leading to the synthesis of new RNA products encoding unknown proteins, or/and to metabolic pathways variations which caused up to 50% changes in measured osmolytes and branched aminoacids.

4.1 Nutritional testing of GM feed with GM soybean in target animal species

Many studies with GM plants were carried out in target species to assess the nutritive value of the feed and their performance potential and were revealed no significant differences in performance indices and quality parameters of meat, eggs or milk, when farm animals were fed diets containing GM or conventional feeds (Aumaitre et al., 2004; Flachowsky et al., 2005a; Świątkiewicz S. & Świątkiewicz M., 2009). These studies have focused on livestock nutrition, in order to confirm nutritional equivalence and to obtain further information concerning the safety of animal products.

As regards monogastric livestock, many feeding studies with 1-day-old broiler chicks have been reported (EFSA, 2008), including GM lines of corn, soybean, canola and wheat and appropriate counterparts. Few experiments are available with laying hens (Aulrich et al., 2001; Halle et al., 2006) where GMOs were compared with near isogenic counterparts. McNaughton et al. (2011) compared the nutritional performance of laying hens fed corn grain (event DP-Ø9814Ø-6) and processed soybean meal (event DP-356Ø43-5), individually or in combination, with the performance of hens fed diets containing conventional corn and soybean meal. The performance (body weight, feed intake and egg production) and egg quality of hens fed GM feeds was comparable with that of hens fed diets formulated with conventional feed. In each study, chemical composition and nutritional value of GM lines and the near-isogenic non-GM lines were found to be comparable without biologically significant differences in the production parameters measured. Experiments with growing and laying quails were carried out to test diets with isogenic or GM Bt 176 corn (Flachowsky et al., 2005b; Halle et al., 2006). Health, hatchability and performance of quails and the quality of meat and eggs were unaffected by the diets. Improvements in livestock performance were noted significant with the diet containing Bt corn compared to diets containing conventional corn grain (Piva et al., 2001a, 2001b). The authors attributed the results to the fact that the use of Bt lines reduced secondary fungal infection and, as a consequence, reduced mycotoxin contamination. Research conducted with growing and finishing pigs (EFSA, 2008) including GM corn grain, sugar beet, soybean meal, rapeseed meal, rice and wheat, showed that when compositional analyses of GM lines and the near-isogenic non-GM line and commercial varieties were comparable, then nutritional equivalence was also established.

As regards ruminants, comparable performance of beef cattle fed corn grain, corn silage or stover from GM plants or from conventional plants is reported, and in dairy cows the inclusion in the diet of feed ingredients derived from a wide range of GM plants unaffected feed intake, milk yield and composition (EFSA, 2008). Milk quality is generally measured as the fat, protein and lactose concentration and as such there is no evidence to suggest that the inclusion of GM feed ingredients affects milk quality. As with other livestock species, studies with lactating dairy cows also showed that once compositional equivalence was demonstrated then nutritional equivalence occurred.

Finally, the production studies carried out with fish provided similar conclusions to those drawn from studies conducted with other livestock species (EFSA, 2008).

	Parameters	Results	References
Poultry	BWG, ADG, FCR, muscle weight	No significant differences	(Hammond et al., 1996)
Pig	BWG, DMI, FCR, carcass quality, sensory score of meat	No significant differences	(Cromwell et al., 2001)
Cow	Milk yield and composition, DMI, nitrogen balance, rumen VFA composition	No significant differences	(Hammond et al., 1996)

Table 3. Some experiments carried out to establish the qualitative and quantitative performance of monogastric or ruminant livestock fed with GM soybean. BWG: Body Weight Gain; ADG: Average Daily Gain; FCR: Feed Conversion Ratio.

5. Fate of transgenic DNA and new protein in animal organs and products

One of the most important questions about the use of GM products in animal nutrition is the possibility that modified DNA could be transferred from plants to animal products or to bacteria, with harmful consequences (FAO, 2004). Other problems regard the ability of transgenic proteins to provoke food intolerance or allergic reaction in susceptible people. Hence, it is necessary to consider the destiny of these molecules within the animal organism (Alexander et al., 2007).

The gastrointestinal tract is constantly exposed to DNA that is released from partially or completely digested food, ingested microbes, and DNA from intestinal microflora. Ingested food is mechanically disrupted and the released DNA, although poorly digested, is cleaved through acid hydrolysis and enzymatic digestion into small DNA fragments. Eventually some of these fragments are converted to single nucleotides. Acid hydrolysis in the gastrointestinal tract is expected to depurinate most adensine and guanine nucleotides of the food DNA (Klinedinst & Drinkwater, 1992). The presence of various phosphatases and deaminases continue to destroy the structural integrity of any free DNA. The breakdown products of DNA are absorbed for using at the cellular level for synthetic processes as they may be found in blood and tissues (McAllan, 1982). All though there were conflicting reports on the fate of GM DNA in the biological system it was observed that DNA could pass through the gut wall into the blood stream and taken up by cells in the blood, liver, spleen and passed through the placenta to the cells of the feoetus and the newborn one (Doerfler & Schubert, 1998).

In ruminants, experimental evidence suggests that more than 80% of DNA is completely disrupted after 2 hours (Wiedemann et al., 2006). However, this degradation is not complete and not immediate (van den Eede et al., 2004).

In animal tissues some fragments of chloroplast DNA have been found. The reason why chloroplast DNA is more frequently detected in animal products is the number of the genes involved and the sensitivity of the PCR method. In transgenic plants, every cell contains hundreds chloroplast genes, but only one transgenic gene (Aumaitre et al., 2002).

Research on the fate of foreign DNA in the mammalian organism showed that PCR products specific to foreign DNA could be detected therein. It was concluded that DNA fragments from the gastrointestinal tract could reach the bloodstream and be transported through the epithelium of the gut and the cells of the Peyer's patches to spleen and liver cells. Such DNA fragments are probably retained for a short while and then digested (Schubbert et al., 1994, 1997, 1998). While the intestinal tract does not seem an absolute barrier against the uptake of macromolecules or even of microorganisms, the mechanism of foreign DNA uptake by the intestinal wall epithelia is unknown. In addition, not much is known about the degradation and integration of the DNA. There is some evidence that fragments of foreign DNA are not digested in the gut and might enter the organism or become incorporated into cells lining the gut wall (Doerfler, 2000; Tony et al., 2003).

Transfer of the plant DNA to bacteria needs several steps, and the expectation seems to be extremely low (Kuiper et al., 2003; Sharma e al., 2004). Bacterial resistance to antibiotics is not a specific problem of genetic engineering. According to Directive 2001/18/EC, use of GMOs containing antibiotic-resistant genes will be forbidden starting from 01/01/2009 (European Commission, 2001).

Data are also available on the fate of recombinant plant DNA in the gastrointestinal tract of humans. By in vitro simulation of human digestion, 80% of the transgene in naked GM soybean DNA was degraded in the gastric simulations, while no degradation of the transgenes contained within GM soybean and corn was observed in these acidic conditions (Martin-Orúe et al., 2002). In the small intestinal simulations, transgenes in naked soybean DNA were degraded. In contrast, the corn nucleic acid was hydrolysed in the small intestinal simulations in a biphasic process in which approximately 85% was rapidly degraded, while the rest of the DNA was cleaved at a low rate of degradation.

The number of transgene copies passing to the small intestine of human ileostomists consuming GM soya were successful quantified, and up to 3.7% of the transgene could survive passage (Neterwood et., 2004).

Finally, another factor that will directly affect gene persistence throughout the digestive tract and therefore indirectly affect the chance of passage across the GIT epithelium is the digestibility of the ingested plant species. Feedstuffs with relatively greater digestibility, such as soybean meal, are likely to have their DNA degraded more rapidly, decreasing the chance of absorption.

A low copy endogenous (soybean lectin) and recombinant (CP4 epsps) gene in longissimus dorsi muscle samples from pigs fed herbicide-tolerant soybean meal, in the grower and finisher phases, respectively were attempted to detect (Jennings et al., 2003). The same results about the fate of the CP4 epsps gene in other species were reported (Klotz & Einspanier, 1998).

The CP4 epsps transgenic gene was not found in muscle and liver of chicken fed herbicide-tolerant soybean up to 7 weeks after ingestion (Khumnirdpetch et al., 2001). According to the authors, GM soybean fragment were degraded in the gastrointestinal tract.

As regards ruminants, chloroplast gene fragments were found in the leucocytes of dairy cows fed small quantities of transgenic soybean meal, while no fragments of the transgenic DNA were found in any tissue examined and in milk (Klotz & Einspanier, 1998). Similarly, high copy chloroplast "rubisco" gene fragments were found in the blood of cattle fed GM and soybean meal, but transgenic sequences were never detected (Phipps et al., 2003).

In fish, soybean meal is used as dietary source of protein in their diet, however, because of the presence of anti-nutritional factors (ANFs) their inclusion levels should be kept low (Olli

et al., 1994). The fate of ingested GM soybean DNA fragments (120 and 195 bp) and a 180 bp fragment of the lectin gene of soybean in Atlantic salmon and their survival through the gastrointestinal tract (GIT) were investigated and the DNA was traced in a variety of fish tissues (liver, muscle and brain) (Sanden et al., 2004). Only the smaller GM DNA fragment (120 bp) was amplified from the content of the stomach, pyloric region, mid intestine and distal intestine, while no transgenic or conventional soybean DNA fragments were detected. The uptake of dietary DNA into blood, kidney and liver of salmon was investigated also by other authors (Nielsen et al., 2005) which determined the DNA fragment size if dietary DNA was detected. Most of the feed (partially digested) was found in the pyloric region, mid intestine, and distal intestine at 4, 8, and 16 h after force-feeding, while the highest concentrations of dietary DNA in liver and kidney were found 8 h after force-feeding, and blood up to 64 h. Finally, the cauliflower mosaic virus 35S promoter fragment (220 bp) of the GM defatted soybean meal was detected in the muscle of rainbow trout receiving both levels of GM soybean (approximately 15 and 30%) diet by nested PCR, but the frequency of detection was greater at the higher inclusion level (Chainark et al., 2006). Additionally, the promoter fragment was not detected by the fifth day after changing the diet to non-GM soybean. Conversely, the promoter fragment was not detected from fish fed with the non-GM SBM diet. Successively, Chainark et al. (2008) traced foreign DNA fragments from genetically modified defatted soybean meal (GM SEM) in rainbow trout by nested polymerase chain reaction (PCR) and located by in situ hybridization. Either a GM or non-GM SBM formulated diet (42% protein) was fed to fish (average weight 50.5 g) for 2 weeks. The degradation results showed that the cauliflower mosaic virus 35S promoter (220 bp) fragment was detected in the contents of digestive system only in fish fed the GM SBM diet, and it was not detected on the third day after changing the diet to the non-GM SBM diet. For the possible transferral results, the promoter fragment was detected in the leukocyte, head kidney and muscle only of fish fed the GM SBM diet; it was not detected on the fifth day after changing the diet to the non-GM SBM diet. These results suggest that a foreign DNA fragment was not completely degraded and might be taken up into organs through the gastrointestinal tract. However, foreign DNA was not detected after the withdrawal period. Thus, the data show that uptake of DNA from GM SBM might not remain in the tissues of fish fed GM SBM diet. Similarly, Ran et al. (2009) found DNA fragments from RR soybean in different tissues and organs of tilapias (*Oreochromis niloticus*, GIFT strain).

Tudisco et al. (2006) in order to evaluate the presence of plant DNA fragments in tissues to follow the fate of plant fed, carried out a research on twenty weaned 30-day-old New Zealand rabbits (10 males and 10 females), individually caged, which were equally assigned to control (C) and treated (T) groups. The animals were given a diet containing soybean meal (solved extracted) which was from conventional or Roundup Ready beans, for group C and T, respectively. The presence of chloroplast DNA was found in tissues and blood from both control and treated groups. The percentage of positive samples were: 50% (blood), 70% (muscle), 80% (heart), 70% (liver) and 80% (kidney). By contrast specific fragments of soybean were not detected in all samples but only in the plant samples. Similarly transgenic fragments gave undetectable results.

Subsequently, the same authors (Tudisco et al. 2010) investigated the presence of DNA fragments in blood and milk from goats fed conventional (control) or Roundup Ready soybean and in blood, skeletal muscle and organs from their offspring. Transgenic target DNA sequences (35S and CP4 EPSPS) were detected in blood and milk from goats that received a diet containing transgenic soybean as well as from some samples of their

offspring, not in the control group. Those findings show plant DNA fragments are likely to survive digestive processes to some extent (Duggan et al., 2003; Einspanier et al., 2004), as well as their transfer to blood and milk. In addition, the detection of plant DNA in tissues and organs of nursed kids could support the hypothesis of a gene transfer through milk.

6. Effects on animal health of GM soybean

In different experiments food and feed derived from GM plants, mixed in animal diets have been fed to rats, mice or other animal species during different periods of administration, and parameters such as body weight, feed consumption, blood chemistry, organ weights, histopathology, etc., have been measured. With respect to recent studies on safety assessment of GM soybeans, the scientific literature shows rather contradictory results.

No immunotoxic activity or an increase in the IgE in serum and histopathological abnormalities were found in the mucosa of the small intestine of rats and mice fed heat-treated GM soybean meal containing the cp4-epsps (Teshima et al., 2000).

In Sprague–Dawley rats, Appenzeller et al. (2008) conducted a subchronic feeding study with the herbicide-tolerant soybean DP-356Ø43-5 (356043). Diets were fed to young adult animals for at least 93 days. Compared with rats fed with the isoline control or conventional reference diets, no biologically-relevant, adverse effects were observed in rats fed diets containing 356043 soybean with respect to body weight/gain, food consumption/efficiency, clinical signs, mortality, ophthalmology, neurobehavioral assessments (sensory response, grip strength and motor activity), clinical pathology (hematology, coagulation, serum chemistry and urinalysis), organ weights, and gross and microscopic pathology. Similarly, Delaney et al. (2008) carried out in Sprague–Dawley rats a subchronic feeding study of high oleic acid soybeans (Event DP-3Ø5423-1). DP-3Ø5423-1 (305423) is a GM soybean produced by biolistic insertion of a gm-fad2-1 gene fragment and the gm-hra gene into the germline of soybean seeds. Compared with rats fed the non-GM control diet, no biologically-relevant differences were observed in animals fed the 305423 diet with respect to body weight/gain, food consumption/efficiency, mortality, clinical signs of toxicity, or ophthalmologic observations. In addition, no diet-related effects were noted on neurobehavioral assessment, organ weights, or clinical or anatomic pathology. Based on the results of these studies, the authors concluded that 356043 and 305423 soybeans were as safe and nutritious as conventional non-GM soybeans. Sakamoto et al. (2007; 2008) conducted 52-week and 104-week feeding studies of genetically modified soybeans in F344 rats. Although in both studies several differences in animal growth, food intake, serum biochemical parameters and histological findings were observed between rats fed the GM (glyphosate-tolerant) soybeans and those fed a commercial diet, body weight and food intake were similar for the rats fed the GM and non-GM soybeans. Gross necropsy findings, hematological and serum biochemical parameters, organ weights, and pathological findings showed no meaningful differences between rats fed the GM and non-GM soybeans. These results indicate that long-term intake (54 and 104 weeks) of GM soybeans at the level of 30% in the diet had no apparent adverse effect in rats.

In a 42-day feeding trial study conducted in broiler chickens (McNaughton et al., 2008), it was also concluded that 356043 soybean was nutritionally equivalent to non-transgenic control soybean with a comparable genetic background.

Finally, also related to GM soybeans, Mathesius et al. (2009) assessed the safety of a modified acetolactate synthase protein (GM-HRA) used as a selectable marker in GM

soybeans. The authors did not find adverse effects in mice following acute oral exposure to GM-HRA at a dose of at least 436 mg/kg of body weight, or in a 28-day repeated dose dietary toxicity study at doses up to 1247 mg/kg of body weight/day. It was concluded that GM-HRA protein is safe when used in agricultural biotechnology.

In contrast to the above results, in a long-term study on female mice fed with a GM modified soybean (insertion of the bacterial CP4 EPSPS gene to confer a high level of tolerance to glyphosate), focused on assessing the effects of this diet on liver of old animals (until 24 months of age) and to elucidate possible interference with aging, Malatesta et al. (2008a) found that GM soybean intake could influence the liver morpho-functional features during the physiological process of aging. Several proteins belonging to hepatocyte metabolism, stress response, calcium signaling and mitochondria were differentially expressed in GM-fed mice, indicating a more marked expression of senescence markers in comparison to controls. Moreover, hepatocytes of GM-fed mice showed mitochondrial and nuclear modifications indicative of reduced metabolic rate. In previous studies on hepatocytes from young and adult (2–8 months of age) female mice fed GM soybeans, nuclear modifications involving structural constituents of the transcription and splicing properties pathways were seen (Malatesta et al., 2002a). Although the cause(s) of the observed alterations could not be conclusively established, it was noted that these modifications disappeared when GM soybean was replaced by a non-GM one in the diet (Malatesta et al., 2005). Since the GM soybean used was tolerant to glyphosate and was treated with the glyphosate-containing herbicide Roundup, the effects observed might be due to herbicide residues. Accordingly, and aiming to verify this hypothesis, Malatesta et al. (2008b) treated rat hepatoma tissue culture (HTC) cells with 1–10 mM Roundup and analyzed cellular features by flow cytometry, fluorescence, and electron microscopy. Under these experimental conditions, the death rate and the general morphology of HTC cells were not affected, as well as most of the cytoplasmic organelles. However, in HTC-treated cells, lysosome density increased and mitochondrial membranes were modified indicating a decline in the respiratory activity. In addition to the above, nuclei underwent morpho-functional modifications suggesting a decreased transcriptional/splicing activity. The authors did not exclude that factors other than the presence of the herbicide residues could be responsible for the cellular modifications described in GM-fed mice. However, they indicated that the concordance of the effects induced by low concentrations of Roundup on HTC cells suggested that the presence of Roundup residues could be one of the factors interfering with multiple metabolic pathways.

Cisterna et al. (2008) investigated the ultrastructural and immunocytochemical features of pre-implantation embryos from mice fed either GM or non-GM soybean in order to verify whether the parental diet could affect the morpho-functional development of the embryonic ribonucleoprotein structural constituents involved in pre-mRNA pathways. Morphological observations revealed that the general aspect of embryo nuclear components were similar in the GM and non-GM soybean-exposed groups. However, immunocytochemical and in situ hybridization results suggested a temporary decrease of pre-mRNA transcription and splicing in 2-cell embryos and a resumption in 4–8-cell embryos from mice fed GM soybean. In addition, pre-mRNA maturation seemed to be less efficient in both 2-cell and 4–8-cell embryos from GM-fed mice than in non-GM-fed animals.

Battistelli et al. (2010) investigated the duodenum and colon of mice fed on genetically modified (GM) soybean during their whole life span (1-24 months) by focusing their attention on the histological and ultrastructural characteristics of the epithelium, the

histochemical pattern of goblet cell mucins, and the growth profile of the coliform population. Even if the GM soybean-containing diet did not induce structural alterations in duodenal and colonic epithelium or in coliform population, the histochemical approach revealed significant diet-related changes in mucin amounts in the duodenum. In particular, the percentage of villous area occupied by acidic and sulphomucin granules decreased from controls to GM-fed animals.

In a previous ultrastructural analysis of testes from mice fed GM soybean conducted by the same research group (Vecchio et al., 2004), it was found that the immunolabelling for Sm antigen, hnRNPs, SC35 and RNA Polymerase II was decreased in 2 and 5 month-old GM-fed mice, and was restored to normal at 8 months. In GM-fed mice of all ages considered, the number of perichromatin granules was higher and the nuclear pore density lower. Moreover, enlargements in the smooth endoplasmic reticulum in GM-fed mice Sertoli cells were also observed. Consequently, all these studies at the microscopic and ultramicroscopic levels showed cellular changes attributable to GM soybean intake.

Magaña-Gómez et al. (2008) conducted a study in Wistar rats, in which the hypothesis was that the intake of GM (SUPRO 500E) soybean could induce pancreatic stress or injury by analyzing the expression of pancreatitis-associated protein (PAP) and trypsinogens by qRT-PCR in rats fed GM soy protein for 30 days. The hypothesis was based on the results of previous investigations showing that mice chronically fed since gestation with GM had problems in synthesis and processing of zymogens by pancreatic acinar cells and reduced nucleoplasmic and nucleolar and perichromatin granule accumulation on pancreatic acinar cell nuclei (Malatesta et al., 2002b; 2003). Magaña-Gómez et al. (2008) did not find differences in nutritional performance among rats fed non-GM and GM diets. The GM diet induced significant zymogen-granule depletion after 15 days feeding, returning to normal levels after 30 days. Acinar disorganization started as early as 5 days after initiation of the GM diet and it recovered after 30 days. Levels of PAP mRNA significantly increased in the GM diet between day 1 and day 3 and decreased to the basal level by day 15. In turn, trypsinogen mRNA peaked at two different times: at day 1 and at day 15, decreasing to basal levels after 30 days, while plasma amylase levels remained unchanged at all times. The authors indicated that GM soy protein intake affected pancreas function, evidenced by the early acute PAP mRNA increased levels and pancreas cellular changes followed by recuperation of acinar cells after 30 days.

Evaluating the GM soybean in Atlantic salmon diet, enlarged spleen and possible impaired spleen function as the number of smaller-sized red blood cells simultaneously increased were indicated (Hemre et al., 2005).

The same authors (Sagstad et al., 2008) reported lower plasma triacylglycerol levels and a significantly larger spleen somatic index in fish groups fed GM soybean compared to groups fed non-GM soybean.

Ermakova (2006) examined the effect of glyphosate-resistant (RR) GM soybean seeds fed to pregnant female rats on the number and weight of pups delivered The study was originally published in Russian, and was heavily criticised for using coated seeds ready for planting instead of beans suitable for feed. The control non-GM soybean was not the isogenic parent line, either. However, because of the possible serious implications of the results of this study for humans and animals it should have been repeated and possibly verified by other scientists with the correct GM soybean diets. Indeed, she has repeatedly pleaded for this but no one dared to try to reproduce her experiments. In this study rats were fed with laboratory rat chow and this diet was complemented with GM or conventional soybean for

two weeks before mating, during the pregnancy and during suckling and the body mass and the number of pups were observed. The data indicated that on the GM soybean-supplemented rat chow significantly fewer pups were born, and with smaller body mass, than on the control non-GM soybeans.

In order to evaluate the possible health effects of a GM diet, Tudisco et al (2006) studied the activity of organ specific enzymes in two groups of New Zealand rabbits, given a diet containing soybean meal which was from conventional or Roundup Ready beans.

Statistical differences were detected in kidney for alanine aminotransferase (ALT), gamma glutamyltransferase (GGT) and lactic dehydrogenase (LDH) (higher activity for group fed GM soybean) whereas in the heart such result was seen only for LDH. No statistical differences were found for serum, liver and skeletal muscle. Significant differences between groups were detected for heart LDH1 and LDH2 and for kidney LDH1, thus confirming the significant increase of the enzyme in these tissues. Moreover, despite no significant differences were found for LDH total activity in liver, a significant increase (LDH1) and decrease (LDH4) were found also in this organ.

Brasil et al. (2009) found that rats fed on GM soy showed altered morphology of the uterus and the ovaries: had greater volume density of endometrial glanular epithelium, reduced follicle number and increased corpus luteum numbers (a tendency to abort or less of a chance to get pregnant). Although the GM diet was not supplemented with cysteine as the other diets, and it is difficult to assess if the results were due to consumption of the transgenic soy itself or were due to the presence of glyphosate (and/or AMPA), always present in GM seeds, the findings are disturbing and warrant further studies.

A recent study found that the hamsters fed with GM Soybean showed the growth of hairs inside the pouches of the mouth and the number of hairy mouthed hamsters was much higher in the third generation of GM soy fed animals than in others (Baranov et al., 2010). According to the authors, it remains unclear why these hair structures appear in the oral cavity of mammals. We may only speculate on the origin of this phenomenon. The gingival pouches may result from paradontitis and paradontosis caused by feeding on compound food in the vivarium, i.e., by a suboptimal diet. This pathology may be exacerbated by elements of the food that are absent in natural food, such as genetically modified (GM) ingredients (GM soybean or corn meal) or contaminants (pesticides, mycotoxins, heavy metals, etc.). Probably, hair growth in the gingival pouches is a protective reaction of the body suppressing the progress of gingival pathology, because the hair bundles are so dense that they prevent food from getting into the pouches and the resultant inevitable inflammation. Hair grows in the parts of the mucosa that, being affected by mechanical factors, acquire the capacity for keratinization.

More recently, Tudisco et al (2010) studied the possible effects on cell metabolism, by determination of several specific enzymes in serum of goats fed conventional or Roundup Ready soybean and in heart, skeletal muscle, liver and kidney of their offspring. Aspartate aminotransferase (AST) and ALT enzyme activity were significantly lower in serum from goats fed GM soybean but enzyme levels were in the normal range. Statistical differences were detected in kid's kidney for GGT and LDH, whereas in the heart and skeletal muscle this result was seen only for LDH. The increase in LDH activity was confirmed by histochemistry. In addition, significant differences between control and treated animals were detected for heart, kidney, muscles and liver LDH isoenzyme distribution, particularly concerning the LDH1, as found previously in the rabbits. Since LDH1 is known to be involved in cell metabolism by favouring the reaction of lactate to pyruvate (Van Hall, 2000), these results could indicate a general increase in cell metabolism.

A summary of experimental studies concerning health of animals fed genetically modified soybean is reported in Table 4.

Animal species	Length of study	Main effects	Reference
BN rats and B10A mice	15 weeks	No immunotoxic activity. No histopathological abnormalities.	Teshima et al. (2000)
Sprague–Dawley rats	> 93 days	No adverse effects on body weight/gain, food consumption, clinical signs, mortality, ophthalmology, neurobehavioral assessment, clinical pathology, organ weights and gross and microscopic pathology	Appenzeller et al. (2008)
Sprague–Dawley rats	–	No adverse effects on body weight/gain, food consumption, and mortality, clinical signs of toxicity or ophthalmological observations, neurobehavioral assessments, organ weights or clinical and anatomic pathology	Delaney et al. (2008)
Mice	28 days	No adverse effects	Mathesius et al. (2009)
F344 rats	52 weeks	No adverse effect in gross necropsy findings, hematological and serum biochemical parameters, organ weights and pathological findings	Sakamoto et al. (2007)
F344 rats	104 weeks	No adverse effect in gross necropsy findings, hematological and serum biochemical parameters, organ weights and pathological findings	Sakamoto et al. (2008)
Broilers	42 days	No adverse effects were found. It was concluded that GM 356Ø43 was nutritionally equivalent to non-GM soybean with comparable genetic background	McNaughton et al. (2008)
Mice	-	Enlargements in the smooth endoplasmic reticulum of Sertoli cells	Vecchio et al. (2004)
Mice	–	Several proteins belonging to hepatocyte metabolism, stress response, calcium signaling and mitochondria were differentially expressed in GM-fed mice indicating a more marked expression of senescence markers in comparison to controls. GM-fed mice showed mitochondrial and nuclear modifications indicative of reduced metabolic rate	Malatesta et al. (2008a)

Mice	–	No morphological differences in embryos of GM and non-Gm soybean-exposed groups. Microscopic and ultramicroscopic cellular changes attributed to GM soybean intake	Cisterna et al. (2008)
Wistar rats	30 days	No adverse effects in nutritional performance. Altered pancreas function evidenced by the early acute PAP mRNA increased levels and pancreas cellular changes	(Malatesta et al., 2002a) and (Malatesta et al., 2002b)
Wistar rats	30 days	Significant zymogen-granule depletion	Magaña-Gómez et al. (2008)
Pregnant Rats	2 weeks	Fewer pups born with smaller body mass	Ermakova et al. (2006)
Rabbits	60 days	Significant alteration of kidney ALT, GGT and LDH activity. Significant alteration of heart LDH activity	Tudisco et al. (2006)
Hamsters	-	Growth of hairs inside the pouches of the mouth and the number of hairy mouthed	Baranov et al. (2010)
Kids	60 days	Significant alteration of kidney GGT and LDH activity. Significant alteration of LDH isoenzyme distribution in heart, kidney, muscle and liver	Tudisco et al. (2010)

Table 4. A summary of experimental studies concerning health of animals fed with genetically modified soybean.

7. Human/animal safety of glyphosate

Glyphosate is not a genetically modified product but because its use in agriculture is inseparable from the cultivation of herbicide-tolerant GM crops in a particular technology package, its effects on health need to be examined also with that of the glyphosate-resistant GM crops. Although the declared aim of the introduction of glyphosate resistant GM crops was that with these crops the amount of herbicide sprayed on the land should decrease, due to the ever increasing area of cultivation of glyphosate-resistant Roundup Ready GM crops, the use of glyphosate has in fact increased (Benbrook, 2004; 2009). The glyphosate-containing sprays destroy all weeds but the growth of the glyphosate-resistant GM crop is protected regardless of how much glyphosate is sprayed on to the land. To make sure that all weeds are destroyed the use of glyphosate and consequently the glyphosate load of the land has been substantially increasing after the first few years of a slight reduction (Benbrook, 2004; 2009). This has happened despite the ever-increasing number of publications showing that glyphosate has many serious and detrimental effects on the environment and biodiversity (Relyea 2005) with the development of herbicide-resistant weeds (Duke, 2005; Owen & Zelaya 2005; Warwick et al., 2008; Zelaya et al., 2007). There is also an urgent need to consider the potentially seriously damaging effects of this total

herbicide on human/animal health, particularly as it is used in large amounts. Indeed, there are a number of recently published papers that all indicate possible damaging effects of glyphosate on health and reproduction which need to be taken seriously. On previous work the findings of Marc et al. (2005) have confirmed and extended their previous results by showing that the main ingredient of commercial Roundup formulations, glyphosate, in a mM concentration range, particularly when used together with the obligatory polyoxyethylene amine surfactant, inhibited the transcription of one of the enzymes involved in hatching of sea urchin embryos and therefore significantly delayed their hatching. When it is considered that farm workers inhale commercial herbicide sprays in which the active ingredient concentration exceeds by about 25 times of that used in the transcription inhibition studies by Marc et al. (2005), health concerns due to the use of glyphosate must be acute. In another study it was shown that in the oral treatment of Wistar rats with increasing concentrations of the herbicide Glyphosate-Biocarb, a formulation used in many countries such as Brazil, the number of Kupffer cells in hepatic sinusoids increased, followed by large deposition of reticulin fibres and the leakage of hepatic aspartate aminotransferase and alanine aminotransferase into the circulation, indicating hepatic damage in these animals (Benedetti et al., 2004). Successively, Richard et al. (2005) and Benachour et al. (2007) showed that glyphosate, particularly as used together with polyoxyethylene amine surfactant in Roundup Ready formulations, was toxic to human placental JEG3 cells at concentrations lower than that used in agricultural practices. Even at subtoxic concentrations RR was an endocrine disruptor on aromatase activity and its mRNA level as glyphosate interacted with the active site of the purified enzyme. It is possible that the pregnancy problems in agricultural workers using Roundup may be traced back to the exposure to this herbicide (Savitz et al., 2000).

Recently, Gasnier et al. (2009) exposed human liver HepG2 cells, a well-known model to study xenobiotic toxicity, to four different formulations and to glyphosate, which is usually tested alone in chronic *in vivo* regulatory studies. They measured cytotoxicity with three assays (Alamar Blue®, MTT, ToxiLight®), plus genotoxicity (comet assay), anti-estrogenic (on ER_, ER_) and anti-androgenic effects (on AR) using gene reporter tests. They also checked androgen to estrogen conversion by aromatase activity and mRNA. All parameters were disrupted at sub-agricultural doses with all formulations within 24 h. These effects were more dependent on the formulation than on the glyphosate concentration. First, the observed a human cell endocrine disruption from 0.5ppm on the androgen receptor in MDA-MB453-kb2 cells for the most active formulation (R400), then from 2ppm the transcriptional activities on both estrogen receptors were also inhibited on HepG2. Aromatase transcription and activity were disrupted from 10 ppm. Cytotoxic effects started at 10ppm with Alamar Blue assay (the most sensitive), and DNA damages at 5 ppm. According to the authors, the direct G action is most probably amplified by vesicles formed by adjuvants or detergent-like substances that allow cell penetration, stability, and probably change its bioavailability and thus metabolism. These detergents can also be present in rivers as polluting contaminants. The type of formulation should then be identified precisely in epidemiological studies of G-based herbicides effects. Of course to drive hypotheses on *in vivo* effects, not only dilution in the body, elimination, metabolism, but also bioaccumulation and time-amplified effects should be taken into account. These herbicides mixtures also present endocrine effects on human cells, at doses far below agricultural dilutions and toxic levels on mitochondrial activities and membrane integrity.

All these findings indicate that there is an urgent need to carry out systematic and direct studies, independent of the biotech industry, on the short- and long-term effects on animal (and human) health of exposure to glyphosate and its more effective commercial formulations alone and/or preferably in combination with the appropriate GM crop. With the presently cultivated huge areas of Roundup Ready crops and the anticipated even larger future extensions of this glyphosate-dependent GM crop technology the potential danger for animal/human health needs to be dealt with in advance and not if or when it occurs. If we consider that RR soybeans may in themselves damage reproduction, a combination of the similar, possibly synergistic effects of the GM crop and glyphosate could be a potential disaster waiting to happen.

8. Conclusions

The debate on the safety of genetically modified organisms (GMOs) used for food and feed is still very lively throughout the world, more than 15 years after their first commercial release. Huge social, economical, and political issues have been raised. Unfortunately, although some stakeholders claim that a history of safe use of GMOs can be upheld, there are no human or animal epidemiological studies to support such a claim as yet, in particular because of the lack of labeling and traceability in GMO-producing countries. As a matter of fact, 97% of edible GMOs among cultivated GMOs (soy, corn and oilseed rape or canola, excluding cotton) are grown in South and North America, where GMOs are not labeled. All these plants have been modified to tolerate and/or produce one or more pesticides, and contain therefore such residues at various levels. Most are Roundup residues (it is a major herbicide used worldwide and tolerated by about 80% of GMOs).

As stated by the EFSA (2008), several aspects have to be investigated when considering whether or not recombinant DNA from GM plants, or the derived proteins can end up in animal tissues and products. These include (i) the fate of the recombinant DNA and protein during feed processing and ensiling; (ii) the fate of the recombinant DNA and protein in the gastrointestinal tract of animals fed with GM feed; (iii) the potential absorption of the digested pieces of DNA or protein into animal tissues/products and (iv) the potential of biological functionality of absorbed DNA and protein fragments.

The mere detection of recombinant DNA fragments in animal organs and tissues could not justify, by itself, public concerns regarding human consumption of products from farm animals fed transgenic crops. However, the persistence of DNA after dietary exposure is one aspect of risk assessment for novel food. Indeed, as concerns the hypothetical horizontal gene transfer of recombinant DNA from GM crop-derived feeds to animal and human gut microflora, Netherwood et al. (2004), found that a small proportion of feed DNA survives passage through the human upper gastrointestinal tract and a very small proportion of the small intestinal microflora containing transgenic feed. According to the authors, even if this result does not indicate a complete transgenic transfer to the prokaryotes, the survival of transgenic DNA during the passage through the small intestine should be considered in future safety assessments of GM foods. In addition, any alteration in cell metabolism should be taken into account in this field. For instance, the modification in LDH synthesis suggests an increase in cell metabolism. Therefore, possible long-term effects of such an alteration need to be elucidated.

In conclusion, taking into account the potential risks related to GMP impact, further researches are needed in this area, including studies to determine DNA transport or entry

mechanisms/processes across the epithelial layer of the gastro-intestinal tract into the bloodstream, as well as degradation or accumulation of foreign DNA in blood or other organs of animal species. In any case, the traceability of products from animals fed on GMOs is crucial.

9. References

Alexander, T.W., Reuter, T., Aulrich, K., Sharma, R., Okine, E.K., Dixon, W.T., & McAllister, T.A. (2007). A review of the detection and fate of novel plant molecules derived from biotechnology in livestock production. *Animal Feed Science and Technology*, 133, pp. 31-62.

Alibhai, M.F. & Stallings, W.C. (2001). Closing down on glyphosate inhibition – with a new structure for drug discovery. *Proceedings of the National Academy of Sciences,* 98, pp. 2944-2946.

Appenzeller, L.M., Munley, S.M., Hoban, D., Sykes, G.P., Malley, L.A., Delaney, B. (2008). Subchronic feeding study of herbicide-tolerant soybean DP-356Ø43-5 in Sprague–Dawley rats. *Food Chem Toxicol*, 46, pp. 2201–13.

Aulrich, K., Böhme, H., Daenicke, R., Halle, I., Flachowsky, G. (2001). Genetically modified feeds in animal nutrition. 1st Com.: Bacillus thuringiensis (Bt) corn in poultry, pig and ruminant nutrition. *Archives of Animal Nutrition*, 54, pp. 183-195.

Aumaitre, A., Aulrich, K., Chesson, A., Flachowsky, G., Piva, G. (2002). New feed from genetically modified plants: substantial equivalence, nutritional equivalence, digestibility, and safety for animals and the food chain. *Livestock Production Science*, 74, pp. 223-238.

Aumaitre, A. (2004). Safety assessment and feeding value for pigs, poultry and ruminant animals of pest protected (Bt) plants and herbicide tolerant (glyphosate, glufosinate) plants: interpretation of experimental results observed worldwide on GM plants. *Italian Journal of Animal Science,* 3, pp. 107-121.

Baranov, A.S., Chernova, O.F., Feoktistova, N., Yu, Surov, A.V. (2010). A New Example of Ectopia: Oral Hair in Some Rodent Species. *Doklady Akademii Nauk*, 431, pp. 559–562.

Battistelli, S., Citterio, B., Baldelli, B., Parlani, C., Malatesta, M. (2010). Histochemical and morpho-metrical study of mouse intestine epithelium after a long term diet containing genetically modified soybean. *European Journal of Histochemistry,* 54, pp. 154-157.

Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., Séralini, G.E. (2007). Time- and dose-dependent effects of roundup on human embryonic and placental cells. *Archives of Environmental Contaminant Toxicology*, 53, pp. 126-133.

Benbrook, C.M. (2004). Genetically engineered crops and pesticide use in the United States. The first nine years. *BioTech InfoNet Technical*, 7.

Benbrook, C. (2009). Impacts of genetically engineered crops on pesticide use: The first thirteen years. The Organic Center, *Critical issues*, pp.1-47.

Benedetti, A.L., Lourdes Vituri, C., de Trentin, A.G., Domingues, M.A.C., Alvarez-Silva, M. (2004). The effects of sub-chronic exposure of Wistar rats to the herbicide Glyphosate-Biocarb. Toxicolology Letters, 153, pp. 227-232.

Benetrix, F. (2000). Evaluation de la valeur technologique, alimentaire et zootechnique de produits et de sous produits issus de plantes genetiquement modifiees, *personnal communication*.

Brake, J. & Vlachos, D. (1998). Evaluation of Transgenic Event 176 "Bt" Corn in Broiler Chickens. *Poultry Science*, 77, pp. 648-653.

Brasil, F.B., Soares, L.L., Faria,T.S., Boaventura, G.T., Barcellos Sampaio, F.J., Ramos, C.F. (2009). The Impact of Dietary Organic and Transgenic Soy on the Reproductive System of Female Adult Rat. *The Anatomical Record,* 292, pp. 587–594.

Center for Environmental Risk Assessment (2010). A Review of the Environmental Safety of the CP4 EPSPS Protein. ILSI Research Foundation. Washington D.C., USA, pp. 1-16.

Chainark, P., Satoh, S., Hino, T., Kiron, V., Hirono, I., Aoki, T. (2006). Availability of genetically modified soybean meal in rainbow trout *Oncorhynchus mykiss* diets. *Fisheries Science*, 72, pp. 1072–1078.

Chainark, P., (2008). Availability of genetically modified feed ingredient II: investigations of ingested foreign DNA in rainbow trout *Oncorhynchus mykiss*. *Fisheries Science*, 74, pp. 380-390.

Cisterna, B., Flach, F., Vecchio, L., Barbino, S.M.L., Battistelli, S., Martin, T.E., Malatesa, M., Biggiogera, M. (2008). Can a genetically modified organism-containing diet influence embryo development? A preliminary study on pre-implantation mouse embryos. European Journal of Histochemistry, 52, pp. 263–267.

Clarke, J.H. & Ipharraguerre, I.R. (2001). Livestock performance: feeding biotech-crops. *Journal of Dairy Science*, 84, pp. E9–E18.

Cromwell, G.L., Lindemann, M.D., Randolph, J.H., Stanisieweski, E.P., Hartnell, G.F. (2001). Soybean meal from Roundup Ready or conventional soybeans in diets for growing-finishing pigs. *J. Anim. Sci.,* 79, pp. 1318.

Delaney, B., Appenzeller, L.M., Munley, S.M., Hoban, D., Sykes, G.P., Malley, L.A. , Sanders, C. (2008). Subchronic feeding study of high oleic acid soybeans (event DP-3Ø5423-1) in Sprague–Dawley rats. *Food and Chemical Toxicology,* 46, pp.3808–3817.

Doerfler, W. (2000). Foreign DNA in Mammalian Systems. Wiley-VCH Verlag GmbH, Weinheim.

Doerfler, W., Schubert, R. (1998). Uptake of foreign DNA from the environment: the gastro-intestinal tract and the placenta as portals of entry. *Wien Klin Wochenschr,* 110, pp. 40-44.

Duggan, P.S., Chambers, P.A., Heritage, J., Forbes, J.M. (2003). Fate of genetically modified corn DNA in the oral cavity and rumen of sheep. *British Journal of Nutrition*, 89, pp. 159–166.

Duke, S.O. (2005). Taking stock of herbicide-resistant crops ten years after introduction. *Pest Management Science,* 61, pp. 211-218.

Einspanier, R., Lutz, B., Rief, S., Berezina, O., Zverlov, V., Scwarz, W., Mayer, J. (2004). Tracing residual recombinant feed molecules during digestion and rumen bacterial diversity in cattle fed transgene corn. *European Food Research and Technology*, 218, pp. 269–273.

Ermakova, I. (2006). Genetically modified soy leads to the decrease of weight and high mortality in rat pups of first generation: preliminary studies. *EcosInfo*., 1, pp. 4-10.

European Commission (2001). Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of

genetically modified organism and repealing Council Directive 90/220/EEC. *Official Journal of Communities*, L106, 1–39.

European Commission (2003). Regulation (EC) 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off J Eur Communities*, L268, 1–23

European Food Safety Authority (EFSA) - GMO Panel Working Group on Animal Feeding Trials (2008). Safety and nutritional assessment of GM plants and derived food and feed: The role of animal feeding trials. Food Chemistry and Toxicology, 46, pp. S2-S70.

FAO/WHO (2000). Safety Aspects of Genetically Modified Foods of Plant Origin. Report of a Joint FAO/WHO Expert Consultation on Foods Derived from Biotechnology. World Health Organization, Geneva.

FAO (2004). The state of food and agriculture 2003-2004. http://www.fao.org/docrep/006/y5160e/y5160e10.html

Faust, M. (2000). Livestock products composition and detection of transgenic DNA/proteins. *Proceeding of Annual Meeting ADAS-ASAS Agricultural and Biotechnology Market*, pp. 1-48, Baltimore MD, USA.

Flachowsky, G., Aulrich, K., (2001). Nutritional assessment of feeds from genetically modified organism (GMO). *Journal of Animal Feed Science*, 10, pp. 181–194.

Flachowsky, A., Chesson, A., Aulrich, K. (2005a). Animal nutrition with feeds from genetically modified plants. *Archive of Animal Nutrition*, 59, pp. 1-40.

Flachowsky, G., Halle, I., Aulrich, K. (2005b). Long term feeding of Bt-corn—a 10 generation study with quails. *Archives of Animal Nutrition*, 59, pp. 449–451.

Francis, G., Makkar, H.P.S. & Becker, K. (2001). Anti-nutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, 199, pp. 197–227.

Franz, J.E., Mao, M.K. and Sikorski, J.A. (1997). Glyphosate: A Unique Global Herbicide. In *ACS Monograph* American Chemical Society (Eds.), Washington D.C., pp. 27-64.

Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M.C., Séralini, G.E. (2009). Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology,* 262, pp. 184–191

Halle, I., Aulrich, K., Flachowsky, G. (2006), Four generations feeding GMO-corn to laying hens. *Proc Soc Nutr Physiol* , 15, 114 (Abstract).

Hammond, B.G., Vicini, J.L., Hartnell, G.F., Naylor, M.W., Knight, C.D., Robinson, E.H., Fuchs, R.L. and Padgette, SR. (1996). The feeding value of soybeans fed to rats, chickens, catfish and dairy cattle is not altered by genetic incorporation of glyphosate tolerance. *Journal of Nutrition*, 126, pp. 717–727.

Hemre, G.I., Sanden, M., Bakke-McKellep, A.M., Sagstad, A., Krogdhal, A. (2005). Growth, feed utilization and health of Atlantic salmon Salmo salar L. fed genetically modified compared to non-modified commercial hybrid soybeans. *Aquaculture Nutrition*, 11, pp.157-167.

Jennings, J.C., Kolwyck, D.C., Kays, S.B., Whetsell, A.J., Surber, J. B., Cromwell, G.L., Lirette, R.P., Glenn, K.C. (2003). Determining whether transgenic and endogenous plant DNA and transgenic protein are detectable in muscle from swine fed Roundup Ready soybean meal. *Journal of Animal Science*, 81, pp. 1447–1455.

Khumnirdpetch, V., Intarachote, U., Treemanee, S., Tragoonroong, S., Thummabood, S. (2001). Detection of GMOs in the broilers that utilized genetically modified soybean meals as a feed ingredient. In *Plant & Animal Genome IX Conference*, Town and Country Hotel, San Diego, San Diego, California, USA.

Klinedinst, D. & Drinkwater, N. (1992). Mutagenesis by apurinic sites in normal and ataxia telangiectasia human lymphoblastoid cells. *Molecular Carcinogenesis*, 6, pp. 32-42.

Klotz, A. & Einspanier, R. (1998). Detection of "novel-feed" in animals? Injury of consumers of meat or milk is not expected. *Mais*, 3, pp. 109–111.

Konig, A., Cockburn, A., Crevel, R.W.R., Debruyne, E., Grafstroem, R., Hammerling, U., Kimber, I., Knudsen, I., Kuiper, H.A., Peijnenburg, A.A.C.M., Penninks, A.H., Poulsen, M., Schauzu, M.,Wal, J.M. (2004). Assessment of the safety of foods derived from genetically modified (GM) crops. *Food Chem. Toxicol.* , 42, pp. 1047–1088.

Kuiper, H.A., Kleter, G.A. (2003). The scientific basis for risk assessment and regulation of genetically modified foods. *Trends of Food Science Technology*, 14, pp. 277-293.

ISAAA (International Service for the Acquisition of Agri-biotech Applications) (2010). Global Status of commercialised Biotech/GM crops: 2010. http://www.isaaa.org/resources/publications

Magaña-Gómez, J.A., López Cervantes, G., Yepiz-Plascencia, G., Calderón De La Barca, A.M. (2008). Pancreatic response of rats fed genetically modified soybean. *J Appl Toxicol*, 28, pp. 217–226.

Malatesta, M., Caporaloni, C., Rossi, L., Battistelli, S., Rocchi, M.B.L., Tonucci, F., Gazzanelli, G. (2002a). Ultrastructural analysis of pancreatic acinar cells from mice fed on genetically modified soybean. *Journal of Anatomy*, 201, 409–415.

Malatesta, M., Caporaloni, C., Gavaudan, S., Rocchi, M.B.L., Serafini, S., Tiberi, C., Gazzanelli, C. (2002b). Ultrastructural morphometrical and immunocytochemical analyses of hepatocyte nuclei from mice fed on genetically modified soybean. *Cell Structur and Function*, 27, pp. 173–180.

Malatesta, M., Biggiogera, M., Manuali, E., Rocchi, M.B.L., Balzelli, B., Gazzanelli, G. (2003). Fine structural analyses of pancreatic acinar cell nuclei from mice fed on genetically modified soybean. *European Journal Histochemical*, 47, pp. 385–8.

Malatesta M., Tiberi C., Baldelli B., Battistelli S., Manuali E., Biggiogera M. (2005). Reversibility of hepatocyte nuclear modifications in mice fed on genetically modified soybean. *European Journal Histochemical*, 49, pp. 237–242.

Malatesta, M., Boraldi, F., Annovi, G., Balzelli, B., Battistelli, S., Biggiogera, M., Quaglino, D. (2008a). A long-term study on female mice fed on a genetically modified soybean: effects on liver ageing. *Histochemistry Cell Biology*, 130, pp. 967–977.

Malatesta, M., Perdoni, F., Santin, G., Battistelli, S., Muller, S., Biggiogera, M. (2008b). Hepatoma tissue culture (HTC) cells as a model for investigating the effects of low concentrations of herbicide on cell structure and function. Toxicology *In Vitro*, 22, pp. 1853–1860.

Marc, J., Le Breton, M., Cormier, P., Morales, J., Bellé, R., Mulner-Lorillon, O. (2005). A glyphosate-based pesticide impinges on transcription. *Toxicol. Appl. Pharmacol.*, 203, pp. 1-8.

Martens, M.A. (2000). Safety evaluation of genetically modified foods. *Int. Arch. Occup. Environ. Health*, 73, pp. S14–S18.

Martin-Orúe, S.M., O'Donnell, A.G., Arino, J., Netherwood, T., Gilbert, H.J., Mathers, J.C. (2002). Degradation of transgenic DNA from genetically modified soya and maize in human intestinal simulations. *British Journal of Nutrition*, 87, pp. 533–542.

Mathesius, C.A., Barnett, J.F., Cressman, R.F., Ding, J., Carpenter, C., Ladics, G.S., Schmidt, J., Layton, R.J., Zhang, J.X.Q. , Appenzeller, L.M., Carlson, G., Ballou, S., Delaney, B. (2009). Safety assessment of a modified acetolactate synthase protein (GM-HRA) used as a selectable marker in genetically modified soybeans. *Regul Toxicol Pharmacol*, 55, pp. 309–320.

McAllan, A.B. (1982). The fate of nucleic acids in ruminants. *Proc Nutr Soc*, 41, pp. 309-317.

McNaughton, J., Roberts, M., Smith, B., Rice, D., Hinds, M., Sanders, C., Layton, R., Lamb, I., Delaney B. (2008). Comparison of broiler performance when fed diets containing event DP-3Ø5423-1, nontransgenic near-isoline control, or commercial reference soybean meal, hulls, and oil. *Poultry Science*, 87, pp. 2549–2561.

McNaughton, J., Roberts, M., Rice, D., Smith, B., Hinds, M., Delaney, B., Iiams, C., Sauber, T. (2011). Nutritional equivalency evaluation of transgenic maize grain from event DP-O9814O-6 and transgenic soybeans containing event DP-356O43-5: laying hen performance and egg quality measures. *Poultry Science*, 90 (2), pp. 377-389.

Neterwood, T., Martin-Orúe, S.M., O'Donnell, A.G., Gockling, S., Graham, J., Mathers, J.C., Gilbert, H.J. (2004). Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. *Nature Biotechnology*, 22, pp. 204-209.

Nielsen, C.R., Berdal, K.J., Bakke-McKellep, A.M., Holst-Jensen, A. (2005). Dietary DNA in blood and organs of Atlantic salmon (Salmo salar L.). *European Food Research and Technology*, 221, pp. 1–8.

Novak, W.K. & Hasleberger, A.G. (2000). Substantial equivalence of anti-nutrients and inherent plant toxins in genetically modified novel foods. *Food Chemistry and Toxicology*, 38, pp. 473–483.

Olli, J.J., Krogdahl, A., van den Ingh, T.S.G.A.M. & Brattas, L.E. (1994). Nutritive value of four soy products in diets for Atlantic salmon (*Salmo salar* L.). *Acta Agriculture Scandinave A Animal Science*, 44, pp. 50–60.

Owen, M.D.K., Zelaya, I.A. (2005). Herbicide-resistant crops and weed resistance to herbicides. *Pest Management Science*, 61, pp. 301-311.

Padgette, S.R., Biest Taylor, N., Nida, D.L., Bailey, M.R., MacDonald, J., Holden, L.R., Fuchs, R.L. (1996). The composition of glyphosate-tolerant soybean seeds is equivalent to that of conventional soybean. *Journal of Nutrition*, 126, pp. 702–716.

Pandey, A., Kamle, M., Yadava, L.P., Muthukumar, M., Kumar, P., Gupta, V., Ashfaque, M., Pandey, B.K. (2010). Genetically modified food: Its uses, future prospects and safety assessments. *Biotechnology*, 9, pp. 444-458.

Phipps, R.H., Deaville, E.R., Maddison, B.C. (2003). Detection of transgenic and endogenous plant DNA in rumen fluid, duodenal digesta, milk, blood, and feces of lactating dairy cows. *Journal of Dairy Science*, 86, pp. 4070–4078.

Piva, G., Morlacchini, M., Pietri, A., Piva, A., Casadei, G., (2001a). Performance of weaned piglets fed insect-protected (MON 810) or near isogenic corn. *Journal of Animal Science*, 79, pp. 106(Abstract) .

Piva, G., Morlacchini, M., Pietri, A., Rossi, F., Grandini, A., (2001b). Growth performance of broilers fed insect-protected (MON 810) or near isogenic control corn. *Poultry Science*, 80 (Suppl. 1), 320 (Abstract).

Ran, T., Mei, L., Lei, W., Aihua, L., Ru, H., Jie, S. (2009). Detection of transgenic DNA in tilapias (*Oreochromis niloticus*, GIFT strain) fed genetically modified soybeans (Roundup Ready). *Aquaculture Research*, 40, pp. 1350-1357.

Relyea, A.A. (2005). The impact of insecticides and herbicides on the biodiversity and productivity of aquatic communities. *Ecological Applications,* 15, pp. 618-627.

Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., Seralini, G.-E. (2005). Differential effects of glyphosate and Roundup on human placental cells and aromatase. Environmental Health Perspective, 113, pp. 716-720.

Sagstad, A., Sanden, M., Krogdahl, A., Bakke-McKellep, A.M., Froystad, M., Hemre, G.-I. (2008). Organs development, gene expression and health of Atlantic salmon (Salmo salar L.) fed genetically modified soybeans compared to the near-isogenic non-modified parental line. *Aquaculture Nutrition*, 14, pp. 556-572.

Sakamoto, Y., Tada, Y., Fukumori, N., Tayama, K., Ando, H., Takahashi, H. (2007). A 52-week feeding study of genetically modified soybeans in F344 rats. *Journal Food Hygiene Society of Japan*, 48, pp. 41–50.

Sakamoto, Y., Tada, Y., Fukumori, N., Tayama, K., Ando, H., Takahashi, H. (2008). A 104-week feeding study of genetically modified soybeans in F344 rats. *Journal Food Hygiene Society of Japan*, 49, pp. 272–282.

Sanden, M., Bruce, I.J., Rahman, M.A., Hemre, G.-I. (2004). The fate of transgenic sequences present in genetically modified plant products in fish feed, investigating the survival of GM soybean DNA fragments during feeding trials in Atlantic salmon, *Salmo salar* L. *Aquaculture*, 237, pp. 391–405.

Savitz, D.A., Arbuckle, T., Kaczor, D., Curtis, K.M. (2000). Male pesticide exposure and pregnancy outcome. *Am. J. Epidemiol,*. 146, pp. 1025-1036.

Saxena, D. & Stotzky, G. (2001). Bt corn has a higher lignin content than non-Bt corn. *Am. J. Bot*., 88, pp. 1704–1706.

Schubbert, R., Lettmann, C., Doerfler, W. (1994). Ingested foreign (phage M 13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. *Molecular Genetics*, 242, pp. 495-504.

Schubbert, R., Renz, D., Schmitz, B., Doerfler, W. (1997). Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. *Proc Natl Acad Sci USA,* 94, pp. 961-966.

Schubbert, R., Hohlweg, U., Renz, D., Doerfler, W. (1998). On the fate of orally ingested foreign DNA in mice: chromosomal association and placental transmission to the fetus. *Molecular Genetics*, 259, pp. 569–576.

Seralini, G.E., Mesnage, R., Clair, E., Gress, S., Spiroux de Vendomois, J., Cellier, D. (2011), Genetically modified crops safety assessments: present limits and possible improvements. *Environmental Sciences Europe,* 23, pp. 10. doi:10.1186/2190-4715-23-10

Sharma, R., Alexander, T.W., John, S.J., Dugan, M.E. R., Aalhus, J.L., Stanford, K., Mcallister, T.A. (2004). Relative stability of transgene DNA fragments from GM rapeseed in mixed ruminal cultures. *British Journal of Nutrition*, 91, pp. 673-681.

Sidhu, R.S., Hammond, B.G., Fuchs, R.L., Mutz, J.-N., Holden, L.R., George, B. & Olson, T. (2000). Glyphosate-tolerant corn: The composition and feeding value of grain from

glyphosate-tolerant corn is equivalent to that of conventional corn (Zea mays L.). *Journal of Agricultural and Food Chemistry*, 48, pp. 2305-2312.

Świątkiewicz S., Świątkiewicz M. (2009). Second generation of genetically modified plants in animal nutrition. *Medycyna Wet*, 65, pp. 460-465.

Teshima, R., Akiyama, H., Okunuki, H., Sakushima, J.-I., Goda, Y., Onodera, H,, Sawada, J.-I. and Toyoda, M. (2000). Effect of GM and non-GM soybeans on the immune system of BN rats and B10a mice. *Journal of Food Hygiene Society Japan*, 41,pp. 188–193.

Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B., France, J. (1994). A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Animal Feed Science and Technology*, 48, pp. 185-197.

Tony, M.A., Butschke, A., Broll, A., Grohman, L., Zagon, J., Halle, I., Danicke, S., Schauzu, M., Hafez, H.M., Flachowsky, G. (2003). Safety assessment of Bt 176 maize on broiler nutrition: degradation of maize-DNA and its metabolic fate. *Archive of Animal Nutrition*, 57, pp. 235–252.

Tudisco, R., Calabro', S., Terzi, V., Cutrignelli, M.I., Bovera, F., Piccolo, V., Zicarelli, F., Piccolo, G., Infascelli, F. (2004). In vitro fermentation kinetics of some genetically modified feeds. *Proceeding of 2nd International Meeting on Veterinary morpho-functional Biotechnologies,* Naples, Italy, Giugno 2004.

Tudisco, R., Lombardi, P., Bovera, F., d'Angelo, D., Cutrignelli, M.I., Ma stellone, V., Terzi, V., Avallone, L., Infascelli, F. (2006). Genetically modified soybean in rabbit feeding: detection of DNA fragments and evaluation of metabolic effects by enzymatic analysis. *Animal Science*, 82, pp. 193-199.

Tudisco, R., Ma stellone, V., Cutrignelli, M.I., Lombardi, P., Bovera, F., Mirabella, N., Piccolo, G., Calabrò, S., Avallone, L., Infascelli, F. (2010). Fate of transgenic DNA and evaluation of metabolic effects in goats fed genetically modified soybean and in their offsprings. *Animal*, 4, pp. 1662-1671.

Van den Eede, G., Aarts, H., Buhk, H.J., Corthier, G., Flint, H.J., Hammes, W., Jacobsen, B., Midtvedt, T., van der Vossen, J., von Wright, A., Wackernagel, W., Wilcks A. (2004). The relevance of gene transfer to the safety of food and feed derived from genetically modified (GM) plants. *Food Chemistry and Toxicology*, 42, pp. 1127-1156.

Van Hall, G. (2000). Lactate as a fuel for mitochondrial respiration. *Acta Physiologica Scandinava*, 168, pp. 643-656.

Vecchio, L., Cisterna, B., Malatesta, M., Martin, T.E., Biggiogera, M. (2994). Ultrastructural analysis of testes from mice fed on genetically modified soybean. *European Journal of Histochemistry*, 48, pp. 448–454.

Wiedemann, S., Lutz, B., Kurtz, H. F., Schwarz, J., Albrecht, C. (2006). In situ studies on the time-dependent degradation of recombinant corn DNA and protein in the bovine rumen. *Journal of Animal Science,* 84, pp. 135-144.

Warwick, S.I., Legere, A., Simard, M.J., James, T. (2008). Do escaped transgenes persist in nature? The case of an herbicide resistance transgene in a weedy Brassica napa population. *Molecular Ecology*, 17, pp. 1387-1395.

Zelaya, I.A., Owen, M.D.K., VanGessel, M.J. (2007). Transfer of glyphosate resistance: Evidence of hybridisation in Conyza (Asteraceae). *Amer. J. Botany*, 94, pp. 660-673.

8

Molecular Markers: Assisted Selection in Soybeans

Eduardo Antonio Gavioli
Taquaritinguense Institute of Higher Education – ITES
Department of Agronomy
Taquaritinga - SP
Brazil

1. Introduction

Modern agriculture seeks increasing gains in productivity, due to great demand for food and the reduction of new agricultural frontiers. A major concern relates to fungal diseases and pest damage, and productivity growth necessarily implies reducing losses caused by these organisms.

Genetic improvement provides plants with different degrees of resistance, which can be used by farmers, making the most economical and efficient management. The process of obtaining resistant cultivars is usually done by the transfer of resistance alleles from exotic sources, which need further evaluation. This strategy has been used successfully in breeding programs for many years.

The evaluation process in plants is an improvement methodology with high cost, complex and subject to environmental variations. Another problem encountered concerns the manipulation of plant pathogens in a place where they occur. As an alternative to overcome the problems mentioned above are used molecular markers. With the development of research in molecular biology, there was the possibility of having one more tool in breeding programs, using DNA as the basic material.

The markers can be classified according to the methodology used to identify them: hybridization - RFLP (Restriction Fragment Length Polymorphism) or amplification of DNA - RAPD (Random Amplified Polymorphic DNA); SCAR (Sequence Characterized Amplified Regions); microsatellites (or SSR - Simple Sequence Repeats) and AFLP (Amplified Fragment Length Polymorphism). The markers are based on natural variation in DNA sequence and have Mendelian segregation.

The use of molecular markers was initiated in the last century, when Bateson & Punnett (1905) indicated the possibility of linkage between genes controlling characteristics of petal color and shape of pollen grain.The strategy of using molecular markers requires basic knowledge about the genetic nature of the trait studied, classifying it as a qualitative or quantitative (Ferreira & Grattapaglia, 1995), whose difference is based on the magnitude of the effect of replacing one allele by another in a given locus.

Molecular markers can be a useful tool to monitor the transfer of alleles of interest. In the early stages of intermediate and improving the process is efficient, but final confirmation is essential in field conditions (Alzate-Marin et al., 2005).

This need for phenotypic analysis requires quality in the polls so that the marker may reflect the field conditions. The test is performed with molecular markers using only a small

sample of plant tissue (usually leaves). No damage occurs and the plant can be conducted normally.

Several breeding programs have used molecular biology techniques, aiming at the marker-assisted selection (Alzate-Marin et al., 2003; Benchimol et al., 2003; Maluf et al., 2008). Features such as disease resistance, pest resistance, genetic purity, gene pyramiding, are some areas with possibilities of action research. Phenotypic characteristics difficult to measure can also be evaluated using molecular markers.

The marker-assisted selection is a process of indirect selection in which the character in question has a high heritability, since not influenced by environmental factors. There is increased efficiency of plant breeding, reducing the number of progenies and the number of generations for the stabilization of the genotypes. The selection can be performed in early generations (Barbosa Neto, 1998; Federizzi, 1998).

This is a useful tool in the genetic improvement of plants introduced differential pricing depending on the type of bullet used. The procedures require specialized laboratories with sophisticated equipment and qualified personnel. There is need to integrate multidisciplinary involving researchers with backgrounds in classical plant breeding, chemistry, biochemistry, plant physiology, statistics, computer science, bioinformatics and others.

The chapter aims to describe the key molecular markers, showing the evolution over recent decades and its practical applications in soybean. It also aims to relate key findings and future possibilities of this tool that are joining forces for the development of soybean.

2. Molecular markers based on DNA hybridization

2.1 RFLP (Restriction Fragment Length Polymorphism)

Changes in DNA caused by changes in the nucleotides can be identified if they occur on a site of a restriction nuclease. If the DNA of plants, which differ in one or more of these nucleotides is digested by these enzymes, fragments of different sizes are generated and can be identified and subsequently cloned (Botstein et al., 1980). These fragments are called RFLP (Restriction Fragment Length Polymorphism).

The polymorphism revealed by restriction fragment analysis allows studies of biological phenomena and processes: characterization of germplasm, genetic map construction and assessment of genetic diversity.

In many studies were identified RFLP markers, which contributed to a broad coverage of the genome. Several genetic maps have been generated, allowing multiple applications within the plant breeding (Nodari et al., 1993). The first research related to RFLP markers were assigned to Helentjaris et al. (1986).

The principle of the technique is the extraction of DNA from a plant and its subsequent fragmentation through different restriction enzymes. Plants have, in most cases, more than one billion base pairs in its genome, and enzymatic digestion produces thousands of fragments that vary in length according to the distribution of restriction sites. The collective analysis is impossible, but the technique allows them to be separate and distinct.

The quality of DNA to be used is essential for the results obtained by RFLP technique. The genetic material is fragmented using restriction enzymes (Ferreira & Grattapaglia, 1995).

The DNA fragments obtained were separated by electrophoresis and subsequently transferred to a membrane using a technique called Southern blotting (Southern, 1975). The identification of the fragments is possible, using radioactive probes, which can be a

fragment of the plant itself, complementary to the fragment of interest. At the end of the process the membranes are exposed to an X-ray film, showing the hybridization due to emission of radiation by the probe. The polymorphism observed among the plants may be related to genetic differences. The marker behaves as co-dominant, where at each locus studied is possible to identify individuals homozygous or heterozygous. The amount of information produced is large and allows the analysis of gene action and interactions between alleles.

The restriction nucleases are enzymes capable of breaking the DNA strand cutting it systematically in specific locations. The first enzyme found in the bacterium *Haemophilus influenzae*, showed ability to cut the genetic material of *Escherichia coli* and was named *HindII*. Enzymes have been isolated from bacterial strains differ by cleavage at specific restriction sites. The enzymes recognize sequences of four to eight bases. Enzymes that recognize restriction sites composed of four base pairs cleave DNA on average every 256 nucleotides ($4^4 = 256$). Those who recognize sites with 6 and 8 bp cleave DNA on average every 4096 and 65536 bp, respectively. However, this average can vary significantly, depending mainly on the base composition of DNA analyzed.

Preliminary evaluations of the cleavage sites, the relationship between the amount of enzyme and DNA, and exposure time are factors that determine the success of this step of RFLP technique. After digestion the samples receiving the loading buffer (0.25% bromophenol blue, 0.25% xylene cianol and 25% ficoll type - 400 - in water) and are subjected to electrophoresis (Ferreira & Grattapaglia, 1995).

The concentration of agarose gel used varies between 0.5% and 2% depending on the size of the fragments generated by digestion. For larger fragments, we use a lower concentration and when the fragments generated have low molecular weight, is used gels with higher concentration. The dye ethidium bromide (0.5 ug/ml) is added to the gel heated in order to promote the visualization of the fragments under ultraviolet light.

At the end of electrophoresis, the fragments should be transferred to a nylon membrane or nitrocellulose filter, according to the methodology Southern blotting (Southern, 1975): 1. Place the gel in alkaline solution (0.5 N NaOH, 1.5 M NaCl) for thirty minutes to break the hydrogen bonds of the double helix and allow hybridization with probes; 2. Transfer the gel to a neutral buffer solution (0.5 M Tris-Cl, 1.5 M NaCl, pH 7.5) for thirty minutes; 3. Transfer the fragments to the membrane by capillary action, which could last more than six hours; 4. Rinse the membrane in 10X SSC solutions and set in oven for thirty seconds ultraviolet and 5. Place the membrane to dry at room temperature and then subjected to treatment in vacuum furnace for three hours at 90°C and 6. Use immediately or store the membrane at 4°C.

The last step of the RFLP technique is the process of hybridization between the fragments generated by enzymatic digestion and the probes and subsequent exposure to X-ray. According to Ferreira & Grattapaglia (1995), the probes can be clones obtained by reverse transcription of mRNA, fragments of genomic DNA, fragments generated by amplification of known sequences or RAPD bands. The selection of clones is critical to the success of the technique. Depending on the type of probe used RFLP markers can show good results within the genome.

The membrane and the probes are placed in a common solution to the homologous sequences may hybridizing. This step is conducted for about twelve hours at 60°C. After this period the membranes are washed in SSC solution, dried and exposed to X-ray.

Through RFLP markers can be generated linkage maps used for mapping other traits of agronomic importance. Through them it is possible to detect associations between markers

and genes of interest. When the model is defined, the selection criterion becomes an RFLP marker and not the phenotype. This procedure is called marker-assisted selection and can be used for difficult to assess characteristics: resistance to nematodes, protein production.
Some researchers have used RFLP technique (Apuy et al., 1988; Doyle, 1988; Doyle & Beachy, 1985) and reported at the time, the low level of polymorphism found in soybean plants. The correct choice of enzymes and determination of time of exposure of DNA to enzymatic action, can determine the degree of polymorphism.
Keim et al. (1990) developed a genetic map for soybean using RFLP marker associated with QTL (Quantitative Trait loci) and highlighted the quality of the technique. The same authors reported on the importance of maps for the improvement and understanding of plant evolution.
The RFLP technique has high consistency and repeatability of results. Several searches were performed for characterization of cultivars (Autrique et al., 1996; Gebhardt et al., 1989; O'Donoughue et al., 1994). Tozuka et al., (1990) conducted in 1097 collecting wild plants of soybean (*Glycine soja*) in Japan and applied the RFLP technique in mitochondrial DNA, in order to separate them into groups using two probes (*coxII* and *atp6*). The authors stressed the quality of technical and plants classified in 18 groups.
The resistance to cyst nematode (*Heterodera glycines* Ichinohe), due to the damage it causes in soybeans, is the subject of several studies. *Rhg4* gene was mapped on linkage group A2 by Weisemann et al. (1992) and Webb et al. (1995). Mahalingam & Skorupska (1995) obtained similar results working with RFLP and RAPD markers. Factors such as the large number of steps and use, in many cases, of radioactive probes, prevent the use of the technique on a large scale.

3. Molecular markers based on DNA amplification

3.1 PCR (Polymerase Chain Reaction)

The technique of polymerase chain reaction (PCR) was developed in the late 80s (Mullis & Faloon, 1987; Saiki et al., 1988) and revolutionized the molecular genetic studies (Watson et al., 1997). The PCR allows the production of a large number of copies of specific DNA sequence without cloning. The impact of the PCR technique and methods derived from it Kary Mullis took to win the Nobel Prize in 1993. Many methods for cloning, sequencing and analysis of DNA polymorphism were accelerated or replaced by the use of the many derivations of the PCR technique (Ferreira & Grattapaglia, 1995).
The reaction has as its basic principle the natural replication of DNA molecules that occur in cells. After opening the double strand, obtained by heating, a primer specific base pairs is the region of interest, and then there is the amplification of a new single strand. The double-stranded DNA serves as template for the synthesis. The reaction is mediated by the action of Taq polymerase and results in amplification of a specific fragment.
According to Watson et al. (1997) PCR technique is relatively simple and versatile. The equipment required for initial reaction is the DNA that contains the sequence to be amplified, two oligonucleotides (primers) that direct the starting point of synthesis, DNA polymerase and the four deoxyribonucleotides (A, T, C, G). The mixture also contains a buffer solution, magnesium chloride and water.
The reaction is performed in an appropriate tube, heated (92-95°C) for 5 minutes, so that the double-stranded molecules are split to form single strands. These single strands serve as

templates for the primers. Then the temperature is reduced (35 to 60°C) providing suitable conditions for pairing between primers and complementary sequences. In the next step the temperature is high (72°C) for up to 5 minutes. At the end of the temperature is again raised to 94°C for 20 seconds, for the separation of short strips of DNA that serve as templates for new cycles. For most protocols, the number of cycles varies between thirty and sixty.
The discovery of the bacterium *Thermus aquaticus* which lives in water temperature of 75°C and isolation of its polymerase (Saiki et al., 1988), enabled the automation of PCR by using thermocyclers (Watson et al., 1997), which are heating blocks that can be programmed to control the time and reaction temperature. They are equipment with the capacity to change the temperature quickly and repeat the cycle according to the protocol and the number of amplified fragments doubles every cycle. The Taq polymerase of bacteria supports high temperatures without being destroyed.
The enzyme requires a double-stranded fragment in the case, provided by specific primers to initiate amplification of the complementary strand of DNA. If two primers complementary to both strands of DNA are used, the amplification occurs in both directions and two new single strands are generated. Each will have as starting point the sequence of an initiator and extend beyond the other's position in initiating complementary strand. The extension process is always from the position 3' of the initiator. Millions of copies of a given follow-up of DNA, typically up to 4000pb can be synthesized using Taq polymerase.
According to Watson et al. (1997) in 1985 there were three research reports with PCR, some years later the PCR was being used in thousands of labs around the world. The technique caused a major revolution in the practice of molecular genetics.

