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Soybean

The Basis of Yield, Biomass and Productivity

Edited by Minobu Kasai

SOYBEAN - THE BASIS OF YIELD, BIOMASS AND PRODUCTIVITY

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http://dx.doi.org/10.5772/63118
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Notice

First published in Croatia, 2017 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 - The Basis of Yield, Biomass and Productivity
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p. cm.

Print ISBN 978-953-51-3117-5

Online ISBN 978-953-51-3118-2

eBook (PDF) ISBN 978-953-51-4850-0

Meet the editor

Minobu Kasai is a professor of the Hirosaki University in Japan. He received his PhD degree from the University of Tokyo. His researches and papers are related to environmental physiology of higher plants including soybean, and they are most summarized in a chapter of an InTech book (2013) and a mini-review of Trends in Photochemistry and Photobiology (2014).The basis of yield, biomass, and productivity in plants including soybean is very important, since it is considered as the basis of living organisms including us.

Contents

Preface

Soybean is one of the organisms largely contributing to our life. Therefore, it is important to know soybean from various aspects. The knowledge and soybean itself will be greatly useful, if they are soundly used.

The chapters constituting this book present reviews and researches especially concerning the basis of yield, biomass, and productivity in soybean. Yield, biomass, and productivity in plants are some of the bases for maintaining or improving our ecosystem which includes our life and surrounding environments. Therefore, this book is expected to be useful for many people.

Of course, more researches and investigations are important to further gain the knowledge concerning the basis of yield, biomass, and productivity and make them useful for our ecosystem.

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Chapter 1

Soybean Yield Responses to Micronutrient Fertilizers

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/67157

Abstract

The availability of commercial products containing micronutrients for the management of crops has increased in recent years, but there are experimental results showing great variability in response to their application. A literature review was made in 28 scientific articles about the answers in the soybean yield in Brazilian agriculture due to the application of fertilizer containing micronutrients. Then, the aim of this chapter is to approach the efficiency of sources, doses, application methods, time, and yield results achieved in recent years by Brazilian research with the application of micronutrients in soybean. Adequate doses and sources of micronutrient increase Brazilian soybean yield, especially in that soil with low micronutrient content. High yields can be obtained in soils that have micronutrient levels considered adequate or high without their application. To right choice of micronutrients fertilizers, the farmer must know about solubility and other characteristics, including easiness to handling and applying and price. In general, the application method does not result in differences in soybean productivity. Thus, when applying micronutrients in the soil, topdressing or seed furrow, and leaf, and seed treatment, the most important aspects seem to be the time and dose to provide the nutrients in adequate amounts the plant requires.

Keywords: manganese, molybdenum, boron, zinc, copper

1. Introduction

The increase in production capacity of Brazilian soybeans farmers is allied to scientific advances and the availability of technologies in the productive sector [1]. The use of mineral fertilizers for soil and foliar application and other technologies has greatly contributed to the production progress. In this context, the most efficient use of micronutrients is essential to achieve high yield.

The availability of commercial products containing micronutrients for the management of crops has increased in recent years, but there are experimental results showing great variability in response to its application [2]. The main sources of micronutrients used in soybean crops vary in their physical form, chemical reactivity, cost, and agronomic efficiency.

Some sources are water soluble, such as chelates, nitrates, sulfates, and chlorides, while others are water insoluble but provide micronutrients to plants when applied to the soil, which are carbonates, phosphates, oxides, and silicates, among others [3]. The main advantage of chelates is the low dissociation in solution, i.e., the binder tends to remain bound to the metal even under conditions in which the metal precipitate or become insoluble (in concentrated solutions with neutral or alkaline reaction). This feature allows Cu, Fe, Mn, and Zn to remain in solution and maintain its availability to plants. Thus, the efficiency of chelates applied to the soil may be two to five times per unit of micronutrient, as compared to the inorganic sources [4].

Micronutrient oxide sources have the lower solubility, therefore generally less costing than the more soluble sources. However, some research work has shown improved efficiency of oxide use in relation to other sources [5,6]. Another group of micronutrient sources has been widely used is oxide nanoparticles in concentrated suspension, in which due to the small particle size, the elements are absorbed by the leaves and, depending on the pH of the cell, the cations (Zn^{2+}, Cu^{2+} and Mn^{2+}) can be released [7].

Despite the recognized importance of fertilizer with micronutrients, there is a need for a literature review that demonstrates more broadly the breakthroughs achieved by scientific research in the fertilizer with micronutrients, particularly for soybean, which is an important agricultural commodity and large consumers of micronutrients in the world.

A literature review was made in 28 scientific articles about the answers in the soybean yield in Brazilian agriculture due to the application of fertilizer containing micronutrients. Detailed descriptions of the treatments and their discussion can be found in the original articles. In this chapter, we seek for the objectivity in the main information related to the application of fertilizers, that is, the yield responses. The data shown in the graphs were compiled from research papers, and their claims should be given to the cited authors.

The hypothesis of this review is that the adequate supply of micronutrient fertilizer can increase soybean yield in Brazilian agriculture. Then, the aim of this review is to approach the efficiency of sources, doses, application methods, time, and yield results achieved in recent years by Brazilian research with the application of micronutrients in soybean.

http://dx.doi.org/10.5772/67157

2. Bases for soybean fertilization with micronutrients in Brazil

The use of the history of the area for soybean cultivation is fundamental to the proper micronutrient fertilization management. Plants cultivated on those areas that receive frequent spray applications with fungicides containing micronutrient rarely develop nutrient deficiency symptoms. However, other factors affect the micronutrient availability to plants, such as soil pH, soil organic matter content, and soil redox potential. The pH increase implies decreases of the Cu, Fe, Mn, and Zn micronutrients in the soil solution and the cation exchange sites. Thus, excessive limestone application can reduce the availability of these micronutrients in the soil and induce deficiency. In addition, the organic matter can decrease the solubility of some micronutrients by the formation of organic complexes constituted by humic acids and Fe, Mn, Cu, and Zn. On the other hand, organic matter may also increase the availability of micronutrients by complexation with fulvic acids and can be a source of micronutrients to soil in conditions favorable to their decomposition, such as heat, moisture, aeration, and high microbial activity. These factors have mainly been related to the increase in B (boron) availability. Oxidation reactions influence in particular Fe and Mn availability. When soybean is grown in regions of high rainfall, if the soil is not well drained, Fe toxicity may occur due to the reduction of the redox potential, which causes reduction of Fe^{3+} to Fe^{2+} and can increase Fe availability in the soil [8].

To interpret micronutrient contents in the soil, it is necessary to perform the soil test. However, each region of Brazil follows its own methodology, because there is no standardization of the methods to be used throughout the territory, especially because the soil and climate characteristics are distinct between the regions. Thus, the interpretation of the micronutrient contents in the soil must be performed according to tables of each state. Soybeans are grown in all regions of the country, with emphasis on the central-west and south regions, where the largest grain quantities are produced. In Rio Grande do Sul and Santa Catarina states, the average soil contents for B (hot water), Cu, Zn (HCl), Mn (Mehlich-1), and Fe (ammonium oxalate) should be between 0.1–0.3, 0.2–0.4, 0.2–0.5, 2.5–5.0, and <5.0 mg dm^{-3}, respectively. Values below or above those mentioned are interpreted as low or high [9]. In Paraná state, B (hot water), Cu, Zn, Mn, and Fe (Mehlich-1) must be between 0.5–0.6, 1.6–2.0, 1.6–2.0, 9.0–12.0, and 40–60 mg dm^{-3}, respectively. And, for biome Cerrado areas, the reference values for average contents are 0.3–0.5, 0.5–0.8, 1.1–1.6, and 2.0–5.0 mg dm^{-3}, respectively, for B (hot water), Cu, Zn, and Mn [10]. It is important to mention that soils with micronutrient contents above critical levels present a low likelihood of response to fertilization.

The evaluation of micronutrient availability can also be done by analysis of soybean leaves. The use of foliar diagnosis is based on the premises that there are direct relations between the dose of the nutrient and the production, dose of nutrient and content in soil and foliar, and foliar content and production. The procedure for sampling soybean leaves for leaf analysis is to collect the third leaf (third trifoliate leaves) from the apex on the main stem with petiole at the time of full bloom (R2). The sample should adequately represent nutritional status of the portion one wishes to evaluate. For soybean, it is suggested to sample 30 plants in each homogeneous field. Dirty soil samples and dry, diseased, or insect-attacked tissues should not be collected. Avoid taking samples before evaporation of dew or when, on previous days, the use of soil or

foliar fertilization or applied defensive. Samples should be sent to the laboratory as soon as possible. The interpretation of the results of the tissue analysis is done by comparing the levels observed in the sample with ranges of concentrations considered adequate, that is, the ranges of sufficiency. The reference values for the micronutrient contents in the soybean crop for B, Cu, Zn, Mn, Fe, and Mo are 21–55, 10–30, 20–50, 20–100, 50–350, and 1.0–5.0 mg kg^{-1} [8, 11].

Amounts of micronutrients recommended vary depending on the region of Brazil. In Rio Grande do Sul and Santa Catarina states, the application of 12–25 g ha^{-1} of molybdenum, via seed, or between 25 and 50 g ha^{-1} of molybdenum, via foliar fertilization, is suggested for soybean cultivation. The Brazilian Agricultural Research Corporation recommends the following doses for the first soybean cultivation in micronutrient-deficient soils, 4–6 kg ha^{-1} of Zn, 0.5–1.0 kg ha^{-1} of B, 0.5–2.0 kg ha^{-1} of Cu, 2.5–6.0 kg ha^{-1} of Mn, 50–250 g ha^{-1} of Mo, and 50–250 g ha^{-1} of Co, all applied in haul and with residual effect for at least 5 years. For application to the groove, ¼ of the doses described is recommended, but the application should be repeated for 4 consecutive years. Mo and Co should be applied by the seed treatment with 12–25 g ha^{-1} of Mo and 1–5 g ha^{-1} of Co, and it requires high solubility products. In the Cerrado region, when soil fertilization is not possible, fertilization via seed applying 3 kg of Cu oxide with 80 kg of moist seeds and then bacteria inoculation with *Rhizobium* is suggested. In addition, Mo and Co should be provided via seed: 50–130 g Na molybdate or 40–90 g ammonium molybdate and 8–20 g cobalt chloride or 9–23 g of cobalt sulfate per 80 kg of seeds. Cobalt is not an essential element in plants. However, it is suggested to be applied in soybean cultivation, because Co is part of the structure of vitamin B12 required for the synthesis of leghemoglobin, a protein that has the function of transporting oxygen for the oxidative metabolism of the enzyme nitrogenase, responsible for the biological fixation of atmospheric nitrogen. The use of inoculation with nitrogen-fixing bacteria completely replaces the application of nitrogen in soybean cultivated in Brazil [8].

Micronutrients can be applied by different methods in soybean cultivation: soil fertilization, foliar fertilization, and seed treatment. The application via soil provides greater use efficiency by plants, because it increases the concentration of the element in the soil solution. The application via soil can be done to the haul with fertilizer incorporation during soil preparation, as occurs in conventional agriculture, and can also be applied to the haul without incorporation, as does not occur in no-till areas. And in both ways, the micronutrient can be separated or mixed with NPK. The most common is the application in the sowing lines, beside and below the seeds, usually mixed with NPK and applied with seeder-fertilizer machines.

3. Soybean yield responses to fertilizer containing manganese

In soil, Mn occurs in three valences, Mn^{2+}, Mn^{3+} ($Mn_2O_3 \cdot nH_2O$), and Mn^{4+} ($MnO_2 \cdot nH_2O$), but is actively absorbed by the plant root system as Mn^{2+} [12, 13]. For foliar applications, the most traditional source of manganese is sulfate. Some other sources present retention in the cuticle, $MnSO_4$ > $MnCl_2$ > Mn-EDTA [14].

The effect of manganese fertilization in the soil and leaf in different soybean crop seasons (*Glycine max* L. Merrill) was studied in Ijaci municipality, Minas Gerais state [15]. The authors

http://dx.doi.org/10.5772/67157

evaluated two cultivars (Conquista and Garimpo), leaf-applied four manganese dosages (150, 300, 450, and 600 g ha^{-1}) and three application times (V4, V8, and V10, respectively, with 4, 8, and 10 trifoliate leaves with unfolded leaflets). Additional treatments consisted of control which had not received foliar application of Mn and Mn application on soil at sowing. For the application on the leaves, commercial product Mangan 10® chelate was used, while for Mn at sowing $MnSO_4 \cdot H_2O$ (manganese sulfate) containing 30% Mn, mixed planting fertilizers was used. Mn foliar applications parceled in V4 and V8 stage at a dose of 450 g or 600 g ha^{-1}, with chelated product containing 10% Mn, were responsible for the higher yields obtained, and it was considered more efficient than applications to the soil (**Figure 1**).

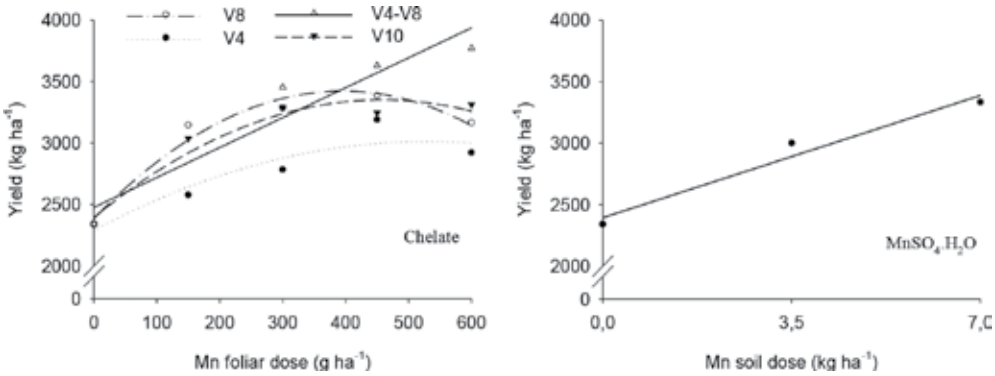

Figure 1. Doses, source, time, and local to apply Mn in soybean plants in Ijaci municipality, Minas Gerais state, Brazil. Note: Adapted from Ref. [14]. Other details are shown in the text.

Relations between limestone and manganese doses in mineral nutrition of soybean (*Glycine max* L. Merrill) were evaluated in Rio Verde, southwest of Goias [16]. The soils (Dystrophic Red-Yellow Latosol (LVd) and Dystrophic Quartz Sand-AQd) were evaluated in natural conditions and showed low fertility and pronounced acidity problems, with calcium and magnesium below the critical level for soybean and manganese contents in toxic levels. The authors concluded that the use of manganese in these soils was unnecessary and harmful to soybean. On the other hand, other researchers have observed manganese deficiency cases in soybean in no-till system, because lime is applied on surface [17].

The hypothesis that tolerant soybean to glyphosate requires further addition of leaf manganese due to changes in absorption, and metabolism of the element was evaluated in Taquaraçu municipality do Sul, in Rio Grande do Sul state (RS) [18]. The Mn source used was a commercial product Profol® with 14% (w/v) manganese soluble in the formulation as chelate form. On the plots that received foliar application of Mn, the dose used was 2.0 L ha^{-1} of commercial product. The conclusion was that although manganese supplementation increases foliar content, there was no increase in soybean productivity. This result showed that in soils with Mn levels considered adequate or high, transgenic soybeans do not require foliar manganese supplementation. Similar results were also obtained by other researchers [19] in the experimental area of São Paulo State University-UNESP Jaboticabal-São Paulo.

The application of Mn and glyphosate at different growth stages of soybean variety BRS 245 RR and its effects on foliar nutrient content and grain yield were assessed in Rio Brilhante municipality, Mato Grosso do Sul state (MS) [20]. The authors evaluated crops without foliar application with Mn, Mn application on V4 soybean growth stage, with Mn application at V4 + V8, with Mn application at V4 + R2, with Mn application at V4 + V8 + R2, with Mn application at V8; with Mn application at V8 + R2, and Mn application on growth stage R2. Each application was sprayed 332 g ha^{-1} Mn on the leaves. The product used was Basfoliar Manganês® (10% Mn), containing Mn sulfate chelated with EDTA. No differences in yield were observed (3000 kg ha^{-1}) as a function of Mn applications.

Chemical forms and manganese availability in soybean yield in soil under no-tillage system were evaluated in Tibagi and Castro municipalities, in Paraná state (PR) [21]. The authors used the $MnSO_4$ and varied the Mn doses from 0 to 48 kg ha^{-1} applying manually to the soil. They did not observe variations in soybean yield (3000 kg ha^{-1}). According to the authors, the lack of effect of Mn on soybean yield in no-tillage system may be due to the complexation of the nutrient by organic matter stable forms, non-available to plants.

The Mn availability to plants depends on many factors relating to the soil, particularly the pH and the organic matter. On the other side, even no-tillage system promotes increased soil pH in the surface layer, which implies less Mn availability in the soil; the research results have not shown soybean response to Mn leaf application.

4. Soybean yield responses to fertilizer containing molybdenum

Mo is a micronutrient less abundant in the soil than other and the least required by crops. In the soil, Mo appears in the anionic form as $HMoO_4^-$ and MoO_4^{2-}. In those soils with pH > 5.0, Mo is absorbed predominantly as MoO_4^{2-} [11], while at pH < 4.3 the predominant forms are protonated species as $HMoO_4^-$, $MoO_3(H_2O)_3$ [13]. Although Mo is considered as a low mobility nutrient in the plant, Mo can be applied to the leaves with good results, since redistribution is good [14].

The effect of foliar application of Mo dose in soybean and beans (*Phaseolus vulgaris* L.—Carioca Perola) at greenhouse was evaluated in Rio Verde municipality, Goias state [22]. The authors varied the Mo doses between 0 and 160 g ha^{-1} and observed no differences in any of the variables in the two species. The authors explained the results by stating that the Mo needed by the plant was supplied with the initial soil reserve. The pH of the soil was around 7.0, which is the pH to the greatest availability of Mo.

Foliar application of Mo and cobalt to soybean crop, CD 214 RR variety, was evaluated in São João (PR) [23]. Foliar application was carried out with cobalt and Mo micronutrients (12.0% sodium molybdate and cobalt sulfate 2.0% commercial product—Basfoliar CoMol HC). Dose which was 0–200% of the dose recommended by the manufacturer is 309 mL ha^{-1}, and the application was carried out 25 days later after crop emergence. The authors found that the application of Mo and cobalt (Co) to the leaves did not affect the soybean development. Application of Mo concentrations at soybean seed treatment and foliar was evaluated in Palotina municipality, Paraná state (PR) [24]. The leaf application was made 25 days after

emergence at doses ranging from 0 to 160 g ha^{-1}, while to seed treatment was used 0.6 g of Mo per seed kg. The authors found no significant differences in yields between treatments. The yield average was 2104 kg ha^{-1}. The authors justified that the absence of response to the addition of Mo may be related to adequate levels of Mo availability in the soil or with concentrations of Mo in the seed sufficient to meet the needs of the plants.

Seed and foliar treatments with zinc (Zn) and Mo to soybean crop were evaluated in Cascavel (PR) [25]. For seed treatment and foliar application, the source of Mo + Zn was the commercial product Booster®: 3.5% Zn and 2.3% Mo. The treatments were distributed as follows: Treatment 01—control (only cultivation with application of insecticides, fungicides, and herbicides), Treatment 02—seed treatment (3 mL kg^{-1} seed), Treatment 03—seed treatment (3 mL kg^{-1} seed) + a foliar application (400 mL ha^{-1}, which were applied when the soybean was at 4–5 trefoil), and Treatment 04—seed treatment (3 mL kg^{-1} seed) + two foliar applications (400 mL ha^{-1}, which was applied when soy was 4–5 trefoil + 400 mL ha^{-1} when the soybean was at the beginning its flowering). The variety used in the experiment was a cultivar of BMX Apollo RR presenting with an early maturity of 5.5 group cycle with unlimited growth, with 340,000 plants per hectare. There was increase in 1100 kg ha^{-1} in yield in the seed treatment (Treatment 02), as compared to the control. There was no difference between Treatment 02, Treatment 03, and Treatment 04 yield, with a mean of 5050 kg ha^{-1} (**Figure 2**).