3.2 RAPD (Random Amplified Polymorphic DNA)

Molecular markers may characterize a plant from plant tissue samples. Welsh and McClelland (1990) and Williams et al. (1990) proposed a technique denominated RAPD (Random Amplified Polymorphic DNA) using primers of arbitrary sequence (10 nucleotides) and identifies polymorphic among different individuals. RAPD molecular markers can be used for studies related to genetic mapping, population genetics, molecular systematics, fingerprint genotypes and marker-assisted selection.
The technique allows the identification of numerous markers without prior notification, which may or may not be associated with traits of interest. If a particular marker is physically linked to a gene of agronomic interest, the selection of this results in indirect selection marker gene (Ferreira & Grattapaglia, 1995). The situation described is characterized as marker-assisted selection. If the distance between the marker and the gene is small, the efficiency of indirect selection will be higher.
The protocols used to show satisfactory results, but generally you should take steps to maintain the quality of DNA and reagents. The type of thermocycler can also be considered as a variable. The observation of the proposed action results in quality bands and the possibility of replication of results.
The principle of the technique is based on the PCR reaction: elevated temperature of the solution to the double-stranded DNA to open in their hydrogen bonds. Then the reduced temperature and allowing the pairing occurs between the primers and the DNA strand. Finally the temperature is again raised to the Taq DNA polymerase to amplify the fragments thereby generating bands that will be visualized in gel electrophoresis.
The temperature variations are possible using a thermal cycler that automatically performs the operations for each step: denaturation (92-95°C), pairing (35-60°C) and amplification

(72°C). The cycle is repeated about 40 times and the final product of amplification can be visualized in agarose gel with ethidium bromide dye under ultraviolet light. RAPD markers behave as dominant, characterized by amplification of fragments or not.

The low temperature necessary for linking the primer to the template makes the process very dependent on the conditions of amplification. Therefore, modifications in the thermocycler used, in the DNA polymerase and other reagents of the reaction mixture can change the pattern of amplification. This may not representing a major problem in a group of researchers who carefully standardize the conditions of amplification, but may prevent data from being compared between different laboratories (Alzate-Marin et al., 2005).

The successful use of RAPD molecular marker is directly linked to quality of genomic DNA, which can be extracted using CTAB protocol. A soybean leaf stage provides enough DNA for several reactions. The leaf collected should be placed in contact with liquid nitrogen and then macerated. DNA can be extracted immediately or the leaves can be stored at – 80°C.

After extraction, the DNA must be quantified for standardization to occur in the reactions, which assures quality of the bands obtained. For RAPD reactions, quantities of DNA in the order of nanograms provide adequate conditions for the success of the process. DNA can be stored at low temperatures (-20°C to -80°C) depending on usage needs. Contaminant compounds such as proteins, polysaccharides and phenolic compounds can affect the quality of DNA and inhibit the action of Taq polymerase.

The observed differential to obtain RAPD markers, in relation to the PCR process is the use of arbitrary primers, and a large number of random amplification. Thus the bands obtained can not be directly related to a gene of interest. The primers are oligonucleotides of known sequence featuring 10bp. Currently a large number of primers is offered on the market, which allows an enormous amount of reactions with the same DNA.

To associate a characteristic phenotypic marker for a particular molecularly was proposed by Michelmore et al. (1991) methodology BSA (Bulked Segregant Analysis).

Once determined the phenotypic trait of interest, plant a segregating F_2 population are classified according to the established criteria, such as resistant or susceptible to a given disease.

The plant DNA can be extracted from a small leaf sample, taken at the beginning of plant development, individually stored refrigerated. All plants of the F_2 population provide DNA for further RAPD reactions.

After the end of the phenotypic evaluations plants should be classified into distinct groups, contrasting in relation to the characteristic under study. Each group is composed of a small number of plants following the theory. For a dominant RAPD marker segreganting in an F_2 population, the probability of a bulk of n individuals having a band and a second bulk of equal size not having a band will be $2(1-[1/4]^n)(1/4)^n$ when the locus is unlinked to the target gene. Therefore low individuals per book are required. For example, the probability of an unlinked locus being polymorphic between bulks of 10 such individuals is 2×10^{-6}. Even when many loci are screened, the chances of detecting an unlinked locus are small. As smaller bulks are utilized, the frequency of false positives will increase. However, as the linkage of all polymorphisms is confirmed by analysis of a segregating populations, bulked segregant analysis with only small numbers of individuals in one or both bulks will provide great enrichment for marker linked to target loci (Michelmore et al., 1991).

Two solutions containing DNA should be prepared in equimolar concentrations, for the continuation of the technique. Example: A group with DNA from plants resistant to disease and another group with the DNA of plants susceptible to disease. Initially the RAPD reactions are performed with the DNA of each group. Thus polymorphism in a group may be associated with the characteristic that distinguishes them.

Once polymorphism observed between the groups, the reactions are being carried out individually with DNA from plants of groups, with the objective to confirm the results.
A marker that shows Mendelian segregation can be considered a gene locus. Segregation of phenotypic characteristics assessed and polymorphic fragments should be individually assessed according to the 3:1 hypothesis.
Then the analysis must be performed by testing the hypothesis of joint 9:3:3:1 segregation. If the hypothesis is rejected, the marker and the allele that determines the desired trait can be on the same chromosome. The question is which the distance between them, which can be determined by the number of recombinant individuals
The smaller the genetic distance between the marker and the allele of interest, the greater the efficiency of molecular markers, to the point that if the distance is close to zero, the marker is the gene itself. So there is a need for an excellent mechanism for phenotypic evaluation and qualified people to apply it, for all subsequent work depends on this step.
Another point that needs to be analyzed is concerned for the fate of a marker allele is not in question but rather a region of chromosome near the gene of interest. The situation may lead to specific markers since the chromosomal region, classified as a molecular marker may not be present in different plants. The absence of amplification can be explained by mutations or rearrangements between the two sites, or on the site of hybridization of the initiator (Paran & Michelmore, 1993). Differences in only one base pair may be sufficient to inhibit amplification, especially at position 3 '(Williams et al., 1990).
It is expected that with advances in functional genomics projects, some markers will lose its practical application, because knowing the sequence of the allele, the job is much more accurate.
Martins Filho et al. (2002) used the technique BSA, in a survey of three F_2 populations of soybean exhibiting resistance to the fungus *Cercospora sojina*. The authors have built on resistant and susceptible groups, with six plants in each one of them and were able to identify RAPD markers in three populations. The DNA of plants assessed individually confirmed the identification observed in the groups, validating the methodology BSA. The distances between the markers found by the authors and the locus studied ranged between 2.3 and 6.7 cM.
Carvalho et al. (2002) identified two RAPD markers linked to gene for resistance to stem canker in soybeans, which were amplified by the same initiator: $OPAB19_{1320}$ present in all plants classified as homozygous resistant (coupling phase) and $OPAB19_{1150}$ present in susceptible plants (repulsion phase), whereas heterozygous plants classified as resistant showed the two fragments. The distance between the gene and the markers, was estimated at 4.7 cm.
Gavioli et al. (2007) worked with F_2 populations of soybean plants resistant and susceptible to the fungus that causes stem canker, which were evaluated in a greenhouse. The authors applied the methodology BSA (seven plants in each group) and identified a polymorphic RAPD band, with 588 bp, within the group of resistant plants. There were two replications of observed distances of 6.0 and 7.4 cM from the RAPD marker and the locus of interest. DNA from plants of each group was analyzed individually and the band found previously was confirmed (Figure 1).
Recently Costa et al. (2008) identified RAPD markers linked to genes conferring resistance to rust caused by *Phakopsora pachyrhizi*. The authors applied the methodology BSA (ten plants in each group) and related markers: $OPBB16_{650}$, $OPAK04_{800}$ $OPR04_{850}$ and distant to 5.1 cM and 6.3 cM and 14.7 cM, respectively, the locus of resistance *Rpp4*. The authors concluded

that the RAPD primers identified in the survey are indicated for assisted selection of soybean genotypes with the same source of resistance in this study. The selection can be performed in the early stages of development to occur without destroying the plants.

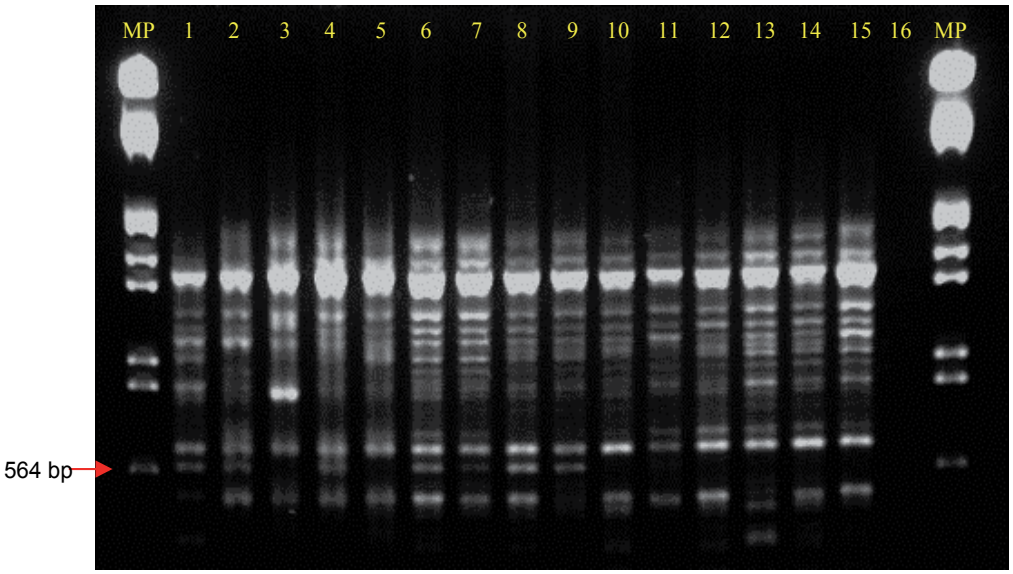


Fig. 1. RAPD polymorphic fragment of 588bp observed in: 1. resistant parent BR92-15454; 2. F_1 plant; 4. resistant bulk and 6-9. F_2 resistants progenies and absent in: 3. susceptible parent IAC-11; 5. susceptible bulk and 10-15. F_2 susceptible progenies. 16. Negative control. Molecular pattern (MP), originating from the digestion of λ with the enzymes *EcoRI* and *Hind III*.

On soybeans, other authors have worked with RAPD markers and were able to identify some of them linked to genes of interest. Heer et al. (1998) identified markers for genes that determine resistance to cyst nematode. Chowdhury et al. (2002) worked with plants resistant to downy mildew and reported the existence of two markers linked to the gene that determines resistance, respectively, 4.9 and 23.1 cM.

Segregating progeny may be evaluated for the presence or absence of a particular marker. The heterozygote cannot be distinguished and this represents a loss of information in relation to RFLP markers. Populations consisting of backcross progeny, recombinant strains and di-haploids do not undergo this loss of information, since the full information available can be obtained in the presence or absence of the marker (Reiter et al., 1992).

3.3 SCAR (Sequence Characterized Amplified Regions)

RAPD reactions can be performed easily, but problems related to standardization of equipment, reagents and protocols, often leads to difficulties for certain searches can be repeated. As referenced above, this may not be a big problem in a group of researchers careful (Alzate-Marin et al., 2005).

To overcome the inconsistent results with RAPD markers, polymorphic fragments obtained, have been cloned, sequenced and converted into SCAR markers (Sequence Characterized Amplified Regions) (Paran & Michelmore, 1992). According to the authors SCAR markers

have advantages: specificity for detecting a single locus and less sensitivity to variations of the reactions. SCAR markers have co-dominant nature and were defined as fragments of genomic DNA, located in a defined locus, which are identified by PCR amplification using a pair of specific oligonucleotides as primers (Ferreira & Grattapaglia, 1998; Nietzsche et al., 2000).

The RAPD polymorphic fragments have to be isolated, cloned and sequenced and then will be used for the synthesis of new primers. Usually the SCAR primers have at their 5 'end, the initiator used in RAPD reactions and its initial 3 'end, the additional bases that will characterize the new initiator.

The SCAR markers can be synthesized starting from a molecular marker RAPD. The isolation of the RAPD fragment is accomplished through a direct cut in the agarose gel containing the desired band. The isolated fragment must be inserted into a vector, usually a plasmid, which will be used in the process of bacterial transformation. Transformed colonies is necessary to separate the fragment that contains, those not containing the desired DNA fragment. After this step the selected colonies are grown for growth and subsequent multiplication of the fragment. Detailed protocols on bacterial transformation can be obtained in Sambrook et al. (1989).

The fragments should be extracted and purified from plasmids. Restriction enzymes are able to cut the plasmid at sites flanking the fragment. The insert should be sequenced to be known that the bases are among the RAPD primers.

For the synthesis of new initiators is considered some parameters such as GC percentage (minimum 50%) and pairing temperature (more than 56°C). Usually the new primers have between 16 and 24 base pairs. There are computer programs that help in developing new primers. Martins Filho et al. (2002) worked with two primers containing 18 nucleotides in each, and determined the temperature from 62°C as an ideal pairing.

At the end of the process, the new SCAR primers should be tested with the plants of the F_2 population and parents to prove the link between the marker and the locus of interest. The confirmation allows the use of marker assisted selection in the process.

Often there is loss of polymorphism of RAPD, when converted into SCAR markers. The problem results from the amplification of two alleles of that locus, which prevents the differentiation between plants. Paran & Michelmore (1992) reported that the polymorphism observed in the RAPD reaction, can be caused by differences in the nucleotide sequence of the site of annealing or rearrangement in the internal sequence of the amplification. A mis-pairing, mainly at the 3' primer, prevented the amplification of a fragment of the genotypes. In a SCAR primer, with the largest number of nucleotides, the end without pairing, is positioned in its middle region and may not interfere with the amplified fragments.

The loss of polymorphism can be solved through the use of restriction enzymes, which promote the cut at specific sites in one allele of a given locus. The technique was successfully used by several authors, among them: Weng et al. (1998), Lahogue et al. (1998), Dax et al. (1998) and Zhang & Stommel (2001).

The choice of restriction enzyme based on the sequencing of the fragments SCAR. The sequences are evaluated within and should be sought restriction sites that can differentiate them. Monomorphic fragments in molecular weight may differ in base sequence.

The fragments are then PCR amplified and digested individually. The result of electrophoresis may reveal again the initial polymorphism. Gavioli et al. (2007) converted a RAPD polymorphic in a SCAR marker. The process resulted in the loss of polymorphism,

which was resolved by digesting the fragments with the enzyme *HincII* (Figure 2). The technique was efficient because alleles from resistant parent were digested by *HincII* enzyme produced two fragments, one of 531 bp and another of 57 bp, while the susceptible parent stayed with the fragment of 588 bp. F_1 plants heterozygous for the locus, showed a pattern of three bands. The same result was observed in F_2 plants classified as heterozygous resistant.

The loss of polymorphism was recovered by enzymatic digestion and plants could be distinguished in homozygous recessive, homozygous dominant and heterozygous. The process becomes more expensive, but allows for the recovery of the polymorphism and the use of SCAR marker.

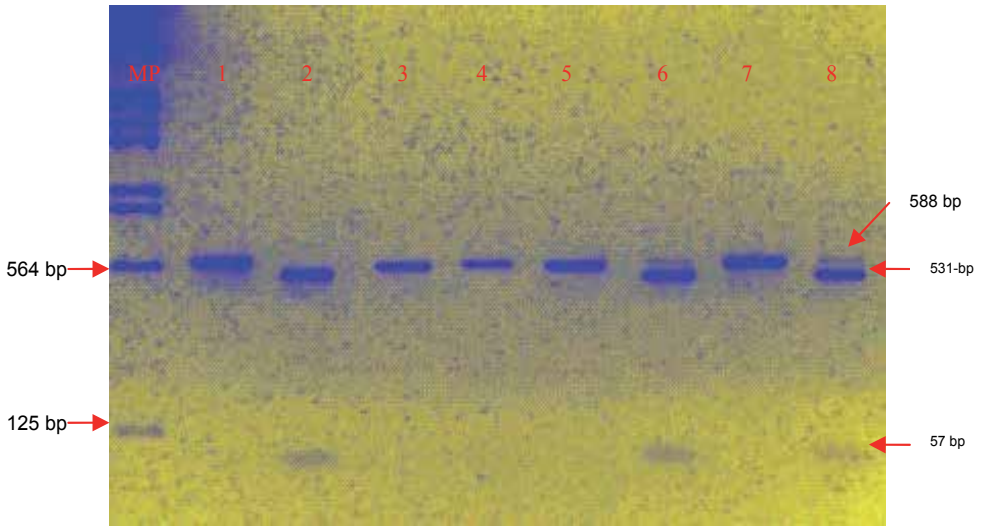


Fig. 2. SCAR monomorphic fragment of 588bp with and without enzymatic digestion (*HincII*): 1. resistant parent BR92-15454; 2. resistant parent BR92-15454 (digested); 3. susceptible parent IAC-11; 4.susceptible parent IAC-11(digested); 5. F_1 plant, 6. F_1 plant (digested); 7. heterozygotic resistant F_2 plants; 8. heterozygotic resistant F_2 plants (digested). Molecular pattern (MP), originating from the digestion of λ with the enzymes *EcoRI* and *Hind III*.

There are numerous studies to obtain SCAR markers linked to disease resistance of crops. In soybean there are the surveys conducted by Heer et al. (1998) who worked with resistance to soybean cyst nematode; Martins Filho et al. (2002), in studies on resistance to the fungus *Cercospora sojina*; Zheng et al. (2003) in relation to the mosaic virus and Carvalho et al. (2002) in the evaluation of soybean plants resistant to stem canker.

3.4 Microsatellites – SSR (Simple Sequence Repeat)

Another type of molecular marker is the microsatellite or simple sequence repeat (SSR). Microsatellites or STR or SSRP (Simple Sequence Repeat Polymorphisms) or STMS (Sequence Tagged Microsatellite Sites) are some of the dominions assigned to these molecular markers. The use of these markers has been increasing, due to the fact that you use the PCR technique, co-dominant and present with a relatively high frequency within the plant genome (Akkaya et al., 1992).

Are sequences consisting of repetitions of one to four nucleotides, which occur naturally in the genome, such as repeated (AT)n, (ATT)n. The genome of plants has, on average, ten times less than the microsatellite genome (Powell et al., 1996). The repeats are more common in plants (AT)n, (GA)n, (AC)n, (AAT)n and (AAC)n (Gupta & Varshney, 2000; Wang et al., 1994). The DNA from organelles has a low frequency of SSRs (1 per 317 Kb) (Wang et al., 1994). Microsatellites are present in coding regions and non-coding (Zane et al., 2002).

The variation of n number of repeated elements generates a great amount of polymorphism. According Brondani et al. (1998), microsatellites have characteristics that result in benefits for the plant breeding: Nature co-dominant and multiallelic; highly polymorphic, allowing precise discrimination even of highly related; abundant and uniformly dispersed throughout the genome plants; can be analyzed by the PCR reaction. In plants one of the first findings was made by Nybom et al. (1992).

Another important point is that the DNA sequences flanking the SSRs are conserved within the same species, allowing the selection of specific primers that amplify via PCR. The amplification using a pair of primers complementary to unique sequences that flank, resulting in an enormous fragment length polymorphism This size variation of PCR products is a consequence of the occurrence of different numbers of repeating units within structure of microsatellites (Ashkenazi et al., 2001; Cregan et al., 1999a; McCouch et al., 1997; Morgante & Olivieri, 1993). Thus, alleles may be determined for a given population. Homozygous individuals have the same number of repetitions in the chromosomes, while heterozygous individuals have different numbers of repeats in both chromosomes. Therefore, the locus is defined by the pair of primers and the various alleles by the size of amplified bands.

Some mistakes during DNA replication in different individuals of the same species, can provide a varying number of repeats within a microsatellite, which are different alleles. Currently, many species of plants already possess a set of microsatellite markers for use in genetic studies (Akkaya et al., 1992; Cregan et al., 1999a).

The practical use of microsatellite markers has occurred in human studies (Litt & Luty, 1989) and attracted the attention of plant breeders, since several studies have shown that microsatellites are widely distributed in the genome of the species (Brunel, 1994). According to Ferreira & Grattapaglia (1995), in eukaryotic genomes, these simple sequences are very frequent, randomly distributed, besides being highly polymorphic genetic locus.

The initial protocols identified the microsatellite locus in clones of total genomic libraries using probes complementary to regions of interest, such as (AC) 10 and (AG) 20 (Rassmann et al., 1991). The amplification products are separated by electrophoresis, which in most cases, should be done on polyacrylamide gel because of the small size difference between fragments. The attainment of the primers is the most expensive step of the process of using microsatellite markers in marker assisted selection in plants.

Each microsatellite locus can be analyzed individually or jointly with another, when the alleles of each locus have sizes sufficiently different to migrate into separate zones in the gel (Lanza et al., 2000).

Microsatellite markers are indicated to various kinds of analysis, because they are polymorphic in soybean, highly reproducible, co-dominant and by their low cost, considering that about 650 pairs of primers specific for soybeans are available on the market (Cregan et al., 1999a).

Registration and the granting of rights to new varieties is usually done based on morphological and physiological characteristics, uniformity and stability, necessitating the

distinction in at least one of them. However, most morphological traits are quantitative traits, with its expression being altered by environmental factors. Moreover, the registration number is increasing rapidly, making it impossible for authorities to compare the new varieties efficiently with existing ones. The marker has been proven useful for identifying varieties and tested for various crops such as soybeans.

The microsatellite markers have been used in soybean for mapping specific genes that determine agronomic traits, and also to identify QTLs (Quantitative Trait Loci) of economic importance, involved in grain yield and genetic resistance to pests and diseases, which are characteristics of complex inheritance (Yuan et al., 2002).

The development of genetic maps is considered one of the applications of greatest impact in the technology of molecular markers for genetic analysis of species and, potentially, in plant breeding. In this context, the genetic maps enable: Full coverage and analysis of genomes; decomposition of complex genetic traits into their Mendelian components; location of genomic regions that control traits of importance; quantification of the effect in these regions studied feature, directing all this information to use in breeding programs.

Some quantitative trait loci (QTL) for resistance to some important diseases of soybean, were mapped in the chromosomal region adjacent to the locus of resistance to leaf rust *Rpp5.* Near the marker Satt009, were mapped QTL for resistance to the pathogen *Sclerotinia sclerotiorum*, the fungus that causes white mold in soybean (Arahana et al., 2001), and an adjacent chromosomal region, is mapped a QTL for resistance to the cyst nematode, which causes the most serious threat to soybean production (Concibido et al., 1997).

Mekesem et al. (2000) constructed a map of high saturation of three genomic regions in soybean, which contains the *Rhg4* and *rhg1* alleles that confer resistance to SCN, and the region containing the *Rfs* allele, which confers resistance to *Fusarim solani* f. sp. *glycines*.

In linkage group N, 3.2 cM from Satt009 marker was mapped a locus of resistance to *Phytophthora sojae*, the pathogen that causes root rot in soybean and marker linked to Satt080, and a QTL for resistance to *Fusarium solani* f. sp. *glycines* (Njiti et al., 2002).

Other microsatellite markers linked to various diseases of soybean have been reported in the literature. Mudge et al. (1997) concluded that microsatellite flanking Satt038 and Satt130 allele *rhg1*. Cregan et al. (1999b) detected the SSR Sat-168 and Satt 309 delimiting *rgh1*. The marker Satt215 was found to be linked to the gene *Rbs1*, which confers resistance to black pod-of-staff, with selection efficiency of 88% (Bachman et al., 2001). A new gene, RCS_{Peking}, which confers resistance to stain-frog-eye, was mapped to 1.1 cM of marker Satt244, on linkage group G (Yang et al., 2001). Funganti et al. (2004) identified the marker Satt114, linked to the nematode resistance locus of the root knot nematode (*Meloydogyne javanica*), in linkage group F.

Garcia et al. (2008) mapped the locus *Rpp5* in three different populations of soybean (PI 200456, PI 471904 and PI 200526), and obtained: six markers linked to this locus in population PI 200456 (Satt530, Sat_208, Sat_166, Sat_275, and Sat_280 Sat_266) and five in PI471904 (Satt530, Sat_208, Sat_166, Sat_275, Sat_280) and three in PI 200526 (Sat_166, and Sat_275 Sat_280).

Morceli et al. (2008) identified two new microsatellite markers potentially associated with resistance of soybean to soybean rust caused by *Phakopsora pachyrhizi*. The authors evaluated the markers on individual plants, and found the link to *Rpp5* gene and are present on linkage group N of soybean. The efficiency of selection was determined for all markers linked to gene *Rpp5*, and the combination of the markers Sat_275 + Sat_280 was 100%.

Schuster et al. (2004) used markers for determining genetic purity of seed lots of soybean. The authors presented a table listing the primers used more frequently in the determination

of genetic purity of seed lots of soybean: Sat_128, Satt070, Sat_085, Satt079, Sat_110, Satt184, Satt005, Satt141, Satt186, Satt146, Sat_064, Sat_094, Satt163, Satt181, Sat_105, Satt162, Satt183, Satt167, Sat_099, Satt156, Satt150, Satt175, and Satt136 Sat_127.

The following is related in detail, the protocol used by Schuster et al. (2004): Total volume of 10 or 25 mL, containing 12.5 mM Tris-HCl (pH 8.3), 62.5 mM KCl, $MgCl_2$, 2.5 mM, 125 mM of each deoxinucleotídios (dATP, dTTP, dGTP and dCTP), 0.2 μM of each primer (sense and antisense), a unit of Taq DNA polymerase and 30 ng of DNA. The total volume of the reaction was 25 mL when the separation was performed in 3% agarose gel and 10 mL when the separation was performed on 10% polyacrylamide gel. The amplifications were performed in Perkin Elmer 9600 thermocycler, programmed for an initial step of 7 min at 72°C, followed by 30 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C. Finally, a step of 7 min at 72°C. The amplified fragments were separated by electrophoresis on 3% agarose gel containing ethidium bromide etídio (0,2 μg/mL) and 1X TBE buffer (90 mM Tris-borate, 2 mM EDTA), or 10% polyacrylamide gel in TBE buffer. In this case, the gels were stained after the run in TBE containing ethidium bromide (2μg/mL).The gels were photographed under ultraviolet light.

The authors concluded that analysis of DNA extracted from seeds, considered atypical by the method of visual analysis with microsatellite markers is effective in determining the genetic purity of seed lots of soybean, and that the use of proteinase K and RNase in the process of extraction allows to obtain greater quantities and better quality of DNA for analysis.

Fuganti et al. (2004) conducted research towards the identification of microsatellite molecular markers for selection of soybean genotypes resistant to the nematode *Meloydogyne javanica*. The authors concluded at the time that the markers, SOYHSP 176 and Satt 114, the linkage group F of soybean, can be considered potential markers to be used in the process of marker assisted selection of soybean genotypes resistant to root knot nematode

Hoshino et al. (2002) performed an extensive review of the microsatellite markers and reported that despite its widespread popularity there are certain limitations: 1. Identification of genotypes: usually performed by determining the length of the PCR product. The polymorphism is attributed to changes in the number of repeating units, however, it is possible that small insertions or deletions occur in adjacent regions that did not necessarily alter the number of repetitions, but change the length of the fragment generated. In such cases, determining the number of repetitions from a cloned fragment and its subsequent use for amplification in other species without sequencing, can lead to mistakes in the estimation of genetic distances; 2. null alleles: the occurrence of point mutations, insertions or deletions at the site of pairing of the initiator can prevent the amplification of a given microsatellite locus. If changes are not fixed in the population, only a portion of the alleles will be amplified; 3. mutation rate: Positive correlations between the length of the microsatellite sequence and mutation rate are not always true, and can cause errors in the estimation of genetic distances; 4. Frequency of different organisms: The application of this marker in a growing number of organisms has revealed that the abundance of repetitive sequences varies significantly between species. While some species contain a sufficient number of microsatellites for population studies, others have few sequences of this kind; 5. Artifacts PCR: Theoretically, the PCR technique allows the amplification of microsatellites from a single cell, but the amplification of microsatellites from small amounts of DNA are associated with high frequency of errors. Two common types of mistakes in this case are: the amplification of the alleles with incorrect length, and no amplification of one allele in

heterozygotes. The authors asserted that this type of marker is a valuable tool for analysis of genetic variability in germplasm of plant species and therefore its maintenance.
Microsatellite markers have many advantages compared to other types of markers (RFLP, RAPD, AFLP) are highly polymorphic and informative; the co-dominant inheritance, which allows discrimination between homozygous and heterozygous; are multi-allelic; occurring abundantly in genomes of eukaryotes; are based on PCR and thus need small amounts of DNA; are highly reproducible; require no radioactivity; are well dispersed in the genome in coding regions and non-coding; loci are often conserved between related species.
The microsatellite markers have been used extensively to the major species of agronomic importance and have potential to occupy a prominent place among the markers of greatest use.

3.5 AFLP (Amplified Fragment Length Polymorphisms)

Since its development and publishing, this technique has been used to characterize genotypes, genetic mapping, especially in species with low DNA polymorphism (Zabeau, 1993). The use of this marker is based on the technique that combines DNA fragmentation with restriction enzymes type II, that cleave DNA at specific sites of rare cutting (recognize sites of 6-8 bases, ex: *ApaI*, *EcoRI*, *HindIII* and *PstI*) and frequent cutting (recognize sites of four bases, ex: *MseI* and *TaqI*) and amplification of these fragments by PCR (Vos et al., 1995).
The use of specific restriction enzymes, allows the knowledge of the cohesive ends generated, in which they are linked adapters that serve as binding sites for primers in a PCR reaction. It is essential that the DNA digestion is complete, because the partial digestion can reveal false polymorphisms. The purity of DNA used is a fundamental requirement for obtaining good results.
In the process of digestion, the two enzymes (unusual cutting / frequent cutting) can be used simultaneously (double digestion) or in two steps, if there is a reaction buffer common to both enzymes. Three classes of fragments are generated: frequent/frequent; unusual/frequent and unusual/unusual.
From the digestion of genomic DNA with *EcoRI* and *MseI* is expected that most of the fragments are cut *MseI*/*MseI* (frequent/frequent). Fragments cut *EcoRI*/*MseI* (unusual/frequent) occur in approximately equal frequency to twice the number of restriction enzyme sites of *EcoRI* and fragments cut *EcoRI*/*EcoRI* (unusual/unusual) occur in low volume (Ferreira & Grattapaglia, 1995).
The fragments generated by enzymatic digestion should be linked to specific adapters that have additional terminals to the ends resulting from cleavage. The process of connecting the adapters involves using ligases that enables DNA fragments to bind to the adapters.
With this procedure, both the sequence of adapters, as the result of the restriction site are known, allowing the construction of specific primers to these sequences for pre-amplification restriction fragment through PCR reactions. The primers consist of a sequence complementary to the adapter, followed by another site-specific restriction enzyme, and an extension of selective nucleotides at the 3' end (Lopes et al., 2002).
In the first stage of amplification, called pre-amplification, a selective nucleotide is used in the 3' end of primers. In the second phase of amplification, called selective amplification, three selective nucleotides are used in the terminal 3' primers.
The second amplification is done with a sample of the first. Therefore, only the fragments that have complementary nucleotides to the selective nucleotides will be amplified. Thus, the alleles of AFLP loci (presence or absence of a specific fragment or band) are from the loss

or gain of a restriction site or the complementary bases selective or not used at the terminals 3 'primers where one starts PCR with the region, which flanks the restriction site (Lopes et al., 2002). The authors presented a table with the restriction sites, sequences of adapters and primers used for six enzymes in AFLP analysis (Table 1).

Restriction enzyme	**Restriction site**	**Adapter /Primer**	**Base sequence**
EcoRI	5′...G↓A A T T C...3′ 3′...C T T A A↑G...5′	Adapter	5'-C T C G T A G A C T G C G T A C C-3' 3'-C A T C T G A C G C A T G G T T A A-5'
		Primer	5'-G A C T G C G T A C C A A T T C **E**-3'
MseI	5′...T↓T A A...3′ 3′...A A T↑T...5′	Adapter	5'-G A C G A T G A G T C C T G A G-3' 3'-T A C T C A G G A C T C A T-5′
		Primer	5'-G A T G A G T C C T G A G T A A **E**-3'
PstI	5′...C T G C A↓G...3′ 3′...G↑A C G T C...5′	Adapter	5'-C T C G T A G A C T G C G T A C A T G C A-3' 3'-C A T C T G A C G C A T G T-5'
		Primer	5'-G A C T G C G T A C A T G C A G **E**-3'
HindIII	5′...A↓A G C T T...3′ 3′...T T C G A↑A...5′	Adapter	5'-C T C G T A G A C T G C G T A C C-3' 3'-C T G A C G C A T G G T C G A-5'
		Primer	5'-G A C T G C G T A C C A G C T T **E**-3'
ApaI	5′...G G G C C↓C...3′ 3′...C↑C C G G G...5′	Adapter	5'-T C G T A G A C T G C G T A C A G G C C-3' 3'-C A T C T G A C G C A T G T-5'
		Primer	5'-G A C T G C G T A C A G G C C C **E**-3'
TaqI	5′...T↓C G A...3′ 3′...AGC↑T...5′	Adapter	5'-G A C G A T G A G T C C T G A C-3' 3'-T A C T C A G G A C T G G C-5'
		Primer	5'-C G A T G A G T C C T G A C C G A **E**-3'

Table 1. Restriction sites, sequences of adapters and primers used for six enzymes in AFLP analysis (E: arbitrary nucleotide used in the pre-amplification).

Fragments cut *EcoRI*/*MseI* are preferentially amplified, and this is due to lower efficiency of hybridization of primers *MseI* compared to that with primers *EcoRI*, and also the fact that the fragments *MseI*/*MseI* have terminal inverted sequence being amplified by a single initiator, enhancing the formation of a loop structure that competes with the annealing of primers (Vos et al., 1995).

For separation and identification of AFLP amplification products, the best method is to electrophoresis in denaturing polyacrylamide gel, which provides a high level of resolution, being effective for detecting single nucleotide differences. The number of fragments visualized in a polyacrylamide gel is variable and can reach values higher than one hundred. Theoretically the larger the genome size, the greater the amount of fragments. The readings of the gels can be manual or automated. The manual reading is performed after the revelation of the banding pattern by staining with silver nitrate or revelation in autoradiographs, in this case using primers labeled with radioisotopes. The second type is performed by DNA analyzers and requires fluorescent labeling.

The AFLP technique detects a greater number of fragments compared to other techniques that reveal molecular markers and provides comprehensive coverage of the genome. The use of restriction enzymes combined with appropriate conditions for hybridization of primers for the amplification reactions combines the robustness of the RFLP technique with the practicality of PCR.

Just as the RAPD technique, the methodology requires no prior information of DNA sequences. AFLP markers present together, the exploratory capacity of RFLP polymorphisms (presence or absence of restriction sites) with the advantage of PCR. AFLP markers are dominant and heterozygous genotypes can not be directly discriminated against the homozygotes.

Malone et al. (2003), analyzing genetic contamination in soybean determined that, in addition to the cultivars presented a high degree of genetic similarity, analysis of the banding pattern obtained by AFLP revealed the presence of additional bands in all samples when compared with the strains pure (seed genetics). The variation in the fingerprinting of cultivars suggested to be related to genetic contamination occurred between the batches studied, and exogenous contamination.

Mertz et al. (2009) tried to verify the effectiveness of the technique of cDNA-AFLP in obtaining fragments of genes differentially expressed in soybean seed coats with contrasting permeability and have concluded that the technique could be a promising alternative for studies aimed at identifying genes related to seed quality. According to the authors, the cDNA-AFLP technique is effective in identifying genes expressed, because it allowed the taking of 47 differentially expressed cDNA fragments between the coats of soybean genotypes CD-202 and TP.

Colombari Filho et al. (2010) undertook a study to evaluate the heterosis for grain production in soybean and its relationship with genetic distances obtained with the AFLP molecular marker. The authors concluded that heterosis for grain production in soybean is correlated with genetic distances obtained with AFLP markers and it is possible to select from crosses from the molecular genetics distance between the parents.

The quality of DNA needed is an important factor for the success of the AFLP technique. During the extraction of a large number of samples of genomic DNA preparations can be varied in quantity and in quality. A DNA of high purity is required to ensure a complete digestion by restriction enzymes in all DNA samples. The quality of the enzymatic digestion can lead to errors of interpretations (Ferreira & Grattapaglia, 1995).

4. Relationship between markers and use practices

The markers presented are some similarities between themselves regarding the level of polymorphism, random distribution in the genome stability, gene expression among others. The comparison between them is presented in Table 2 adapted from Ferreira & Grattapaglia (1995).

Characteristics	RFLP	RAPD	Microsatellites	AFLP
Level of polymorphism	Low - High	Low - High	Very High	Very High
Environmental stability	High	High	High	High
Number of locus	High	High	High	High
Gene expression	Co-dominant	Dominant	Co-dominant	Dominant
Number of alleles per locus	Multiallelic	Two	Multiallelic	Two
Distribution in genome	Multiple	Random	Random	Random
Accessibility Technology	Moderate	Very High	Very Low	Moderate
Identification of genotypes	High	Very High	Very High	Very High
Application in breeding	Mean cost	Low Cost	Expensive	Low Cost
Specific mapping	Moderate	Very High	Moderate	Very High
Evaluation of germplasm	High	High	High	Very High
Genetic mapping	High	High	Very High	High
Genetics autogamy	Moderate	High	Very High	Very High
Genetic allogamous	Moderate	High	Very High	Very High
Phylogenetic analysis	Very High	Moderate	High	Moderate

Table 2. Comparative analysis for some characteristics of molecular markers.

The molecular markers reported so far in the chapter became important tools in the genetic improvement of plants. The following is a brief account made of the possible uses for these markers.

Construction of genetic maps: The Mendelian segregation observed in molecular markers, allows them to be used to construct genetic maps of connection and enable the location of genes within the chromosomes. QTLs mapping;

Characterization of genetic variability: the polymorphism generated from DNA and observed in the form of bands, allows us to study and confirm the genetic variability among plants;

Monitoring of genes: Genes may be screened in breeding programs that use the technique of backcrossing. Some applications of molecular markers linked to breeding programs can be facilitated through the use of this tool. In programs of backcross markers can maximize the efficiency of programs by increasing the probability of conversion of individuals and

reducing the time required to obtain an acceptable recovery of the recurrent parent (Borém & Miranda, 2005). The proportion of recurrent parent obtained in the first backcross generation, after selection of a marker in each of the chromosomes of tomato, roughly equals the proportion in three backcross generations in the absence of selection (Tankskey & Rick, 1980);

Genetic characterization: Description of a plant gene in their DNA. Molecular methods allow the varietal characterization with high discrimination power. Several studies have shown the quality of information on the molecular identification of germplasm (Lee et al.,1989; Smith & Smith, 1991).

Marker-assisted selection: The possibility of identifying genes of interest, to be strongly linked to molecular markers allows the selection can be made indirectly. In cases where gene expression occurs only in the final phase of the cycle of the species, such as the lipoxygenase in soybeans, the possibility of indirect selection through molecular markers becomes attractive (Borém & Miranda, 2005). The same authors reported useful for screening individuals who have two or more genes whose expression produces similar phenotypes, as is the case of pyramiding genes conferring resistance to a pathogen.

Great prospects for the area of molecular markers have been discussed. The potential usefulness of these techniques will be achieved, for example, through indirect selection based on markers: when the desired character has low heritability or present difficult evaluation, in situations where there are undesirable genetic correlations between traits, and especially the practice of early selection in crops perennials. The isolation, cloning and manipulation of individual genes that control quantitative traits of economic importance is a goal, theoretically attainable.

5. New markers

The possibility of manipulation of genetic material, derived from discoveries as plasmids, restriction enzymes and the enzyme reverse trasncriptase led to the development of RFLP markers. The PCR technique revolutionized the field of studies on molecular markers and allowed the studies that led to the development of RAPD, microsatellites and AFLP, described in the chapter.

The speed of evolution of knowledge, provided by the large number of studies, development of equipment, reagent delivery and qualification of researchers put it, every day, new tools available to the scientific community. No doubt the generation of knowledge is beneficial and desirable, but the constant changes lead to the need for updates, especially laboratories, which is not always possible. In this line of reasoning, are presented some of the newer molecular markers.

The development of markers in the studies reveals the possibility of treating areas with occasional changes, such as a molecular marker. A Single Nucleotide Polymorphism or SNP is a small change or variation that may occur in a DNA sequence in a significant portion (more than 1%) of a population.

SNPs are the most frequent forms of genetic variations. Approximately 90% of human genetic variations are SNPs that occur every 300 ~ 600 nucleotides and has become markers of choice for its high abundance and development of technologies for large-scale genotyping as microarray hybridization, primer extension, and connection. SNPs are changes in DNA that are maintained in future generations, the mutation was distinguished by being present with a range greater than 1%. When the mutation is set at a minimum frequency of 1% is considered a SNP (Kwok & Gu, 1999).

Variations in the sequences are the result of mutations and SNPs are mutations that have spread over generations and may occur either in coding regions as in non-coding genomes. They are classified as non-synonymous when they occur in coding regions and can result in an amino acid substitution in the protein sequence. Classification as synonymous SNPs is given the changes that occur in non-coding region. These markers can be used in genotyping and as a new strategy for identification of polymorphism in species with low degree of polymorphism.

Molecular markers are very important in structural and functional genomics of animal species, plants and microorganisms. The marker developed by Hu & Vick (2003) was named Region Amplification Polymorphism Target (TRAP). It's is a fast and efficient using bioinformatics tools and data from EST's to generate polymorphic markers around targeted candidate gene sequences based on PCR. It is considered a dominant marker, which combines the ease of the RAPD markers with polymorphism of AFLP markers.

The polymorphism is generated from a combination of a fixed primer designed from an EST sequence of interest, and an arbitrary primer. Recent studies have reported the use of TRAP markers to access genetic diversity in sugarcane and identification of possible candidate genes for cold tolerance and metabolic pathway involved in sucrose (Alwala et al., 2006). The technique is extremely simply practice involving the extraction of genomic DNA and a PCR reaction.

Möller (2010) evaluated 286 F_2 plants from crosses of IAC-100 and CD-215 in order to identify markers linked to genes conferring resistance to sucking bugs of soybean seeds. For the marker TRAP, 11 primer combinations fixed / arbitrary generated 230 brands, and 31 (13.48%) polymorphic, an average of 2.82 marks per polymorphic combination. Despite the lower number of different polymorphic by combining the percentage of polymorphism obtained with the marker TRAP (0.13) was higher than for AFLP (0.10).

Finally the marker Nucleotide Binding Site (NBS) which has been successfully used in potato, tomato, lettuce, barley (Van der Linden et al., 2004), allowing the identification of areas of resistance genes highly conserved between species. The technique consists of amplification of specific regions using degenerate primers homologous to conserved sequences of resistance genes (R-genes).

The visualization of fragments generated by molecular markers, is usually performed using gel electrophoresis, which can hinder the precise correlation between the bands and allelic variations. Technological developments put at the disposal of researchers, hybridization methods such as microarrays. The high cost restricts their use in most laboratories. Alternatively, we developed a methodology called: Diversity Arrays Technology (DArT), which allows analysis of genomic representations without the need for sequencing.

The technique is based on the construction of panels of diversity and hybridization microarray. Its use allows the visualization of the presence or absence of specific fragments in the genome.

6. Conclusion

Molecular markers have facilitated the studies of genetics, taxonomy and evolution of plants delivering breakthrough in scientific knowledge. New possibilities of genetic manipulation emerged, directly benefiting the plant breeding.

The selection methods were largely enhanced by the use of molecular markers, and the success of marker-assisted selection depends on the degree of association between him and

the characteristic of interest: the greater the association, the lower the chance of recombination between the marker and gene controlling the trait, with a higher selection efficiency. The use of molecular markers presents several advantages over morphological markers: lack of phenotypic analysis, which has high cost and difficulty in performing; no need for special environments (drought and inoculation with pathogens); allows for subjective evaluations (performance organoleptic); there is no destruction of the plant evaluated; allowing the early identification of features found only in advanced stages of plant development.

In plant breeding, there are some challenges, and obtain individuals with the gene combination desirable for the features of interest, presented as one. Two factors are involved in resolving the issue: the existence of variability and the selection process. The identification of individuals requires an efficient method and skill of the investigator.

Molecular markers may help in the selection process for individuals as well as in the process of reviewing the existing diversity. The applications follow some peculiarities and the challenge of reducing costs is a objective to be achieved.

The advantages and limitations of these tools were described in the chapter and serve as a basis for further studies and investigated. The speed in the generation of new knowledge requires much intellectual and financial investment from the professionals working in the field of molecular marker-assisted selection.

7. Acknowledgments

I especially thank my wife Rita de Cássia and daughter Mariana for the encouragement and support so that I could write this chapter.

I thank my family for their confidence in my work.

I thank the authors cited in the chapter, because without their research, it would be impossible to accomplish this work.

8. References

Akkaya, M.S., Bhagwat, A.A. & Cregan, P.B. (1992) Length polymorphisms of simple sequence repeat DNA in soybean. *Genetics,* Vol.132, pp. 1131-1139.

Alwala, S., Suan, A., Arro, J.A., Veremis, J.C. & Kimbeng, C.A. (2006) Target region amplification polymorphism (TRAP) for assessing genetic diversity in sugarcane germplasm collections. *Crop Science*, Vol.46, pp. 448-455.

Alzate-Marin, A.L., Costa, M.R., Arruda, K.M., Barros, E.G. & Moreira, M.A. (2003) Characterization of the anthracnose resistance gene present in Ouro Negro (Honduras 35) common bean cultivar. *Euphytica*, Vol.133, pp. 165-169.

Alzate-Marin, A.L., Cervigni, G.D.L., Moreira, M.A. & Barros, E.G. (2005) Seleção assistida por marcadores moleculares visando ao desenvolvimento de plantas resistentes a doenças, com ênfase em feijoeiro e soja. *Fitopatologia Brasileira*, Vol.30, pp. 333-342.

Apuya, N., Frazier, B.L., Keim, P., Roth, E.J. & Lark, K.G. (1988) Restriction length polymorphisms as genetic markers in soybean, *Glycine max* (L.) Merr. *Theoretical and Applied Genetics*, Vol.75, pp. 889–901.

Arahana, V.S., Graef, G.L., Specht, J.E., Steadmen, J.R. & Eskridge, K.M. (2001) Identification of QTLs for resistance of *Sclerotinia sclerotiorum* in soybean. *Crop Science*, Vol.41, pp.180-188.

Ashkenazi, V., Chani, E., Lavi, U., Levy, D., Hillel, J. & Veilleux, R.E. (2001) Development of microsatellite markers in potato and their use in phylogenetic and fingerprinting analyses. *Genome*, Vol.44, pp. 50-62.

Autrique, E., Nachit, M.M., Monneveux, P. Tanksley, S.D. & Sorrells, M.E. (1996) Genetic diversity in durum wheat based on RFLPs, morphological traits and coefficient of parentage. *Crop Science*, Vol.36, pp. 735-742.

Bachman, M.S., Tamulonis, J.P., Nickell, C.D. & Bent, A.F. (2001) Molecular markers linked to brown stem rot resistance genes, *Rbs1* and *Rbs2*, in soybean. *Crop Science*, Vol.41, pp.527-535.

Barbosa Neto, J.F. (1998) Seleção assistida por marcadores moleculares. In: *Marcadores moleculares em plantas*, Milach, S.C.K., pp.75-80. UFRGS, Porto Alegre.

Bateson, W. & Punnett, R.C. (1905) Experimental studies in the physiology of heredity. *Reports to Evol. Comm. Royal Soc.* Vol.2. pp. 1-131.

Benchimol, L.L., Lanza, L.L.B., Souza, J.R. & Souza, A. P. (2005) Microsatellite-assisted backcross selection in maize. *Genetics and Molecular Biology*, Vol.28, No.4, pp. 789-797.

Borém, A. & Miranda, G.V. (2005) *Melhoramento de Plantas*. 4 ed. UFV, Viçosa.

Brondani, R.P.V., Brondani, C., Tarchini, R. & Grattapaglia, D. (1998) Development, characterization and mapping of microsatelite markers in Eucaliptus grandis and E. urophylla. *Theoretical and Applied Genetics*, Vol.97, pp. 816-827.

Brunel, D. (1994) A microsatellie marker in Helianthus annuus L. *Plant Molecular Biology*, Vol.24, pp. 297-400.

Carvalho, G.A., Sediyama, T., Marin, A.L.A., Barros, E.G. & Moreira, M.A. (2002) Identification of RAPD markers linked to a soybean stem canker resistance gene. *Brazilian Phytopathology*, Vol.27, pp. 474-478.

Colombari FIlho, J. M., Geraldi, I. O. & Barona, M. A. A. (2010) Heterose e distâncias genéticas moleculares para a produção de grãos em soja. In: *Ciência e Agrotecnologia*, Vol.34, No. 4. pp. 940-945. Access in: 19/03/2011, Available from: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1413-70542010000400020&lng=en&nrm=iso>.

Concibido, V.C., Lange, D.A., Denny, R.L., Orf, J.H. & Young, N.D. (1997) Genome mapping of soybean cyst nematode resistance genes in "Peking", PI 90763, and PI 88788 using DNA markers. *Crop Science*, Vol.37, pp.258-264.

Costa, M.M., Unêda-Trevisoli, S.H., Pinheiro, J.B, Kiihl, R.A.S., Calvo, E.S. & Di Mauro, A.O. (2008) Marcadores RAPD para detecção de resistência à ferrugem-asiática-da-soja. In: *Pesquisa agropecuária brasileira*, Vol.43, No.12, pp. 1733-1739. Access in: 19/03/2011, Available from: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-204X2008001200013&lng= en&nrm=iso>.

Chowdhury, A.K., Srinives, P., Saksoong, P. & Tongpamnak, P. (2002) RAPD markers linked to resistance to downy mildew disease in soybean. *Euphytica*, Vol.128, pp. 55-60.

Cregan, P.B., Jarvik, T., Bush, A.L., Soemaker, R.C., Lark, K.G. Kahler, A.L., Kaya, N., Vantoai, T. T., Lohnes, S.D.G., Chung, J. & Specht, J.E. (1999a) An integrated genetic linkage map of the soybean genome. *Crop Science*, Vol.39, pp.1464–1490.

Cregan, P.B., Mudge, J., Fickus, E.W., Danesh, D., Denny, R. & Young, N.D. (1999b) Two simple sequence repeat markers to select for soybean cyst nematode resistance conditioned by the *rhg1* locus. *Theoretical and Applied Genetics*, Vol. 99, pp. 811-818.

Dax, E., Livneh, O., Aliskevicius, E., Edelbaum, O., Kedar, N., Gavish, N., Milo, J., Geffen, F., Bluementhal, A., Rabbinowich, H.D. & Sela, I. (1998) A SCAR marker linked to the to MV resistance gene, *Tm22*, in tomato. *Euphytica,* Vol.101, pp. 73-77.

Doyle, J.J. (1988) 5s Ribosomal gene variation in the soybean and its progenitor. *Theoretical and Applied Genetics*, Vol. 75, pp. 621-624.

Doyle, J.J. & Beachy, R.N. (1 985) Ribosomal gene variation in soybean (*Glycine*) and its relatives. *Theoretical and Applied Genetics*, Vol. 70, pp. 369-376.

Federizzi, L.C. (1998) Estrutura de um programa de melhoramento de plantas e possíveis aplicações de marcadores moleculares: visão do melhorista. In: *Marcadores Moleculares em Plantas*, Milach, S.C.K., pp. 3-15. UFRGS, Porto Alegre.

Ferreira, M.E. & Grattapaglia, D. (1995) *Introdução ao uso de marcadores RAPD e RFLP em análise genética*. EMBRAPA/CENARGEN, Brasília.

Ferreira, M.E. & Grattapaglia, D. (1998) *Introdução ao uso de marcadores moleculares em análise genética*. 3. ed. EMBRAPA/CENARGEN, Brasília.

Fuganti, R., Beneventi, M.A., Silva, J.F.V., Arias, C.A.A., Marin, S.R.R., Binneck, E. & Nepomuceno, A.L. (2004) Identificação de Marcadores Moleculares de Microssatélites para a Seleção de Genótipos de Soja Resistentes a *Meloydogyne javanica*. *Nematologia Brasileira*, Vol.28, No.2, pp 125-130.

Garcia, A., Calvo, E.S., Kiihl, R.A.S., Harada, A., Hiromoto, D.M. & Vieira, L.G. (2008) Molecular mapping of soybean rust (*Phakopsora pachyrhizi*) resistance genes: discovery of a novel locus and alleles. *Theoretical and Applied Genetics*, Vol.117, pp. 545-553.

Gavioli, E. A., Di Mauro, A. O., Centurion, M. A. P. C. & Di Mauro, S. M. Z. (2007) Development of SCAR marker linked to stem canker resistance gene in soybean. *Crop Breeding and Applied Biotechnology*, Vol.7, pp.133–140.

Gebhardt, C., Blomendahl, C., Schachtschabel, U., Debener, T., Salamini, F. & Ritter, E. (1989). Identification of 2n breeding lines and 4n varieties of potato (*Solanum tuberosum* ssp. tuberosum) with RFLP fingerprints. *Theoretical and Applied Genetics*, Vol. 78, pp. 16-22.

Gupta, P.K. & Varshney, R,K. (2000) The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on breas wheat. *Euphytica*, Vol.113, pp. 163-185.

Helentjaris, T., Slocum, M., Wright, S., Schaefer, A. & Nienhuis J. (1986) Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. *Theoretical and Applied Genetics*, Vol.72, pp. 761-769.

Heer, J.A., Knap, H.T., Mahalingam, R. Shipe, E.R., Arelli, P.R. & Matthews, B.F. (1998) Molecular markers for resistance to *Heterodera glycines* in advanced soybean germplasm. *Molecular Breeding*, Vol.4, pp. 359-67.

Hoshino, A.A., Palmieri, D.A., Bravo, J.P., Pereira, T.E.B., Lopes, C.R. & Gimenes, M.A. (2002) Aplicação de marcadores moleculares do tipo microssatélite na caracterização e conservação de recursos genéticos mantidos em bancos de germoplasma. *Biotecnologia Ciência & Desenvolvimento*, Vol.5, No. 29, pp. 146-150.

Hu, J., Vick, B.A. (2003) Target region amplification polymorphism: a novel marker technique for plant genotyping. *Plant Molecular Biology Reporter*, No. 21, pp.289-294.

Keim, P., Diem, B.W., Olson, T.C. & Shoemakert, R.C. (1990) RFLP Mapping in Soybean: Association Between Marker Loci and Variation in Quantitative Traits. *Genetics*, Vol.126, pp. 735-742.

Kwok, P.Y. & Gu, Z. (1999) Single nucleotide polymorphism libraries: why and how are we building them? *Molecular Medicine Today*, Vol.5, No.12, pp. 538-543.

Lahogue, F., This, P. & Bouquet, A. (1998) Identification of a codominante Scar marked linked to the seedlessness character in grapevine. *Theoretical and Applied Genetics*, Vol.97, pp. 950-959.

Lanza, M. A., Guimarães, C. T. & Schuster, I. (2000) Aplicação de marcadores moleculares no melhoramento genético. *Informe Agropecuário*, Vol.21, No.204, pp. 97- 108.

Lee, M., Godshak, E.B., Lambey, K.R. & Woodman, W.L. (1989) Association of restriction fragment length polymorphisms among maize inbreds with agronomic performance of their crosses. *Crop Science*, Vol.29, pp. 1067-1071.

Litt, M. & Luty, J.A. (1989) A hypervariable microsatellite revealed by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics*, Vol.44, pp. 398-401.

Lopes, R., Lopes, M.T.G., Figueira, A.V.O., Camargo, L.E.A., Fungaro, M.H.P., Carneiro, M.S. & Vieira, M.L.C. (2002) Marcadores Moleculares Dominantes (Rapd e Aflp). Aspectos técnicos e interpretação genética. *Biotecnologia Ciência & Desenvolvimento*, Vol.5, No. 29, pp. 56-60.

Mahalingam, R. & Skorupska, H.T. (1995) DNA markers for resistance to *Heterodera glycines* Race 3 in soybean cultivar Peking. *Breeding Science*, Vol.45, pp. 435-443.

Malone, G., Zimmer, P.D., Branco, J.S.C., Mattos, L.A.T., Kopp, M.M., Cervieri Filho, E., Carvalho, F.I.F. & Oliveira, A.C. (2003) Identificação de contaminação genética em cultivares comerciais de soja (*Glycine max*) utilizando a tecnologia de marcadores moleculares AFLP. In: *Congresso Brasileiro de Melhoramento de Plantas 2,* Porto Seguro. Resumos... Porto Seguro: SBMP. (Available in CD).

Maluf, M.P., Guerreiro Filho, O. & Fazuoli, L.C. Biotecnologia: aporte tecnológico ao melhoramento do cafeeiro no IAC. Available from: www.iac.sp.gov.br/New/Oagronomico/532/05_biotecnologia.pdf. Access in: 20/01/2011.

Martins Filho, S., Sediyama, C.S., Moreira, M.A & Barros, E.G. (2002) RAPD and SCAR markers to resistance to frogeye leaf spot in soybean. *Genetics and Molecular Biology*, Vol.25, No.3, pp. 317-321.

McCouch, S.R., Chen, O., Panaud, S., Temnykh, Y., Xu, Y.G., Cho, N., Huang, T. & IshiiI, M. (1997) Microsatellite marker development, mapping and applications in rice genetics and breeding. *Plant Molecular Biology*, Vol.35, pp. 89-99.

Meksem, K., Zobrist, K., Ruben, E., Hyten, D., Quanzhou, T., Zhang, H. B. & Lightfoot, D.A. (2000) Two large-insert soybean genomic libraries constructed in a binary vector: application in chromosome walking and genome wide physical mapping. *Theoretical and Applied Genetics*, Vol.101, pp.747-755.

Mertz, L.M., Henning, F.A. & Zimmer, P. D. (2009) cDNA-AFLP na identificação de genes relacionados a qualidade fisiológica. *Revista Brasileira de Sementes* [online]. Vol.31, No.2, pp. 48-53. Access in: 19/03/2011, Available from:

<http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0101-31222009000200005&lng=en&nrm=iso>. ISSN 0101-3122. doi: 10.1590/S0101-31222009000200005.

MicheImore, R.W., Paran, I. & Kesseli, R.V. (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segreganting populations. *Proceedings of the National Academy of Sciences of the United State of America*, Vol.88, pp. 9828-9832.

Möller, M. (2010) Mapeamento de locos de resistência quantitativa da soja ao complexo de percevejos. Available from: http://www.academico.com/tesedissertacao/mapeamento-de-locos-de-resistencia-quantitativa-da-soja-ao-complexo-de-percevejos. Access in: 20/03/2011.

Morceli, T. G. S., Unêda Trevisoli, S.H., Morceli Jr., A.A., Kiihl, R.A.S., Calvo, E.S., Di Mauro, A.O. & Garcia, A. (2008) Identificação e validação de marcadores microssatélites ligados ao gene *Rpp5* de resistencia a ferrugem-asiatica-da-soja. In: *Pesquisa Agropecuária Brasileira*, Vol.43, No. 11, pp. 1533-1541. Access in: 19/03/2011, Available from: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-204X2008001100011&lng=pt&nrm=iso>.

Morgante, M. & Olivieri, A.M. (1993) PCR-amplified microsatellites as markers in plant genetics. *The Plant Journal*, Vol.3, pp.175-182.

Mudge, J., Cregan, P.B., Kenworthy, J.P., Kenworthy, W.J., Orf, J.H. & Young, N.D. (1997) Two microsatellite markers that flank the major soybean cyst nematode resistance locus. *Crop Science*, Vol.37, pp.1611-1615.

Mullis, K. & Faloona, F. (1987) Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction. *Methods Enzymol*. Vol.55, pp. 335-350.

Nietsche, S., Borém, A., Carvalho, G.A., Ocha, R.C., Paula Jr, T.J., Barros, E.G. & Moreira, M.A. (2000) RAPD and SCAR markers linked to gene confering resistance to Angular Leaf Spot in Common Bean. *Journal Phytopathology*, Vol.148, pp.117-121.

Njiti, V.N., Meksem, M., Iqbal, J., Johnson, J.E., Kassem, M.A., Zobrist, F., Kilo, V.Y. & Lightfoot, D.A. (2002) Common loci underlie fiels resistance to soybean sudden death syndrome in Forrest, Pyramid, Essex, and Douglas. *Theoretical and Applied Genetics*, Vol.104, pp.294-300.

Nodari, R.O., Tsai, S.M., Gilbertson, R. & Gepts, P. (1993) Towards an integrated map of common bean. II. Development of an RFLP-basead genetic linkage map. *Theoretical and Applied Genetics*, Vol.85, pp. 513-520.

Nybom, H., Ramser, J., Kaemmer, D., Kahl, G. & Weising, K. (1992) Oligonucleotide DNA fingerprinting detects a multiallelic locus in box elder (*Acer negundo*). *Molecular Ecology*, Vol.1, pp. 65-67.

O'Donoughue, L.S., Souza, E., Tanksley, S.D. & Sorrells, M.E. (1994) Relationships among North American oat cultivars based on restriction fragment length polymorphisms. *Crop Science*, Vol.34, pp. 1251-1258.

Paran, I. & Michelmore, R.W. (1992) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theoretical and Applied Genetics*, Vol.85, pp. 985-93.

Rassmann, K., Schlötterer, C. & Tautz, D. (1991) Isolation of simple sequence loci for use in polymerase chain reaction-based DNA fingerprinting. *Electrophoresis*, Vol.12, pp. 113-118.

Reiter, R.S., Williams, J., Feldman, K.A., RAfalski, J.A., Tingey, S.V. & Skolnick, P.A. (1992) Global and local genome mapping in *Arabidopsis thaliana* by using recombinant imbred lines and random amplification polymorphic DNAs. *Proc. Natl. Acad. Sci.*, Vol.89, pp. 1477-1481.

Saiki, R.K., Gelfand, D.H., Stoeffel, S., Shcarf, S.J., Higuchi, G., Horn, G.T., Mullis, K.B. & Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, Vol.239, pp. 487-491.

Sambrook, J., Fritsch, E.F. & Maniatis T. (1989) *Molecular cloning: a laboratory manual*. 2nd ed. Cold Spring Harbor Laboratory Press.

Smith, J.S.C. & Smith, O.S. (1991) Restriction fragment length polymorphisms can differentiate amog U.S. maize hybrids. *Crop Science*, Vol.31, pp. 893-899.

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by electrophoresis. *Journal of Molecular Biology*, Vol.98, pp.503-517.

Tanksley, S.D. & Rick, C.M. (1980) Isozymic gene linkage map of the tomato; applicatiosn in genetics and breeding. *Theoretical and Applied Genetics*, Vol.57, pp. 161-170.

Tozuka, A., Fukushi, H., Hirata, T., Ohara, M. Kanazawa, A. Mikami, T. & Shimamoto, Y. (1998) Composite and clinal distribution of Glycine soja in Japan revelead by RFLP analysis of mitochondrial DNA. *Theoretical and Applied Genetics*, Vol.96, pp. 170-176.

Van Der Linden, C.G., Wouters, D.C.A.E., Milalka, V., Kochieva, E.Z., Marinus, J.M., Smulders, M.J.M. & Vosman, B. (2004) Efficient targeting of plant disease resistance loci using NBS profiling. *Theoretical and Applied Genetics*, No.109, pp. 384-393.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. & Zabeau, M. (1995) AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Research*, Vol.23, pp. 4407-4414.

Wang, Z., Weber, J.L., Zhong, G. & Tanksley, S.D. (1994) Survey of plant short tandem DNA repeats. *Theoretical and Applied Genetics*, Vol.88, pp.1-6.

Watson, J. D. , Gilman, M., Witkowski, J. & Zoller, M. (1997) E. H. Babá, (Trad.). *O DNA Recombinante*. 2 ed. UFOP, Ouro Preto.

Webb, D. M., Baltazar, B. M., Rao-Arelli, A.P., Schupp, J., Clayton, K., Keim, P. & Beavis, W. D. Genetics mapping of soybean cyst nematode race-3 resistance loci in the soybean PI 437.654. *Theoretical and Applied Genetics*, Vol.91, pp. 574-581.

Weisemann, J.M., Matthews, B.S. & Devine, T.E. (1992) Molecular markers located to the soybean cyst nematode resistance gene *Rhg4*. *Theoretical and Applied Genetics*, Vol.85, pp.136-138.

Welsh, J. & McClelland, M. (1990) Figerprinting genomes using PCR with arbitrary primers. *Nucleic Acid Research*, Vol.18, pp. 7213-7218.

Weng, C., Kubisiak, T.L. & Stine, M. (1998) Scar markers in a longleaf pine x slash pine F_1 family. *Forest Genetics*, Vol.5, pp. 239-247.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. & Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Research*, Vol.18, pp. 6531-6535.

Yang, W., Weaver, D.B. , Nielsen, B.L. & Qiu, J. (2001) Molecular mapping of a new gene for resistance to frogeye leaf spot in soybean in "Peking". *Plant Breeding*, Vol.120, pp.73-78.

Yuan, J., Njiti, V.N., Meksen, K., Iqbal, M.J., Triwitayakorn, K., Kassem, M.A., Davis, G.T., Schimdt, M.E. & Lightfoot, D.A.(2002) Quantitative trait loci in two soybean

recombinant inbred line populations segregating for yield and disease resistance. *Crop Science*, Vol.42, pp.271-277.

Zabeau, M. (1993) Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application no.0534858 A1.

Zane, L., Bargelloni, L. & Patarnello, T. (2002) Strategies for microsatellite isolation: a review. *Molecular Ecology*. Vol.11, pp.1-16.

Zhang, Y. & Stommel, J.R. (2001) Development of SCAR and CAPS markers linked to the *Beta* gene in tomato. *Crop Science*, Vol.41, pp. 1602-1608.

Zheng, C., Chang, R., Qiu, L., Chen, P., Wu, X. & Chen, S. (2003) Identification an characterization of a RAPD/SCAR marker linked to a resistance gene for soybean mosaic virus in soybean. *Euphytica*, Vol.132, pp. 199-210.

9

Identification and Application of Phenotypic and Molecular Markers for Abiotic Stress Tolerance in Soybean

Berhanu Amsalu Fenta[1], Urte Schluter[1], Belen Marquez Garcia[2], Magdeleen DuPlessis[1], Christine H. Foyer[2] and Karl J. Kunert[1]
[1]*Forestry and Agricultural Biotechnology Institute, Plant Science Department, University of Pretoria, Hillcrest, Pretoria;*
[2]*Centre for Plant Sciences, Faculty of Biology, University of Leeds, Leeds, LS2 9JT,*
[1]*South Africa*
[2]*UK*

1. Introduction

Soybean is a sub-tropical crop, however, its present cultivation range extends from temperate regions to the tropics. The sustainability and predictability of soybean crop production can therefore be severely restricted by environmental stresses. Of these, drought stress is considered to be the cause of major limitations in yield, particularly for soybean crops grown in rain-fed areas (Manavalan et al., 2009; Siddique et al., 2001). The detrimental effects of drought on plant metabolism arise largely from osmotic constraints particularly to the cytoplasm (Lopes et al., 2011). Varieties that are able to grow well under stressful conditions and retain high yields have therefore great potential economic importance. Ideally, therefore, such varieties must be able to sustain growth under limited water supply, conditions that also cause nutrient deprivation and exacerbate the production of reactive oxygen species (Lopes et al., 2011; Foyer & Shigeoka, 2011).

The production of drought-tolerant soybean varieties is a major goal of many plant breeders but progress to date remains slow. Intensive research efforts have identified a variety of genes and processes that are affected by drought in soybean (see for example, Chen et al., 2007 a, b). Similarly, much is known about how drought-induced changes in plant metabolism and gene expression influence plant growth, development and yield. However, sustained increases in soybean yield under stressful conditions will require improved crop management practices as well as new soybean varieties with enhanced drought tolerance.

Many research groups world-wide are involved in the identification of phenotypic and molecular markers for application in marker-assisted breeding programs. A range of robust phenotypic and molecular markers are required to assist cultivar evaluation for stress tolerance. Ideally, any selected markers should be able to discriminate between stress-tolerant and sensitive soybean cultivars using rapid, inexpensive methods. It is an advantage to have markers that do not require destruction of the plants or plant organs, particularly as the assessment of non-destructive markers allows greater consistency in measurements over time. The routine use of molecular markers in soybean breeding

strategies is vital to the understanding of the nature of the different mechanisms that can contribute to drought tolerance and sustained crop yields under field conditions. Only then can desired traits be incorporated in an informed manner in soybean improvement programs. Drought-tolerant varieties must also yield well under both optimal and drought conditions. Markers have therefore to work well under field conditions as well as in the laboratory or in controlled environments, where there is absolute control of other parameters such as temperature, soil moisture, light, and day-length. In this regard, testing potential markers under fixed or portable rain-fed shelters is often considered to be a first step in the assessment of the potential of a marker under semi-natural conditions over the plant life cycle.

The application of a potential marker in the field must also take into account the existing infrastructure of the sites where the marker will be used and the technical expertise required for accurate assessment. While some technologies, such as the molecular markers or "omics" approaches, are excellent analytical tools in the laboratory, they are technically demanding, often costly and often require specific skills. These factors are often not compatible with the requirements of the agro-industrial environment, where any useful marker has to be cheap, simple in application and should potentially be usable in automated systems for high throughput screening. We consider here some of the achievements to date with regard to the identification of a useful marker for drought tolerance in soybean and provide insights from our own research concerning the identification and application of a phenotypic or molecular marker in soybean. We focus particularly on the identification of useful shoot and root parameters, as well as symbiotic nitrogen fixation and its relationship to photosynthesis under optimal and stress conditions.

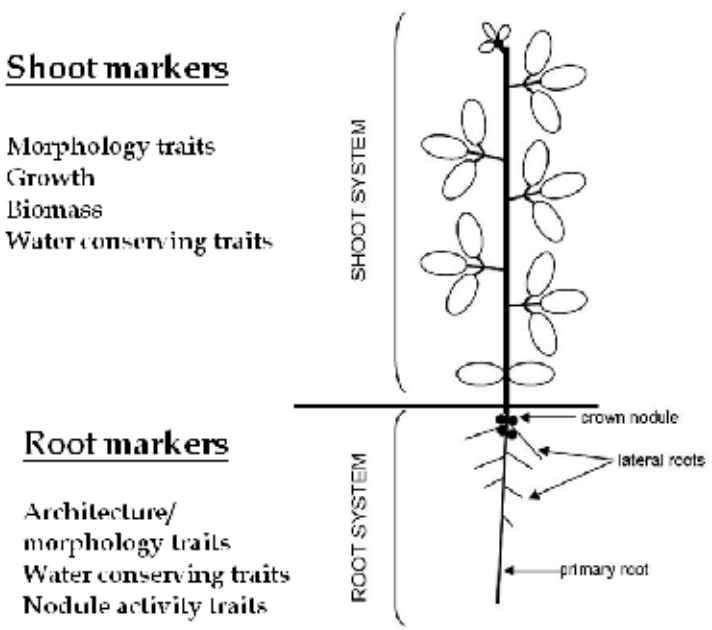


Fig. 1. Phenotypic soybean markers. Classes of phenotypic markers for evaluation of soybean plant performance under drought mentioned in the text.

2. Shoot markers for drought

2.1 Morphological and physiological markers

Of the wide range of possible morphological characteristics that can be used in the selection of soybean varieties for enhanced drought tolerance (Figures 1 and 2), shoot parameters are generally considered to be the easiest to assess under field conditions. Shoot markers remain major targets in breeding programs, particularly in developing countries, where variations in shoot morphology are often determined subjectively under field or glasshouse conditions. Often this involves visual monitoring of easily detectable plant characteristics such as the number of leaves per plant or the shoot height. These simple parameters can be measured easily in soybean at different intervals during the growing period, and they can be assessed together with a range of other less easily determined parameters such as dry matter yield per plant (Udensi et al., 2010) or photosynthetic capacity and water use efficiency (Gilbert et al., 2011). Rapid growth is often directly related to the supply of water during the growing season. Hence, early maturity, early vigour, stomatal regulation, leaf area maintenance and osmotic adjustment of roots and shoots are generally considered to be useful and effective markers of good plant performance under drought conditions.

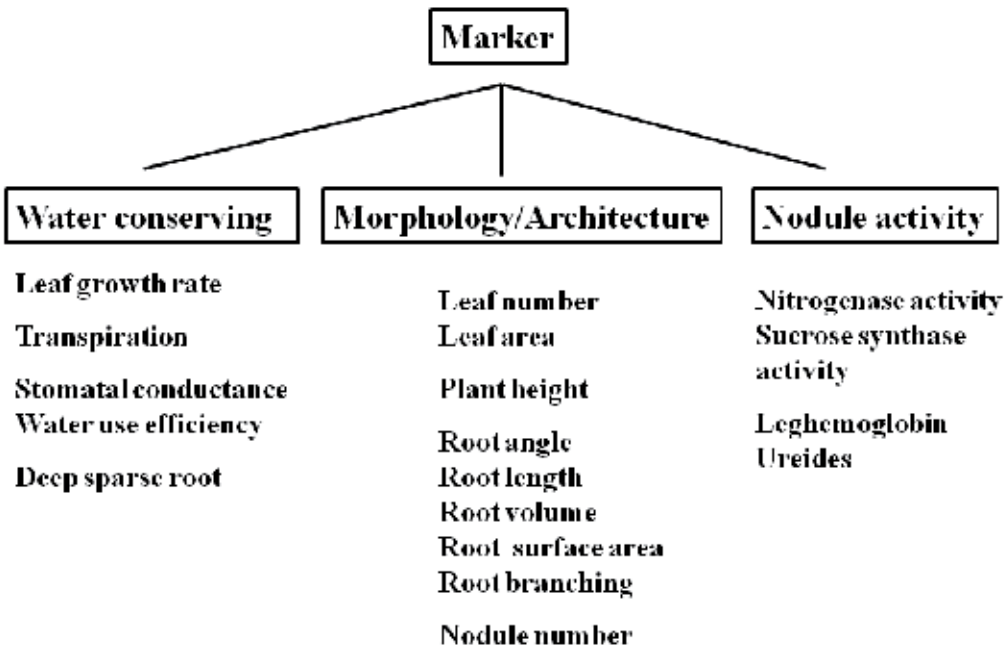


Fig. 2. Measurable phenotypic markers for drought tolerance.

A wide range of morphological, architectural, and physiological parameters are associated with water conservation and these can be used as markers for drought tolerance in soybean. Water deprivation leads to changes in turgor, osmotic pressure, leaf water potential, stomatal conductance and transpiration (Basra et al., 1999; Earl, 2002; Ribas-Carbo et al., 2005). Stomatal closure as a result of leaf water loss results in higher leaf water potentials

and allows better maintenance of the leaf water (Nakayama et al., 2007). However, stomatal closure also results in reduced CO_2 movement for carboxylation within the chloroplast and this can be a major cause of drought-induced decreases in CO_2 assimilation capacity, particularly in C3 plants (Chaves & Oliveira, 2004; Flexas et al., 2006; Warren, 2008). General plant performance parameters, such as biomass production, shoot and root length or seed yield but also leaf movements and phenological plasticity as an adaptation to drought (Acosta-Gallegos & White, 1995; Pastenes et al., 2005) might be useful as indicators for drought tolerance. Such physiological traits are therefore potential candidates for development as breeding markers for the selection of superior drought tolerant varieties. However, breeders can often be reluctant to use such traits, particularly if the associated measurements are time-consuming and technically demanding.

2.2 Water use efficiency

Traits that serve to conserve water (conservative traits), including low stomatal conductance, low leaf growth rate, high water use efficiency (WEU), or deep but sparse root systems, favour better water use efficiencies. Research efforts in this regard have often tended to focus on the use of instantaneous water use efficiency (IWUE) values as a physiological marker for drought tolerance. The IWUE of a plant is determined from the ratio between CO_2 assimilation rate and stomatal conductance values (Soares-Cordeiro et al., 2009). It is considered that higher IWUE values provide an indication of improved drought tolerance because varieties with high IWUE values are better able to assimilate carbon at low stomatal conductance and hence attain a greater yield using less water than other varieties. Genetic variation has been observed in soybean with regard to the amount of dry matter produced per given amount of water (Hufstetler et al., 2007). Moreover, soybean genotypes with a quick recovery after drought stress were more productive than genotypes with slower recovery (Hufstetler et al., 2007). Rapid recovery after drought stress is considered as an important trait in varieties grown under drier conditions with periods of drought. Water use efficiency (WUE) is increased by reduced transpiration and water use and biomass production is tightly linked to transpiration, WUE, and nitrogen accumulation. Breeding plants for high WUE under drought might actually result in low-yielding genotypes (Blum, 2011). It has been argued biomass production under most drought conditions can only be enhanced by an effective use of water which will allow maximal soil moisture capture for transpiration and minimizing water loss by soil evaporation (Blum, 2011).

Breeding efforts to improve drought tolerance in soybeans must ensure that a high level of water use efficiency is maintained in new varieties. In a study carried out in our group we compared a range of physiological shoot and root traits under optimal and drought conditions in three soybean varieties: Prima 2000, a commercial variety registered in South Africa that is suitable for cultivation in areas with longer growing seasons but suffering water stress during the growth period; A5409RG, a commercial variety that harbours a glyphosate-resistance gene and that is better suited to areas that do not experience drought or where crops are grown under irrigation; and Jackson, a drought-escaping cultivar with a shorter life cycle (Chen et al., 2007 a,b; Sall & Sinclair, 1991). Of the three genotypes studied, Prima had the highest IWUE values and shoot biomass under both well-watered and drought conditions (Fenta et al., 2011, Figure 3). Prima maintained highest photosynthetic CO_2 assimilation rates under drought. The IWUE values in Prima leaves experiencing long term drought stress were twice those measured under water-replete conditions. The drought-induced increase in IWUE values observed in Jackson was smaller under drought,

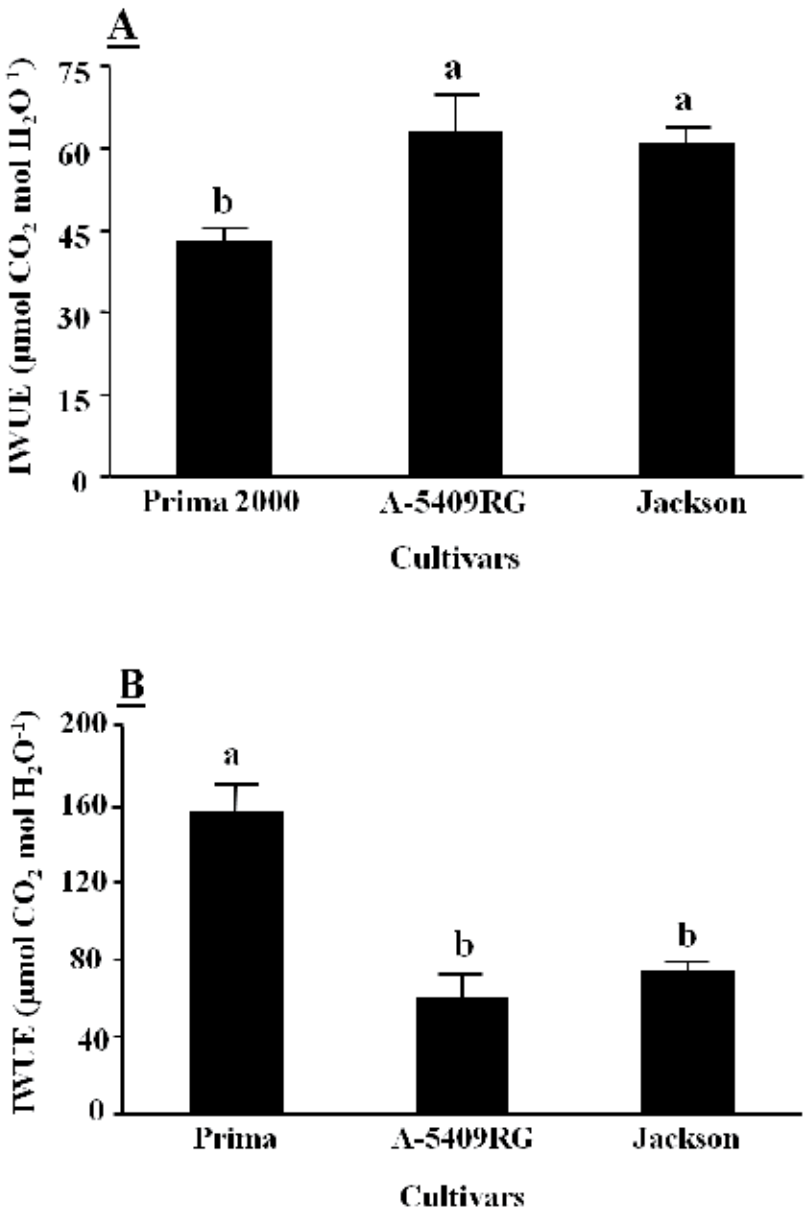


Fig. 3. Instantaneous water use efficiency of soybean. Comparison of instantaneous water use efficiency (IWUE) values measured in three soybean cultivars at day 0 (A) and after 18 days growth under drought conditions (B). Data are the means ±SEM of four independent replicates. Means with the same letter above the bars are not significantly different.

even though Jackson had lower stomatal conductance rates than Prima. In contrast, no significant differences ($P > 0.05$) in IWUE values were found in A-5409RG either under water-replete or drought conditions.

3. Root markers for drought

Although much information is already available on potential markers for shoot performance, there is relatively little information available on markers for root architecture or root morphology. Several of the root-related traits, such as deep penetration and dense roots, have been found to be related to improved growth under stress. For example, slow wilting in soybean may be associated to deep rooting (Hufstetler et al., 2007). The ability of the plant to extract water from deeper soil profiles is considered to be important in enabling plants to maintain optimal water relations as well as carbon assimilation under drought stress. Deep root systems often allow changes in carbon allocation patterns before water limitation adversely affects growth (Jordan et al., 1983; Jones & Zur, 1984; Blum, 1985; O'Toole & Bland, 1987; Sponchiado et al., 1989; Sinclair & Muchow, 2001; Campos et al., 2004; Manschadi et al., 2006, 2008; Reynolds et al., 2007; Lopes & Reynolds, 2010). For example, bean genotypes with deeper roots were found to have better seed yields and crop growth (Sponchiado et al., 1989).

Improving root traits could contribute to sustainable productivity under water-limited conditions. Root architecture traits, such as root angles and branching, are considered to play a basic role in water acquisition. However, measurements of such parameters under environment conditions is not always representative of the conditions experienced in the field, particularly in situations where in root growth is restricted in pot experiments. Measurements of root traits under field conditions are further very difficult. Moreover, such traits, when measured, are not positively related to yield. While root traits are considered to be important in selection of improved plant performance under drought, most researchers are reluctant to use them particularly when dealing with complex root systems.

Flowering plants have two main types of a root system: an allorhizic root system is found in dicotyledonous species, as illustrated in Figure 4 (Osmont et al., 2007) and a homorhizic root system is typical for monocotyledonous species. Adventitious roots are rare in allorhizic root systems but they occasionally emerge from hypocotyls or stems. Soybean has a typical allorhizic root system with two root types, a primary root (or tap root) and lateral roots (Figure 4). The first root that emerges from the hypocotyls in the soybean root system dominates the lateral roots, which can also produce branches.

Our recent research on soybean has focused on the development of a root morphology marker for drought tolerance. In the study, we applied particularly imaging analysis to determine various root parameters in field-grown plants (Figure 5). This analysis enabled the determination of a number of root traits including total length, total surface area and total volume, as well as tap root parameters and lateral root branching density. In this study, the drought tolerance of the three soybean genotypes discussed above was tested under field conditions using a randomized complete block design. Over the first four weeks of the study, all plants were watered regularly (8 mm/day) using a pivot sprinkler irrigation in addition to rain to allow optimal growth with soil moisture status at near field capacity. In some blocks (controls), plants were always grown with this adequate water supply. In other blocks, water stress was initiated one month after sowing by withholding irrigation for one month. Rain fell on only three days of this latter experimental period. This study showed

that Prima 2000 and Jackson have deeper roots with a greater branching density than A-5409RG under drought conditions (Figure 5). A-5409RG had a shallower and thicker root system under drought conditions, with lower overall numbers of roots and a lower branching density. This cultivar also had the highest shoot to root ratio under drought conditions. The production of a lower root biomass compared to the shoot would result in a lower water harvesting capacity. Prima 2000 and Jackson 2000 also had larger root systems under drought than A-5409RG with a greater root length, root surface area and a greater root volume (Figure 6). Both varieties were therefore able to extend the root system in response to drought and so withstand water stress. This study indicates the importance of root traits in the field performance of different soybean cultivars under drought conditions. The association of root traits with the higher productivity of Prima 2000 under drought conditions suggests that these parameters could be used as a marker for drought tolerance in the field.

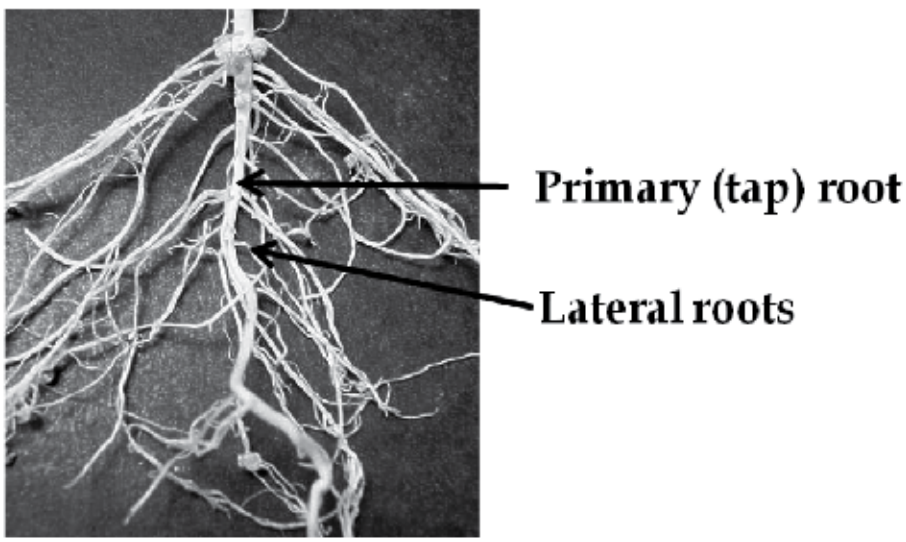
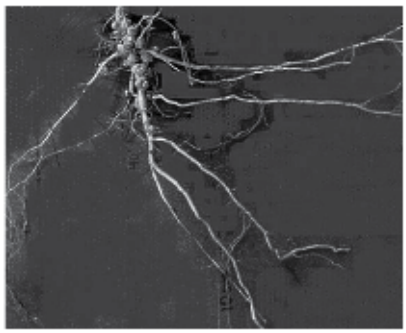


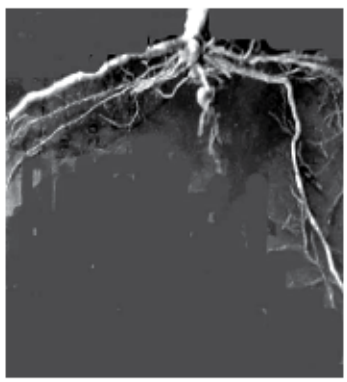
Fig. 4. Soybean root system architecture. Schematic representation of the allorhizic root system architecture of soybean.

3.1 Nodule markers

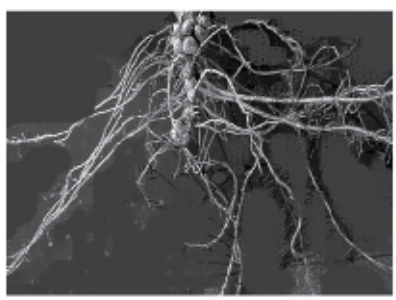
For soybean, the ability to maintain not only high rates of photosynthesis but also symbiotic nitrogen fixation under drought can be important "in terms of yield". Symbiotic nitrogen fixation is rapidly inhibited by drought and thus soybean plants exposed to drought are also deprived of nitrogen (Sinclair et al., 2007). Like photosynthesis, nitrogenase activity can therefore be used as a sensitive marker for drought tolerance. Metabolic markers, such as nitrogenase activity, detect the early plant responses to drought and they are more sensitive than morphology markers. Soybean genotypes can be selected for increased yield on the basis of leaf or nodule numbers (Udensi et al., 2010). Other nodule parameters such as size, leghemoglobin content, ureide accumulation can be used as markers for nitrogen fixation capacity and hence plant performance.



Jackson



A-5409RG



Prima 2000

Fig. 5. A comparison of the root phenotype in three soybean cultivars grown under conditions of drought. A representative shoot phenotype is shown for each of the three soybean cultivars studied: Jackson, A-5409RG and Prima 2000. Photos were taken after plants had been exposed to drought for 1 month under field conditions.

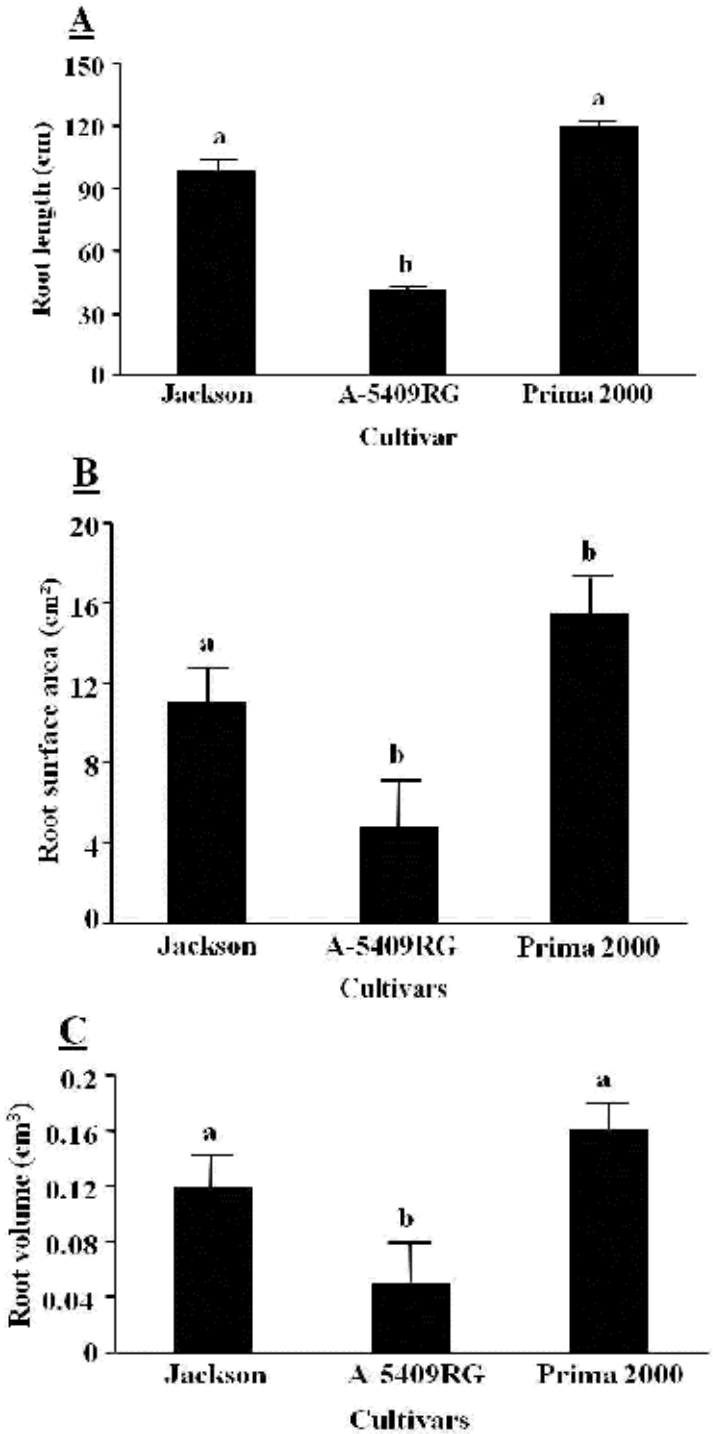


Fig. 6. A comparison of root morphology traits in three soybean cultivars after plants had been exposed to drought for 1 month under field conditions.

Root length (A), root surface area (B) and root volume (C) were measured in soybean cultivars, Jackson, A-5409RG and Prima 2000. Roots were harvested in the field to a depth of 60 cm under drought conditions. The data were obtained from the roots as illustrated in Figure 7 and are the mean ± SE of 3 individual root systems per cultivar. Scanned root images were analyzed using the winRHIZO 2008a software which is an image analysis system specially designed for root measurement in different forms (Regent Instruments Canada Inc.). Using this software, root morphological data of root length, average diameter, total area and volume were determined. Furthermore, nodules were counted from the root images. Significance was determined using mean separation student's t-test ($\alpha = 0.05$) Means with the same letter above the bars are not significantly different.

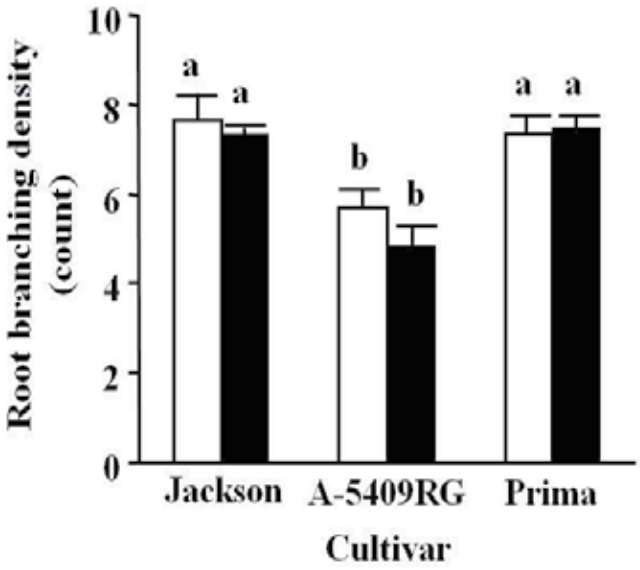


Fig. 7. A comparison of the root architecture traits in three soybean cultivars: Jackson, A-5409RG and Prima 2000 (Prima). Measurements of primary (tap) root branching (open bars) and lateral root branching density (closed bars) were performed after plants had been exposed to drought for 1 month under field conditions. Data represent the mean ± SE for values obtained from 3 individual plants. The student's t-test ($\alpha = 0.05$) was applied. The letters above the bars indicate significant differences.

When a large collection (> 3,000) of soybean lines was screened for variations in leaf ureide contents under drought only eight were found to maintain low ureide contents under conditions of water deprivation (Sinclair et al., 2000). Thus, leaf and nodule ureide contents could be used as markers for alterations in nitrogen fixation under drought.

Of the factors that are important in preventing the inhibition of nitrogen fixation under drought perhaps the most important is the maintenance of the oxygen diffusion barrier. Other important factors include a continued supply of assimilated carbon from the leaves, together with high nodule sucrose synthase activities. In addition the export of ureides from the nodules is also important in preventing the accumulation of these metabolites and also amino acids in the nodules. Increases in nodule ureide or amino acid levels would lead to an

inhibition of nitrogenise activity. (Durand et al., 1987, Gonzalez et al., 1998, Arrese-Igor et al., 1999, King & Purcell, 2005). There is a close relationship between leaf and nodule water potentials (Durand et al., 1987). Water stress directly inhibits nitrogenase activities because of increased resistance to oxygen diffusion in the nodule. The increase in oxygen diffusion resistance is often linked to a decrease in nodule respiration as well as nitrogenase activity. In these conditions respiratory substrate accumulate as do oxidized lipids, and there is enhanced expression of antioxidant genes suggesting that impaired respiratory activity in the bacteroids under drought leads to enhanced oxidation prior to effects on sucrose synthesis or leghemoglobin (Naya et al., 2007).
In an earlier study on the stress tolerance of different soybean varieties, we investigated the responses of symbiotic nitrogen fixation to dark chilling (van Heerden et al., 2008). Nodule structure, respiration and carbon-nitrogen interactions were compared in the two soybean genotypes that differed in chilling sensitivities: PAN809 (PAN) is chilling sensitive and Highveld Top (HT) is chilling resistant. We found that nodule numbers and the abundance of nitrogenase and leghemoglobin proteins were unaffected by dark chilling. However, the chilling stress caused a large decrease in nodule respiration rates and nitrogenase activities. The large chilling-dependent decrease in ureide contents observed in the PAN nodules was linked to decreases in respiration and an inability to maintain the oxygen diffusion barrier (van Heerden et al., 2008). These data suggest that nodule respiration and mitochondrial markers could be useful tolls in assisting conventional breeding efforts aimed at the development of higher yielding soybean genotypes with better chilling tolerance.

4. Molecular markers for drought

Accurate phenotypic markers must ultimately be associated with molecular markers to aid and accelerate current plant breeding efforts to select improved soybean varieties with better stress tolerance. The effectiveness of morphological and physiological markers can vary greatly according to the growth stage of the plant and the many variables in the environment, particularly under field conditions. The selection of molecular markers is based either on variations in genomic DNA or on variations in gene expression (transcriptome) patterns. Comparisons of the transcriptome and also proteome signatures of organs or tissues under stress conditions can provide a direct assessment of processes and/or components than can be developed into a useful marker for stress tolerance in breeding programs. In general, a molecular marker might identify variations in plant responses to stress at the gene level, or in certain regions of DNA, the composition of DNA or in the degree of DNA methylation. While the DNA content of a cell is independent of environmental conditions, organ specificity or growth stage, DNA methylation can be regulated by these endogenous and environmental conditions and so alter also the patterns of gene expression. Variable regions of the genome (transposons), which can include single copy and repetitive genome regions, can also have considerable impact on plant stress tolerance. Such genomic regions can be functional or 'silent' without any obvious function. More recently, next generation sequencing with the identification of single nucleotide polymorphisms, is increasingly being considered as a tool to identify useful molecular markers for stress tolerance.
Further, Quantitative trait loci (QTLs) are chromosomal regions (genomic loci) that might regulate the expression levels of proteins. QTLs can be associated with DNA segments that are considered to make a significant contribution to the expression of complex phenotypic traits

such as stress tolerance. Quantitative traits are usually affected by more than one gene and by the environment. The association of morphological or physiological phenotypic markers with molecular markers in QTL analysis is considered to ultimately be the ideal approach to accelerate plant breeding programs in marker-assisted selection (MAS). MAS is the process whereby the identified markers of all types (morphological, physiological, biochemical or molecular) are used for indirect selection of required traits. MAS approaches however are far from trivial and they are often not cost effective in current breeding programs. Drought tolerance is a mutagenic trait, often influenced by large numbers of minor QTLs, rather than one or more major QTLs. This means that the development and effective large-scale application of MAS is still technically challenging, costly and time-consuming.

The identified molecular DNA markers should ideally be applicable using relatively simple methods that are amenable to automation and high throughput. However, the isolation DNA from plants is not technically demanding and can be carried out in any laboratory that has basic DNA isolation and characterisation equipment. Potential markers can be first evaluated in small sample subsets in order to confirm that the desired loci are present. Such methodologies normally require large sample collections or population sizes because the chosen markers have be applied to large segregating populations in order to determine whether the markers are linked to the required traits (Ribaut et al., 2002).

Relatively few markers for drought tolerance have been identified in soybean. However, progress in this regard will be accelerated once large collections of potential DNA sequences have been established for marker development (Shinozaki, 2007). Accurate genetic and physical maps of the soybean genome are also essential for the development of useful molecular markers for drought tolerance in soybean. Towards this goal, a total of 318 AFLP, 121 SSR, 108 RFLP, and 126 STS markers have been integrated into a linkage map composed of 509 RFLP, 318 SSR, 318 AFLP, 97 AFLP-derived STS, 29 BAC-end or EST-derived STS, 1 RAPD, and five morphological markers (Hisano et al., 2007, Xia et al., 2007). A further very useful advance has come from the sequencing of the soybean (Glycine max (L.) Merr. 'Williams 82') genome. Some 66,153 protein-coding loci are now available at: http://www.phytozome.net/soybean. Moreover, 3290 microsatellites (SSRs) identified from BAC end sequences of clones (comprising the 'Williams 82' physical map) were screened and two hundred and sixty-five SSRs were genetically mapped in at least one mapping population (Shoemaker et al., 2008).

4.1 Identification of markers using microarrays

Microarrays involve the immobilisation of single-stranded DNA on a solid support that is hybridised with a single-stranded DNA or RNA population (Rockett and Dix, 1999). In microarray analysis gene expression and regulation patterns can be monitored on a large scale (Quackenbush, 2001). The technology facilitates screening for differently expressed genes in different plant varieties. Differently expressed genes can potentially be useful in MAS providing that they encode proteins involved in the traits of interest.

A spotted soybean cDNA microarray containing 36,000 elements derived from EST libraries is available that covers a wide range of tissues and organs at different developmental stages under optimal and stress conditions (Vodkin et al., 2004). Expressed sequence tags (ESTs) are coding regions within the DNA that can also be used for MAS or mapping purposes. The isolation of 6570 full-length sequences of soybean cDNAs derived from tissues exposed to different abiotic stresses will aid marker development (Umezawa et al., 2008) as will the isolation of ESTs from drought-stressed soybean root tips (Valliyodan & Nguyen, 2008).

We have analysed the crown nodule transcriptome at two stages of development (i.e. on 7 and 11 week-old plants) in order to identify possible markers for nodule development/senescence that might underpin plant performance. A comparison of the crown nodule transcriptomes was performed on two cultivars (PAN809 and Highveld Top) after 7 and 11 weeks of growth. PAN 809 is generally recommended for use under long growing seasons and this variety is often used in moderate and hot regions of Southern Africa because it is well-suited to irrigated- and rain-fed cultivation conditions. Highveld Top was developed specifically to withstand the low night temperatures that are often experienced during the growing season at high altitude in Southern Africa. The first nodule harvest point (7 weeks) was chosen because the measured capacity of symbiotic nitrogen fixation was highest at this point of nodule development. The second harvest point (11 weeks) was chosen because symbiotic nitrogen fixation had decreased by 50% at this time point relative to week 7. Figures 8 and 9 illustrate the degree of genotypic variation in the nodule transcriptome signatures observed in the nodules of 7 and 11 week-nodules under optimal growth conditions. While 702 transcripts were changed in abundance in Highveld Top nodules harvested at week 11 compared to week 7, 1737 transcripts were differentially expressed in PAN809 nodules under the same conditions (Figure 8). Of these, 226 transcripts were identical and showed similar patterns of increase or decrease at week 11 relative to week 7 in both cultivars (Figure 8). Of the transcripts that were differentally expressed in PAN809 and Highveld Top nodules at week 7, the PAN809 nodule transcriptome had a much higher number of transcripts encoding proteins involved in cell wall and stress metabolism than Highveld Top (Figure 9). For example, an extensin-like protein and proteins involved in disease resistance were much more abundant in PAN 809 nodules than Highveld Top nodules at week 7. Moreover, transcripts encoding the extensin-like protein were enhanced to a much greater extent in PAN 809 than Highveld Top. Transcripts encoding disease resistance proteins were also much higher in PAN 809 nodules than Highveld Top nodules at 11 weeks. The biological relevance of these findings is currently under investigation in relation to the different performance of these two cultivars.

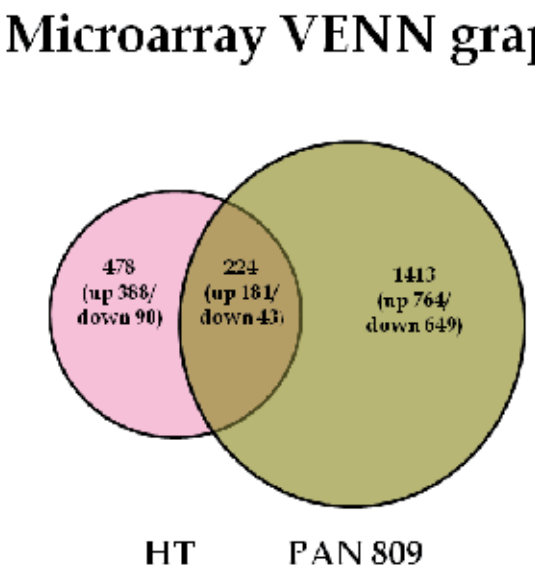


Fig. 8. A comparison of differential gene expression in Highveld Top (HT) and PAN 809 nodules harvested from 7- and 11-week old plants.

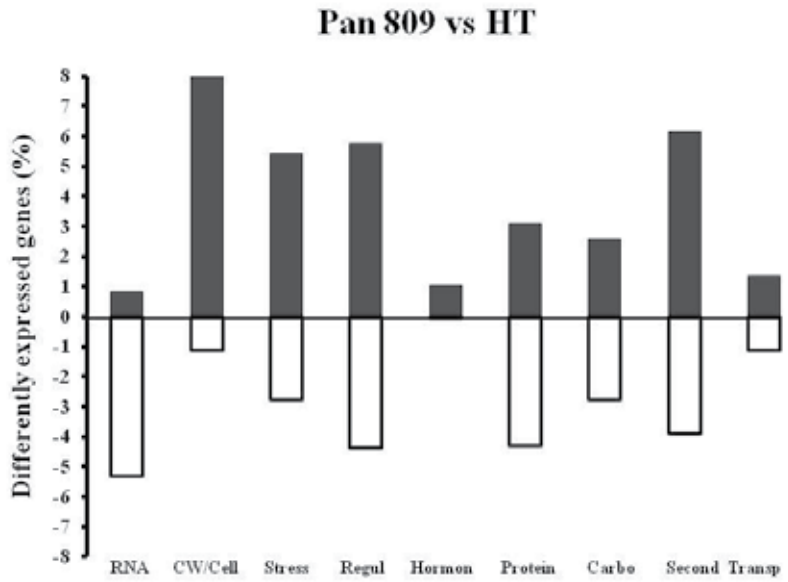


Fig. 9. A comparison of the relative expression of different transcripts in the crown nodules from Pan 809 and Highveld Top. Positive values represent relative increases in transcript abundance while negative values represent decreases in the abundance of transcripts encoding proteins involved in RNA/signaling (RNA), cell wall metabolism (CW/Cell), defense or stress responses (Stress), regulation (Regul), hormone metabolism (Hormon), protein metabolism (Protein), primary metabolism (Carbo), secondary metabolism (Second) and transport (Transp).

5. Conclusions

The fixation of atmospheric nitrogen in leguminous plants through the symbiotic union with soil bacteria (rhizobia) and powered ultimately by photosynthesis is an important driver of ecosystem sustainability in the face of climate change. Grain legumes such as soybean are rich in protein, starch, fibre and other essential nutrients and valuable in the production of foodstuffs and feed. They are also used as a natural nitrogen source in agriculture, particularly in Africa. Nodule development is a complex process that has been intensively studied for many years but many questions remain concerning the orchestration of bacterial infection, nodule development and nodule senescence in relation to the senescence of the whole plant. As with other major crops, grain legume production can be severely restricted by environmental stresses. Our studies, which have been summarized here, have largely focused on the effects of environmental stress on nodule senescence in soybean. Our aim has been to determine the contribution that stress-induced nodule senescence makes to the overall sensitivity of soybean plants to drought. Furthermore, we have characterised a range of physiological, metabolic and molecular parameters that could be useful in selection of genotypes for enhances stress tolerance. The conclusions from are studies are discussed below.

5.1 Characterization of chilling-induced tolerance traits in soybean

We compared nodule structure, carbon/nitrogen interactions and respiration in PAN, which is chilling-sensitive and in HT, which is more chilling-resistant (van Heerden et al., 2008). Under optimal growth conditions, SNF began to decline after 9 weeks in PAN nodules and after 11 weeks in HT. A transcriptome analysis was performed on PAN and HT nodules harvested from plants at 3 until 15 weeks after germination. Of the genes that showed the same developmental pattern in both varieties transcripts encoding a cysteine proteinase gene (Gma.8481.1.S1_at) that belongs to a subgroup a vacuolar processing enzymes (legumains) was up-regulated in the senescent nodules. Nodule numbers were unaffected by dark chilling in both genotypes. The abundance of the nitrogenase and leghemoglobin proteins was not changed as a result of dark chilling but nodule respiration rates, nitrogenase activity and NifH and NifK mRNAs were decreased while nodule starch, sucrose and glucose were increased. Chilling-induced decreases in nodule respiration continued in PAN nodules after return to optimal temperatures but respiration recovered in HT by the end of the chilling period. This recovery was associated with a large decrease in the area of the intercellular spaces in the nodule cortex and infected zone in HT. This acclimatory response was not seen in PAN nodules. We conclude that the ability to regulate the oxygen diffusion barrier is an important component of ability of nodules to tolerate stress (van Heerden et al., 2008). The HT nodules were able to regulate both respiration and the area of the intercellular spaces during chilling and so control the oxygen diffusion barrier. We conclude that chilling-induced inhibition of SNF in PAN nodules was caused by the inhibition of respiration coupled to the failure to regulate the oxygen diffusion barrier effectively (van Heerden et al., 2008). Furthermore, the stress-induced limitations in SNF make an important contribution the greater chilling-induced inhibition of photosynthesis in PAN than HT.

5.2 Characterization of drought tolerance traits in soybean

The characterisation of simple but accurate phenotypic markers for enhanced drought tolerance is important because drought is considered to be the most important factor limiting soybean productivity in the field. A comparison of shoot, root and nodule parameters in three genotypes: Prima 2000, glyphosate-resistant A5409RG and Jackson revealed a positive correlation between SNF and photosynthesis under optimal and drought conditions (Fenta et al., 2011). Considerable genotypic variation was observed in the responses of photosynthesis to drought. While Jackson and Prima performed better than A-5409RG in short-term drought, SNF in Jackson nodules was equally inhibited in all cultivars under long term drought conditions. Drought-induced decreases in shoot to root ratios occurred in all three cultivars, together with a reduction in whole plant biomass (Fenta et al., 2011). However, the shoot to root ratios under drought were significantly higher in A-5409RG than Jackson or in Prima, showing that there is considerable genotypic variation in the control of shoot to root ratios in soybean in response drought. We conclude that that the ability to sustain shoot biomass under the nitrogen limitation caused by impaired SNF could used as a marker for drought tolerance in soybean (Fenta et al., 2011).

5.3 A role for CLAVATA3/Embryo Surrounding Region (CLE) peptide signalling in soybean nodule development

Nodule development is an energy demanding process and so it might be more economical for the plant, to be able to form few larger nodules compared to many small ones. Indeed,

auto-regulation mutants that have an abundant number of nodules do not necessarily fix more nitrogen and often have a reduced shoot growth. Thus, by understanding primordium formation and meristem activity in soybean, we might use this information to alter nodulation architecture leading to less energy demanding nodule formation. CLE peptides are small secreted peptides derived from the C-terminal region of pre-proproteins. They control the balance between stem cell proliferation and differentiation in plant developmental processes and fulfil as yet largely undefined roles in nodule development. A genome-wide survey of CLE peptides in soybean resulted in the identification of 39 GmCLE genes (Mortier et al., 2011). Two different CLE expression patterns were identified; one of these was linked with nodule primordium development and the other was linked with nodule maturation. We conclude that group-III CLE peptides are produced in the nodules and that they are involved in primordium homeostasis and in auto-regulation of nodulation (Mortier et al., 2011).

5.4 Marker assisted selection

Phenotypic and molecular markers can be equally important in plant breeding programmes. The identification of "perfect" marker gene(s) conferring the required traits related to enhanced drought tolerance might prove to be elusive because abiotic stress tolerance is a multi-genic trait. Much current research effort in soybean breeding is focused on this goal. There is an urgent need of validated linked markers for stress tolerance. The usefulness of the different markers discussed above in MAS depends on many factors, not least the available infrastructure, technical expertise and the relevance of the technology to the traits under consideration. New and improved technologies for molecular marker selection are developing rapidly but the application of such technologies to plant breeding programmes remains slow. Unfortunately, there is still a wide gulf between advances in basic knowledge of the genes and proteins that underpin stress tolerance mechanisms and the successful application of this knowledge through MAS approaches in plant breeding programmes.

6. Acknowledgments

MDP was funded by the South African Protein Research Trust and the National Research Foundation in South Africa and BMG was funded by Subprograma Estancias de movilidad posdoctoral en centros extranjeros (2009), Ministerio de Educación (Spain). BAF was financially supported by CIAT, Colombia. This work was funded by EU FP7 (PIRSES-GA-2008-230830: Legume Improvement).

7. References

Acosta-Gallegos, J.A. & White J.W. (1995). Phenological plasticity as an adaptation by common bean to rainfed environment. *Crop Science*, Vol. 35, No 1, pp. 1238-1238, ISSN 1435-0653

Arrese-Igor, C., Gonzalez, E.M., Gordon, A.J., Minchin, F.R., Galvez, L. & Royuela, M. (1999). Sucrose synthatse and nodule nitrogen fixation under drought and other environmental stresses. *Symbiosis,* Vol. 27, No. 3/4, pp. 189–212, ISSN 0334-5114

Basra, S.M.A., Ahmad N. & Khaliq, A. (1999). Water relation studies in water stressed sugarcane (*Saccharum officinarum* L.). *International Journal of Agricultural Biology,* Vol. 1, No. 1/2, pp. 1–4, ISSN 1813-2235

Blum, A. (1985). Breeding crop varieties for stress environments. *Critical Reviews in Plant Sciences,* Vol. 2, No. 3, pp. 199-238, ISSN 0735-2689

Blum, A. (2011). Plant Breeding for Water-Limited Environments. DOI 10.1007/978-1-4419-7491-4_3, Springer Science+Business Media, LLC 2011.

Campos, H., Cooper, A., Habben, J.E, Edmeades, G.O., & Schussler J.R. (2004). Improving drought tolerance in maize: a view from industry. *Field Crops Research,* Vol. 90, No. 1, (November 2004), pp. 19-34, ISSN 0378-4290

Chaves, M.M. & Oliveira, M.M. (2004). Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. *Journal of Experimental Botany*, Vol. 55, No. 407, (November 2004), pp. 2365-2384, ISSN 0022-0957

Chen, P., Sneller C.H., Purcell L.C., Sinclair T.R., King C.A. & Ishibashi, T. (2007a). Registration of soybean germplasm Lines R01-416F and R01-581F for improved yield and nitrogen fixation under drought stress. *Journal of Plant Registrations*, Vol. 1, No. 2pp. 166-167, ISSN 1936-5209

Chen, M., Wang, Q.Y., Cheng, X.G., Xu, Z.S., Li, L.C. & Ye, X.G. (2007b). GmDREB2, a soybean DRE-binding transcription factor, conferred drought and high-salt tolerance in transgenic plants. *Biochemical Biophysical Research Communications,* Vol. 353, No. 2, (February 2007), pp. 299 – 305, ISSN 0006-291X

Durand, J.L., Sheehy, J.E. & Minchin, F.R. (1987). Nitrogenase activity, photosynthesis and nodule water potential in soybean plants experiencing water deprivation. *Journal of Experimental Botany,* Vol. 38, No. 2, (February 1987), pp. 311–321, ISSN 0022-0957

Earl, H.J. (2002). Stomatal and non-stomatal restrictions to carbon assimilation in soybean (Glycine max) lines differing in drought use efficiency. *Environmental and Experimental Botany*, Vol. 48, No. 3, (December 2002), pp. 237–246, ISSN 0098-8472

Fenta, B.A., Driscoll, S.P., Kunert, K.J. & Foyer, C.H. (2011). Characterization of drought tolerance traits in nodulated soybeans: The importance of maintaining photosynthesis and shoot biomass under drought-induced limitations on nitrogen metabolism. *Journal of Agronomy and Crop Science*, submitted, ISSN 0931-2250

Flexas, J., Ribas-Carbó, M., Bota, J., Galmés, J., Henkle, M., Martínez-Cañellas, S. & Medrano, H. (2006). Decreased Rubisco activity during water stress is not induced by decreased relative water content but related to conditions of low stomatal conductance and chloroplast CO_2 concentration. *New Phytologist*, Vol. 172, No. 1, (October 2006), pp. 73-82, ISSN 0028- 646X

Foyer, C.H. & Shigeoka, S. (2011). Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiology,* Vol. 155, No. 1, (January 2011), pp. 93-100, ISSN 0032-0889

Gilbert, M.E., Zwieniecki, M.A. & Holbrook, N.M. (2011). Independent variation in photosyntheitc capacity and stomatal conductance leads to differences in intrinsic water use efficiency in 11 soybean genotypes before and during mild drought. *Journal of Experimental Botany,* doi:10.1093/jxb/erq461.

Gonzalez, E.M., Aparicio-Tejo, P.M., Gordon, A.J., Minchin, F.R., Royuela, M. & Arrese-Igor C. (1998). Water-deficit effectson carbon and nitrogen metabolism of pea nodules. *Journal of Experimental Botany,* Vol. 49, No. 327, (October 1998), pp. 1705–1714, ISSN 0022-0957

Hisano, H., Sato, S., Isobe, S., Sasamoto, S., Wada, T., Matsuno, A., Fujishiro, T., Yamada, M., Nakayama, S., Nakamura, Y., Watanabe, S., Harada, K. & Tabata, S. (2007).

Characterization of the soybean genome using EST-derived microsatellite markers. *DNA Research,* Vol. 14, No. 6, pp. 271–281, ISSN1340-2838

Hufstetler, E.V., Boerma, H.R., Carter, T.E. & Earl, H.G. (2007). Genotypic variation for three physiological traits affecting drought tolerance in soybean. *Crop Science*, Vol. 47, No. 1, pp. 25–35, ISSN 1435-0653

Jones, J.W. & Zur B. (1984). Simulation of possible adaptive mechanisms in crops subjected to water stress. *Irrigation Science,* Vol. 5, No. 3, (October 1984), pp. 251-264, ISNN 0342-7188

Jordan, W.R., Dugas, W.A. & Shouse P.J. (1983). Strategies for crop improvement for drought-prone regions. *Agricultural Water Management,* Vol. 7, No. 1-3 (September 1983), pp. 281-289, ISSN 0378-3774

King, C.A. & Purcell, L.C. (2005). Inhibition of N_2 fixation in soybean is associated with elevated ureides and amino acids. *Plant Physiology,* Vol. 137, No. 4, (April 2005), pp. 1389–1396 ISSN 0032-0889

Lopes, M.S., Araus, J.L., van Heerden, P.D.R. & Foyer, C.H. (2011). Enhancing drought tolerance in C_4 crops. *Journal of Experimental Botany,* 10.1093/jxb/err105, ISSN 0022-0957

Lopes, M.S. & Reynolds, M.P. (2010). Partitioning of assimilates to deeper roots is associated with cooler canopies and increased yield under drought in wheat. *Functional Plant Biology,* Vol. 37, No. 2 (February 2010), pp. 147-156, ISSN 1445-4408

Manavalan, L.P., Guttikonda, S.K., Tran, L.P. & Nguyen H.T. (2009). Physiological and molecular approaches to improve drought resistance in soybean. *Plant and Cell Physiology,* Vol. 50, No. 7, pp. 1260–1276, ISSN 0032-0781

Manschadi, A.M., Christopher, J., deVoil, P. & Hammer, G.L. (2006). The role of root architectural traits in adaptation of wheat to water-limited environments. *Functional Plant Biology,* Vol. 33, No. 9 (September 2006), pp. 823-837, ISSN 1445-4408

Manschadi, A.M., Hammer, G.L., Christopher, J.T. & deVoil, P. (2008). Genotypic variation in seedling root architectural traits and implications for drought adaptation in wheat (*Triticum aestivum* L.). *Plant and Soil,* Vol. 303, No. 1/2, (February 2008), pp. 115-129, ISSN 0032-079X

Mortier, V., Fenta, B.A, Martens, C., Rombauts, S., Holsters, M., Kunert, K., & Goormachtig, S., (2011). Search for nodulation-related CLE genes in the genome of *Glycine max*. *Journal of Experimental Botany,* doi:10.1093/jxb/erq426.

Nakayama, N., Saneoka1, H., Moghaieb, R.E.A. Premachandra, G.S. & Fujita, K. (2007). Response of growth, photosynthetic gas exchange, translocation of 13C-labelled photosynthate and N accumulation in two soybean (*Glycine max* L. Merrill) cultivars to drought stress. *International Journal of Agricultural Biology,* Vol. 9, No. 5, pp. 669–674, ISSN 1813-2235

Naya, L., Ladrera, R., Ramos, J., Gonzalez, E.M., Arrese-Igor, C., Minchin, F.R. & Becana, M. (2007). The response of carbon metabolism and antioxidant defenses of alfalfa nodules to drought stress and to subsequent recovery of plants. *Plant Physiology,* Vol. 144, No.2, (June 2007), pp. 1104–1114, ISSN 0032-0889

Osmont, K.S., Sibout, R. & Hardtke, C.S. (2007). Hidden branches: Developments in root system architecture. *Annual Review of Plant Biology*, Vol. 58, (June 2007), pp. 93-113, ISSN 1543-5008

O'Toole, J.C. & Bland, W.L. (1987). Genotypic variation in crop plant root systems. *Advances in Agronomy,* Vol. 41, pp. 91-145, ISBN 9780120007417

Pastenes, C., Pimentel, P. & Lillo, J. (2005). Leaf movements and photoinhibition in relation to water stress in field-grown beans. *Journal Experimental Botany,* Vol. 56, No. 411 (January 2005), pp. 425-433, ISSN 0022-0957

Quackenbush, J. (2001). Computational analysis of microarray data. *Nature Reviews: Genetics,* Vol. 2, No. 6, (June 2001), pp. 418–427, ISSN 1471-0056

Reynolds, M.P., Dreccer, F. & Trethowan R. (2007). Drought adaptive traits derived from wheat wild relatives and landraces. *Journal of Experimental of Botany,* Vol. 58, No.2, (January 2007), pp. 177-186, ISSN 0022-0957

Ribas-Carbo, M., Taylor, N.L., Giles, L., Busquets, S., Finnegan, P.M., Day, D.A., Lambers, H., Medrano, H., Berry, J.A. & Flexas, J. (2005). Effects of water stress on respiration in soybean leaves. *Plant Physiology,* Vol. 139, No1, (September 2005), pp. 466–473, ISSN 0032-0889

Ribaut, J.M., Jiang, C. & Hoisington, D. (2002). Simulation experiments on efficiencies of gene introgression by backcrossing. *Crop Science,* Vol. 42, No.2, pp. 557–565, ISSN 1435-0653

Rockett, J.C. & Dix, D.J. (1999) Application of DNA arrays to toxicology. *Environmental Health Perspectives*, Vol. 107, No. 8, (August 1999), pp. 681–685, ISSN 0091-6765

Sall, K. & Sinclair, T.R. (1991). Soybean genotypic differences in sensitivity of symbiotic nitrogen fixation to soil dehydration. *Plant and Soil,* Vol. 133, No. 1, (May 1991), pp. 31-37, ISSN 0032-079X

Shinozaki, K. (2007) Acceleration of soybean genomics using large collections of DNA markers for gene discovery. *DNA Research,* Vol. 14, No. 6, p. 235, ISSN 1340-2838

Soares-Cordeiro, A.S., Driscoll, S.P., Pellny, T.K., Olmos, E., Arrabaça, M.C. & Foyer, C.H. (2009) Variations in the dorso-ventral organization of leaf structure and Kranz anatomy coordinate the control of photosynthesis and associated signalling at the whole leaf level in monocotyledonous species. *Plant, Cell and Environment* Vol. 32, pp.1833-1844.

Shoemaker, R.C., Grant, D., Olson, T., Warren, W.C., Wing, R., Yu, Y., Kim, H., Cregan, P., Joseph, B., Futrell-Griggs, M., Nelson, W., Davito, J., Walker, J., Wallis, J., Kremitski, C., Scheer, D., Clifton, S.W., Graves, T., Nguyen, H., Wu, X., Luo, M., Dvorak, J., Nelson, R., Cannon, S., Tomkins, J., Schmutz, J., Stacey, G. & Jackson, S. (2008). Microsatellite discovery from BAC end sequences and genetic mapping to anchor the soybean physical and genetic maps. *Genome,* Vol. 51, No. 4, pp.294–302.

Siddique, K.H.M., Regan, K.L., Tennant, D. & Thomson, B.D. (2001). Water use and water use efficiency of cool season grain legumes in low rainfall Mediterranean-type environments. *European Journal of Agronomy,* Vol. 15, No. 4, pp. 267-280, ISSN 1161-0301

Sinclair, T.R. & Muchow, C.R. (2001). System analysis of plant traits to increase grain yield on limited water supplies. *Agronomy Journal,* Vol. 93, No. 2, pp. 263-270, ISSN 0002-1962

Sinclair, T.R., Purcell, L.C., Vadez, V., Serraj, R., King, C.A. & Nelson, R. (2000). Identification of soybean genotypes with N_2 fi xation tolerance to water deficits. *Crop Science,* Vol. 40, No. 6, pp. 1803–1809, ISSN 1435-0653

Sinclair, T.R., Purcell, L.C., King, C.A. , Sneller, C.H., Chen, P. & Vadez, V. (2007). Drought tolerance and yield increase of soybean resulting from improved symbiotic N fixation. *Field Crops Research,* Vol. 101, No.1, (February 2007), pp. 68–71, ISSN 0378-4290

Sponchiado, B.N., White, J.W., Castillo, J.A. & Jones, P.G. (1989). Root growth of four common bean cultivars in relation to drought tolerance in environments with contrasting soil types. *Experimental Agriculture,* Vol. 25, No. 2, (April 1989), pp. 249-257, ISSN 0014-4797

Udensi, O., Ikpeme, E.V., Markson, A.A., Edu, E.A.B., Umana, E.J. & Urua, I.S. (2010). Selection of soybean genotypes using morphological markers. *International Journal of Current Research,* Vol. 7, (August, 2010) pp. 5-8, ISSN 0975-833X

Umezawa, T., Sakurai, T., Totoki, Y., Toyoda, A., Seki, M. & Ishiwata, A. (2008). Sequencing and analysis of approximately 40,000 soybean cDNA clones from a full-length-enriched cDNA library .*DNA Research,* Vol. 15, No. 6, (December 2008) pp. 333-346, ISSN1340-2838

Valliyodan, B. & Nguyen, H.T. (2008). Genomics of abiotic stress in soybean. In: *Genetics and Genomics of Soybean*, G. Stacey, (Ed.), 343–372, Available from Springer, New York

Van Heerden, P.D.R., Kiddle, G., Pellny, TK., Mokwala, P.W., Jordaan, A., Strauss, A.J., de Beer, M., Schlüter, U., Kunert, K.J. & Foyer, C.H. (2008). Regulation of respiration and the oxygen diffusion barrier in soybean protect symbiotic nitrogen fixation from chilling-induced inhibition and shoots from premature senescence *Plant Physiology*, Vol. 148, No. 1, (September 2008), pp. 316-327, ISSN 0032-0889

Vodkin, L.O., Khanna, A., Shealy, R., Clough, S.J., Gonzalez, D.O. & Philip, R., Zabala, G., Thibaud-Nissen,F., Sidarous, M., Strömvik, M.V., Shoop, E., Schmidt, C., Retzel, E., Erpelding, J., Shoemaker, R.C., Rodriguez-Huete, A.M., Polacco, J.C., Coryell, V., Keim, P., Gong, G., Liu, L., Pardinas, J. & Schweitzer, P. (2004). Microarrays for global expression constructed with a low redundancy set of 27,500 sequenced cDNAs representing an array of developmental stages and physiological conditions of the soybean plant. *BMC Genomics,* Vol. 5, (September 2004), p. 73, ISSN 1471-2164

Warren, C.R. (2008). Stand aside stomata, another actor deserves centre stage: the forgotten role of the internal conductance to CO_2 transfer. *Journal of Experimental Botany,* Vol. 59, No. 7, (May 2008), pp. 1475-1487, ISSN 0022-0957

Xia, Z., Tsubokura, Y., Hoshi, M., Hanawa, M., Yano, C. & Okamura, K. (2007). An integrated high-density linkage map of soybean with RFLP, SSR, STS, and AFLP markers using a single F2 population. *DNA Research,* Vol. 14, No. 6, pp. 257–269, ISSN1340-2838

10

Identification and Confirmation of SSR Marker Tightly Linked to the Ti Locus in Soybean [*Glycine max* (L.) Merr.]

Jongil Chung
Department of Agronomy, Gyeongsang National University, Chinju, Republic of Korea

1. Introduction

Soybean [*Glycine max* (*L.*) Merr.,] is considered a high quality source of oil and protein for food and feed. However, the several antinutritional factors (lipoxygenase, trypsin inhibitor, lectin, and P34 allergen protein) present in raw mature soybean seeds. Soybean Kunitz trypsin inhibitor (KTI) protein has been proposed as one of the major antinutritional factor (Westfall and Hauge, 1948). KTI protein is a small, monomeric and non-glycosylated protein containing 181 amino acid residues. This 21.5 kDa non-glycosylated protein was first isolated and crystallized from soybean seeds by Kunitz (1945). KTI protein can cause the induction of pancreatic enzyme hypersecretion and a fast stimulation of pancreas growth, which is histologically described as pancreatic hypertrophy and hyperplasia (Liencer, 1995). Also, KTI may cause unfavorable physiological effects (Vasconcelos et al., 2001) and decrease weight gain in animals (Palacios et al., 2004). Proper heat processing is required to destroy KTI protein. However, excessive heat treatment may lower amino acid availability. The genetic removal of the KTI protein will improve the nutritional value of soybean. From the USDA germplasm collection, two soybean accessions (PI157440 and PI196168) lacking the KTI protein have been identified (Orf and Hymowitz, 1979). Based on the availability of soybean null lines lacking the KTI protein, it was suggested that KTI protein is not essential for soybean growth or development. Five electrophoretic forms of KTI have been discovered. The genetic control of four forms, *Ti* ^{a}, *Ti* ^{b}, *Ti* ^{c}, and *Ti* ^{d}, has been reported as a codominant multiple allelic series at a single locus (Singh et al., 1969; Hymowitz and Hadley, 1972; Orf and Hymowitz, 1979). Orf and Hymowitz (1979) found that the fifth form does not exhibit a soybean trypsin inhibitor-A2 band and is inherited as a recessive allele designated *ti*. Studies of amino acid and nucleotide sequences of polymorphic variants of KTI have revealed that there is a large sequence differences in nine amino acid residues between *Ti* ^{a} and *Ti* ^{b} (Song et al., 1993; Wang et al., 2004). Each *Ti* ^{c}, *Ti* ^{d} and *Ti* ^{e} differ by only one amino acid from *Ti* ^{a} type and *Ti* ^{f} differs by one amino acid from *Ti* ^{b} type (Wang et al., 2004). The *Ti* locus has been located on linkage group 9 in the classical linkage map of soybean (Hildebrand et al., 1980; Kiang, 1987), which is integrated in molecular linkage map A2 (chromosome number 8) of the USDA/Iowa State University soybean molecular linkage map (Cregan et al., 1999).

DNA markers have become fundamental tools for research involving soybean improvement programs. Microsatellites or simple sequence repeat (SSR) markers are highly polymorphic, abundant, and distributed throughout the genome (Cregan et al., 1999). With the development and public release of SSR primers, SSR markers have become available on molecular soybean linkage group (Cregan et al., 1999). Molecular markers tightly linked to desired genes are a valuable tool to detect genotypes of interest, saving time and resources. Marker assisted selection (MAS) using DNA markers instead of phenotypic assays reduces cost and increases the precision and efficiency of subsequent selection steps applied in breeding. To date, detection of the KTI protein free genotypes has been based on SDS-PAGE gel electrophoresis analysis of crude protein from mature seeds, however, with this method, test samples are restricted to proteins from mature soybean seeds. This is a time-consuming process, which is not possible in the early seedling stages of the corresponding population. SSR markers tightly linked to the *Ti* locus were identified and confirmed in soybean populations for marker assisted selection. If a marker linked to the *Ti* locus can be confirmed, then selection for KTI protein free genotypes might be performed at early seedling stages with relative ease.

2. Identification of SSR marker

2.1 Plant genotypes

Soybean genotype C242 (clark derived near isogenic line) has the *ti* allele and lacks a soybean kunitz trypsin inhibitor. C242 was a generous gift from J. Specht, professor of Agronomy, University of Nebraska-Lincoln, USA. Cultivar Jinpumkong2 and Clark has kunitz trypsin inhibitor protein band (*TiTi*). Two mapping populations were developed. Population 1 was derived from a cross between cultivar Jinpumkong2 and C242. Population 2 was made from a mating between cultivar Clark and C242. The F_1 plants from two populations were grown in the greenhouse to produce F_2 seeds.

2.2 Determination of kunitz trypsin inhibitor genotype

98 F_2 seed from F_1 plants for population 1 and 243 F_2 seed from F_1 plants for population 2 was analysed electrophoretically to determine the presence (SKTI- ` + ') or absence (SKTI- 'null ') of kunitz trypsin inhibitor. A piece of cotyledon from each F_2 seed was removed and the remaining embryo germinated to given a F_2 mapping population. The separated cotyledon tissue was incubated for 30 min (room temperature) in 1 ml Tris-HCl, pH 8.0, containing 1.56 % v/v β-mercaptoethanol. After centrifugation, 50 µl of the supernatant were added to an equivalent amount of 5X sample buffer [10% w/v sodium dodecyl sulphate (SDS), 50% v/v glycerol, 1.96% v/v β-mercaptoethanol, 1 M Tris-HCl, pH 6.8]. The samples were boiling at 97°C for 5 min and then centrifuged. Two microlitre of the supernatant were used for electrophoresis on 12% acrylamide SDS polyacrylamide gel electrophoresis (SDS-PAGE) medium gels in Owl Separation Systems. Inc(Model: P9DS, Portsmouth, NH USA). Electrophoresis was practiced at 120 V for 7 hr. Gels were stained overnight in an aqueous solution of 0.25 g coomassie brilliant blue R250, 10% acetic acid, 45% methanol and destaining solution (5% acetic acid, 14% methanol) for several hours. A Wide-Range SDS-PAGE molecular mass standard (Sigma Marker™, Product Code : M4038) containing the 21.5kDa soybean trypsin inhibitor protein, was used to aid recognition of samples lacking the kunitz trypsin inhibitor.

2.3 DNA extraction and DNA marker analysis

F_2 seeds tested for kunitz trypsin inhibitor protein were planted in the field on May, 2004. Young leaves were collected from the 94 individual F_2 plants germinated among 98 F_2 seeds and parent plants in population 1. In population 2, random 97 F_2 seeds among 243 F_2 seeds were planted in the greenhouse on April, 2005. Young leaves were collected from the 94 individual F_2 plants. Genomic DNA was extracted from finely ground leaf tissue by means of a modified CTAB procedure (Saghai Maroof et al., 1984). For the analysis of random amplified polymorphic DNA (RAPD) markers, One-thousand 10-mer oligonucleotide primers were obtained from Operon Technologies (Alameda, U.S.A). For the analysis of simple sequence repeat (SSR) marker, total 35 SSR primers were selected from the A2 soybean molecular linkage map (Cregan et al., 1999) that contains *Ti* locus. Satt primers selected were synthesized by Bioneer, Inc. (Korea). For the analysis of amplified fragment length polymorphic (AFLP) markers, 342 primer sets were used. Amplification and electrophoresis for RAPD, SSR, and AFLP markers was performed as described by Kim et al., (2003). Based on the results of F_1 seed genotype for kunitz trypsin inhibitor, the present and absent bulk populations from F_2 plant population were made (Michelmore et al., 1991). The present and absent bulk population contained twenty F_2 individuals each, which were selected on the basis of the kunitz trypsin inhibitor protein electrophoresis, respectively. RAPD, SSR, and AFLP markers were used in population 1. Only the markers linked in population 1 including *Ti* locus were used in population 2.

2.4 Genetic linkage analysis

Primers that distinguished the bulks and the parents were tested on the entire F_2 population. Marker (RAPD, AFLP, and SSR) data obtained from 94 F_2 progenies of population 1 and 2 were used to construct genetic linkage map including *Ti* locus using the computer program MAPMAKER v. 3.0 (Lander et al., 1987). Markers were assigned to group using the "Group" command, with a LOD score of 4.0 and maximum recombination distance of 50 cM. Once markers were assigned to a given linkage group, the most linkage marker order within the group was determined using the "Compare" command. Marker orders within each linkage group were ascertained by use of "Ripple" command. Map distance (cM) were computed using the Kosambi (Kosambi, 1944) mapping function.

2.5 Detection of Satt228 marker

The banding patterns of kunitz trypsin inhibit protein (SKTI) that appeared in the parents and F_2 seeds from the cross between cultivar Jinpumkong2 and C242 (population 1) are shown in Figure 1. Jinpumkong2 parent had band in 21.5 KDa position and the band was segregated in F_2 seeds. The observed data for population 1 were 72 seeds with SKTI protein band and 26 seeds with no SKTI protein band (χ^2=0.12, P=0.70-0.80). For population 2, the observed data were 185 seeds with SKTI protein band and 58 seeds with no SKTI protein band (χ^2=0.17, P=0.70-0.80). These observations fit the expected 3 : 1 ratio for the presence or absence of the SKTI protein band. Earlier studies have shown that the null phenotype of SKTI is inherited as a recessive allele designated *ti* (Orf and Hymowitz, 1979). The segregation ratios of 3 : 1 observed in the F_2 seed (population 1 and 2) and the Chi-square values strongly suggest that kunitz trypsin inhibitor protein band is controlled by a single recessive gene.

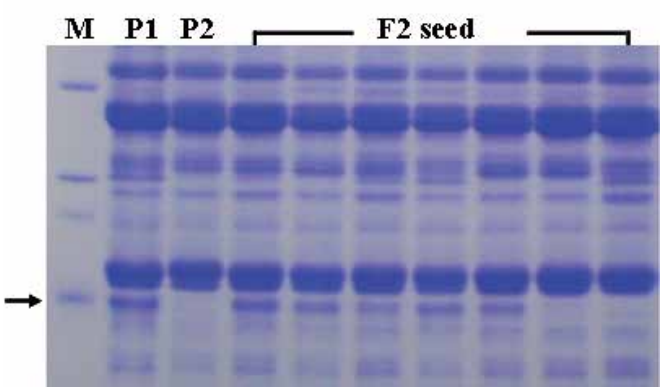


Fig. 1. Polyacrylamide gels of protein extracted from parents and F_2 seeds. P1 (Jinpumkong2) and P2 (C242) are parents and arrow points to the Kunitz trypsin inhibitor band (21.5 KDa).

Of the 1,000 RAPD primers tested on two parents of population 1 (Jinpumkong2 and C242), approximately 12 % (124) primers produced polymorphic DNA fragment differences between the parents. Only 35 primers were identified as being polymorphic between bulked DNA samples with SKTI protein and bulked DNA samples with no SKTI protein. Of those 35 primers, only 16 also exhibited polymorphism between parents. Among 342 primer sets of AFLP analysis, only 10 primers were shown polymorphism between parents. Three SSR primers (Satt409, Satt228 and Satt429) among 35 primers selected were shown polymorphism between parents. Total 48 markes (35 RAPD, 10 AFLP, and 3 SSR) were used to obtain segregation data from 94 F_2 individuals of population 1. Figure 2 represents some example of segregating DNA fragment for SSR markers (Satt228) in parents, bulked samples and F_2 population.

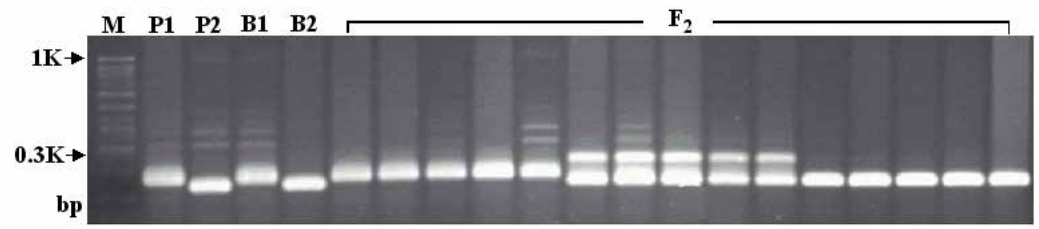


Fig. 2. Patterns of segregating DNA fragment for SSR primer Satt228 in parents, bulked samples, and F_2 population. P1 is Jinpumkong2 (*TiTi*) and P2 is C242 (*titi*). B1 is bulked of present kunitz trypsin inhibitor protein individuals and B2 is bulked of absent individuals.

A genetic map was constructed from the 48 segregating DNA markers and *Ti* locus. A total 11 DNA markers (4 RAPD, 4 AFLP, and 3 SSR) and *Ti* locus was found to be genetically linked in population 1. Three SSR markers, Satt409, Satt228, and Satt429 linked with *Ti* locus within 10 cM (Figure 3). Satt228 marker was very tightly linked with *Ti* locus at 0 cM. Three SSR markers linked with *Ti* locus in population 1 were applied in population 2. Only two SSR markers, Satt228 and Satt409 were linked with *Ti* locus. Satt228 marker was linked with *Ti* locus in 3.7 cM distance (Figure 3). The order and map distances of SSR markers and *Ti* locus differed between populations 1 and 2.

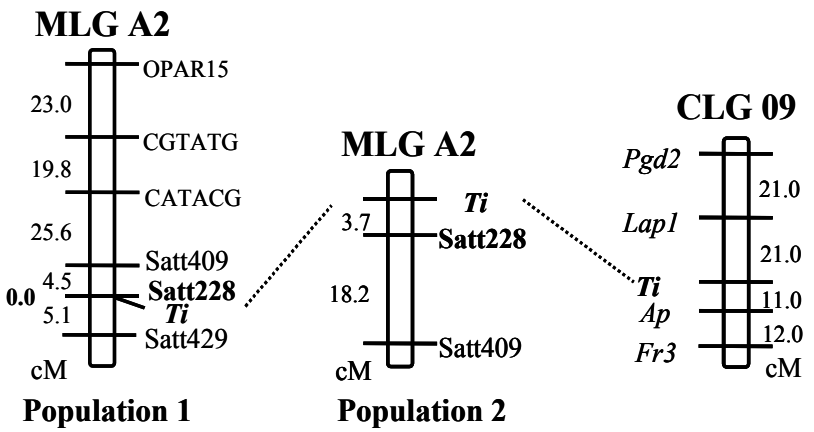


Fig. 3. Molecular linkage map A2 (Cregan et al., 1999) of *Ti* locus defined using population 1 and population 2. Population 1 was derived from the cross of Jinpumkong2 (*TiTi*) and C242 (*titi*). Population 2 was derived from the cross of Clark (*TiTi*) and C242 (*titi*). Map was constructed using MAPMAKER/EXP (LOD 4.0 maximum distance 50 cM). Marker loci names are on the right and kosambi map distances are on the left. CLG09 is the classical linkage group 9 (Hildebrand et al., 1980; Kiang 1987).

3. Confirmation of Satt228 marker

3.1 Screening of *titi* genotype using Satt228 marker

Only two genotypes (PI 157440 and PI 196168) and two near isogenic lines (C242, W60) have been known as soybean genotypes with lacking Kunitz trypsin inhibitor protein (*titi* genotype). C242 is a near isogenic line derived from cultivar 'Clark' and W60 is a near isogenic line derived from cultivar 'William'.