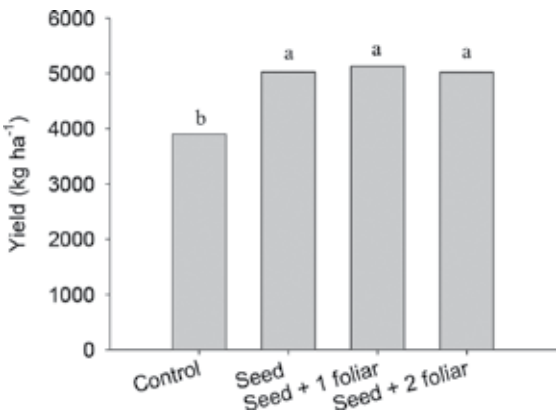

Figure 2. Soybean yield, due to the use of zinc and molybdenum. Note: Adapted from Ref. [25]. Other details are shown in the text.

The effectiveness of different molybdenum sources using the products, Nectar (225 g L^{-1} Mo + 22,5 g L^{-1} Co), Molybdate (254 g L^{-1} Mo + 262 g L^{-1} P_2O_5), and MIQL-Mo (250 g L^{-1} Mo) in the development and productivity of soybean cultivar BR-16, was evaluated in Santa Maria (RS) [26]. Before sowing, the molybdenum sources applied to soybean seeds using the following doses, Nectar, 0.15 g Mo kg^{-1} seed; Molybdate, 0.15 g Mo kg^{-1} seed; and MIQL, 0.15 g Mo kg^{-1} seed. Seed inoculation with *Bradyrhizobium japonicum* inoculum through supplying 6 g kg^{-1} of seed was also performed using the product with trade name "Emerge®." The difference in yield between treatments with Mo and those without Mo was approximately 1600 kg ha^{-1}. However, the authors found no significant differences in productivity (3570 kg ha^{-1}) between different sources of Mo.

Technical and economic feasibility of the application of Mo, Co (cobalt), and B (boron) to increase soybeans yield (cultivar RS-10) was determined in Coronel Bicaco (RS) [2]. The treatments were the following combinations: CoMo, CoMo + Mo, CoMo + Mo + Mo, CoMo + Mo + P30 (30% P_2O_5, 5% N and 1.2% Mg), CoMo + B, B, Mo, Mo + Mo, and control treat. Product used to CoMo combinations was the commercial product CoMo Plus 250® (1.7% Co and 17% Mo) at a dose of 0.09 L ha^{-1} applied via seed. In treatments in which Mo was used separately, the source used was sodium molybdate (39.5% Mo) at a dose of 0.12 kg ha^{-1}, applied to the leaves together with herbicides at 30 days after emergence (DAE). When Mo was applied two times, it was performed at 30 and 60 DAE; P, N, and Mg were applied with the commercial product Nutijá P30 at a dose of 2.0 L ha^{-1} applied at 60 DAE. B was applied with the commercial product Solubor (20.5% B) at a dose of 1.0 kg ha^{-1}, applied to the leaves at 60 DAE of soybean plants. The highest yield (3596 kg ha^{-1}) and economic viability (net return U$ ha^{-1} 49.19) were obtained with the application of CoMo + Mo + Mo (**Figure 3**).

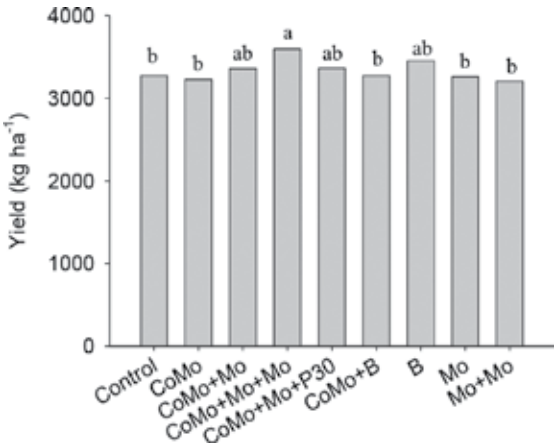

Figure 3. Soybean grain yield, under the application of micronutrients in crop year 2001/02. Note: Adapted from Ref. [2]. Other details are shown in the text.

Molybdenum and cobalt applications on soybean nodulation, cultivar COODETEC 201, and their effects on grain yield were evaluated in Ponta Grossa (PR) [27]. Mo was applied at two doses (0 and 48 g ha^{-1}), and Co was applied at four doses (0, 2, 4, and 8 g ha^{-1}) to seeds. The sources of molybdenum and cobalt were sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) and cobalt sulfate ($CoSO_4 \cdot 7H_2O$), respectively. Molybdenum treatment decreased the iron content in the leaves, but did not affect soybean yield (3000 kg ha^{-1}). There was a linear decrease in plant height, leaf zinc concentration, and yield with increasing dose of cobalt applied. The authors concluded that the molybdenum application to soybean is not required in soil pH 5.2 ($CaCl_2$ 0.01 mol L^{-1}) and that cobalt applied to the seed at doses greater than 3.4 g ha^{-1} is toxic to soybean.

Due to the small quantities required by the soybean crop and partial mobility in the plant, the application of molybdenum to leaf or seed treatment has shown satisfactory results in increasing the productivity. Regarding Co, the provision should be made with caution, because Co excess in the soil can cause toxicity to plants and reduces Fe and Mn absorption, leading to deficiency of these micronutrients [12].

http://dx.doi.org/10.5772/67157

5. Soybean yield responses to fertilizer containing boron

Boron (B) is probably absorbed by the roots of the plants in the undissociated form as boric acid (H_3BO_3), which is the main soluble form in the soil. In the same way as calcium, boron undergoes a unidirectional transport in the xylem, via transpiration stream from roots to shoots; in phloem, B is practically immobile. Thus, boron is not redistributed in plants, and causes the appearance of withdrawal symptoms primarily in younger organs and in growth regions [12]. However, there is a statement in the literature that plants containing appreciable amounts of polyols (with cis-hydroxyls) that bind to the boron present the mobility of B in the phloem. Soy, for example, contains large amounts of the cis-diol pintol molecule, which may result in the phloem mobility of B [14].

After zinc, boron is the micronutrient whose deficiency occurs more widely in the areas of Cerrado, Brazil. Applying it at soil is the most efficient way to provide B. However, Ca and B foliar spraying is very widespread at the time of flowering. Supposedly, this procedure favors better fertilization of the flowers and grain formation by the B effect and reduces the abortion of the newly formed pods due to the presence of Ca [28]. The productivity of four soybean cultivars was evaluated as a function of foliar mineral fertilizer application containing 8% calcium and 2% boron in R1 stage (early flowering 50% of flowering plants) and R3 (final flowering, pod up to 1.5 cm in length) [1]. The productivity was significantly higher when the solution based on Ca and B was applied in R3. The BRS MG 705S RR showed the best performance among cultivars, reaching an average yield of 6506 kg ha^{-1} with the fertilizer of 1.0 kg ha^{-1} of fertilizer in Selvíria (MS) (**Figure 4**). However, the response of soybeans to leaf-borated fertilizer at different stages and application rates was not observed in Borrazópolis (PR) [29]. The borated foliar fertilization did not affect the productivity of soybeans. However, the application of 1 kg ha^{-1} of B in soybean development V4 stadium reduced the leaf N content compared to the control treatment. Application of 2 kg ha^{-1} of B in R2 stadium resulted in an increase in the fertilization efficiency for potassium.

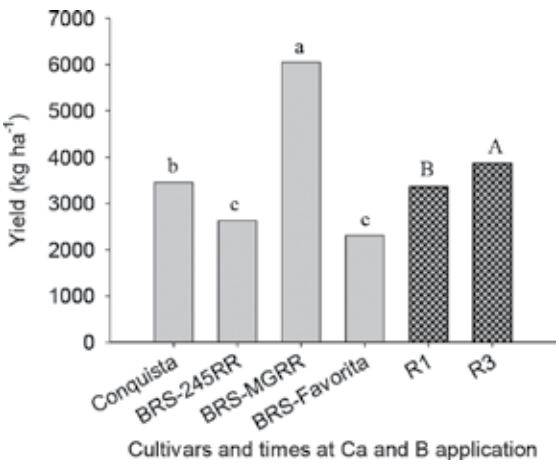

Figure 4. Effect of foliar application of Ca and B on productivity of soybean cultivars and application time of Ca and B to the leaves in Selvíria municipality, Mato Grosso do Sul state, Brazil, 2007. Note: Adapted from Ref. [1]. Other details are shown in the text.

The effect of B applied in different doses and stages by foliar spray on the morphological characteristics, production and physiological quality of soybean seeds of M-SOY 8411 variety, was evaluated in Santa Carmem (MT) [30]. The seeds were treated with fungicide Fludioxonil + metalaxyl-M + and molybdenum and cobalt using liquid inoculants of 100 mL, 150 mL, and 300 mL, respectively, per 100 kg of seed. Boron doses ranged from 0 to 400 g ha^{-1} (0–4 L ha^{-1} of the commercial product Basfoliar Boron 10%). There were no yield differences between different B doses or application at soybean growth stage (V5, V9, and R3).

Overall, the research work of foliar application of B in soybean showed no yield responses due to the nutrient application. It may be related to the nutrient content in the soil. The nutrient might be sufficient to the crop need in places where the studies were carried out.

6. Soybean yield responses to fertilizer containing zinc and copper

Zinc deficiencies have occurred in a wide variety of soils around the world and in Brazil. Zn deficiencies are the most common among the micronutrients, especially in sandy soils and savannah. Zinc is absorbed predominantly as a divalent cation (Zn^{2+}); at high pH, it may be absorbed as monovalent cation ($ZnOH^{+}$) [13]. The zinc sulfate application has been considered the standard for the nutrient. However, zinc nitrate, zinc chloride, or sulfate mixed with zinc chloride has shown good results. The zinc chelated with EDTA has shown better absorption than zinc sulfate form [14], while zinc oxides are materials which have a lower solubility.

Productivity and yield of soybean cultivar "Spring" fertilized with different zinc doses in field conditions were evaluated in Palotina (PR) [31]. The Zn doses tested were 0, 2, and 4 kg ha^{-1}, applied at sowing as zinc sulfate ($ZnSO_4$). Zn doses applied did not influence significantly the yield. The authors attributed the result to the Zn content in the soil prior to application. The Zn content in the layer 0–20 cm was presented as medium (1.4–2.0 mg dm^{-3}) and therefore considered somewhat responsive to fertilizer. In the same municipality, differences in yield when NPK (02-20-18) + 0.3% Zn was applied in the sowing using different commercial products as source of Zn were not observed [32]. On the other hand, there was response to Zn doses applied. The soil Zn content at the beginning of the experiment was 3.65 mg kg^{-1}, even so the authors observed yield increase of 679 kg ha^{-1} when they applied twice the dose suggested for that soil.

Copper and zinc fertilizer doses on the soybean yield were evaluated in Assis Chateaubriand municipality, Paraná state (PR) [33]. The authors cultivated soybeans without application of micronutrients, with application of copper and zinc oxide to seed, with application of copper and zinc oxide via seed and leaf, and applying copper and zinc oxide only to leaf. The copper and zinc oxide doses applied in seed treatment were 1.88 mL kg^{-1} and 4.24 mL^{-1}, respectively, and foliar sprays at 35 days after emergence were 109 mL ha^{-1} of copper oxide and 245 mL ha^{-1} of zinc oxide. Regardless of the application mode, copper and zinc micronutrient supply provided an increase in 600 kg ha^{-1} in soybean yield compared with control treatment. On the other hand, there was no significant difference in soybean yield (3100 kg ha^{-1}) when Cu and Zn were applied to seed, seed and leaf, and leaf (**Figure 5**).

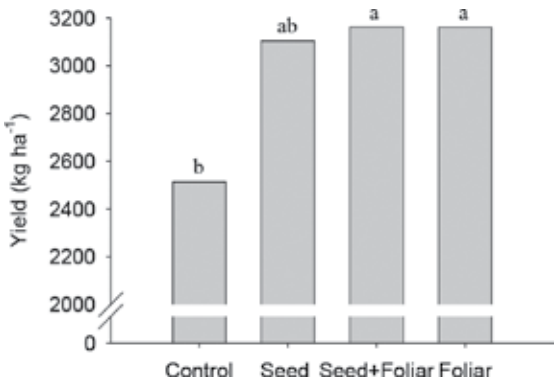

Figure 5. Soybean yield as a function of using zinc and copper. Note: Adapted from Ref. [33]. Other details are shown in the text.

Copper doses and application methods in soybean cultivation were evaluated in Planaltina municipality, Federal District (DF_ [34]. The authors tested Cu applications via soil, topdressing (0 – 4.8 kg ha^{-1}), and drilling (1.2 and 2.4 kg ha^{-1}) using copper sulfate pentahydrate. They cultivated soybean three times after applications and did not reapply copper in the second and third crops to assess the residual effect. All plots received 3880 kg ha^{-1} of limestone (229 g kg^{-1} of Ca 72 g kg^{-1} of Mg) to raise base saturation to 50%, 1031 kg ha^{-1} of agricultural gypsum, 240 kg $ha^{-1}$of P_2O_5 in the form of superphosphate triple, 100 kg ha^{-1} of K_2O using potassium chloride and a mixture with 2 kg ha^{-1} of B (borax), 6 kg ha^{-1} Zn (sulfate), 3 kg $ha^{-1}$of Mn (sulfate), 0.25 kg ha^{-1} of Mo (ammonium molybdate), and 0.3 kg ha^{-1} of Co (chloride). In the first crop after Cu applications, the average yield was 2320 kg ha^{-1} of grains, and they did not observe difference between treatments. In the second crop after application, there was increase of soybean yield at 600 kg ha^{-1} in the treatments without Cu fertilizer or received only 0.4 kg ha^{-1} at topdressing and 1082 kg ha^{-1} in soybean yield to the other treatments. In the third crop after Cu applications, the control plot (without Cu) and the plot that just received 0.4 kg ha^{-1} produced 548 kg ha^{-1} less soybean grains compared to those that received from 1.2 to 4.8 kg ha^{-1} a haul and 1.2 to 2.4 kg ha^{-1} in the planting furrow. For these last treatments mentioned, the yield average was 3168 kg ha^{-1}, without significant difference between them.

The scientific papers generally have not shown differences in soybean yield due to application method when Zn and Cu are applied in the soil to the furrow, to the haul, to the leaves, or in seed treatment, but the right doses are important to obtain high yield, especially when contents of Cu and Zn in soil are below critical levels.

7. Conclusions

Adequate doses and sources of micronutrient increase soybean yield especially in that soil with low micronutrients content. However, high yields can be obtained in soils that have micronutrient levels considered adequate or high without their application.

To right choice of micronutrients fertilizers, the farmer must know about solubility and other characteristics as easiness to handling and applying and price.

In general, the application method does not result in differences in soybean productivity. Thus, when applying micronutrients in the soil, topdressing or seed furrow, and leaf or seed treatment, the most important aspects seem to be the time and dose to provide the nutrients in adequate amounts the plant requires.

Acknowledgements

The authors thank CAPES, ALTAGRO plant nutrition, and ABC Agricola for the financial support.

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Chapter 2

Soybean Architecture Plants: From Solar Radiation Interception to Crop Protection

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/67150

Abstract

The soybean plant architecture in relation to better solar radiation interception and production gain is an aspect that requires a better understanding, since soybean is an important crop worldwide. The genetic traits, management and environmental conditions are points that further extend the range of issues on crop productivity. The light quality is measured by the red/far-red (R/FR) ratio (R ~ 660 nm, FR ~ 730 nm). This affects the plant growth and morphological developments in different ways. The plant leaves change their angle during the day to better intercept radiation. This heliotropic movement and some computational models together have been used to enhance some agricultural practices. Soybean plant is dependent on the interaction between genotype and environment. Thus, the enhanced understanding in relation to photosynthetic activity, grain yield by light interception efficiency and culture protection managements in soybean are covered.

Keywords: productivity, light, management, canopy, heliotropism

1. Introduction

The greatness of agriculture is natural transformation of solar radiation into fiber, oil, protein and carbohydrates. Management of plant arrangement, space organ distribution and plant density have to be appropriate for the maximum interception of photosynthetically active radiation in soybean plant canopy and for maximizing the dry matter accumulation during vegetative growth and early reproductive stages. The knowledge about the plant growth and development and their interactions with environment are essential for maintaining and increasing crop yield. More information is useful in plant management and breeding programs.

This chapter exposes the productive aspects of soybean plant and canopy architecture. Topics concern the characterization and definition of plant architecture, interception of solar radiation, CO_2 assimilation and yield, crop product deposition under the variations of plant/canopy architecture, crop managements and their impacts on architecture modifications. The definition of soybean plant canopy architecture aiming at aggregating knowledge and enhanced understanding of the increment in photosynthetic activity as well as the influence to grain yield of light interception efficiency and crop protection managements in soybean crop are assessed.

2. Characterization and definition of soybean plant architecture

Architecturally, soybean plants can be regarded as a continuum born from the successive production of metameric units. One metameric unit consists of an internode, a trifoliate leaf and the associated reproductive branch born at respective node. The structure of the whole plant can suffer modifications. These are due to the genetically or environmentally induced modification of the structure of each individual metamere or of its number [1].

Plant architecture refers to the organization of plant components in space which can change with time. Furthermore, architecture can be defined by topological and geometric information. Topology is related to the physical connections between plant components. Geometric information includes the shape, size, orientation and spatial location of the components. Thus, the geometry is mostly involved in plant environment exchanges and resource capture. However, the topology can be used to build up biological sequences embedded in axes or still can be considered as the basis for internal fluxes for energy, mass and information [2].

In relation to size and shape of leaflet, Zheng and Chen [3] proposed three classes for leaflet size (small, intermediate and large) and four categories for leaflet shape that has been lately corrected by Chen and Nelson [4] considering five categories for leaflet shape—oval, ovate, lanceolate, linear and ultra linear. Length/width ratio and length are chosen to define leaflet shape and leaflet size, respectively. Values of length/width ratio ranged from 1.3 to 6.2 and those of length from 3 to 14 cm [4].

The soybean plant morphology and architecture are determined by branching and internode length [5], whereas its growth and development are affected significantly by a cultivar-specific temperature regime [6]. Thus, flowering time, number of pods, maturity and plant morphology are complex traits controlled by genetic and external factors. These characteristics have considerable effects on the adaptation and grain yield of soybean. The identification of novel genes and an understanding of their molecular basis and mechanisms involved are critical to improve soybean productivity. Characteristics associated with soybean yield components and plant architecture are substantially correlated with both genotype and phenotype [7].

The involvement of the single nucleotide polymorphisms (SNPs) in the plant architecture and yield component traits has been established [7]. These traits are severely influenced by environmental factors. The *ln* locus (*ln* locus named after narrow leaflet) was identified as a regulator of leaflet shape and number of seeds per pod in soybean. This suggested positive

applications to soybean breeding [8]. The IPA1 locus (ideal plant architecture 1) [9] has an ability to generate an ideal plant architecture with reduced tiller number. Recent studies suggest that the genetic modulation of brassinosteroid (BR) receptor genes can alter plant architecture [10]. In soybean, investigations are still needed to confirm the relationship between BR receptor genes and plant architecture. However, the transcript abundance of BR receptor genes in nodules, apical buds, cotyledons, epicotyls, hypocotyls, leaves, lateral roots and primary roots was demonstrated, implying that the genes play an important role in soybean growth and development [10].