Satt228 marker very tightly linked to *Ti* locus at distance of 0 cM was used to screen germplasms with *titi* genotype (Kunitz trypsin inhibitor protein absent) for marker confimation and testing the possibility of marker-assisted selection (MAS). Amplification patterns obtained from Satt228 marker using genomic DNA of four soybean strains (PI157440, PI196168, W60, and C242) with *titi* genotype (Kunitz trypsin inhibitor protein absent) and three cultivars ('Jinpumkong2', 'Clark', and 'William') with *TiTi* genotype (Kunitz trypsin inhibitor protein present) are shown in Figure 4-1A. Also, polyacrylamide gel banding patterns of protein extracted from random 10 seeds of these seven germplasms used are shown in Figure 4-1B. *TiTi* genotypes ('Jinpumkong2', 'Clark', and 'William') had allele1, however, *titi* genotypes (PI196168, C242, W60 and PI157440) had allele2 in the result of PCR by Satt228 marker (Figure 4-1A). *TiTi* genotypes ('Jinpumkong2', 'Clark', and 'William') had 21.5 kDa band that indicates Kunitz trypsin inhibitor protein, however *titi* genotypes (PI196168, C242, W60 and PI157440) did not have the band in of protein gel electrophoresis from the mature seed (Figure 4-1B). From the comparison of gel electrophoresis for Kunitz trypsin inhibitor protein (Figure 4-1B) and banding pattern amplified by Satt228 marker from the genomic DNA (Figure 4-1A), there was a strong agreement between protein band (21.5 kDa) for Kunitz trypsin inhibitor protein and banding pattern by Satt228 marker. All *TiTi* genotypes ('Jinpumkong2', 'Clark', and 'William') which shown 21.5 kDa protein band in protein electrophoresis of mature seed had

the allele1 amplified by Satt228 marker from the genomic DNA. However, all *titi* genotypes (PI196168, C242, W60 and PI157440) which shown no 21.5 kDa protein band in electrophoresis of mature seed had allele2 amplified by Satt228 marker from the genomic DNA.

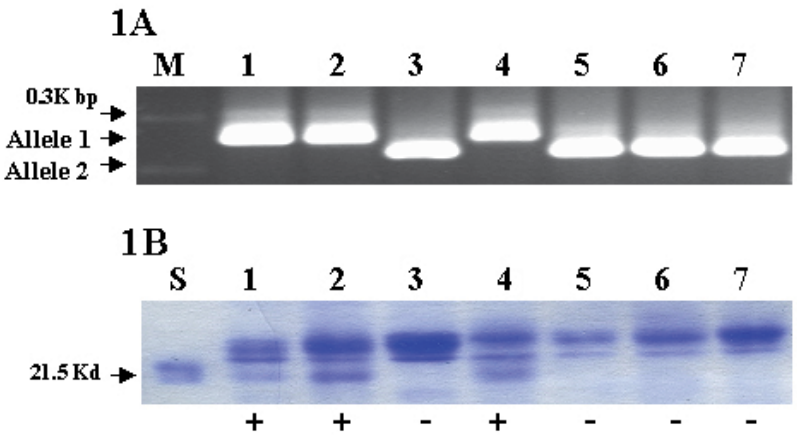


Fig. 4. Pattern of genomic DNA amplification by Satt228 marker using leaf tissue of germplasms (1A) and pattern of polyacrylamide protein gel electrpophoresis extracted from 10 random seeds harvestes (1B). M; molecular marker, S; Kunitz trypsin inhibitor protein (Sigma, product number: T6522). 1:Jinpumkong2(*TiTi*), 2:Clark (*TiTi*), 3:PI196168 (*titi*), 4: William (*TiTi*), 5: C242 (*titi*), 6: W60 (*titi*), 7: PI157440 (*titi*). +: present of KTI protein and -: absent of KTI protein.

Moraes et al. (2006) reported specific DNA marker designed to detect the absence of SKTI protein. For markers to be most useful in breeding programs, they should reveal polymorphism in different genetic backgrounds, which is referred to as marker validation (Sharp et al., 2001). Specific DNA marker designed to detect the absence of SKTI protein reported by Moraes et al. (2006) was not valid between germplasms of *TiTi* (SKTI protein present) and *titi* (SKTI protein absent) genotype used in this study. No polymorphism was observed among germplasms used. However, Cosegregation between allele of Satt228 marker and presence or absence of SKTI protein in several soybean germplasms of *TiTi* and *titi* genotyes was observed (Figure 4-1A and 1B). This results indicate that selection of germplasms or lines with lacking Kunitz trypsin inhibitor protein is possible by Satt228 marker analysis.

3.2 Confirmation of Satt228 marker in four different soybean populations

Cosegregation between Satt228 marker and Ti locus was confirmed in four different populations. Two cultivars (Jinpumkong2, Hannamkong) and two landraces (GS06, 20M183) have Kunitz trypsin inhibitor protein (*TiTi* genotype) in their mature seeds. The C242 parent is a clark-derived near isogenic line and does not have Kunitz trypsin inhibitor protein (*titi* genotype) in the mature seeds. Four different populations were developed. Four female parents (Jinpumkong2, Hannamkong, GS06, 20M183) and one male parent C242 were crossed in the greenhouse in June 2002. F_1 seeds from the cross of Jinpumkong2 x C242, Hannamkong x C242, GS06 x C242, and 20M183 x C242 were obtained and planted in

the greenhouse. F_2 seeds per each cross were harvested from several F_1 plants in November 2002. All F_2 seeds per each cross were planted in the field in May 2003. F_2 plants per each cross were harvested individually. Random F_3 seeds from individual F_2 plant per each cross were tested by SDS-PAGE protein analysis to detect Kunitz trypsin inhibitor protein. Individual F_2 plants (F_3 seeds) with free Kunitz trypsin inhibitor protein (*titi* genotype) per each cross were planted in the greenhouse and harvested individually in June 2004. Random F_4 seeds from individual F_3 plant harvested per each cross were planted in the field in June 2004. At maturity, F_4 plants (F_5 seeds) were harvested individually per each cross in November 2004. Random F_5 seeds from individual F_4 plant harvested per each cross were planted in the field in May 2005. Five parents and individual F_5 plants per each cross were used to confirm the SSR marker tightly linked to *Ti* locus. Agronomical traits except for the Kunitz trypsin inhibitor protein were not considered in each generation. The pedigree for the development of the four populations lacking the Kunitz trypsin inhibitor protein is summarized in Figure 5.

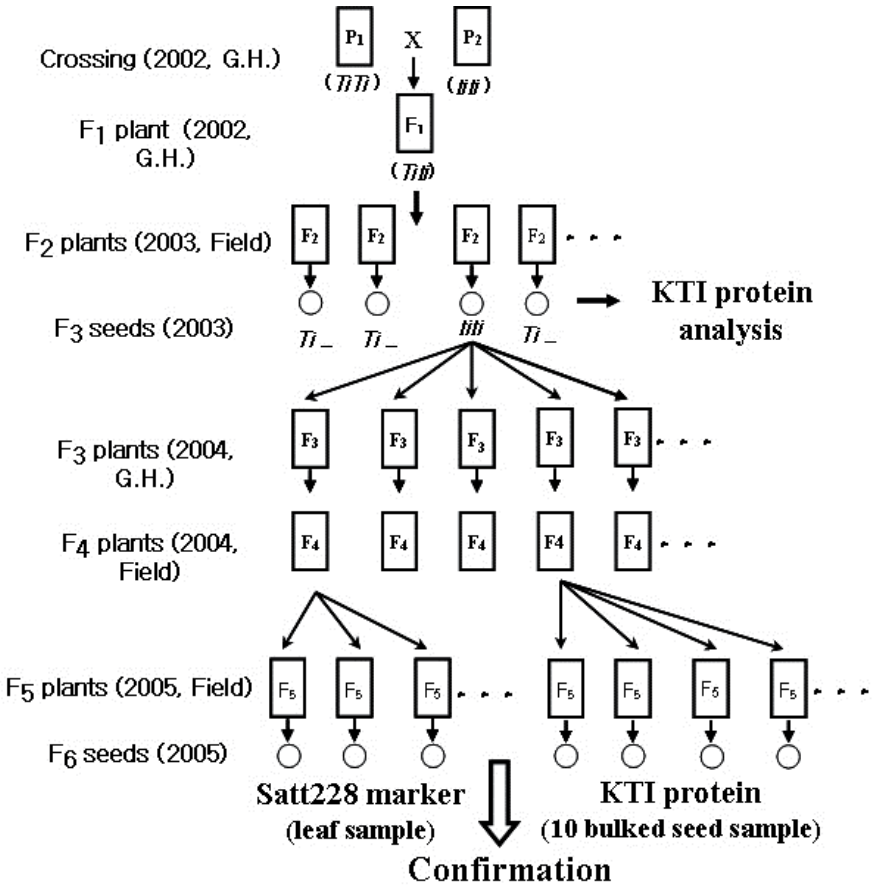


Fig. 5. The pedigree of the four population development to confirm cosegregation between marker Satt228 and the *Ti* locus. F_2 plants lacking the KTI protein from each population were selected and advanced to the next generation. All F_5 plants have the *titi* genotype (lacking Kunitz trypsin inhibitor protein). G.H is greenhouse.

Segregation patterns of genomic DNA amplification by the Satt228 marker using young leaf tissue of four parents and several individual F_5 plants (A, C, E, G) and patterns of polyacrylamide protein gel using protein extracted from 10 random seeds of four parents and individual F_6 harvested (B, D, F, H) are shown in Figure 6. The bands amplified by the Satt228 marker are clearly detecting the AA and BB genotypes. The seed protein band by the SDS-PAGE is a little different in color density according to each population, staining time of Coomassie blue and protein content. However, the detection of the presence or absence of the Kunitz trypsin inhibitor protein was very clear because the Kunitz trypsin inhibitor protein is controlled by single gene and is not influenced by environment.

Satt228 marker analysis was conducted on the genomic DNA of the parents and the 273 individual F_5 plants lacking the Kunitz trypsin inhibitor protein derived from the cross of 'Jinpumkong2' (*TiTi*) and C242 (*titi*). After harvesting at maturity, SDS-PAGE electrophoresis using crude protein extracted from ten random F_6 seeds of each F_5 plants and parents was performed to detect Kunitz trypsin inhibitor protein of size 21.5 kDa. DNA banding pattern of the Satt228 marker and polyacrylamide gel banding patterns of the protein is shown in Figure 6 (A and B). The P_1 parent (Jinpumkong2) had the AA genotype (allele 1) and the P_2 parent (C242) had BB genotype (allele 2) for Satt228 marker. All 273 individual F_5 plants were shown only to have the BB genotype (A of Figure 6). This indicated all 273 F_5 progenies had the *titi* genotype and contained no Kunitz trypsin inhibitor protein. Also, the P_1 parent had Kunitz trypsin inhibitor protein of 21.5 kDa size and the P_2 parent did not have the KTI protein (B of Figure 6). All 273 individual F_5 plants did not have the Kunitz trypsin inhibitor protein of 21.5 kDa size (B of Figure 6). Amplification patterns obtained from the Satt228 marker using genomic DNA of 17 individual F_5 plants derived from the cross of Hannamkong (*TiTi*) and C242 (*titi*) and polyacrylamide gel banding patterns of protein extracted from the mixture of 10 random F_6 seeds harvested from each F_5 plant are shown in Figure 6 (C and D). The P_1 parent (Hannamkong) had a AA genotype while the P_2 parent (C242) had the BB genotype (C of Figure 6). All 17 individual F_5 plants derived from cross of Hannamkong and C242 showed only the BB genotype pattern for Satt228 marker analysis (C of Figure 6). This indicated all 17 F_5 progenies had the *titi* genotype and no Kunitz trypsin inhibitor protein. For the protein analysis, the P_1 parent had the 21.5 kDa Kunitz trypsin inhibitor protein while the P_2 parent did not have the KTI protein in polyacrylamide protein (D of Figure 6). All 17 individual F_5 plants did not have the 21.5 kDa Kunitz trypsin inhibitor protein within their F_6 seed samples (D of Figure 6). Amplification patterns by the Satt228 marker using genomic DNA of 45 individual F_5 plants derived from the cross of GS06 (*TiTi*) and C242 (*titi*) and polyacrylamide gel banding patterns of protein extracted from the mixture of 10 random F_6 seeds harvested from each F_5 plant are shown in Figure 6 (E and F). The P_1 parent (GS06) had the AA genotype and the P_2 parent (C242) had the BB genotype (E of Figure 6). All 45 individual F_5 plants derived from the cross of GS06 and C242 displayed only the BB genotype pattern for marker Satt228 (E of Figure 6). Also, the P_1 parent had the 21.5 kDa Kunitz trypsin inhibitor protein and the P_2 parent did not have the KTI protein in polyacrylamide protein gel from mature seeds (F of Figure 6). All 45 individual F_6 seeds harvested from same individual F_5 plants did not have Kunitz trypsin inhibitor protein of 21.5 kDa size (F of Figure 6). This indicated all 45 F_5 progenies had the *titi* genotype and contained no Kunitz trypsin inhibitor protein. Using marker Satt228, amplification patterns from 56 individual F_5 plants derived from the cross of 20M183 (*TiTi*) and C242 (*titi*) and

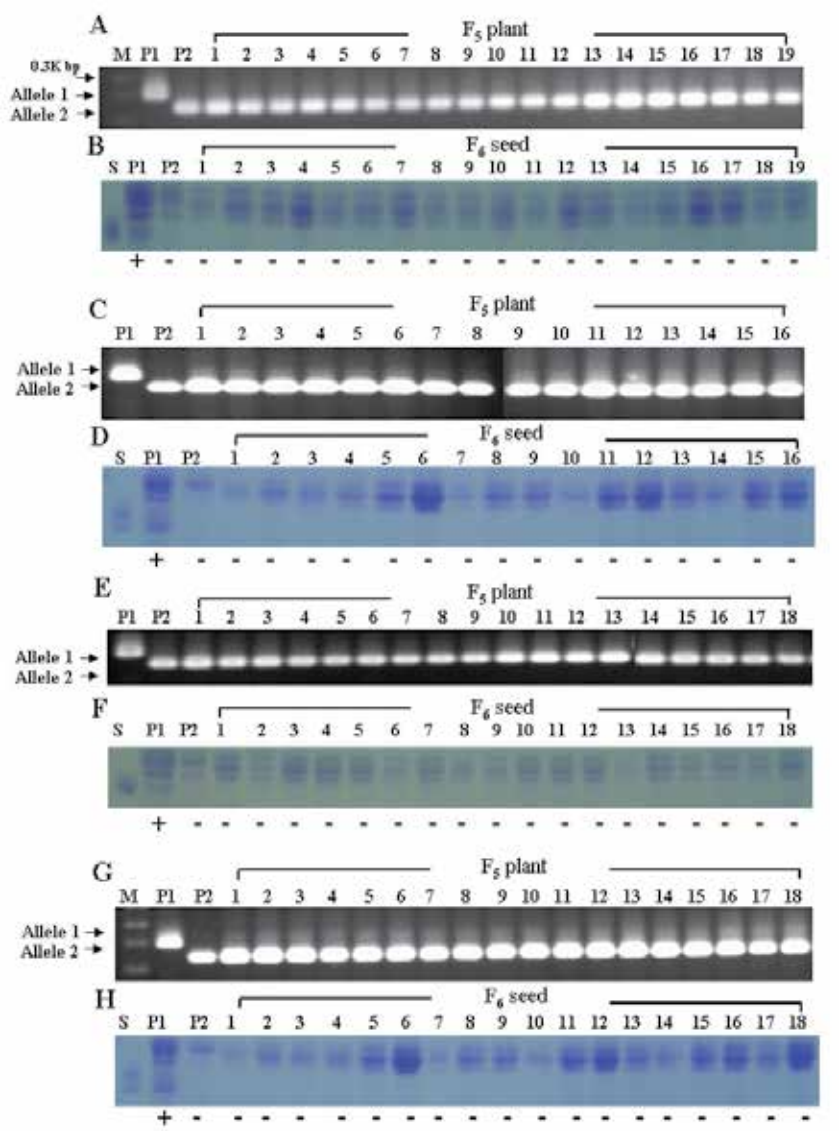


Fig. 6. Pattern of genomic DNA amplification by Satt228 marker using leaf tissue of parent and individual F_5 plants (A, C, E, G) and pattern of polyacrylamide protein gel using protein extracted from parents and 10 random seeds of individual F_6 seed harvested (B, D, F, H). A and B, P1: 'Jinpumkong2' and P2: C242; C and D, P1: 'Hannamkong' and P2: C242; E and F, P1:GS06 and P2: C242; G and H, P1:20M183 and P2: C242. M; molecular marker, S; Kunitz trypsin inhibitor protein (Sigma, product number: T6522), +; present of KTI protein, -; absent of KTI protein.

polyacrylamide gel banding patterns of protein extracted from the mixture of 10 random F_6 seeds harvested from each F_5 plant are shown in Figure 6 (G and H). The P_1 parent (20M183) had the AA genotype while the P_2 parent (C242) had the BB genotype (G of Figure 6). All 56 individual F_5 plants were shown only to have the BB genotype pattern for the Satt228

marker (G of Figure 6). This indicated all 56 F_5 progenies had the *titi* genotype and contained no Kunitz trypsin inhibitor protein. For the protein analysis the P_1 parent had the Kunitz trypsin inhibitor protein of 21.5 kDa, while the P_2 parent did not have the KTI protein (H of Figure 2). All 56 individual F_5 plants did not have the 21.5 kDa Kunitz trypsin inhibitor protein based upon their 10 random F_6 seed samples (H of Figure 6).
Segregation patterns of genomic DNA amplification by the Satt228 marker using young leaf tissue of four parents and several individual F_5 plants (A, C, E, G) and patterns of polyacrylamide protein gel using protein extracted from 10 random seeds of four parents and individual F_6 harvested (B, D, F, H) are shown in Figure 6. Satt228 marker analysis showed the four female parents had the allele 1 (AA genotype) while the C242 male parent has the allele 2 (BB genotype). In seed, four parents had KTI protein and C242 had not KTI protein of 21.5 kDa. A total of 391 F_5 plants derived from the four crosses (273 plants from Jinpunkong2 x C242, 17 plants from Hannamkong x C242, 45 plants from GS06 x C242, and 56 plants from 20M183 x C242) all have the allele 2 (BB genotype) for Satt228 marker. The 391 individual F_6 seeds harvested from same individual F_5 plants are also absent of the KTI protein. Complete cosegregation between the Satt228 marker allele and the *Ti* locus was observed in these four different populations (Kim et al., 2008).

4. Conclusion

Soybean Kunitz trypsin inhibitor (KTI) protein is a small, monomeric and non -glycosylated protein containing 181 amino acid residues and is responsible for the inferior nutritional quality of unheated or incompletely heated soybean meal. Ti gene controls the presence or absence of KTI protein. SSR marker tightly linked to the Ti locus was identified and was confirmed in two ways. Two mapping populations were developed. Population 1 was derived from a cross between cultivar Jinpumkong2 (*TiTi*) and C242 (*titi*). Population 2 was made from a mating between cultivar Clark (*TiTi*) and C242. Each F_2 seed from F_1 plants was analysed electrophoretically to determine the presence of the KTI protein band. Twelve DNA markers (4 RAPD, 4 AFLP, and 3 SSR) and *Ti* locus were found to be genetically linked in population 1 consisted with 94 F_2 individual plants. Three SSR markers (Satt409, Satt228, and Satt429) were linked with *Ti* locus within 10 cM. Satt228 marker was tightly linked with *Ti* locus. Satt228 marker was tightly linked within 0 - 3.7 cM of the *Ti* locus. Using several germplasms with *TiTi* or *titi* genotypes, Satt228 marker was confirmed. *TiTi* genotypes ('Jinpumkong2', 'Clark', and 'William') had allele1 and *titi* genotypes (PI196168, C242, W60 and PI157440) had allele2 in Satt228 marker analysis. 'Jinpumkong2', 'Clark', and 'William' (*TiTi* genotype) had Kunitz trypsin inhibitor protein of 21.5 kDa size and PI196168, C242, W60, and PI157440 (*titi* genotype) did not have the band in protein gel electrophoresis from the mature seed. Cosegregation between KTI protein (21.5 kDa size) and allele of Satt228 marker was observed in seven germplasms with different genetic background. This result indicates that Satt228 marker may effectively utilized to select the plant with *titi* genotype. Also, Satt228 marker tightly linked to the *Ti* locus was confirmed in four different F_5 populations. Four female parents (*Glycine max* L. cv. Jinpumkong2, Hannamkong, GS06, 20M183) of *TiTi* (KTI protein present) genotype and one male parent C242 of *titi* (KTI protein absent) genotype were used. Four different populations of F_2 plants free of KTI protein were advanced to the F_5 generation. Satt228 marker analysis showed the four female parents had the allele 1 (AA genotype) while the C242 male parent has the allele 2 (BB genotype). In seed, four parents had KTI protein and C242 had not KTI protein of 21.5 kDa. A total of 391 F_5 plants derived from the four crosses (273 plants from Jinpunkong2 x C242,

17 plants from Hannamkong x C242, 45 plants from GS06 x C242, and 56 plants from 20M183 x C242) all have the allele 2 (BB genotype) for Satt228 marker. The 391 individual F_6 seeds harvested from same individual F_5 plants are also absent of the KTI protein. Complete cosegregation between the Satt228 marker allele and the *Ti* locus was observed in these four different populations. The objective of this research was to identify and to confirm a SSR marker tightly linked to the *Ti* locus for MAS breeding in different genetic populations and germplasms. So far, KTI free new soybean cultivars (Gaechuck#1, Gaechuck#2 and Jinnong#1) have been developed using Satt228 marker.

5. Acknowledgment

Soybean genotype C242 and W60 was a generous gift from James E. Specht, professor of Agronomy, University of Nebraska-Lincoln, U.S.A.

6. References

Cregan, P.B., T. Jarvik, A.L. Bush, R.C. Shoemaker, K.G. Lark, A.L. Kahler, N. Kaya, T.T. VanToai, D.G. Lohnes, Jongil Chung & J.E. Specht, 1999. An integrated genetic linkage map of the soybean genome. Crop Sci. 39:1464-1490.

Hidebrand, D.F., J.H. Orf & T. Hymowitz. 1980. Inheritance of an acid phosphatase and its linkage with the Kunitz trypsin inhibitor in seed protein of soybeans. Crop Sci. 20: 83-85.

Hymowitz, T. & H.H. Hadley, 1972. Inheritance of a trypsin inhibitor variant in seed protein of soybeans. Crop Sci. 12:197-198.

Kim, M.S., Y.J. Cho, D.J. Park, S.J. Han, J.H. Oh, J.G. Hwang, M.S. Ko & J.I. Chung, 2003. Construction of genetic linkage map for korean soybean genotypes using molecular markers. KoreanJ. Crop Sci. 48(4):297-302.

Kim, MS, Hur MR, Jeong WH, Park MS, Lee KJ, Shim SI, Kim MC, Jung WS, Lee JH, and Chung JI. 2008. Confirmation of Satt228 marker tightly linked to the Ti locus in four different soybean populations. Genes &Genomics 30(4):329-336.

Kiang, Y.T., 1987. Mapping three protein loci on a soybean chromosome. Crop Sci. 27: 44-46.

Kosambi, D.D., 1944. The estimation of map distance from recombinationAnn. Eugen. 12: 172-175.

Kunitz, M., 1945. Crystallization of a soybean trypsin inhibitor from soybean. Science 101:668-669.

Lander, E., P. Green, J. Abrahamson, A. Barlow, M.J. Daly, S.E. Lincoln & L. Newburg, 1987. MAPMAKER: An interactive computer package forprimary genetic linkage maps of experiment and natural populations. Genomics 1:174-181.

Liencer, I.E. (1995) Possible adverse effects of soybean anticarcinogens. Journal of Nutrition 125: 744-750.

Michelmore, R.W., J. Paran & R.V. Kesseli, 1991. Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregation populations. Proc. Natl. Acad. Sci. 88:9828-9832.

Moraes, R., Soares, T., Colombo, L., Salla, M., Barros, J., Piovesan, N., Barros, E. and Moreira, M. (2006)Assisted selection by specific DNA markers for genetic eliminationof the kunitz trypsin inhibitor and lectin in soybean seeds. Euphytica149:221-226.

Orf, J.H., & T. Hymowitz, 1979. Inheritance of the absence of the Kunitz trypsin inhibitor in seed protein of soybeans. Crop Sci. 19:107-109.

Palacios, M., Easter, R.A., Soltwedel, K.T., Parsons, C.M., Douglas, M.W. and Hymowitz, T. (2004) Effect of soybean variety and processing on growth performance of young chicks and pigs. Journal of Animal Science 82(4):1108-1114.

Saghai Maroof, M.A., K.M. Soliman, R.A. Jorgensen & R.W. Allard, 1984.Ribosomal DNA spacer-length polymorphisms in barley: Mendelianchromosomal location and population dynamics. Proc. Natl. Acad. Sci. 81:8014-8018.

Sharp, P.J., Johnston, S., Brown, G., McIntosh, R.A., Pallotta, M., Carter, M., Bariana, H.S., Khartkar, S., Lagudah, E.S., Singh, R.P., Khairallah, M., Potter, R., Jones, M.G.K. 2001. Validation of molecular markers for wheat breeding. Aust J Agric Res 52:1357-1366.

Singh, L., C.M. Wilson & H.H. Hadley, 1969. Genetic differences in soybean trypsin inhibitors separated by disc electrophoresis. Crop Sci. 9:489-491.

Song, S.I., Kim, C.H., Baek, S.J., Choi, Y.D. 1993. Nucleotide sequences of cDNAs encoding the precursors for soybean (*Glycine max*) trypsin inhibitors (Kunitz type). Plant Physiol. 101:1401–1402.

Vasconcelos, I.M., Maia, A.A.B., Siebra, E.A., Oliveira, J.T.A., Carvalho, A.F.F.U. and Melo, V.M.M. (2001) Nutritional study of two Brazilian soybean cultivars differing in the contents of antinutritional and toxic proteins. Journal of Nutritional Biochemistry 12(1):55-62.

Westfall, R.L., & S.M. Hauge, 1948. The nutritive quality and the trypsin inhibitor content of soybean flour heated at various temperatures. J. Nutr. 35:379-389.

Wang, K.J., Yamashita, T., Watanabe, M., Takahata, Y. 2004. A *Ti*^{b}-derived variant of Kunitz trypsin inhibitor in wild soybean (*Glycine soja*). Genome 47:9–14.

Part 2

Modern Techniques and Technologies

11

Spectral Remote Sensing of the Responses of Soybean Plants to Environmental Stresses

Dora Krezhova
Space and Solar-Terrestrial Research Institute, Bulgarian Academy of Sciences
Bulgaria

1. Introduction

Precision agriculture, site-specific application of inputs tailored to the needs of the crop, is one of the new ways that modern agriculture could potentially maintain or enhance crop yields and minimize environmental pollution. Knowledge about variations in vegetation species and community distribution patterns, alterations in vegetation phenological cycles, and modifications in the plant physiology and morphology provide valuable insight into the climatic, edaphic, geologic, and geophysical characteristics of Earth's areas (Janetos & Justice, 2000). During the past decade remote sensing techniques have been widely used to monitor crops throughout their growing period to help in making decisions for good agricultural practices. Spectral remote sensing methods provide the possibility for early, efficient, objective, and non-destructive evaluation of plant responses to different stress factors of the environment (Campbell et al., 2007; Govender et al., 2009; Li et al., 2010). Field remote sensing applications addressed agriculture and forestry survey, fire detection and fire-fuel mapping, mineral mapping, and atmospheric modelling. Airborne, space-borne and hand-held technologies are commonly used to investigate the spectral responses of plants. Hyperspectral remote sensing makes possible to enhance significantly the spectral measurement capabilities over conventional remote sensing sensor systems, as well as to improve the spectral information content. This entails detailed assessment of the changes in the physiological stage of plants in response to the changes in the environment (Zarco-Tejada et al., 2002; Steele et al., 2008a), detecting of early-stage vegetation stress (Krezhova et al., 2005; Ouyang et al., 2007), discriminating land cover types (Flamenco-Sandoval et al., 2007), leaf pigment concentrations (Coops et al., 2003), modelling quantitative biophysical and yield characteristics of agricultural crops (Delalieux et al., 2009a; Chatzistathisa et al., 2011).

Ground-truth is essential for detecting plant stress, and two commonly used ground-based optical methods, leaf spectral reflectance and chlorophyll fluorescence, are reviewed for their usefulness and practical application. When these methods were combined with remarkable advances in Global Positioning System (GPS) receivers, geographic information systems (GIS), and enhanced crop simulation models, remote sensing technology has the potential to transform the ways that growers manage their lands and implement precision farming techniques (Upchurch, 2003; Hatfield, et al., 2008; Shuanggen & Komjathy, 2010). To obtain accurate and complementary comparative assessments for plant responses to the environmental changes, methods have been applied from different research fields - remote

sensing, plant physiology, biochemistry, virology, etc. Early detection of stress could identify plant physiological condition at larger spatial and temporal scales before visible effects are apparent (Krezhova et al., 2009a; Chatzistathisa et al., 2011).

Soybean *(Glycine max L.)* is one of the most important and valuable agricultural species of legume, as its high protein content is of primary importance for human food and animal feed. Soybean is the leading oilseed crop produced and consumed worldwide. Fat-free soybean meal is a primary, low-cost, source of protein for animal feeds and most pre-packaged meals, as well as a good source of protein for the human diet. The soy vegetable oil is another valuable product of processing the soybean crop and a biofuel feedstock (FAOSTAT, 2011).

Soybean yields have steadily increased in the past 30 years owing to a combination of genetic and management improvement. Rapid soybean demand increases in the last decade challenge the reliability of supply, stock levels, and reasonable pricing. In order to meet the demand, there are two alternatives: increase planted hectares or increase yield. Increasing soybean hectares by substituting for other crops (e.g. sunflower in Argentina or cotton in the United States), utilizing pasture (e.g. Santa Fe, Argentina or Mato Grosso, Brazil) or replacing native vegetation (e.g. cerrado in Brazil) has been the most expedient manner to increase soybean output (Masuda & Goldsmith, 2009). Going forward available farmland for soybean production will be limited by decreasing quantities of land not already in production, increased farmland loss for urbanization, heightened sensitivities about agricultural uses of land, and weak property rights in regions such as Africa that constrains the employment of modern agricultural methods (Goldsmith, 2008). Although soybean use for biodiesel production may require expansion of land area devoted to soybean in some parts of the world, such an expansion is not likely in Europe and North America. Hence, yield increases will become the major source for sustaining further increases in soybean production, particularly in these two significant regions of the world. The design of soil and crop management strategies that fully exploit the climatic and genetic yield potential of soybean remains a key challenge to achieve this goal (USDA, 2009).

Gene transformation and genetic engineering are likely to be of assistance in increasing crop yields worldwide, particularly in less-developed areas affected by low crop productivity and malnutrition. Crop transformations restricting the influence of biological pests could contribute to increased crop productivity (Miflin, 2000). Once pests are controlled, either using genetically improved plants or various management options, the further step could be to increase the inherent yielding capability of plants. Yield potential may be increased by improving of the overall physiological capacity of plants and by preventing the negative consequences of abiotic stresses. Increasing leaf photosynthetic rates appear to be a straightforward way of increasing crop yields. Considerable physiological research has been carried out to select and breed for genotypes with superior photosynthetic rates, and was successful in identifying such cultivars in maize, wheat and soybean (Masclaux et al., 2001; Habash et al., 2001; Sinclair et al., 2000). In soybean, the trait is inherited quantitatively (Sall & Sinclair, 1991).

To achieve high yield potential, soybean must sustain high photosynthesis rates and accumulate large amounts of nitrogen (N) in seeds. It exists in leaves primarily as ribulose biphosphate carboxylase/oxygenase and there is generally a strong relationship between N per unit leaf area and photosynthesis (Sinclair, 2004). Biological nitrogen fixation (BNF) and mineral soil or nitrogenous fertilizers are the main sources of meeting the N requirement of high-yielding soybeans. However, antagonism between nitrate concentration in the soil

solution and the nitrogen fixation process in the nodules is the main constraint the crop faces in terms of increasing N uptake when no other abiotic stress that reduces BNF activity occurs. A number of reviews have been published on BNF in legumes (Unkovich & Pate, 2000; Hardarson & Atkins, 2003) and soybean in particular (Hungria et al., 2005, Hungria et al., 2006). However, these summaries were mostly qualitative and did not emphasize the role of BNF and inherent soil fertility in high-yielding soybean systems. Likewise, many studies evaluating the response of soybean to N fertilization show conflicting results that make it difficult to draw a general conclusion about soybean response to N fertilizer (Ray et al., 2005; Osborne & Riedell, 2011).

In sustained agronomic systems, both the BNF and an adequate management of the organic matter, play important roles. However, the BNF importance as a source of N for agriculture has diminished in recent decades as increasing amounts of fertilizer N have been used for the production of food and cash crop. Currently, it's of great practical importance because the use of nitrogenous fertilizers has resulted in unacceptable levels of water pollution (increasing concentrations of toxic nitrates) and eutrophication of lakes and rivers (Barker & Sawyer, 2005; Salvagiotti et al., 2008). Thus, legumes are also essential to improve the soil fertility and quality of agricultural lands and to reclaim eroded or barren areas, making them crucial for agricultural and environmental sustainability (Saikia & Jain, 2007). However, legume BNF in crop species is very sensitive to environmental constraints such as salinity, drought, and light in particular (Ibanez et al., 2008; Salehi et al., 2008; USDA, 2009).

Many fundamental studies are dedicated on how plants detect and respond to stresses in their environment. The stress factors cause changes in the normal physiological processes of all cultural and wild plants. They influence the metabolism, photosynthesis and enzyme activity, and lead to a dramatic reduction of yields and to deterioration of the output quality. The physiological condition of plants is indicative of plant productivity and adaptability to stress and it is a general indication of the environment in which they grow (Alia et al., 2006; Gray et al., 2010). Research on biotic stresses includes the molecular mechanisms used by viruses, bacteria, fungi, and nematodes to incite disease and those used by plants to resist infection (Li et al., 2008; Yang et al., 2009; 2008; Delalieux et al., 2009b). Research on abiotic stresses includes molecular mechanisms by which plants resist such unfavourable conditions as drought, flooding, chilling, light, excess salts, toxic metals, and pollutants (Flawers, 2004; Jones, 2007; El-Nahry & Hammad, 2009).

Soil salinity is one of the widespread environmental factors and the major factor limiting plant production in many areas of the world. This is especially true in arid and semi-arid regions of the world like some regions of Bulgaria. Salinity influences almost every aspect of the physiology and biochemistry of plants (Arida & Das, 2005). High exogenous salt concentrations affect seed germination, water deficit, cause ion imbalance of the cellular ions resulting in ion toxicity and osmotic stress (Yousfi et al., 2007; Singha et al., 2010). As with most cultivated crops, the salinity response of legumes varies greatly and depends on such factors as climatic conditions, soil properties and the stage of growth. One of the important impacts of salinity on plants is that it essentially creates a physiological drought in plants (Munns, 2002). The ability to monitor or evaluate the efficiency of cropping production systems in saline areas can be significantly improved by applying remote sensing techniques (Thenkabail et al., 2004; Campbell et al., 2007).

Light is one of the most important environmental factors regulating plant development and the expression of plant genes. A plant's ability to maximize its photosynthetic productivity depends on its capacity to sense, evaluate, and respond to light quality, quantity, and

direction. Stratospheric ozone depletion has led to elevated levels of ultraviolet-B (UV-B) radiation (280-320 nm) on the surface of the Earth. Increased UV-B levels have negative effects on human health (Norval et al., 2006) as well as on the plant development, morphology, and physiology (Jia Gio & Wang, 2008). Low influence UV-B radiation stimulates distinct responses, such as the accumulation of UV-absorbing pigments. Low influence of UV-B was also found to stimulate the transcript levels of a robust set of genes involved in stress responses (Rock, 2000). Although the effects of UV-B on plants are well characterized at the physiological level, little is known about the effects of UV-B on underground (root) physiology, particularly in interaction with other environmental factors. An increasing number of studies have been designed to test the interactions of environmental factors on plants, such as the interaction between UV-B and water stress (Cechin et al., 2008), interaction between salinity and Fe deficiency (Zancan et al., 2006), and interaction between UV-B radiation and Fe deficiency (Zancan et al., 2008).
The aim of this chapter is to show some aspects of the recent applications of non-destructive remote sensing techniques, hyperspectral leaf reflectance and chlorophyll fluorescence, for detection and discrimination of the effects of some environmental stresses (salinity and enhanced UV-B radiation) on young soybean plants, as well as the influence of the biological nitrogen fixation on the spectral responses of the plants to stress. To evaluate the effects of a given stress a comparative analysis was performed between the changes of the leaf spectral reflectance and fluorescence data and the stress markers such as phenols, malondialdehyde, thiol groups, proline and hydrogen peroxide, and chlorophyll content that were estimated by biochemical methods.

2. Remote sensing methods

Generally, remote sensing refers to the activities of recording, observing, and perceiving (sensing) objects or events at far away (remote) places. Remote sensing is defined as a science and technology by which the characteristics of objects or events of interest can be identified, measured or analyzed without direct contact with the sensors. The spectral information relies on the properties of the light after multiple interactions, i.e., reflections, transmissions, and absorptions with the object. The information needs a physical carrier to travel from the objects/events to the sensors through an intervening medium. The electromagnetic radiation which is reflected or emitted from an object is the usual source of remote sensing data. However any media such as gravity or magnetic fields can be utilized. Remote sensing is a technology to identify and understand the object or the environmental condition through the uniqueness of their spectral responses. This technology offers advantages such as viewing parts of the Earth at different scales (synoptic view), monitoring of regions that are very remote or with restricted access, ability to obtain imagery of an area of the Earth at regular intervals over many years and to evaluate changes in the landscape as well as capability to distinguish anthropogenic effects.
A basic assumption made in remote sensing is that specific targets (soils of differed types, water with varying degrees of impurities, rocks of differing lithologies, or vegetation of various species) have an individual and characteristic manner of interacting with incident radiation that is described by the spectral response of that target. Different materials reflect and absorb visible (VIS) and infrared light differently at different wavelengths. They have different colours and brightness when seen under the sun. Thus, the targets can be differentiated by their 'spectral reflectance signatures', a term used to describe the spectral

response of a target. The variety of earth's surface materials is enormous, and therefore the recording of their spectral signatures (also known as spectral library) requires substantial financial and time investments. With the development of hyperspectral technology, the spectral resolution of hyperspectral sensors have reached less than 10 nm, which is sufficient for creating a continuous spectral curve from 350-2500 nm to detect subtle changes in the spectral behaviour of the earth objects. For years, efforts have been made to establish such datasets and pool them for general use through spectral libraries. Such spectral libraries are maintained by many organizations including the Johns Hopkins University (JHU), the Jet Propulsion Laboratory (JPL), and the United States Geological Survey (USGS). Many of these datasets are made available with commercial remote sensing image processing software packages.

2.1 Spectral reflectance

Methods based on reflectance makes use of VIS, near infrared (NIR), and short-wave infrared (SWIR) sensors to form images of the earth's surface by detecting the solar radiation reflected from targets on the ground. These methods rely on making measurements simultaneously in one or more wavebands. Spectrophotometers offer the simplest solution for spectral reflectance measurements. They measure spectrum of light reflected from the whole (mostly circular) field of view of the instrument but not provide any spatial information on the pattern of reflection (West et al., 2003). Earlier studies utilized multispectral sensors with low spatial (60 m to 80 m) and spectral resolution commonly collected in four to seven spectral bands in the VIS and NIR regions. Spectral resolution refers to the number and width of the portions of the electromagnetic spectrum measured by the sensor. A sensor may be sensitive to a large portion of the electromagnetic spectrum but have poor spectral resolution if it captures a small number of wide bands. Spatial resolution defines the level of spatial detail depicted in an image and it is directly related to image pixel size. The spatial property of an image is a function of the design of the sensor in terms of its field of view and the altitude at which it operates above the surface (Smith, 2001a). Early airborne systems included a multispectral camera mounted on board a light aircraft. Spectrometers at this time were bulky, heavy instruments which were not easily transportable in the field and most measurements were taken in laboratories.

Remote sensing technologies have advanced significantly over the past 10 to 15 years. With the development of hyperspectral remote sensing technologies, researchers have benefited from significant improvements in the spectral and spatial properties of the data, allowing for more detailed plant and environmental studies (Thenkabail et al., 2004; Blackburn, 2007). These technologies acquire many hundreds of spectral bands across the VIS, NIR, and mid-infrared portions of the electromagnetic spectrum from 350 nm to 2500 nm, using satellite, airborne or hand-held devices. Advances in spectrometry and information technologies have resulted in state-of-the-art portable field instruments which allow for the collection of hand-held hyperspectral signatures. There are certain problems in the area of hyperspectral analysis connected with the optimal selection of bandwidth, number of bands and spatial as well as spectral resolutions and some constraints like data storage, communication bandwidth, discrimination/classification accuracy, minimum signal-to-noise ratio, sensor selection, data acquisition procedures and the cost factor.

The spectral reflectance responses are affected by factors such as soil nutrient status, the growth stage of the vegetation, the colour of the soil (which may be affected by recent weather conditions). In some instances, the nature of the interaction between incident

radiation and earth's surface materials will vary in time during the year, such as might be expected in the case of vegetation as it develops from the leafing stage, through growth to maturity and, finally to senescence. These responses also depend upon such factors as the orientation of the Sun, the height of the Sun in the sky (solar elevation angle), direction in which the sensor is pointing relative to nadir (the look angle), the topographic position of the target in terms of slope orientation, the state of health of vegetation if that is the target, and the state of the atmosphere.

Vegetation has a unique spectral signature which enables it to be distinguished readily from other types of land cover in an optical/infrared part of the electromagnetic spectrum. The spectral responses of vegetation are governed primarily by scattering and absorption characteristics of the leaf internal structure and biochemical constituents, such as pigments, water, nitrogen, cellulose and lignin (Sims & Gamon, 2002; West et al., 2003). In recent years, there has been an expanding body of literature concerning the relationship between the spectral reflectance properties of vegetation and the structural characteristics and pigment concentration in leaves (Gitelson et al., 2003; Blackburn, 2007; Sun et al., 2008; Hatfield et al., 2008). Chlorophyll pigment content is a major factor that dictates the amount of energy reflected or emitted and can be good indicator of crop health (Wu et al., 2008).

The function describing the dependence of the ratios of the intensity of reflected light to the illuminated light on wavelength in VIS (400-700 nm), NIR (700-1200 nm), and SWIR (1200-2400 nm) spectral ranges is the spectral reflectance characteristic (SRC) of the target. Fig.1 presents the typical spectral reflectance characteristics of green vegetation. The labelled arrows indicate the common wavelength bands used in optical remote sensing of vegetation: A - blue band, B - green band, C - red band, D - NIR band, and E - SWIR band. Reflectance is low in both the blue (450 nm) and red (670 nm) regions of the spectrum, due to absorption by chlorophyll for photosynthesis, also known as the chlorophyll absorption bands. It has a peak at the green region (550 nm) which gives rise to the green colour of vegetation. In the NIR region, the reflectance is much higher than that in the VIS band due to the cellular structure in the leaves. Hence, vegetation can be identified by the high NIR but generally low VIS reflectance. The reflectance of vegetation in the SWIR region is more varied, depending on the types of plants and the plant's water content. Water has strong absorption bands around 1.45, 1.95 and 2.50 μm. The SWIR band can be used in detecting plant drought stress and delineating burnt areas and fire-affected vegetation.

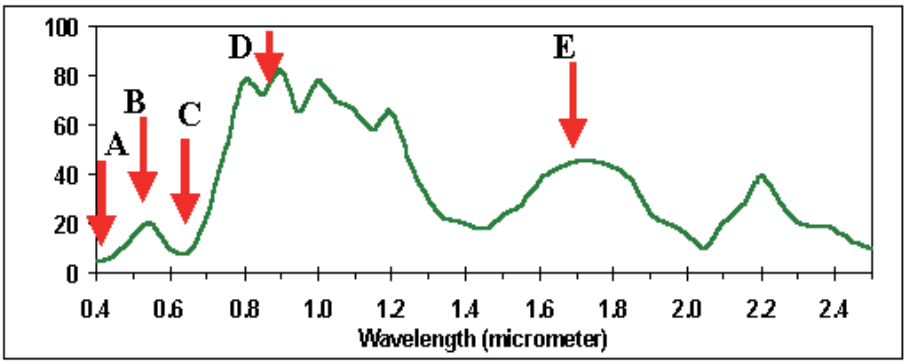


Fig. 1. Typical spectral reflectance characteristic of green vegetation in the VIS, NIR and SWIR ranges.

The shape of the reflectance spectrum is used for identification of vegetation type. For the same vegetation type, the reflectance spectrum also depends on other factors such as the leaf moisture content and health of the plants. Fig. 2 shows typical reflectance spectra of some species of green vegetation compared to a spectral signature for senescent leaves (Smith, 2001b).

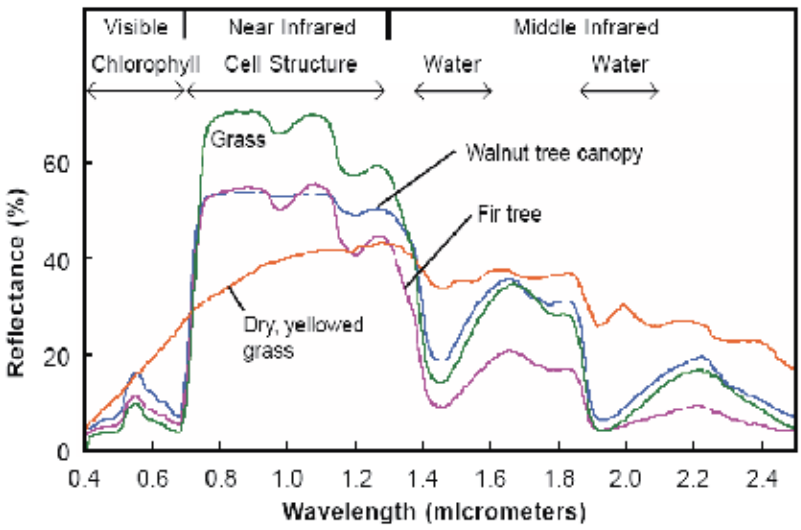


Fig. 2. Leaf reflectance spectra of different vegetation types.

In past decade, the research efforts were focused on the elucidation of some aspects of the link between the spectral responses and the physiology of plants under stress. In stressed vegetation, leaf chlorophyll content decreases, thereby changing the proportion of light-absorbing pigments, leading to a reduction in the overall absorption of light (Zarco-Tejada et al., 2000; Clay et al., 2006; Gang et al., 2010). These changes affect the spectral reflectance signatures of plants through a reduction in green reflection and an increase in red and blue reflections, resulting in changes in the normal spectral reflectance patterns of plants (Zarco-Tejada et al., 2000; Campbell et al., 2007).

More recent works have highlighted the importance of more specific narrow-band regions such as the red edge (maximum slope of vegetation reflectance from 680 nm to 720 nm) for predicting plant stress (Fitzgerald et al., 2006; Blackburn, 2007; Steele et al., 2008). The reflectance around red edge is sensitive to wide range of crop chlorophyll content and leaf internal scattering (Dawson & Curran, 1998). Experimental and theoretical studies show that red edge position shifts according to changes of chlorophyll content, N content, biomass and hydro status, age, plant health levels, and seasonal patterns (Filella & Penuelas, 1994; Pu et al., 2003; Hatfield et al., 2008;). These observations on red edge position can effectively be used to classify and distinguish different vegetation types and ages in the study.

Mathematical functions of two or more spectral bands are used rather than direct reflectance data to minimize the negative impact of interfering factors, such as the surrounding land cover, bare soil, or climatic/atmospheric conditions (McDonald et al., 1998; Huete et al., 2002). These functions are called vegetation indices (VIs), each designed for optimal correlation with a particular vegetation feature. The capacity of vegetation indices to

characterize natural canopies and agricultural crops has been demonstrated in numerous studies aimed at seasonal phenology (Carter, 1998; Qi et al., 2000), biomass prediction (Broge and Leblanc, 2001; Haboudane et al., 2004), mapping chlorophyll content (Haboudane et al., 2002). Numerous studies have documented the use of vegetation indices such as ratio vegetation index (RVI) and normalized difference vegetation index (NDVI) in the detection of crop stress (Kobayashi et al., 2001; Vigier et al., 2004; Yang et al., 2009). Combining individual spectral reflectance bands as simple ratio vegetation indices (SRVI) has been a common approach in remote sensing because it generally reduces the effects of spectral noise and allows for better temporal comparisons due to minimization of atmospheric effects (Carter & Miller, 1994). Commonly, SRVIs have consisted of the ratio of blue to red wavebands in an effort to detect responses due to changes in chlorophyll a and b concentrations. Gitelson et al. (2003, 2006) suggested the use of empirical vegetation indices, calculated from the reflectance of three wavelengths that were highly correlated with chlorophyll (Chl), carotenoid, and anthocyanin concentrations to estimate the content of foliar pigments in single leaves. Furthermore, various statistical and artificial intelligence methods have been used to analyze the remotely sensed data in agricultural crops. Among many, popular approaches include cluster analysis (Holden & LeDrew, 1998), principal component analysis (Zhang et al., 2002; 2003), partial-least square regression (Huang & Apan, 2006), artificial neural networks (Liu et al., 2008).

With the advent of hyperspectral remote sensing technology, more detailed data are potentially available. Therefore the extracting meaningful relationships of the overwhelming quantity of data are necessary. Currently, a variety of techniques have been used including a number of different vegetation indices, band absorption analysis, spectral mixture analysis, "red edge" position, statistical analysis, wavelet transform and neural networks (Thenkabail et al., 2004; Delalieux et al., 2007; Steele et al., 2008b).

2.2 Chlorophyll fluorescence

In recent years, chlorophyll fluorescence (ChlF) analysis has become one of the widely used techniques available to plant physiologists and has participated increasingly in plant ecology and physiology studies (Rolando & Little, 2003; Chaerle et al., 2004; Gielen et al., 2006). This analysis has been used more extensively to provide considerable information on the organization and function of the photosynthetic apparatus (Campbell et al., 2007). The chlorophyll molecule has the ability to absorb light energy and transfer it into the photosynthetic apparatus. Excess energy can be dissipated as heat or re-emitted as light at longer wavelength, i.e. chlorophyll fluorescence. The increase in efficiency of one of these three processes (absorption, fluorescence and thermal emission) will result in a decrease in yield of the other two. As such, the relative intensities of ChlF are strongly related to the efficiency of photochemistry and heat dissipation (Papageorgiou & Govindjee, 2004; Lichtenthaler et al., 2007; Delalieux et al. 2009b) and may provide additional data to detect plant stress in an early stage. Generally, fluorescence yield is highest when photochemistry and heat dissipation are lowest.

Chlorophyll a (Chl a) is contributing largely to plant fluorescence emission. Excitation energy for this fluorescence is delivered from accessory antenna chlorophylls (Chl a and Chl b), absorbing light of blue and red wavelengths, and from carotenoids, absorbing photons of blue wavelengths. At room temperature, Chl a emits fluorescence in the red and NIR spectral regions between 650–800 nm, in two broad bands with peaks at λ_{max1} (684–695 nm) and λ_{max2} (730–740 nm) (Franck et al., 2002). The shorter wavelength emission is attributed to Chl a

mostly associated to PSII (Dekker et al., 1995), whereas the longer wavelength emission originates from antenna chlorophyll of both PSI and PSII (Agati et al., 2000; Buschmann, 2007). Several environmental factors, including water, salinity, light and nutrients, affect the process of photosynthesis and may lead to plant stress. Changes in chlorophyll function take place before changes in chlorophyll content, before any physical signs of tissue or chlorophyll deterioration are manifested in the plant, and therefore alterations in the fluorescence signal occur before any visible signs are apparent (Cambpell et al., 2007; Li et al., 2010). Under conditions of stress, some plant mechanisms for disposing of excess energy do not work efficiently, thus causing changes in the competing reactions of photochemistry, heat loss and fluorescence. Although the total amount of chlorophyll fluorescence is very small (only 2 or 3% of total light absorbed), measurement is quite easy. The spectrum of fluorescence is different to that of absorbed light with the peek of fluorescence emission being at longer wavelength than that of absorption. Therefore, fluorescence yield can be quantified by exposing a leaf to light of defined wavelength and measuring the amount of light re-emitted at longer wavelengths (Maxwell & Johnson, 2000).

Various fluorescence intensity ratios, combining the emissions at blue (F440), green (F520), red (F690), and NIR (F740) wavelengths, were proposed for probing the vegetation vitality status and stress responses (Buschmann et al., 2000; Mishra & Gopal, 2008). The red ChlF emission between 684-695 nm is strongly reabsorbed by the Chl pigments in the upper layer leaf cells (Agati et al., 1993; Dau, 1994), while the NIR ChlF between 730–740 nm is re-absorbed to a much smaller extent. Consequently, the ratio between the red and far-red ChlF bands (e.g. F690/F740) decreases with increasing leaf Chl content in a curvilinear relationship, which can be used as a good inverse indicator of Chl content changes due to plant growth or stress events (Buschmann, 2007). Finally, the UV excited blue-to-red/NIR fluorescence intensity ratios (F440/F690 and F440/F740) were proposed as indicators of the leaf physiological development (Stober et al., 1994; Meyer et al., 2003), but also as marker of the nutrition availability and stress occurrence (Heisel et al., 1996).

The red and NIR fluorescence emissions by Chl a are highly dynamic, being modulated by photochemical and non-photochemical quenching. These dynamic phenomena yielded important insights into the molecular processes of photosynthesis that occur within time-scales ranging from femtoseconds to minutes depending on the power of an actively applied actinic light (Govindjee, 1995; Nedbal & Koblizek, 2006; Baker, 2008). Most widely used field observations are active, using devices exciting the photosynthetic machinery with a measuring light and recording the induced fluorescence. Introduction of the pulsed amplitude modulation (PAM) fluorometer allowed non-imaging outdoor measurements in broad daylight (Schreiber et al., 1986). Fluorescence imaging was introduced in the laboratory by Omasa et al. (1987) and modified for field surveys in the mid-1990s by Nedbal et al. (2000). The laser pulses of actinic light, which can be discriminated from static and panchromatic background light, are applied to elicit fluorescent transients when measuring fluorescence from a distance (Cecchi et al., 1994; Corp et al., 2006). The footprint of such a light detection and ranging (LIDAR) laser beam can be expanded from several centimetres up to metres to cover larger observation areas or to decrease the power of the excitation source (Saito et al., 2005). The first field laser-induced vegetation fluorescence was observed by Measures et al., (1973). Lately, an eye-safe outdoor laser-induced fluorescence transient (LIFT) fluorometer has been constructed. This device is able to measure the fluorescence parameters and non-photochemical quenching or electron transport rate from a distance of about 30-50 m (Ananyev et al., 2005; Kolber et al., 2005). A new generation active field fluorescence

instrument, developed by Raimondi et al. (2007), was successfully employed in summer 2007 during the joint CarboEurope, FLEX, and Sentinel-2 ESA mission campaign. These ground-based active fluorescence-sensing techniques can be used whenever temporal monitoring of fluorescence transients is required regardless of the appearance of cloud cover.
Lately, chlorophyll fluorescence techniques proved to be a non-intrusive, fast and reliable attractive tool in ecophysiological studies, and have extensively been used in assessing plant responses to environmental stress.

3. Materials and treatments

3.1 Plant material

The experiments were carried out on young soybean plants (*Glycine max L., cultivar Pavlikeni 101)*. They were grown as water cultures in a growth chamber under controlled conditions (16/8 photoperiod day to night, photon flux density 90 μmol m^{-2} sec^{-1}, humidity 60-70%, and temperature 25 ± 1 °C). Soybean seeds were surface sterilized with 70% ethanol and washed afterwards several times in distilled water. Then, they were let to germinate on a damp filter paper at 24 °C for three days in dark. On the 4th day seeds were transferred in plastic vessels with half strength well aerated Helrigel nutrient solution (Helrigel, 1898) and were put to growth clamber.

3.2 Induction of stresses

The first part of experiments was carried out to investigate the influence of a single environmental stress factor (salinity) on the spectral properties and physiological state of soybean plants. Plants were grown in nutrient solution with constant nitrate levels at all development stages (1st to 4th trifoliate expanded leaves). They were divided into three groups. These one kept only in nutrient solution were used as control (untreated). At the growth stage of 2nd trifoliate expanded leave to the nutrient solutions of the other two groups was added NaCl at concentrations 40 mM and 80 mM. Some of the investigated leaves are shown in Fig. 3. They are from plants salinity treated for 14 days.

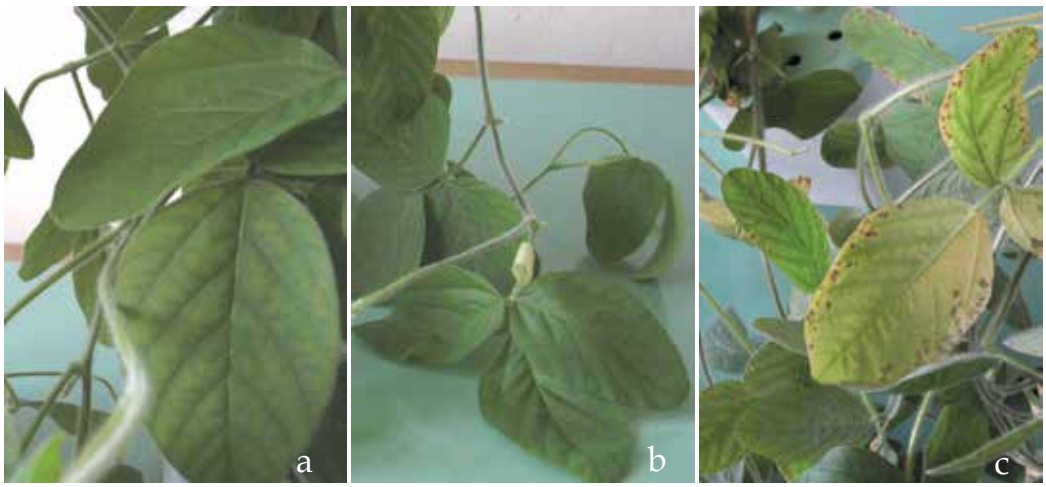


Fig. 3. Leaves from control (a) and treated with 40 mM NaCl (b) and 80 mM NaCl (c) soybean plants.

The second part of the experiments was focused on studying of the effect of the salinity on nitrogen fixing soybean plants. Three day's seedlings were inoculated with adding of 108 cells ml^{-1} suspension of Bradyrhizobium japonicum strain 273. After that they were transferred into plastic vessels with Helrigel nutrient solution. The nitrogen in the nutrient solution was equal to ¼ of the full dose until the growth stage of fully expanded 2nd trifoliate leaf, i.e. up to nodule forming and beginning of effective nitrogen fixation. The plants were salinity treated during the vegetative stage of growth from 2nd to 4th trifoliate expanded leaf for 14 days. Salinity was performed by means of adding in nutrient solution NaCl at two concentrations (40 mM and 80 mM). The control plants were kept only in nutrient solution. Figs. 4, 5, and 6 show the roots and nodules of nitrogen fixing control and treated with two NaCl concentrations plants on 14th day after the salinity.

The third part of experiments was aimed to investigate the effect of salinity stress and two consecutive stress factors (salinity and UV-B radiation) on young nitrogen fixing soybean plants. They were grown, nitrogen fixing, and salinity treated in the same way as the plants of the previous experiments.

Fig. 4. Control nitrogen fixing soybean plants: a) leaves; b) roots.

The investigated plants were divided into six groups. The first group consisted of untreated (control) plants. The second and third groups were only salinity treated by two NaCl concentrations. The plants of the fourth group were control (not salinized). The plants of fifth and sixth groups were treated for 14 days with 40 mM and 80 mM NaCl, respectively. Together with the control group they were irradiated with UV-B light for two hours. As a light source a lamp HPQ type with intensity 64.4 µmol m^{-2} s^{-1} was used at a distance of 25 cm. Fig. 7 a), b) shows some of investigated leaves from first (control for salinity treatment) and forth (control for treatment with UV-B radiation) groups. Fig. 8 a), b) and Fig. 9 a), b) show some leaves from plants treated with 40 mM NaCl and (40 mM NaCl + UV-B), and 80 mM NaCl and (80 mM NaCl + UV-B), respectively.

Fig. 5. Nitrogen fixing soybean plants treated with 40 mM NaCl: a) leaves; b) roots.

Fig. 6. Nitrogen fixing soybean plants treated with 80 mM NaCl: a) leaves; b) roots.

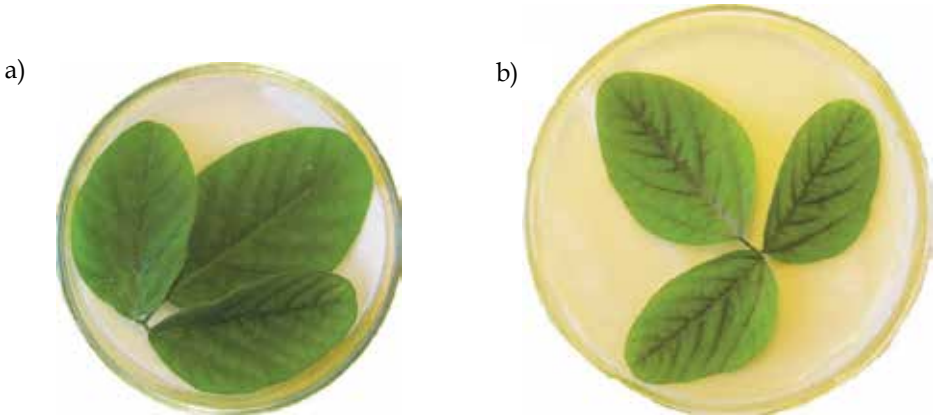


Fig. 7. Leaves from plant groups: a) first (control); b) fourth (control +UV-B).

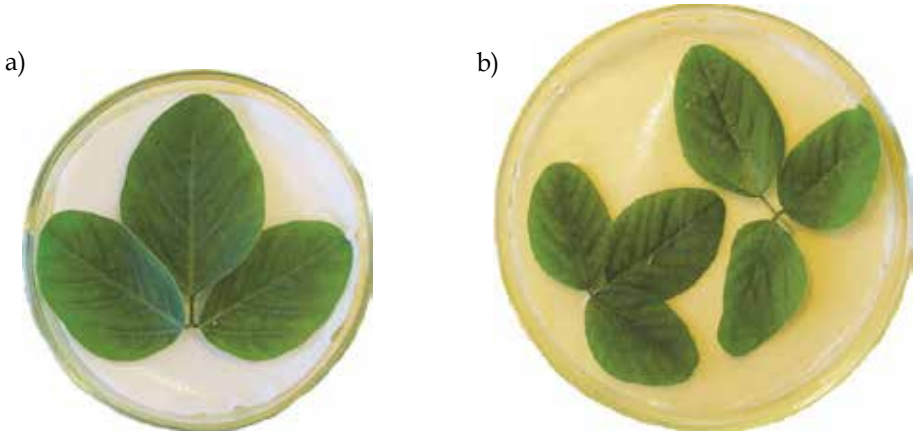


Fig. 8. Leaves from plant groups: a) second (40 mM NaCl salinity; b) fifth (40 mM NaCl + UV-B).

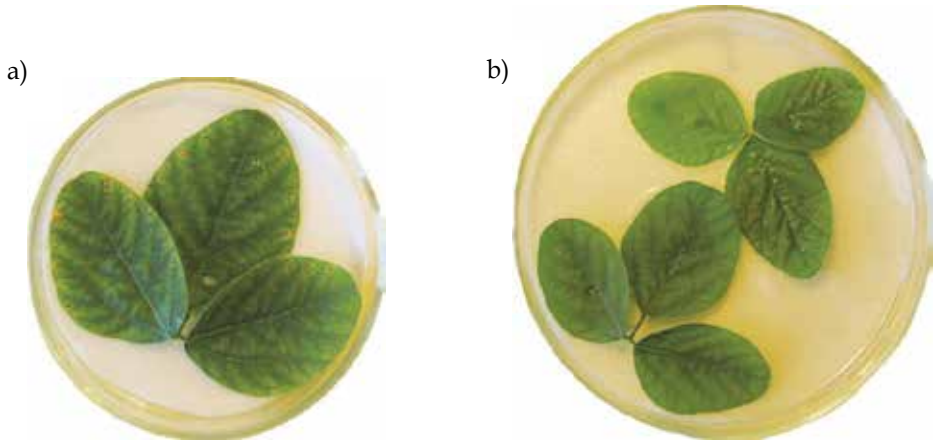


Fig. 9. Leaves from plant groups: a) third (80 mM NaCl salinity; b) sixth (80 mM NaCl + UV-B)

4. Biochemical methods

The biochemical parameters, stress markers such as phenols, proline, malondialdehyde (MDA), thiol groups, hydrogen peroxide (H_2O_2), glutathione, and leaf pigments (chlorophyll a and b) were determined to analyze the physiological stage of the plants and to perform a comparative analysis with the results from the spectral remote sensing methods. All stress markers were measured using a spectrophotometer Multiskan Spectrum (Thermo Electron Corporation). Fresh leaf materials (0.2 g) of soybean plants were homogenized at 4 ^{o}C in 0.1% cold trichloroacetic acid. The homogenates were centrifuged at 15000 g for 30 min. Then the supernatants obtained were used for determination of the stress markers after applying different methods.

Phenols and proline are important protective components of the plant cells and they accumulate when cells are in stress conditions. The antioxidant properties of phenols are mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (Rice-Evans et al., 1997; Khan et al., 2002). Plants generally accumulate some of osmolytes such as proline under salt and drought stress (Delauney & Verma, 1993), which protect the proteins against denaturation and also act as osmotic balancing agents (Sivakumar et al., 2000). Content of total free phenols was measured according to the method developed by Swain and Goldstein (1959) using Folin - Ciocalteau reagent. Caffeine acid was used as a standard. The absorbance was registered at 725 nm. Plants generally accumulate some of osmolytes such as proline under salt and drought stress, which protect the proteins against denaturation and also act as osmotic balancing agents (Chadalavada et al., 1994; Kavi Kishor et al., 2005). Proline content was determined by measuring the absorbance at 520 nm after reaction with a mixture glacial acetic acid and ninhydrin according the method of Bates et al., (1973). Proline concentration in the samples was determined from the standard curve calibrated with different concentrations of the standard proline.

Hydrogen peroxide is known to damage various cellular components and evoke structural modifications of proteins, lipids and DNA under stress (Halliwell & Gutteridge, 2002). Hydrogen peroxide and malondialdehyde contents are routinely estimated together with electrolyte leakage measurements to assess the extent of oxidative stress. The injurious impact of reactive oxygen species on the membranes of cells is realized by lipid peroxidation. The basic damage products by this process are aldehydes and mainly MDA. The accumulation of MDA is an especially sensitive marker of stress. For determination of the amount of MDA as a final product of lipid peroxidation, the method of Kramer et al. (1991) was used. The absorbance was measured at 532 nm and 600 nm. The content of endogenous free thiol groups was determined using the Elman's reactive according to the method of Edreva & Hadjiska, (1984). Hydrogen peroxide absorbance was measured at 390 nm after reaction with KJ according to Alexieva et al., (2001). Content of H_2O_2 was calculated using the standard curve with known amount of KJ. Glutathione content was measured according to Gronwald et al., (1998).

Compounds bearing a free thiol groups (-SH), such as low molecular cell metabolites like glutathione, as well as a number of enzymes, which are active only in a reduced state, play a key role in important cellular functions, and the massive oxidation of -SH groups could be regarded as an aspect of oxidative toxicity (Haugard, 2000). The relationship between the SH-state in plants and their resistance to various stress factors is well known. Glutathione is major fraction of the SH pool in the cells (Haugard, 2000; Noctor et al., 2002).

Pigment contents (chlorophyll a and b) were calculated after the extraction of leaf material with 80% acetone according to the method of Arnon (1949). The extinction of chlorophyll a and chlorophyll b was determined at 663 nm and 845 nm using a spectrometer Specol 11.

5. Spectral measurements

5.1 Leaf spectral reflectance

Hyperspectral reflectance data were collected in VIS and NIR spectral ranges (450-850 nm) by using a portable fibre-optics spectrometer USB2000 (http://www.OceanOptics.com). In the range investigated the main part of the reflected from leaves radiation is concentrated. Data were obtained at 1170 spectral bands with a step of 0.3 nm and a spectral resolution of 1.5 nm.

The reflectance measurements were carried out on an experimental setup in laboratory (Fig. 10). The entrance lens, a standard screen WS1 (diffuse reflectance white plastic with Lambertian reference surface) and plant leaves were set on a special adjustable platform, which provides an accurate relative positioning of all equipment components. The fibre-optic cable was located at nadir view (perpendicular to leaf surface). As a light source a halogen lamp providing homogeneous illumination of measured leaf surfaces was used. The acquisition and processing of data were carried out by means of portable computer using specialized software. The spectral reflectance characteristics were obtained as a ratio of the reflected radiation from the leaves and the reflected radiation from WS1 screen. The measurements were performed on fresh, immediately picked off soybean leaves at the stage of 4th trifoliate expanded leaf from up to 25 plants from each investigated group.

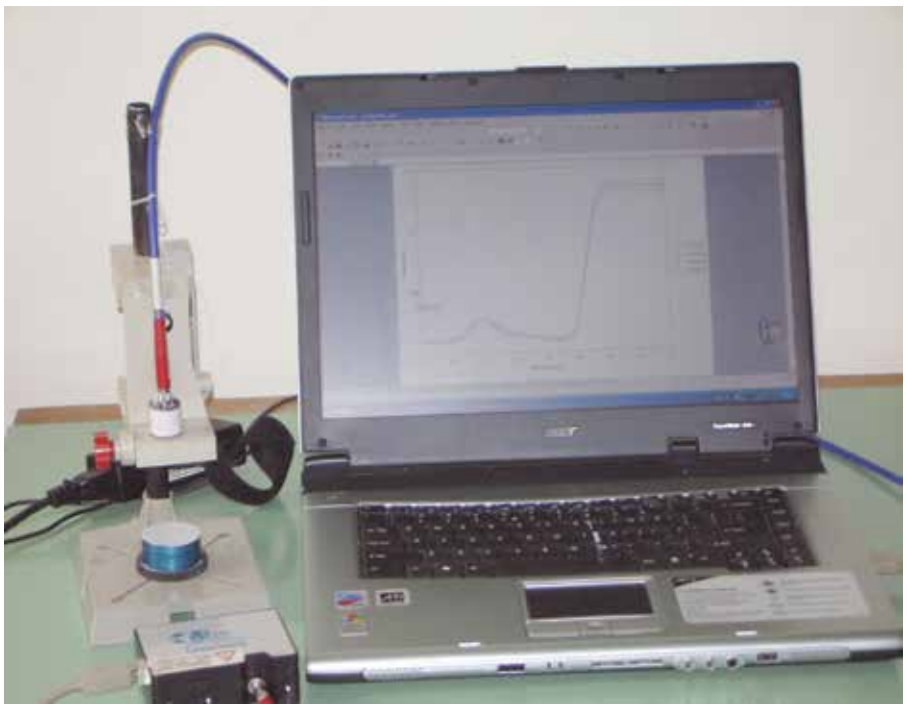


Fig. 10. Experimental setup for spectral reflectance measurements.

5.2 Chlorophyll fluorescence

The spectral measurements of the chlorophyll fluorescence were carried out under laboratory conditions using the same portable fibre-optics spectrometer (USB2000). Data were collected in the VIS and NIR spectral ranges (600-900) nm in 910 spectral bands with a step of 0.3 nm where the main part of the emitted from the plants fluorescence radiation is concentrated. As a source of actinic light, a LED diode with light output maximum at 470 nm and light intensity 507 $\mu mol\ m^{-2}\ s^{-1}$ was used. The tested leaves were dark adapted before the measurements for ten minutes. The abaxial side of the leaves was irradiated with actinic light and the exited fluorescence was measured from the adbaxial leaf surface. The control of the spectrometer and the acquisition and processing of data were carried out by means of specialized software. The measurements were conducted on fresh detached leaves from by 20 plants of the each group of plants in the 4^{th} trifoliate expanded leave node on the 14^{th} day after the salinity treatment.

5.3 Statistical analysis

The hyperspectral reflectance and fluorescence data of the control and treated plants were subjected to statistical analysis through the Student's t-criterion and linear stepwise Discriminant Analysis (DA). Because 1170 reflectance and 910 fluorescence values were available to be used as classification features, it was computationally efficient to select a subset of bands on the basis of discriminant capability.

The reflectance analysis was performed in four most informative for investigated plants spectral ranges: green (520-580 nm, maximal reflectivity of green vegetation), red (640-680 nm, maximal chlorophyll absorption), red edge (680-720 nm, maximal slope of the reflectance spectra) and the NIR (720-770 nm) (Krezhova et al., 2005, 2007). The statistical significance of the differences between SRC of control and treated plants was examined in eight spectral bands (wavelengths) chosen to be disposed uniformly over the above mentioned ranges (λ_1 = 524.29 nm, λ_2 = 539.65 nm, λ_3 = 552.82 nm, λ_4 = 667.33 nm, λ_5 = 703.56 nm, λ_6 = 719.31 nm, λ_7 = 724.31 nm, and λ_8 = 758.39 nm).