Class I KNOX homeobox family genes are involved in the plant growth and development, especially in the growth and development of leaves, flowers and pods [11]. *GmSBH1* is a homeobox gene isolated from soybean, which showed diverse expression patterns in cotyledon, embryo, seed coat, seedling stem, seedling root, flower and pod. The overexpression of *GmSBH1* in *Arabidopsis* altered the leaf and stoma phenotypes. This result demonstrates that *GmSBH1* is required for maintaining growth and development in soybean [11]. Overexpression of a flowering time-related *APETALA2-like* gene *GmTOE4a* caused late flowering and altered soybean plant morphology (increased stem thickness and reduced plant height, internode length and leaf size) [12], demonstrating that the gene plays a role in the regulation of the photoperiodic flowering pathway in soybean. The miR156 and miR172, microRNA genes, are known to be associated with vegetative phase change. In soybean, it was shown that miR156 and miR172 genes are involved in the change from juvenile to adult phase, thus demonstrating that the genes play an important role in plant development [13]. In soybean plants overexpressing miR156b, flowering time was suppressed and other genes were negatively regulated [14]. These results, in near future, may facilitate the development of new soybean cultivars with high yield potential as well as more adapted cultivars to environmental conditions.

The soybean varieties with determinate growth permit the lower number of metamere units per main stem and lower competition between the vegetative and reproductive growth and maturation, leading to higher grain production [15]. The indeterminate type varieties have much more internodes at the main stems, which are consequently longer than those of determinate cultivars. Indeterminate type varieties continue to elongate their stems for about 1 month after the beginning of flowering, while determinate ones stop their elongation after 10 days from flowering [16].

The search for an ideotype that has a good genetic potential to overcome the environmental adversities and presents high productivity is a challenge for agriculture worldwide. One genotype may have a good performance in a given region, which may not be ideal for another. Likewise, in different years of cultivation in the same place, the response may vary. Therefore, genotype and environment corroborate specific responses. **Table 1** shows the variation between soybean genotypes for some agronomic traits that define plant architecture.

The light quality is measured by the red/far-red (R/FR) ratio (R ~ 660 nm, FR ~ 730 nm). Light that has passed through a leaf canopy is rich in FR light but poor in R light [23]. The R/FR ratio decreases from ~1.2 in full sunlight to 0.05 in closed canopies, with a decrease occurring before canopy closure by absorption of red light by photosynthetic pigments [24]. Plants that detect a low R/FR ratio will initiate some physiological changes and like this, plants express

shade avoidance characteristics [25, 26]. In an experimental system of modification in the R/FR ratio under relay intercropping with maize and soybean, decreases in stem diameters (20.3 and 21.3%) and root length (23.5 and 30.5%) and an increase in the seedling height (approximately 89.8 and 86.9%) were observed for two cultivars, as compared to those under sole cropping [27].

Characters	References
Plant height (cm)	
34.3–62.4	[17]
51.15–58.35	[18]
44.87–82.75	[19]
76.0–102.3	[20]
First pod insertion height (cm)	
4.41–12.77	[17]
11.15–11.95	[18]
7.85–12.50	[19]
Internode length (cm)	
2.47–2.83	[18]
Number of branches*	
1.87–1.88	[18]
3.25–6.77	[19]
3.0–7.4	[20]
Length of primary branches (cm)	
22.92–24.53	[18]
Number of nodes on the main stem	
19.0–24.7	[21]
Leaf area index (leaf area per unit ground surface area)	
3.3–6.6	[20]
5.6–9.8	[22]

*Average number of branches in different soybean cultivars; Ref. [18]—considered the branches over 5 cm; Refs. [19, 20]—considered the total branches of plant.

Table 1. Range of the values (minimum and maximum) of agronomic traits of different genotypes of soybean.

The competition between plants in the canopy is detected by an alteration in R/FR light ratio. This alteration affects the apical dominance and the growth of lateral organs. It occurs because light signals are perceived by the phytochrome, which has a function in detecting the level of competition plants will encounter [28]. In general, there is an increase in the plant stature in response to the decrease in light quality. It is important to emphasize that phytochromes

(PHY) are a small family of R/FR light photoreceptors which regulate several of important developmental responses in plants, such as rapid biochemical events and slower morphological changes. A pair of PHYA paralogs (*GmPHYA1* and *GmPHYA2* genes) of the soybean has been explored. The expression pattern of the genes varied among tissues. The high transcript abundance was in the soybean seedling and hypocotyl, suggesting that the PHYA products could be involved in aspects of seedling establishment and photomorphogenesis. In addition, the transcripts showed abundance in the younger leaves [29].

The plant growth to light direction is called phototropism, which is a photomorphogenic response. Plants are cultivated under a source of a directional light curve themselves to the light direction to maximize the light absorption and this response is mediated by the blue light [30]. There are proteins involved in the phototropism, which are named phototropins (phot1 and phot2). Besides the phototropism, these proteins are involved in chloroplast movement, quick growth inhibition of etiolated plants, leaf expansion and regulation of stomatal aperture [30].

The reduction in the ozone layer has an effect to increase ultraviolet radiation reaching the earth's surface, especially the radiation of ultraviolet-B (UV-B). High levels of ultraviolet radiation influence negatively on carbon assimilation rate and growth of plants [31]. The alterations in plant height, branching pattern and leaf size of soybean plants were observed in UV-excluded sunlight when compared to control plants. The exclusion of UV radiation increased leaf dry weight (43%), leaf fresh weight (22%) and leaf area (54%). In addition, the exclusion of solar UV-B and UV-B/A radiation increased the plant height (30% for exclusion of UV-B and 60% for exclusion of UV-B/A). Thus, the solar spectrum causes changes in soybean growth and morphological developments [31]. The exclusion of the UV-B/A radiation also caused elongated internodes in soybean plants, resulting in greater plant height. Increases in the main stem length were also observed for exclusion of both UV-B and UV-B/A (45 and 237% in one cultivar and 52 and 198% in other cultivar). The number of the branches was not affected by the UV treatments and the total leaf area was less in plants exposed to UV radiation [32].

The leaves of some plants including soybean have an important characteristic of altering their angle during the day aiming to adjust the intercepted radiation. This movement is called heliotropism, which is induced by the blue light (400–500 mm) [33]. The heliotropism is divided into two leaf movements which are called diaheliotropism and paraheliotropism [34]. Diaheliotropism is a movement maintaining the leaf blades perpendicular to the solar rays, maximizing light interception with carbon gain [35]. Paraheliotropism is a movement maintaining the leaf blades parallel to the solar rays and reducing the effects of hydric stress [36], photoinhibition [37] and high leaf temperature [38]. Genotypes respond differently to heliotropism and besides that, those responses differ during the cultivation cycle and under stressing conditions [39].

The heliotropic movement can be used to enhance some agricultural practices and hence, some computational models have been adopted. Computer modeling has become an important tool to enhance understanding of development and growth of the plants. In the modeling development, the functional-structural plant modeling refers to models describing the

development over time of the 3D architecture or structure of plants as guided by physiological processes which, in turn, are driven by environmental factors [40]. Thus, simulation models can be used to predict the outcome of plant trait modifications resulting from the genetic variation and also its interaction with the environment on plant performance, contributing to plant breeding process [41].

3. Solar radiation interception, carbon fixation and grain yield

Soybean presents high levels of carbon fixation with the maximum air temperature of around 30°C and photosynthetically active radiation leading to saturation is proximally 2000 μmol m^{-2} s^{-1} [42]. These may be reasons why the origin of soybean is East Asia. However, some models demonstrate a high photosynthetic activity at a temperature of 35°C with a photon flux density of 1200 μmol m^{-2} s^{-1} and internal CO_2 concentrations above 800 μmol CO_2 mol^{-1} [43]. Historically, with genetic improvement in soybean crop, there have been considerable modifications in plant architecture components with the goal of improving mechanization efficiency principally on the height of insertion of pods. At the same time, harvesting technology has also advanced. However, a number of cultivars continue with the insertion of the first legumes higher than what is necessary to actual reality of the field.

Photosynthetically active radiation interception (PARint, μmol m^{-2} s^{-1}) is highly variable depending on plant population in the fields, the environmental conditions and plant genotypic characteristics including plant structure. In **Figure 1**, we can observe the PARint and the relation with grain yield in two equal parts of four soybean genotypes cultivated in Brazil. We can also observe significant differences in plant architecture between the four cultivars. There is also a reflex on solar radiation interception in the under part of vegetative canopy, which is divided into two equal parts. Generally, plants that receive more solar radiation in the upper part have higher grain yield in the upper and medium part (**Figure 1**) (Müller 2016, unpublished data). Nevertheless, it must be highlighted that this is not always true; there is a dependency on a number of definition factors of yield, especially about drainage capacity of photoassimilates for the grains that deal with a variable characteristic between genotypes, signalized in a larger scale by cytokinin hormone [44].

The lower availability of solar radiation can be reflected in the grain yield due to net fixation of carbon. The quality of light is also important, especially considering the relation between red wavelength and extreme red that can speed up the process of senescence of the soybean leaves. Burkey and Wells [45] observed the influence of light on the acceleration of senescence process of soybean leaves. As a result, it is clear that the light of solar radiation is important in the production of photoassimilates and the maintenance of photosynthetic activity.

In sun plants, as soybean, the light compensation point (the amount of photosynthetically active radiation where net fixation is zero) is located in 10–20 μmol m^{-2} s^{-1} [30]. Thus, solar radiation above this value is necessary in order to have increment in carbon fixation and reflection on growth and yield. There is a strict relationship between the availability of nitrogen and photosynthetic activity [46]. Variations of nitrogen in the plant were obviously

http://dx.doi.org/10.5772/67150

related to its availability in soil [47] and the variations can be attributed to the vegetative canopy, especially involving the age of the leaf and the availability of solar radiation [48]. Models demonstrate relations between lower light interception and the fall of the protein content in the vegetative canopy as well as variations of the protein content with the availability of direct and diffuse radiation [49].

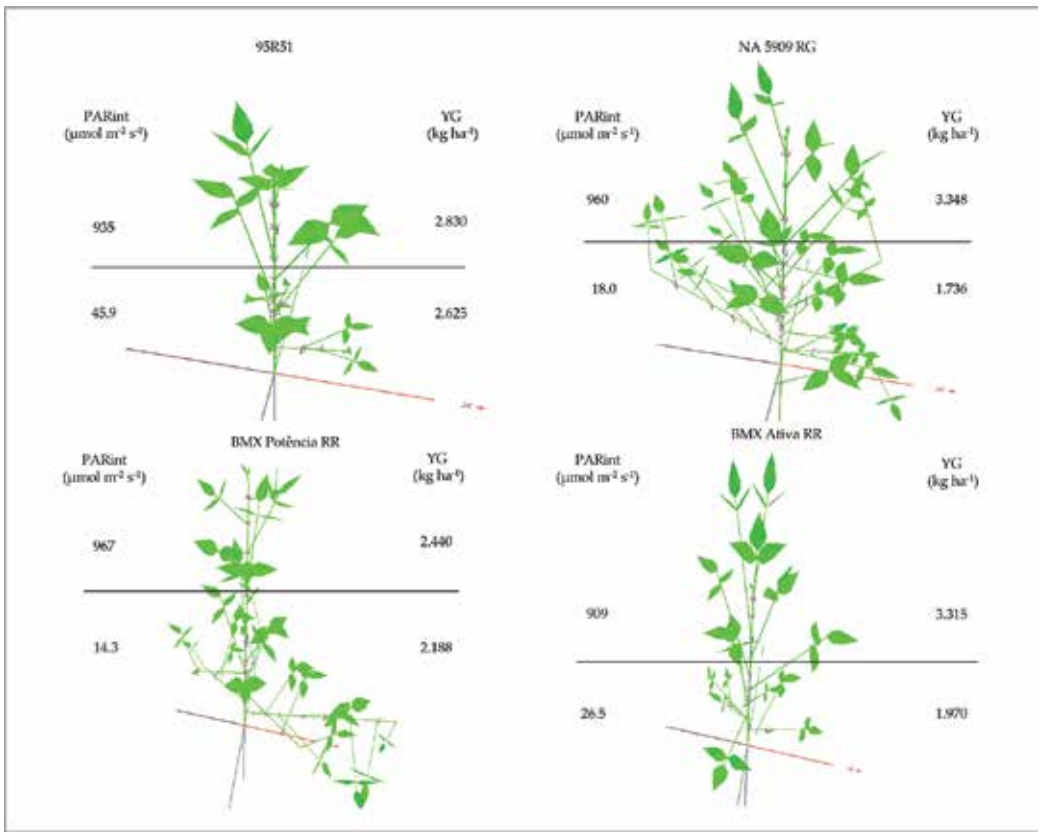

Figure 1. Architectures of vegetative canopies of four cultivars of soybean and photosynthetically active radiation interception (PARint, μmol m^{-2} s^{-1}) and grain yield (YG, kg ha^{-1}) (Müller 2016, unpublished data).

The capacity of nitrogen use by the plants is intimately connected to the availability of light in relation to the activity of the photochemical stage of photosynthesis [49]. In this stage, reducing agents are generated that will reduce nitrate to nitrite and afterwards, to ammonia that will be assimilated in the form of amino acid. This process known as "photoassimilation" is crucial for the metabolism of the plants and therefore, it is easily comprehended that as long as the quantity of light is reduced through the vegetative canopy, the process of photoassimilation will also be reduced [48]. One of the possibilities for increasing photosynthetic production through genetic breeding, challenge is the obtainment of the soybean cultivars that have a major activity and amount of RuBisCO as well as of other enzymes involved in

the photosynthesis, which would result in a larger demand for nitrogen [50]. Thus, the light interception would have to become more efficient to support cultivars with larger demands of nitrogen and subsequently more photoassimilation.

In soybean, symbiotic nitrogen fixation and how the energy used in this process originates in the photosynthesis are very important. The variation in the solar radiation interception due to the architecture can influence these processes. For the production of 1000 kg of grain are necessary around 85 kg N ha^{-1}, of which around 60–90% are available by the symbiotic nitrogen fixation through diazotrophic bacteria. However, around 14% of the photoassimilates produced by soybean plants are used for this process [51]. This fact strongly suggests that the architecture of the plants that can define the capacity of solar radiation interception has a strict relationship with the capacity of symbiotic bacteria in the soybean plants fixing atmospheric nitrogen.

The light extinction coefficient (k) is an attribute that is related to the solar radiation interception and the architecture of the plants mainly in the vertical distribution of the leaves [52]. The k provides information about the transmissibility of solar radiation through the vegetative canopy not only by the relation of leaf area but also by the variations that may result from distinct plant architectures. The extinction coefficient (k) is defined as $\ln(1-\varepsilon_{int}) = -k$. LAI, ε_{int} = efficiency of the solar radiation interception, determined from quotient between the solar radiation intercepted and total incident on the canopy and LAI = leaf area index, total leaf area per unit ground surface area [53].

In soybean crop, the high capacity of the solar radiation interception was observed with values of 0.52 and 0.93 before and after flowering, demonstrating high potential of interception of solar radiation per unit of LAI [54]. Ebadi et al. [55] studying 17 soybean genotypes observed minimum and maximum values of 0.44 and 0.62, respectively. It is highlighted that phenomenological stages exist in soybean crop in which the exposure to solar radiation presents more influence on the determination of grain yield. Later stages of the vegetative period and of the beginning of the flowering are determinant for definition of the number of legumes [56]. The restriction of 25% of solar radiation when compared with the environmental conditions in the beginning of the flowering can modify the availability of photoassimilates at the point of influencing effective fructification and compromise the grain yield [57].

Studies of genetic dissection and relations of the plant architecture with yield composition demonstrate positive correlations among the number of branches in the main stem, total number of nodes, diameter of the stem in the third node with a number of legumes, number of grains per legume and total grains per plant [7]. Soybean presents a high relation between production of biomass in shoot part and interception of solar radiation even in the phenological stage of physiologic maturation [58]. The efficiency of the use of radiation is the result of the gain of dry mass in relation to PARint accumulated [59]. The literature presents varied values of efficiency of the use of radiation in the range from 1.23 to 2.53 g MJ^{-1} PAR [60, 61]. In general, it is known that higher values of efficiency of the use of radiation are observed in the reproductive period of the soybean crop.

Considering that the composition of the soybean grain yield is defined by the fertility of the nodes, that is, effective fructification, the number of legumes and number and mass of the

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grains, the production of sugars originating in the photosynthesis is fundamental to compensate this energetic cost. Among the great challenges of the increase in grain yield in soybean crop, fructification is highlighted. A number of factors are involved in this process, from the hormonal action by the definition of drainage organs and the formation of structures [44], the mineral action by movement of sugars (K, Mg and B), structuring (Ca, B and Mg) [62] and the availability of sugars for production of carbon skeletal and ATP [63]. To last factor, the availability of solar radiation is a primary function for photosynthetic activity to produce photoassimilates.

The availability of solar radiation is reflected in the environment temperature. In some soybean production regions that are exposed to higher temperatures, it is common to observe that in some years there is less solar radiation available, which results in major gains in yield. This is likely to be related to the decrease in metabolic losses in the processes of respiration and photorespiration that results from lower temperature.

The breeding programs should be more emphatic in studies related to the architecture of soybean plants. In general, a number of important actions are taken to deal with biotic (pests and diseases) and abiotic (drought resistance) stresses. However, the selection of materials aiming at the photosynthetic efficiency by means of architectural variations is little explored. Koester et al. [64] evaluating 24 soybean cultivars liberated from 1923 to 2007 in the United States observed that maximum photosynthesis, stomatal conductance and nocturnal respiration were not significantly modified throughout these years. Nevertheless, the authors also observed that the daily biomass gain was higher in newer cultivars and suggested that this better performance was associated with a higher content of chlorophyll and the drainage capacity in reproductive organs. Ustun et al. [65] verified that the masses of the cultivars between 1940 and 1970 were considerably small, demonstrating that there was increase in drainage capacity of reserve for the reproductive organs in the cultivars.

4. Spray deposition under variations of plant architecture

With the aim of increasing and improving agricultural production, the number of applications of phytosanitary products during the cultivation cycle has been elevated, burdening with production costs and risks of contaminating the operator and the environment. In this sense, the application technology has been improving, permitting an improvement in the deposition of active ingredients in the desired target.

The application success depends on many factors. Some are controlled by the farmer and some not. Among the possible control factors are type of product, type of equipment, volume of solution, droplet size, application frequency and moment of application. However, some factors have a direct influence on pulverization quality of the phytosanitary products and cannot be controlled by the farmer, such as the architecture, the phenological stage and anatomical and morphological characteristics of the plants.

The variability among different soybean cultivars in relation to the architecture influences the deposition of droplets in the plant strata. The low penetration of active ingredients in the

plant canopy interior is mainly due to the so-called umbrella effect of the superior leaves. This contributes to the spread of diseases, because beyond not having protection, the lower part (one-third) has a microclimate that favors the occurrence of diseases, especially fungal diseases.

The efficacy of the application technology to disease control can be determined by the number and size of the droplets that reach the target per cm^2. The quantities of droplets depend on the characteristics of the product to be applied. For an effective control, a coverage of around 30–40 droplets and coverage of around 50–70 droplets per cm^2 are needed for systemic fungicides and protective fungicides, respectively [66]. Even considering the variation in the types of tips and flows, the volume deposited in the lower part (one-third) is significantly inferior to the amount deposited in the higher part (one-third) [67]. This amount can be up to three times higher than the lower part (one-third) [68]. Wolf and Daggupati [69] observed only 10% coverage of the tissue in the lower part.

Leaf diseases reduce the healthy photosynthetic leaf area, decreasing the solar radiation intercepted and the capacity of radiation use, which consequently may cause the leaf fall. It is easily inferred that the grain mass is negatively affected by the premature leaf fall and therefore, losses occur in the yield and in the quality of the final product. The visual appearance of the leaf diseases does not always represent the impact in the photosynthesis. When plants infected with the fungus *Phakopsora pachyrhizi* were evaluated, the disease impact in photosynthetic activity was larger than the visual estimation [70].

The application of phytosanitary products in alternate hours of the day can be an alternative to make this practice more efficient with more active ingredient in the inferior part of the canopy. It is observed that superior leaves are less affected by the "umbrella" effect in certain times of the day, due to angulation change of the leaves by the heliotropic process. The angulation of the leaves in diaheliotropism in the early morning and late afternoon can hamper the droplet penetration in the plant inferior stratum. However, the angulation of the leaves in paraheliotropism in the late morning and early afternoon can decrease the physical barrier of the plant higher stratum in the penetration of phytosanitary products.

Climatic factors such as temperature, relative humidity and wind speed must be monitored to avoid evaporation and droplet drift [71]. These environmental conditions cannot be favorable to pulverizing in the moment when the plant superior leaves are in greater angulation compared with the soil. Thus, we believe that when possible to conciliate the leaves angulation with environmental condition in the pulverization moment can increase disease control efficiency. Architecture characters that mostly influence droplet deposition in the plant are stature, the number of branches and size and number, format and orientation of the leaves. Plants with larger stature, a higher number of branches and higher LAI present the higher variations in droplet deposition [20].

Biotic and abiotic factors are variable conditions between systems and years of cultivation, to which the farmer needs to optimize the crop management adequately. Hence, the following question is proposed: which is the architecture that is proportionate to a better efficacy in phytosanitary product deposition, resulting in an effective participation of all parts (all three equal parts) of the plant in the grain yield among different cultivation conditions?

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5. Crop managements and their impacts on architecture modification

Excessive growth in soybean plants has caused lodging, damaging the harvest, besides hampering phytosanitary managements and light interception in the canopy interior. This excessive growth is due to fertilization, climatic conditions, sowing density and season and the own characteristics of cultivars used. Plants with higher stature do not mean higher yield and many times occurs the contrary, since the plant uses much energy to produce green mass instead of sending this energy for grain production and filling, besides causing self-shading.

With the aim of making soybean plant architecture more efficient in the use of environmental resources, growth regulators have been used. These substances are applied exogenously and influence the physiological processes, stimulating and/or inhibiting cell elongation or division.

Apical bud growth can inhibit axillary bud growth due to the apical dominance caused by the auxin hormone. Inhibitors of auxin transport, such as the 2,3,5-triidobenzoic acid (TIBA), can eliminate the inhibition of axillary buds [30]. TIBA when applied in stage V5 of soybean in which fourth trifoliate leaves are completely developed [72] reduced the plants stature without negatively influencing yield-related parameters [73].

Some vegetal regulators can inhibit the synthesis route of gibberellic acids synthetized by vegetables. Plants treated with these substances can present characteristics of agronomic interest different from those not treated, which can benefit some cultivation traits. Trinexapac-ethyl doses did not interfere in the components of soybean yield, but altered the plant stature and stem diameter [74]. Nonetheless, other authors who applied the same regulator found a reduction of 12% in the yield [75].

The use of chlormequat chloride and chlorocholine chloride in soybeans presented significant differences in reduction in stature and in effects on flower dry mass, root dry mass and root/aerial part ratio and in the number of flowers, besides reducing the leaf area, the dry mass of legumes and the total dry mass of plants [75]. The use of growth regulators when applied in the right moment and in the right doses has provided positive results in soybeans. Growth regulators can modify the architecture in a way that light interception and phytosanitary management are beneficial, making it possible for the farmer to make strong fertilizations and use genotypes with a high genetic potential for grain yield, even though they present exaggerated growth.

The adjustment in plant arrangement through sowing density and width can be proportionate to positive results due to a better soil coverage, a greater weed control, a decrease in intraspecific competition, an increase in the use of water and nutrients, the interception of solar radiation and better phytosanitary management. Adjusting sowing density is important to optimize the cultivation growth and the time necessary for the canopy closure. Shading caused by plants' high density especially in the plant lower part (one-third) harms the cultivation productive potential. This is due to the lack of collaboration of these shading structures in carbon assimilation and consequent decrease in the maintenance and grain filling.

Soybean plant width and density vary according to the characteristics of cultivars and environments. Hence, the potential of cultivars can be optimized by the adjustment of width

through the plant density, growth habit and climatic conditions. Soybean has the capacity to adjust itself to different environments and managements due to its plasticity [76].

Sowing density recommended above by the breeder might cause the development of higher plants with a reduced stem diameter, generating the plants more vulnerable to lodging. The number of branches and legumes, grain yield and thousand grain mass per plant decreases with the increase in plant density [77]. An increase in density maximizes the competition between plants of the canopy, being a possible factor for the reduction in branches per plant. A reduction in the number of branches per plant from 0.26 to 0.05 in two cultivars for each additional plant per m^{-2} was observed [78]. Meanwhile, low plant density might have increased the number of branches with a major contribution of them with the total yield. A small plant population results in larger grain yield through the increase in the number of fertile legumes per m^2 and higher grain weight due to a decrease in intraspecific competition [79].

The period of sowing must be planned having in mind the effort to avoid unfavorable environmental conditions principally in the critical period, which starts at the flowering and goes up to the grain filling. Any kind of stress in this period can affect negatively the crop yield components. However, cultivars response to photoperiod and temperature are distinct, being some more sensitive than others. Thus, the most appropriate time of sowing for each cultivation and environment must be evaluated.

Early sowing can increase LAI and increase grain yield. However, it can also promote self-shading and lodging by excessive vegetative growth [80]. When the photoperiod is prolonged in the moment of grain filling, it permits a higher duration of this phase, enabling an increase in the seeds through a higher number of nodes and more legumes per node [81]. In late sowing occur the early flowering, reduction in cycle and stature of the plant as well as a negative association between emergence date, maturation and stature of the plant [82]. The crop will present lower vegetative growth due to high temperatures and shortening of days in the beginning of the cycle, inducing the flowering even in small plants and lower LAI. Thus, grain yield can be harmed by alterations in the plant morphology and architecture [76]. In the southeast of Brazil sowing on November 11 and 26, reduction in the plant stature at flowering and maturation and in the number of days for maturation was observed when comparing the second season with the first. Also, the delay of 15 days in the sowing resulted in an increase in the height of insertion of the first legume, while the weight of 100 grains and the yield were not influenced by the sowing period [83].

Soybean is a plant highly dependent on the interaction between genotype and environment. Soybean can change its cycle and vegetative growth, depending on this interaction. Soybean cycle duration depends on the floral induction's photoperiod and temperature. These factors are reflected in plant architecture, cycle and crop yield potential.

6. Final considerations

The soybean has a high genetic potential for grain yield. However, there are several interferences during the cycle that can compromise the potential. We believe that the soybean plant architecture directly affects the final yield of the culture through the low efficiency in

interception of solar radiation and the difficulty of controlling disease, especially in the lower strata of plants.

Improving plant architecture can bring the benefit for further exploring natural ingredients that are the sun's raw materials. Improvement in phytosanitary management can reduce the number of the applications during the cycle, with consequent reduction in production costs and environmental risks. Thus, it is suggested that studies are carried out to evaluate the architecture in relation to interception of solar radiation and deposition of active ingredients. This will contribute to breeding programs to develop the plants that adjust the morphology in relation to these aspects, contributing to a genetic gain and social.

Acknowledgements

The authors are grateful to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support. PNPD/CAPES for scholarship of A.C and Prosup/CAPES for scholarship M.M.

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Chapter 3

Strategies of Chemical Protection for Controlling Soybean Rust

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/67454

Abstract

Asian soybean rust (*Phakopsora pachyrhizi*) is an aggressive and destructive disease that undermines the current 34 million hectares of soybean production system in Brazil. The disease is present throughout the entire cultivated area. The disease control has required a combination of several practices in order to avoid losses. In the last 15 harvests, the application of fungicides has been shown as an effective alternative for the producer in the control of this aggressive disease. Since the first fungicides emergency recommended for the 2002/03 season (azoxystrobin, difenoconazole, fluconazole, pyraclostrobin + epoxiconazole, and tebuconazole), a large number of new formulations were added to the arsenal to control rust. There are today recorded in MAPA (Ministry of Agriculture and Supply) about 45 active ingredients (alone or in combination are about 120), trademarks, and formulations for the rational use against rust. Among fungicides, there are differences in efficacy, residual period, metabolic stability, and translocation rate, requiring care from the producer and technical assistance in the choice of the product to be used in each situation. In this review, the chemical control of rust is analyzed in Brazil from 2001/02 to 2013/14; its economic importance, strategic variables for the rational fungicides practice, factors that complicate the chemical control and the risk of resistance to the main chemical groups.

Keywords: Soybean, Asian rust, chemical control, active ingredient, triazole, strobilurin

1. Introduction

Soybean rust is caused by two species*: Phakopsora meibomiae* genus, which causes American rust, which naturally occurs in several legumes from Puerto Rico, Caribbean, the Southern State of Paraná (Ponta Grossa), and *Phakopsora pachyrhizi*, which causes Asian soybean rust, present in most countries that grow soybeans and from the 2000/01 season, also in Brazil and Paraguay. A distinction between the two species is made by teliospores morphology and DNA analysis [1–3].

Asian soybean rust caused by *P. pachyrhizi* has been a serious disease in Asia for many decades. It appeared in Africa in 1997, and appeared in the Americas fields in 2001. In the USA, it was first found in the continent, in late 2004, probably brought in by a hurricane; it was considered such a threat that it was listed as a possible weapon of bioterrorism. Soybean rust cannot overwinter in areas with freezing temperatures, but it can spread by wind rapidly over such large distances, its development can be so explosive, and it can cause such rapid loss of leaves that it is now one of the most feared diseases in the world's soybean-growing areas.

Asian soybean rust (P. pachyrhizi) is a very destructive disease that undermines the current soybean production system in Brazil. ASR can cause yield losses of up to 90%. The disease was first reported in Brazil in open field areas in 2001. The disease importance can be judged by its rapid expansion, virulence, and amount of losses (**Table 1**). This situation was common in the Cerrado and South regions, where the climate favors the disease, makes its control difficult and the large extensions of crops represent one more challenge in spraying [4–7].

Crop season	Grain loss[(1)]	Rust cost[(2)]	Observations
2001/2002	569.2 thousand tons (US$ 125.5 million)[(a)]	US$ 177 million	First year with the disease occurrence on commercial areas. No fungicides registered for soybean rust. Economic losses were observed in the states of Rio Grande do Sul, Paraná, Mato Grosso do Sul, Mato Grosso, and Goiás.
2002/2003	3.4 million tons (US$ 737.4 million)[(b)]	US$ 1.16 billion	Rust occurred in 80% of Brazilian cultivated area, receiving three spray applications, on average. Five commercial fungicides were registered as an emergency. Major losses in the state of Bahia. Rust was reported in all producer states.
2003/2004	4.6 million tons (US$ 1.22 billion)[(c)]	US$ 2.08 billion	Soybean rust occurred in 70% of the cultivated area, receiving 3.5 sprays per hectare on average. Lack of fungicides to spray. Rust reported in all producer states, except in Roraima and Pará, and in Distrito Federal.

http://dx.doi.org/10.5772/67454

Crop season	Grain loss[1]	Rust cost[2]	Observations
2004/2005	Losses not estimated; only localized occurrences	US$ 1.215 billion Control cost: US$ 1.215 billion (US$ 32.6/spray × 2 sprays – 80% of cultivated area)	Drought in most of the regions; rust did not have significant impact. Mato Grosso was the most affected state. No disease was registered in Distrito Federal or in the states of Bahia, Piauí, Roraima, and Pará.
2005/2006	2.9 million tons (US$ 640 million[a] + 10% taxes)	US$ 2.124 billion Control cost: US$ 1.42 billion (US$ 40/spray × 2 sprays – 80% of cultivated area)	Off-season soybean sowing increased rust incidence in the crop season. Rust was reported in all producer states, except in Piauí, Roraima, and Pará, and in Distrito Federal.
2006/2007	2.67 million tons (US$ 615.7 million)[d]	US$ 2.19 billion Control cost: US$ 1.58 billion (US$ 33/spray × 2.3 sprays – 99% of cultivated area)	The soybean-free period implemented in the states of Tocantins, Goiás, and Mato Grosso reduced early onset of rust. Rust reported in all producer states, except in Roraima and Pará, and in Distrito Federal.
2007/2008	418.5 thousand tons (US$ 204.5 million)[e]	US$ 2.38 billion Control cost: US$ 1.97 billion (US$ 43/spray × 2.2 sprays)	Soybean-free period implemented in the states of Tocantins, Goiás, Mato Grosso, Mato Grosso do Sul, Minas Gerais, São Paulo, and Maranhão. At the end of the growing season, a lower efficiency of DMI fungicides was reported. Rust reported in all producer states, except in Piauí, Pará, and Roraima, and in Distrito Federal.
2008/2009	571.8 thousand tons (US$ 71.7 million)[f]	US$ 1.74 billion Control cost: US$ 1.67 billion (US$ 30/spray × 2.4 sprays)	Soybean-free period also implemented in the state of Paraná. Drought in most of the growing regions. Rust reported in all producer states, except in Pará and Roraima, and in Distrito Federal. Epidemics in the state of Bahia.
2009/2010	Losses not estimated; only localized occurrences	US$ 2.09 billion Control cost: US$ 2.09 billion (US$ 33/spray × 2.7 sprays)	The rainy winter was favorable for the survival of the fungus in volunteer soybean plants, and the weather was favorable during the crop season for epidemics. Fungicide sprays avoided losses. Rust reported in all producer states, except in Pará and Roraima.
2010/2011	Losses not estimated; only localized occurrences	US$ 2.10 billion Control cost: US$ 2.10 billion (US$ 35/spray × 2.5 sprays)	Dry winter helped to decrease the fungus population. The Anti-Rust Consortium started recommending only the application of a premix of DMI and QoI fungicides due to the lower efficiency of DMIs in all regions. Fungicide sprays avoided losses. Rust reported in all producer states, except in Piauí, Maranhão, Tocantins, Pará, and Roraima, and in Distrito Federal.

Crop season	Grain loss[1]	Rust cost[2]	Observations
2011/2012	363.5 thousand tons (US$ 191.6 million)[g]	US$ 1.73 billion Control cost: US$ 1.54 billion (US$ 22/spray × 2.8 sprays)	La Niña weather condition: drought in the southern region and in the state of Mato Grosso, with lower incidence and severity of soybean rust. Losses in Mato Grosso. Rust reported in all producer states, except in Piauí, Maranhão, Tocantins, and Roraima.
2012/2013	Losses not estimated; only localized occurrences	Control cost: US$ 1,94 billion (US$ 25/spray × 2.8 sprays)	Volunteer soybean plants with rust overwinter in Mato Grosso. El Niño weather condition: irregular rain occurrence. Low disease severity in the South of the country. Rust reported in all producer states, except in Piauí, Tocantins, Pará, and Roraima.
2013/2014	Losses not estimated; only localized occurrences	Control cost: US$ 2.2 billion (US$ 25/spray × 3 sprays)	Low disease pressure on South and Southeast regions due to below-average rainfall and high temperature. In the Center-West region, late-sowed soybean had high severity (with rains in February). Lower efficiency of QoI and of a premix of QoI + DMI. Rust reported in all producer states, except in Piauí, Pará, and Roraima.

Source: Consórcio Antiferrugem (2015).

[1]Calculated considering soybean price: (a) US$ 220.50 per ton; (b) US$ 220 per ton; (c) US$ 266 per ton; (d) US$ 230.6 per ton; (e) US$ 488.72 per ton; (f) US$ 230.65 per ton; and (g) US$ 527.07 per ton.

[2]Control cost + grain losses. DMIs, demethylation inhibitor; and QoI, quinone outside inhibitor. By Godoy et al, 2016 (19).

Table 1. Estimated grain losses and costs due to Asian soybean rust control in Brazil, since the 2001/2002 crop season [19].

The fact that Asian soybean rust is a disease of recent occurrence (from 2001) and the limited availability of information about the climatic influences of soybean cultivation regions could have an influence on the severity of the disease each year, which makes the generic control recommendation that satisfies all the regions difficult. The continuous periods of leaf wetness, because of 8h of rain or dew, and daily temperatures ranging from 15 to 30° C favor the development of the disease [4, 8–11]. Control strategies for Asian soybean rust require a combination of management practices to avoid or minimize losses [7, 10, 12]. The main measures adopted must be: (1) to increase the rotation area with non ASR host crops (corn, cotton per example), (2) to sow earlier maturity groups cultivars, concentrating sowings in the beginning of the period indicated for each region: earlier sowings usually develop under conditions less favorable to rust, (3) to avoid planting in various times and late cultivars, because soybean planted later (or of long cycle) will be more damaged by the load of spores multiplied in the first crops, (4) to sow soybean with plant density that favors good leaf aeration in order to optimize the penetration and leaf coverage by fungicides, (5) do not sow soybean in the off-season period and eliminate as much of volunteers or guaxa soybean as possible, (6) rational use of fungicides following epidemiological criteria and resistance management strategies, and (7) to sow cultivars with genetic resistance (partial resistance—slow rust) to ASR.

http://dx.doi.org/10.5772/67454

2. Importance of the chemical control for soybean rust

Soybean cultivation methods done by farmers increase the occurrence of biotrophic pathogen such as *P. pachyrhizi*, the chemical control of the soybean rust has been shown feasible and efficient. However, problems with correct disease management, lack of information about the biology, and pathogenesis of the disease under environmental conditions in Brazil and operational limitations of spraying in extensive cultivation areas have hampered the performance of various control programs implemented on a commercial scale [13, 14]. The chemical control of Asian soybean rust is the most widely used method for controlling the disease. Fungicide application has been shown to be an effective alternative for the producer in the management of this aggressive disease. Fungicides from chemical groups of triazoles, strobilurins, carboxamides, and, from the last three seasons, the protectants are the mostly used to control the disease, with difference in the preventive and curative efficiency between the active ingredients within each group [8, 15–18].

Under the technical, epidemic and economic point of view, the application numbers used to control rust are from two to five applications of fungicides. Over the 16 years managing soybean rust in Brazil, the fungicides management changed according to compounds' evolution and also according to soybean rust resistance to fungicides. In 2007/08 season, triazole + strobilurin mixtures dominated the fungicide application market for the control of the Asian soybean rust. In 2013 was launched the first compound with carboxamides (fluxapyroxad, solatenol, or benzovindiflupyr) fungicides in mixture. Also, with fungicides efficacy reduction, the adoption of integrated measures to control the disease will be important for the sustainability of the crop [19]. This review aims to discuss the main features of triazoles, strobilurins, carboxamides, protectants, and their mixtures, groups of systemic and protectants fungicides most important and used to control the rust, as their chemical structure, biological activity, translocation in the plant, and synergism between mixtures. In addition, some biological properties of these fungicides indispensable for the treatment of rust in adverse control situations will be discussed in this work, among them: penetration, translocation, curative effect, and absolute residual period. The control programs adopted in different Brazilian soybean regions will also be discussed, as well as the problem of decreased sensitivity of the fungus to fungicides and the risk of resistance to these products.

3. Main chemical groups of fungicide for soybean rust

3.1. Multisite fungicides

The protective fungicides are intended to ensure the protection of plants before pathogen attack. They must be applied before pathogens infect, forming a protective barrier toxic for fungi and bacteria in plants. When applied to the surface of plant organs, exert a toxic barrier preventing the penetration of fungi by inhibiting the spores germination process Syngenta

[20]. The characteristic of the contact protective fungicides as not to penetrate the plant is essential that they do not become phytotoxic to plants.

3.2. Biological properties of the multisites

Recently, after problems in efficacy with the two most used fungicides group DMI's and QoI's due to sensitivity reduction of Asian rust in soybeans, some multisite groups as copper-based, dithiocarbamates, and chloronitriles products have been tested in combination with more specific systemic products to the disease in order to improve the effectiveness and resistance management.

Fungicides copper base are contact products and are characterized by forming a toxic barrier that prevents the germination of spores on the surface of the sheet, as altered metabolism and inhibits proteinic and enzymatic action over 20 mechanisms impeding the penetration of the fungus in the tissue leaf. A low risk of resistance due to the large number of work sites in the pathogen. It is necessary to caution in the preparation and application of fungicides, because in some situations can cause phytotoxicity or burning the plants. Other care and constant hustle to keep the product in suspension evenly and avoid settling in the application tank bottom is fundamental.