The fluorescence spectra were analyzed in five characteristic spectral bands, chosen at wavelengths: λ_1 (at the middle of the forefront edge), λ_2 (first maximum), λ_3 (at the middle between first and second maximum), λ_4 (second maximum), and λ_5 (at the middle of the rear slope). They are illustrated in Fig. 11 for a typical fluorescence spectrum of green vegetation.

The Student's t-criterion and linear DA were applied for determination of the statistically significance of differences at $p<0.05$ between the means of sets of the values of the reflectance and chlorophyll fluorescence of control and treated plants in the above mentioned wavelengths. They were further regarded as discriminative features. The Student's t-criterion was utilized under the prerequisite for the existence of numerous, independent and approximately of one and the same order factors of small impacts on the variables under examination. DA was used to increase classification accuracy. One output of the method is the determination of the posterior probability that spectral data of a given leaf belongs to the class of control or treated plants. For this purpose discriminant analysis will be implemented in one dimensional spaces defined by the features examined. In some of the cases DA was performed in two or three-dimensional spaces for enhancement of the discriminative possibility.

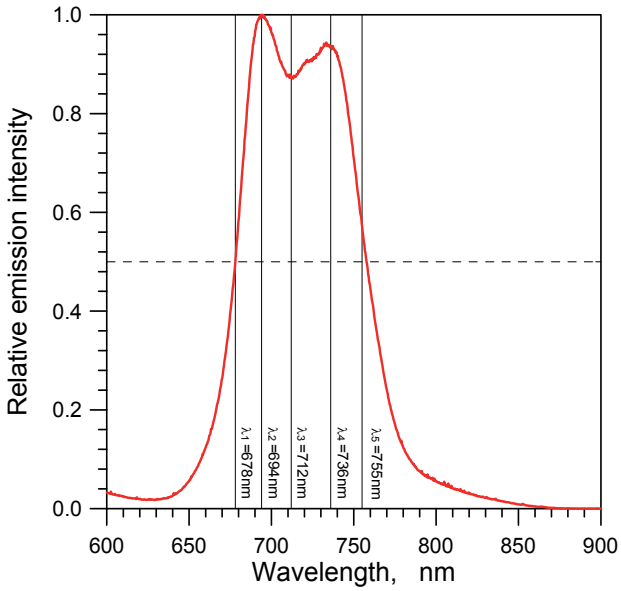


Fig. 11. Characteristic wavelengths chosen for statistical analysis of fluorescence.

All statistical analyses were conducted using the STATISTICA software, Version 6.1, 2002, (http://www.statsoft.com).

6. Results and discussion

6.1 Influence of a single stress factor at soybean plants

The effect of salinity on leaf spectral reflectance of soybean plants for the first part of the experiments (see Fig. 3) is shown in Fig. 12 (Krezhova et al., 2009a). The SRC were averaged over all studied areas (pixels) of leaves of control and treated with each of the two salt concentrations plants. The discrepancy (lack of coincidence or very small differences) between the characteristics of control and treated by 40 mM NaCl plants was observed in the green (520-580 nm, maximal chlorophyll reflection) and NIR ranges.

At 80 mM NaCl the values of the average SRC of treated leaves with respect to control decrease significantly in both the green and red (640-680 nm) ranges. In the red edge (680-720 nm) it is observed a shift to longer wavelengths (8 nm) indicating the occurrence of stress. Necrosis spots were seen on some of the leaves of plants treated with this NaCl concentration (Fig. 3 c). The red edge position changed significantly with the increase in NaCl concentration applied to the plants and it is a consequence of the decreased chlorophyll content determined by biochemical method. In the NIR range the reflectance changed non-significant against the control.

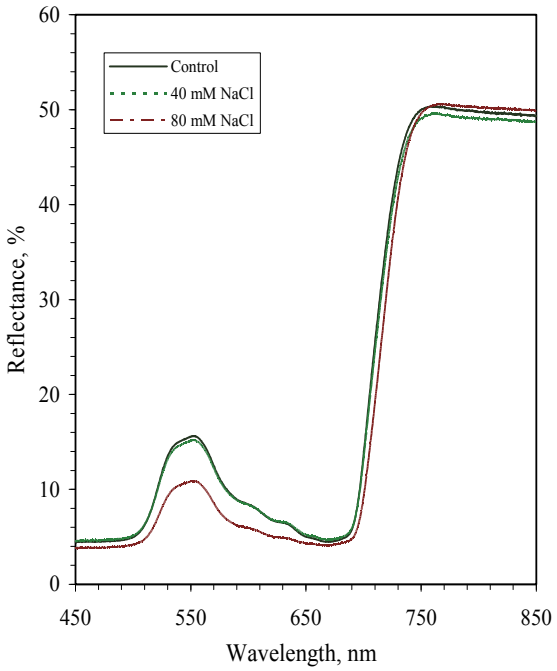


Fig. 12. Averaged spectral reflectance characteristics of control and treated with 40 mM NaCl and 80 mM NaCl soybean plants.

The results (p-level of the difference between SRC means of treated plants and SRC means control plants at a given λ) from the statistical analysis of the spectral data are set out in Table 1 and Table 2. In Table 1 p_{st} stands for the significance p-level of the Student's t-criterion. In Table 2 p_{DA} designates the significance p-level of the DA model. The index c stands for reflectance or fluorescence data of control plants. Statistically significant differences between SRC means of control and treated at 80 mM NaCl concentration were detected at $p<0.05$ by means of the Student's t-criterion in each wavelength with the exception of λ_8 in the NIR range. The impact of 40 mM NaCl salinity is not sufficient to provoke detectable changes in SRC in the wavelengths examined.

Student's t-criterion			
40 mM NaCl		80 mM NaCl	
Pairs compared	p_{st}	Pairs compared	p_{st}
λ_1/λ_{1c}	0.328	λ_1/λ_{1c}	<0.001
λ_2/λ_{2c}	0.210	λ_2/λ_{2c}	<0.001
λ_3/λ_{3c}	0.185	λ_3/λ_{3c}	<0.001
λ_4/λ_{4c}	0.061	λ_4/λ_{4c}	<0.001
λ_5/λ_{5c}	0.125	λ_5/λ_{5c}	<0.001
λ_6/λ_{6c}	0.120	λ_6/λ_{6c}	<0.001
λ_7/λ_{7c}	0.082	λ_7/λ_{7c}	<0.001
λ_8/λ_{8c}	0.285	λ_8/λ_{8c}	0.94

Table 1. Significance p-level of the Student's t-criterion in the cases of 40 mM NaCl and 80 mM NaCl salinity.

Similar results are obtained through linear DA. The grouping variable used on the first stage of DA implementation was designed to consist of only two groups - control and by one of the treated with different NaCl concentration plants. Since the DA significant p-level for the case of 40 mM NaCl turned out to be >>0.05 with all wavelengths (0.12<p<0.98) the p_{DA} are not shown in the Table 2. Anyway, if for example the three dimensional space (λ_4, λ_6, λ_7) is used, the p_{DA} level is p<0.001 while the incorrectly classified cases are 12 from 69.

Discriminant analysis, 80 mM NaCl		
One dimensional spaces	p_{DA}	Number of incorrectly classified cases
λ_1	<0.001	0
λ_2	<0.001	0
λ_3	<0.001	0
λ_4	<0.001	18 from 69
λ_5	<0.001	1 from 69
λ_6	<0.001	6 from 69
λ_7	<0.001	11 from 69
λ_8	0.95	29 from 69

Table 2. Significance p-level of the Discriminant analysis model in the case of 80 mM NaCl salinity.

The contents of the evaluated stress markers and chlorophyll a and b are shown in Table 3. The values of control plants were taken as 100%. The 40 mM NaCl treatment did not provoke changes in the phenol content in soybean leaves, while 80 mM NaCl caused a decrease of the content by about 33.6%. Such sharp decrease of phenols content in the leaves treated by 80 mM NaCl gives grounds to consider the phenols as playing the role of endogen antioxidant in plants.

Stress markers, pigments	Control	40 mM NaCl	80 mM NaCl
Phenols, (μM/gDW)	123.2 ±2.1	122.9 ±1.1 2%	81.8±0.9 33.6%
Proline, (nmol/gDW)	30.8±0.7	34.6±2.3 12.3%	48.1±1.9 56.2
MDA (nmol/g DW)	97.6±5.3	114.9±6.8 18%	140.2±5.6 44%
H_2O_2 (μM/gDW)	6.8±0.06	5.1±0.04 25%	6.3±0.02 7%
Thiol groups (μM/gDW)	2.49±0.01	3.59±0.04 42%	4.23±0.03 70%
Chlorophyll a (mg/g FW)	1.39±0.03	1.09±0.02 21%	0.71±0.03 49%
Chlorophyll b (mg/g FW)	0.60±0.01	0.46±0.03 23%	0.35±0.01 42%
GSSG/ TG (%)	52.4	64.1 23%	76.3 46%

Table 3. Values of the biochemical parameters of salinity treated soybean plants.

A number of authors have observed that most plant species exhibit a remarkable increase in their proline content in consequence of the action of different kinds of stress such as UV-radiation, drought, salinity, etc. (Sivakumar et al., 2000; Jogeswar et al., 2006; Sun et al., 2008). Characteristic changes in proline content at the salinity stress are described in roots and leaves of alfalfa and pea (Tramontano & Jouve, 1997), and in leaves of cotton and bean (Brankova et al., 2005). Our results show that an increase of proline content by about 12% takes place under the influence of salinity at the lower concentration. A more significant increase of proline by about 56% was observed at 80 mM NaCl concentration.

An increase in hydrogen peroxide and MDA contents upon salt stress has been reported for different plant spices (Yang et al., 2008). This increase was shown to be related to the amount of stress and well correlated with lipid membrane damage. Our results show that salinity at 40 mM NaCl leads to an increase of MDA by about 18% in comparison with the control. A more substantial increase is observed at 80 mM NaCl that reaches 44%. These results agree with the findings of Jogeswar et al. (2006) who have established a significant increase of MDA at treatment of sorghum (Sorghum bicolour) with 150 mM NaCl.

Unexpectedly, in our experiments the H_2O_2 content was found lower by about 25% at the low salinity and by about 7% at the high salinity. This finding provides grounds to continue our investigations in order to determine the activity of enzymes from the antioxidant system (catalase and peroxidase) using H_2O_2 as substrate.

The measurements of thiol groups observed an increase by about 40% at the treatment with 40 mM NaCl. The salinity at 80 mM NaCl lead to about doubling (by 70%) of the free -SH groups. By our opinion, the increase of thiol groups might serve as a marker of damages induced by salinity stress. The ratio of oxidized form glutathione to total glutathione (GSSG/TG) is much higher than the control at 80 mM NaCl which is evidence of a strong reduction of the capacity of antioxidant system for the plants under study.

The averaged fluorescence spectra over 20 leaves of the control and by 20 treated leaves with each of the two NaCl concentrations of the same soybean plants used in the first part of the experiments are shown in Fig. 13 (Iliev et al., 2009a). All spectra are normalized to their second maximum at λ_m which in this case coincided with the wavelength of 738 nm. Changes in the fluorescence spectra of treated plants against the control were predominantly observed in the arising forefront. Curve 2 (the averaged leaf spectrum of plants treated with 40 mM NaCl) slightly differs against control curve 1 in the spectral range 640-680 nm. Curve 3 (80 mM NaCl treatment) differs against curve 1 significantly within the spectral range 600-740 nm.

The Student's t-criterion and linear DA were applied to estimate the statistical significance of the differences between the means of the indices chosen to characterize the fluorescence spectrum (halfwidth, wavelength at the first maximum, area) defined as, (see Fig. 11):

- halfwidth of the fluorescence spectrum ($\lambda_5 - \lambda_1$); λ_1 (relative emission intensity, REI = 0.5 in the forefront) and λ_5 (REI = 0.5 in the rear slope);
- REI at wavelength $\lambda_2 = \lambda_{1m}$ (at first maximum of the fluorescence spectrum);
- fluorescence spectrum area *S* (between wavelengths 640 nm and 840 nm).

The main results of the statistical analysis are summarized in Table 4 and Table 5. It is seen in Table 4 that the changes of the indices under the conditions of 80 mM NaCl concentration could be detected at $p<0.05$ by means of each of the indices. Also, it is clear that the impact of 40 mM NaCl is not sufficient to induce detectable changes in any of the fluorescence indices.

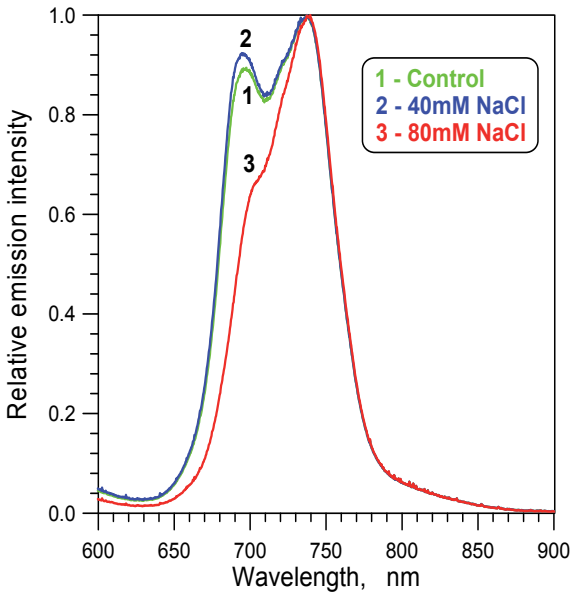


Fig. 13. Averaged fluorescence spectra of control and treated with: 40 mM NaCl and 80 mM NaCl soybean leaves.

Student's t-criterion			
Salinity 40 mM NaCl		Salinity 80 mM NaCl	
Pairs compared	p_{st}	Pairs compared	p_{st}
λ_1/λ_{1c}	0.955	λ_1/λ_{1c}	<0.001
$\lambda_{1m}/\lambda_{1mc}$	0.045	$\lambda_{1m}/\lambda_{1mc}$	0.007
λ_5/λ_{5c}	0.257	λ_5/λ_{5c}	0.009
$(\lambda_5-\lambda_1)/(\lambda_5-\lambda_1)_c$	0.202	$(\lambda_5-\lambda_1)/(\lambda_5-\lambda_1)_c$	<0.001
S/S_c	0.012	S/S_c	<0.001

Table 4. Significance p-level of the t-criterion for the set of fluorescence indices.

Similar results are obtained through linear discriminant analysis by making use of one dimensional spaces defined by each of the indices herein used, the p_{DA} level is p<0.05 while the incorrectly classified cases are not more than 5 cases from 24. The two indices λ_m and S also indicated perspective possibilities for detection of salinity injures on soybean plants.

The results from the chlorophyll fluorescence analysis revealed that the low NaCl concentration applied does not produce statistically significant changes in the leaf fluorescence. Applying of high NaCl concentration lead to significantly changed forefront of the fluorescence spectra due to caused salinity stress in the soybean plants.

In summary, the results from the first part of experiments have shown that there is a difference in the spectral reflectance characteristics in response to different salt

concentration treatment of soybean plants. The shift of the red edge position correlated with increased concentration of the salinity. Low NaCl concentration (40 mM) caused insignificant changes in the SRC and led to salinity tolerance whereas high NaCl concentration (80 mM) induced considerable SRC changes implying presence of salinity stress in soybean plants. This finding was in agreement with the outcome from the chlorophyll fluorescence analysis carried out on the same plants and evaluated biochemical stress markers such as phenols, proline, malondialdehyde, thiol groups, hydrogen peroxide, and leaf pigment contents (Chl a and Chl b).

Discriminant Analysis				
	Salinity 40 mM NaCl		Salinity 80 mM NaCl	
One dimensional spaces	p_{DA}	Number of incorrectly classified cases	p_{DA}	Number of incorrectly classified cases
λ_1	0.996		<0.001	0
λ_{1m}	0.044	5/22	0.0132	5 from 24
λ_5	0.256		0.019	3 from 24
$(\lambda_5 - \lambda_1)$	0.199		<0.001	1 from 24
S	0.0123	8/22	<0.001	0

Table 5. Significance p-level of the Discriminant analysis model for the set of fluorescence indices.

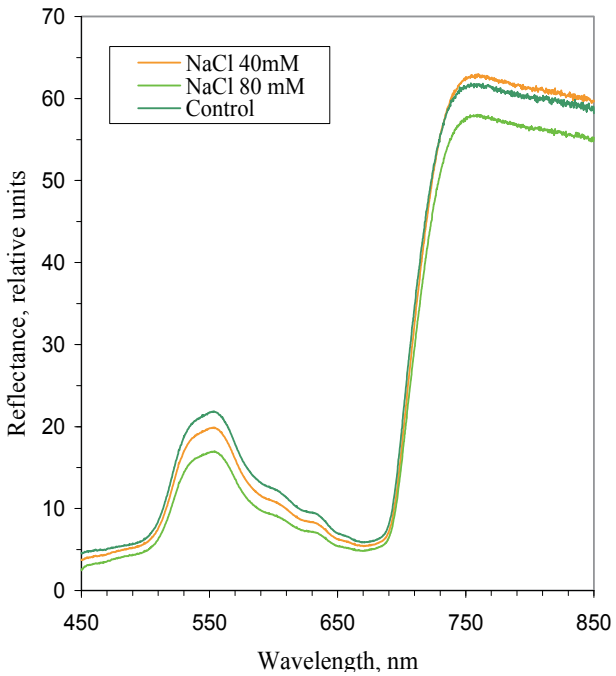


Fig. 14. Averaged spectral reflectance characteristics of control and treated with 40 mM and 80 mM NaCl nitrogen fixing soybean plants.

The second part of experiments was aimed at studying of the salinity effect on nitrogen fixing soybean plants. The measurements were performed on 25 areas (pixels) of randomly picked off leaves from each group of plants. The averaged SRC are displayed in Fig. 14 (Krezhova et al., 2009b). It is seen that the values of the SRC of the treated leaves with respect to control decrease significantly in both the green and red (450-680 nm) spectral ranges. In the red edge region it is observed a shift to longer wavelengths in correspondence with the increase in NaCl concentration applied, which is indicating occurrence of plant stress. For the case of 40 mM NaCl the shift is 2 nm while for the high salt concentration it is 6 nm. In the NIR range, the reflectance at low NaCl concentration increased while at high NaCl concentration it decreased due to the changes of water and nitrogen content in the leaves.

The results of application of the Student's t-criterion are presented in Table 6. Statistically significant are differences for which $p<0.05$ and only the differences in wavelengths λ_7 and λ_8 at 40 mM NaCl salinity are non-significant.

	40 mM NaCl		80 mM NaCl		Control
Pairs compared	p_{st}	mean	p_{st}	mean	mean
λ_1/λ_{1c}	<0.001	14.41	<0.001	12.10	15.47
λ_2/λ_{2c}	<0.001	18.96	<0.001	16.13	20.74
λ_3/λ_{3c}	<0.001	19.86	<0.001	16.93	21.74
λ_4/λ_{4c}	0.0027	5.46	<0.001	4.87	5.92
λ_5/λ_{5c}	<0.001	22.95	<0.001	20.76	25.22
λ_6/λ_{6c}	0.0298	44.98	<0.001	41.15	46.32
λ_7/λ_{7c}	0.3117	50.50	<0.001	46.27	51.15
λ_8/λ_{8c}	0.3048	62.61	0.001	57.98	61.76

Table 6. Significance p-level of the t-criterion in the case of 40 mM NaCl and 80 mM NaCl salinity.

Linear DA was implemented making use of one-dimensional spaces defined by each one of the wavelengths. Table 7 shows that probability levels p_{DA} coincide with that of the Student's t-criterion as the grouping variable consisted for each concentration of only two classes: control and treated plants (40 mM NaCl or 80 mM NaCl) at a given wavelength. At λ_7 and λ_8 (the same as for the t-criterion) statistically significant differences between SRC means for control and tread plants were not observed. Therefore the number of incorrectly classified cases was maximal at these wavelengths.

To illustrate better discriminative DA possibilities we performed DA in a two-dimensional space defined by the wavelengths λ_7 and λ_8, which manifested worst results when applied separately. Making use of data for concentration 40mM NaCl the p-level turned out to be <0.001 and the number of incorrectly classified cases was only 12.

The contents of the evaluated biochemical parameters - stress markers (phenols, proline, MDA, H_2O_2, free thiol groups, ratio of oxidized to total glutathione GSSG/TG) and content of chlorophyll a and b, are shown in Table 8. The values for control plants were taken as 100%. The 40 mM NaCl concentration lead to a phenol content decrease of about 19%, whereas the 80 mM NaCl salinity treatment provoked their much stronger decrease (by 59%). At 40 mM NaCl the proline content increased with 8%. A considerable increase of the proline (73%) was observed at 80 mM NaCl concentration. Under salinity stress most plant

	40 mM NaCl	40 mM NaCl	80 mM NaCl	80 mM NaCl
One dimensional spaces	p_{DA}	Number of incorrectly classified objects	p_{DA}	Number of incorrectly classified objects
λ_1	<0.001	14 from 50	<0.001	5 from 50
λ_2	<0.001	13 from 50	<0.001	5 from 50
λ_3	<0.001	13 from 50	<0.001	6 from 50
λ_4	0.0027	15 from 50	<0.001	7 from 50
λ_5	<0.001	12 from 50	<0.001	7 from 50
λ_6	0.0298	21 from 50	<0.001	8 from 50
λ_7	0.3117	22 from 50	<0.001	10 from 50
λ_8	0.3048	24 from 50	0.001	15 from 50

Table 7. Significance p-level of the linear DA in the case of 40 mM NaCl and 80 mM NaCl salinity of nitrogen fixing soybean plants.

species exhibit a remarkable increase in the proline content. MDA is an indicator of free radical production and potential to withstand and recover after membrane injury under stress. In our experiment, we established that 40 mM NaCl salinity induced a reduction of the MDA content with 17%, while 80 mM NaCl salinity lead to a decrease of the MDA content with 55%. When measuring the thiol groups, an increase of about 108% of their content was observed at concentration 40 mM NaCl. A much higher free thiol groups' content increase of 151% was established at concentration 80 mM NaCl. The enlargement of the content of free thiol groups is a marker for the presence of injures caused by salinity stress. The H_2O_2 levels in our experiment indicate that under the conditions of salinity stress the H_2O_2 content becomes larger; it became 126% at the high salt concentration of 80 mM NaCl. After salinity treatment of soybean plants the ratio of oxidized to total glutathione GSSG/TG increased with approximately 13% at the lower NaCl concentration and of the order of 26 % at the higher concentration. This brings to decreasing of the nitrogen fixing capacity and the plant sustainability.

Stress markers, pigments	Control	40 mM NaCl		80 mM NaCl	
Phenols, µmol/gDW	131.3 ±2.1	106.7 ±1.1	81% (19↓)	54.6±0.9	41.6% (59↓)
Proline, nmol/gDW	21.9±0.7	23.7±2.3	108% (8↑)	37.9±1.9	173% (73↑)
MDA, nmol/gDW	141.3±5.3	117.5±6.8	83.1% (17%↓)	62.7±5.6	44.3% (55↓)
H_2O_2 , µmol/gDW	2.8±0.06	3.41±0.04	121% (21↑)	6.35±0.02	226% (126↑)
Thiol Groups, µmol/gDW	1.03±0.02	2.15±0.07	(108% ↑)	2.59±0.08	(151%↑)
Chlorophyll a, mg/g FW	1.22±0.03	1.19±0.02	98%(2↓)	0.94±0.03	79% (21↓)
Chlorophyll b, mg/g FW	0.54±0.01	0.38±0.03	70.3%(30↓)	0.34±0.01	63% (37↓)
GSSG/TG, %	57.2	64.6	113%(13↑)	21.3	126% (26↑)

Table 8. Values of the biochemical parameters of nitrogen fixing soybean plants.

The decrease of the leaf chlorophyll content under salinity stress is a main phenomenon of the plant sensitivity. In our experiments, both the content of chlorophyll a and chlorophyll b decreased at the two salinity levels, the decrease under 80 mM NaCl being larger.

Concluding, it was found that the results from the implementation of the two methods, leaf spectral reflectance and biochemical analysis, revealed that both the NaCl concentrations

bring to salinity stress in the nitrogen fixing soybean plants and to decline of the biological nitrogen fixation. The red edge shift to longer wavelengths is an indicator of stress and is correlated with the decreased chlorophyll content and salinity rate.

The course of the averaged fluorescence spectra over 20 control and 20 salinity treated leaves of nitrogen fixing soybean plants used for the second part of the experiments is shown in Fig. 15 (Iliev et al., 2009b). All spectra were normalized against their second maximum. Changes in the spectra of treated leaves against the controls were significant in the forefront and in the spectral range between first and second maximums (680-740 nm).

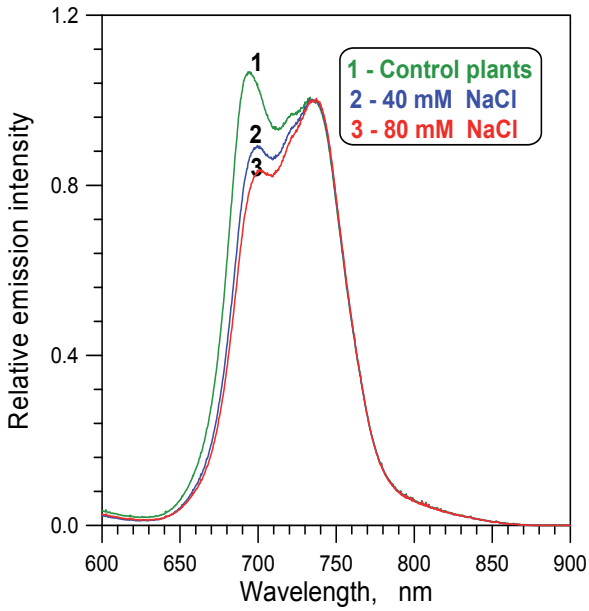


Fig. 15. Averaged fluorescence spectra of control and treated with 40 mM and 80 mM NaCl nitrogen fixing soybean plants.

The results of the Student's t-criterion and linear DA are displayed in Table 9 and Table 10. For analysis, five fluorescence values for each spectrum (from all 910) in characteristic wavelengths in the spectral range 600-900 nm were selected: λ_1 (at the middle of the forefront), λ_2 (first maximum), λ_3 (at the middle between first and second maximum), λ_4 (second maximum) and λ_5 (at the middle of rear slope), see Fig. 11.

Pairs	40 mM NaCl	40 mM NaCl	80 mM NaCl	80 mM NaCl	Control
compared	p_{st}	mean	p_{st}	mean	mean
λ_1/λ_{1c}	<0.001	911.6	<0.001	758.1	1345.8
λ_2/λ_{2c}	<0.001	2100.6	<0.001	1878.4	2672.0
λ_3/λ_{3c}	0.0016	2097.0	<0.001	1994.9	2332.9
λ_4/λ_{4c}	0.318	2436.9	0.2305	2408.1	2506.8
λ_5/λ_{5c}	0.480	1494.2	0.4919	1490.1	1525.1

Table 9. Significance p-level of the t-criterion for the set of amplitudes of the fluorescence spectra.

Statistically significant differences between data means at wavelengths λ_i and λ_{ic}, i = 1,…, 5 were established by the Student's t-criterion at $p<0.05$ for the data at the first three wavelengths and for both the NaCl concentrations. DA confirmed these findings in one dimensional spaces defined separately by each of the five wavelengths.

	40 mM NaCl	40 mM NaCl	80 mM NaCl	80 mM NaCl
One dimensional spaces	p_{DA}	Number of incorrectly classified objects	p_{DA}	Number of incorrectly classified objects
λ_1	<0.001	8 from 40	<0.001	4 from 34
λ_2	<0.001	10 from 40	<0.001	5 from 34
λ_3	0.0016	11 from 40	<0.001	8 from 34
λ_4	0.318	15 from 40	0.2305	14 from 34
λ_5	0.480	18 from 40	0.4919	12 from 34

Table 10. Significance p-level of the linear DA for the set of amplitudes of the fluorescence spectra.

The results revealed that the two NaCl concentrations applied produce statistically significant changes in the forefront of leaf fluorescence spectra of the nitrogen fixing soybean plants. This corresponds to the salinity stress disclosed by the biochemical parameters (stress markers and pigments) and by the spectral reflectance in the VIS and NIR spectral ranges evaluated for the same soybean plants. The two remote sensing techniques (chlorophyll fluorescence and spectral reflectance) independently detected that both the NaCl concentrations bring to salinity stress in the nitrogen fixing soybean plants.

6.2 Influence of the combined impact of stresses on soybean plants

The third part of experiments aimed at assessing of the impact of the single stress factor salinity and combined stress factors salinity and enhanced UV-B radiation on young nitrogen fixing soybean plants. Spectral data were taken for 25 areas (pixels) of leaves from each group of investigated plants. Fig. 16 shows the averaged spectral reflectance characteristics of the control group and salinity treated at two NaCl concentrations nitrogen fixing soybean plants (Krezhova et al., 2011a). It is seen that changes in SRC for the salinized plants are noticeable in all investigated spectral ranges. The effect of the salinity was manifested by decreasing of the values in the green (520-580 nm) and red (640-680 nm) ranges and increasing in the NIR (720-770 nm). The red edge position of the SRC at low and high NaCl treatments is shifted to longer wavelengths (1 nm and 5 nm, respectively). On some of the leaves treated with 80 mM NaCl concentration the necroses spots were observed (Fig. 9a).

The results from the statistical analysis applying Student's t-criterion are shown in Table 11. Statistically significant differences are obtained at $p<0.05$ in the two cases of salinity treatment with the exception of 40 mM NaCl concentration in λ_6 and λ_8, and 80 mM NaCl in λ_7.

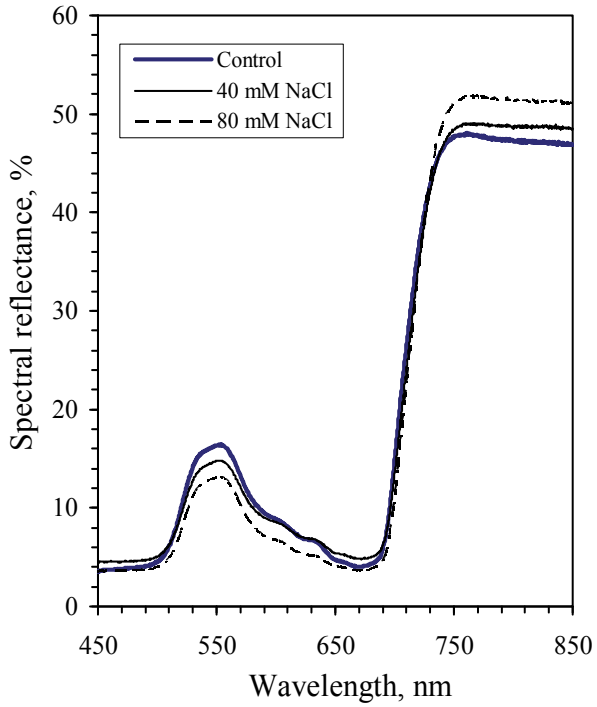


Fig. 16. Averaged spectral reflectance characteristics of control and treated with 40 mM and 80 mM NaCl nitrogen fixing soybean plants.

Pairs	Control	40 mM NaCl		80 mM NaCl	
compared	mean	p_{St}	mean	p_{St}	mean
λ_1/λ_{1c}	11.93	<0,001	10.92	<0.001	9.28
λ_2/λ_{2c}	15.74	<0.001	14.21	<0.001	12.61
λ_3/λ_{3c}	16.40	<0.001	14.83	<0.001	13.18
λ_4/λ_{4c}	4.08	<0.001	4.83	<0.001	3.65
λ_5/λ_{5c}	19.27	<0.001	17.23	<0.001	15.37
λ_6/λ_{6c}	35.59	0.029	33.59	0.001	33.60
λ_7/λ_{7c}	39.47	0.011	38.06	0.167	38.79
λ_8/λ_{8c}	47.91	0.152	48.88	0.007	51.73

Table 11. Significance p-level of the t-criterion in the cases of 40 mm NaCl and 80 mm NaCl salinity.

Fig. 17 shows the averaged SRC of leaves of plants from the second set of three groups including the control (treated only with UV-B radiation) and the other two groups on which the combined action of stresses, salinity at two concentrations + UV-B radiation, was applied. The values of the averaged spectral characteristics of treated leaves with respect to control decrease significantly in the green and red (520-660 nm), and NIR ranges. For these SRC it is observed an approaching of the red edge position nearer to the control (2 nm), which is an indicator for diminishing effect of the salinity stress. Averaged SRC after (80 mM NaCl + UV-B) treatment is very close to the one after (40 mM NaCl + UV-B) treatment.

The results from the statistical analysis concerning the combined impact of stresses to soybean plants are set out in Table 12. They indicate the increasing number of wavelengths in which the differences of the SRC against the control are non-significant. In the case of (40 mM NaCl + UV-B) the differences are non-significant in the whole NIR range. For (80 mM NaCl + UV-B) treatment they are non-significant in four of the wavelengths.

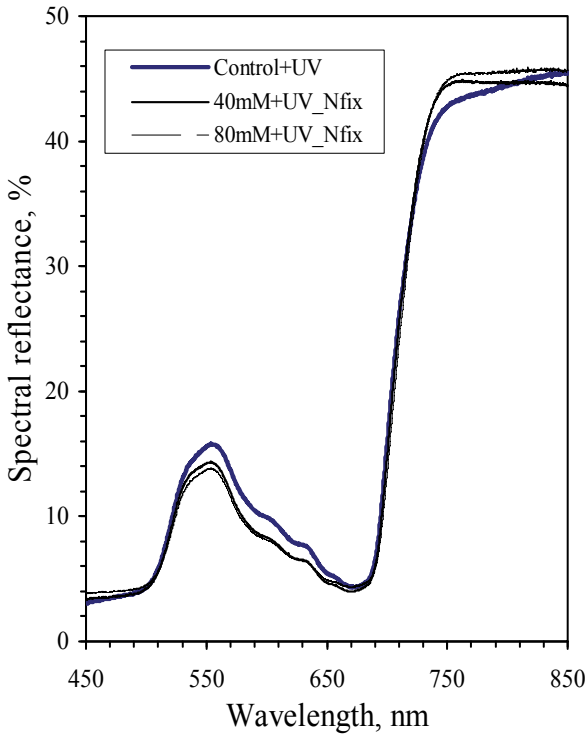


Fig. 17. Averaged spectral reflectance characteristics of control and treated nitrogen fixing soybean plants with: 40 mM NaCl + UV-B and 80 mM NaCl + UV-B.

Pairs compared	Control mean	40 mM NaCl +UV-B p_{St}	40 mM NaCl +UV-B mean	80 mM NaCl + UV-B p_{St}	80 mM NaCl + UV-B mean
λ_1/λ_{1c}	11.03	<0.001	10.38	<0.001	9.92
λ_2/λ_{2c}	14.63	<0.001	13.60	<0.001	13.06
λ_3/λ_{3c}	15.47	<0.001	14.30	<0.001	13.80
λ_4/λ_{4c}	4.41	<0.001	3.99	0.541	4.34
λ_5/λ_{5c}	19.79	0.006	19.71	<0.001	17.08
λ_6/λ_{6c}	33.09	0.733	33.37	0.067	32.23
λ_7/λ_{7c}	35.99	0.344	36.87	0.835	36.09
λ_8/λ_{8c}	43.22	0.214	44.80	0.102	45.29

Table 12. Significance p-level of the t-criterion in the cases of (40 mM NaCl +UV-B) and (80 mM NaCl + UV-B) treatment.

The effect of combined stress action of salinity + UV-B radiation was assessed by biochemical parameters: phenols, proline, malondialdehyde, and hydrogen peroxide. The contents of the evaluated stress markers are shown in Table 13.
The content of phenols decreased with 34.2% and 17% for low and high NaCl concentration, respectively. This is an indicator for decreasing of the action of salinity stress on the plants from the group treated with 80 mM NaCl +UV-B. The production of the MDA is a very sensitive stress marker. When its content is decreased it is a sign of the induction of sustainability to the applied stress. The increase in H_2O_2 and MDA contents was shown to be related to the amount of stress and well correlated with lipid membrane damage (Demiral & Turkan, 2005). In our experiment the amount of MDA increased for 40 mM NaCl + UV-B treatment with 4.5% and decreased in the other case with 7.6%. The hydrogen peroxide levels indicate that under the action of salinity stress + UV-B radiation the H_2O_2 content becomes higher with the increasing NaCl concentration. Hydrogen peroxide is known to damage cellular components and provoke structural modifications of proteins and lipids (Mandhania et al., 2006). Our experiment shows that a considerable increase of proline content takes place under the influence of salinity + UV-B at the lower concentration. By contrast, at 80 mM NaCl a significant decrease of proline by about 10.4% was observed.

Stress Markers, Pigments	Control +UV-B	40 mM NaCl + UV-B	80 mM NaCl +UV-B
Phenols (µM/g DW)	5.173 ±0.07	3.442±0.9 34.2%	4.289 ±0.04 17%
Proline (nmol/g DW)	8.942±0.87	22.955±1.9 156.7%	8.017±0.66 10.4%
MDA (nmol/g DW)	24.68±0.3	25.78±0.82 4.5%	22.80±0.66 7.6%
H_2O_2 (µM/g DW)	8.752±0.06	20.528±0.04 134.5%	50.903±0.02 481.6%

Table 13. Values of the biochemical parameters for the combined treatment of nitrogen fixing soybean plants.

Spectral data analysis revealed that in the case of the action of a single salinity stress on the young nitrogen fixing soybean plants there were statistically significant differences between the reflectance spectra of the leaves of control and treated plants at the two NaCl concentrations in all of the ranges examined with the exception of two wavelengths in NIR at 40 mM NaCl concentration and one wavelength at 80 mM NaCl concentration. In the SRC this effect was manifested by decreasing of the values in the green and red ranges and increasing in NIR. The results indicated that the plants were under conditions of stress which has been better pronounced for the higher NaCl concentration where necrotic and chlorotic lesions on the leaves have appeared due to chlorophyll degradation. This finding was established by evaluated biochemical markers of stress: phenols, proline, MDA, and H_2O_2.
In the case of treatment with (40 mm NaCl + UV-B) and (80 mm NaCl + UV-B) the results indicated that stress in the plants was also present but the influence of UV-B radiation after salinity reduces the consequences of salinity stress especially at the higher NaCl concentration. In the spectral reflectance characteristics this effect is manifested through an

increase of SRC values for treated plants and their approaching to the control SRC. This finding was established by the analysis of the evaluated biochemical stress markers.
The averaged fluorescence spectra of leaves of the same nitrogen fixing soybean plants treated with the two NaCl concentrations are displayed in Fig. 18 (Krezhova et al., 2011b). All spectra are normalized to their second maximum, which in this case is at $\lambda_4=\lambda_m=$ 738 nm. Changes in the spectra of treated plants (second and third groups) against the control (first group) increased with increasing NaCl amount and were observed in the spectral range spanning from arising forefront to the second maximum (640-740 nm). At their rear slope, the fluorescence spectra were with almost equal values.

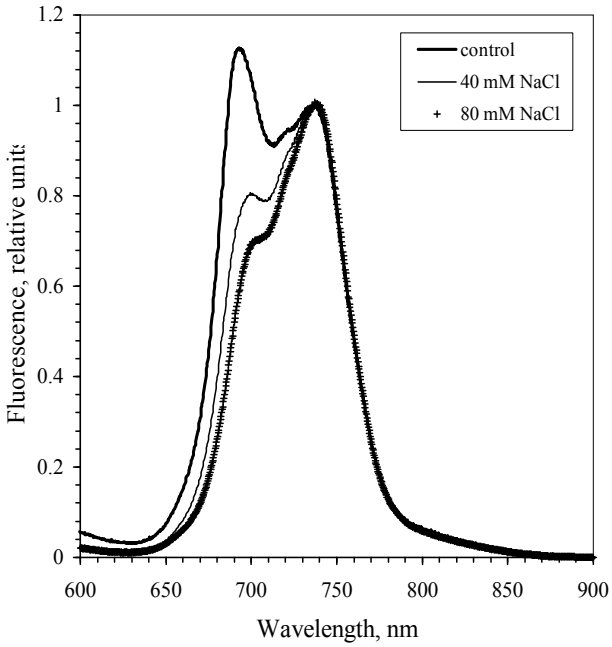


Fig. 18. Normalized average fluorescence spectra of control and treated with 40 mM NaCl and 80 mM NaCl nitrogen fixing soybean plants.

The results from the statistical analysis are presented in Table 14. Statistically significant differences were evaluated by Student's t-criterion at $p<0.05$ for the data at first three wavelengths for the two NaCl concentrations.

Pairs compared	Control mean	40 mM NaCl p_{St}	40 mM NaCl mean	80 mM NaCl p_{St}	80 mM NaCl mean
λ_1/λ_{1c}	0.335	<0.001	0.565	<0.001	0.221
λ_2/λ_{2c}	1.122	<0.001	0.770	<0.001	0.613
λ_3/λ_{3c}	0.912	<0.001	0.803	<0.001	0.735
λ_5/λ_{5c}	0.610	0.987	0.630	0.874	0.610

Table 14. Significance p-level of the t-criterion in the cases of 40 mM NaCl and 80 mM NaCl salinity of nitrogen fixing soybean plants.

The averaged normalized fluorescence spectra of the nitrogen fixing soybean plants subjected to combined stress factors (salinity + UV-B radiation) for the two NaCl concentrations are displayed in Fig. 19. It is observed that the differences between the spectra are less pronounced than those at single salinity stress impact. For the combined action of (40 mM NaCl+UV-B) the averaged spectrum has higher values than control due to induced tolerance to salinity stress from the action of UV-B radiation. For (80 mM NaCl+UV-B) treatment the values are lower and more close-set to the control due to the positive action of UV-B radiation expressed through the decreasing effect of salinity.

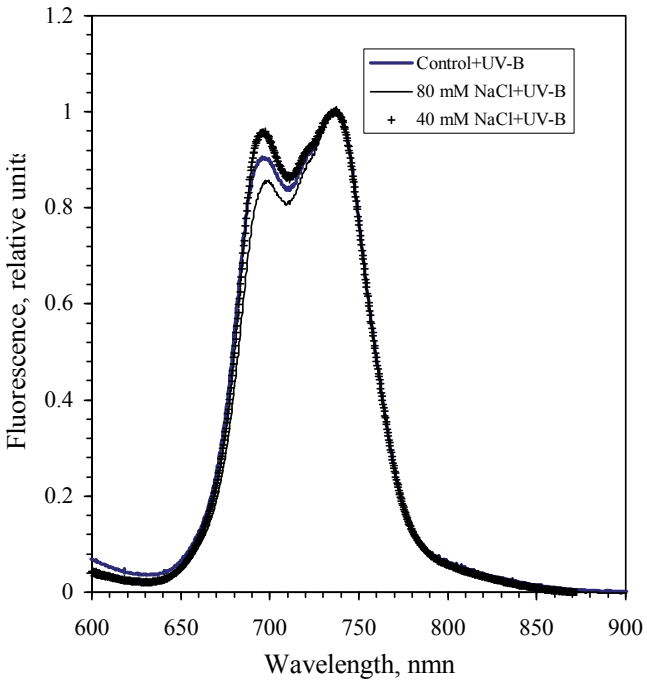


Fig. 19. Normalized average fluorescence spectra of control and treated with (40 mM NaCl + UV-B) and (80 mM NaCl + UV-B) nitrogen fixing soybean plants.

Table 15 shows the results from the Student's t-criterion for the combined stress treatment of the soybean plants. The differences against the control spectra are statistically significant at two of the investigated wavelengths for the two cases.

The results revealed that the salinity treatment at two NaCl concentrations significantly changed the fluorescence spectra of the nitrogen fixing soybean plants in the spectral range spanning from arising forefront to the second maximum (640-740 nm) due to salinity stress caused in the plants. In the case of initial salinity treatment of the plants followed by their irradiation with UV-B light the differences between the fluorescence spectra decreased due to the favourable effect of the UV-B light. These results were in compliance with the findings concerning the leaf spectral reflectance and biochemical parameters measured on the same nitrogen fixing soybean plants, treated with 40 mM and 80 mM NaCl concentrations and salinity + UV-B radiation.

Pairs compared	Control +UV-B mean	40 mM NaCl+UV-B p_{St}	40 mM NaCl+UV-B mean	80 mM NaCl+UV-B p_{St}	80 mM NaCl+UV-B mean
λ_1/λ_{1c}	0.440	0.967	0.441	0.832	0.380
λ_2/λ_{2c}	0.878	0.028	0.985	0.039	0.821
λ_3/λ_{3c}	0.836	0.010	0.873	0.041	0.811
λ_5/λ_{5c}	0.616	0.853	0.615	0.789	0.621

Table 15. Significance p-level of the t-criterion in the cases of 40 mM NaCl+UV-B and 80 mM NaCl+UV-B treatment for nitrogen fixing soybean plants.

7. Conclusions

In general this chapter illustrates the capability of hyperspectral remote sensing technologies in environmental stress studies and for timely making informed decisions in vegetation management. Our results demonstrate the potential of hyperspectral remote sensing methods, spectral reflectance and chlorophyll fluorescence in particular, for detection, discrimination and assessment of the effects of single and combined environmental stresses (salinity and enhanced UV-B radiation). A comparative analysis was performed between the changes of the leaf spectral reflectance characteristics, fluorescence spectra and values of the stress markers (phenols, malondialdehyde, thiol groups, proline, hydrogen peroxide), and chlorophyll content that were estimated by biochemical methods. As a result we obtained accurate and complementary benchmarking for plant responses to the environmental stress investigated.

The research and technological advances in the field of remote sensing have greatly improved the ability to detect and quantify environmental stresses that affect the productivity of agricultural vegetation. Hyperspectral remote sensing has made big progress with the advance of technique that has also increased the demand of its application for conducting, easily and without damage, rapid health condition assessments of vegetation cover. Further progress can be expected through extension of the inter-comparison of techniques, the parallel refinement of experimentally derived approaches and modelling, and by defining the optimum strategies reflecting different user requirements for scaling methods up to the canopy level. Modern management of agricultural resources is a complex endeavour that is now benefiting from a convergence of technical advances in information sciences, geographic positioning capabilities, and remote sensing systems. Using hyperspectral remote sensing as a tool for precision agriculture is a new field of research. Future work is necessary to further explore the full potential of this technology. The more programs and projects conducted in the recent years, the more models developed or improved for hyperspectral data processing to promote its applications.

8. References

Agati, G., Fusi, F., Mazzinghi, P., & Dipaolo, M. L. (1993). A simple approach to the evaluation of the reabsorption of chlorophyll fluorescence-spectra in intact leaves. *Journal of Photochemistry and Photobiology, B-Biology,* Vol. 17, pp. 163–171.

Agati, G., Cerovic, Z. G., & Moya, I. (2000). The effect of decreasing temperature up to chilling values on the in vivo f685/f735 chlorophyll fluorescence ratio in phaseolus

vulgaris and pisum sativum: the role of the photosystem i contribution to the 735 nm fluorescence band. *Photochemistry and Photobiology,* Vol. 72, No 1, pp. 75-84.

Alexieva, V., Ivanov, S., Mapelli, S., & Karanov, E. (2001). The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant Cell Environment*, Vol. 24, pp. 1337-1344.

Alia, A. J., Xua, J. L., Ismail, A. M., Fu, B. Y., Vijaykumar, C. H., Gao, M., Domingo, Y. M., Maghirang, J., Yu, R., Gregorio, S. B., Yanaghihara, S., Cohen, M., Carmen, B., Mackill, D., & Li, Z. K. (2006). Hidden diversity for abiotic and biotic stress tolerances in the primary gene pool of rice revealed by a large backcross breeding program. *Field Crops Research,* Vol. 97, pp. 66–76.

Ananyev, G., Kolber, Z. S., Klimov, D., Falkowski, P. G., Berry, J. A., Rascher U., Martin, R., & Osmond, B. (2005). Remote sensing of heterogeneity in photosynthetic efficiency, electron transport and dissipation of excess light in Populus deltoides stands under ambient and elevated CO_2 concentrations, and in a tropical forest canopy, using a new laser-induced fluorescence transient device. *Global Change Biology,* Vol. 11, pp. 1195–1206.

Arida, P. A. K., & Das, A. B. (2005). Salt tolerance and salinity effects on plants: a review. *Ecotoxicology and Environmental Safety*, Vol. 60, pp. 324-349.

Arnon, D. I. (1949). Copper enzymes in isolated cloroplasts. Polyphenoloxidase in Beta vulgaris, *Plant Physiology*, Vol. 24, pp. 1-15.

Baker, N. R. (2008). Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annual Review of Plant Biology,* Vol. 59, pp. 89–113.

Barker, D. W., & Sawyer, J. E. (2005). Nitrogen Application to Soybean at Early Reproductive Development. *Agronomy Journal*, Vol. 97, pp 615–619.

Bates, L., Waldren, R., & Teare, I. (1973). Rapid determination of free proline for water-stress studies. *Plant Science,* Vol. 39, pp. 205-207.

Blackburn, G. A. (2007). Hyperspectral remote sensing of plant pigments. *Journal of Experimental Botany,* Vol. 58, pp. 855-867.

Brankova, L., Ivanov, S., Alexieva, V., & Karanov, E. (2005). Salt induced alteration in the levels of some oxidative parameters and unspecific defence compounds in leaves of two plant species (cotton and bean) with different sensitivity to salinity. *Compt. rend. Acad. bulg. Sci.*, Vol. 58, No 11, pp. 1307-1312.

Broge, N. H., & Leblanc, E. (2001). Comparing prediction power and stability of broadband and hyperspectral vegetation indices for estimation of green leaf area index and canopy chlorophyll density. *Remote Sensing of Environment,* Vol. 76, pp. 56–172.

Buschmann, C., Langsdorf, G., & Lichtenthaler, H. K. (2000). Imaging of the blue, green, and red fluorescence emission of plants: an overview. *Photosynthetica,* Vol. 38, pp. 483–491.

Buschmann, C. (2007). Variability and application of the chlorophyll fluorescence emission ratio red/far-red of leaves. *Photosynthesis Research*, Vol. 92, pp. 261–271.

Campbell, P. K. E., Middleton, E. M., Mcmurtrey, J. E., Corp, L. A., & Chappelle, E. W. (2007). Assessment of vegetation stress using reflectance or fluorescence measurements. *Journal Environmental Quality,* Vol. 36, pp. 832-845.

Carter, G.A., & Miller, R. L. (1994). Early detection of plant stress by digital imaging within narrow stress-sensitive wavebands. *Remote Sensing of Environment*, Vol. 50, No 3, pp. 295–302.

Carter, G.A. (1998). Reflectance wavebands and indices for remote estimation of photosynthesis and stomatal conductance in pine canopies, *Remote Sensing of Environment,* Vol. 63, pp. 61–72.

Cecchi, G., Mazzinghi, P., Pantani, L., R, Tirelli, D., & Deangelis, P. (1994). Remote sensing of chlorophyll a fluorescence of vegetation canopies. 1. Near and far-field measurement techniques. *Remote Sensing of Environment,* Vol. 47, pp. 18–28.

Cechin, I., Corniani, N., Terezinha De Fátima Fumis, & Cataneo, A. C. (2008). Ultraviolet-B and water stress effects on growth, gas exchange and oxidative stress in sunflower plants. *Radiation and Environmental Biophysics,* Vol. 47, No 3, pp. 405-413.

Chaerle, L.; Hagenbeek, D., De Bruyne E., Valcke, R., & Van Der Straeten, D. (2004). Thermal and chlorophyll-fluorescence imaging distinguish plant–pathogen interactions at an early stage. *Plant Cell Physiology*, Vol. 45, pp. 887–896.

Chadalavada, S., Rajendrakumar, B., Reddy, & Reddy, V. A. (1994). Proline-protein interactions: Protection of structural and functional integ rity of M4 lactate dehydrogenase, *Biochemical and Biophysical Research Communications*, Vol. 201, pp. 957-963.

Chatzistathisa, T. A., Papadakisa, I. E., Theriosa, I. N., Giannakoulab, A., & Dimassi K. (2011). Is chlorophyll fluorescence technique a useful tool to assess manganese deficiency and toxicity stress in olive plants?. *Journal of Plant Nutrition*, Vol. 34, No 1, pp. 98 – 114.

Clay, D. E., Chang K. K. J., Clay, S. A., & Dalsted K. (2006). Characterizing water and nitrogen stress in corn using remote sensing. *Agronomy Journal*, Vol. 98, pp. 579-587.

Coops, N., Stone, C., Culvenor, D. S., Chisholm, L. A., & Merton, R. N. (2003). Chlorophyll content in eucalypt vegetation at the leaf and canopy scales as derived from high resolution spectral data. *Tree Physiolo*gy, Vol. 23, pp. 23-31.

Corp, L. A., Middleton, E. M., McMurtrey, J. E., Campbell, P. K. E., & Butcher, L. M. (2006). Fluorescence sensing techniques for vegetation assessment. *Applied Optics,* Vol. 45, pp. 1023–1033.

Dau, H. (1994). Short-term adaptation of plants to changing light intensities and its relation to photosystem II photochemistry fluorescence emission. *Journal of Photochemistry and Photobiology B-Biology,* Vol. 26, pp. 3–27.

Dawson, T. P., & Curran, P. J. (1998). A new Technique for Interpolating the Reflectance Red Edge Position. *International Journal of Remote Sensing*, Vol. 19, pp. 2133-2139.

Dekker, J.P., Hassoldt, A., Pettersson, A., van Roon, H., Groot, M. L., & van Grondelle, R. *(*1995). On the nature of the F695 and F685 emission of Photosystem II. *In:* Mathis P, *editor. Photosynthesis: from light to biosphere,* Dordrecht, The Netherlands*:* Kluwer Academic Publishers,. pp. 53-56.

Delalieux, S., Auwerkerken, A., Verstraeten, W. W., Somers, B., Valcke, R., Lhermitte, S., Keulemans, J. & Coppin, P. (2009a). Hyperspectral Reflectance and Fluorescence Imaging to Detect Scab Induced Stress in Apple Leaves. *International Journal of Remote Sensing,* Vol. 1, pp. 858-874

Delalieux, S., Somers, B., Verstraeten, W.W., van Aardt, J.A.N., Keulemans, W., & Coppin, P. (2009b). Hyperspectral indices to diagnose leaf biotic stress of apple plants considering leaf phenology. *International Journal of Remote Sensing,* Vol. 30, pp. 1887–1912.

Delalieux, S., van Aardt, J. A. N., Keulemans, W., Schrevens, E., & Coppin, P. (2007). Detection of biotic stress (Venturia inaequalis) in apple trees using hyperspectral data: Non-parametric statistical approaches and physiological implications. *Europian Journal of Agronomy,* Vol. 27, pp. 130–143.

Delauney, A., & Verma, D. (1993). Proline biosynthesis and osmoregulation in plants. *Plant Journal,* Vol. 4, pp. 215-223.

Demiral, T., & Turkan, I. (2005). Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. *Environmental and experimental botany*, Vol. 53, pp. 247–257.

Edreva, A., & Hadjiska, E. (1984). On the determination of the content of sulphydryc (thiol) groups in plant material, (in Bulgarian). *Plant Fiziology,* Vol. X , No 3, pp. 73-82.

El-Nahry, A. H., & Hammad, A. Y. (2009). Assessment of Salinity Effects and Vegetation Stress, West of Suez Canal, Egypt Using Remote Sensing Techniques, *Journal of Applied Sciences Research*, Vol. 5, No 3, pp. 316-322.

FAOSTAT Database on Agriculture. (2011). http://www.fao.org/library/

Filella, I., & Penuelas, J. (1994). The red edge position and shape as indicators of plant chlorophyll content, biomass and hydric status. *International Journal of Remote Sensing,* Vol. 15, No 7, pp. 1459-1470.

Fitzgerald, G. J., Rodriguez D., Christensen, L. K., Belford, R., Sadras, V. O., & Clarke, T. R. (2006). Spectral and thermal sensing for nitrogen and water status in rainfed and irrigated wheat environments. *Precision Agriculture,* Vol. 7, pp. 233-248.

Flamenco-Sandoval, A., Ramos, M. M, & Masera, O. R. (2007). Assessing implications of land-use and land-cover change dynamics for conservation of a highly diverse tropical rain forest. *Biological comservation,* Vol. 138, pp. 131–145.

Flowers, T. (2004). Improving crop salt tolerance. *Journal of Experimental Botany*, Vol. 55, pp. 307–319.

Franck. F., Juneau, P., & Popovic, R. (2002). Resolution of the Photosystem I and Photosystem II contributions to chlorophyll fluorescence of intact leaves at room temperature. *Biochim. Biophys. Acta*. Vol. 1556, No 2-3, pp. 239-46.

FAOSTAT http://www.faostat.org, Food and Agriculture Organization (FAO) of the United Nations.

Gielen, B., Vandermeiren, K., Horemans, N., D'Haese, D. D., Serneels, R., & Valcke, R. (2006). Clorophyll a fluorescence imaging of ozone-sressed *Brassica napus L.* plants differing in glucosinolate concentrations. *Plant Biology,* Vol. 8, pp. 698–705.

Gitelson, A. A., Gritz, Y., & Merzlyak, M. N. (2003). Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves. *Journal of Plant Physiology,* Vol. 160, pp. 271-282.

Gitelson, A. A., Keydan, G. P., & Merzlyak, M. N. (2006). Three-band model for noninvasive estimation of chlorophyll, carotenoids, and anthocyanin contents in higher plant leaves. *Geophysical Research Letters,* Vol. 33, L11402.

Goldsmith, P. D. (2008). Economics of Soybean Production, Marketing and Utilization. *In Soybeans: Chemistry, Production, Processing, and Utilization,* Eds.

Govender, M., Dye, P. J., Weiersbye, I. M., Witkowski, E. T. F., & Ahmed, F. (2009). Review of commonly used remote sensing and ground-based technologies to measure plant water stress, *Water SA*, Vol. 35 No. 5, pp. 741-752, http://www.sabinet.co.za.

Govindjee (1995). Sixty-three years since Kautsky: Chlorophyll a fluorescence. *Aust. J. Plant Physiol.* Vol. 22, pp. 131-160.

Gray, S. B., Dermody, O., & DeLucia, E. H. (2010). Spectral reflectance from a soybean canopy exposed to elevated CO_2 and O_3. *Journal of Experimental Botany,* pp. 1-10, doi:10.1093/jxb/erq244.

Gronwald, J. W., & Plaisance, K. L. (1998). Isolation and Characterization of Glutathione S-Transferase Isozymes from Sorghum. *Plant Physiology,* Vol. 117, No. 3, pp. 877–892.

Habash, D. Z., Massiah, A. J., Rong, H. L., Wallsgrove, R. M., & Leigh, R. A. (2001). The role of cytosolic glutamine synthetase in wheat. *Annual Applied Biology,* Vol. 138, pp. 83–89.

Haboudane, D., Miller, J. R., Tremblay, N., Zarco-Tejada, P. J., & Dextraze, L. (2002). Integrated narrow-band vegetation indices for prediction of crop chlorophyll content for application to precision agriculture. *Remote Sensing of Environment,* Vol. 81, pp. 416–426.

Haboudane, D., Miller, J. R., Pattey, E., Zarco-Tejada, P.J., & Strachan, I. B. (2004). Hyperspectral vegetation indices and novel algorithms for predicting green LAI of crop canopies: modeling and validation in the context of precision agriculture. *Remote Sensing of Environment,* Vol. 90, pp. 337–352.

Halliwell, B., & Gutteridge, J. (2002). *Free radicals in biology and medicine.* Third edition, Oxford Univ. Press.

Hardarson, G., & Atkins, C. (2003). Optimising biological N_2 fixation by legumes in farming systems. *Plant Soil*, Vol. 252, pp. 41–54.

Hatfield, J., Gitelson, A., Schepers, J. & Walthall, C. (2008). Application of Spectral Remote Sensings for Agronomic Decision. *Celebrate the Centennial [A Supplement to Agronomy Journal*], pp. 117-131.

Haugard, N. (2000). Reflections on the role of the thiol group in biology. *Annual NY Academy Science*, Vol. 899, pp. 148-158.

Heisel, F., Sowinska, M., Miehe, J.A., Lang, M., & Lichtenthaler, H. K. (1996). Detection of nutrient deficiencies of maize by laser induced fluorescence imaging. *Journal of Plant Physiology,* Vol. 148, pp. 622-631.

Helrigel, H. (1898). Arbeiten der Deutschen Landwirtschafts Gesellschaft, Vol. 34, pp. 7-19.

Holden, H., & LeDrew, E. (1998). Spectral discrimination of healthy and non-healthy corals based on cluster analysis, principal component analysis, and derivative spectroscopy. *Remote Sensing of Environment,* Vol. 65. No 2, pp. 217-224.

Huang, J. F., & Apan, A. (2006). Detection of Sclerotinia rots disease on celery using hyperspectal data and partial least squares regression. *Journal of Spatial Science*, Vol. 52, No. 1, pp. 131-144.

Huete, A., Didanm K. Miura, T., Rodriguez, E. P., Gao, X., & Ferreira, L. G. (2002). Overview of the radiometric and biophysical performance of the MODIS vegetation indices. *Remote Sensing of Environment,* Vol. 83, pp. 195–213.

Hungria, M., Franchini, J., Campo R., & Graham, P. (2005). The importance of nitrogen fixation to soybean cropping in South America. In: D. Werner and W. Newton, Eds, *Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment,* Springer, Netherlands (2005), pp. 25–42.

Hungria, M., Franchini, J., Campo R., Crispino, C., Moraes, J., Sibaldelli, R., Mendes, I., & Arihara, J. (2006). Nitrogen nutrition of soybean in Brazil: contributions of

biological N_2 fixation and N fertilizer to grain yield. *Canadian Journal of Plant Science,* Vol. 86, pp. 927–939.

http://www.OceanOptics.com

http://www.statsoft.com

Ibanez, F., Taurian, T., Angelini, J., Tonelli, M. L., & Fabra, A. (2008). Rhizobium giardinii and Rhizobium tropici isolated from peanut nodules in Central Argentina. *Soil Biology and Biochemistry*, Vol. 40, pp. 537–539.

Iliev, I., Krezhova, D., Yanev, T., Kirova, E., & Alexieva, V. (2009a). Response of chlorophyll fluorescence to salinity stress on the early growth stage of the soybean plants (Glycine max L.). *IEEE Proceedings of International conference of Recent Advances in Space Technologies,* Istanbul, Turkey, June 2009, pp. 403-407.

Iliev, I., Krezhova, D., Yanev, T., & Kirova, E. (2009b). Effects of salinity on chlorophyll fluorescence of nitrogen fixing soybean plants *(Glycine max L.)*, *AIP Proceedings of 7th General Conference of the Balkan Physical Union,* ISSN: 0094-243X, Alexandroupolis, Greece, September 2009,pp. 606-611.

Janetos, A. C., & Justice, C. O. (2000). Land cover and global productivity: A measurement strategy for the NASA programme. *International Journal of Remote Sensing,* Vol. 21, No. 6, pp. 1491-1512.

Jia Guo, W. H., & Wang, M. H. (2008). Ultraviolet and environmental stresses involved in the induction and regulation of anthocyanin biosynthesis: A review. *African Journal of Biotechnology,* Vol. 7, No. 25, pp. 4966-4972.

Jogeswar, G., Pallela, R., Jakka, N. M., Reddy, P. S., Venkateswara R. J., Sreenivasulu, N., & Kavi Kishor, P. B. (2006). Antioxidative response in different sorghum species under short term salinity stress. *Acta Physiol. Plantarum*, Vol. 28, No. 5, pp. 465-475.

Jones, H. G. (2007). Monitoring plant and soil water status: established and novel methods revisited and their relevance to studies of drought tolerance. *Journal of Experimental Botany*, Vol. 58, pp.119-130.

Kavi Kishor, P. B., Sangam, S., Amrutha, R. N., Laxmi, P. S., Naidu, K. R., Rao, K. R. S. S., Sreenath Rao, Reddy, K. J., Theriappan, P. & Sreenivasulu, N. (2005). Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plant growth and abiotic stress tolerance. *Current Science*, Vol. 88, pp. 424-438.

Khan, M. L., Singha, K. S. H., & Panda, S. (2002). Changes in antioxidant levels in Oryza sativa L. roots subjected to NaCl-salinity stress. *Acta Physiology Plant,* Vol. 24, pp. 145–148.

Kolber, Z., Klimov, D., Ananyev, G., Rascher, U., Berry, J., & Osmond, B. (2005). Measuring photosynthetic parameters at a distance: laser induced fluorescence transient (LIFT) method for remote measurements of photosynthesis in terrestrial vegetation. *Photosynthesis Research,* Vol. 84, pp. 121–129.

Kramer, G., Norman, H., Krizek, D., & Mirecki, R. (1991). Influence of UV-B radiation on polyamines, lipid peroxidation and membrane lipids in cucumber. *Phytochemistry,* Vol. 30, pp. 2101-2108.

Krezhova, D., Yanev, T., Alexieva, V., & Ivanov S. (2005). Early detection of changes in leaf reflectance of pea plants (Pisum sativum L.) under herbicide action. *Proceedings of 2nd International conference of Resent Advances in Space Technologies,* Istanbul, Turkey, June 2005, pp. 636-641.

Krezhova, D., Alexieva, V., Yanev, T., & Ivanov, S. (2007). Remote Sensing Study of the Influence of Herbicides fluridone and acifluorfen on the Spectral Reflectance of Pea Plant Leaves (Pisum sativum L.), *IEEE Proceedings of 3rd International conference of Recent Advances in Space Technologies,* Istanbul, Turkey, 2007, pp. 326-330.

Krezhova, D., Kirova, E., Iliev, I., & Yanev, T. (2009a). Assessment of the effect of salinity on the early growth stage of soybeam plants (Glycine max L.). *IEEE Proceedings of International conference of Recent Advances in Space Technologies,* Istanbul, Turkey, June 2009, pp. 397-402.

Krezhova, D., Kirova, E., Yanev, T., & Iliev, I. (2009b). Effects of salinity on leaf spectral reflectance and biochemical parameters of nitrogen fixing soybean plants (Glycine max L.), *AIP Proceedings of 7th General Conference of the Balkan Physical Union,* ISSN: 0094-243X, Alexandroupolis, Greece, September 2009, pp. 694-699.

Krezhova, D., & Kirova, E. (2011a). Hyperspectral remote sensing of the impact of environmental stresses on nitrogen fixing soybean plants (Glycine max L.), *IEEE proceedings* of *5th International Conference of Recent Advances in Space Technologies,* ISBN: 978-1-4244-9617-4, Istanbul, Turkey, June 2011, pp. 172-177.

Krezhova, D., Iliev, I., & Kirova, E. (2011b). Chlorophyll fluorescence of nitrogen fixing soybean plants (Glycine max L.), *IEEE proceedings of 5th International Conference of Recent Advances in Space Technologies,* ISBN: 978-1-4244-9617-4, Istanbul, Turkey, June 2011, pp. 184-187.

Kobayashi, T., Kanda, E., Kitada, K., Ishiguro, K., & Torigoe, Y., (2001). Detection of rice panicle blast with multispectral radiometer and the potential of using airborne multispectral scanners. *Phytopathology*, Vol. 91, No. 3, pp. 316-323.

Li H., Payne, W.A., Michels, G. J., & Charles, M. R. (2008). Reducing plant abiotic and biotic stress: Drought and attacks of greenbugs, corn leaf aphids and virus disease in dryland sorghum. *Environmental and Experimental Botany,* Vol. 63, pp. 305–316.

Li G., Wan S., Zhou J., Yang Z., & Qin, P. (2010). Leaf chlorophyll fluorescence, hyperspectral reflectance, pigments content, malondialdehyde and proline accumulation responses of castor bean (Ricinus communis L.) seedlings to salt stress levels. *Industrial Crops and Products*, Vol. 31 pp. 13–19.

Lichtenthaler, H. K., Alexander, A. C., Marek, M. V., Kalina, J. & Urban, O. (2007). Differences in pigment composition, photosynthetic rates and chlorophyll fluorescence images of sun and shade leaves of four tree species. *Plant Physiology and Biochemistry*, Vol. 45, No 8, pp. 577-588.

Liu, Z. Y., Huang, J.F., Tao, R.X., & Zhang, H. Z. (2008). Estimating rice brown spot disease severity based on principal component analysis and radial basis function neural network. *Spectroscopy and Spectral Analysis*, Vol. 28, No. 9, pp. 2156-2160.

Mandhania, S., Madan, S. & Sawhney, V. (2006). Antioxidant defense mechanism under salt stress in wheat seedlings. *Biologia Plantarum,* Vol. 50, No 2, pp. 227-231.

Masclaux, C., Quillere, I., Gallais, A., & Hirel, B. (2001). The challenge of remobilization in plant nitrogen economy. A survey of physio-agronomic and molecular approaches. *Annual Applied Biology*, Vol. 138, pp. 69–81.

Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany,* Vol. 51, No 345, pp. 659–668.

Masuda, T., & Goldsmith, P. D. (2009). World Soybean Production: Area Harvested, Yield, and Long-Term Projections. *International Food and Agribusiness Management Review,* Vol. 12, No. 4, pp.143-162.

McDonald, A. J., Gemmell F. M., & Lewis, P.E. (1998). Investigation of the utility of spectral vegetation indices for determining information on coniferous forests. *Remote Sensing of Environment,* Vol. 66, pp. 250–272.

Measures, R. M., Houston, W., & Bristow, M. (1973). Development and field-tests of a laser fluorosensor for environmental monitoring. *Canadian Aeronautics and Space Journal,* Vol. 19, pp. 501–506.

Meyer, S., Cartelat, A., Moya, I., & Cerovic, Z. G. (2003). UV-induced bluegreen and far-red fluorescence along wheat leaves: a potential signature of leaf ageing. *Journal of Experimental Botany,* Vol. 54, pp. 757–769.

Miflin, B. (2000). Crop improvement in the 21st century. *Journal of Experimental Botany*, Vol. 51, pp. 1–8.

Mishra, K. B., & Gopal, R. (2008). Detection of nickel-induced stress using laser-induced fluorescence signatures from leaves of wheat seedlings. *International Journal of Remote Sensing*, Vol. 29, No 1, pp. 157-183.

Munns, R. (2002). Comparative physiology of salt and water stress. *Plant, Cell and Environment,* Vol. 25, pp. 239-250.

Nedbal, L., & Koblızek, M. (2006). Chlorophyll fluorescence as a reporter on in vivo electron transport and regulation in plants. In: Grimm B., Porra R., Rüdiger W, Scheer H., eds. *Biochemistry and biophysics of chlorophylls.* Dordrecht, The Netherlands: Springer, pp. 507–519.

Nedbal, L., Soukupova, J., Kaftan, D., Whitmarsh J., & Trtílek M. (2000). Kinetic imaging of chlorophyll fluorescence using modulated light. *Photosynthesis Research,* Vol. 66, pp. 3–12.

Noctor G., Gomez, L., Vanacker, H., & Foyer, C. (2002). Interactions between biosynthesis,compartmentation and transport in the control of glutathione homeostasis and signaling. *Journal of Experimental Botany*, Vol. 53, No 372, pp. 1283-1304.

Norval, M., Cullen, A. P., de Gruijl, F. R., Longstreth, J., Takizawa, Y., Lucas, R. M., Noonan, F. P. & van de Leun, J. C. (2006). The effects on human health from stratospheric ozone depletion and interactions with climate change. In: Environmental Effects of Ozone Depletion and Its Interactions with Climate Change: 2006 Assessment. The Environmental Effects Assessment Panel Report. United Nations Environmental Program, Nairobi, pp. 25-63.

Omasa, K., Shimazaki, K. I., Aiga, I., Larcher, W., & Onoe, M. (1987). Image-analysis of chlorophyll fluorescence transients for diagnosing the photosynthetic system of attached leaves. *Plant Physiology*, Vol. 84, pp. 748–752.

Osborne, S. L., & Riedell, W. E. (2011). Impact of low rates of nitrogen applied at planting on soybean nitrogen fixation. *Journal of Plant Nutrition*, Vol. 34, No. 3, pp. 436 – 448.

Ouyang, B., Yang, T., Li, H., Zhang, L., Zhang, Y., Zhang, J., Fei, Z., & Ye, Z. (2007). Identification of early salt stress response genes in tomato root by suppression subtractive hybridization and microarray analysis. *Journal of Experimental Botany*, Vol. 58, No. 3, pp. 507–520.