Phthalonitriles (chlorothalonil) are characterized by benzene ring formed only by carbon. In cyclic structures from group lying one nitrogen atom and may also be a sulfur atom depending on the formulation and active ingredient. These fungicides are rapidly metabolized in plants, and become constituent proteins. The mode of action of the heterocyclic nitrogen is of the interference of DNA and RNA synthesis exhibits good protective action depending on the concentration used.

The dithiocarbamates fungicides mark the beginning of the use of organic fungicides. They are derivatives of carbamic acid compounds and generally have a broad action being one of the most used fungicides consumption. Dithiocarbamates were originally used in the rubber production process. The first dithiocarbamate fungicide known was patented in 1934. Since then, new generations of dithiocarbamates base metal salts (ferbam) showed good control levels in diseases in ornamentals. This group is currently performing as a very important tool in resistance management in various pathosystems. The dithiocarbamates act primarily through inhibition of enzymes of the power production cycle of the pathogen cells, and makes them unavailable for the body of metal ions such as copper and iron.

3.3. Fungitoxicity

The protectant fungicides cupric copper base are widely used for the control of downy mildew, rusts, blights, and bacterial spots and other diseases caused by pathogenic fungi and bacteria. In crops such as tomatoes and peppers, use is often seen as the protection profile of these products and illnesses caused by bacteria in these cultures.

Dithiocarbamates fungicides group had good acceptance due to the lower level of phytotoxicity in comparison to copper fungicides and sulfur. In the early 1960s, EBDC (manganese

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ethylene bisdithiocarbamate) fungicides were considered the most important and versatile group of organic fungicides. The mancozeb, for example, is used in more than 70 cultures, 120 countries, and many pathosystems. Main multisites under development and registered for soybean rust as the protectants research and use in Brazil to control soybean rust is quite new, the number of registered is small (mancozeb, mancozeb + azoxystrobin, chlorothalonil + tebuconazole and copper oxyclhoride). But regarding the field trial tests by antirust consortium diverse mixtures with protectants and systemic compounds are under development as important soybean diseases management tool.

4. Demethylation inhibitors fungicides

According to FRAC (Fungicide Resistance Action Committee) [21] International, the group of sterol biosynthesis inhibitors, based on the mechanism of action of compounds, includes four classes of fungicides, but only three of them, G1, G2, and G3 are used as fungicides in agriculture: DMIs, amines (formerly called morpholine), and hydroxyanilides. The compounds that inhibit the ergosterol biosynthesis are very effective as agents to control plant diseases. They are systemic and have protective, curative, and eradicative properties [22, 23]. The first class shows inhibitors of the demethylation of C14 from the sterol synthesis. DMI fungicides are also known as sterol biosynthesis inhibitors reaction (SBIs). They are commonly known as pyrimidines, pyridines, piperazines, imidazoles, triazoles, and conazoles, and these extremely effective and versatile compounds were responsible for the emergence of the world's largest market of agricultural fungicides, especially for cereal crops. They are found within this group of fungicides with broad and restricted spectrum of action, high systemicity, high fungitoxicity, selectivity, chemically stable, and with long residual effect. The second class of DMIs is composed of amines (current name for morpholine), compounds that inhibit Δ8-Δ7 isomerase and Δ14 reductase in sensitive fungi. The third class consists of hydroxyanilides, compounds that inhibit C3 keto-reductase.

4.1. Triazole fungicides

Triazoles are versatile organic fungicides of broad spectrum, with apoplastic preferential systemicity, eradicative/curative action and long residual effect. Chemically, they are formed by the addition of different radicals to a basic molecule of 1,2,4-triazole. They are classified as (a) triazoles with keto: triadimefon radicals; (b) triazole with ketal: propiconazole radicals and etaconazole; (c) triazoles with hydroxy: triadimenol radicals, bitertanol, and dichlobutrazole; (d) triazoles without other functional groups: fluotrimazol [23].

According to Hewitt [22] and Azevedo [16], the importance and use of fungicides in agriculture has increased rapidly in recent years due to the combination of a series of biological qualities, among them: high fungitoxicity to several pathogens that cause major diseases such as rusts, powdery mildew, and leaf spots, especially in cereals, quick penetration and translocation in plant tissues with uniform distribution; eradicative/curative action on infections already begun, being used based on preestablished control levels, avoiding costs with preventive

applications, often unnecessary and with prolonged residual effect, enabling the use of lower doses and/or longer intervals between applications, thereby reducing the number of sprays.

4.2. Biological properties of triazoles

4.2.1. Fungitoxicity

The fungitoxicity of some triazoles such as tebuconazole, frutriafol, and cyproconazole has been one of the main safety reasons of these compounds in the control of the Asian soybean rust. The curative and eradicative action of the products on the structures of this destructive fungus has allowed success in the control and protection of the culture, even in field curative situations.

4.2.2. Systemicity

Soybean rust has caused, among other things, a real movement in the research field, development, and registration of agrochemical companies. Several works have been conducted not only in research and development of new products, but mainly in the pursuit of effectiveness of products in more appropriate dosages, in the timing of application and in the phenological stadiums more favorable to applications [16].

One of the first works to demonstrate the difference in translocation of recommended fungicides to control soybean rust was conducted by Fundação MT, in the person of Dr. Arlindo Harada. The study was conducted with cultivar BR 154, the fungicides applied were in 06/23/03 and the assessment on 07/15/03. The crop was at phonological stadium from R2 to R3. The systemicity of different triazole fungicides and their mixtures was evaluated when applied in different regions of the leaf (center, base, apex, and petiole). The results showed a better systemic effect of flutriafol, followed by tebuconazole and epoxiconazole + pyraclostrobin mixture [24].

The systemicity for specific fungicides (strobilurin and triazole) to control soybean rust has been demonstrated in an experiment conducted under controlled conditions. Analyzing the behavior of strobilurins, it could be observed that azoxystrobin has a mild redistribution throughout the leaf, moving through the xylem, following the transpiration stream, thus proving its systemic effect. Pyraclostrobin is only visible in nervures and at low concentrations; not spreading to the rest of the leaf, showing no significant systemic effect [25]. Cyproconazole presents a fast movement at high concentrations throughout the leaf, shortly in the first days after treatment, moving through the xylem, following the transpiration stream, demonstrating its systemic effect; epoxiconazole has a slower translocation, with initially high concentration in the nervures, which will be diluted to the rest of the leaf over time.

The easiness through which fungicides penetrate and translocate within the plant is due in part to its physicochemical properties. This easiness can be measured based on the ability of the fungicide to distribute between alcohol (octanol) and water when applied to form a mixture of two substances. It is the so-called partition coefficient or log P value. All systemic fungicides with log P value of 3.2 or less move fast in the plant. Systemic fungicides with values greater than 3.2 do not move very fast, although they enter the plant [24]. The

http://dx.doi.org/10.5772/67454

octanol-water partition coefficient (K ow) or Log P has been used as a parameter to measure the translocation rate or the systemicity of systemic fungicides, when applied to plants [26] (**Table 2**). It has been accepted that compounds with lower Log P values are faster and will control the disease with higher efficiency. In the specific case of soybean rust, with frequent use of triazoles alone or in mixtures with strobilurin, fungicides that have lower Log P values such as flutriafol and cyproconazole (**Table 2** and **Figure 1**) translocate faster in soybean leaves and the advantage is only when these products are curatively applied in the fungus postinfection phase. When applied preventively and according to the phenological stadium more favorable to rust, this advantage disappears. Another factor that influences the systemicity use of systemic fungicides is their solubility in water expressed in ppm [16, 26].

Active ingredient	Log P or Ko/w
Flutriafol	2.3
Cyproconazole	2.9
Tetraconazole	3.1
Triadimenol	3.3
Epoxiconazole	3.4
Tebuconazole	3.7
Propiconazole	3.7
Hexaconazole	3.9
Difenoconazole	4.3

Table 2. Log *P* values or Kow of different triazoles, some of them used for soybean rust.

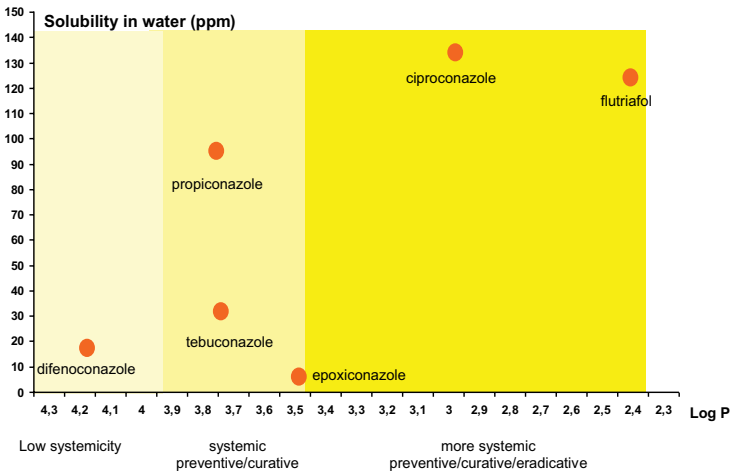

Figure 1. Solubility and Log P values of different triazoles.

4.3. Main triazoles registered for Asian soybean rust

The main triazoles registered in Brazil to control soybean rust are cyproconazole, difenoconazole, epoxiconazole, fluquinconazole, flutriafol, tebuconazole, tetraconazole, metconazole, and prothioconazole. There are some triazoles + triazole mixture registered propiconazole + cyproconazole, cyproconazole + difenoconazole. The main of triazoles and strobilurin mixtures registered for the control of soybean rust are: azoxystrobin + cyproconazole, azoxystrobin + tebuconazole, azoxystrobin+flutriafol, epoxiconazole + pyraclostrobin, trifloxystrobin + cyproconazole, trifloxystrobin + prothioconazole, trifloxystrobin + tebuconazole, picoxystrobin + cyproconazole, and picoxystrobin + tebuconazole. As main triazoles and benzimidazoles mixtures to control soybean rust are: methyl-thiofanate + flutriafol, epoxiconazole + methyl-thiofanate, carbendazim + flutriafol and tebuconazole + carbendazim. In 2016 was launched the first triple ready mix with triazole + carboxamide and strubilurin.

5. Strobilurin fungicides: fungicides inhibitors of fungal mitochondrial respiration (QoI)

Fungicides known as QoIs (quinoline outside inhibitors) are broad-spectrum fungicides and include three families of strobilurin fungicides and two other represented by compounds fenamidone and famoxadone [27]. The mechanism of action of strobilurins occurs through inhibition of the mitochondrial respiration, which blocks the electron transfer between cytochrome b and c_1 at the Qo site, interfering with the ATP production. Strobilurins are referred to as "QoI" or Group II fungicides, which is simply a reference to their unique mode of action [16].

According to Hewitt [22] one of the most significant advances in the discovery of fungicides was the introduction of synthetic analogues of strobilurin A, a compound produced by *Strobilurus tenacellus* basidiomycete. It is a successful and recent example of the use of natural products as a model of discovery and development of new systemic fungicides. In 1969, it was discovered that culture extracts of the *Oudemansiella mucida* basidiomycete fungus that grows on decomposing wood, had antifungal activity, or were capable of killing fungus. In the decade of the 1970s, several compounds of the extract of this fungus were isolated, including the one responsible for the antifungal activity, mucidicin. This discovery led other researchers to investigate the chemical composition of various basidiomycete fungi, and in 1978, a compound with fungicidal properties called strobilurin was isolated from *Strobilorus tenacellus*. The studies showed that the compound initially called mucidicin had the same structure of strobilurin. In view of the good results so far found, around 20 species of basidiomycetes were studied, and this resulted in the synthesis of a large number of new compounds with fungicide activity. These compounds, although different, had some similarity from the chemical point of view, i.e., the presence of the structural unit derived from the B-metoxy acrylic acid [25, 28].

5.1. Fungitoxicity and field uses

The fungitoxicity of strobilurins has been one of the reasons for using these compounds in programs aimed to control diseases in plants. In the specific case of soybean rust, these

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fungicides have been widely used in mixtures with triazoles. The use of simple formulations for this disease is unusual, although there are registered products [9, 29]. From the Products tested and registered for soybean rust, pure, or in mixtures with triazoles, it could be observed that the dosages of these compounds range from 0.20 to 0.5 L of cyproconazole (**Table 3**).

5.2. Systemicity

In a study by Embrapa [2] the azoxystrobin + cyproconazole and pyraclostrobin + epoxiconazole systemicity was compared in soybean leaves. Fungicides were brushed on the basis of leaf area (at concentrations recommended for use in the field), which were inoculated with rust (*P.pachyrhizi*) in the next day and incubated in greenhouse. After 16 days, the percentage of area above the point of application on which products controlled the disease was assessed. Azoxystrobin + cyproconazole fully controlled the disease throughout the extension of leaves, while the pyraclostrobin + epoxiconazole did not, showing that the faster translocation of azoxystrobin + cyproconazole reflects in better control (inoculation 1 day after the application of fungicides).

The translocation of strobilurins certainly is not one of the most important features of this chemical group of fungicides. The fungitoxicity, the action spectrum and the effective residual period are peculiar characteristics of this group of products, but there are differences in the translocation of these compounds when applied to control soybean rust (**Table 4**) [16].

The main strobilurins registered in Brazil to control soybean rust are: azoxystrobin, pyraclostrobin, trifloxystrobin, and picoxystrobin.

Active ingredient	Commercial product name	Dose L or kg cp*.ha^{-1}
Azoxystrobin	Priori	0.20
Picoxystrobin	Aproach	0.20
Cyproconazole + azoxystrobin	Priori Xtra	0.30
Cyproconazole+trifloxystrobin	SphereMax	0.30
Epoxyconazole+pyraclostrobin	Opera	0.5
Propiconazole+trifloxystrobin	Stratego	0.4
Tebuconazole+ trifloxystrobin	Nativo	0.5
Prothioconazole+ trifloxystrobin	Fox	0.4
Azoxystrobin+benzovindiflupyr	Elatus	0.2
Fluxapyroxad+piraclostrobin	Orkestra	0.35
Piraclostrobin+epoxiconazole+fluxa pyroxad	Ativum	0.8

*Commercial product.

Table 3. Fungicides from the strobilurin group, pure or in mixtures and their respective doses for the control of soybean rust (*P. pachyrhizi*).

Active ingredient	Log P or Ko/w
Azoxystrobin	2.5
Kresoxim-methyl	3.4
Pyraclostrobin	4.0
Trifloxystrobin	4.5

Table 4. Translocation rate of different strobilurins used to control soybean rust.

6. Fungicides inhibitors of succinate dehydrogenase (SDHIs)

Fungicides known as SDHIs include eight different chemical groups of carboxamides represented by phenyl-benzamides, phenyl-oxo-ethyl thiofene amide, pyridinyl-ethyl-benzamide, furan-carboxamides, oxathiin-carboxamides, thiazole-carboxamides, pyrazol-carboxamides, and pyridine-carboxamides. The mechanism of action of carboxamides occurs on the target enzyme succinate dehydrogenase (SDH, so-called complex II in the mitochondrial respiration chain), which is a functional part of the tricarboxylic cycle and linked to the mitochondrial electron transport chain. SDH consists of four subunits (A–D) and the binding site of the SDHIs (the ubiquinone binding site) is formed by the subunits B–D. Carboxamides are broad-spectrum fungicides that inhibit fungal cell respiration, which prevents energy production and leads to rapid cell death. While it may not be critical to know how carboxamides work, it is important to recognize the SDHI designation and be aware that all carboxamides have the same mode of action. The new broad-spectrum fungicides class has been quickly adopted by the market, which may lead to a high selection pressure on various pathogens. All of the 17 marketed SDHI fungicides bind to the same ubiquinone-binding site of the SDH enzyme. Their primary biochemical mode of action is the blockage of the TCA cycle at the level of succinate to fumarate oxidation, leading to an inhibition of respiration.

The Fungicide Resistance Action Committee has developed resistance management recommendations for pathogens of different crops in order to reduce the risk for resistance development to this class of fungicides. These recommendations include preventative usage, mixture with partner fungicides active against the current pathogen population, alternation in the mode of action of products used in a spray program, and limitations in the total number of applications per season or per crop [30].

6.1. Biological properties of SDHI fungicides

SDHI fungicides were discovered more than 40 years ago. Due to the limited disease and application spectrum of the "first generation" carboxamides, resistance under commercial conditions remained limited to a few crop/pathosystems (primarily Basidiomycetes), e.g., *Puccinia horiana*, chrysanthemum rust, and Ustilago nuda, loose smut in barley. In addition to these "first generation" molecules, SDHIs with increased spectrum and potency were launched starting in 2003 and new ones continue to be launched today. This modern generation of SDHIs is

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rapidly achieving market share in many crops and new SDHIs are currently in development. They are classified by FRAC (Fungicide Resistance Action Committee) activity group code number 7 [21].

The market adaptation to the new technology and its penetration has been to be very fast around the world and it was not different for soybean rust control in Brazil. The reason for the rapid adoption of SDHIs is their high level of activity and the lack of effective alternative control options. Many soybean pathogens have developed resistance to the QoI's, and reduced sensitivity to the demethylation inhibitor (DMI) fungicides, which generates increased challenges for the farmers to efficiently control diseases and maintain or increase crop yield and quality. The class of compounds inhibiting complex II of fungal respiration was originally called carboxamide fungicides, with the earliest compound in this class, carboxin, being first marketed in 1966. This narrow-spectrum fungicide was used mainly as a seed treatment to control basidiomycete pathogens such as smuts. Thereafter, benodanil, fenfuram, mepronil, flutolanil, furametpyr, and thifluzamide followed between 1971 and 1997; however, these compounds gave only slightly broader-spectrum control compared with carboxin. The first carboxamide with truly broad-spectrum foliar activity was boscalid, launched in 2003. FRAC is currently listing 19 SDHI compounds (benodanil, benzovindiflupyr, bixafen, boscalid, carboxin, fenfuram, fluopyram, flutolanil, fluxapyroxad, furametpyr, isofetamida, isopyrazam, mepronil, oxycarboxin, penflufen, penthiopyrad, pydiflumetofen, sedaxane, and thifluzamide), belonging to different chemical types. Currently the "overall" spectrum of SDHI fungicides is extremely broad, being comparable with the QoI spectrum, with the exception of oomycete activity, which is still lacking. Adepidyn™ (pydiflumetofen) is the first member of a new chemical group among the succinate dehydrogenase inhibitor fungicides (SDHI, FRAC Group 7), the N-methoxy-(phenyl-ethyl)-pyrazole-carboxamides. The common name for Adepidyn™ is pydiflumetofen. Adepidyn™ has a wide range of plant pathogen species.

6.1.1. Fungitoxicity

The fungitoxicity of carboxamides has been one of the reasons for using these compounds in programs aimed to control diseases in plants. Generation II SDHIs are intended for use in integrated disease management programs, or as mixing or alternation partners to prevent fungicide resistance. Fungicides from this class are effective against various diseases of cereals, fruits, and vegetables. In the specific case of soybean rust, these fungicides have been firstly used in mixtures with QoIs. Due to the detection of F129L resistance to QoIs in 2013/14 season (officially informed by FRAC), the fungicides research against soybean rust advanced for another mixture partners. In 2016 were launched epoxiconazole, pyraclostrobin, and fluxapyroxade, fungicide triple mixture containing in ready mixture to offer in Brazilian Market.

6.1.2. Systemicity

SDHI fungicides are derived from a diverse range of chemistry and, depending on the host and pathogen, have protectant, translaminar, or systemic activity.

7. Residual period of fungicides for soybean rust

According to Balardin et al. [4] and Azevedo [31], the effective residual period of a fungicide is beyond the intrinsic features that the active ingredient presents under experimental conditions may vary depending on the relationship between its pathogenesis and the general physiological conditions of the plant. The application of fungicides after the infection onset undergoes more drastic reductions in the residual according to the population density of the pathogen at the application time.

The residual period of a fungicide, being systemic, mesostemic, or protective, is a biological property quite peculiar to the various chemical groups. According to Balardin et al. [4], it is the maintenance of the active ingredient within the plant tissues at sufficient concentration to inhibit or delay the infection caused by a pathogen. In this case, the period of time that fungicide can provide protection to the plant is considered as absolute residual, i.e., benzimidazoles have an absolute residual around 15 days, triazoles, between 22 and 25 days, and strobilurins between 27 and 30 days. Considering mixtures of fungicides from different groups, the synergistic effect may cause an absolute residual period longer than that observed for products used alone. When there is no synergism between the components of the mixture, it is expected that the residual of the mixture is the same as the product with higher residual.

Under current field conditions, there are big differences between the effective and absolute residual of some systemic fungicides. According to Balardin et al. [4] and Yorinori [6], the residual period of a particular active ingredient is only one reference, since, for all that is done from the viewpoint of a culture management and population dynamics of the pathogen, the effective residual becomes the residual actually achievable under field conditions. The effective residual period of a fungicide exceeds the intrinsic characteristics that the active ingredient presents under experimental conditions, which may vary depending on the reaction between the pathogenesis and the general physiological conditions of the plant. Overall, the effective residual period is the result of strategy and tactics established in the form of an integrated management planning and may be influenced by factors as diverse as the time of fungicide application in relation to plant development or its pathogenesis, the population density of the pathogen, age, nutrition, and various components of the plant phytotechnical management, or even the expression of minor genes associated with partial resistance.

8. Strategies of chemical control for soybean rust

The strategies of chemical control for soybean rust should be based on five main points: (1) disease monitoring, (2) phenological stages of the culture, (3) choice of the fungicide, (4) application timing, and (5) application technology [16, 29, 32].

The disease monitoring and its identification in the early stages are essential for the efficient use of the chemical control and the frequent inspection of the tillage should be carried out. The protecting of plants must occur before the appearance of the first lesions (preventive) or at the beginning

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when the inoculum potential is still low. The spraying should reach maximum leaf area, and fungicides with longer residual period and systemicity should be selected [4, 16, 29].

According to Azevedo [16], the spraying programs based on phenological stages can also be used for major crops such as soybeans, corn, bean, and rice. The most illustrative and practical example is the soybean culture. For diseases of the aerial parts, there is what is called the critical period of protection. This period runs from the end of the vegetative period until R6 stadium. It changes between cultivars, and a difference of 15 days between early and intermediate cycles is common. Fungicide applications should be made within this period, especially respecting the critical stage of each disease and the residual period of several products. The protection of the culture against rust will always require the observation of the phenological stadium, and stage from the beginning of flowering until full flowering is currently considered as critical period (R1 | R2) for the first spraying of fungicides [4, 5, 10].

The success of a phytosanitary treatment program for the control of several diseases primarily depends on the use of a fungicide of proven efficiency and of a technology developed for its application. The influence of uncontrollable meteorological, biological, and agronomic factors should also be considered [16, 33]. Fungicides manufactured to control soybean rust are effective. However, success will largely depend on proper application. Proper application starts with selecting the right equipment, specifically nozzles, and spraying the right amount of fungicide uniformly across the field before the disease is detected. Pesticide manufacturers have invested heavily to determine the most effective as well as economical application rate for the fungicides labeled for soybean rust.

Spraying the right amount of fungicide on each acre of soybean is not enough to achieve effective pest control. How uniformly the fungicide is deposited on the spray target is as important as the amount deposited. Each nozzle produces a unique spray pattern. Some nozzles require precise overlapping of patterns from adjacent nozzles. Setting the proper boom height for a given nozzle spacing is extremely important in achieving proper overlapping. A low boom does not allow proper overlap while a boom set too high causes overdosed areas. Other situations that cause improper overlapping and poor uniformity include: clogged nozzles, misaligned nozzles spraying at different directions, and mixing nozzles with different spray angles. All these common errors contribute to nonuniform coverage.

The control of Asian soybean rust is a major concern for soybean producers in Brazil [9, 32, 34]. Considering the plant development stage at the time of applications, often with complete closure and large leaf area, it is generally agreed that the application techniques need to provide droplets with good penetration and coverage of leaves, even for fungicides with systemic action [35]. In the case of systemic fungicides for control of soybean rust, there is a false assumption that the application technology and the spraying programs are not as important as the implementation of protective products, because less coverage and amount of deposits would be needed, since they are products that penetrate and translocate on the leaf surface. However, most systemic fungicides display only partial translocation, usually from the leaf base to apex, with no translocation from lower leaves to the upper ones. This fact alone reinforces the value of application technology, which is able to make these products to penetrate into the body of plants through the use of small and medium-sized droplets [16].

Another important point concerns the timing (timing of application). Since the appearance of rust, the control with preventive applications proved to be more efficient than the curative applications. This recommendation is now considered standard [9, 16, 29] and most technical recommendations for the rust control is based on the following procedure: "giving preference to preventive applications from the flowering stadium (R1), opting for curative applications only if rust appears still in vegetative stages." Despite these recommendations, what has been observed in recent harvests was a significant number of curative applications, especially in regions where inoculum pressure was too high, as in the region of Primavera do Leste/MT, for example. These curative applications occurred due to two basic factors: (1) early beginning of rust in the crop, with the appearance of symptoms in the vegetative stadiums, and (2) inadequate applications, with errors regarding both the technique and the time of application, which compromised the control. These facts led to drastic reduction in the residual period of products, resulting in the need for a greater number of applications for the disease control.

Another reason that supports preventive applications concerns the epidemiology of the disease. Systemic fungicides, even those with curative and eradicative properties should not be curatively recommended, because in practical control situations, it is extremely difficult to determine the disease intensity threshold for which the "eradication" with systemic fungicides is effective. This fact became very evident at the time of the resurgence of the soybeans rust in Brazil, early in the first control recommendations. Triazoles have been and still are used to control this disease, many times in curative applications, out of the "biological timing," in some cases with 10–20% of rust infection, which lead to failure situations, with irreversible damage to the producer [16, 36].

Soybean rust has its greatest development after flowering, with large leaf area, so it is difficult for the fungicide to penetrate in the mass of leaves [34]. Field experiments have shown that the average coverages in the soybean canopy in the application of fungicides for rust control are: 70–90% for the upper canopy, 15–40% for the medium canopy, and 1–15% for the lower canopy [35, 37]. Leaf coverage tests conducted with sensitive paper showed that the deposition of fungicides on the inside of the leaf decreases from top to bottom of plants, whatever is the application technology and volume. This indicates subdose deposition on lower leaves, which may not affect the fungus or have a very short effect, thus allowing the rust resurgence in a few days. According to Yorinori [29], Balardin [4], and Antuniassi [32], this is the main reason for complaints about the reduction of the residual period of a fungicide that should be active for 25–30 days.

Soybean is cultivated in many regions of the country, with quite diverse weather conditions. The diversity of climatic conditions from one year to another, in different regions of Brazil, makes it impossible to formulate a package of chemical protection (cake recipe) that meets the needs of the entire country. Up to the present moment, there is no chemical protection strategy for the management of the Asian rust that meets efficiency, cost, and operability of all producing regions, but rather application programs based on researches carried out by public and private institutions, which are being improved every year, according to the rust occurrences [17, 38, 39].

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A good example is the latest recommendations summarized by the Antirust consortium in Brazil, where differences between the spraying programs recommended in different soybean cultivation regions in Brazil could be observed (**Table 5**).

State	Criterion adopted	Chemical group	Average number of applications
Rio Grande do Sul	Flowering ǀ Control Control ǀ Calendar	Strobilurin + Triazole Strobilurin	1.5
Paraná	Flowering ǀ Control Control ǀ Calendar	Strobilurin + Triazole Triazole Strobilurin + Triazole	1.7
Minas Gerais	Flowering (preventive)	Strobilurin + Triazole Strobilurin + Triazole	1.6
Bahia	Flowering (preventive) Control ǀ Calendar	Strobilurin + Triazole Strobilurin + Triazole	2.0

Table 5. Chemical fungicide group, criterion adopted by state and average number of applications summarized by the latest Antirust Consortium Londrina, 2008.

9. Efficacy of fungicides for soybean rust

Since the first fungicides recommended in emergency situations for the 2002/03 harvest (azoxystrobin, difenoconazole, fluquinconazole, epoxiconazole + pyraclostrobin and tebuconazole), a large number of new formulations have been added to the current arsenal to control rust. There are currently registered in MAPA [3, 9, 29] about 45 active ingredients (alone or in combination), trademarks, and formulations for the rational use against rust. Among fungicides, there are differences in efficacy, residual period, metabolic stability, and transportation rate, demanding from producer, researcher, and technical assistance, criteria in choosing the product to be used in each situation. Another every important point: in addition to rust, it is necessary to take into account the occurrence of other diseases such as anthracnose, late season diseases (target leaf spot, leaf blight, and powdery mildew), which may require a combination of different active ingredients.

Fungicides have greatly reduced their effectiveness when applied after the establishment of soybean rust [10, 40]. These facts hinder the implementation of a control system based on levels of disease severity. Data from this research indicate that soybean rust can only be detected by the naked eye from a severity level of 5%, which is very high and risky to start the chemical control. Results obtained by Andrade and Andrade [41], in the chemical control of rust, showed that a delay of seven days in the fungicide application (after detection of the disease), is already enough for an increase in defoliation of 82%, when compared to fungicide treatment performed when the disease appears. When the delay in the beginning of spraying was by 14 days, defoliation increased by 155%.

Juliatti et al. [42] studied the efficacy of fungicides to control Asian soybean rust and found proven efficiency of the strobilurine + triazoles mixture, even after 10% of leaf area infected with rust.

Godoy et al. [12] tested the protective, curative, and eradicative effects of azoxystrobin, carbendazim, tebuconazole, difenoconazole and epoxiconazole + pyraclostrobin fungicides in the control of Asian soybean rust in greenhouses. Except for carbendazim, all fungicides had a protective effect with control over 90%, up to 8 days after treatment. No product showed eradicative effect when applied during the incubation period of the disease; however, all treatments reduced disease severity and the viability of urediniospores. Azevedo [8] tested in greenhouse conditions, different fungicides from chemical groups of triazole and strobilurin, preventively, and curatively applied to control rust. The best results were obtained with flutriafol, azoxystrobin + cyproconazole, tebuconazole and pyraclostrobin + epoxiconazole preventively applied. When curatively applied, the best results were with tebuconazole and flutriafol.

According to Godoy [9] the use of fungicides has been intensified in soybean crops due to the resurgence of rust and lack of resistant varieties, therefore, the information on the efficiency of fungicides for the control of various diseases are increasingly required to guide their proper use in the field. The various tests for the disease control in soybeans emerged during the XXV Soybean Research Meeting of the Central Region of Brazil, held in 2003, in Uberaba-MG (Minas Gerais State from Brazil), whose objective was to provide research results that could be used throughout the country to help the technical assistance in choosing the correct fungicide for the control of different diseases that affect the culture. The tests were not intended to evaluate the timing of application and the residual of different products, but rather to compare the efficacy of products in the same situation. Trials comparing different registered products, and those in registration phase, are performed by public and private research institutions, foundations, universities and cooperatives [39]. In studies to assess the efficacy of products carried out in 2002/03 and 2003/04 harvest by the official tests network, a different behavior of fungicides was observed as for their efficacy to control Asian rust in different regions of soybean cultivation. As for fungicides recommended in XXV and XXVI Soybean Research Meeting of the Central Region of Brazil, held in Uberaba, Minas Gerais, and Ribeirão Preto, SP (São Paulo State from Brazil), respectively, the best results were obtained by the products from the chemical groups triazole and strobilurin [2, 36]. On average, there are currently two sprayings with pure fungicide (triazoles) or mixed (triazoles + strobilurins) to control the disease [9, 12, 43–45].

During the 2006/2007 harvest, experiments were conducted to evaluate the efficiency of products registered and of those under registration phase for the control of soybean rust. In function of the number of products, treatments were divided into two tests, according to the group of fungicides, including a list of triazoles and strobilurins, mixtures of triazoles with strobilurins and mixtures of triazoles with benzimidazoles in another list. The results obtained in different regions of the country have confirmed the efficacy of mixtures of triazoles with strobilurins as the most effective fungicides for the control of soybean rust [9].

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10. The risk of resistance of *Phakopsora pachyrhizi* to fungicides

Resistance is a stable and heritable change in a fungal population in response to the application of a fungicide, resulting in a reduction of sensitivity to the product [46]. With the introduction of systemic fungicides with specific mechanism of action, the problem worsened and since then, several plant pathogens of economically important crops have shown resistance to a variety of groups of fungicides. The inherent risk of resistance depends on several factors that may be associated with the product (persistence in the plant, mechanism of action, monogenic resistance, among others) and with the target (life cycle, genetic variability, mutation potential, existence of cross-resistance, adaptability or fitness, among others). These factors do not necessarily operate alone and do not apply in all cases. The agronomic risks should also be considered, i.e., crops over large areas with short rotation, monoculture, use of transgenic plants with genes expressing pesticide activity, geographic isolation of populations, and high population densities.

The resistance mechanisms may vary, but involve mainly changes in the primary site of action of the fungicide on the plant pathogen. According to FRAC International, the group of sterol biosynthesis inhibitors comprises four classes of fungicides, but only three of them, G1, G2, and G3 are used as fungicides in agriculture: DMI's, amines (formerly called morpholines), and hydroxyanils. They all act in fungi by inhibiting the sterol biosynthesis, but differ with respect to the target site. Fungicides called triazoles belong to the group of products that act by interrupting the functions of the cell membrane of fungi. They act by inhibiting the sterol biosynthesis, more specifically, ergosterol, which is an important substance for maintaining the integrity of the cell membrane of fungi [22]. Sterol biosynthesis inhibitors (SBIs) are divided into two distinct groups: C14-demethylation inhibitors (DMIs), of which main representatives are triazoles, and inhibitors of enzymes A-isomerase and A-reductase represented by morpholine fungicides. In the case of DMIs, the resistance mechanisms are not yet fully understood. Mutations in the CYP51 site have been identified in plant pathogens of cereals and related to loss of sensitivity to triazoles [47]. Regarding the likelihood of resistance emergence, in general, they are classified as with intermediate risk.

Fungicides known as QoIs (Quinone outside inhibitor) include three families of fungicides: strobilurin and two others represented by fenamidone and famoxadone. These fungicides act on the inhibition of mitochondrial respiration. The mechanism of action of strobilurins occurs through inhibition of mitochondrial respiration, which blocks the electron transfer between cytochrome b and c_1 (cytochrome 1), at the Qo (Quinone oxydase) site, interfering with the production of ATP. In most cases, resistance is conferred by a single mutation point in the cyt (cytochrome) gene, leading to a change in position 143 of the amino acid from glycine to alanine (G143A). There are species such as *Pythium aphanidermatum* (Edson) Fitzp and *Pyricularia grisea* Sacc, in which the change is from phenylalanine to leucine at position 129 F129L, also conferring resistance to QoIs, but to a lesser degree than G143A [48, 49]. The resistance of *Blumeria graminis* in wheat and barley to fungicides from the QoIs group (strobilurins, famoxadone and fenamidone) is related to mutation at a specific point, namely the replacement of glycine by alanine at position 143 of cytochrome b [50]. Several

mutations in mitochondrial cytochrome b have been reported, however, only two-G143A and F129L have occurred in field populations, and mutation G143 is the main responsibility for failures in disease control. This group, according to Brent and Hollomon [51], presents a high risk of resistance.

Worldwide, there are no reports of resistance of fungi that cause rust to triazoles and strobilurins, but populations or races with different requirements regarding the same fungicide (more or less sensitive) [52].

According to the model proposed by Brent and Hollomon [51], the risk of appearance of resistant of fungi that cause rust in general to the group of strobilurins is low, and is even lower for the group of triazoles. Due to this, the sensitivity monitoring is only recommended in cases where there are suspicions. Based on genetic information, rusts such as *Puccinia spp., Uromyces appendiculatus, P. pachyrhizi, Hemileia vastatrix* cannot acquire resistance to the group of QoIs based on mutation in the cyt b gene-position G143A due to specific genetic structure that does not allow the mutation directly after position 143. These fungi are therefore considered of low risk for the development of resistance [27].

The fungicides most widely used for the chemical management of soybean rust are strobilurins and triazoles, both with specific site inhibitors. In this case, the occurrence of mutation in the target site of the plant pathogen may lead to high levels of resistance (higher gene resistance), with consequent loss of agronomic efficiency of products [53]. The risk of emerging resistant populations of *P. pachyrhizi* to fungicides currently in use exists; however, the results obtained in monitoring the sensitivity of the pathogen populations that have been carried out by two pesticides manufacturers show that there is no resistance in *P.pachyrhizi* populations to tebuconazole, azoxystrobin, and cyproconazole [27, 52].

Since the 2005/2006 harvest, the companies Syngenta Crop Protection and Bayer CropScience have conducted surveys to monitor the sensitivity of *P. pachyrhizi* populations. The method used to quantify the fungus sensitivity and the definition of different population profiles is a bio-assay in detached soybean leaves (detached leaf test), through which the effective fungicide concentration to control 50% of the population (EC50) is determined in values expressed in parts per million (ppm) [54].

According to Singer et al. [52], for tebuconazole in the 2005/2006 harvest, the lowest EC50 value obtained was 0.016 ppm, and the highest was 0.52 ppm. These values are considered extremely low, since the dose practiced in field is 500 ppm. Moreover, the set of results showed that there were no differences in sensitivity of the pathogen among the various producing regions of the country. Therefore, it could be concluded that the predominant populations of the fungus this season proved to be very sensitive to triazole.

In the 2006/2007 harvest, the lowest EC50 value obtained for tebuconazole was 0.08 ppm, and the highest was 1.85 ppm. Only three sites showed higher values, namely 1.3, 1.74, and 1.85 ppm. The set of results showed a marked predominance of populations very sensitive to tebuconazole; however, the few higher values found can already mean the occurrence of populations with different sensitivity to the fungicide.

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In the last harvest (2007/2008), which represents the third year of monitoring, the lowest EC50 value obtained for tebuconazole was 0.04 ppm, i.e., similar to values of the last 2 years. Values between 1.04 and 1.8 ppm were also observed, which represented the highest for the previous crop and now are considered as average values.

According to Singer et al. [52] and based on existing information, in monitoring systems and also considering the baseline values for other fungi such as the pathogen that causes wheat leaf rust (*Puccinia triticina*), it could be concluded that from the results obtained in the last three harvests, the standard values for populations more or less sensitive to *P. pachyrhizi* have already been established. In this context, values between 0.01 and 10 ppm could be acceptable, and values between 0.01 and 1.0 ppm refer to very sensitive populations, values from 1.0 to 2.0 ppm characterize populations of intermediate sensitivity, and from 2.0 to 10 ppm, represent the less sensitive.

According to Buzzerio [27], following the recommendations of the FRAC International and also FRAC Brazil, the company Syngenta Crop Protection monitors *P.pachyrhizi* populations for active ingredients azoxystrobin and cyproconazole using *in vitro* and *in vivo* sensitivity bioassay methods. According to results obtained in the 2005–06 harvest for active ingredient azoxystrobin (*in vitro* method) the estimated lethal concentration (CL) 90 was between 0.0103 and 0.4945 ppm. In the 2006–07 harvest, the estimates were between 0.0861 and 0.5065 ppm for the same product. For active ingredient cyproconazole (*in vivo* method) the estimated CL 90 was between 0.0934 and 0.5007 ppm. According to results obtained and taking into account that the recommended dose of azoxystrobin is 300 ppm and cyproconazole is 120 ppm, when used in combination, it could be concluded that for both active ingredients, there is no change in sensitivity of the fungus that causes rust. The variation between estimates can be considered within the natural range of populations.

Soybean rust arrived in Brazil in 2001, and severe epidemics outbreaks resulted in heavy applications of triazoles alone. This led to warnings by concerned chemical companies and scientists of the risks of soybean rust fungicide resistance developing against the triazoles. Sure enough, in the first quarter of 2008, a lower than expected efficacy of triazoles was observed in Mato Grosso and Mato Grosso do Sul states. However, no sensitivity change was detected in the triazole-strobilurin mixtures which continued to perform very well.