Papageorgiou, G. C., & Govindjee, J. (2004). Chlorophyll a fluorescence, a signature of photosynthesis. *Advances in Photosynthesis and Respiration*; Springer: Dordrecht, The Netherlands.

Pu, R., Gong, P., Biging, G., & Larrieu, M. R. (2003). Extraction of Red Edge Optical Parameters from Hyperion Data for Estimation of Forest Leaf Area Index. *IEEE Transactions on geoscience and remote sensing*, Vol. 41, No. 4, pp. 916-921.

Qi, J., Kerr, Y. H., Moran, M. S., Weltz, M., Huete, A. R., Sorooshian, S., & Bryant, R. (2000). Leaf area index estimates using remotely sensed data and BRDF models in a semiarid region. *Remote Sensing of Environment,* Vol. 73, pp. 18–30.

Raimondi, V., Palombi, L., Lognoli, D., Cecchi, G., & Masotti, L. (2007). Design and development of a new high speed performance fluorescence imaging lidar for the diagnostics of indoor and outdoor cultural heritage. Proceedings *of LACONA VII: 7th International Conference on Lasers in the Conservation of Artworks*, Madrid, September 2007. Taylor and Francis.

Ray, J. D., Heatherly, G., & Fritschi, F. B. (2005). Influence of large amounts of nitrogen on nonirrigated and irrigated soybean. *Crop Science*, Vol. 46, pp. 52–60.

Rice-Evans C., Miller, N. & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trend in Plant Science*, Vol. 2, No 4, pp. 152-159.

Rock, D. C., (2000). Pathways to abscisic acid-regulated gene expression. *New Phytology*, Vol. 148, pp. 357-396.

Rolando, C. A., & Little, K. M. (2003). Using chlorophyll fluorescence to determine stress in Eucalyptus grandis seedlings. *South African Forestry Journal*, Vol. 197, pp. 5-12.

Saikia, S. P., & Jain, V. (2007). Biological nitrogen fixation with non-legumes: An achievable target or a dogma?. *Current science,* Vol. 92, No 3, pp. 317-322.

Saito, Y., Matsubara, T., Koga, T., Kobayashi, F., Kawahara, T. D., & Nomura, A. (2005). Laser-induced fluorescence imaging of plants using a liquid crystal tunable filter and charge coupled device imaging camera. *Review of Scientific Instruments,* Vol. 76, pp. 106–103.

Salehi, M., Foroozan, S., Kazem, P., & Heidari-Sharifabad, H. (2008). The Effect of Salinity on the Nitrogen Fixation in 4 Cultivars of Medicago sativa L. in the Seedling Emergence Stage. *Research Journal of Agriculture and Biological Sciences,* Vol. 4, No 5, pp. 413-415.

Sall, K., & Sinclair, T. R. (1991). Soybean genotypic differences in sensitivity of symbiotic nitrogen fixation to soil dehydration. *Plant Soil*, Vol. 133, pp. 31–37.

Salvagiotti, F., Cassman, K. G., Specht, J. E., Walters, D. T., Weiss, A., & Dobermann, A. (2008). Nitrogen uptake, fixation and response to fertilizer N in soybeans: A review. *Field Crops Research*, pp. 1-18, doi: 10.1016/ j.fcr.2008.03.001.

Schreiber, U., Schliwa, U., & Bilger, W. (1986). Continuous recording of photochemical and nonphotochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynthesis Research,* Vol. 10, pp. 51–62.

Shuanggen, J. & Komjathy, A. (2010). GNSS Reflectometry and Remote Sensing: New Objectives and Results. *Advances in Space Research,* Vol. 46 , pp. 111–117.

Sims, D. A., & Gamon, J. A. (2002). Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment*, Vol. 81, pp. 337-354.

Sinclair, T. R., Purcell, L. C., Vadez, V., Serraj, R., King, C. A., & Nelson, R. (2000). Identification of soybean genotypes with N_2 fixation tolerance to water deficit. *Crop Science,* Vol. 40, pp. 1803–1809.

Sinclair, T. R., Purcell, L. C., & Sneller, C. H. (2004). Crop transformation and the challenge to increase yield potential. *Trends in Plant Science*, Vol. 9, No 2, pp. 70-75.

Singha, A. L., Hariprasannaa, K., Chaudharia, V., & Gora, H. K. (2010). Identification of groundnut (arachis hypogaea l.) cultivars tolerant of soil salinity. *Journal of Plant Nutrition*, Vol. 33, No 12, pp. 1733–1743.

Sivakumar, P., Sharmila, P., & Pardha Saradhi P. (2000). Proline alleviates salt stress induced enhancement in ribulose 1,5-bisphosphate oxigenase activity. *Biochemical and Biophysical Research Communications*, Vol. 279, pp. 512-515.

Smith, R. B. (2001a). Introduction to remote sensing of the environment. www.microimages., (Accessed 24/03/2006).

Smith, R. B. (2001b). Introduction to hyperspectral imaging. www.microimages.com, (Accessed 11/03/2006).

Stober, F., Lang, M., & Lichtenthaler, H. K. (1994). Blue-green, and red fluorescence emission signatures of green etiolated and white leaves. *Remote Sensing of Environment,* Vol. 47, 65–71.

Steele, M., Gitelson, A. A., & Rundquist, D. (2008a). Nondestructive Estimation of Leaf Chlorophyll Content in Grapes, *American Society for Enology and Viticulture,* Vol. 59, pp. 299-305.

Steele, M., Gitelson, A. A., & Rundquist, D. (2008b). A Comparison of two techniques for nondestructive measurement of chlorophyll content in grapevine leaves. *Agronomy Journal,* Vol. 100, pp. 779-782.

Sun P., Grignetti A., Liu S., Casaccia R., Salvatori R., Pietrini F., Loreto F., & Centritto M. (2008). Associated changes in physiological parameters and spectral reflectance indices in omiv *(Olea europea L.)* leaves in response to different levels of water stress. *International Jurnal of Remote Sensing*, Vol. 29, pp. 1725–1743.

Swain, T., & Goldstein, (1959). Phenolic constituent of Prunus domestika. I. The quantative analysis of phenolic constituents. *Jurnal of Science Food and Agriculture,* Vol. 10, pp. 63-68.

Tramontano, W. A., & Jouve, D. (1997). Trigonelline accumulation in salt-stressed legumes and the role of other osmoregulators as cell cycle control agents. *Phytochemistry*,Vol. 44, No 6, pp.1037-1040.

Thenkabail, P.S., Enclona, E.A., Ashton, M. S., & Van Der Meer, B. (2004). Accuracy assessments of hyperspectral waveband performance for vegetation analysis applications. *Remote Sensing of Environment*, Vol. 91, pp. 354–376.

Unkovich, M. J., & Pate, J. S. (2000). An appraisal of recent field measurements of symbiotic N_2 fixation by annual legumes. *Field Crops Research,* Vol. 65, pp. 211–228.

Upchurch, P. (2003). Remote Sensing for Crop Management. *Photogrammetric Engineering & Remote Sensing,* Vol. 69, No 6, pp. 647–664.

USDA (United States Department of Agriculture). (2009). Agricultural Projections to 2018. Long-term Projections Report OCE-2009-1, February, 2009.

Vigier, B. J., Pattey, E., & Strachan, I. B. (2004). Narrowband vegetation indexes and detection of disease damage in soybeans. *IEEE Geoscience and Remote Sensing Letters*, Vol. 1, No 4, pp. 255-259.

West, J. S., Bravo C., Oberti, R., Lemaire, D., Moshou, D., & McCartney, H. A. (2003). The potential of optical canopy measurement for targeted control of field crop diseases. *Annual Review of Phytopathology*, Vol. 41, pp. 593-614.

Wu, C. Y., Niu, Z., Tang, Q., & Huang, W. J. (2008). Estimating chlorophyll content from hyperspectral vegetation indices: Modeling and validation. *Agricultural and Forest Meteorology*, doi:10.1016/j.agrformet.2008.03.005

Yang, S., Su, X., Nagendra Prasad, K., Yang, B., Cheng, G., Chen, Y., Yang, E., & Jiang, Y. (2008). Oxidation and peroxidation of postharvest banana fruit during softening. *Pakistan Journal of Botany,* Vol. 40, No 5, pp. 2023-2029.

Yang, Z., Rao, M. N., Elliott, N. C., Kindler, S. D., & Popham, T. W. (2009). Differentiating stress induced by greenbugs and Russian wheat aphids in wheat using remote sensing. *Computers and Electronics in Agriculture,* Vol. 67, No 1-2, pp. 64-70.

Yousfi, S., Wissal, M., Mahmoudi, H., Abdelly, C., & Gharsalli, M. (2007). Effect of salt on physiological responses of barley to iron deficiency. *Plant Physiology and Biochemistry*, Vol. 45, pp. 309-314.

Zancan, S., Cesco, S. & Ghisi, R. (2006). Effect of UV-B radiation on iron content and distribution in maize plants, *Environmental and Experimental Botany*, Vol. 55, pp. 266-272.

Zancan, S., Suglia, I., La Rocca, N., & Ghisi, R. (2008). Effects of UV-B radiation on antioxidant parameters of iron-deficient barley plants. *Environmental and Experimental Botany*, Vol. 63, pp. 71-79.

Zarco-Tejada, P., Miller, J., Mohammed, G., Noland, T., & Sampson, P. H. (2002). Vegetation stress detection through chlorophyll a and b estimation and fluorescence effect on hyperspectral imagery. *Journal Environmental Quality*, Vol. 31, pp. 1433–1441.

Zarco-Tejada, P. J., Miller, J. R., Mohammed, G. H., & Noland, T. L. (2000). Chlorophyll fluorescence effects on vegetation appar- ent reflectance. I. Leaf-level measurements and model simulation. *Remote Sensing of Environment,* Vol. 74, pp. 582-595.

Zhang, M., Liu, X., & O'Neill, M. (2002). Spectral discrimination of Phytophthora infestans infection on tomatoes based on principal component and cluster analysis. *International Journal of Remote Sensing*, Vol. 23, No 6, pp. 1095-1107.

Zhang, M., Qin, Z., Liu, X., & Ustin, S. L. (2003). Detection of stress in tomatoes induced by late blight disease in California, USA, using hyperspectral remote sensing. *International Journal of Applied Earth Observation and Geoinformation*, Vol. 4, No 4, pp. 295-310.

12

Polarization Sensitive Optical Imaging and Characterization of Soybean Using Stokes-Mueller Matrix Model

Shamaraz Firdous
National Institute of Lasers & Optronics (NILOP), Islamabad, Pakistan

1. Introduction

Light polarimetry is a useful tool by which to analyze the modification in the shape and orientation of the field vectors of the electromagnetic radiation which propagates through scattering medium. Among the methods available to analyze turbid media, the use of polarized light has attracted much attention recently, as it has been discovered that multiply scattered photons still maintain partial polarization. [1-4] A typical experiment entails launching a known polarization state in light into a turbid sample and measuring the polarization properties of the reemitted light. The detected signal depends on many variables, including the number and nature of scattering events, the incident polarization state, and the detection geometry. [5-7] In the past few years, several groups have shown how polarization sensitive scattering measurements can be used to measure certain properties of turbid medium such as the average particle size, [8] scattering coefficient, anisotropy factor of particle suspensions , [9] optical material characterization, [10-11] and the study of biological materials. [11-13] An optical polarizers and retarders are rotated to provide additional incident and analyzed polarization states to enable the reconstruction of the 2-D Mueller matrix of various biological sample [14-15]. It has also been shown that the benefits of using polarized light can be combined with different optical modalities. For example, the benefit of using of polarized light in optical coherence tomography (OCT) measurements can significantly improve image contrast. [16-17]

Furthermore, the measurement of polarization parameters of the light scattered benefits from a relatively simple, fast, and convenient data acquisition procedure, [18-19] which motivates the ongoing efforts aimed at further developing the scattering polarization imaging technology. If some of the light retained its polarization properties upon multiple scattering at 180^0 transmittion mode and this effect could be quantified and exploited, potentially useful measurements could be made in almost any clinical situation. Since light in the visible and infrared regions of the electromagnetic spectrum is not harmful to biological tissues at moderate flounce levels, has a penetration depth of several millimeters, and has a reasonable chance of scattering out of the tissue and being detected, it would be ideal for making noninvasive measurements. Other practical reasons for studying the behavior of light at 180° would be for the possibility of spatial imaging to map out the locations of sample structures and compositions, and to gain a better general understanding of turbid systems. [20-22]

A comprehensive understanding of light propagation and scattering for the most general case of highly scattering media is yet to be attained. An analysis based on the Stokes vector and Mueller matrix approach provides a theoretical framework, which can be directly related to the experimentally measurable parameters [23-24]. The Stokes vector - Mueller matrix approach for scattering has been extended to characterization of spatially varying polarization patterns for scattered light. In this approach, determining 16 components of the Mueller matrix for the studied object gives a comprehensive description of scattering properties of a sample or a medium in the spatial domain. Rather then being just one number, each of the 16 components of the Mueller matrix is, in fact, a two-dimensional (2D) array of numbers, corresponding to different spatial locations across the surface of the object or medium.[25]

In this study, we consider experimental Mueller matrix of soybean oil (highly tissue like phantom) for their polarization and depolarization observations. The transmitted photons preserve their polarization memory and Mueller matrix represents this information in the form of matrix array and intensity patterns.

2. Optical properties of the scatterer that influence polarization

The scattering through soybean oil is the principle mechanism that modifies the initial polarization state of the incident light. The polarization state of light after a single scattering event depends on the direction of scattering and incident polarization state. [26-27] In many turbid media such as tissue, scattering structures have a large variance in size and are distributed or oriented in a complex and sometimes apparently random manner. Because each scattering event can modify the incident polarization state differently, until finally the polarization state is completely randomized. An important exception is when the media consists of organized linear structures, such as birefrengent soybean oil, and then the phase retardation between orthogonal polarization components is proportional to the distance traveled through the birefrengent medium. The phase retardation of the scattering medium is given as.

$$\delta = \frac{2\pi \Delta n x}{\lambda} \tag{1}$$

The phase retardation measurement through turbid media is aimed at retrieving useful information from such multiply scattered light. The behavior of light in random media is well-known from the extensive study of wave propagation. Light traveling in a random medium can be classified into three categories, the ballistic, the snake and the diffuse light. The ballistic light either remains unscattered, or undergoes coherent forward scattering in the medium. This light travels undeviated and has the shortest path length in the medium. The snake light is that which undergoes near-forward scattering, and follows path that undulate about the ballistic path.[28] The diffuse photons largely exceed the other two categories in number and undergoes multiple scattering. We considered all three kinds of photons in our study.

A large number of different experiments are possible if one wants to study the polarization dependent scattering properties of turbid media. The probing light may be linearly polarized at various angles, right and left-hand circularly, or elliptically polarized. Light coming from the scattering medium can be analyzed in the same numerous ways. However, only a few measurements are needed to completely characterize the optical properties of

any material. The necessary procedure is elegantly demonstrated by the Stokes-vector Mueller matrix approach to polarization and light scattering.

3. Stokes vector-Mueller matrix formulism

The research of polarized scattered light deals with the entire scattering process in the context of Stokes-Mueller matrices and polarizations. [29] An introduction to optical polarization often starts with a description of the optical elements which physically act as polarizers and retarders. The Stokes vector-Mueller matrix- calculus is then used to show mathematically how these optical elements affect a light beam.

A Stokes vector, a 4 x 1 vector, is a mathematical representation of the polarization state of light. [30] It can be represented as a set of six intensity measurements recorded through a set of various polarizing filters. The Stokes vector is composed of four elements, *I, Q, U*, and *V* and provides a complete description of the light polarization state. If the total irradiant intensity I_t incident on the sample and $I_{0}0$, $I_{90}0$, $I_{+45}0$, $I_{-45}0$, I_{rc} , and I_{lc} the irradiances transmitted by a polarizer-retarders are focused to the detector, then, the Stokes parameters are defined by:[30]

$$S=\begin{bmatrix} I \\ Q \\ U \\ V \end{bmatrix}=\begin{bmatrix} S_0 \\ S_1 \\ S_2 \\ S_3 \end{bmatrix}=\begin{bmatrix} I_t \\ I_{0}0-I_{90}0 \\ I_{+45}0-I_{-45}0 \\ I_{rc}-I_{lc} \end{bmatrix}=\begin{bmatrix} \left\langle E_{0x}^2 \right\rangle+\left\langle E_{0y}^2 \right\rangle \\ \left\langle E_{0x}^2 \right\rangle-\left\langle E_{0y}^2 \right\rangle \\ 2\left\langle E_{0x}E_{0y}\cos\delta \right\rangle \\ 2\left\langle E_{0x}E_{0y}\sin\delta \right\rangle \end{bmatrix} \tag{2}$$

S_{11}(Parallel x Parallel)	S_{12} (horizontal x Parallel)	S_{13} (45^{0}x Parallel)	S_{14} (L.circulerx Parallel)
I_{00}	I_{h0}-I_{v0}	I_{+0}-I_{-0}	I_{L0}-I_{R0}
S_{21} (Parallel x horizontal)	S_{22} (horizontalx horizontal)	S_{23} (45^{0}x horizontal)	S_{24} (L.circularx horizontal)
I_{0h}-I_{0v}	(I_{hh}+I_{vv})-(I_{vh}+I_{hv})	(I_{+h}+I_{-v})-(I_{-h}+I_{+v})	(I_{Lh}+I_{Rv})-(I_{Rh}+I_{Lv})
S_{31} (Parallel x 45^0)	S_{32} (horizontal x45^0)	S_{33} (45^{0}x45^0)	S_{34} (L.circularx45^0)
(I_{0+}-I_{0-})	(I_{h+}+I_{v-})-(I_{v+}+I_{h-})	(I_{++}+I_{--})-(I_{-+}+I_{+-})	(I_{L+}+I_{R-})-(I_{R+}+I_{L-})
S_{41} (Parallel x L.circular)	S_{42}(horizontal x L.circular)	S_{43}(45^{0}xL.circular)	S_{44}(L.circularxR.circuler)
(I_{0L}-I_{0R})	(I_{hL}+I_{vR})-(I_{vL}+I_{hR})	(I_{+L}+I_{-R})-(I_{-L}+I_{+R})	(I_{LL}+I_{RR})-(I_{RL}+I_{LR})

Table 1. A matrix array showing the polarization measurements, necessary to measure each particular matrix element of the different configurations (polarizer and analyzer) setup.

Where *Ex* and *Ey* are the electric field vectors along x and y direction and δ is angle. After normalizing the Stokes parameters by the irradiance *I*, *Q* describes the amount of light polarized along the horizontal (*Q*= +1) or vertical (*Q*= -1) axes, U describes the amount of light polarized along the +45° (*U*= +1) or -45° (*U*= -1) directions, and V describes the amount of right (*V*= +1) or left (*V*= -1) circularly polarized light.

A Mueller matrix, a 4 x 4 matrix, is a mathematical description of how an optical sample interacts or transforms the polarization state of an incident light beam and given as [30]

$$[M] = \begin{bmatrix} m_{11} & m_{12} & m_{13} & m_{14} \\ m_{21} & m_{22} & m_{23} & m_{24} \\ m_{31} & m_{32} & m_{33} & m_{34} \\ m_{41} & m_{42} & m_{43} & m_{44} \end{bmatrix} \tag{3}$$

where, M is the 4 x 4 Mueller matrix of the media or sample and can be experimentally measured through the application of various incident polarization states and then by analyzing the state of polarization of the light leaving the sample. Since a Mueller matrix contains 16 elements (*mij*) of the matrix M and reconstruction requires 49 independent polarization measurements according to different polarizer and wave plate orientation as shown in table.1 and Fig.1. [31] The Mueller matrix can be thought of as the "optical fingerprint" of a sample. This matrix operates directly on an input or incident Stokes vector, thus resulting in an output 4 x 1 Stokes vector that describes the polarization state of the light leaving the sample. This is described mathematically by the equation given as [32]

$$[S_{out}] = [M_{system}][S_{in}] \tag{4}$$

where $[S_{out}]$ the output Stokes vector, $[S_{in}]$ the Stokes input vector and $[M_{system}]$ is the Mueller matrix representing the entire experimental optical system given as

$$[M_{system}] = [QW][A_M][M][QW][P_M] \tag{5}$$

The output stokes vector $[S_{out}]$ can be calculated by relation in Eq. 4, putting the values of the Mueller matrix for optics and the Mueller matrix of the system. [33] The complete characterization of the polarization state of light by means of the Stokes parameters permits the calculation of the degree of polarization (DOP), defined as

$$DOP = \sqrt{\frac{Q^2 + U^2 + V^2}{I}} = \sqrt{\frac{S_1^{\,2} + S_2^{\,2} + S_3^{\,2}}{S_0^{\,2}}} \tag{6}$$

For purely polarized light, the degree of polarization is unity i.e. 1, and the Stokes parameters obey the equality $I^2=Q^2+U^2+V^2$, while for partially polarized light, the degree of polarization is smaller than unity, leading to $I^2>Q^2+U^2+V^2$. An input beam can be decomposed into purely polarized beams. After propagation through an optical system, the Stokes parameters of the purely polarized beam components are added to give the Stokes parameters for the original input beam. [34]

4. Determining the Mueller matrix

If the Mueller matrix is not known, all the elements can be determined experimentally. It can be shown that 49 intensity measurements with various orientations of polarizers and analyzers are necessary to obtain the 16 elements of the Mueller matrix. [25]

$$
\begin{aligned}
m_{11} &= I_{00} \\
m_{12} &= I_{h0} - I_{v0} \\
m_{13} &= I_{+0} - I_{-0} \\
m_{14} &= I_{l0} - I_{ro} \\
m_{21} &= I_{0h} - I_{0v} \\
m_{22} &= (I_{hh} + I_{vv}) - (I_{vh} + I_{hv}) \\
m_{23} &= (I_{+h} + I_{-v}) - (I_{-h} + I_{+v}) \\
m_{24} &= (I_{lh} + I_{rv}) - (I_{rh} + I_{lv}) \\
m_{31} &= I_{0+} + I_{0-} \\
m_{32} &= (I_{h+} + I_{v-}) - (I_{v+} + I_{h-}) \\
m_{33} &= (I_{++} + I_{--}) - (I_{-+} + I_{+-}) \\
m_{34} &= (I_{l+} + I_{r-}) - (I_{r+} + I_{l-}) \\
m_{41} &= I_{0l} - I_{0r} \\
m_{42} &= (I_{hl} + I_{vr}) - (I_{vl} + I_{hr}) \\
m_{43} &= (I_{+l} + I_{-r}) - (I_{-l} + I_{+r}) \\
m_{44} &= (I_{ll} + I_{rr}) - (I_{rl} + I_{lr})
\end{aligned}
\tag{7}
$$

Where the first term represents the input polarization state while the second the output polarization state of light. The states are defined as: h = horizontal, v = vertical, + = +45°, - = -45°, r = right circular and l = left circular. Once all 16 elements of the matrix are obtained, the medium is completely described in terms of its optical properties.

5. Error analysis of Mueller matrix polarimeter

For the retardations close to 0^0 or 90^0 the background noise on the detectors introduces a significant and systematic error of 15^0 at a signal to noise ratio of 10 dB. [35] The coherent detection scheme which calculates the Stokes parameters has better immunity to the system. in the calculation of the Q parameter the spectral density in one polarization channel is subtracted from the spectral density in the orthogonal polarization channel, thus eliminating constant background noise terms, and the U and V parameters are calculated from the cross correlation between the orthogonally polarized channels, eliminating autocorrelation noise. Noise will decrease the degree of polarization, since it will be present as autocorrelation noise in the Stokes parameter I. In the incoherent detection scheme only V is measured and the error in the phase retardation is introduced by the decrease of the amplitude of oscillations with increasing depth. In the coherent detection scheme, the Stokes parameters Q, U, and V can be renormalized on DOP, restoring the amplitude of the oscillations, and thus eliminating the systematic error.

We have analyzed system errors introduced by the extinction ratio of polarizing optics and chromatic dependence of wave retarders, and errors due to dichroism, i.e., the differences in

the absorption and scattering coefficients for polarized light in soybean oil. System errors can be kept small by careful design of the system with achromatic elements, but can never be completely eliminated. Dichroism is a more serious problem when interpreting the results as solely due to birefringence. However, Mueller matrix polarimetry measurements have shown that the error due to dichroism is relatively small. [36] The variance in the computed Stokes vectors of transmitted light (excluding effect of birefringence) is due to multiple scattering, speckles, and shot noise (i.e., optimized system). At some depth, the detected signals are limited by shot noise. At shallower depths (i.e., before the shot noise limit) variance in the Stokes parameters is primarily due to the effects of multiple scattering and speckle. Multiple scattering scramble the polarization mainly in a random manner and this offers some means to distinguish it from birefringence. Thus, birefringence induced changes are relatively slow, and the Stokes parameters change according to the Mueller matrix of a linear retarder. [37] However, an optic axis that varies with depth will give changes in the polarization state that will be difficult to distinguish from the random manner of multiple scattering. More research is necessary on this complex problem. We use coherent light source and standard optical filters to minimize these errors for our Mueller matrix polarimeter.

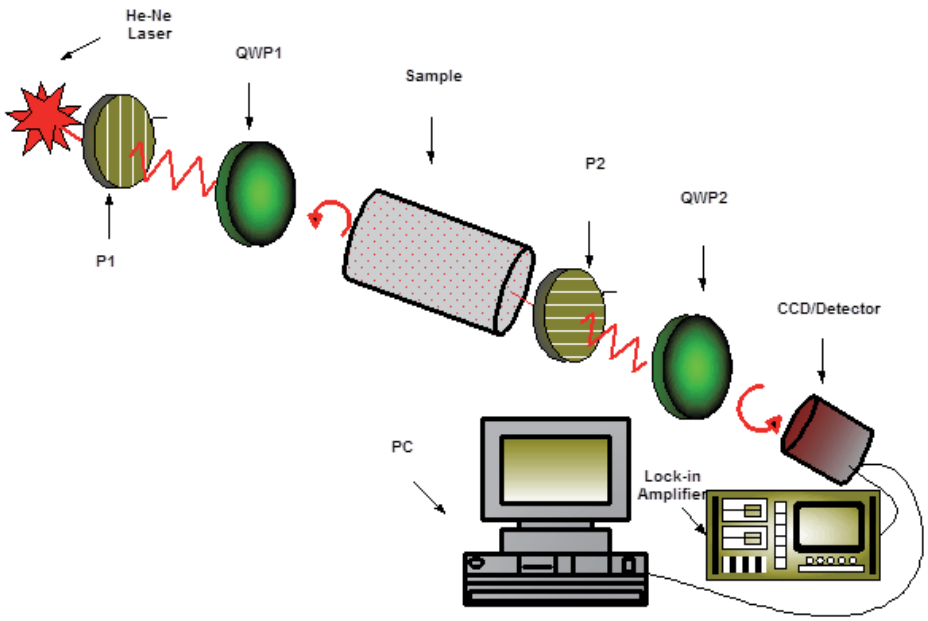


Fig. 1. Experimental setup for measurements of transmitted Mueller matrix elements. A He-Ne laser beam with an output power of 5 mW at a wavelength of 632.5 nm is used as the light source. The laser light is focused on polarizer P1 for obtaining linearly polarized light. The circularly polarized light is generated, by inserting a quarter mica retardation plates behind the linear polarizer. The output polarized light is focus to soybean oil and again pass through linear polarizer, quarter wave plate and recorded on photodiode detector/CCD camera, which is controlled and operated with Lab software. The soybean oil is used as scattering phantom.

The experimental setup for Mueller matrix polarimetry is shown schematically in Fig. 1. A light source with spot size is less than 2 mm passes through a Polarizer (P1) and quarter wave-plate (QWP1) and impinges on the sample. The scattered light which emerges from the sample passes through a analyzer (P2) and quarter wave-plate(QWP2), and is then recorded by the detector connected to lock in amplifier or CCD camera system (Pico Star, Lab Vision). The CCD resolution is 12 bit and the lab view software is used for data analysis. The scattering medium (soybean oil) is placed in a cylindrical thin-walled quartz cell (3x2 cm). Soybean oil is an inexpensive, non-toxic liquid with dielectric properties similar to very low-water-content fatty tissue. For this reason, it is used as the tissue phantom. [38]

6. Experimental results

In this study the soybean oil is used as tissue like phantom. We recovered optical information by selectively detecting a transmitted component of the scattered photon flux that has its initial polarization state preserved. These photons transmitted through or re-emitted from a multiply scattering medium by using relatively inexpensive Mueller matrix polarimeter provides the basis for several potential applications. The measurement technique is based upon an operational principle, which involves the modulation of a polarization state. The resulting modulated light signal is collected by the detector/CCD camera and is analyzed pixel by pixel to calculate individual intensity patterns, which correspond respectively to the 16 components of the scattering Mueller matrix. In brief, the two polarizers and quarter-wave plates inserted in the probing and analyzing beam paths, are generate a periodic signal, this signal carries information about the properties of the medium which induces the transformation of the polarization state of the modulated probing light. The experimental procedure requires collecting of 16 intensity images at various orientations of the polarizing components. The described procedure provides the possibility to calculate the scattering Mueller matrix for a given sample.

The Stokes parameter I of the system in Fig.2 represents the magnitude of the intensity of the scattered light. Thus, any abrupt change in the detected signal indicates strong discontinuity in the refractive index of the specimen. Along with I, other Stokes parameters, *Q, U* and *V* can be used to detect structural changes that are not simply detected from I. Other Stokes parameter images of *U* and *V* show supplement information that there is no apparent level of stress inside the scatterer. Therefore, by analyzing the corresponding series of the polarization patterns one can trace scattering events of different order. Specifically, the data analysis of Fig. 3, suggests several interesting observations regarding the general properties of the scattering Mueller matrix. First, the magnitude of the off-diagonal components of the scattering Mueller matrix is significantly smaller than the magnitude of the diagonal components.

In the present experiments, we are able to trace the polarization patterns and to verify that the existing magnitude distributions are preserved. The magnitude and the sign of most of the spatial extent of the matrix components for Fig. 3, 4 closely resemble the form of the Mueller matrix for scattering medium. The next important observation is that the experimental results clearly display several symmetry properties of certain matrix components for homogeneous scattering medium. The seven out of sixteen are independent and other can be calculated through symmetry relation. [39] By comparing the images of Fig. 3, 4, and 5, one can identify the unique features of the Mueller matrix for scattering medium. The axial symmetry of the system provides relation between all the Mueller matrix

components and reduces the number of measurements, which reduces the observation time. These symmetry relations describe that the seven independent elements are, [40]

$$m_{11}(\theta,\varphi), m_{12}(\theta,\varphi), m_{14}(\theta), m_{22}(\theta,\varphi), m_{23}(\theta,\varphi), m_{24}(\theta,\varphi), m_{44}(\theta), \quad (8)$$

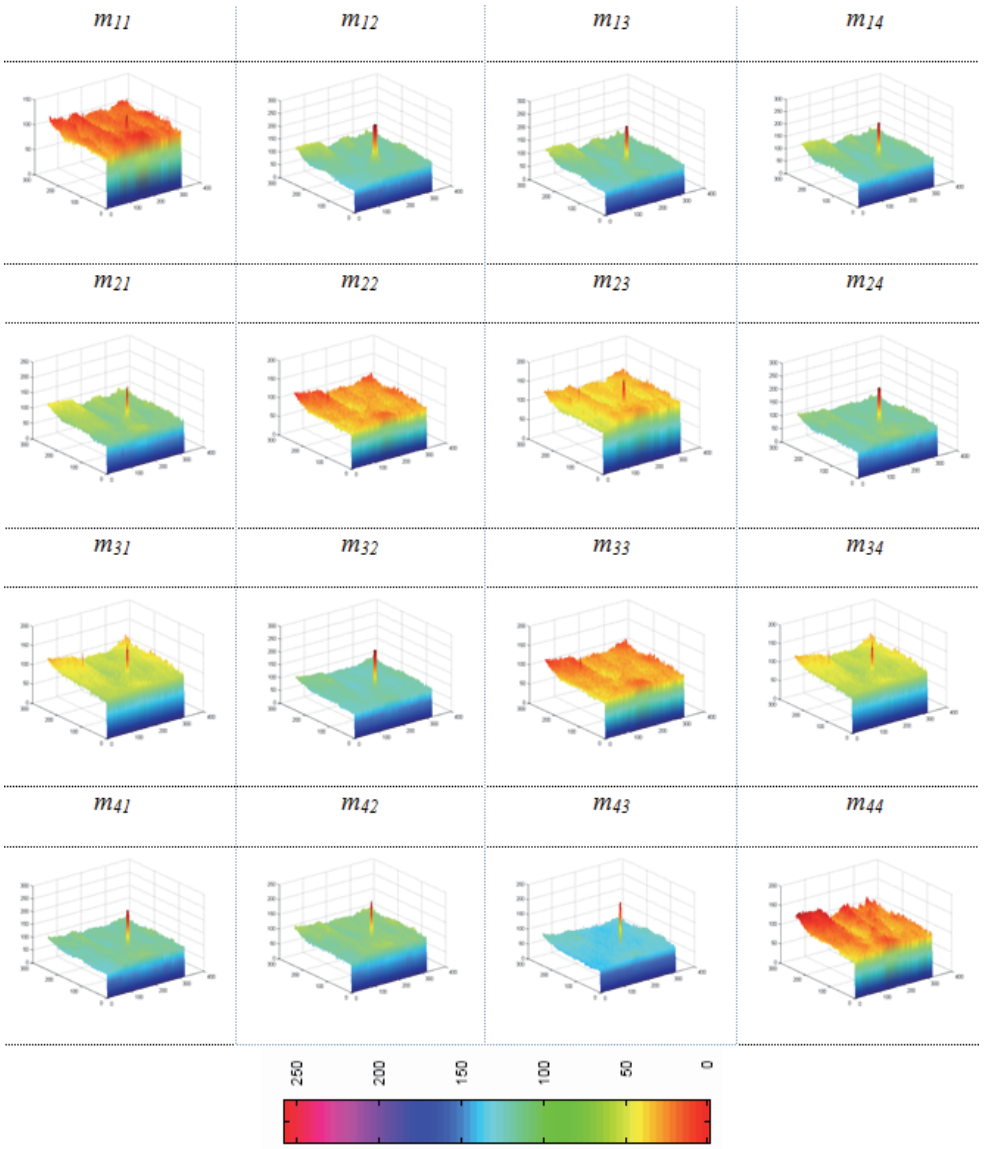


Fig. 2. Transmitted Mueller matrix components (3D) corresponding to a 16 images of scattering medium. The images are taken with the experimental setup in Fig.1. The scale bar is adjusted so that red represent the maximum irradiance, yellow to middle one, green for minimum irradiance and blue means "no light" or component change by an order of magnitude. All displayed images are 3x3 cm.

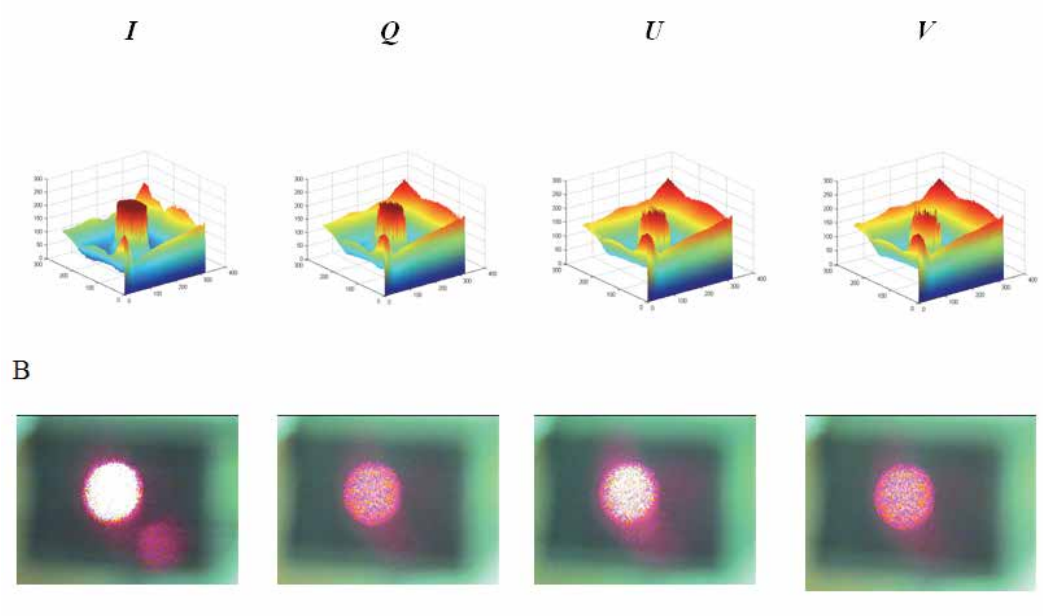


Fig. 3. the Stokes vector I, Q, U, and V are shown, I present the total irradiant power profile, Q for horizontal polarized light, U for $+45^0$ polarized incident light and V the right circular polarized light. (a) Represents the 3-D images and (b) represent the direct transmitted intensity for all the Stokes vectors.

Where θ is the rotation angle of the transmission axis of the polarizer, φ is the phase shift of the retarder and the other nine dependent elements are:

$$\begin{matrix} m_{13}(\theta,\varphi) = m_{12}(\theta,\varphi+\frac{\pi}{4}) \\ m_{21}(\theta,\varphi) = m_{12}(\theta,\varphi) \end{matrix},$$

$$m_{31}(\theta,\varphi) = -m_{13}(\theta,\varphi) = m_{12}(\theta,\varphi-\tfrac{\pi}{4}),$$

$$m_{32}(\theta,\varphi) = -m_{23}(\theta,\varphi) = m_{23}(\theta,\varphi\pm\tfrac{\pi}{4}),$$

$$m_{33}(\theta,\varphi) = -m_{22}(\theta,\varphi-\tfrac{\pi}{4}),$$

$$m_{34}(\theta,\varphi) = m_{24}(\theta,\varphi-\tfrac{\pi}{4}),$$

$$\begin{matrix} m_{41}(\theta,\varphi) = m_{41}(\theta) = m_{14}(\theta) \\ m_{42}(\theta,\varphi) = m_{24}(\theta,\varphi) \end{matrix},$$

$$m_{43}(\theta,\varphi) = -m_{34}(\theta,\varphi) = m_{24}(\theta,\varphi+\tfrac{\pi}{4}) \tag{9}$$

Fig. 6. displays the matrix array, which represents the detector reading specific to a linear polarizer lie in the first row, first column elements, those specific to a quarter wave plate lie in the fourth row, fourth column elements.

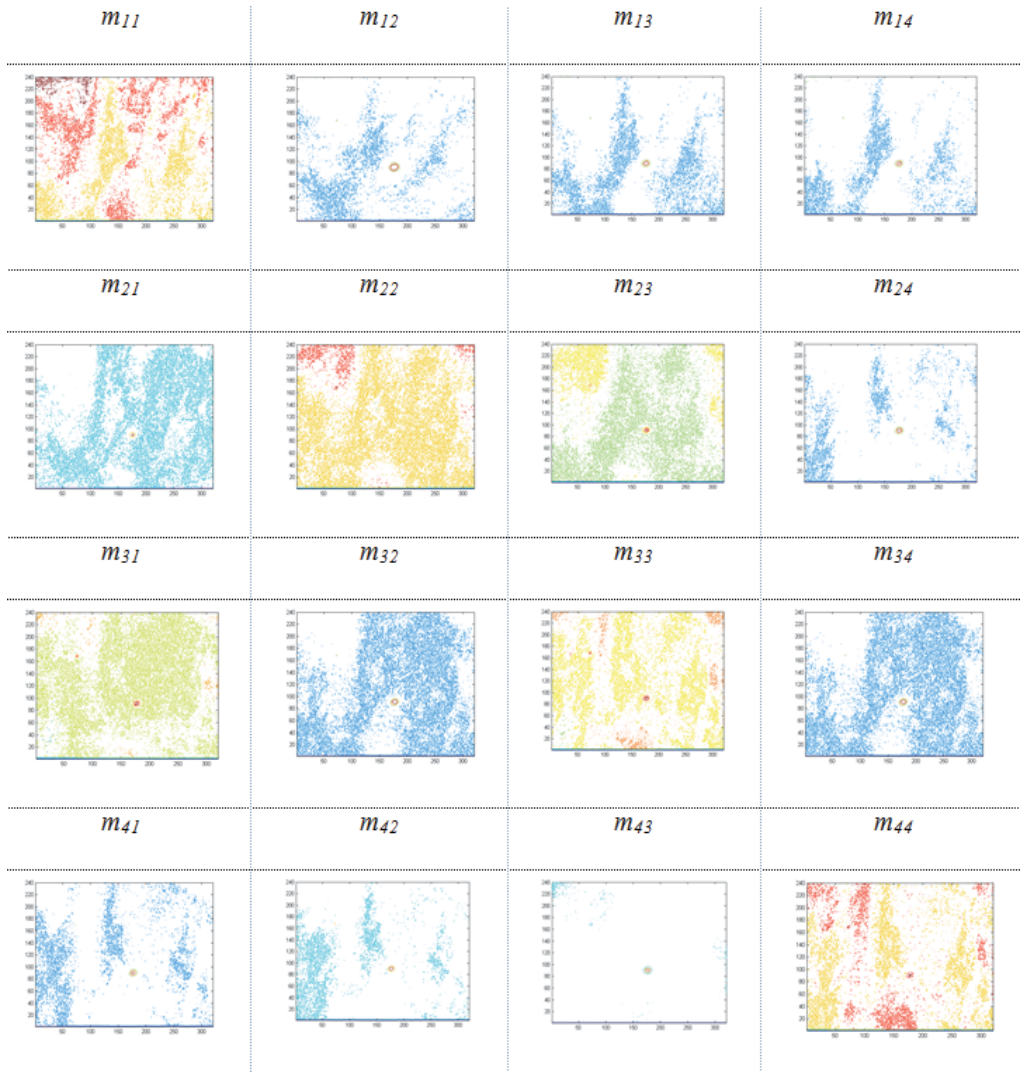


Fig. 4. Transmitted Mueller matrix components (2D) corresponding to a scattering. These 2-D images are derived through 49 measurements of Mueller matrix polarimeter. The central spot presents the output transmitted irradiance by scatterer along with scattering pattern.

Making the above 49 measurements of polarized light from a scatterer will produce 16 matrix element pattern. Each one is an electric field dependent intensity measurement for a particular arrangement of input-output optics. These 16 curves contain all the information that can be learned from a scattering experiment. Choosing input-output optical combinations, different than described above, will produce a set of patterns drastically different in appearance but not fundamentally different in information content. When the 16 matrix elements are measured the data is ready for analysis. For certain perfect particles like spheres, fibers, and mixtures of perfect particles, the matrix elements can be exactly predicted. So the set of 16 measurements will stand as the signature of the scatterer as described by polarized scattered light.

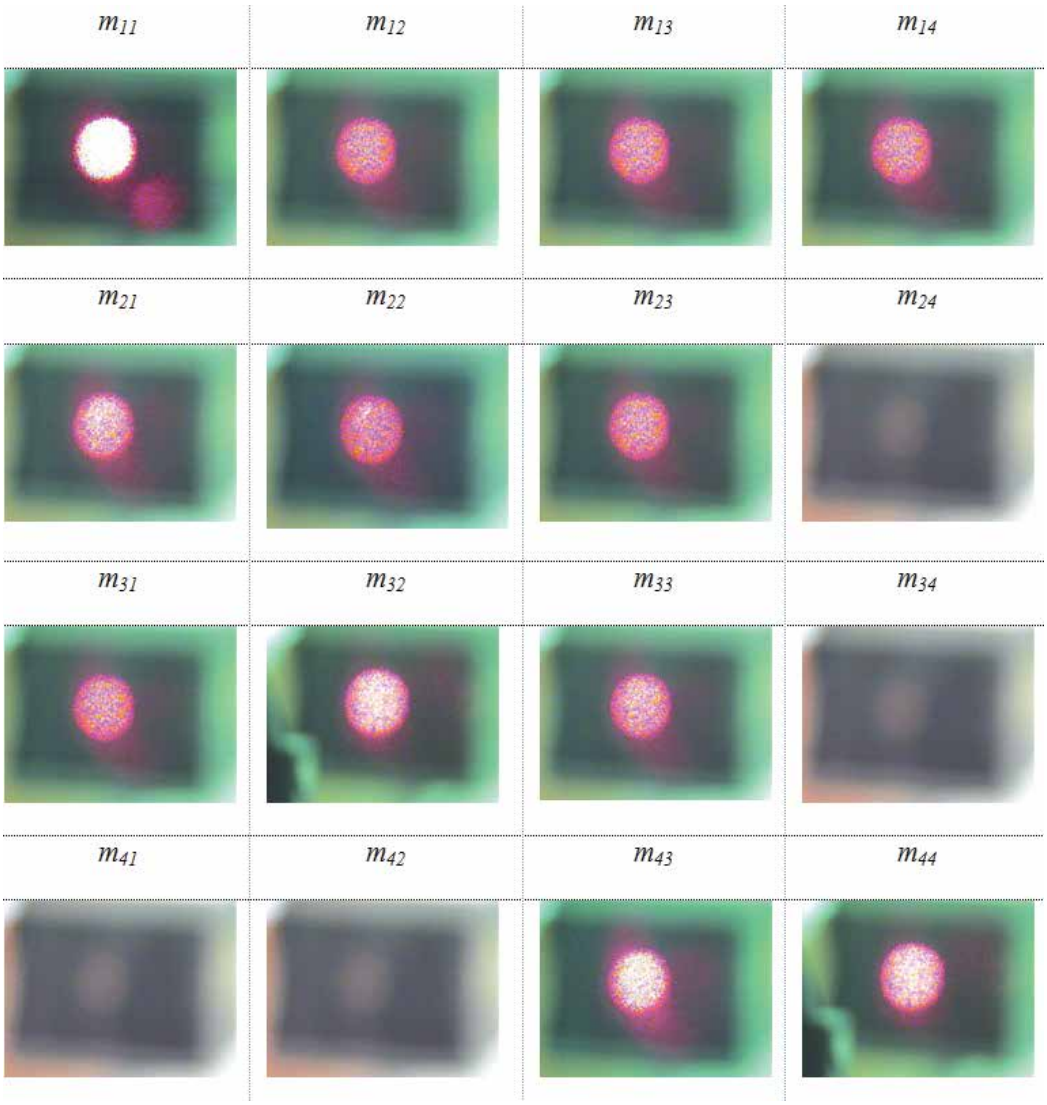


Fig. 5. Experimental transmitted polarization images of Mueller matrix components corresponding to a scattering medium of randomly distributed particles. The images corresponds the direct scatterer irradiance, measured through polarization discrimination technique.

Mueller matrix polarimetric pattern analysis predicts interesting information about the medium. The ballistic, snake and diffuse photons reaching the camera contributes to the formation of direct image. The diffuse photons have suffered multiple scattering before exiting the scattering medium. Ballistic photons completely preserve the polarization properties of the irradiant light after passing through scattering medium. The snake photons recorded by the detector are partially polarized but to the smaller degree then the ballistic photons, because snake photons partially preserve polarization. The diffuse photon depolarized for thick sample. The collective image of these three scattered photon provides

useful information and characterizes the scattering medium in term of size shape under Raleigh and Mie scattering theory.

The diagonal elements of Mueller matrix consist on linear and circular polarization pattern. The Mueller matrix m_{11} describe the properties of the total irradiance of light source and provides less information comparing to other elements of the matrix array, but all other elements are normalized through it. The m_{22} composed of linear horizontal and vertical polarization state. The liner polarization preserve through longer distance in the scattered as compared to circular light The concentration , size and shape of the particles in scattering medium can be predicted through careful analysis of this element alomgwith other linear elements of the matrix. If the value of this element is zero or below, then the medium obey Raleigh theory and the size of the particle is small as compared to the irradiated wavelength. If its value is greater than zero than it can be explain through Mie theory and particles of the scattering medium are larger in size. m_{33} depend on $\pm 45^0$ linear polarization state and describes the properties almost close to m_{22} element. The last element m_{44} of the Mueller matrix compose on circular polarization. If the size of scatterer is larger, then the magnitude of this element will be in negative otherwise greater than or equal to zero. The difference between normal and malignant biological tissues can be characterized through this element. But for larger scattered concentration, it is less informatics because the data is taken through diffuse photon and the preservation of circular polarization is not dominant in this medium. If this element is measured through the ballistic and snake photons contribution then it reviles a significant role in characterization of biological tissues. In our case the experimental data shows decline in the diagonal elements from top to bottom that conforms the preservation of linear polarization in diffuse medium for longer distance compared to circular one. As m_{44} is greater than zero, which predicts that the size of scattered is larger than the irradiating wavelength. The size of the particle can be numerically calculated through Mie scattering theory. [41]

0.986	0.007	0.004	0.0003
0.007	1.003	-0.007	0.009
0.008	-0.007	0.992	-0.003
0.003	-0.006	-0.007	0.989

a

0.910	0.657	-0.314	0.097
0.739	0.732	0.793	0.083
0.435	0.243	0.620	-0.213
0.133	0.421	0.136	0.751

b

Fig. 6. Mueller matrix data for transmitted polarized laser beam (a) no sample (air) and (b) for scattering turbid sample.

First row and first column of the Mueller matrix except m_{14} and m_{41} describe the linear polarization pattern. Each and every element of this group is very informatics, and describes the structure of the dense diffuse scattered. From Fig. 3, 4, and 5 we see that the intensity contrast reduces from right to left and top to bottom of the Mueller intensity matrices except m_{13}, which tells about the enriched optical activity and highly birefringence of the sample. The higher value of element m_{13} is due to randomization of the sample molecules, when the

incident light is of $\pm 45^0$ polarized. From analysis of the elements of this group the normality and abnormality of the medium can easily be defined. In Fig.7 the depolarization of linearly polarized light through scattered is represented and it increases with the depth of soybean oil.

The elements in the middle of this group m_{22}, m_{23}, m_{32}, and m_{33} can obtain through $\pm 45^0$ linear polarization. If some properties of the scattered cannot be obtain through the elements of other group they can be characterize through it. The elements m_{23} and m_{32} decline for dense scattering medium and the scattering angle for these elements is very small. The reduction of these elements directly related to the variation in structure of the scattered.

The last row and last column of this matrix set consist on circular polarization pattern. This group exhibits the depolarization properties of the medium. The depolarization is faster in dense as in case of circular one. The light is equally right and left-hand polarized and the effect is strongest in the center, near the laser entry point. Here the scattered light has undergone only a few scattering events and the polarization effects are strongest. With increasing distance from the point of light incident, the number of scattering events increases and eventually the polarization information is lost, the value of the m_{44} approaches zero. The majority of the elements of this group shows decline in the magnitude for dense medium and predict that the scattering cross-section of medium is small for this wavelength and the circular polarization preservation of light is weaker.

The next important observation is that the experimental results in Mueller matrix array display several symmetry properties and relations among them. This can be seen in Fig. 3-6 and these derived symmetry relations hold, of course, if the scattering medium contains one kind of randomly distributed asymmetrical particles or optically active. Some elements of the matrix have same behavior and other one are of same shape but rotated through 90^0 as indicated in equation 8, 9 of this paper. All sixteen Mueller matrix components together provide a "finger print" of the scattering medium under investigation. As just shown, looking at the entire Mueller matrix often enables one to distinguish qualitatively between two media. Lot of information about particle size, refractive index, particle shape etc. has to be found in the Mueller matrix by careful analysis of the matrix elements. However, further information may be gained, for example, by measuring the diffuse backscattering and back-reflectance at different incident and observation angles, or time-dependent polarization effects.

In this study we presented a polarization discrimination scattering experiment and have taken care to establish unambiguously the coordinate systems involved, the redundancy of certain measurements and the importance of particular orientations of optical element combinations. We believe that these concepts are important for understanding and fully appreciating optical polarization and that this approach is attractive because it discusses the inexpensive and non invasive procedures that are equally valid.

7. Conclusion

We describe the Mueller matrix polarization discrimination (MMPD) technique for characterization of highly scattering media(soybean oil) through laser beam. In our experiments, the scattering regime was adjusted to be at the incipient transition between single and multiple scattering. From an experimental standpoint the scattering is most challenging and on the other hand, it is rich in information content because the low-order scattering events are responsible for non-trivial polarization features. Our results

demonstrate that the Mueller matrix components satisfy symmetry relations. These measurements provide detailed information about the changes in the magnitude and sign of Mueller matrix components. This should offer more insight and could lead to novel procedures for characterizing scattering phenomena. We discuss the entire experimental measured Mueller polarized matrix in detail for extracting the taking information about the structure, size, and shape of the scattering particles in term of its output polarization. This has the potential of characterization of turbid sample for their optical properties through polarized laser radiations.

We concluded that soybean oil is optical active, less retardence and highly depolarizing. All these characteristic describe soybean oil an accurate tissue like phantom with low absorption and higher scattering coefficient. Further study and characteristic of soybean oil can be deduced with application of Raleigh and Mie scattering models.

8. References

[1] A. Vitkin, R. D. Laszlo, and C. L. Whyman, "Effects of molecular asymmetry of optically active molecules on the polarization properties of multiply scattered light", Opt.Exp. 10, 222-229 (2002).

[2] S. Firdous, M. Fuzail, M. Atif, and M.Nawaz Polarimetric Characterization of Ultra-High Molecular Weight Polyethylene (UHMWPE) for Bone Substitute Biomaterials, Optik 122 (2) 99-104 (2011)

[3] S. Firdous, W. Waqas, M. Idrees,M. Atif, M. Nawaz, and M. Ikram, Polarization Sensitive Optical Imaging of Biomaterials using Mueller matrix Polarimetric Algorithm, Lasers in Medical Science (submitted 2011)

[4] R. R. Ansari, S. Bockle, and L. Rovati, "New optical scheme for a polarimetric- based glucose sensor", J. Biomed. Opt. 9, 103–115 (2004).

[5] D. Ge, Y. Cai, and Q. Lin, "Propagation of partially polarized light Gaussian Schell-model beam in dispersive and absorbing media", Opt. Commun. 229, 93-98(2004).

[6] S. Firdous, K. Hassan, and M. Ikram, Formulation of Mueller Matrix and Modeling of depolarizing and scattering of nitrobenzene in a Kerr Cell, Appl. Opt. 44, 7,1171-1177 (2005)

[7] C. Cote, I. A. Vitkin, "Balanced detection for low noise precision polarimetric measurements of optically active multiply scateering tissue phantoms", J. Biomed. Opt. 9, 213-220 (2004).

[8] A. H. Hielscher, J. R. Mourant, and I. J. Bigio,"Influence of particle size and concentration on the diffuse backscattering of polarized light from tissue phantoms and biological cell suspensions,'' Appl. Opt. 36, 125–135 (1997).

[9] P. Yang, H. Wei, G. W. Kittawar, Y. X. Hu, D. M. Winker, C. A. Hostetler, and B. A. Baum, "Sensitivity of the backscattering Mueller matrix to particle shape and thermodynamic phase", Appl. Opt. 42, 4389-4395 (2003).

[10] L. Jin, T. Hamada, Y. Otani, and N. Umeda, "Measurement of charactristic of magnatic fluid by the Mueller matrix imaging polarimeter", Opt. Eng. 43, 181-185(2004).

[11] J. T. Oh, S. W. Kim, "Polarization optical coherence tomography for photoelasiticty testing of glass/epoxy composites", Opt. Exp. 11, 1669-1676 (2003).

[12] S. L. Jacques, M. R. Ostermeyer, L. Wang, and D. V. Stephens, ''Polarized light transmission through skin using video reflectometry: toward optical tomography of superficial tissue layers,'' Proc. SPIE 2671, 199–210 (1996).

[13] M. C. Pierce, J. Strasswimmer, B. H. Park, and J. F. Deboer, "Birefrangence measurement in human skin using polarization-sensitive optical coherence tomography", J. Biomed. Opt. 9, 287-291 (2004).
[14] S. Jiao, G. Yao, and L.V.Wang, " Depth-resolve two-diamentional Stokes vector backscattered light and Mueller matrix of bialogical tissues measured with optical coherence tomography", App. Opt. 39, 6318-6324 (2000).
[15] I. Berezhnyy, and A. Dogariu, "Time-resolved Mueller matrix imaging polarimetry" Opt.Exp. 12, 4635-4649 (2004).
[16] J. F. Deboer, T. E. Milner, "Review of polarization sensitive optical coherence tomography and stokes vector determination", J. Biomed. Opt. 7, 359-371 (2002).
[17] J. L. Pezzaniti, R. A. Chipman, "Mueller matrix imaging polarimetry", Opt. Eng. 34, 1558-1568 (1995).
[18] V. Sankarn, J. T. Walsh, and D. J. Maitland, "polarized light propagation through tissue phantom, ehms containing densely packed scatterers", Opt. Lett. 25, 239-241 (2000).
[19] R. Cross, B. Heffner, and P. Hernday, " Polarization measurement goes automatic", Lasers and Optronics, 10, 25-26 (1991).
[20] A. Nezhuvingal, Y. H. Anumula, and B. D. Cameron, "Mueller matrix optical imaging with application to tissue diagnistics", Proc. SPIE. 4961, 1-10 (2003).
[21] F. R Brehonnet, B. L. Jeune, P. Elies, J. Carious, and J. Lotrain, "Optical media and target charactrization by Mueller matrix decomposition", J. Phy. D. 29, 34-38 (1996).
[22] G. W. Kittawar, D. J. Gray, "Mueller matrix imaging of target in turbid media:effect of the volume scattering function", App. Opt. 42, 7225-7230 (2003).
[23] J. S. Baba, J. R .Chung, A. H. DeLaughter, B. D. Cameron, and G. L. Cote´, "Development and calibration of an automated Mueller matrix polarization imaging system", J. Biomed. Opt. 7, 341–349 (2002).
[24] S. G. Demos and R. R. Alfano, ''Optical polarization imaging,'' Appl. Opt. 36, 150–155 (1997).
[25] W. S. Bickel and W. M. Bailey, ''Stokes vectors, Mueller matrices, and polarized scattering,'' Am. J. Phys. 53, 468–478 (1985).
[26] F. Delplancke, ''Automated high-speed Mueller matrix scatterometer,'' Appl. Opt. 36, 5388–5395 (1997).
[27] W. S. Bickel, A.J. Watkins, and G.Videen," The light-scattering Mueller matrix elements for Rayleigh, Rayleigh–Gans, and Mie spheres," Am. J. Phys. 55, 559-561 (1987).
[28] E. Collett, Polarized Light: Fundamentals and Applications, Marcel Dekker, New York (1993).
[29] E. Hecht, Optics, 3rd ed., pp. 322–323, 366–367, 370, Addison Wesley Longman, Reading, MA (1998).
[30] C. Brosseau, Fundamentals of Polarized Light: a Statistical Optics Approach, Wiley, New York (1998).
[31] V. Sankarn, M. J. Everett, D. J. Maitland, and J. T. Walsh, "Compression of polarized light propagation in bialogical tissue and phantom", Opt. Lett. 24, 1044-1046 (1999).
[32] D. H. Goldstein, "Mueller matrix dual-rotating retarder polarimeter", Appl. Opt. 31, 6676-6683 (1992).
[33] D. S. Kilger, J. W. Lewis, and C. Einterz Randall, Polarized Light in Optics and Spectroscopy, Academic, San Diego, CA (1990).

[34] S. Firdous and M. Ikram, Laser stokes polarimetry for the Characterization of bio-materials using liquid crystal variable retarders, Proc. SPIE 6632 (2007).
[35] E. A. West, M. H. Smith, "Polarization errors associated with birefrangent waveplates", Opt. Eng. 34, 1574-1580 (1995).
[36] D. H. Goldstein, R. A. Chipman, "Error analysis of a Mueller matrix polarimeter", J. Opt. Soc. Am. A. 7, 693-700 (1990).
[37] P. A. Williams, A. H. Rose, and C. M. Wang, "Rotating-polarizer polarimeter for accurate retardance measurement", Appl. Opt. 36, 6466-6472 (1997).
[38] S. T. Flock, S. L. Jacques, B. C. Wilson, W. M. Star, and M. V. Gemert, "Optical properties of intralipid: A phantom medium for light propagation studied", Laser Surg. Med.12, 510-519 (1992).
[39] M. j. Rakovic, G. W. Kittawar, M. Mehrubeoglu, B.D.Cameron, L. V. Wang, S.Resteger, and G.L.Cote., "Light backscattering polarization pattern from turbid media:theory and experiment",Appl. Opt.38, 3399-3408 (1999)
[40] S. Firdous, M. Ikram, " Charactrization of soybean oil through diffusely backscattered polarized light with matrix calculus-II",IEEE proceeding INCC 2004,115-123 (2004)
[41] H. C. Van de Hulst, Light Scattering by Small Particles, Dover, New York (1981).

13

Distant-Graft Mutagenesis Technology in Soybean

Pan Xiang-Wen[1], Cao Chang-Yi[1], Zhang Qiu-Ying[1],
Li Yan-Hua[1], Wu Xiu-Hong[2], Wang Guo-Dong[1] and Du Wei-Guang[1]
[1]Key Laboratory of Mollisols Agroecology, National Observation Station of Hailun Agroecology System, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Harbin,
[2]Jiamusi Branch of Heilongjiang Academy of Agricultural Sciences, Jiamusi China

1. Introduction

As we know, sexual hybridization is the primary means of creating genetic variation in the conventional breeding methods, all kinds of new varieties are mainly developed through this means. However, sexual hybridization can only be carried out between species. The distant-graft mutagenesis technology developed rapidly in recent years just made it possible to bypass the natural obstacles of incompatibility or hybrid embryos sterile of sexual hybridization between distantly-related species and integrate the different sources of rootstock and scion organically, and allow the scion to grow, develop and fruit normally. As the sequence of it, a wide range of mutations can be induced under the adverse conditions of distant grafting and all kinds of the unique new plant types can also be created. Qian (1993), the well-known Chinese scientist, had ever pointed out: "We should develop a technical science---The graft-transformed science of plants, whose role must be no less than genetic engineering."

Compared to other mutagenesis technologies, the distant-graft mutagenesis technology can integrate the advantages of rootstock and scion and further expand the genetic base of target crop. Besides, it characters as: (1) the mutated traits stabilize quickly, which may shorten the breeding period; (2) the operation technique is simple and easy to grasp; (3) the required test conditions and input are comparatively lower than other mutagenesis technologies. Now, the distant-graft mutagenesis technology has been applied in resources innovation and genetic improvement of crops such as wheat, millet, cotton, peanut, mungbean, corn, and soybean etc, and a lot of new plant materials have been created, from which many new varieties with the strong resistance to stress and diseases or the improved yield and quality have been bred out. For example, sweet potato/mungbean, castor/cotton, yam/soybean, sweet potato/soybean and castor/soybean etc are all bred out from the progeny of distant graft. Here we introduce the history, definition, types of distant graft, the procedure and key influencing factors for soybean distant-graft mutagenesis, the types and the possible mechanism of soybean distant-graft mutagenesis and prospect of soybean distant-graft mutagenesis, hoping that this technology can be popularized to widely use in resources

innovation and genetic improvement of soybean and to promote the process of soybean new varieties.

2. History of distant graft

There has been a long history of over 1400 years for application of distant-grafting to agricultural production. The earliest records was found in sixth century encyclopedia of Chinese agricultural knowledge-- "Essential skill to benefit the Qi-people", where it was recorded that the graft union with high survival rate between pearl and mulberry tree yielded the poor quality fruits while the graft union with low survival rate between pearl and jujube or pomegranate tree yielded the good quality fruits. After that, the grafts between different trees, flowers, vegetables and crops were recorded in many agriculture-related books such as "Compendium of Materia Medica" and journals such as "Studies in the History of Natural Sciences" and so forth.

From the purpose of distant graft, it could be found that grafting was originally used to improve plant tolerance to cold, resistance to diseases and enhance quality of fruits in the woody trees cultivation and propagation. Since the discovery of inheritable variation induced by grafting, the distant grafts have been used to innovative plant resources and improve plant varieties. Now, the distant graft technology is not only an effective approach for creating new resources and breeding new varieties, but also an important means in plant physiology, pathology, genetic research.

3. Definition and methods of distant graft

3.1 Definition of distant graft

Broadly speaking, grafting is that the branch or bud or stem of a plant is grafted onto the stem or root of another plant, so that the two parts coming from different plants are connected together and grow into a complete plant. Distant-grafting is that the grafting is carried out among different families/genus/species of plants.

The survived grafted plant, the whole scion and stock form a uniform conductive system, the stems and leaves developed by the scion and the root system of rootstock exchange for nutrient. Because the grafted plant is composed by two different individuals, where the roots of rootstock not only function to absorb water and minerals, but also function to synthesize organic acids and amino acids while the leaves of scion are the main organ for the synthesis of organic nutrients, both two parts are not mechanically combined but are an interdependent organic whole. Rootstock and scion each possess a specific function necessary for survival, and also affect the function of each other, including the synthesis of metabolites and the development of reproductive organs, which is consequently expressed in the progeny of scion seeds.

3.2 Methods of distant graft

There have been many different graft methods developed from the long-term graft practices. According to the resource of scion, the graft methods can be classified into three categories: bud graft, branch graft and stem graft. But according to the grafting modes of stock and scion, the graft methods can be divided into three main categories: plug graft, cleft graft and splice graft. For plug graft, the lower or upper of stem of rootstock seedlings is made a hole by using of bamboo sticks, where the scion is inserted after its stem end is whittled into

wedge shape, so that the rootstock and scion can develop a new plant; cleft grafting is that the scion with wedge-shaped end is inserted the spliced mouth of the stem of rootstock; As for splice graft, all the stems of rootstock and scion are cut into the inclined planes with the same angle and length, and then they are bounded together. Other graft methods such as whip and tongue graft, saddle graft, bridge graft, inarch graft etc. are sometimes used in practices. In addition, according to the combination pattern of rootstock and scion, the graft methods are including single Shoot to root grafts, Y shaped grafts with one scion, interstock grafts, Y shaped grafts with two scions, Y shaped grafts with Y shaped shoots and A shaped grafts with two rootstocks (Jia and Han, 2010).

4. Procedure for soybean distant-graft mutagenesis

4.1 Selection and cultivation of rootstock

4.1.1 Choice of rootstock

The plants with tuber roots are suitable for distant grafting as rootstocks, such as ginger, lily, sweet potato, potato, yam, etc. In addition, other plants with thick stem are also appropriate for grafting as rootstocks, for example, castor, sunflower, sesame, maize, sorghum and so on.

4.1.2 Cultivation of rootstock

The stem node near the surface of soil is often chose for graft site by the way of side-plug when grafting, so it is first assured that stem of rootstock is thick enough to prevent splitting of stem while the scion is inserting the hole of rootstock. In order to increase the stem diameter of rootstock, some necessary methods such as ahead of sowing or cutting off the main stem are generally adopted.

4.2 Appropriate grafting age of rootstocks and soybean scions

4.2.1 The grafting age of rootstocks

Under normal circumstances, the rootstocks are sowed earlier 40-45 days than the scion of soybean. But the climate conditions and the rootstocks types may influence the growth and development of rootstocks, the best age of rootstocks are about 50 days after emergency (DAM) under good growth season.

4.2.2 The grafting age of soybean scions

7-9 DAM soybean seedlings is best for grafting according to our practices, and that seedling age is too small or too big all have important effect on the graft survival rate.

4.3 Grafting operation

4.3.1 Drilling a hole at the stem of rootstock

When grafting, the surface soil near to the stem of rootstock is first scraped away a little with the bamboo blade. Second the nearest internode from the topsoil is decided to use as graft site. Then the surgical blade is sterilized with alcohol cotton and cut off the section of the internode vertically. After that, the sterilized bamboo stick with the wedged end is rotatedly inserted into the cut section at an angle of 30-45 degree along the stem. The size and depth of drilled hole is depended on the stem thickness of scion seedling and the length of wedged end of the scion seedling. Generally, the depth of hole is 1.0-1.5 cm and the diameter of the hole is about 0.2-0.3 cm.

4.3.2 Whittling the stem of scion into wedge end

The robust soybean seedlings are selected as scions. The roots are first rinsed with tap water, and then dried out with the filter paper. After that, the stem of soybean seedling is shaved down to the wedged end from the top root hairs with the wallpaper blade, and the wedge length is generally 1.0-1.5 cm.

4.3.3 Inserting the scion into the rootstock

After pulling out the bamboo stick from the hole of rootstock, the prepared scion is immediately inserted into the hole of rootstock slowly with moderate force, assuring that the scion end just reaches the bottom of the hole.

4.3.4 Embedding the joint site with the clay

After the scion is inserted into the hole of the rootstock, the joint site and scion are immediately embedded and fixed using the clay with a humidity of 60-70%.

4.4 Management measures

4.4.1 Shading the graft union

In order to enhance the survival rate of graft, the graft union must be shaded for about 45 days. The strength of shaded light gradually goes down, and finally drops close to natural condition.

4.4.2 Supplying water for the scion

During the shading period, the rootstock is not only supplied with enough water for normal growth, but also the scion plant is offered necessary water one time per day using the micro-sprayer.

4.4.3 Removing the lateral roots from the scion

7 days after grafting, the lateral roots may grow from the scion. It is necessary to check the newly-grown lateral roots of scion daily, and promptly remove them with a surgical blade. These works will continue to 45 days after grafting so that the scion can host on the rootstocks.

4.4.4 Removing the main stem of rootstock

At 45 days after grafting, the part above the surface soil 10cm of the main stem of the rootstock is cut down.

4.4.5 Later management

After removing the main stem of rootstock, the graft union is transferred to normal growth conditions until the scion plant can mature regularly and the seeds (G0 generation) are gained.

4.5 Identification of mutants

From the G1 generation to G3 generation of grafted scion, there are different types of mutants to take on. The mutated traits include the morphological and physiological traits. Before these mutants are used for genetic research and breeding materials, they had better

be subject to molecular identification through different molecular maker methods. The detailed methods for identification of soybean mutants refer to the methods of Li *et al*. (2003).

5. Main factors influencing distant-graft mutagenesis technology in soybean

Soybean distant-graft mutagenesis technology is originated from the graft practices that enhance resistance to stress or diseases of crops and all the operative steps may influence the survival rate and the mutants-induced rate. Hence, we should pay attention to the key steps when grafting.

5.1 Main factors influencing the survival rate of grafting

5.1.1 Affinity between rootstock and soybean scion

The high or low affinity between rootstock and scion directly affects the survival rate of grafting, so choosing a distant-related plant materials whose affinity with soybean is high will provide an effective safeguarding for distant grafting mutagenesis in soybean.

5.1.2 Viability of rootstock and soybean scion

Viability of the rootstock and soybean scion is critical for success of the graft. Generally, we use plant materials with strong resistance and adaptability as rootstocks and choose soybean varieties with better integrated traits as scion, so that soybean scion can gain enough water and nutrition from rootstock during the process of grafting and the survival rate of grafting is improved.

5.1.3 Methods of graft and level of technology

There are different survival rates of grafting for different graft methods. Though to some extent, selection of graft methods depends on the type of scion and purposes of research, the bud graft is better than the stem graft and branch graft, and the plug is better than the cleft graft and splice graft. For distant grafting in soybean, the side-plug graft on the base of stem is generally an ideal approach. In addition, the skill level of grafting technology has an important impact on the survival rate of graft. If the wedge-shaped stem end of the soybean scion is subject to damage or doesn't get to the bottom of the hole on the base of stem of rootstock when grafting, the seedling of soybean scion would wither to death for lack of sufficient water and nutrient.

5.1.4 The level of management after grafting

Management after grafting can not be ignored. Water supply and shading time must be controlled timely and accurately after grafting. Insufficient shading time and water supply all have an obvious impact on the survival rate of graft. Especially, when checking and removing the lateral roots of scion, the operator do not hurt the root of rootstock.

5.2 Main factors affecting the mutation-induced rate of graft

5.2.1 Combination of rootstock and soybean scion

The grafting combinations between different rootstock and soybean may induce different types of mutant. In our study, it is found that the grafting of different combinations will produce different types of mutations. For example, grafting between soybean and castor often induces seed coat color and oil content mutants in their offspring.