According to Godoy et al. [19], this efficiency reduction of triazoles is mainly due to overexposure of soybean rust to triazole fungicides used by themselves. Tebuconazole is cheap in Brazil and is sold in competition with a number of generic brands. Many farmers in the Mato Grosso used up to four applications of tebuconazole per season, despite the FRAC recommendation of only two applications per season. Although tebuconazole is a recommended fungicide, its single site mode of action makes it vulnerable to resistance. The use of a strobilurin-triazole mixture is a major strategy to manage resistance, promoted by the agrochemical industry, for reducing risk of resistance towards both fungicide groups. These two active ingredients are complimentary in their action because strobilurins inhibit fungal respiration and consequently inhibit spore germination, whereas triazoles inhibit germ tube elongation, fungal penetration, and mycelial growth.

11. Antiresistance strategies for fungicides in soybean

Just like its use in the field, the antiresistance strategies for fungicides should always be applied in a preventive way [16]. The most safe and ideal situation would be the use of an antiresistance strategy before the occurrence of the problem, because once the pathogen population has become resistant, the only control possibility would be the application of another fungicide with a different mechanism of action, or a nonchemical control method. This keeps happening most of the times in the field. It is the fungicide syndrome. This no longer works; let us switch to another. The resistance problem has become so serious that the agrochemical companies have considered the problem from the screening of new molecules, with the information on the risk of the group to which the product belongs as criterion [55]. The way it is launched to market, including registration and usage recommendations, and monitoring of the product are also designed following antiresistance strategies and have greatly contributed to the decrease of the resistance problems in Brazil.

The availability of a large number of commercial products for the control of soybean rust does not necessarily mean the existence of several chemical groups. The main fungicides registered are restricted to only two groups of active ingredients: strobilurins and triazoles. This fact is reason of concern due to the possibility of the fungus to develop mutants resistant to these chemical groups [16, 29, 38, 53]. The chemical control strategies are based almost exclusively on the use of these products. Therefore, the vulnerability exists and the antiresistance strategies should be increasingly implemented.

Management strategies: use of fungicides in mixtures rather than products used alone, restriction on the number of treatments applied per harvest, use of the dose recommended by the manufacturer, use of integrated disease management, avoid eradicative use, and increased chemical diversity through the use of other fungicides in subsequent treatments should be implemented to minimize or even avoid the problems of fungicide resistance.

The development or evolution of resistance can be minimized through antiresistance strategies [21]. The following antiresistance strategies are mentioned: minimize the use of fungicides and particularly repeated applications of fungicides from the same chemical group; restrict the number of fungicide applications per season and chemical group, and apply only whenever needed, implement the use of rotation of fungicides from different chemical groups or the use of ready formulations or tank mixtures always following the manufacturer's recommendations, always use fungicides at doses recommended by the manufacturer, use of integrated disease management such as to eliminate the primary sources of inoculum, use of resistant varieties, crop rotation, sanitation of tools, etc., and baseline studies and sensitivity monitoring. Monitoring methods have been described in various publications [56–58]. In an attempt to standardize the testing internationally, FAO and FRAC [40, 59] show in details the recommended methods for the major groups of fungicides. In Brazil, almost all companies that produce and market fungicides make the monitoring of fungal populations, while introducing some new molecule or existing products.

These strategies are general. However, in the case of soybean rust, treatment programs that address the rotation of active ingredients is a basic foundation for the sustainability

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of the system and an official recommendation from the FRAC. Furthermore, it should be complemented by other measures such as preventive control of diseases, use of the correct dosage specified by the manufacturer, to follow the sanitary standards, and adoption of good agricultural practices. Recently in Brazil we are using multisite fungicides to soybean rust control using copper compounds and dithiocarbamates, ex. Mancozeb to control in vegetative stages mixed triazoles and strobilurins because after 2013 the fungi resistance increased in Brazil's fields after the massive use of triazoles and strobilurins and curative uses.

12. Conclusions

Plant diseases reduce production and decrease the quality of agricultural products, requiring more and more practical and effective strategic chemical programs of phytosanitary control and treatment. When these treatments are not applied correctly, the agricultural production may suffer losses and damages ranging from negligible percentages up to a total loss, depending on the virulence potential of the pathogens involved, environment, and crop resistance against them. The influence of humans should also be stressed, which has been great in the technological management of cultures that occupy large areas and require the observation of details in choosing the adequate spraying program of products.

In cases of diseases that occur as epidemics caused by exotic or resurgent pathogens as is the case of *P. pachyrhizi*, the first control alternative always used has been the use of fungicides, especially those with systemic and curative activity. At first glance, chemical control has provided satisfactory results; however, there is need for its integration with other control methods. In the case of epidemics, the alternative is the resistant cultivars adoption or at least those with some level of tolerance. Another important point is the registration of products from different mode of action from those available in the market. This is an urgent need, given that the *P. pachyrhizi* populations are changing the sensitivity to key triazole used in their control. Finally, the importance of developing new technologies for application of fungicides or the refinement of existing techniques for the application of products in large areas of cultivation should also be stressed. Undoubtedly, the need to control soybean rust in curative situations in large areas of cultivation and in adverse climate conditions triggered a series of studies and research studies in the sector, so far unprecedented in the current agricultural environment. Many field results have proven the effectiveness of terrestrial and air applications, techniques such as LOV (low oil volume); however, further advances are still needed in this field of study, with the development of field techniques that allow the placement of the product at the bottom of the soybean crop. The higher risk of developing resistance or loss of sensitivity of the fungus in Brazil to carboxamides group of fungicides associated with strobilurin would be no use of multiple site fungicides in the field's crops. In these conditions the evolution of resistance or sensitivity loss will be fast.

Studies on soybean rust in Brazil are still very recent and insufficient There is still lack of information on fungus biology, epidemiology, variability, and host conditions on weeds

that remain between seasons together with soybean, in addition to the offer of some cultivars with high resistance potential, and even the correlation of varieties with different responses regarding the efficacy of fungicides, data sowing, and application times. **Figure 2** shows the main points of action of fungicides in the life cycle of the Asian soybean rust (ASR) (black arrow). The definition of data sowing per state and sanitary rules they are the most important strategy of ASR in Brazil joint combination of systemic and multisite fungicides uses and genotypes with partial resistance. The future shows the combination of this kind of strategy after the carboxamides fungicides (mutation in ASR) resistance discovered by FRACC in 2017.

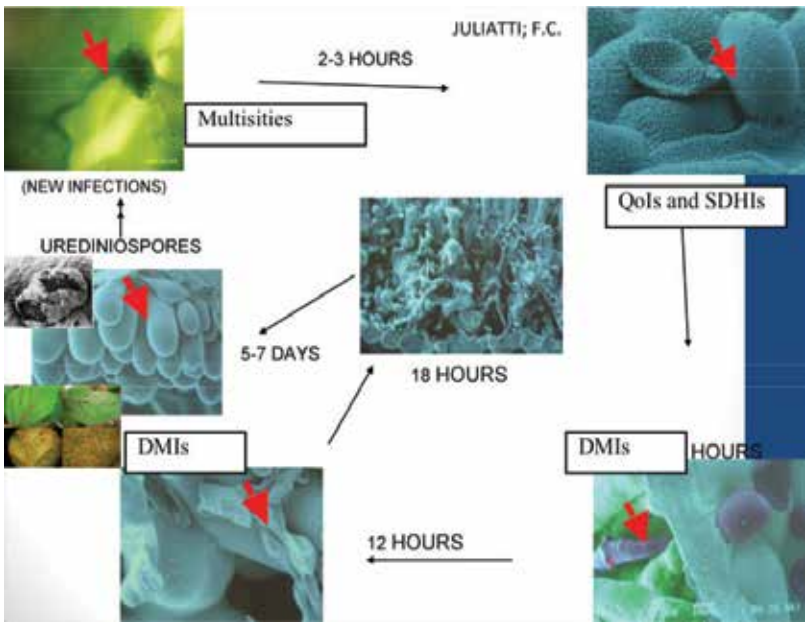

Figure 2. Asian soybean rust (*Phakopsora pachyrhizi*) lifecycle and main fungicide chemical groups used to control rust action.

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http://dx.doi.org/10.5772/67454

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Chapter 4

Inoculation with *Bradyrhizobium* sp. and *Azospirillum brasilense* Associated with Application of Cobalt and Molybdenum on Nutrition and Soybean Yield

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66572

Abstract

Considering the main current limitations and potential of biological fixation of N_2 (BNF) in soybean crop and benefits attributed to various crops by inoculation with *Azospirillum brasilense* (diazotrophic bacteria with free life), with emphasis on larger development of the root system and consequently greater absorption of water and nutrients, we can infer that co-inoculation with both microorganisms of *Bradyrhizobium* sp. and *A. brasilense* can improve the crop performance in an approach that meets the current demands of agricultural, economic, and environmental sustainability. Thus, important researches are needed to evaluate the nutritional status, production components, and the soybean yield affected by cobalt and molybdenum application mode and co-inoculating seeds with bradyrhizobia and *A. brasilense*. We found that seed inoculated with *A. brasilense* and application of cobalt and molybdenum provided higher N concentration in leaf and mass of 100 grains, with a positive impact on the grain yield of soybean, with an increase of 1007 kg ha^{-1} of grain, equivalent to 18.4% more than the control (only inoculated with rhizobia). This research demonstrated that co-inoculation with *Bradyrhizobium* sp. and *A. brasilense* associated with the application of cobalt and molybdenum is beneficial for nutrition and soybean yields.

Keywords: co-inoculation, diazotrophic bacteria, mineral fertilization, nutritional status, foliar diagnosis

1. Introduction

The soybean is the major source of vegetable protein, an essential component in the production of animal feed, in addition to increasing use for human consumption [1].

This explains why the soybean plant is very demanding on nitrogen (N). It is estimated that 80 kg of N is needed to produce 1000 kg of soybean grains. Therefore, to obtain high yields, the biological fixation of N_2 (BNF) should be as efficient as possible [2–7].

The process of BNF in Brazil is responsible for nitrogen accumulated by plants; it represents about 200 kg ha^{-1} N [8], which is no longer applied via mineral fertilizers. It reduces the cost of production [9].

In addition, the use of selected and efficient bradyrhizobia inoculant and cobalt (Co) and molybdenum (Mo) nutrition contributes decisively in the BNF [10]. Cobalt and molybdenum are essential for BNF [11]. The first B12 vitamin is essential for the processing of BNF and other parts of the molybdoenzymes, used in absorption and metabolism of nitrogen [12]. The application of Mo and especially Mo + Co increases BNF [13].

In Brazil, soybean generally responds positively to fertilization with Mo in soils of low fertility and in fertile soils depleted of Mo due to long-term cropping. The micronutrient can be supplied by seed treatment. However, the toxicity of Mo sources to *Bradyrhizobium* strains applied to seed as inoculant has been observed resulting in bacterial death and reductions in nodulation, N_2 fixation, and grain yield [14].

Considering the main current limitations and potential of BNF in soybean crop and benefits attributed to various crops by inoculation with *Azospirillum brasilense* (diazotrophic bacteria with free life), with emphasis on larger development of the root system and consequently greater absorption of water and nutrients, we can infer that co-inoculation with both the microorganisms can improve the crop performance in an approach that meets the current demands of agricultural, economic, and environmental sustainability [15].

Bacteria promoters of plant growth (BPPG) correspond to a group of beneficial microorganisms to plants due to the ability to colonize the surface of roots, rhizosphere, phyllosphere, and internal plant tissues [16, 17]. The BPPG can stimulate plant growth in several ways. The most relevant are BNF capacity [18], increase in nitrate reductase activity when the BPPG grows endophytically plants [19], production of hormones such as auxins, cytokinins, gibberellins, and ethylene, and a variety of other molecules [20], phosphate solubilization [21], and act as biological control agent of pathogens [22]. In general, it is believed that the benefit of BPPG to plant growth is caused by a combination of all these mechanisms [23].

A. brasilense can act in relations between rhizobia and legumes, promoting increases in plant growth, grain yield, and total nitrogen biologically fixed as well as improvements in nitrogen use by plant through symbiosis with rhizobia [24].

Based on the above information and the lack of research about the interaction between co-inoculation with *Bradyrhizobium* sp. and *A. brasilense* associated with the application of cobalt

http://dx.doi.org/10.5772/66572

and molybdenum in soybean crop, researches to evaluate the nutritional status, production components, and the soybean yield affected by cobalt and molybdenum application mode and co-inoculating seeds with bradyrhizobia and *A. brasilense* are important.

2. Materials and methods

The experiment was conducted in the 2014/2015 season in an experimental area that belongs to the UNESP Engineering Faculty located in Selvíria, MS/Brazil, with the following geographical coordinates, 20°22′S and 51°22′W and an altitude of 335 m. The experimental area soil was classified as Distroferric Red Oxisol with clay texture (the granulometric analysis indicated values of particle size of 420, 50 kg^{-1}, and 530 g of sand, silt, and clay, respectively), according to Embrapa (2013) [25], which has been cultivated with annual cultures over 27 years, with the last 10 years in the direct tillage system. Before soybean sowing, corn was cultivated in the area. The annual average temperature was 23.5°C, the annual average pluvial precipitation was 1370 mm, and the annual average relative air humidity was between 70% and 80%.

The experimental design was carried out in a randomized blocks with six treatments and four replications. The treatments were as follows: (1) control (without soybean inoculation with *A. brasilense* and without application of cobalt and molybdenum); (2) cobalt and molybdenum application on the seed with commercial product (15% Mo (195 g L^{-1}) and 1.5% Co (19.5 g L^{-1})) at a dose of 150 ml ha^{-1}, based on Sfredo et al. (2010) [10] recommendation; (3) seed inoculated with *A. brasilense* at a dose of 200 ml ha^{-1} (strains Abv5 Abv6 with guaranteed 2×10^8 colonies forming units (CFU) per ml) and application of cobalt and molybdenum in the abovementioned dose; (4) leaf application of *A. brasilense* in the V3 stage of soybeans, in the abovementioned dose; (5) leaf application of cobalt and molybdenum in the V3 stage in the aforementioned dose; and (6) leaf application of cobalt and molybdenum along with foliar inoculation with *A. brasilense* in V3 stage in the aforementioned doses.

In all treatments, the inoculation with Rhizobium was performed in seeds at a dose of 200 ml ha^{-1} (strains: SEMIA 5019 (*B. elkanii*) and SEMIA 5079 (*B. japonicum*) with 5×10^9 guarantee of viable cells per ml). Each plot consisted of seven lines of 5-m soybean, spaced by 0.45 m, totaling 15.75 m^2. Useful area of the plot was considered to be the three central lines, discounting the simple surround, totalling effective sampling area of 6.75 m^2 per plot.

Chemical properties of the soil in the tillable layer were determined before 2014, before the soybean experiment began. The methods proposed by Raij et al. [26] provided the following results: 10 mg dm^{-3} of P (resin), 5 mg dm^{-3} of $S\text{-}SO_4$, 22 g dm^{-3} of organic matter (OM), $pH(CaCl_2)$ of 5.3, 2.4 $mmol_c$ dm^{-3} of K^+, 21.0 $mmol_c$ dm^{-3} of Ca^{2+}, 18.0 $mmol_c$ dm^{-3} of Mg^{2+}, 28.0 $mmol_c$ dm^{-3} of H+Al, 3.2 mg dm^{-3} of Cu, 22.0 mg dm^{-3} of Fe, 24.2 mg dm^{-3} of Mn, 1.2 mg dm^{-3} of Zn (diethylenetriaminepentaacetic acid (DTPA)), 0.16 mg dm^{-3} of B (hot water), and 60% base saturation. Based on soil analysis and soybean crop fertilization recommendation [27], the fertilization was done in the seed furrows with 96 kg P_2O_5 ha^{-1} (in the form of triple superphosphate) and 70 kg ha^{-1} K_2O (in the form of potassium chloride).

The seeds were treated with the fungicide Thiram + Carbendazim at a dosage of 30 + 70 g active ingredient (a.i.) per 100 kg seed, respectively, after drying the seeds, and were inoculated with Rhizobium, and depending on the treatment the seed was inoculated with *A. brasilense* just before soybean planting in the shade. We used the soybean cultivar BMX Power RR, with a spacing of 0.45 m between lines, with 17 seeds per meter.

The experiments were conducted in a no-tillage system. The area was irrigated by a central pivot sprinkler system when necessary. The water coverage was 14 mm over a period of around 72 h. The control of weeds, pests, and diseases prevention was carried out when necessary in soybean crop. The plants were harvested 120 days after soybean emergence.

Concentrations of N, P, K, Ca, Mg, S, Cu, Fe, Mn, and Zn were measured in soybean plant leaves. The third upper trifoliate leaves (30 plants) in the flowering soybean plants (R2 stage) were collected according to the methodology described in Ambrosano et al. [27]. The determination of nutrients was carried out as described by Malavolta [28]. The leaf chlorophyll index (LCI) was determined indirectly after application of the treatments and when the plants were in the flowering (R2 stage), in 10 plants per plot through readings in the third upper trifoliate leaves, using a digital chlorophyll CFL 1030 Falker (Falker Agricultural Automation, Porto Alegre, Brazil).

The leaf area of 10 leaves per plot was measured using the software ImageJ 1:45 (2011), according to the methodology described by Bauermann [29]. At the time of harvest, 10 soybean plants representing were collected for counting the number of grains per pod, grains per plant, and mass of 100 grains. The mass was determined on a precision scale of 0.01 g and corrected for 13% moisture (wet basis). The soybean was harvested from the plants in the useful area of each plot and grain yield was calculated after mechanical threshing. Data were transformed into kg ha^{-1} and corrected for 13% moisture (wet basis). The results of all the evaluations were subjected to analysis of variance and the Tukey test at 5% probability to compare the averages of treatments, using the Sisvar program.

3. Results and discussion

The seed inoculated with *A. brasilense* and the application of cobalt and molybdenum provided higher N concentration in the leaf, significantly differing from the control only inoculated with Rhizobium (**Table 1**), indicating that the biological nitrogen fixation was potentiated by these treatments. On average, the leaf N concentrations, which are considered suitable, were shown to be 40–54 g kg^{-1} of dry matter (D.M.), according to Ambrosano et al. [27]. However, the co-inoculation with *A. brasilense* associated with cobalt and molybdenum led to the higher leaf N concentration than the concentrations considered suitable, regardless of modes of application and inoculation.

Increases in total nitrogen biologically fixed by plant through symbiosis with rhizobia, associated with *A. brasilense,* have also been reported in other researches [24, 15]. However, Zuffo et al. [30] observed that the use of *A. brasilen*se alone or in co-inoculation with *B. japonicum* does not have significant effect on leaf N concentration.

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The treatments in this research provided similar leaf concentrations of P, K, Ca, and S (**Table 1**). However, there was a higher concentration of Mg in the leaves when Co and Mo and *A. brasilense* were applied in the leaves, although leaf Mg concentration did not differ significantly between most other treatments. It is worth noting that the foliar concentrations of P, K, Ca, Mg, and S (**Table 1**) were within the ranges of 2.5–5.0, 17–25, 4–20, 3.0–10.0, and 2.1–4.0 g kg^{-1} D.M., respectively, which were recommended by Ambrosano et al. [27] as the ideal.

The leaf chlorophyll index (LCI) and leaf Fe and Cu concentrations were not affected by treatments (**Table 2**). This can be explained by adequate leaf N concentrations obtained for soybean crop. Zuffo et al. [30] also observed that the use of *A. brasilense* alone or in co-inoculation with *B. japonicum* does not have significant effect on LCI.

The results are different from those found by other authors using corn plants, who found that the LCI was higher in the treatments with diazotrophs than in the treatments without inoculation. Corn plants that were inoculated with *A. brasilense* had greater LCI than those that were not inoculated, in two crop seasons [31]. Kappes et al. [32] and Quadros et al. [33] found that plants inoculated with *A. brasilense* had improved LCI. These divergent results can be explained by the fact that *A. brasilense* increases reductase activity of nitrate when they grow endophytically plants [19], a fact of minor importance for soybean plant that spends more metabolic energy (ATP) to biologically fix N_2 in the root nodules in relation to nitrate reduction assimilation.