5.2.2 The lateral roots of soybean scion

The newly-grown lateral roots of soybean scion may absorb access water and nutrient from the soil, which will weaken the stress, caused by distant grafting and be not conducive to the induction of mutation. As we noticed, the presence or absence of the lateral roots of scions after grafting is related to whether the mutants occur or not and which generation the mutants occur. When removing the lateral roots of scion in time, the mutated traits can be observed on the grafting generation (G0). When retaining a lateral root of scion, the emergence of mutated traits may be postponed to the G1 to G3 generation. When retaining two or more lateral roots of scion, the grafted progeny may not have any mutants.

5.2.3 The number of retained leaves of soybean scion

The retained leaves numbers of scion when grafting may also have an important impact on the mutation rate. In general, the retained leaves numbers of scion negatively correlate with the mutation rate of grafted progeny. According to our experience, retaining 1-2 leaves of soybean scion is the best option for the early 30 days after grafting.

6. Mutation types induced by distant grafting in soybean

In our research on distant–graft in soybean, we have observed the same morphological mutation as those in mungbean reported by Zhang *et al*. (2002). These mutation consist of the variation of growth habit, growth period, seed size, seed coat color, leaf shape, seed germination capacity and so on, which offer the basic materials for soybean breeding and gene mining. Through the directional selection on the progeny of distant-grafting, we have gained the excellent soybean lines characteristic of good quality, early mature, tolerance to drought and chilling, resistance to stress, lodging and diseases.

The results of karyotype analysis revealed that there was ploidy variation of chromosomes in the offspring of distant grafting in soybean. About 20% of the root tip cell showed chromosomal abnormalities (2n=36 or 38) for some individuals in G1 generation (unpublished). Certainly, there will be other variation such as photosynthetic capacity, isozymes and genes waiting for further research.

7. Possible mechanism of distant-graft mutagenesis in soybean

It is initially thought that the genetic variation induced by grafting is due to integration of genetic material of rootstock into the genome of scion (Taller *et al*., 1998). However, recent research in molecular biology have identified that though there are indeed great changes for the DNA sequence of scion, the genetic material of rootstock can be detected in the genome of scion. Meanwhile, it is found that the stress-related retrotransposons in the progeny of distant graft have undergone transposition (Xiao, 2005), which as we know is one of the important mechanisms to induce genome rearrangement and gene mutation. In addition, there are also other evidences that the nucleic acid materials of rootstock can be transmitted to the scion through grafting (Stegemann and Bock, 2009), in which the small RNA might silence the special genes or affect the special genes expression of the scion.

Taken our preliminary study and the results of recent studies together, we believe that stock's genetic material is not integrated into the soybean genome, and speculate that distant graft-induced heritable variation in soybean may be the result that the nucleic acid substances of rootstock as a signal molecule is transmitted into the plant of soybean during the distant grafting process, which initiate the special small RNA interfere system and lead

to the genetic mutation, or the stress conditions caused by distant grafting stimulate the stress-related transposable element transposition, leading to the genome rearrangement or gene mutation.

8. Prospects

It is of practically important significance for putting distant-graft technology into innovating excellent soybean materials, broadening genetic base of soybean and promoting breeding of new soybean with good quality, high yield and resistance to stress. However, there is yet no comprehensive system of distant grafting mutagenesis up to now. In particular, the affinity mechanism and the mutation mechanism of distant grafting are not clear, which leads many scholars to disbelief in the distant-graft mutagenesis technology and soybean mutants induced by distant-grafting. Thus, the most important task on distant graft now is to check on genotypes of the known existing soybean lines derived from distant-graft through molecular approach, validating the reliability of applying asexual hybridization (grafting) to soybean breeding. On the basis of it, the affinity mechanism and the mutation mechanism of distant grafting will be exploring, highlighting as soon as possible the true nature of distant-grafting mutagenesis. At the same time, the grafting technology is integrated with the conventional identification and screening approaches of the mutated traits to create soybean germplasm resources with good nitrogen fixation ability, resistance to drought, salt, diseases and aging, and to identify the genetic controlling loci of the related traits. Besides, Grafting technique can be applied in revealing the physiological process mechanisms involved by the signal transduction materials and becomes irreplaceable means to deep understand the interaction of different organs of plant. We believe in the coming future, the distant-grafting mutagenesis technology will be paid more attention by scholars, and play an increasing role on the soybean resources innovation, soybean breeding and gene mining and so forth.

9. Acknowledgements

The authors would like to thank all the researchers in the field of distant-graft mutagenesis for offering us the technological instructions on the methods of research. This work has been funded by the project for scientific and technological achievements transformation of Chinese science and technology department (2010GB24910701), the project for special foundation of technological innovation of Harbin (2008RFQYN127) and the project for forefront innovation of Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences (KZCX3-SW-NA09-6).

10. References

Jia Si-Xie. Essential skill to benefit the Qi-people [M]. Annotated by Liao Qi-Yu. Beijing: China Agriculture Publishing Company. 1998.

Jia zhen and Han Tian-Fu. Application of grafting technology in soybean physiology and breeding [J]. Soybean sciences, 2010, 29(1):136-142, 150.

Li Xue-Hua. Preliminary construction of soybean mutant library and identification of mutation types [XL]. Master thesis, Nanjing Agricultural University, 2003.

Stegemann S and Bock R. Exchange of genetic material between cells in plant tissue grafts [J]. *Science*, 2009, 324: 649-651.

Taller J, Hirata Y, Yagishita N, Kita M, Ogata S. Graft-induced genetic changes and the inheritance of several characteristics in pepper (*Capsicum annuum* L.) [J]. *Theor Appl Genet*, 1998, 97:705-713.

Xiao Wei-Min. Isolation and analysis of the Ty1/copia retrotransposons of mungbean and its possible role on the variation of graft [XL]. Doctorial thesis, Beijing University. 2005.

Zhang Dan-Hua, Meng Zhao-Huang, Xiao Wei-Ming, *et al*. Graft-induced inheritable variation in mungbean and its application in mungbean breeding [J]. *Acta Botanica Sinica*, 2002, 44(7):832-837.

14

Transformation of Soybean Oil to Various Self-Assembled Supramolecular Structures

Arumugam Gnanamani, Varadharajan Kavitha,
Ganesan Sekaran and Asit Baran Mandal
Central Leather Research Institute (CSIR), Adyar, Chennai
India

1. Introduction

Today's research worlds try to bring everything in nanosize and the tremendous development on nanosize and technology introduced numbers of molecules with immense applications. Though nanostructures from numbers of metals and materials are being synthesized, supramolecular structures attracts the research group at increasing level, because of the interest and urge to know the origin of life. Hence, research groups at global level are making attempts on how the self-assembly and the supramolecular structures have been formed from the single and /or from the combination molecules.

Thus the design and the construction of supramolecular assembly/structures are quite interesting and various hypothetical theories have been developed to substantiate the origin of life. Supramolecular structures are large molecules fashioned by binding of smaller molecules mutually and it often to develop molecules of preferred form including 2D triangles, squares, pentagons, hexagons and 3D octahedrons, cubes and some irregular shapes. Self-assembly is the most prevailing methodology in the design of large, distinct, ordered structures.

The objects of supramolecular chemistry are defined on one hand by the nature of the molecular components and on the other by the type of interactions that hold them together. Three major steps are involved in supramolecular systems; (i) selective binding, (ii) growth of the components in the correct relative orientation and (iii) termination requiring a built in feature which signifies the end process. The chemistry of supramolecular structure is a constitutional dynamic chemistry due to the reversibility of the connecting events. The kinetic liability confers the self-assembling systems to undergo annealing and self-healing of defects and to manifest tunable degree of polymerization and cohesive properties. In contrast, covalent linked, nonlabile type cannot heal spontaneously and the defects are permanent (Lehn, 2005).

According to Murakami, synthesis of supramolecular structure is based on the principle of molecular recognition and molecular self-assembly realized due to the formation of noncovalent interaction towards the cooperation of many weak bonds including electrostatic interaction, Van der Waals forces, dipole interaction, hydrogen bonding, hydrophobic interaction, and π–π interaction. Recently the interest was drawn to a new topological form of supramolecular structures by self-assembly and also by weak interactions.

Generally, supramolecular solid structures are commonly prepared by different templates; polymers, polystyrene, silica and some other metal nanoparticles. Vesicles and microemulsions are used as template to develop on an attractive and stable supramolecular structure. However, in the soft template approach, the control on the size and mechanical stability of the supramolecular structures could become a problem. Further, as pointed out by Shelnutt and his co-workers, it is not easy to prepare stable or large sized (e.g., > 100 nm in diameter) and thickly walled supramolecular spheres based on the soft template approach. Therefore, a new protocol using biological materials through which the rigid structure with controllable size and thickness can be made easily is of great interesting.

Amphiphilic and/or non-polar components further increase the structural diversity to include sponge and microemulsion phases, and even stable multiphase colloidal dispersions of one complex fluid in another – cubosomes and hexosomes. Many aspects of these nanostructures remain under exploited because self-assembled structures exist in dynamic equilibrium, and hence respond to changes in solution conditions. A great deal could potentially be achieved if amphiphilic self-assemblies could be rendered more robust *in situ.* One method for achieving this is to "lock-in" the self-assembled structure using polymerizable surfactants.

Simple, single-chain fatty acids have long been known to self-assemble into supramolecular structures such as micelles and vesicles (Gebicki & Hicks, 1973; Gebicki & Hicks, 1976). Fatty acids in a bilayer membrane are in rapid exchange with the aqueous environment (Walde et al., 1994). Such amphiphiles can also interact with solid surfaces. The interaction of amphiphiles with solid surfaces often involves adsorption due to chemical or physico-chemical forces through covalent bonds, hydrogen bonds, ion exchange, Van der Waals forces, and hydrophobic effects (Giles, 1982; Evans, 1986).

The interactions of simple, single-chain amphiphiles with many different surfaces results in the organization of membranes and the formation of vesicles. This effect could have played a key role in the organization and formation of the first cell-like structures on the early earth. Since mineral particles have been implicated in very early chemistries and polymerization reactions (Bernal, 1951; Wachtershauser, 1988; Ferris and Hill et al., 1996; Sowerby et al., 2001; Sowerby et al., 2002; Monnard, 2005), it is intriguing that minerals might have also been involved in the formation of yet another essential component of life-the cellular membrane. Mineral-mediated vesicle formation occurs with many disparate types of minerals and is therefore a more general property than clay-catalyzed RNA polymerization.

1.1 Current scenario

In general, as described, formation of supramolecular structures are mediated through individually or in combination of heating, cooling, hydration, solvent addition, refluxing, agitation, stirring, shaking and/or hydrothermal reaction. Further, the size, structure and reproducibility of supramolecular structures depend on the methods followed and the substrates employed in the study.

With regard to substrates, most of the researchers synthesized supramolecular structures using the following synthetic molecules; viz., Poly ethylene amine, 2,4,diaminopyrimidine-nitrobenzoate (Stanley et al., 2005), pyrrole-2-carboxylate dimer (Yin et al., 2006), Trichloromethane (Durov et al., 2006), polyoxovanadate (Duan et al., 2006), Cyclodextrin (zeng-guo & Sanping, 2003), Nitrobenzoic acid with ethylenediamine (Srinivasan & Rane, 2009), p-tert-butylcalix[6]arene, ammonium cations, 1-alkynlyl(phenyl) tetrafluroborate-

iodanes, 18 crown-6 (Ochiai et al., 2003) and etc. Similarly, semisynthetic or the combinational substrates such as galactocerebroside containing long chain unsaturated fatty acids, tris(hydroxylmethyl)- aminomethane based biosurfactant, hyperbranched polyethelenimine and fatty acids, glycolipid derivative with hydrogenated fluorinated mixed lipid tail, synthetic spingolipids, block copolymers and etc., are also in use.
In addition, a complete bio-based supramolecular structures from milk fat protein, lipids, DNA, RNA complex, nucleotides, aminoacids or doublechain aminoacids, phospholipids, glycolipids, peptides, gluconamides, bolamphiphilies, lipopeptide and biological amphiphilies compounds are also introduced by various researchers.

1.2 Vegetable oil / fatty acid based supramolecular structures

Even though supramolecular assemblies from above said molecules are in reports with varied hypothetical explanation, however, still the story behind the assembly of biological molecule is unclear. To understand the theory of self-assembly and supramolecular formation, researchers initiated the self-assembly studies using fatty acids and its derivatives. Montarnal et al., (2008) reported self-healing supramolecular rubber like material using vegetable oil, unsaturated fatty acid derivatives, combined with diethylene triamine and urea. Vegetable oil based supramolecular organogel is synthesized by Rogers et al., (2007). Chen et al., (2005) prepared supramolecular nanocapsules by electrostatic interaction between fatty acids palmitic acid and polyethylenimine. A mixture of fatty diacid and triacid is condensed first with diethylene triamine and then reacted with urea giving an oligomeric supramolecular self-assembled thermoreversible rubber having self-healing property (Cordier et al. 2008). Novales et al., (2008) reported self-assembly of fatty acids and hydroxyl derivative salts to form supramolecular assembly.
Maximum reported supramolecular assemblies involve complete synthetic or hybrid systems and or semi biological system. Complete biological means of supramolecular assemblies demand more time and the process of synthesize is a challenging task.

2. Transformation of soybean oil to supramolecular structures- a biomediated process

The present chapter covers the formation of various supramolecular structures from vegetable oils by microbial product mediated process. The experiments conducted in our laboratory revealed *in situ* transformation of vegetable oils to self-assembled different supramolecular structures viz., vesicle, stable vesicle, supramolecular capsules, colloidosomes, self-healing material, supramolecular rubber like material, organogels, sheet/ flims, bioadhesives, etc., mediated through microbial products. Three different experimental setups were run. In the first set of experiments, microbes were directly used for the transformation of vegetable oil to supramolecular structures. For the second set up, instead of microbes, microbial products were used. The third sets of experiments were executed without any microbes and microbial products but prior to exposure to the medium, the oil was heated at 100 °C.
Soybean and sunflower oil are directly procured from the manufacturers and used as a source for triglycerides. Microbes used in the present study are from the varied sources; marine and clinical origin. Microbial products are also from media suppliers; HiMedia Laboratories Pvt. Ltd and MERCK Ltd. The media used for present study are (i) mineral medium comprises of 1 g NH_4NO_3; 2.55 g NaH_2PO_4; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.1 g $CaCl_2 \cdot H_2O$; 0.02 g $MnSO_4 \cdot H_2O$; 1 g Peptone; 0.5 g Glucose; Zobell marine broth containing 5g Peptic

digest of animal tissues; 1g yeast extract; 0.1g ferric citrate; 19.45g sodium chloride; 8.8g magnesium chloride; 3.24g sodium sulphate; 1.80g calcium chloride; 0.55g potassium chloride; 0.16g sodium bicarbonate; 0.08g potassium bromide; 0.034g; strontium chloride; 0.022 boric acid; 0.004 sodium silicate; 0.0024g sodium fluorate; 0.0016g ammonium nitrate; 0.008g disodium phosphate.

The selective microorganisms; marine *Bacillus* sps and clinical strain *Candida albicans* are cultured in the selective medium with triglycerides at different volumes (0.5, 1.0, 1.5, 2.0, 2.5, 4, 6, 8 and 10% w/v) and at cell concentration of 1X 10^5 cells per ml and incubated for the period of 5-10 days under shaking/ agitation at 180-200 rpm. Followed by inoculation, observations on cell growth, pH profile, hydrolytic enzyme production, fatty acids and glycerol release, surface-active agent production, transformation of oil, micelle formation, nano vesicle to macrosize supramolecular structures were made. The descriptions on various supramolecular structures are briefly explained below:

2.1 Transformation of soybean oil to fatty acid vesicles

In our study, the selected microbes, here in, *Candida* sp. grown in the medium containing carbohydrates, peptone, mineral salts and soybean oil at different percentages (w/v) for the period of more than 5-9 days at 37°C under agitation at 180-200rpm, we observed the slow transformation of these oils to micelle and then to vesicles. Though different concentrations of oils were attempted, only at higher concentrations (>5%) we observed the final transformed product as fatty acid vesicles. In addition to these observations, the physico-chemical analysis of the medium on different days clearly illustrates the steps involved in the process of transformations. We found, the microbes, in order to utilize the substrates released the enzymes and surface-active agents externally. Analysis of enzyme release suggests, lipase was released from 12 hours onwards at the rate of 3.5 U/ml. The produced lipase interacted with the oil present in the external aqueous medium and hydrolyzes it into fatty acids with in 48 hours. In the meantime, the organism produces surface-active agents (33 ±2 mN/m), which further starts interacting with the hydrolyzed fatty acids. Thus, a simultaneous reaction takes place between the fatty acids released upon enzymatic hydrolysis and the surface-active agents produced. Figure 1 illustrates the vesicle formed in the growth medium.

Fig. 1. Fatty acid vesicle from soybean oil.

Though, we couldn't observe the complete hydrolysis of oil with that of the lipase produced, however, the unhydrolyzed oil further solubilized by the released surface-active agents and interact with the already formed vesicles and helps in the assembly of vesicles. In addition, in the case of the chosen oils, the presence of lecithin increases the supramolecular self-assembly results with the increased gellation with multilamellar vesicles. The release of amino acids during the growth of the microorganism may also involved in the transformation of vegetable oil to vesicles.

2.2 Transformation of soybean oil to multilamellar stable vesicles (MLSV)

Multilamellar stable vesicles with different shapes and size formation starts with the micelle formation in the zobell marine broth during the growth of marine *Bacillus* sp. Though internal component facilitates the bilayer formation it has been followed by transformation to multilayer and then thickening of oil. In the present study, the external physical agitation (200 rpm), accelerates the transformation of multilayer vesicles (microscopic) to stable macroscopic structures. The varied macroscopic morphological patterns and the stability observed (spherical to cylindrical shapes) might be due to the available interactions between the components of the medium. The cylindrical morphology observed in the present study may be due to the aggregation of micelles followed by transformation to multilamellar vesicles or the bilayer formation followed by aggregates rolled and transformed to rod like giant vesicle with concentric rings.

According to Yan et al., (2009), bipolar nature of amphiphilic is mostly responsible for multilamellar vesicles. The hydrophobicity of released fatty acids mediates the close arrangement with biosurfactants molecule or it may be due to the non-ionic nature of biosurfactants leads to the layer-by-layer formation resulting with multilayer structures. Further, difference in the ratio of number of molecules in the monolayer or bilayers also decides the shape of the vesicles. The reasons for the different macroscopic structures generated could also be explained by (i) decrease in electrostatic interaction and other forces at the bifurcation time; (ii) presence of sensitive reaction diffusion system and (iii) presence of gravity or electric or magnetic fields; (iv) other factors responsible for the weak orientation and finally (v) mismatching of hydrophilic heads of biosurfactants. In addition, self-assembly formed in the experiments is neither uni-directional nor multi-directional, due to the continuous generation of biosurfactants, fatty acids, protein and carbohydrates resulting with assumed morphological features in the solutes. Jiang et al., (2006) reported macroscopic self-assembly of hyper-branched polyester by simple solvent volatilizing route and obtained multi-walled strucutres with millimeter in diameter and centimeters in length. They also found nature of the solvent ratio of the solution, temperature, and molecular concentrations are the parameters deciding the self-assembly of macroscopic structures. Nevertheless, the surface of the self-assembly obtained in the present study is uniform, nonsticky and flexible, emphasizes, formation of MLV initiated with the formation of uni-layer by the components as explained above followed by multilayer formation by action of external agents like agitation and finally transformed to stable macroscopic structures. The less bound water (<3%) in the individual macro-structures may be due to the dehydration of head groups, results with the decrease in effective area per molecule at interface (Singh et al., 2009). Further, we observed, salts present in the medium, also responsible for the stability of macroscopic structures.

Fig. 2. Multilamellar stable vesicles (MLSV) from soybean oil.

Further, most of the reports suggests only alkaline pH mediates the self-assembly processes (Wang et al., 2004). However, in the experiments concerned, we observed stable vesicle formation at acidic pH (>4.0). The formation of fused structures may also due to the counter ions exist in the growth media. According to Lei & MacDonald (2003), because of the counter ions, there is compression in the bilayer. More the compression more the packing, which reduces the entering of outer molecules to the inner core of the vesicle, increases the diameter of the vesicle to the maximum size and the additional bilayer will leads to fusion of vesicles. Though Singh et al. (2009) reported, presence of cationic surfactant increases the packing of lamellar structures, in our study the produced biosurfactants is a non-ionic and the complete packing of lamellar structure may be due to the accumulation effect. The molecular network formation between fatty acids and biosurfactants provides high thermal stability to the macroscopic structures observed.

2.3 Transformation of sunflower oil to supramolecular capsules

Similar to the multilamellar stable vesicle, transformation of vegetable oil to supramolecular capsules is observed in the microbial mediated process of transformation. The selected microbe marine *Bacillus* sps., when grown in mineral medium with sunflower oil at 5% (v/v) at 37°C under 180-200 rpm agitation. As summarized above, hydrolysis of oil takes place at initial stage by enzymes released during the growth of the organism. The release of fatty acids and the unspent peptides mediates the self-assembly process results with the transparent capsule like structures and opaque structures of different shapes after 5 days and the percentage of transparency decreases and at final stage, more than 90% of capsules are opaque. Though two different shapes were observed during the experimental period, the one with spherical shape initially in the form of transparent spheres and upon increasing the incubation period it becomes opaque. The following figure demonstrates the transparent and opaque supramolecular structures of sunflower oil. The reason for the transparent and the opaque self-assembly structures may be due to the orientation of amphiphilic molecules assembly processes and the lecithin present at appreciable level. Furthermore, the dynamic molecular recognition, the reorientation and the packing parameters might have played the role in the formation of capsule like supramolecular assemblies.

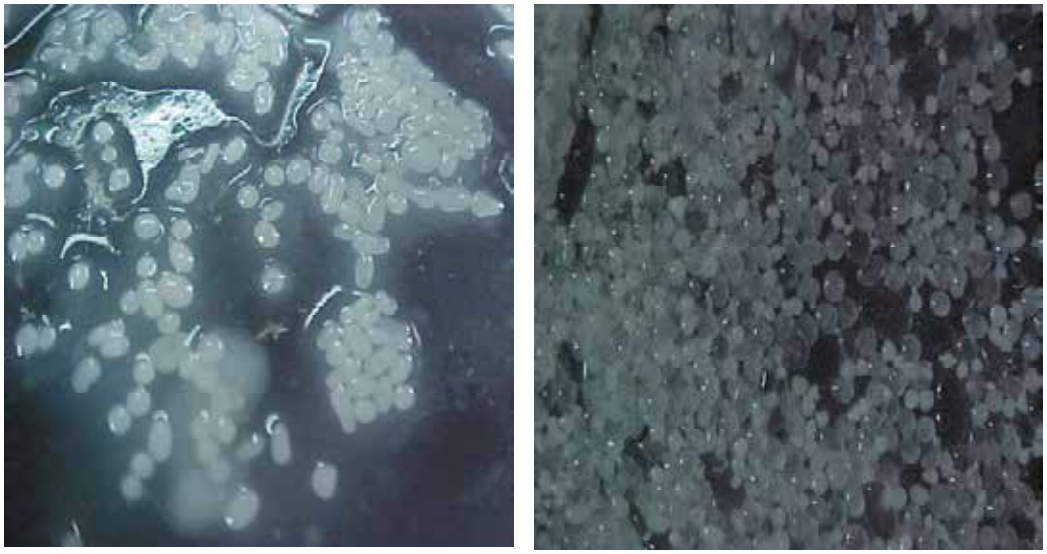


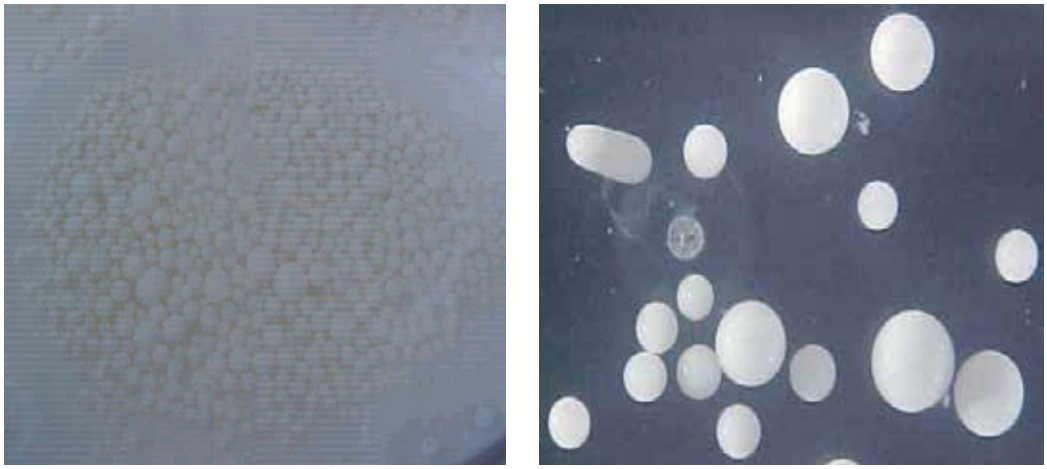
Fig. 3. Supramolecular capsules from sunflower oil.

2.4 Transformation of soybean and sunflower oil to colloidosomes

Colloidal particles are elementary to nature and technology. Self-assembly of colloids at liquid - liquid interface is well documented. Recently, there has been mounting concern in using this self-assembly system to form efficient superstructures, such as emulsions, microcapsules, particles and colloidal crystals. One of the most significant applications of this method is to formulate microcapsules known as colloidosomes, whose shells are composed of coagulated or fused colloid particles.

Based on this method, noval colloidosomes with coagulated or colloidal particles were produced in the presence of oil in water emulsion. Mineral medium with sunflower oil in the presence of marine *Bacillus* sp. and its hydrolytic enzyme and biosurfactants transform the oil into emulsion. Presence of Janus particle (amphiphilic compound nothing but the biosurfactant) stabilizes the emulsion and transform into stable colloidosomes. The presence of the particles at the water/oil interface minimizes the surface energy and therefore stabilizes the emulsion. The wettability and mobility of the particles will determine the stability of the emulsion and those factors are highly dependent on the hydrophobic/ hydrophilic character of the particles and transform into self-assembled supramolecular colloidosomes.

The largest yield of colloidosomes is obtained when using a surfactant to stabilize the oil/water interface. This introduces an electrostatic driving force to take the latex particle to the emulsion interface. The hydrophobicity of the latex particles is the driving force in the surfactant free case but it is evident that the particles are held a short distance apart, presumably due to an electrostatic repulsion in the plane of the emulsion. This repulsion is well known and is thought to operate through the organic phase. The following figure demonstrates the morphological features of colloidosomes obtained from soybean oil (A) and sunflower oil (B).



A B

Fig. 4. Colloidosomes from (A) Soybean oil; (B) Sunflower oil.

2.5 Transformation of soybean oil to self-healing material

An attempt was made on preparation of self–healing material using dimerized fatty acids. In the present study, dimerization of fatty acids has been initiated by use of soy triacylglycerol (STAG) in the presence of lipase (Fungal lipase, Sigma, USA), metal salts (alkali, alkaline earth and transition; Merck, India), monosaccharide and oligopeptides (HiMedia, India) in addition to biosurfactants and under agitation condition (200 rpm, Remi, India). And then self-assembly of dimers in the presence of biosurfactants (Lipopepetide, Sigma, USA) results with the transformation STAG to STLM (Soft Tissue like material).

Fig. 5. Self-healing pattern of delamination damages of STLM.

The obtained STLM was creamy-white in colour with soft nature, partially soluble in polar and non-polar solvents, however, completely soluble in ethylacetate and methanol. STLM showed layered, honeycombed, porous structure with channeled network in Scanning electron microscope. Different damages mode such as cracking, punching, cutting and delamination were made. Since minor damages like cracking healed at the very faster rate (24 hours) and however, healing of punching, cutting and delamination damages, took more than 96 hours and the healing rate differs with the depth of the damage. Figure 5 illustrates the self-healing pattern of delamination damages of STLM.

However, STLM obtained in the present study, heal upon various damages (punching, delamination and cutting) without any inducers and also demonstrate more flexibility upon ageing (more than six months). Further, we found, surface rearrangement, diffusion and reunion of the self-healing material in the presence of aqueous atmosphere as evidenced through spread test conducted. The lower surface tension moieties at the end of the chain make the molecules migrate to the surface, and the diffusion and the interface results in the cross-linking and self-healing property. As reported by Wool (2008), healing of polymers proceeded with various stages of healing mechanisms, viz., surface rearrangement, wetting, diffusion and randomization and also suggest, the fibrillar morphology, nature of molecules at the end of the chain and *in situ* oxidation – reduction reactions.

Further, wetting and spreading of the fluid on the surface of the material, enhances the healing process (Brochard, 1986). In the present study, while damaging the self-assembled tissue like material, the released imbibed materials (hydrophobic and hydrophilic) diffuse through cut ends and trigger the repairing and healing process. The attractive force between the molecules present inside the material and on the surface of the material assembles by itself due to the Van der Waals forces. Here, the driving forces were the hydrophobic components (free fatty acid and unhydrolyzed oil) present in multilayered channeled structures.

With regard to wetting and dewetting processes of semi solid and liquid materials, in general, wetting makes the material to spread over the surface of the water, and in dewetting, the material shrinks and again wetting, spreading of a material transform the substrate to a very thin film (Scheludko, 1968 and lsraelachvili, 1985). However, in contradict to the said natural phenomena of wetting and dewetting, in the present study, we observed, a complete wetting (soaking), make STLM to shrink and the partial wetting, make it to spread. In the partial wetting state, (i) hydrostatic pressure makes the polymer to migrate towards the edge of the petriplate, which, (ii) further triggered by non-covalent bonding between the layers of the material and the hydrophobic components imbibed; (iii) the interface between the water and the material also acting as a driving force; (iv) Surface tension between the air and water interface also pull the material towards the edge. Presence of intramolecular bonding between the dimerized molecules gives the stability and it helps in the structure retrieval.

2.6 Transformation of soybean oil to organogel

We herein report, transformation of soybean oil to macro-sized self-assembled organogel in the aqueous medium containing mineral salts, glucose and peptone. Experiments were carried out at 37°C under agitation (200 rpm) for the period of 240 hours. Though different concentrations (0, 2.5, 5.0 and 10% (w/v)) of soybean oil was examined, the formation of macro-sized organogel was observed only with 10% concentration and the flasks receiving

lower concentrations displayed only the micellar structures. In the first stage, we visualized the transformation of soybean oil to micelle formation similar to the micelle of experimental flasks receiving 2.5 and 5.0% concentrations. Initial micellar formation could be due to agitation and the mineral salts present in the medium. Increasing the incubation period, further (after 6 days) results with vesicle formation and then to gellation. The formation of vesicle and gellation could be due to the primary aggregation of micelles. Similar to the report on self-assembly of peptides, in the present study, presence of glucose, peptone and the prevailing temperature (37°C) may initiate the primary aggregations. In addition, lecithin concentration and the phosphate moiety induce gellation and stabilization of micelles.

When the incubation period further increased to beyond 10 days we observed a formation of a single macro-sized organogel. The gel was spherical in shape with 2 cm in diameter. The inner structure of the cross sections showed multiple layers arranged like onion rings with no hallow space with tissue like appearance and feel in the center core. The thickness of the outer membrane was measured as 1 mm. According to Ai *et al*., (2003) and Liu *et al*., (2003) the layered assembly has molecular scale thickness and a few tens of layers can be easily achieved and the total layered area can be extended above the micrometer scale. Similarly, we observed continuous layering results with a macro-size organogel of 2 cm in diameter. Since, the directional and differential orientation of self-assembly decides the final size and structure of the secondary aggregates, in the present study, layering continues with directional orientation resulting with spherical size. Additional hydrogen bonding and the external agitation force may be responsible for the directionality, and the weak intermolecular forces (Van der Waals attractions) play the major role in the force balance, which results with multiple layering of bilayers in the secondary aggregations. Other than the above said driving forces, pH of the medium (6.0 ± 0.5), presence of divalent cations and anions (transition metal salts), and temperature may also instigate the secondary aggregations and the co-operation between the primary and the secondary aggregations.

There is a possibility of two different modes of primary aggregations for the existence of macrosized organogel. If the primary aggregation was a layered type, then the secondary aggregation could be a multi-layered with the space in between them able to hold either water or lipophilic compound depends on the nature of gel. In other words, if the self-aggregation was a fusion of micelles then the flower like aggregations takes place results with the circular/ spherical layered structures with same kind of holding nature.

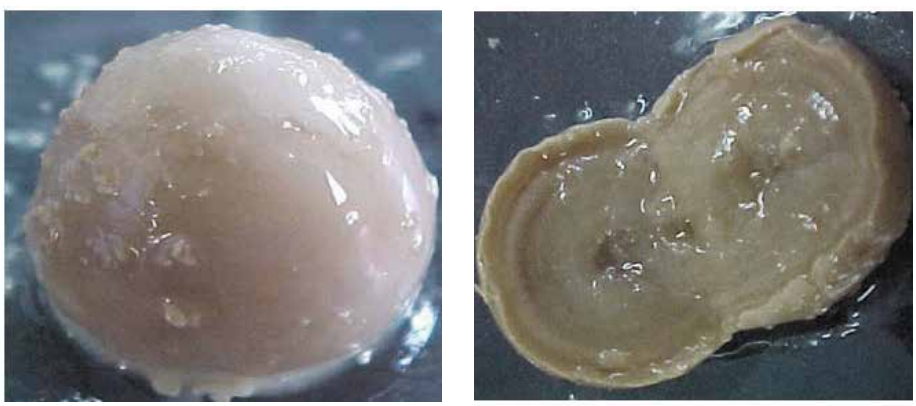


Fig. 6. Macrosized self-assembled organogel from soybean oil.

2.7 Transoformation of soybean oil, sunflower oil to bioadhesive

In the present study, dimerization of fatty acids of triglycerides have been realized during microbial growth. Growing the chosen marine microorganism in the medium increases the multiplication of cells and the doubling time (30 minutes). Though, triglycerides were given at varied concentrations (2 to 10 weight percent), only with >5.0 weight percent concentration, visible changes in the oil phase of the medium were observed.

At >5.0 percent concentration of oil we visualized the transformation of oil phase within 24 to 48 h of incubation. This might by due to the rapid *in situ* hydrolysis of triglycerides by the lipolytic enzymes produced by the organisms. Similarly, an increase in glycerol content in the cell free broth also substantiates the enzymatic process of the cleavage of triglycerides and the presence of a biosurfactant with appreciable surface activity of cell free broth may also contribute to the formation of thread like structures.

The presence of free fatty acids at trace levels evidenced throughout the experimental period in the form of oleic or linoleic acid further confirmed the hydrolysis of oil and the percentage of linoleic acid has been found at higher level compared to oleic acid. The formation of thread like structures further condensed to develop in to a solid mass with adhesive nature when the incubation period increases beyond 120-h.

In situ generation of biosurfactants plays a vital role in the condensation and polymerization as reported by Kestelman and Veselovsky (2001). The increased adhesive nature observed in the product of the present study might be due to the biosurfactants available in the broth during the formation of the adhesive product and its involvement in the intramolecular network of the product. Markevich et al., (1986) detailed the role of surfactant in increasing the adhesive nature of the product, where, they employed synthetic surfactants.

The thread like structures formed during the growth of the organism has been considered as the dimers of fatty acids or triglycerides polymers and these dimers are the precursors for the product formation, however, we couldn't ascertain the nature of dimers formed. Followed by dimerization, the reaction may further preceded and provide the product with adhesiveness. Dimerization followed by product formation might be affected by the presence of (i) biosurfactant; (ii) available amino acids (iii) unspent metal ions (iv) free glycerol and (v) monomers of triacylglycerols in the medium. With regard to dimerization in aqueous medium, Lyons (1969), Wheeler and Godfrey (1974) and Isbell (1994) reported less percentage of water increases the yield of dimers. However, in the present study, dimerization was evidenced in the aqueous medium. The following figure illustrate the adhesive nature of the supramolecular self-assembly structures of soybean (A) and sunflower (B).

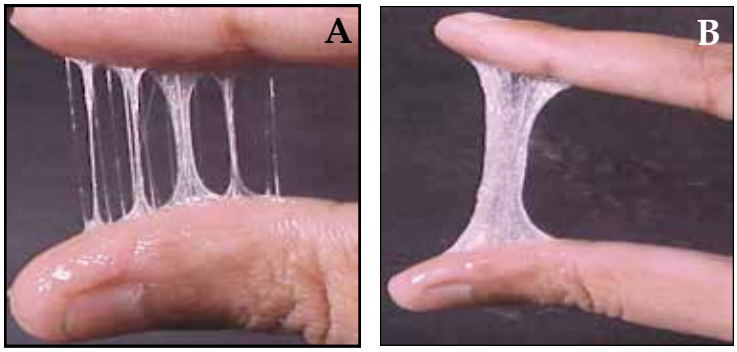


Fig. 7. Bioadhesive from soybean oil and sunflower oil.

2.8 Transformation of vegetable oils to other supramolecular structures

In addition to the above described wide numbers of supramolecular structures, we also received rubber like, membrane/sheet like and microemulsion structures from vegetable oil under insitu conditions. The following figure depicts the images of rubber like (a), membrane/sheet like (b) and microemulsion structures (c).

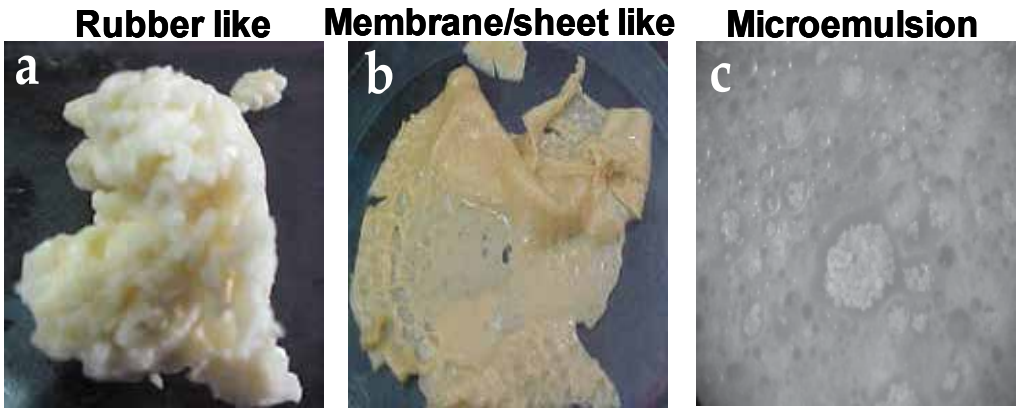


Fig. 8. Different supramolecular structures from vegetable oil (a) rubber like;
(b) membrane/sheet like; (c) microemulsion structures.

The major differences in the above said three structures are lies with the nature of the vegetable oil chosen. The rubber like material is obtained when the oil is heated near boiling point and then cooled and then incorporated in the mineral medium. The important message obtained from this material preparation is, the whole process is proceeded with out any microbes. Hence, self-assembly would have been takes place by mere self-assembly of the saturated fatty acids.

With reference to membrane/sheet like material and microemulsion structures, marine *Bacillus* sps is involved in the presence of mineral salts.

All these supramolecular structures received from vegetable oils under *in situ* condition during the growth of organisms pose a serious question on whether application of all the microbial products separately without microbes will provide the same kind of materials or not. Thus experiments were conducted separately with the selected oils with the enzyme lipase obtained from Sigma and the biosurfactants separated from the species used in the present study, mineral medium and the environmental conditions like agitation at 180-200 rpm. We received supramolecular structures of vesicles, multilamellar vesicles and self-healing materials. Other said structures could not be received and implies they will be formed only in the presence of microbes.

The following schematic representation revealed the formation of various supramolecular structures from vegetable oils and the transformation phases observed in the presence and the absence of microbes.

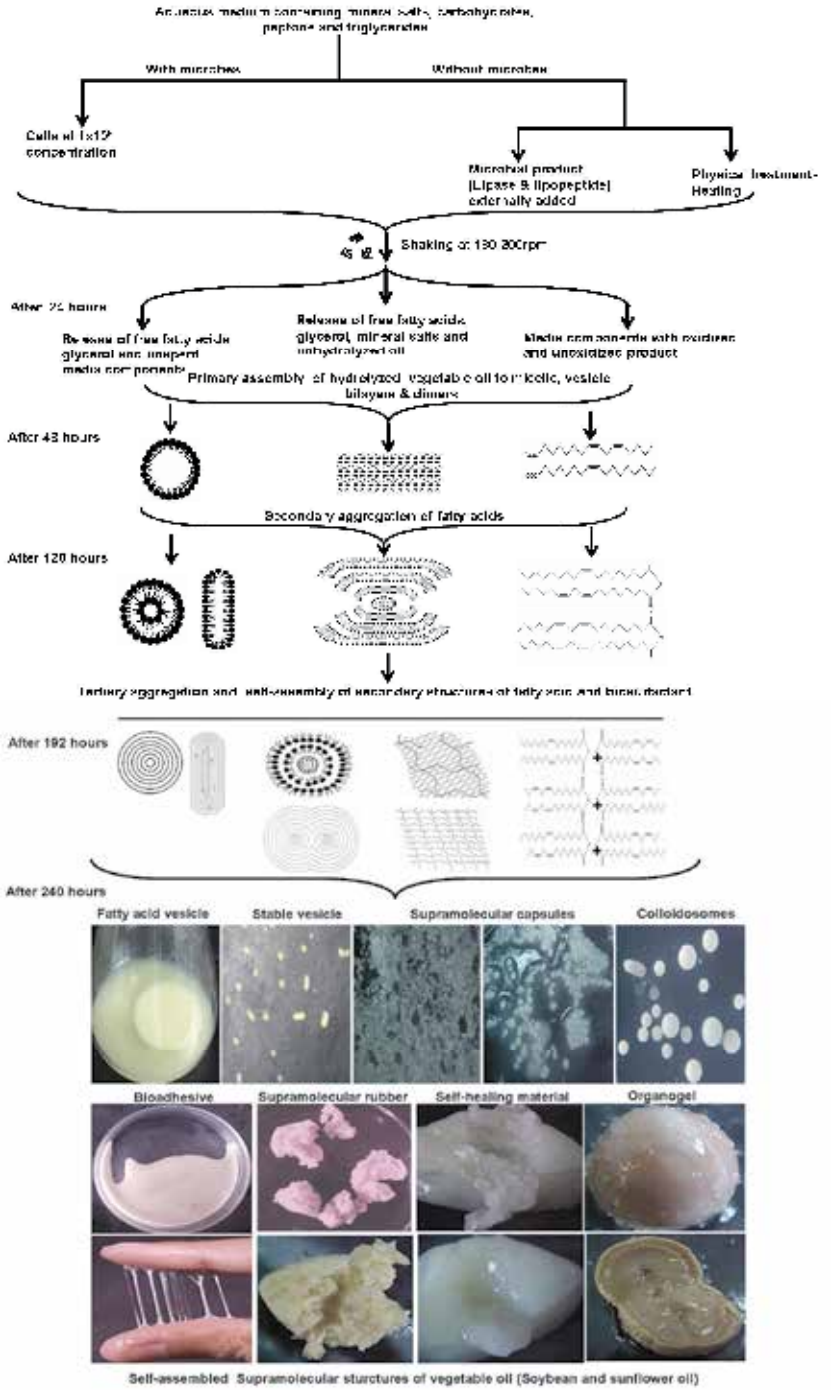


Fig. 9. Schematic representation of different self-assembled supramoelcular structures using plant triglycerides with microbes, with microbial products and by simple physical treatments.

3. Conclusion

As summarized in the introduction, self-assembled supramolecular structures and research on self-assembly of amphiliphilc molecules are always in limelight, since, any one of the hypothetical theories will provide the answer for the question on how the life is originated? Though supramolecular structures of synthetic chemicals and polymers were made under various environmental conditions, preparation of biological supramolecules needs intensive research. The results of our study emphasize the role of microbes and the microbial products on transformation of simple vegetable oils to supramolecular structures under *in situ* conditions. These findings have the pathway for the new approaches on how the live cells are involved in the formation of supramolecular structures. The reproducibility of the results of our study exemplifies, more avenues for future research.

4. Acknowledgment

The authors greatly acknowledge Director General, Dr. Sameer Bramachari, Council of Scientific and Industrial Research, New Delhi, for the support.

5. References

Ai, H. Jones, S. Lvov, Y. (2003). Biomedical applications of electrostatic layer-by-layer nano-assembly of polymers, enzymes, and nanoparticles. *Cell Biochem Biophys,* Vol.39, pp. (23-43).

Bernal, JD. (1951). *The physical basis of life*. Routledge, London.

Brochard, F. (1986) Spreading of liquid drops on thin cylinders the manchon droplet transition*, J Chem Phys*, Vol. 84, No. 8, pp. (4664– 4672).

Chen, Y. Shen, Z. Frey, H. Prieto, JP. Stiri, J. (2005). Synergistic assembly of hyperbranched polyethylenimine and fatty acids leading to unusual supramolecular nanocapsules. *Chem Commun*, Vol.6, pp. (755–757).

Cordier, P. Tournilhac, F. Ziakovic, C. Leibler, L. (2008). Self-healing and thermoreversible rubber from supramolecular assembly. *Nature*, Vol. 451, No.21, pp.(977-980).

Duan, WJ. Cui, XB. Xu, Y. Xu, J. Yu, H. Yi, Z. Cui, Y. Wang, TG. (2007). Two new hydrogen bond-supported supramolecular compounds assembly from polyoxovanadate and organoamines. *J Solid State Chemistry*, Vol. 180, pp. (1875–1881).

Evans, DF. Ninham, BW. (1986). Molecular forces in the self-organization of amphiphiles. *J Phys Chem*, Vol.90, No. 2, pp. (226–234).

Ferris, JP. Hill, AR. Jr. Liu, R. Orgel, LE. (1996). Synthesis of long prebiotic oligomers on mineral surfaces. *Nature*, Vol. 381, No.6577, pp. (59– 61).

Garcia, RM. Song, Y. Dorin, RM. Wang, H. Li, P. Qiu, Y. Swol, F. Shelnutt, JA. (2008). Light-driven synthesis of hollow platinum nanospheres. *Chem Commun*, 22, pp.(2535–2537).

Gebicki, JM. Hicks, M. (1973). Ufasomes are stable particles surrounded by unsaturated fatty acid membranes. *Nature*, Vol.243, pp. (232–234).

Gebicki, JM. Hicks, M. (1976). Preparation and properties of vesicles enclosed by fatty acid membranes. *Chemistry and Physics of Lipids*, Vol.16, No.2, pp. (142-160).

Giles, CH. (1982). Forces operating in adsorption of surfactants and other solutes at solid surfaces: a survey. Plenum, New York.

Isbell, TA. (1994). Acid catalyzed condensation of oleic acid into estolides and polyestolides. *J Am Oil Chem Soc*, Vol.71, pp. (169-174).

Jiang, G. Wang, L. Yu, H. Chen, C. Dong, X. Chen, T. Yang, Q. (2006). Macroscopic self-assembly of hyperbranched polyesters. *Polymer*, Vol.47, pp. (12–17).

Kestelman, V. Veslovsky, R. (2001). *Adhesion of polymer*, Kindle Edition, McGraw –Hill publishers, pp. 22-97.

Lehn, J.(2005).Supramolecular polymer chemistry- Scope and perspectives. In. Supramolecular polymers ed.Alberto Ciferri, CRC Press, Taylor and Francis, FL,USA pp.1-27.

Lei, G. MacDonald, RC. (2003). Lipid Bilayer Vesicle Fusion: Intermediates Captured by High-Speed Microfluorescence Spectroscopy. *Biophysical Journal*, Vol.85, No. 3, pp.(1585–1599).

Liu, S. Volkmer, D. Kurth, DG. (2003). Functional polyoxometalate thin films via electrostatic layer-by-layer self-assembly. *J Cluster Science*, Vol. 14, No. 3, pp. (405-419).

Lsraelachvili, J. (1985). *Intermolecular and surface forces* (New York: Academic)

Lyons, JP. (1969). *Power steering process and lubricating composition*. US Patent 3,429,820

Markevich, MA. Rytov, BL. Vladimirov, LV. Shashkin, DP. Shiryaev, PA. Solovev, AG. (1986). Structural organization in epoxy polymers and oligomers. *Vysokomolek Soedin A*, Vol. 28 No.8, pp.(1595–1602).

Monnard, PA. (2005). Catalysis in abiotic structured media: an approach to selective synthesis of biopolymers. *Cell Mol Life Science*, Vol. 62, No.5, pp.(520–534).

Montarnal, D. Cordier, P. Ziakovic, CS. Tournilhac, F. Leibler, L. (2008). Synthesis of self-healing supramolecular rubbers from fatty acid derivatives, diethylene triamine, and urea. *Journal of Polymer Science*: *Part A: Polymer Chemistry*, Vol. 46, pp.(7925–7936).

Novales, B. Navailles, L. Axelos, M. Nallet, F. Douliez, JP. (2008). Self-assembly of fatty acids and hydroxyl derivative salts. *Langmuir*, Vol. 24,pp.(62-68).

Ochiai, M. Miyamoto, K. Suefuji, K. Shiro, M. Sakamoto, S. Yamaguchi K. (2003). Synthesis and structure of supramolecular complexes between 1-alkynyl (phenyl) (tetrafluoroborato)-l3-iodanes and 18-crown-6. *Tetrahedron*, Vol. 59, pp.(10153–10158).

Rogers, A. Smith, AK. Wright, AJ. Marangoni, AJ. (2007). Novel cryo-SEM technique for imaging vegetable oil based organogels. *J Am Oil Chem Society*, Vol. 84, pp. (899–906).

Scheludko, A. (1968). Thin liquid films. *Adv Coll Inter Science*, Vol.1, pp. (391-464).

Singh, K. Marangoni, DJ. Quinn, JG. Singer, RD. (2009). Spontaneous vesicle formation with an ionic liquid amphiphile. *Journal of Colloids and Interface Science*, Vol.335, pp. (105–111).

Sowerby, SJ. Cohn, CA. Heckl, WM. Holm, NG. (2001). Differential adsorption of nucleic acid bases: relevance to the origin of life. *Proc Natl Acad Sci USA*, Vol. 98, No.3, pp. (820–822).

Sowerby, SJ. Petersen, GB. Holm, NG.(2002). Primordial coding of amino acids by adsorbed purine bases. *Orig Life Evol Biosphere*,Vol. 32, No. 1. pp.(35– 46).

Srinivasan, BR. Rane, GK. (2009). Synthesis, properties and supramolecular structure of di(aqua) bis(ethylenediamine) nickel(II) bis(4-nitrobenzoate). *J Chem Science*, Vol. 121, No. 2, pp. (145–153).

Stanley, N. Muthiah, PT. Geib, SJ. Luger, P. Weber, M. Messerschmidt, M. (2005). The novel hydrogen bonding motifs and supramolecular patterns in 2,4-diaminopyrimidine–nitrobenzoate complexes. *Tetrahedron*, Vol.61, pp. (7201–7210).

Wachtershauser, G. (1988). Before enzymes and templates: theory of surface metabolism. *Microbiological Reviews*, Vol. 52, No. 4, pp. (452– 484).

Walde, P. Wick, R. Fresta, M. Mangone, A. Luisi, P.L. (1994). Autopoietic self-reproduction of fatty acid vesicles. *Journal of American Chemical Society*, Vol. 116, No.26, pp. (11649–11654).

Wang, J. Kuang, M. Duan, H. Chen, D. Jiang, M. (2004). pH-dependent multiple morphologies of novel aggregates of carboxyl-terminated polymide in water. *European Physical Journal E*, Vol. 15, No. 2, pp. (211–215).

Wheeler, CC. Godfrey, AW. (1975). *Lubricating oil compositions*. US Patent 3,909,423

Wool, RP. (2008). Self -healing materials: a Review. *Soft Matter*, Vol. 4, pp. (400–418).

Yan, Y. Lu, T. Huang, J.(2009). Recent advances in the mixed systems of bolaamphiphiles and oppositely charged conventional surfactant. *Journal of Colloids and Interface Science*, Vol. 337, pp. (1–10)

Yin, Z. Li, Z. (2006). Pyrrole-2-carboxylate dimer: a robust supramolecular synthon for crystal engineering. *Tetrahedron Letters*, Vol. 47, pp. (7875–7879).

Zeng-guo, F. Sanping, Z. (2003). Synthesis and characterization of biodegradable hydrogels based on photopolymerizable acrylate-terminated CL-PEG-CL macromers with supramolecular assemblies of a-cyclodextrins. *Polymer*, Vol. 44, pp. (5177–5186).

15

Soybean: Plant Manipulation to *Agrobacterium* Mediated Transformation

Muhammad Zia
Department of Biotechnology, Quaid-i-Azam University; Islamabad
Pakistan

1. Introduction

Revolution in plant biotechnology can be categorized into in vitro culture and genetic transformation. Plant regeneration was successfully achieved in 1950's while production of transgenic plant was accomplished in 80's. For production of transformants, in vitro culturing strategies are prerequisite.
Soybean has been cultured through organogenesis and embryogenesis but still it is considered recalcitrant. Many explant types has been subjected for shoot induction but immature cotyledons and cotyledonary node of mature seeds got attention in recent years due to high number of shoot production in less time period. But still nature of culture media, application of plant growth regulator and environmental conditions affect on regeneration efficiency. If all constrains are consistent, genotype dependency along with age of explants can not be neglected.

2. In vitro manipulation of plants

Plant cell and tissue culture or in vitro manipulation of plant is the key of modern plant biotechnology. Whole plant can be regenerated under aseptic conditions (in glass vessels) using tissues and even cell when provided balanced nutritional conditions. This technology successfully lead to production of elite cultivars, conservation of endangered plant, production of virus free plant, safeguarding of germplasm and production of secondary metabolites. Beside all these, establishment of culturing protocol is main principle in near about all transgenic plant production strategies. Ability of cell to generate into whole organism is attributed to totipotency and plant cells are unique in this case. However, understanding culture conditions with regard to plant species and explant type is critical for development of reliable system. The physiology of explant is more important because stage and age of explant respond differentially under same conditions. While, some plant species can be easily propagated and some species demand variability in growth regulator(s) concentration(s).
The development of successful tissue culture procedure demand appropriate physiological and chemical conditions. Physiological settings include temperature, pH, light and humidity. As a matter of concern, plant cells and tissues have capability to accommodate minor variations in these parameters. However, regarding chemical environment, that include growth medium and hormone, a little variation may wrench the ability of

regeneration. Growth medium consists of appropriate level of essential minerals (major, minor and trace elements), vitamins, carbon source (monosaccharide or disaccharide) and in some specific cases additives such as charcoal, amino acids, specific chemical etc. Now a number of media are commercially available for plant tissue culture such as MS (Murashige & Skoog, 1962); B5 (Gmaborg et al., 1968); SH (Schenk & Hildebrandt, 1972); LS (Linsmaier & Skoog, 1965); White, 1963 and many more. The choice of suitable media depends upon a number of factors such as plant specie, explant type, explant age, geographical distribution of plant and even season if explant is picked from in vivo condition. These basal medium are designed to keep the plant tissue alive and somewhat proliferative. However, for callus induction, shoot and root differentiation, plant growth regulators are required for these developmental programs. Most common classes of growth regulators include auxins, cytokinins and gibberellins either natural or synthetic. For all the stages of development from a cell to whole plant, appropriate type and concentration of these hormones is required that is selected only on hit and trial basis.

2.1 Callogenesis to organogenesis

Callus is mass of undifferentiated cells that develop when explant is grown on appropriate medium. Callogenesis is basically absence of organogenesis. Callus often produces organs and in this situation callus proliferation is halted. Organ production is dependent upon level of cytokinin in the medium. Such differentiation that lead to bud or shoot formation is also termed as direct organogenesis. However, depending upon hormone type and concentration, callus may undergo different developmental stages that lead to somatic embryogenesis (indirect organogenesis). Organ formation is hooked on the balance of auxin and cytokinin and even ability of cell to develop shoot or root. During culture in the presence of suitable phytohormones, cell become competent that leads to differentiation and lastly morphogenesis occurs.

Sometime cell irrespective to plant tissue or callus may undergo embryo formation. These somatic embryos like zygotic embryos pass through different developmental stages as bipolar, globular, torpedo and cotyledonary. These somatic embryos can be successfully bred into whole plant even in the absence of growth hormones.

2.2 Soybean tissue culture strategies

Meristemic tissue formation from cotyledons of immature embryos of *Glycine max* through somatic embryogenesis first time was observed by Lippmann & Lippmann, 1984. Age of explant and concentration of auxin in the medium strongly affect the development of somatic embryos. However, addition of cytokinin along with 2,4-dichloro phenoxy acetic acid (2,4-D) and higher concentration of sugar inhibited embryo formation. Li and co-workers (Li et al., 1985) obtained thousands of plantlets and somatic embryoids from single cell of young embryo when cultured on Murashige and Skoog (MS) medium containing 6-benzyl amino purine (BAP) and indole acetic acid (IAA) under low light conditions. Single cells obtained in this case converted into proembyos in liquid medium leading to somatic embryos formation and hence plantlet on agar containing medium. Further, Lazzeri et al. (1985) presented a reliable system for the regeneration from somatic tissues of soybean. They predicted that formation of somatic embryos from immature cotyledons of soybean looks imitated process that occurs over a range of culture conditions; and the efficiency of embryogenesis depends upon physiological and chemical conditions mostly plant growth regulators. Surface and subsurface cells of cotyledons can be converted into somatic

embryos at high concentration of auxin (Naphthalene acetic acid; NAA), however, the germination of soybean somatic embryos usually do not require exogenous growth regulators and young immature cotyledons have great tendency to give rise somatic embryos. After embryo development and in secondary stage of plantlet formation desiccation perform positive role for better recovery (Parrott et al., 1988; Finer, 1988). Lazzeri et a*l*. (1987) furhter reported that embryo initiation in soybean system is predominantly multicellular and 2,4-D plays a major role in it. However, efficiency of process can be enhanced by NAA and these induced embryos were closely related to zygotic embryos. Subculturing also influence frequency of normal embryo development during somatic embryogenesis. Although, complete cotyledon is considered to produce embryos, Hartweck et al. (1988) reported that epidermal and sub-epidermal cells at distal periphery of cotyledon and geterogeneous embryogenic tissues in central region of cotyledons can produce embryos in the presence of NAA and 2,4-D, respectively. Later on Liu et al. (1992) stated that epidermal cells produce somatic embryos without intervening callus phase (direct organogenesis) and presence of 2,4-D and NAA play major role in this histo-differentiation. Different developmental stages of somatic embryo formation initiate from proembryo while secondary embryogenesis and chimeric embryo development occur during differentiation (Gyulai et al., 1993). The differentiation process takes place in 4-6 weeks, initiated by three and four cell embryo leading to development of globular and heart shape embryo. Abaxial side of explant facing the medium resulted in faster formation of somatic embryos from subepidermal tissue in the presence of silver nitrate, irrespective to pH conditions and high light intensity causes faster production of somatic embryos (Santarem et al., 1997; Hofmann et al., 2004). Meurer et al. (2001) & Fermando et al. (2002) worked on soybean somatic embryogenesis from immature zygotic cotyledons from different locations. They found that genotype and location strongly affect soybean primary embryo development and to develop somatic embryos one should be able to realize acceptable level of embryo initiation of each cultivar. Influence of genetic variations in soybean on embryo initiation from immature cotyledons has been well established but upturn in weight, volume, embryo developmental stages and plant recovery can partially be overcome by modifying protocols. The use of ethylene inhibitor, low concentration of nitrogen and sucrose, desiccation, spermidine and alteration in nitrogen source, polyethylene glycol and sorbitol, reported by different researchers, significantly enhanced embryoid formation and their maturation to plant. Conclusively, in addition to above mentioned factors, breeding line; immature embryo age, quality and appropriate choice and concentration of hormone is essential for significant results.

Besides using immature cotyledons and embryos, a lot of work has also been carried out using cotyledonary node explants from seeds or plantlets after few days of germination. First report of plant regeneration from soybean cotyledonary node segment of seedlings grown in the presence of BAP was by Cheng et al., 1980. They obtained multiple shoot bud formation on medium containing high concentration of BAP but better bud growth was noticed when cultures were transferred to low concentration of BAP. Wright and co-workers also reported that BAP is an essential component of media for shoot induction from cotyledonary node explants. Carbon source (sucrose or fructose) and salt concentration (full MS, ½ MS or 1/4MS) have different effects even hormone concentration is kept constant. They further reported that seedlings germinated on water agar medium were not so responsive for shoot induction (Wright et al., 1986). BAP treatment to embryonic axes does not allow the cell to remain quiescent and cells are reprogrammed to produce multiple somatic foci (Buising et al., 1994). Presence of cytokinins (BAP) interrupts chromosomal

DNA replication in large number of cells in shoot apex that ultimately leads to formation of multi cell loci leading to shoot development. Thidiazuron (TDZ) induce adventitious shoots more efficiently than BAP and hypocotyls proved better than cotyledonary nodes for multiple bud formation while plating method (hypocotyls ending in contact to media) and cutting of explant also effects adventitious shoot formation from mature soybean seed hypocotyl (Zia et al., 2010a). However, after shoot bud induction, placement of explant on zeatin riboside containing medium allow the shoots to increase in length more as compared with other cytokinins. Sairam et al. (2003) developed an efficient protocol for callogenesis and embryogenesis from cotyledonary node explant on MS medium containing 2,4-D and BAP. According to them regeneration efficiency was genotype dependent and the best choice of carbon source might be sorbitol for callus induction and maltose for organogenesis. Addition of other growth regulators such as TDZ and Kinetin in MS or B5 medium varied embryo or shoot formation in different soybean genotypes from mature half seed's nodal segment. However, different stages of proliferation and regeneration also vary depending upon genotype. Such variability's can partially be overcome by some modifications in embryogenesis and regeneration protocols (Bailey et al., 1993). Recently Loganathan et al. (2010) reported the somatic embryogenesis from immature embryonic shoot tips on MS medium containing 6% sucrose, 2,4-D and amino acids. The embryos efficiently regenerated into shoots on hormone free MS medium containing charcoal. While, 72-96hr desiccation positively influenced on plantlet formation.

There are very few reports of soybean regeneration from other explants. In 1977 Beversdorf & Bingham reported callogenic response from hypocotyls and ovaries as explant on semi solid and liquid medium. They failed to regenerate shoots; however, they observed structures similar in appearance to embryos in liquid medium. Primary leaf explant turned into callus when cultured on B5 medium. Indirect organogenesis was successfully achieved when callus was further cultured on modified medium containing pyroglutamic acid that greatly enhanced regeneration capability (Wright et al., 1987). Kim et al. (1994) stated that addition of proline in the medium increased the number of shoots but decreased the length of generated shoots. They also reported that cobalt and zinc also play an effective role in shoot induction from primary leaf nodes. Droste et al. (1993) cultured primary leaf less meristem on organic enriched medium and find microscopic bud like structure within two weeks; however, very few plants were developed from these buds. Reichert et al. (2003) and Tripathi & Tiwari (2003) demonstrated that regeneration efficiency from hypocotyls, epicotyl and primary leaf explants is also genotype maturity dependent. The shoots regenerate from acropetal end and/or central region of cotyledonary node tissue. They further concluded that explant, inoculation medium and appropriate concentration/ combination of growth hormone are also essential for better regeneration efficiency. Stem node segments were also cultured on different basal medium for shoot bud formation (Saka et al., 1980). Combination of MS salts and B5 vitamins supplemented with BAP was found better choice to produce shoot buds. However, bud growth stimulated on medium containing low BAP concentration and replacement of sucrose with fructose.

Protoplast culture; isolated from immature cotyledons has also been reported. Dhir et al. (1991) cultured these protoplast in the liquid medium in the presence of combination of cytokinins (BAP, Kinetin, Zeatin) and observed 21% multiple shooting response from compact calli. The regeneration efficiency increased upto 30% when glutamine, aspragine and Gibberellic acid (GA3) were added in the medium. However, medium supplemented with different amino acids and their derivatives as nitrogen source was found better for

plant recovery from protoplast derived calli. However, composition of medium varies embryogenic calli initiation and then somatic embryo differentiation (Zhang & Komatsuada 1993). Zhao et al. (1998) reported that TDZ plays an important role in embryo induction and germination during soybean anther culture but plant differentiation rate was quite low. Addition of 2,4-D in the medium and culturing in light significantly increased the morphogenic response of anther walls and connective tissues. No androgenic response was observed in anther culture of four soybean genotypes but somatic embryogenesis was observed from the epidermis and the middle layer (Rodrigues et al., 2004, 2005). Higher concentration of 2,4-D during anther culture results in plasmolysis of microspores. Time of culture was also found effective for induction of somatic embryos derived from anther culture. Frequencies of binucleate symmetrical grains and multinucleate / multicellular structure formation were also found significant in the day of culture and cultivar interaction (Cardoso et al., 2007).

3. Plant transformation: a prospective to revolution

Transformation is the alteration in genetic makeup of a cell due to incorporation of a foreign DNA fragment that expresses in the cell resulting variation in physiochemical properties. Plant transformation is now a routine practice and carried out through different approaches including *Agrobacterium* mediated, gene gun, electroporation, microinjection and few more. More than 120 diverse plant species have been transformed. Now in most of the developed countries transgenic crops are cultivated with improved nutritional quality and tolerance to biotic and abiotic stresses. This not only improved food quality and quantity for humans and animals but also somewhat has positive influence on environment. Even after a lot of advancement in transformation technologies, many plant species including soybean is considered recalcitrant to transformation.

Agrobacterium mediated transformation of soybean has shown significant improvement and enabled public and private sector for production of commercial cultivars with transgenic traits. A number of reports describe condition standardization for T-DNA delivery, effect of *Agrobacterium* strain and choice of cultivar and conditions to produce high yield of transformants. Beside all above mentioned conditions, soybean cultivar susceptibility to *Agrobacterium* can not be overlooked. Although, protocols for production of transgenic plant have been standardized but all seems ineffective. We are far away from getting transformants from a single experiment especially in case of soybean that is still considered obstinate to transformation.

3.1 Biological way to introduce DNA into plant cell

Nature has offered *Agrobacterium* the ability to transfer some part of DNA from plasmid to plant cell. This T-DNA (transfer DNA) naturally causes callus formation on plant's parts termed as crown gall disease. However, this is multifarious procedure that involves two biological systems; bacteria and plant cell and success is subjected to compatibility. Unsurprisingly virulence story of *Agrobacterium* is the key for tumor induction. This virulence provokes by simple carbohydrates and phenolic compounds that are released by injured plant tissue. After this initiative, vir genes activate and produce proteins. These proteins hold the charge of transformation that include scratch of T-DNA, carry, direct towards the plant cell and finally integrate into plant genome. Naturally this T-DNA contains genes that are involved in biosynthesis of plant hormones that are involved in uncontrolled proliferation of plant cell leading to callus formation.

Engineering technologies and molecular mindsets expiated asset of *Agrobacterium* to transfer the genes of interest into plant cell. This revolution lighted the pathway to break inert kingdom genetic exchange restrictions. They terminated the property of *Agrobacterium* to cause tumor but did not change the belongings that are involved in T-DNA transfer mechanism. Finally, plant biotechnological era came to revolution to produce transgenic plant species with desired characters. However, all the barriers could not be departed productively. Factors responsible for production of transformants have been studies worldwide and are found more or less same for all genotypes even plant or Agrobacterium. These factors, at *Agrobacterium* flank, include genotype, plasmid constrains, T-DNA length and signaling mechanism. While at plant cell side, the factors include type, age, genetic makeup and welcome address to T-DNA. The welcome discourse also depends upon physical and chemical conditions that finally lead to produce whole plant from a single transformed cell.

Although initially dicots were considered host for *Agrobacterium* but advancement in procedures commanded *Agrobacterium* to display same role in monocots as in dicots. The process of plant transformation is a routine matter in most of the labs but some plant species are still considered recalcitrant to transformation.

3.2 Susceptibility of soybean to *Agrobacterium*

Soybean genotype susceptibility for tumor induction was studied by Pedersen et al., 1983 and Owens & Cress in 1984 on infection with *Agrobacterium*. According to their reports, crown gall formation is dependent upon soybean genotype and *Agrobacterium* strain used as well as on environmental conditions. Physiological age of soybean cotyledons also exert great influence on tumor initiation and tumor morphology. Owens and Smigocki (1988) indicated that transformed soybean cells could be recovered by co-infecting with super-virulent strain and addition of phenolic compounds (Acetosyringone or Syringaldehyde) in inoculation medium increase transformation efficiency. It is also possible to produce tumorigenic genotype by crossing non-tumorigenic with highly tumorigenic genotype in soybean so conventional crossing may help to transform non-susceptible genotypes. Luo et al. (1994) observed production of transformed calli from mature seed cotyledons working on transformation friendly genotype "Peking" with *Agrobacterium* strain A281 harboring pZA-7 (UidA + nptII). They mentioned that production of transformed calli is a simple tool to test constructs designed for soybean transformation. Genotype of *Agrobacterium* (nopaline, agropine, octopine) also plays an important role in infection and T-DNA inheritance (Mauro et al., 1995). Acetosyringone may facilitate tumor formation significantly but not for all *Agrobacterium* strains. However, strain/genotype difference was observed significant while older plant parts showed less susceptibility to tumor formation. Transformation event occurs in number of cells but poor selection and non-regenerable callus formation attribute to poor recovery of transformed plants (Donaldson & Simmond, 2000). A new *Agrobacterium tumefaciens* strain KAT23 isolated from peach root also found effective to induce callus at soybean tissues (Yukawa et al., 2007). This nopaline type strain can transform T-DNA of Ti plasmid and of binary vector efficiently to many legumes including soybean.

3.3 Soybean *Agrobacterium* mediated transformation

Hinchee and his colleagues first time reported soybean transformation with *Agrobacterium* strain pTiT37-SE harboring pMON9749 (GUS + nptII) and pMON894 (nptII + glyphosate tolerance). They successfully regenerated plants on media containing kanamycin and

glyphosate (Hinchee et al., 1988). Modification to regeneration protocol is essential to get high level of transformants. Greater number of mitotic cycles are required before embryo initiation and production of plants with transformed germ lines cells. EHA101 was found more potent to transform soybean immature cotyledons and recovery of transformed plants over LBA4404 (Parrott et al., 1989). However, McKenzie & Cress (1992) were able to get transformed plants from cotyledon and hypocotyl explants from 10 days old seedlings working with LBA4404 harboring pBI121. Transformation efficiency is not dependent only on Agrobacterium genotype but soybean cultivar, age of explant and other conditions also influence. Trick & Finer (1997) introduced sonication assisted *Agrobacterium* mediated transformation (SAAT) system. SAAT permits efficient delivery of T-DNA to large number of plant cell in a variety of different plant tissues. In soybean, GUS expressing surface area increased upto 79.9% by SAAT treatment for 10 sec. Other tissues that are considered difficult to transform can be subjected to SAAT that permit Agrobacterium to infect deep within the plant tissue. While SAAT treatment was not found effective at post co-cultivation period with decreased shoot proliferation from cotyledonary node of some soybean genotypes (Meurer et al., 1998). They also reported that inoculum OD_{600} 1.0 gave better transient expression but no interaction was found between SAAT, *Agrobacterium* strain and soybean genotype. Micro-wound in plant tissues due to SAAT treatment release compounds that facilitate growth and accumulation of bacteria under aerobic conditions so facilitate transformation efficiency (Finer & Finer, 2000). However, longer sonication time may damage plant tissue (Santarem et al. 1998). Way of placement of explant (adaxial side incontact with medium) on medium (Ko et al., 2003); exposure of soybean explants to $AgNO_3$ throughout shoot induction and shoot elongation (Olhoft et al., 2004); explant preparation in the presence of *Agrobacterium* culture and varying level of kanamycin during selection and regeneration (Zia et al., 2010b) are important for better recovery of transformants.

Instead of kanamycin resistant plant, glufosinate resistant (bar gene) plants were produced by Zhang et al. (1999) and Clemente et al. (2000) using cotyledonary node explants of 5 days old seedlings. Glyfosinate selection regime is important to get rid of non-transformed plants and to minimize chimerism. Yan et al. (2000) analyzed that immature zygotic size 8-10mm and co-cultivation for short period increase transient expression while selection by direct replacement at low concentration of hygromycin also increase somatic embryo development and plant regeneration. Cystine present in co-cultivation medium increase transformation efficiency due to presence of thiol group and polyphenol oxidase and peroxidase inhibition (Olhoft & Somers, 2001; Olhoft et al., 2001). Copper and iron chelators were also found effective for better expression. Olhoft and his colleagues successfully transformed soybean by cot node method (Olhoft et al., 2003). High frequency upto 16.4% was observed due to presence of cystine, Dithiothreitol (DTT) and thiol compound in infection and co-cultivation medium. Beside this, addition of Silwet-77 as surfactant; co-cultivation at 22°C also played significant role in transformation (Liu et al., 2007). Donaldson & Simmonds (2000) demonstrated that competent cells, in the case of cotyledonary node transformation, are few so has low transformation competency therefore using cotyledonary nodes as explants present low transformation efficiency. Tight selection procedure (selection of explants on selective agent before infection) increases transformation efficiency and occurrence of less escape (Chen, 2004).

Xing and his colleagues produced marker free plants by introducing two T-DNA binary systems (Xing et al., 2000). Integration of two T-DNA followed by their independent

segregation in progeny is a viable mean to produce maker free soybean transgenic plants. Transformation efficiency was observed upto 15.8% using embryonic tips of soybean pre grown on MS medium containing BAP (Liu et al., 2004). They also observed that shoot regeneration and transformation efficiency increased using embryonic tips over hypocotyls and cotyledons. Embryonic tips were also found sensitive against kanamycin treatment at level higher then 10 mg/l. Addition of antioxidant in co-cultivation medium result in significant decrease in browning and necrosis of hypocotyls and increased GUS expression (Wang & Xu, 2008). Embryogenic tips showed better response for hypervirulent strain KYRT1 than EHA105 and LBA4404 when infected for 20 hours (Dang & Wei, 2007). While co-cultivation for 5 days in dark at 22°C in acidic medium (pH 5.4) also enhanced transformation efficiency. Paz et al. (2004) concluded that use of high vigor seed and minimum seed sterilization also raise transformation efficiency from cotyledonary node of 5-6 days seedling plants. Cystine and DDT during co-cultivation increase T-DNA delivery while glyfosinate selection over bialaphos during shoot induction and shoot elongation also increase transformation efficiency. Ko & Korban (2004) reported that size of immature cotyledon (5-8 mm in length), concentration of bacterial culture and co-cultivation for 4 days significantly increase transformation efficiency. However, they failed to get transformants in the presence of kanamycin during selection. Paz et al. (2006) used cotyledonary node of half seeds as an explant. Use of half seed explants ranged transformation efficiency 1.4 to 8.7% and this system is simple and does not require deliberate wounding of explants. Use of thin 30 fibers needle to wound cotyledonary node cells of half seeds also increased transformation efficiency up to 12% confirmed by gfp activity and L- Phosphinothricin (PPT) selection (Xue et al. 2006). Organogenic callus induced from axillary nodal tissue of soybean was also subjected for *Agrobacterium* mediated transformation (Hong et al., 2007). Moderate concentration of TDZ was required for induction of organogenic calli while low concentration of BAP proved best for organogenic response from callus. They also observed that young callus was more competent to T-DNA delivery and multiple shoot regeneration. Olhoft et al. (2007) tested two disarmed *Agrobacterium* strains for soybean transformation. Regeneration frequency was not significantly different when inoculated with *A rhizogenes* strain SHA17 and *A tumefaciens* strain AGL1 while infection with SHA17 increased transformation efficiency upto 3.5 folds.

3.4 Soybean transformation with *Agrobacterium rhizogenes*

Instead of *Agrobacterium tumefaciens*, soybean transformation also been studied by *Agrobacterium rhizogenes* to study efficiency of strain, properties of roots and resistance against nematodes. Cho et al. (2000) got transformed hairy roots by *A. rhizogenes* strain K599 harboring pBI121 (gus + nptII) and pBINm-gfp5-ER (nptII and gfp). They observed that cyst nematode may complete their life cycle in transformed hairy root cultures containing these genes but concluded that such system can be ideal for testing genes that might impart resistance to soybean against nematodes. RNAi silencing was also studied by *A. rhizogenes* mediated transformation to cotyledon explants of soybean (Subramanian et al., 2005). More than 50% roots were transformed with RNAi construct that exhibited more then 95% silencing. Kereszt et al. (2007) reported that infection of *A. rhizogenes* at cotyledonary node of few days seedling might produce 5-7 roots at infection site with 70-100% efficiency. These roots fully support the plants, are capable of nodulation, have phenotype as determined by genotype of shoot. This can further be used for high throughput transformation, to test high

number of genes, different biological processes and symbiotic relation etc. Klink et al. (2008) introduced a new soybean variety MiniMax with a rapid and short life cycle that produced hairy roots under non-axenic conditions when infected with *A. rhizogens* strains K599 harboring disarmed vector pKSF3. These transgenic roots were capable of compatible reactions with several *Heterodera glycines* races.

3.5 In planta *Agrobacterium* mediated transformation

The development of the in planta transformation system (Floral-dip method and Vacuum infiltration) radically accelerated research in basic plant molecular biology. These methods have been targeted mostly for meristems or other tissues that ultimately give rise to gametes.

Soybean transformation also has been subjected by infecting partially germinated seeds with *Agrobacterium* to vacuum infiltration with high frequency (de Ronde et al., 2001). In planta soybean transformation has also been carried out by Lei et al, (1991); Liu et al., (1996) and Hu & Wang (1999). They introduced foreign DNA by pollen tube pathway and by ovarian injection. Such procedures pass tissue culture steps but for routine transformation physiological conditions of recipient plant, type and concentration of DNA, location of ovary etc are critical factors. By such methods, they produced new varieties that yield batter protein and oil contents. But Li et al. (2002) were not able to produce positive results by pollen tube pathway. They reported that DNA was inside the cell but not integrated into soybean genome. Shou et al., 2002 also performed pollen tube pathway transformation procedure using different soybean cultivars. They observed that only 2% progenies were partially resistant to herbicide. However, no plant was confirmed by Southern blotting carrying transformed T-DNA as well as by histochemical GUS assay. They concluded that pollen tube pathway transformation technique is not reproducible for soybean.

4. Conclusions

Plant tissue culture has attained a lot of attention in recent years because it is a gateway to modern plant biotechnology including plant genetic transformation. Although soybean in vitro manipulation and transformation has passed more then thirty years but still establishment of acceptable protocol is far behind that could be used for all cultivars all over the word. The work is going on to overcome the limitations but soybean genotype could not be overlooked in all methodologies. Now destiny is near where new genetically modified varieties of soybean like Roundup ready will be produced globally by following the established protocols.

5. References

Bailey, M.A.; Boerma, H.R. & Parrott, W.A. (1993). Genotype-specific optimization of plant – regeneration from somatic embryos of soybean. *Plant Science*, Vol.93, No.1-2, pp. 117-120, ISSN 0168-9452

Beversdrof, W.D. & Bingham, E.T. (1977). Degree of differentiation obtained in tissue cultures of *Glycine* species. *Crop Science*, Vol,17, pp. 307-311, ISSN 0011-183X

Buising, C.M.; Shoemaker, R.C. & Benbow, R.M. (1994). Early events of multiple bud formation and shoot development in soybean embryonic axes treated with the cytokine. *American Journal of Botany*, Vol.81, No.11, pp. 1435-1448, ISSN 0002-9122

Cardoso, M.B.; Bodanese-Zanettini, M.H.; de Mundstock, E.C. & Kaltchuk-Santos, E. (2007). Evaluation of gelling agents on anther culture: Response of two soybean cultivars. *Brazilian Archives of Biology Technology*, Vol.50, No.6, pp. 933-939, ISSN 1516-8913

Chen, S.Y. (2004). High-efficiency *Agrobacterium*-mediated transformation of soybean. *Acta Botanica Sinica*, Vol.46, No.5, pp. 610-614, ISSN 0577-7496

Cheng, T.Y.; Saka, H. & Voqui-Dinh, T.H. (1980). Plant regeneration from soybean cotyledonary node segment in culture. *Plant Science Letter*, Vol.19, No.2, pp. 91-99, ISSN 0304-4211

Cho, H.J.; Farrand, S.K.; Noel, G.R. & Widholm, J.M. (2000). High efficiency induction of soybean hairy roots and propagation of the soybean cyst nematode. *Planta*, Vol.210, pp. 195-204, ISSN 0032-0935

Clemente, T.E.; LaVallee, B.J.; Howe, A.R.; Conner-Ward, D.; Rozman, R.J.; Hunter, P.E.; Broyles, D.L.; Kasten, D.S. & Hinchee, M.A. (2000). Progeny analysis of Glyphosate selected transgenic soybean derived from *Agrobacterium*-mediated transformation. *Crop Science*, Vol.40, pp. 797-803, ISSN 0011-183X

Dang, W. & Wei, Z.M. (2007). An optimized *Agrobacterium*-mediated transformation for soybean for expression of binary insect resistance genes. *Plant Science*, Vol.173, pp. 381-389, ISSN 0168-9452

De Ronde, J.A.; Cress, W.A. & van der Mescht, A. (2001). *Agrobacterium*-mediated transformation of soybean (Glycine max) seed with beta-glucuronidase marker gene. *South African Journal of Science*, Vol.97, No.9-10, pp, 421-424, ISSN 0038-2353

Dhir, S.K., Dhir, S. & Widholm, J.M. (1991). Plant regeneration from immature cotyledon protoplast of soybean (*Glycine max* L). *Plant Cell Reports*, Vol.10, No.1, pp. 39-43, ISSN 0721-7714

Donaldson, P.A. & Simmonds, D.H. (2000). Susceptibility to *Agrobacterium tumefaciens* and coptyledonary node transformation in short-season soybean. *Plant Cell Reports*, Vol.19, pp. 478-484, ISSN 0721-7714

Droste. A.; Bodanesezanettini, M.M. & Hu, C.Y. (1993). Meristem culture of Brazilian soybean cultures. *Revista Brasilerra De Genetica*, Vol.16, No.1, pp. 135-144, ISSN 0100-8455

Fernando, J.A.; Vieira, M.L.C.; Geraldi, I.O. & Appezzato-da-Gloria, B. (2002). Anatomical study of somatic embryogenesis in *Glycine max* (L.) Merrill. *Brazilian Archive of Biology and Technology*, Vol.45, No.3, pp. 277-286, ISSN 1516-8913

Finer, J.J. (1988). Apical proliferation of embryogenic tissue of soybean [*Glycine max* (L.) Merrill.*J. Plant cell Reports*, Vol.7, pp. 238-241, ISSN 0721-7714

Finer, K.R. & Finer, J.J. (2000). Use of Agrobacterium expressing green fluorescent protein to evaluate colonization of sonicated-assisted *Agrobacterium*-mediated transformation treated soybean cotyledons. *Letter in Application Microbiology*, Vol.30, pp. 406-410, ISSN 1472-765X

Gamborg, O.L.; Miller, R.A. & Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, Vol.50, pp. 151–158, ISSN 0014-4827

Gyulai, G.; Kiss, E.; Csillag, A. & Heszky, L.E. (1993). Developmental analysis primary and secondary somatic embryogenesis in soybean tissue-culture. *Acta Biologica Hungarica*, Vol.44, No.2-3, pp. 189-196, ISSN 0236-5383

Hartweek, L.H.; Lazzeri, P.A.; Cui, D.; Collins, G.B. & Williams, E.G. (1988). Auxin-orientation effects on somatic embryogenesis from immature soybean cotyledons. *In Vitro Cellular and Developmental Biology-Plant*, Vol.24, No.8, pp. 821-828, ISSN 1054-5476

Hinchee, M.A.W.; Connor-Wood, D.V.; Newwll, C.A.; Mcdonnell, R.E.; Sato, S.J.; Gasser, C.S.; Fischhoff, D.A., Re, D.B.; Fraley, R.T. & Horsch, R.B. (1988). Production of transgenic soybean plant using *Agrobacterium*-mediated DNA transfer. *Nature Biotechnology*, Vol.6, pp. 915-922, ISSN 1087-0156

Hofmann, N.; Nelson, R.L. & Korban, S.S. (2004). Influence of media components and pH on somatic embryo induction on three genotypes of soybean. *Plant Cell Tissue and Organ Culture*, Vol.77, No.2, pp. 157-163, ISSN 0167-6857

Hu, C.Y. & Wang, L. (1999). In planta soybean transformation technologies developed in China: pro 1054-5476 cedure, confirmation and field performance. *In Vitro Cellular and Developmental Biology-Plant*, Vol.35, pp. 417-420, ISSN 1054-5476

Kereszt, A.; Li, D.; Indrasumunar, A.; Nguyen, C.D.T.; Nontachaiyapoom, S.; Kinkema, M. & Gresshoff, P.M. (2007). *Agrobaterium rhizogenes*-mediated transformation of soybean to study root biology. *Nature Protocols*, Vol.2, No.4, pp. 948-952, ISSN 1754-2189

Kim, J.H.; Hack, E. & Lamotte, C.E. (1994). Synergistic Effect of proline and inorganic micronutrients and effects of individual micronutrient on soybean (*Glycine max*) shoot regeneration In Vitro. *Journal of Plant Physiology*, Vol.144, No.6, pp. 726-734, ISSN 0176-1617

Klink, V.P.; MacDonald, M.H.; Martins, V.E.; Park, S.C.; Kim, K.H.; Baek, S.H. & Mathews, B.F. (2008). MiniMax, a new diminutive *Glycine max* genotype with a rapid life cycle, embryogenic potential and transformation capabilities. *Plant Cell Tissue and Organ Culture*, Vol.92, pp. 183-195, ISSN 0167-6857

Ko, T.S. & Korban, S.S. (2004). Enhancing the frequency of somatic embryogenesis following *Agrobacterium*-mediated transformation of immature cotyledons of soybean [*Glycine max* (L.) Merrill]. *In Vitro Cellular and Develomental Biology-Plant*, Vol.40, pp. 552-558, ISSN 1054-5476

Ko, T.S., Lee, S.; Krasnyanski, S. & Korban, S.S. (2003). Two critical factors required for efficient transformation of multiple soybean cultivars: *Agrobacterium* strains and orientation of immature cotyledonary explant. *Theoretical and Applied Genetics*, Vol.107, pp. 439-447, ISSN 0040-5752

Lazzeri, P.A.; Hildebrand, D.F. & Collins, G.B. (1985). A procedure for plant regeneration from immature cotyledon tissue of soybean. *Plant Molecular Biology Reporter*, Vol.3, No.4, pp. 160-168, ISSN 0735-9640

Lazzeri, P.A., Hildebrand, D.F. & Collins, G.B. (1987). Soybean somatic embryogenesis: Effect of hormones and culture manipulations. *Plant Cell Tissue and Organ Culture*, Vol.10, No.3, pp. 197-208, ISSN 0167-6857

Lei, B.J.; Yin, G.C.; Lu, C.H.; Qian, H.; Zhang, K.W.; Zhou, S.J.; & Wang, S.L. (1991). Study of exogenous DNA directly transferred into soybean. *Soybean Science*, Vol.10, pp. 58-63, ISSN 1000-9841

Li, B.J.; Langridge, W.H.R. & Szalay, A.A. (1985). Somatic embryogenesis and plantlet regeneration in the soybean *Glycine max*. *Plant Cell Reports*, Vol.4, No.6, pp. 344-347, ISSN 0721-7714

Li, Z.; Nelson, R.L.; Widholm, J.M. & Bent, A. (2002). Soybean transformation via the pollen tube pathway. *Soybean Genetics News letter*, Vol.29, pp. 1-11, ISSN 1054-2116

Linsmaier, E.M. & Skoog, F. (1965). Organic growth factor requirements of tobacco tissue cultures. *Plant Physiology*, Vol.18, pp.100–127, ISSN 0032-0889

Lippmann, B. & Lippmann, G. (1984). Induction of somatic embryos in cotyledons tissue of soybean *Glycine max* (L.) Merrill. *Plant Cell Reports*, Vol.3, No.6, pp. 215-218, ISSN 0721-7714

Liu, G.Y.; Yang, X.Y.; Song, L.J.; He, S.T.; Lei, B.J.; Lu, C.H.; Qian, H.; Li, X.C. & Lu, Y.B. (1996). Breeding a new strain D89-9822 of soybean from introduction of exogenous total DNA and preliminary probing of breeding value. *Soybean Science*, Vol.15, pp. 353-356, ISSN 1000-9841

Liu, H.K.; Yang, C. & Wei, Z.M. (2004). Efficient *Agrobacterium tumefaciens*-mediated transformation of soybean using an embryonic tip regeneration system. *Planta*, Vol.219, pp. 1042-1049, ISSN 0032-0935

Liu, S.J.; Wei, Z.M. & Huang, J.Q. (2007). The effect of co-cultivation and selection parameters on *Agrobacterium*-mediated transformation of Chinese soybean varieties. *Plant Cell Reports*, Vol.27, No.3, pp. 489-498, ISSN 0721-7714

Liu, W.N.; Moore, P.J. & Collins, G.B. (1992). Somatic embryogenesis in soybean via somatic embryo cycling. *In Vitro Cellular Developmental Biology-Plant*, Vol.28, No.3, pp. 153-160, ISSN 1054-5476

Loganathan, M.; Subbiyan, M.; Ling, Y.S.; Wei, C.L.; Wen, H.H.; Pei, F.L.; Chih, W.Y. & Chin, H.L. (2010). Regeneration of soybean (*Glycine max* L. Merrill) through direct somatic embryogenesis from the immature embryonic shoot tip. *In Vitro Cellular and Developmental Biology-Plant*, Vol.46, pp.265–273, ISSN 1054-5476

Luo, G.; Hepburn, A. & Widholm, J. (1994). A simple procedure for the expression of genes in transgenic soybean callus tissue. *Plant Cell Reports*, Vol.13, pp. 632-636, ISSN 0721-7714

Mauro, A.O.; Nobrega, J.C.; Baraldi, G.L. & Collins, G.B. (1995). Susceptibility of some Brazilian soybean genotype to 3 strains of *Agrobacterium* tumefaciens. *Revista Brasileria De Genetica*, Vol.18, No.3, pp. 417-420, ISSN 0100-8455

McKenzie, M.A. & Cress, W.A. (1992). The evaluation of South African cultivars of soybean for their susceptibility to *Agrobacterium tumefaciens* and the production of transgenic soybean. *South African Journal of Science*, Vol.88, No.4, pp. 193-196, ISSN 0038-2353

Meurer, C.A.; Dinkins, R.D.; Redmond, C.T.; McAllister, K.P.; Tucker, D.T.; Walker, D.R.; Parrott, W.A.; Trick, H.N.; Essig, J.S.; Frantz, H.M.; Finer, J.J. & Collins, G.B. (2001). Embryogenic response of multiple soybean [*Glycine max* (L.) merr.] cultivars across three locations. In Vitro Cellular and Developmental Biology-Plant, Vol.37, No.1, pp. 62-67, ISSN 1054-5476

Murashige, T. & Skoog, F. (1962): A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Plant Physiology*, Vol.15, pp. 473–497, ISSN 0032-0889

Olhoft, P.M.; Bernal, L.M.; Grist, L.B.; Hill, D.S.; Mankin, S.L.; Shen, Y.; Kalogerakis, M.; Wiley, H.; Toren, E.; Song, H.S.; Hillebrand, H. & Jones, T. (2007). A novel *Agrobacterium rhizogenes*-mediated transformation method of soybean [*Glycine max* (L.) Merrill] using primary-node explants from seedlings. *In Vitro Cellular Developmental Biology-Plant*, Vol.43, No.6, pp. 536-549, ISSN 1054-5476

Olhoft, P.M.; Flagel, L.E. & Somers, D.A. (2004). T-DNA locus structure in a large population of soybean plant transformed using the *Agrobacterium*-mediated cotyledonary-node method. *Plant Biotechnology Journal*, Vol.2, No.4, pp. 289-300, ISSN 1467-7644

Olhoft, P.M.; Flagel, L.E.; Denovan, C.M. & Somers, D.A. (2003). Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. *Planta*, Vol.216, pp. 723-735, ISSN 0032-0935

Olhoft, P.M.; Lin, K.; Galbraith, J. & Nielsen, N.C. (2001). The role of thiol compounds in increasing *Agrobacterium*-mediated transformation of soybean cotyledonary-node cells. *Plant Cell Reports*, Vol.20, pp. 731-737, ISSN 0721-7714

Owens, D.L. & Cress, D.E. (1984). Genotypic variability of soybean response to *Agrobacterium* Strains harboring the Ti or Ri Plasmid. *Plant Physiology*, Vol.77, pp. 87-94, ISSN 0032-0889

Owens, D.L.& Smigocki, A.C. (1988). Transformation of soybean cells using mix strains of *Agrobacterium tumifaciens* and Phenolic compounds. *Plant Physiology*, Vol.88, pp. 570-573, ISSN 0032-0889

Parrot, W.A., Dryden, G.; Vogt, S.; Hildebrand, D.F.; Collins, G.B. & Willaims, E.G. (1988). Optimization of somatic embryogenesis and embryo germination in soybean. *In Vitro Cellular Developmental Biology-Plant*, Vol.4, No.8, 817-820, ISSN 1054-5476

Parrott, W.A.; Hoffman, L.M.; Hildebrand, D.F.; Williams, E.G. & Collins, G.B. (1989). Recovery of primary transformants of soybean. *Plant Cell Reports*, Vol.7, pp. 615-617, ISSN 0721-7714

Paz, M.M.; Shou, H.; Guo, Z.; Zhang, Z.; Banerjee, A.K. & Wang, K. (2004). Assessment of conditions affecting *Agrobacterium*-mediated soybean transformation using the cotyledonary node explant. *Euphytica*, Vol.136, pp.167-179, ISSN 0014-2336

Pedersen, H.C.; Christiansen, J. & Wyndaele, R. (1983). Induction and in vitro culture of soybean crown gall tumors. Plant Cell Rep*orts,* Vol.2, No.4, pp. 201-204, ISSN 0721-7714

Reichert, N.A.; Young, M.M. & Woods, A.L. (2003). Adventitious organogenic regeneration from soybean genotypes representing nine maturity groups. *Plant Cell Tissue and Organ Culture*, Vol.75, No.3, pp. 273-277, ISSN 0167-6857

Rodrigues, L.R.; Forte, B.D.; Oliveira, J.M.S.; Mariath, J.E.A. & Bodanese-Zanettini, M.H. (2004). Effect of light condition and 2,4-D concentration in soybean anther culture. *Plant Growth Regulation*, Vol.44, No.2, pp. 125-131, ISSN 0167-6903

Rodrigues, L.R.; Oliveira, J.M.S.; Mariath, J.E.A. & Bodanese-Zanettini, M.H. (2005). Histology of embryonic responses in soybean culture. *Plant Cell Tissue and Organ Culture*, Vol.80, No.2, pp. 129-137, ISSN 0167-6857

Sairam, R.V.; Franklin, G.; Hassel, R.; Smith, B.; Meeker, K.; kashikar, N.; Parani, M.; Alabed, D.; Ismail, S.; Berry, K. & Goldman, S.L. (2003). A study on the effect of genotypes, plant growth regulators and sugars in promoting plant regeneration via organogenesis from soybean cotyledonary nodal callus. *Plant Cell Tissue and Organ Culture*, Vol.75, pp. 79-85, ISSN 0167-6857

Santarem, E.R.; Pelissier, B. & Finer, J.J. (1997). Effect of explant orientation, pH, solidifying agent and wounding on initiation of soybean somatic embryos. *In Vitro Cellular Developmental Biology-Plant*, Vol.33, No.1, pp.13-19, ISSN 1054-5476

Santarem, E.R.; Trick, H.N.; Essig, J.S. & Finer, J.J. (1998). Sonication-assisted Agrobacterium-mediated transformation of soybean immature cotyledons: optimization of transient expression. *Plant Cell Reports*, Vol.17. pp. 752-759, ISSN 0721-7714

Schenk, R.U. & Hildebrandt, A.C. (1972). Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany*, Vol.50, pp.199–204, ISSN 0008-4026

Shou, H.; Palmer, R.G. & Wang, K. (2002). Irreproducibility of the soybean pollen tube pathway transformation procedure. *Plant Molecular Biology Report*, Vol.20, pp. 325-334, ISSN 0735-9640

Subramanian, S.; Graham, M.Y.; Yu, O. & Graham, T.L. (2005). RNA Interference of soybean isoflavone synthase gene leads to silencing in tissue distal to the transformation site and to enhanced susceptibility to Phytophthora sojae. *Plant Physiology*, Vol.137, No.4, pp. 1345-1353, ISSN 0032-0889

Trick, N.H. & Finer, J.J. (1997). SAAT: sonication-assisted Agrobacterium-mediated transformation. *Transgenic Research*, Vol.6, pp. 329-336, ISSN 0962-8819

Tripathi, M. & Tiwari, S. (2003). Epigenesist and high frequency plant regeneration from [*Glycine max* (L.) Merrill.]. hypocotyls. *Plant Tissue Culture*, Vol.13, No.1, pp. 61-73

Wang, G. & Xu, Y. (2008). Hypocotyl-based *Agrobacterium*-mediated transformation of soybean (Glycine max) and application for RNA Interference. *Plant Cell Reports*, Vol.27, pp. 1177-1184, ISSN 0721-7714

White, P.R. (1963). The Cultivation of Animal and Plant Cells, 2nd ed. Ronald Press Company, New York.

Wright, M.S.; Koehler, S.M.; Hinchee, M.A. & Carnes, M.G. (1986). Plant regeneration by organogenesis *in Glycine max*. *Plant Cell Reports*, Vol.5, pp. 150-154, ISSN 0721-7714

Wright, M.S.; Ward, D.V.; Hinchee, M.A.; Carnes, M.G. & Kaufman, R.J. (1987). Regeneration of soybean (*Glycine max*) from cultured primary leaf tissue. *Plant Cell Reports*, Vol.6, No.2, pp. 83-89, ISSN 0721-7714

Xing, A.; Zhang, Z.; Sato, S.; Staswick, P. & Clemente, T.E. (2000). The use of the two T-DNA binary system to derive marker-free transgenic soybeans. *In Vitro Cellular Developmental Biology-Plant*, Vol.36, pp. 456-463, ISSN 1054-5476

Yan, B.; Reddy, M.S.S.; Collins, G.B. & Dinkins, R.D. (2000). *Agrobacterium tumefaciens* – mediated transformation of soybean [*Glycine max* (L.) Merrill] using immature zygotic cotyledon explants. *Plant Cell Reports*, Vol.19, pp. 1090-1097, ISSN 0721-7714

Yukawa, K.; Kaku, H.; Tanaka, H.; Koga-Ban, Y. & Fukuda, M. (2007). Enhanced soybean infection by the legume "supervirulent" *Agrobacterium tumefaciens* strain KAT23. *Bioscience Biotechnology and Biochemistry*, Vol.71, pp. 1676-1682, ISSN 0916-8451

Zhang, X.Z. & Komatsauda, T. (1993). Plant-regeneration from soybean (*Glycien max* L) protoplast via somatic embryogenesis. *Science in China Series B-Chemistry*, Vol.36, No.12, pp. 1476-1482, ISSN 1006-9291

Zhang, Z.; Xing, A.; Staswick, P. & Clemente. T.E. (1999). The use of glufosinate as a selective agent in *Agrobacterium*-mediated transformation of soybean. *Plant Cell Tissue and Organ Culture*, Vol.56, pp. 37-46, ISSN 0167-6857

Zhao, G.L.; Liu, Y.Z.; Lin, A.P. & Li, J.B. (1998). Germination of embryo in soybean anther culture. *Chinese Science Bulletin*, Vol.43, No.23, pp. 1991-1995, ISSN 1001-6538

Zia, M.; Rizvi, Z.F.; Riaz-ur-Rehman & Chaudhary, M.F. (2010). Micropropagation of two Pakistani soybean (*Glycine max* L.) cultivars from mature seeds cotyledon nodes. *Spanish Journal of Agricultural Research*, Vol.8, No.2, pp. 448-453, ISSN 1695-971X

Zia, M.; Rizvi, Z.F.; Riaz-ur-Rehman & Chaudhary, M.F. (2010). *Agrobacterium* mediated transformation of soybean (*Glycine max* L.): Some conditions standardization. *Pakistan Journal of Botany*, Vol.42, No.4, pp. 2269-2279, ISSN 0556-3321

16

Salt-Tolerant Acid Proteases: Purification, Identification, Enzyme Characteristics, and Applications for Soybean Paste and Sauce Industry

Xiao Ting Fu[1] and Sang Moo Kim[2]
[1]*Ocean University of China*
[2]*Gangneung-Wonju National University*
[1]*China*
[2]*Korea*

1. Introduction

Soybean, the most cultivated plant in the world, is rich in proteins (40–50%) and contributes many essential nutrients and health-promoting bioactive compounds. Fermented soybean products including soybean paste and soybean curd, *etc.* are consumed in considerable amounts in Asian countries such as China, Japan, and Korea for a long history. Nowadays, they are considered as healthy foods and soy sauce become quite popular all over the world. The fermentation process improves the nutritional quality of the soybeans and contributes to the elimination of trypsin inhibitors (Kim et al., 2010). They are known to be highly digestible and nutritious, and affect a number of physiological activities such as antioxidative activity (Lin et al., 2006), fibrinolytic activity, lowering of blood pressure, and prevention of osteoporosis (Kim et al., 2011). Fermentation starters are essential for the production of fermented soybean products. Microorganisms including *Bacillus*, *Aspergillus*, and *Rhizopus* species and proteases are commonly used as a starter for fermenting soybeans. Proteases produced from microorganisms are widely used for baking, photographic, brewing, fermentation, protein hydrolysates, gelatin industries, meat, leather, and detergents, *etc.* (Murakami et al., 1991). In this study, salt-tolerant acid proteases and their application in the manufacture of fermented soybean products are discussed.

2. Fermented soybean product

2.1 Soybean paste

Soybean paste was originated in China about 2,500 years ago. It is commonly known as Jiang in China, Miso in Japan, Doenjang in Korea, Tao-tjo in Indonesia and Thailand, and Tao-si in Philippines. There are series of soybean paste products in each country. Though they are made with soybean and cereals in the presence of salt, they have special tastes and flavors because of the different ratio of substrates, salt concentration, and the length of fermentation and aging (Fukushima, 1979). The salt concentration of fermented soybean paste is ranged from 4-11%, and the pH is about 5 (Shibasaki and Hesseltine, 1962). Soybean

paste is consumed in large different ways in different countries. It is used as the base for sauces served with meat, seafood, poultry, or vegetable dishes in China, while it is used as the base for soups in Japan and Korea (Fukushima, 1979).

2.2 Soy sauce

Soy sauce is recognized as Jiang-you in China, Shoyu in Japan and Ganjang in Korea. There are two types of soy sauce based on the color. One type of soy sauce is an all purpose liquid seasoning, characterized by a strong aroma, a myriad flavor, and a deep red-brownish color. The other type is in a lighter brown color used mainly for cooking when the original flavor and color of the foodstuff need to be preserved. The difference in aromas observed between Japanese and Chinese styles of soy sauce are ascribed to the different ratios of wheat and soybeans used and the existence of pasteurized process (Fukushima, 1979). The salt concentration of soy sauce is around 16-17%, and the pH is around 4-5 (Shibasaki and Hesseltine, 1962).

2.3 Fermented whole soybean products

Fermented whole soybean products are known as Dou-chi in China, Natto in Japn, and Tempeh in Indonesian (Ogawa et al., 2004), which are much different from their counterparts. These products are solid, and the shape of cooked whole soybean particles is kept as it is. Although these products from different countries share this common characteristic, they have distinctive qualities because of the different bacterial and fungal communities in these products (Kim et al., 2010).

2.4 Soybean curd

Sufu (Fu-ru, or Dou-fu-ru in Chinese) is a fermented soybean curd and a highly flavored, soft creamy product originating in China. It is a cheese-like product consumed as a side dish mainly with breakfast rice or steamed bread (Han et al., 2001). It has a long history and written records date back to the Wei Dynasty 220–265 AD in China. Several types of sufu can be distinguished, according to processing method or according to color and flavor.

3. Starters for the fermented soybean product

Fermentation of soybean is complicated and time-consuming. High amount of salt used for the manufacture and the acid environment inhibit the growth of microorganism and enzyme activity. Therefore, starter is used to shorten the natural fermentation period. Soybean is full of protein and carbohydrate, thus the starter should produce or contain various hydrolytic enzymes such as protease and amylase with strong activity in solid or submerged cultures.

3.1 Microorganisms

Microorganisms used as soybean fermentation starters include bacteria, molds and yeasts. The first procedure for soybean fermentation is the preparation of Qu (in Chinese) or called koji (in Japanese). Koji is a source of microorganisms and their enzymes for converting the carbohydrates and proteins into sugars, peptides, and amino acids, etc. contained in the materials. The nutrients which were produced through the action of these enzymes will be used by yeasts and lactic acid bacteria during further fermentation period (Abe et al., 2006).

3.2 Soybean paste

Cultures of *Aspergillus oryzae*, which known as a seed mold, are commonly used as starter for the production of soybean paste (Fukushima, 1979). Rice or barley is steamed and inoculated with *Aspergillus oryzae* to make koji. After completion of fermentation, the resulting koji is mixed with salt, cooked whole soybeans, pure cultured yeasts, lactic acid bacteria, and water, and then kept for an appropriate period for the second fermentation. The resulting aged mixture is mashed and packaged as miso. In general, miso is a paste of bright yellow to dark brown color. Whiter miso contains more rice than soybeans, whereas the darker miso contains 50 to 90% soybeans (Abe et al., 2006). The molds produce amylases and proteases which hydrolyze the cereal starch and the proteins in soybeans and cereals.

3.3 Soy sauce

Cultures of *Aspergillus oryzae* are also used as starter for the production of soy sauce. The manufacturing of soy sauce is carried out as follows (Fukushima, 1979). Soybeans, or defatted soybean flakes, are moistened and cooked under pressure until they are sufficiently soft. The cooking was done in a batch type or a continuous cooker which allows a high pressure. On the other hand, the wheat is roasted by continuous roasting and then cracked into four to five pieces. The cooked soybean and wheat are mixed and then inoculated with a pure culture of *Aspergillus oryzae* to make koji. After two or three days, the well fermented koji is mixed with yeasts, lactic acid bacteria in the presence of more than 18% salt water which excludes undesirable microorganisms in the second period fermentation. The pH drops from an initial value of 6.5-7.0 down to 4.7-4.8. After fermentation, the product is filtered, pasteurized and packaged as soy sauce. The salt concentration of soy sauce from different country ranges from 10-21% (Stute et al., 2002).

3.4 Fermented whole soybean products

Fermented whole soybean products are made of microorganisms with nindehulled soybean. *Bacillus natto* (Murooka and Yamashita, 2008) and *Bacillus subtilis* (Kim et al., 2011) are the bacteria used for Natto production in Japan and Chungkookjang in Korea, respectively. *Rhizopus oligosporus* is the principal fungus used for the Tempeh preparation in Indonesia. *Bacillus subtilis* and *Rhizopus microsporus* are used in different kinds of Dou-chi production in China (Wang et al., 2008).

3.5 Soybean curd

Actinomucor spp., i.e. *A. elegans* and *A. taiwanensis*, and *Rhizopus* spp., i.e. *Mucor sufu* and *Mucor wutungkiao* are used as starters for high quality sufu making (Han et al., 2001). After preparation of tofu from soybean, one or more types of the above microorganisms are inoculated to ferment pehtze. The pehtze is transferred to salt-saturated solution to adsorb salt until the salt content of pehtze reaches about 16%, which takes 6–12 days. After maturation with different kinds of dressing mixture, different kinds of sufu are produced.

3.6 Enzymes

Manufacturing processing of fermented soybean product requires to uniform of the quality of the product. However, it is difficult to control the natural fermentation according to the traditional method using microorganisms as starters. Because high salt concentration is needed in fermentation of soybean products, salt-tolerant hydrolytic enzymes, especially

protease with high proteolytic activity is used as starter to uniform the quality of the product, and shorten the ripening periods of fermentation. During the fermentation, the pH value decreased down to lower than 5 (Fukushima, 1979), thus the salt-tolerant acid protease is more valuable for the production of fermented soybean products.

4. Salt-tolerant acid proteases

4.1 Microorganisms for the production of salt-tolerant proteases

Salt-tolerant proteases were produced from halotolerant microorganisms; Halobacterium salinarium, Bacillus sp., Halobacterium halobium, Halomonas sp., Halobacillus sp., Virgibacillus sp., Oceanobacillus sp., Saccharomyces cerevisiae, Aspergillus sp.,etc (Barbosa et al., 2006; Jeong et al., 2001; Kim et al., 2004). However, few of them have been purified and characterized to be acid proteases. They are proteases produced by B. subtilis JM3 from anchovy sauce (W.J. Kim and S.M. Kim, 2005), B. megaterium KLP-98 from fermented squid (Fu et al., 2008), Rhizopus japonicus (Chung, 1984), and A. oryzae LK-101 from the traditional Korean soybean paste (Hwang et al., 2010).

4.2 Production of salt-tolerant acid protease

4.2.1 Production of salt-tolerant acid protease by *B. subtilis* JM-3

For the isolation of protiolytic bacteria, anchovy sauce fermented at 15 ± 3 °C for 3 years was inoculated on a brain–heart infusion (BHI) agar and incubated at 27.5 °C. The highest transparent colony was isolated and identified by the genetic mapping method to be *B. subtilis*. Five hundred milliliters of the medium in a 1-L wide-mouth culture flask was inoculated with 10 mL of *B. subtilis* JM-3 suspension prepared from BHI broth media cultivated at 37 °C for 3 days. The culture was incubated at 37 °C for 8 days in a shaking incubator at 150 rpm. The optimal protease production reached 1500 U/L (W.J. Kim and S.M. Kim, 2005).

4.2.2 Production of salt-tolerant acid protease by *B. megaterium* KLP-98

Proteolytic bacteria from the fermented squid were isolated by BHI agar. After inoculating 0.1 mL of fluid of the squid with 10% NaCl concentration fermented at room temperature for 2 months, the culture media were incubated at 37 °C for 48 h. The highest transparent colony on the culture medium was isolated, identified and inoculated in BHI broth media with 10% NaCl. 500 mL of medium in a 1 L wide-mouth culture flask was inoculated with 10 mL suspension prepared from BHI broth media cultivated at 37 °C for 72 h. The culture was incubated at 37 °C for 5 days in a shaking incubator at 150 rpm. The optimal protease production reached 520 U/L (Hwang et al., 2010).

4.2.3 Production of salt-tolerant acid protease by *A. oryzae* LK-101

Proteolytic bacterium was isolated from traditional Korean soybean paste on agar plates incubated for 2 to 4 weeks. Spores suspension was transferred to the seed culture medium (pH 3.0) containing glucose, corn steep liquor, Hyflo Super-Cel, and a defoaming agent. The bacterium was identified by rDNA sequencing method and and named as *A. oryzae* LK-101. For the production of protease, *A. oryzae* LK-101 was cultivated in a 500 mL flask containing 200 mL of 2% defatted soybean flour culture broth at 27 °C for 4 days in a shaking incubator at 150 rpm. The optimal protease production reached 973 U/L (Fu et al., 2008).

4.3 Extraction and purification of salt-tolerant acid protease

Five kinds of reported salt-tolerant acid proteases were extracellular enzymes. Thus, they were extracted and purified from the culture media of each corresponding microorganism.

4.3.1 Purification of salt-tolerant acid protease of *B. subtilis* JM-3 and *B. megaterium* KLP-98

As shown in Table 1 and 2, proteases produced by *B. subtilis* JM3 and *B. megaterium* KLP-98 were purified by a similar procedure. Proteases in the culture media were precipitated by ammonium sulfate. The precipitates were dissolved, dialyzed, and applied to a DEAE-Sephadex ion exchange column. Proteins were eluted with an increasing gradient of NaCl. Fractions containing greater than 50% of maximal peak activity were pooled, dialyzed and applied to a Sephadex G-75 gel filtration column, and eluted with sodium acetate buffer (pH 5.5). Proteases produced by *B. subtilis* JM3 and *B. megaterium* KLP-98 were purified by 35.56 folds with 5.33% of yield and 18.83 folds with 15.3% of yield, respectively.

Steps	Volume (mL)	Total Protein (mg)	Total activity (U)*	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2,000	600.0	3000.0	5.0	100.0	1.0
Ammonium sulfate	10	13.1	320.5	24.5	10.68	4.9
Ultrafiltrate	16	8.5	235.2	27.7	7.84	5.5
DEAE-Sephadex	35	2.2	185.2	84.2	6.17	16.8
Sephadex G-75	50	0.9	160.0	177.8	5.33	35.56

Table 1. Purification of *Bacillus subtilis* JM-3 protease from anchovy sauce (W.J. Kim and S.M. Kim, 2005).

Steps	Volume (mL)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	2,000	1,234	1040	0.84	100.0	1.00
Ammonium sulfate	14	814	1000	1.23	96.2	1.46
DEAE-Sephadex	40	32	172	5.36	16.5	6.37
Sephadex G-75	40	10	159	15.86	15.3	18.83

Table 2. Purification of *Bacillus megaterium* KLP-98 protease from fermented squid (Fu et al., 2008).

4.3.2 Purification of salt-tolerant acid proteases of *Rhizopus japonicus*

As shown in Table 3, two acid proteases were produced by *R. japonicus*. They were precipitated by ammonium sulfate, and purified by twice applications of CMC column (Fig. 1). Proteases I and II were purified by 165.5 folds with 61.6% of yield and 176.5 folds with 2.9% of yield, respectively.

Purification Steps		Total activity (Unit)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme		385000	25300	15.2	100	1.0
Ammonium sulfate		225000	3150	71.4	58.4	4.7
First CMC column		26880	20.5	1311	7	86.25
Second CMC column	Protease I	6050	2.4	2521	1.6	165.5
	Protease II	11000	4.1	2683	2.9	176.5

Table 3. Purification of acid salt-tolerant proteases from *Rhizopus japonicus* (Chung, 1984).

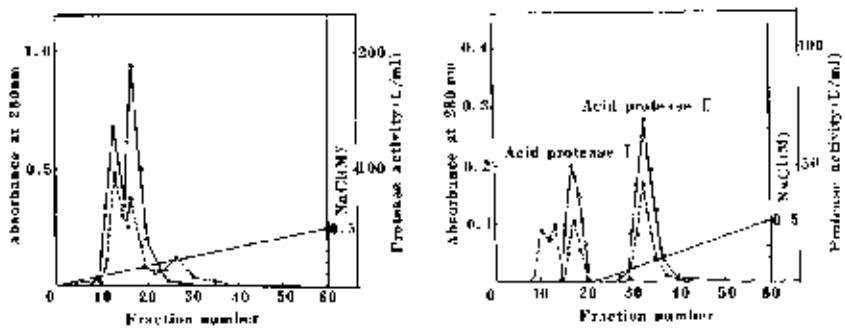


Fig. 1. First (A) and second (B) CMC column chromatography. - - - - Abs 280nm, — — protease activity (Chung, 1984).

4.3.3 Purification of salt-tolerant acid proteases of *A. oryzae LK-101*

As shown in Table 4, purification of protease produced by *A. oryzae* LK-101 was carried out as follows. Culture medium was centrifuged. Ammonium sulfate was added to precipitate the protein. The precipitate was dissolved, dialyzed, and concentrated by ultrafiltration. The resulting concentrate was purified by DEAE-Sephadex ion exchange column and Sephadex G-100 gel filtration column consequently. Protease produced by *A. oryzae* LK-101 was purified by 11.6 folds with 6.8% of yield.

Purification steps	Total activity (Unit)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
Crude extract	97,270	490.7	198.2	100.0	1.00
Ammonium sulfate	21,229	19.4	1,094.3	21.8	5.52
Ultrafiltration	13,351	10.8	1,236.2	13.7	6.23
DEAE-Sephadex	8,913	5.24	1,700.9	9.2	8.57
Sephadex G-100	6,581	2.86	2,301.0	6.8	11.59

Table 4. Purification of *A. oryzae* LK-101 from the traditional Korean soybean paste (Hwang et al., 2010).

4.4 Determination of protease activity

Protease activity was determined according to the modified method of Anson (1939). One mL of enzyme solution was added to 5 mL of 0.6% casein solution in 1/15 M phosphate buffer, pH 6.5 and reacted at 37 °C for 10 min. The reaction was stopped by adding 5 mL of 0.44 M trichloroacetic acid (TCA) and then stood for 30 min. The solution was then filtered with Whatman No. 2. Two mL of the filtrate was mixed with 5 mL of 0.55 M Na_2CO_3 solution and 1 mL of 1 N Folin reagent, and then stood for at room temperature for 30 min. The absorbance was measured at 660 nm with spectrophotometer, and then converted to the amount of tyrosine equivalent based on a standard curve. One unit (U) of protease activity was defined as the amount of enzyme releasing 1 μmol of tyrosine equivalent per 10 min. Protein concentration was determined according to the method of Lowry (Lowry et al., 1951) with egg ovalbumin as the standard. During column chromatography, protein concentration in the fractions was estimated by measuring the absorbance at 280 nm.

4.5 Characterization of salt-tolerant acid protease

Characteristics of proteases purified from the culture medium of *B. subtilis* JM3, *B. megaterium* KLP-98 and *A. oryzae* LK-101 were well studied, while those of the protease from *Rhizopus japonicus* except specific activity were not reported. Thus, the characteristics of the three kinds of proteases were compared and discussed in this study.

4.5.1 Enzyme activity

As shown in Table 5, the protease from different origin varied a lot in specific activity (W.J. Kim and S.M. Kim, 2005; Fu et al., 2008; Chung, 1984; Hwang et al., 2010). The specific activity of the *R. japonicus* protease II was the highest among these five proteases, and that of the *B. megaterium* KLP-98 was the lowest.

Source of protease		Specific activity (U/mg)
Bacillus subtilis JM3		177.8
Bacillus megaterium KLP-98		15.86
Rhizopus japonicus	Protease I	2521
	Protease II	2683
Aspergillus oryzae LK-101		2301

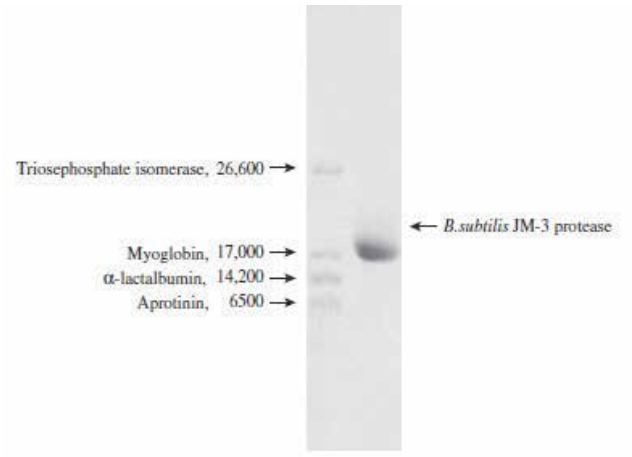
Table 5. Comparison of specific activities of purified salt-tolerant acid proteases.

4.5.2 Molecular mass

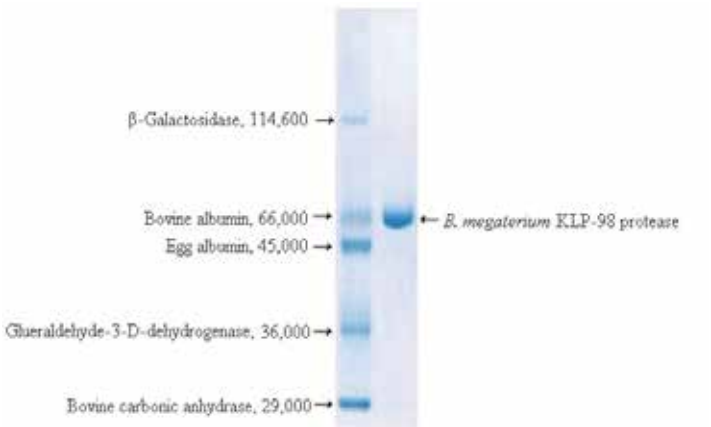
The molecular masses and purities of the protease were determined by SDS-PAGE electrophoresis (Fig. 2) (W.J. Kim and S.M. Kim, 2005; Fu et al., 2008; Hwang et al., 2010).

Source of protease	Mr (kDa)
Bacillus subtilis JM3	17
Bacillus megaterium KLP-98	64
Aspergillus oryzae LK-101	25

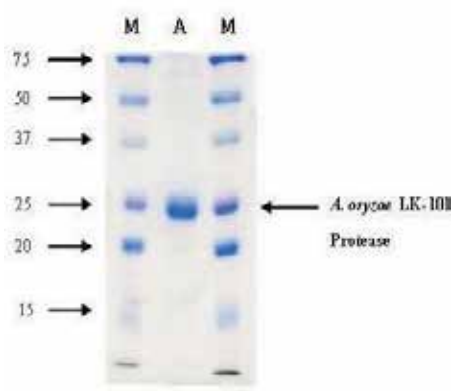
Table 6. Comparison of molecular masses of purified salt-tolerant acid proteases.



A



B



C

Fig. 2. SDS-PAGE electrophoresis of proteases produced by *B. subtilis* JM3, *B. megaterium* KLP-98 and *A. oryzae* LK-101.

Each purified protease was homogeneous on SDS-PAGE. As shown in Table 6, different protease had different molecular masses ranging from 17 kDa to 64 kDa.

4.5.3 Effects of temperature on enzyme activity and stability

As shown in Table 7, the optimal temperature for the activity of protease produced by *A. oryzae* LK-101 was lower than those of the other two proteases, while the stability of proteases produced by *A.* LK-101 and *B. megaterium* KLP-98 was better than that of the protease produced by *B. subtilis* JM3 (W.J. Kim and S.M. Kim, 2005; Fu et al., 2008; Hwang et al., 2010).

Source of protease	Optimal temperature for enzyme activity (°C)	Temperature range for enzyme stability (°C)
Bacillus subtilis JM3	60	≤ 30
Bacillus megaterium KLP-98	60	≤ 40
Aspergillus oryzae LK-101	50	≤ 40

Table 7. Effects of temperature on activity and stability of purified salt-tolerant acid proteases.

4.5.4 Effects of pH on enzyme activity and stability

Relative activity increased up to pH 5.5 and decreased rapidly at higher pH (Fig. 3A). The optimal pH for the hydrolysis of azocasein was 5.5. Therefore, the *B. subtilis* JM-3 protease was classified as an acid protease. *B. subtilis* JM-3 protease showed the optimal stability at pH 5.5 (Fig. 3B) of weak acidic condition, but was unstable above pH 6.0.

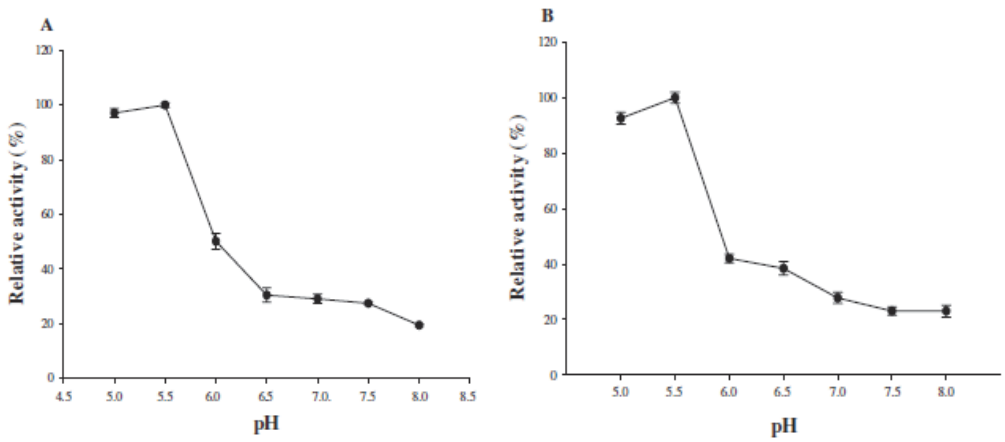


Fig. 3. Effects of pH on activity (A) and stability (B) of purified *Bacillus subtilis* JM3 protease (W.J. Kim and S.M. Kim, 2005).

Relative activity increased up to pH 5.5 and then decreased significantly (Fig. 4A). The optimal pH for the hydrolysis of azocasein was 5.5, and it had high proteolytic activity at weak acidic conditions (pH 5.0-6.0). The optimal pH of *B. megaterium* KLP-98 protease was similar to those of *B. subtilis* JM-3 protease. *B. megaterium* KLP-98 protease was stable around

pH 4.0-5.5 of weak acidic conditions. The pH stability range of *B. megaterium* KLP-98 protease and *B. subtilis* JM-3 protease was narrow.

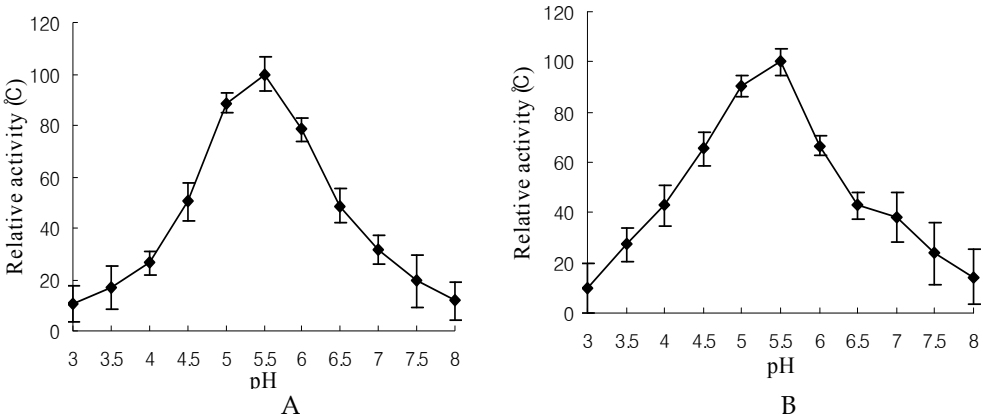


Fig. 4. Effects of pH on activity (A) and stability (B) of purified *Bacillus megaterium* KLP-98 protease (Fu et al., 2008).

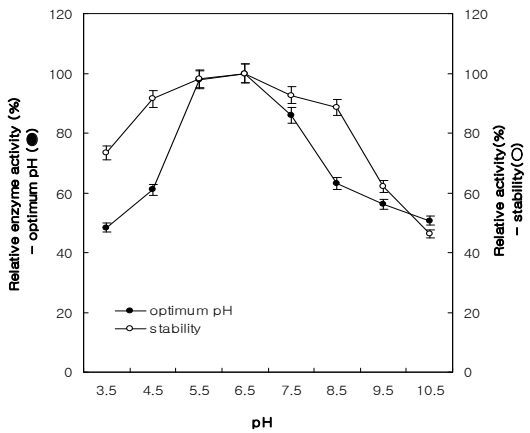


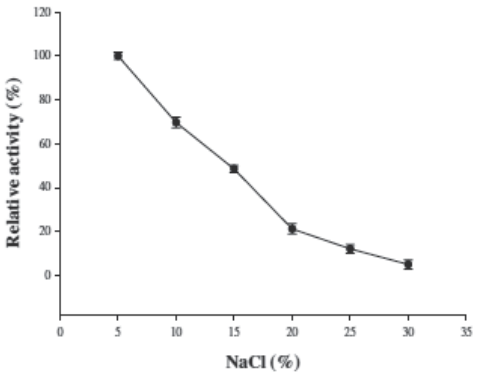
Fig. 5. Effects of pH on activity (A) and stability (B) of purified *Aspergillus oryzae* LK-101protease (Hwang et al., 2010).

The optimum pH for *A. oryzae* AOLK-101 protease activity was determined to be 6.5. It had higher proteolytic activity at weak acidic conditions than at the alkaline region. Thus, it was an acid protease. It remained more than 80% of activity in the region of pH 4.5-8.5 (Fig. 5). Thus the stability of this protease was better than those of *B. subtilis* JM-3 and *B. megaterium* KLP-98 proteases.

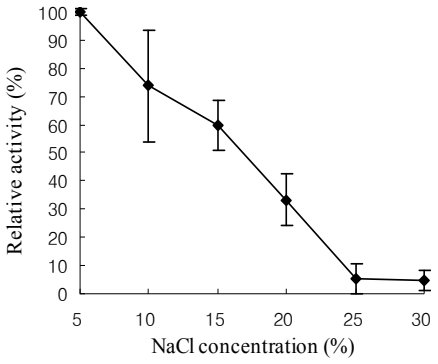
4.5.5 Effects of NaCl concentration on enzyme activity

The relative activities of purified proteases decreased as NaCl concentration increased. However, the protease produced by *B. subtilis* JM3, *B. megaterium* KLP-98 and *A. oryzae* LK-

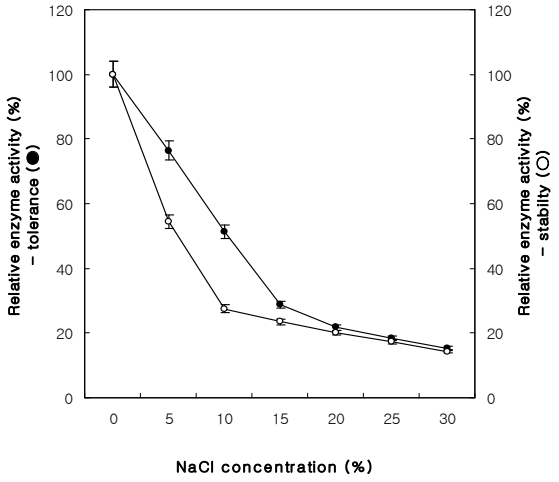
101 still remained 65, 75 and 50% activity at 10% NaCl concentration and 21, 35 and 22% at 20% NaCl concentration, respectively (W.J. Kim and S.M. Kim, 2005; Fu et al., 2008). Thus, these proteases were salt-tolerant proteases which can be used in soybean paste and soy sauce productions.



A



B



C

Fig. 6. Effects of NaCl concentration on the activity of purified *Bacillus subtilis* JM3 protease (A), *Bacillus megaterium* KLP-98 protease (B), and stability and activity of purified *Aspergillus oryzae* LK-101protease (C).

4.5.6 Substrate specificity

The relative activities of the *B. subtilis* JM-3 protease on specific substrates for trypsin-like serine proteases, i.e. BSA, casein, azocasein and L-BApNA, were 120.81, 98.07, 100.00 and 119.24%, respectively. But it did not hydrolyze BTEE, which was the specific substrate for chymotrypsin-like protease. The *B. subtilis* JM-3 protease was therefore presumed to be a trypsin-like serine protease (W.J. Kim and S.M. Kim, 2005).

Substrates with higher specificity for serine and cysteine proteases were also used. *B. megaterium* KLP-98 protease had high affinity to Z-Phe-Arg-NMec (95.56%), which is ideal substrate for cysteine proteases, but also some trypsin-like serine proteases can hydrolyze it. However, *B. megaterium* KLP-98 did not hydrolyze TAME and BTEE, which were model substrates for trypsin and serine protease. Based on the above results, *B. megaterium* KLP-98 protease was, therefore, presumed to be a cysteine protease (Fu et al., 2008).

4.5.7 Effect of inhibitors on enzyme activity

The *B. subtilis* JM-3 protease was strongly inhibited by specific inhibitors for trypsin-like protease, i.e. MTLCK, PMSF, STI and DTT. It was moderately inhibited by inhibitors for chymotrypsin-like proteases, i.e. 2-mercaptoethanol and TPCK. It was not inhibited by inhibitors for cysteine proteases, i.e. NEM and PCMB (Ninojoor, 1985), and inhibitor for metalloproteases, i.e. EDTA. Therefore, the *B. subtilis* JM-3 protease was classified as a trypsin-like serine protease (W.J. Kim and S.M. Kim, 2005).

The *B. megaterium* KLP-98 protease was almost inhibited by NEM, E-64, and egg white cystatin, the specific inhibitors for cysteine protease. Serine protease inhibitors of TLCK and TPCK, aspartic acid protease inhibitor of pepstatin showed no reduction in its activity. Reducing agents such as DTT and 2-mercaptoethanol, and metalloprotease inhibitor, EDTA, even could increase its activity. Based on the results of the substrate specificity and inhibitor studies, *B. megaterium* KLP-98 protease was classified as a cysteine protease (Fu et al., 2008).

EDTA was the strongest inhibitor followed by in order of PMSF, o-phenanthroline, and iodoacetic acid with the inhibition rate of 52, 47, 16 and 8%, respectively. It was moderately inhibited by inhibitors for chymotrypsin-like proteases, i.e. 2,4-dinitrophenol and 2-mercarptoethanol. PMSF is very specific inhibitor for serine protease. Therefore, AOLK-101 protease is classified as a serine protease based on its sensitivity to PMSF. Hence, this indicates that AOLK-101 protease is a serine protease (Hwang et al., 2010).

4.5.8 Kinetic parameters

In order to evaluate the kinetic constants for the purified protease, the initial velocities of the enzyme reactions were determined at various concentrations of the casein substrate. The kinetic constants, K_m and V_{max} values of the protease were calculated from the Lineweaver-Burk plot. The V_{max}/K_m value, which is the physiological or catalysis efficiency value, was calculated. The higher V_{max}/K_m value means the stronger catalytic activity to hydrolyze the substrate. Therefore the protease produced by *B. subtilis* JM3 exhibited the strongest catalytic activity among the three salt –tolerant acid proteases.

Source of protease	K_m (mg/mL)	V_{max} (U/L)	V_{max}/K_m (U mL / L/mg)
Bacillus subtilis JM3	1.75	318	181.6
Bacillus megaterium KLP-98	2.10	285	135.7
Aspergillus oryzae LK-101	1.04	125	119.2

Table 8. Kinetic parameters of the purified protease produced by *Bacillus subtilis* JM3, *Bacillus megaterium* KLP-98, and *Aspergillus oryzae* LK-101 (W.J. Kim and S.M. Kim, 2005; Fu et al., 2008; Hwang et al., 2010).

5. Applications

The applications of the purified proteases to soybean paste and soy sauce fermentation haven't been investigated yet. However, the characteristics of the proteases indicated their potential application. The specific activities were high, and the kinetic parameters were excellent. These proteases were stable below temperature 30 and 40 °C, respectively. For the fermentation of soybean paste and soy sauce, the temperature was around 30 to 35 °C, thus these proteases should be stable during the fermentation. The optimal pH for the activity of the proteases was 5.5 and 6.5, respectively, and they were also stable near this pH value. During the fermentation of soybean paste and soy sauce, the pH decreased to around 5. Therefore, these proteases will exhibit high activities and remain stable during the fermentation. These proteases showed high activities at 10% NaCl concentration and still remain moderate activities at 20% NaCl concentration. Different categories of the fermented soybean paste and soy sauce have different salt concentrations ranging from 10-21%, thus these proteases will be salt-tolerant enough during the fermentation.

The application of the salt-tolerant acid protease produced by *B. megaterium* KLP-98 to the anchovy sauce processing was investigated (Fu et al., 2008). Anchovy sauce was made by mixing anchovy with 20% NaCl at 30 °C for 2 months. The purified *B. megaterium* KLP-98 protease was lyophilized (15.8 U/mg) and thoroughly mixed with 2 month-ripened anchovy sauce in a ratio of 1 mg/100 g. Samples were stored in screwed boxes in dark at 40 °C for two days and various quality characteristics of the liquid fraction were determined every 12 hours. The degree of hydrolysis (DH), which is defined as the percentage of the free amino group cleaved from protein, was calculated. The yield was determined as the volume of liquid fraction obtained per 100 g fish. The final production was obtained with satisfactory color, flavors and taste (data not shown). The improvement of various parameters related to fish protein hydrolysis was discussed. All parameters of the control samples slightly changed during two days fermentation. However, all the parameters of the fish sauce samples with *B. megaterium* KLP-98 protease were greatly changed. The values of pH, TN, DH of the commercial fish sauces are: Vietnam (5.75, 2590 mg/100 mL, 61.6%), China (6.15, 1490 mg/100 mL, 57.8%) and Korea (5.49, 1270 mg/100 mL, 68.2%) (Park et al., 2001). These values of the anchovy sauces in this study are 5.17, 1252 mg/100 mL, 57.4%, which are slightly lower than the commercial products; however, the fermentation period decreased from one year of the commercial production to 2 months and 2 days.

The principle of fish sauce fermentation is somewhat similar to the soybean product fermentation. The successful application of the *B.* megaterium KLP-98 protease to anchovy sauce fermentation indicated the potential application of salt-tolerant acid protease to soybean paste and soy sauce fermentation.

6. Conclusions

From the collective information on salt-tolerant acid proteases, we conclude that proteases are important enzymes naturally purified from variety of halotolerant microorganisms. A few salt-tolerant acid proteases, i.e. proteases produced by *Bacillus subtilis* JM3, *Bacillus megaterium* KLP&98, and *Aspergillus oryzae* LK&101, have a remarkable production and possess excellent properties such as high specific activity, excellent temperature and pH stability and salt-tolerant ability, which enlighten their potential applications in sauce bean paste and soy sauce fermentation.

7. Acknowledgment

This research was supported by the Regional Research Centers Program of the Ministry of Education, Science and Technology (MEST), and the grant of No. RTI05-01-02 from the Regional Technology Innovation Program of the Ministry of Knowledge Economics (MKE), Republic of Korea. Xiao Ting Fu was a recipient of a graduate fellowship provided by Brain Korea (BK21) program sponsored by the Ministry of Education, Science and Technology, Republic of Korea.

8. References

Abe K.; Gomi K.; Hasegawa F. & Machida M. (2006). Impact of *Aspergillus Oryzae* Genomics on Industrial Production of Metabolites. *Mycopathologia*, Vol. 162, No.3, (September 2006), pp. 143–153, ISSN 1573-0832

Anson ML. (1939). The Estimation of Pepsin, Trypsin, Papain, and Cathepsin with Hemoglobin. *Journal of General Physiology*, Vol. 22, No.1, (January 1939), pp. 79-91, ISSN: 0022-1295

Barbosa D.C., Weid I.V.D., Vaisman N. & Seldin L. (2006). Halotolerant Spore-Forming Gram-Positive Bacterial Diversity Associated with Blutaparon Portulacoides (St. Hill) Mears, a Pioneer Species in Brazillian Coastal Dunes. *Journal of Microbiology and Biotechnology*, Vol. 16, No.2, (February 2006), pp. 193-199, ISSN 1017-7825

Chung M.J. (1984). Studies on the Production of Acid Protease by *Rhizopus japonicus* and Purification of the Enzyme. *Korean Journal of Applied Microbiology and Bioengineering,* Vol. 12, No.1, (January 1984), pp. 45-50, ISSN 1389-1723

Fu X.T.; You S.G. & Kim S.M. (2008). Characterization of a Salt-Tolerant Acid Protease Produced by *Bacillus megaterium* KLP-98 and Its Potential as a Fermentation Starter for the Manufacture of Fish Sauce. *Journal of Food Biochemistry*, Vol. 32, No.6, (May 2008), pp. 279-298, ISSN 2092-6456

Fukushima D. (1979). Fermented Vegetable (Soybean) Protein and Related Foods of Japan and China. *Journal of the American Oil Chemists' Society*, Vol. 56, No.3, (March 1979), pp. 357-362, ISSN 0003-021X

Han B.Z.; Rombouts F.M. & Nout M.J. R. (2001). A Chinese Fermented Soybean Food. *International Journal of Food Microbiology*, Vol. 65, No.1, (April 2001), pp. 1-10, ISSN 0168-1605

Hwang J.Y.; Choi S.H.; Lee S.K. & Kim S.M. (2010). Purification and Characterization of *Aspergillus oryzae* LK-101Salt-Tolerant Acid Protease Isolated from Soybean Paste. *Food Science and Biotechnology*, Vol. 19, No.2, (March 2010), pp. 327-334, ISSN 2092-6456

Jeong S.C.; Hyun K.W, & Lee J.S. (2001). Isolation of a Halotolerant Yeast and the Production of Extracellular Protease. *Kor. J. Biotechnol. Bioeng*. Vol. 16, No.2, (April 2001), pp. 158-162, ISSN 1225-7117

Kim D.C.; Oh N.S. & In M.J. (2004). Effect of Carbon and Nitrogen Sources on Cell Growth and Halotolerant Alkaline Protease Production in *Halomonas Marisflava* Isolated

from Salt-Fermented Food. *Food Science and Biotechnology*, Vol. 13, No.3, (April 2001), pp. 837-840, ISSN 2092-6456

Kim E.J.; Hong J.W.; Yun N.R. & Lee Y.N. (2011). Characterization of *Bacillus Phage*-K2 Isolated from Chungkookjang, A Fermented Soybean Foodstuff. *Journal of Industrial Microbiology and Biotechnology*, Vol. 38, No.1, (January 2011), pp. 39-42, ISSN 1367-5435

Kim T.W.; Lee J.H.; Park M.H. & Kim H.Y. (2010). Analysis of Bacterial and Fungal Communities in Japanese and Chinese-Fermented Soybean Pastes Using Nested PCR–DGGE. *Current Microbiology*, Vol. 60, No.5, (May 2010), pp. 315-320, ISSN 0343-8651

Kim W.J. & Kim S.M. (2005). Purification and Characterization of *Bacillus Subtilis* JM-3 Protease from Anchovy Sauce. *Journal of Food Biochemistry*, Vol. 29, No.5, (April 2005), pp. 591-610, ISSN 2092-6456

Lin C.H.; Wei Y.T. & Chou C.C. (2006). Enhanced Antioxidative Activity of Soybean Koji Prepared With Various Filamentous Fungi. *Food Microbiology*,Vol. 23, No.7, (October 2006), pp. 628-633, ISSN 0740-0020

Lowry O.H.; Rosebrough N.J.; Farr A.L. & Randull R.J. (1951). Protein Measurement with the Folin Phenol Reagent. *Journal of Biological Chemistry*, Vol. 193, No.1, (December 1951), pp. 265-275, ISSN: 0021-9258

Murakami K.; Ishida Y. & Atsushi M. (1991). Isolation and Characterization of Alkaline Protease Gene of *Aspergillus oryzae*. *Agricultural Biology and Chemistry*, Vol. 55, No.1, (November 1991), pp. 2807-2811, ISSN 0002-1369

Murooka Y. & Yamashita M. (2008). Traditional Healthful Fermented Products of Japan. *Journal of Industrial Microbiology and Biotechnology*, Vol. 35, No.8, (May 2008), pp. 791–798, ISSN 1367-5435

Ninojoor V. & Srivastava P.N. (1985). Partial purification and characterization of alkaline proteinases from the rabbit testis. Gamete Resource. Vol. 11, No.1 (January 1985), pp. 181-183, ISSN 0148-7280

Ogawa Y.; Tokumasu S. & Tubaki K. (2004). An Original Habitat of Tempeh Molds. *Mycoscience*, Vol. 45, No.4, (August 2004), pp. 271–276, ISSN 1340-3540

Park J.N.; Fukumoto Y.; FujitA.E.; Tanaka T.; Washio T.; Otsuka S.; Shimizu T.; Watanabe K. & Abe H. (2001). Chemical Composition of Fish Sauces Produced in Southeast and East Asian Countries. *Journal of Food Composition and Analysis*, Vol. 14, No.2, (April 2001), pp. 113-125, ISSN: 0889-1575eong S.C.; Hyun K.W. & Lee J.S. (2001). Isolation of a Halotolerant Yeast and the Production of Extracellular Protease. *Korean Journal of Biotechnology and Bioengineering*, Vol. 16, No.2, (February 2001), pp. 158-162, ISSN 1226-8372

Shibasaki K. & Hesseltine C.W. (1962). Miso Fermentation. *Economic Botany*, Vol. 16, No.3, (July-September 1962), pp. 180-195, ISSN 1367-5435

Stute R.; Petridis K.; Steinhart H & Biernoth G. (2002). Biogenic Amines in Fish and Soy Sauces. *European Food Research and Technology*, Vol. 215, No.2, (August 2002), pp. 101-107, ISSN 1438-2385

Wang S.H.; Zhang C.; Yang Y.L. Diao M. & Bai M.F. (2008). Screening of A High Fibrinolytic Enzyme Producing Strain and Characterization of the Fibrinolytic Enzyme Produced from *Bacillus subtilis* LD-8547. *World Journal of Microbiology and Biotechnology*, Vol. 24, No.4, (April 2008), pp.475-482, ISSN 0959-3993

Edited by Dora Krezhova

This book presents the importance of applying of novel genetics and breading technologies. The efficient genotype selections and gene transformations provide for generation of new and improved soybean cultivars, resistant to disease and environmental stresses. The book introduces also a few recent modern techniques and technologies for detection of plant stress and characterization of biomaterials as well as for processing of soybean food and oil products.

ISBN 978-953-51-5156-2

Photo by fotokostic / iStock

IntechOpen