Leaf application of Co and Mo and foliar inoculation with *A. brasilense* provided the largest concentration of Mn in the leaves (**Table 2**), while only the foliar inoculation with *A. brasilense* stood out with the highest Zn concentration in leaf. However, averages of leaf concentrations of Cu, Fe, Mn, and Zn were also suitable as described by Ambrosano et al. [27], being 10–30, 50–350, 20–100, and 20–50 mg kg^{-1} D.M., respectively.

Treatments	N	P	K	Ca	Mg	S
	---------- g kg^{-1} ----------					
Control	48.65 b	4.01 a	19.12 a	9.70 a	5.67 ab	3.06 a
Co, Mo seed	50.91 ab	3.75 a	19.98 a	8.94 a	4.55 b	3.08 a
Co, Mo + Azos seed	56.21 a	4.16 a	20.42 a	8.64 a	4.73 ab	3.16 a
Azos foliar	49.00 b	4.75 a	19.92 a	9.51 a	5.63 ab	3.36 a
Co, Mo foliar	55.95 a	4.01 a	18.38 a	8.74 a	5.32 ab	3.35 a
Co, Mo + Azos leaf	54.30 ab	4.28 a	18.70 a	8.99 a	5.83 a	3.64 a
Overall average	52.50	4.16	19.42	9.09	5.29	3.28
CV (%)	4.34	9.42	5.42	8.75	7.77	12.73
LSD (5%)	6.46	1.11	2.99	2.26	1.17	1.18

Means followed by the same letter in the column do not differ by the Tukey test at 5%. CV: coefficient of variation; LSD: least significant difference.

Table 1. Leaf concentrations of Cu, Fe, Mn, and Zn of soybean affected by cobalt and molybdenum application mode and *Azospirillum brasilense* inoculation mode.

Treatments	LCI	Cu	Fe	Mn	Zn
	---------- mg kg^{-1} ----------				
Control	43.79 a	9.00 a	156.33 a	90.00 ab	47.67 b
Co, Mo seed	44.12 a	8.33 a	163.33 a	71.00 b	50.00 ab
Co, Mo + Azos seed	44.52 a	8.67 a	189.67 a	70.33 b	47.67 b
Azos foliar	43.56 a	10.33 a	212.33 a	85.00 ab	53.33 a
Co, Mo foliar	44.23 a	9.33 a	191.00 a	84.67 ab	46.00 b
Co, Mo + Azos leaf	44.15 a	10.67 a	184.00 a	97.67 a	50.00 ab
Overall average	44.06	9.39	182.78	83.11	49.11
CV (%)	3.11	9.46	27.89	11.06	3.89
LSD (5%)	1.55	2.52	144.60	26.08	5.42

Means followed by the same letter in the column do not differ by the Tukey test at 5%. CV: coefficient of variation; LSD: least significant difference.

Table 2. Leaf chlorophyll index (LCI) and leaf concentrations of Cu, Fe, Mn, and Zn of soybean affected by cobalt and molybdenum application mode and *Azospirillum brasilense* inoculation mode.

The leaf area of soybean was greater in treatment with the application of *A. brasilense*, differing significantly from the application of Co and Mo in the seed (**Table 3**). This bacterium can influence the plant growth by producing auxins, gibberellins, and cytokinins, which provide improved root growth [34] and consequently greater absorption of water and nutrients [22], resulting in more vigorous and productive plant [35, 36].

The control treatment provided greater number of grains per pod, and the number of grains per pod did not differ between treatment with Co and Mo of the leaf and treatment of inoculation of the seed with *A. brasilense* and seed application of Co and Mo. The number of grains per plant showed no difference between treatments. These explain why the smaller mass of 100 grains was obtained for control and leaf application of Co and Mo, in other words, there was less filling grain due to the higher number of seeds per pod.

Seed inoculated with *A. brasilense* and seed application of Co and Mo provided higher mass of 100 grains and grains yield of soybean, with an increase of 1007 kg ha^{-1} of grain, equivalent to 18.4% more than the control (only inoculated with rhizobia), corroborating with Hungria et al. [15] showing that co-inoculation with *A. brasilense* increased yield of soybeans in 16.1% compared to isolated use of *Bradyrhizobium* strains.

These results may be due to several mechanisms, which are the anticipation in the BNF of the nodes, an increase in the dry weight of nodes, promoting the occurrence of nodulation heterologous through the increased formation of hair root and secondary roots, an increase in infection sites, inhibition of plant pathogens and production of phytohormones and influences in the partition of dry matter between the roots and shoots [24]. Yet, pondering Hungria et al. [15], these results caused by co-inoculation bacteria promoters of plant growth and Rhizobia appear to be

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Treatments	Leaf area (cm^2)	Grains per pod	Grains per plant	Mass of 100 grains (g)	Grains yield ($kg\ ha^{-1}$)
Control	64.05 ab	3.00 a	176.30 a	14.68 b	5550 b
Co, Mo seed	62.90 b	2.55 b	145.20 a	15.88 ab	6083 ab
Co, Mo + Azos seed	68.35 ab	2.78 ab	155.67 a	16.10 a	6557 a
Azos foliar	77.80 a	2.65 b	156.90 a	14.80 ab	5355 b
Co, Mo foliar	69.75 ab	2.80 ab	185.07 a	14.60 b	5685 ab
Co, Mo + Azos leaf	67.50 ab	2.65 b	138.47 a	14.88 ab	5602 ab
Overall average	68.39	2.74	159.60	15.15	5805
CV (%)	4.89	4.87	14.61	3.76	7.27
LSD (5%)	14.26	0.31	66.14	1.31	970

Means followed by the same letter in the column do not differ by the Tukey test at 5%. CV: coefficient of variation; LSD: least significant difference.

Table 3. Leaf area, grain per pod, grains per plant, mass of 100 grains, and grains yield of soybean affected by cobalt and molybdenum application mode and *Azospirillum brasilense* inoculation mode.

under the influence of specific signals among bacterial genotypes involved and the genotype of the host plant. It is important to do more related studies on the response of the co-inoculation depending on the genotypes, aiming at the development of more responsive genotypes.

In an important research by Campos et al. [13], they concluded that there are no Mo and Co effects on nodulation in soil with established *Bradyrhizobium* population, soil application of Mo and Co does not supply the Mo and Co necessary to the plant and to the BNF, application of Mo and especially that of Mo and Co increase BNF, as in the present study, application of Mo and Co on leaves has the same effect on BNF as seed applications, as in the present study, and seeds with high concentration of Mo show higher BNF than those with low Mo contents, as in the present study. Also, Hungria et al. [37] reported increases in grains yield of soybean (20%) by application of Mo and Co associated with *Bradyrhizobium* compared to treatment inoculated with *Bradyrhizobium*.

4. Final consideration

Leaf application of Co and Mo and foliar inoculation with *A. brasilense* provided the largest concentration of Mg and Mn in the leaves, while only the foliar inoculation with *A. brasilense* stood out with the highest Zn concentration in leaf and leaf area.

Seed inoculated with *A. brasilense* and seed application of Co and Mo provided higher N concentration in leaf and mass of 100 grains, with a positive impact on the grain yield of soybean, with an increase of 1007 $kg\ ha^{-1}$ of grain, equivalent to 18.4% more than the control (only inoculated with rhizobia).

This research demonstrated that co-inoculation with *Bradyrhizobium* sp. and *A. brasilense* associated with the application of cobalt and molybdenum is beneficial for nutrition and soybean yields. Therefore, as inoculation with *A. brasilense* is a low-cost technique, easy to apply and use, non-polluting, and the technique falling within the desired sustainable context at present, the trend is that this technology be increasingly used in soybean crop.

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Chapter 5

Challenges of In Vitro and In Vivo Agrobacterium-Mediated Genetic Transformation in Soybean

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66708

Abstract

Agrobacterium tumefaciens-mediated genetic transformation of plants is a natural process. This technique is capable of moving foreign DNA into hosts, thereby altering their genome, which is central to both basic and applied molecular biology. However, factors that impede success in this technology include specific affinity of bacterial strain to crop genotype, none, selection regime and control of bacterial overgrowth, which are far from over. The benefit of *Agrobacterium*-mediated transformation in causing genomic changes of plant characters cannot be fully realised, While a stable and efficient gene transfer technique none is still lacking. Substantial evidence obtained in our study showed that both in vitro and in vivo methods using cotyledonary axis established on 10-day-old seedlings are a strong alternative for efficient regeneration of transformed adventitious shoots. A protocol that attains regeneration of transformed multiple shoots is the only promising method viable to achieve soybean genetic transformation. High shoot regeneration of 60.0%, 63.3% and 76.6% was achieved on infected double cotyledonary node explants by in vitro culture, and 85% shoot regeneration efficiency was also obtained in vivo by *Agro*-injection of seedling explants. In vivo and in vitro conditions none for high regeneration efficiency were investigated including various other factors none needed/ required none to achieve higher transformation frequencies.

Keywords: soybean, *Agrobacterium tumefaciens*, in vitro, in vivo, double coty-nodes, single coty-nodes

1. Introduction

Soybean (*Glycine max* L. Merrill.) is one of the world's most important agronomic crops used in the production of edible oil and high protein formulations for health and nutritional

benefits. The soybean has components that have the potential to prevent diseases, such as prostate cancer, heart disease and osteoporosis [15, 58]. However, the growth and productivity of this crop are adversely affected by various, abiotic and biotic, stress factors, such as drought, high temperatures, pests and pathogens. Conventional breeding has been used to produce cultivars that can tolerate these factors. Nevertheless, conventional breeding has limitations due to the narrow gene pool of the crop. The narrow gene pool is a result of over 3000 years of cultivation. Modern breeding techniques like genetic transformation are nowadays employed to bring some improvement into the crop. This process allows for the transfer of genes across non-related organisms, which is an advantage over conventional breeding. Other disadvantages associated with conventional breeding such as low heritability of high yield genes, long breeding periods and long wait periods to release new cultivars has led to the pursuit of genetic transformation as an alternative breeding tool. Breeders acknowledge the ability of genetic transformation to circumvent the shortcomings of sexual reproduction such as the inability to regenerate fertile plants from sterile and vegetatively propagated crops [54]. The most commonly applied methods for plant transformation include (1) *Agrobacterium tumefaciens*-mediated transformation, (2) microprojectile bombardment-mediated transformation, (3) electro and chemical cell surface poration and (4) direct protoplast-mediated DNA transfer [1, 11, 19]. However, genetic transformation still has its own limitations such as genotype specificity, low transformation frequencies and the lack of a routinely used protocol for improvement of recalcitrant crops such as soybean [34, 53].

2. Genetic transformation in soybean

The soybean has become one of the widely cultivated and most valuable oil crops in all parts of the world. The World Health Organisation (WHO) [65] estimated in 2005 that over 20% of the world's population primarily rely on soybean as a raw and processed food source. Gandhi [22] and Lee et al. [32] outlined the domestication of soybean as feed, forage, fibre, oil and protein use in addition to the proprietary production of this crop. This clearly indicates the growing importance of soybean in many countries for subsistence/commercial farming and industrial purposes. The increasing use of soybean for various industries creates a demand for the development and use of new genetically transformed, stress resistant soybean cultivars with improved growth and yield characteristics.

Genetic transformation in soybean started in the late 1980s [13, 23]. The former author used particle bombardment (biolistic) method and the latter authors used *Agrobacterium*-mediated method. *Agrobacterium*-mediated genetic transformation is a technique already used for the development of soybean cultivars tolerant to agrochemicals, pathogens and pests. An example is a Roundup Ready genetically modified (GM) soybean that currently dominates the market, accounting for 83%, 94% and 100% of production in the United States (US), Brazil and Argentina, respectively [7]. This herbicide tolerant Roundup Ready GM soybean contributes more than 60% to the total soybean production, estimated to reach 533 million tons for 2016/2017 as compared to 251.5 million tons in 2011 [64]. Soybean cultivars that meet farmer's needs to circumvent production losses and reduced amount of agrochemicals

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application without generating health, economic and ecological toxicity, and those that cope well under water deficit still need to be developed. In general, genetic transformation technology requires meristematic cells that will take in the introduced DNA segment, a means of the delivery of the DNA segments and a means of selecting transformed cells [56]. Although it is close to three decades since the pioneering works on the genetic transformation of the soybean mentioned above, transformation frequency in the soybean is still low. This led to the genetic transformation in soybean to be regarded as recalcitrant [24, 33].

3. Factors affecting in vitro-based genetic transformation in soybean

Recalcitrance to genetic transformation in soybean is said to be due to (i) the low infection rates of *A. tumefaciens* into the plant cells and (ii) the low rates of regeneration of plants from infected tissues [16, 20, 49]. In addition, genetic transformation in soybean is genotype specific. That is, the success achieved with one cultivar does not guarantee success in other cultivars. The infection rates of plant cells by the *Agrobacterium* depend on the strains of the plasmid and *Agrobacterium* used. On the other hand, the regeneration rates depend on the embryogenic tissue used—its totipotency and health. The health of the tissues is affected by the presence of reactive oxygen species which cause oxidative stress of the explants. Other factors include tissue culture conditions and media used. These factors have been the subject of research since the start of genetic transformation in soybean. According to Paz et al. [48], things that need to be carried out in order to improve soybean transformation efficiency are as follows: (i) optimisation of the selection system; (ii) the enhancement of explant-pathogen interaction and (iii) the improvement of culture conditions to promote the regeneration and recovery of transformed plants.

3.1. Agrobacterium and vectors

Agrobacterium-mediated transformation takes advantage of the natural ability of the *Agrobacterium* to transfer its T-DNA into host plant cells. The commonly used bacterial strain is EHA 101. The vector used is a binary vector pTF101.1 transformed with (i) the *bar* gene for herbicide phosphinothricin (PPT) resistance, (ii) a broad host origin of replication, (iii) spectinomycin resistance gene (aaAda), (iv) double 35S promoter of the cauliflower mosaic virus (CaMV) and (v) construct ST 19 and ST 22 (where ST stands for sequence type) derived by inserting a gene of interest in the multiple cloning site of the pTF 101 vector [38]. Paz et al. [48] found that glufosinate is a better selective agent leading to the recovery of more transformed plants than Bialaphos.

3.2. The choice of explant

3.2.1. Single cotyledonary nodes

Successful genetic transformation depends on the totipotency of the explant. This is because transformed plants should be regenerated from individual cells. The most com-

monly used explant in the genetic transformation of the soybean is the (coty) node explant developed from seedlings [43, 47]. This takes advantage of the meristematic tissue found at the axil of the cotyledon and epicotyl. At the axil, the axillary together with associated auxiliary buds can also be initiated. The axillary shoot, however, should be immediately cut-off after development. This is performed because the axillary bud is already developed when shoot regeneration is initiated. Removal of the axillary shoot promotes development of the auxiliary buds in the same way as cutting-off of the apical bud removing apical dominance. The initiated auxiliary buds stand a better chance of transformation than the axillary bud.

3.2.2. Double cotyledonary nodes

Double coty-node explants can be prepared by excising out the epicotyls at the cotyledonary junction and cutting-off the hypocotyls 4–5 mm beneath the cotyledons. They are prepared by not splitting evenly the cotyledons and still contain meristematic tissues as for the single cotyledonary nodes. Soybean cotyledonary nodes obtained from matured 10-day-old seedlings developed on Murashige and Skoog [44] culture medium supplemented with 2.0 mg/l 6-benzylaminopurine (BA) showed high shoot multiplication [39]. Shoot regeneration can be improved by the development of explant source in soybean transformation from BA pretreated seeds. However, the advantage of using double coty-node or single coty-node explants is the efficient proliferation of higher shoot numbers [39].

3.3. In vitro culture of soybean

Regeneration of transformed soybean plants through tissue culture consists of the following steps: (i) preparation of plant tissue culture medium, (ii) sterilisation and preparation of explants, (iii) infection and co-culture of explants with *Agrobacterium*, (iv) shoot induction, (v) elongation, (vi) rooting and (vii) acclimatisation of rooted plantlets (**Figure 1**) [38].

Plant tissue culture medium: Important step in in vitro plant transformation is to select culture medium suitable for soybean culture. Murashige and Skoog basal medium with various types and concentrations of plant growth regulators (PGR) was reported to be effective for transformed shoot regeneration in soybean [48, 62]. Gamborg's B5 medium [21] is highly recommended as well for the re-initiation of bacterial culture to be used for transformation. In our study, MS and B5 basal culture media were used to initiate soybean cotyledonary node and bacterial cultures [38]. More descriptions of the different types of in vitro culture media which could be applied can also be found in the practical manual by Pierik [51]. The type of plant tissue culture medium selected for plant transformation also depends upon the species to be cultured. Different species have different requirements for both mineral salts and plant growth regulators. Comparison of the culture medium composition of several most commonly used plant tissue culture media can be found for shoot and callus initiation [61] including the use of sulphur-containing compounds such as L-cysteine that increase *Agrobacterium* transfer and expression. In a similar experiment, Paz et al. [48] investigated the effects of dithiothreitol and cysteine (sulphur-containing compounds also called thiols) on the susceptibility of soybean cultivars, ten in number. The results showed that the addition of both dithiothreitol and

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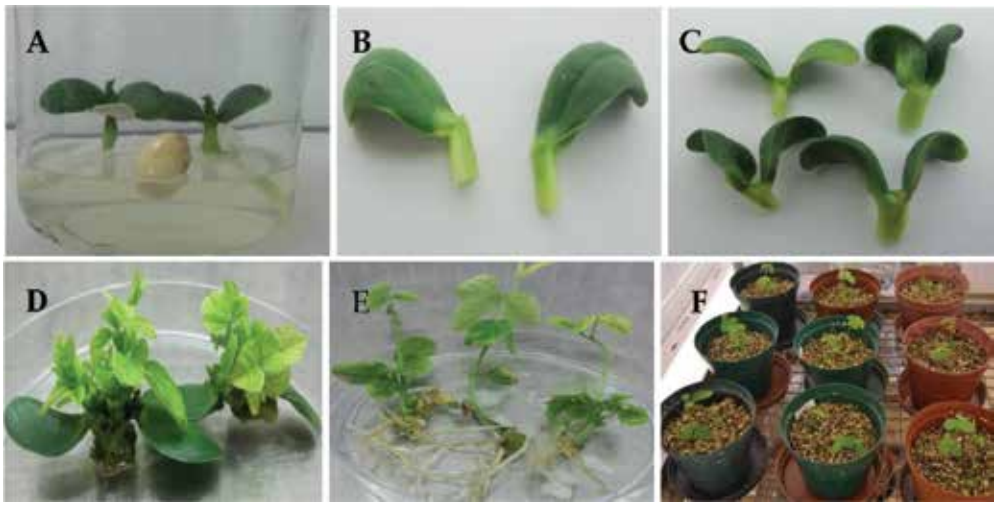

Figure 1. Examples of steps for *in vitro* transformation using cotyledonary node explants of soybean: A—aseptically produced seedlings to serve as explant source, B—single coty-node explants, C—double coty-node explants, D—adventitious shoot induction on double coty-node explants infected with *A. tumefaciens*, E—rooted shoots obtained from PGR-free MS medium with some callus at the base and F—ex vitro acclimatised plantlets.

cysteine led to 95% successful infection rates of the ten cultivars. These compounds prevent oxidative stress in the explants.

Sterilisation of cultures: Success of in vitro regeneration cultures requires good disinfection of plant material. The use of chlorine gas proved effective for surface sterilisation of soybean seeds in our studies [39]. Other sterilising methods include chemical sterilisation of the plant material using 70% alcohol for a few seconds, and 1% sodium hypochlorite (NaClO) containing few drops of Tween 20 for 10–30 min.

Factors influencing sterility of culture:

Factors influencing the rate of contamination in in vitro culture are directly related to the working conditions and the plant materials used. For production of completely aseptic cultures, factors that must be considered regarding the explants selection must include the physiological or ontogenic age of the organ that is to serve as the explant source, season in which explants are obtained, and size and location of the explants. In addition to the above mentioned factors, the quality of the source plant and ultimately the goal of cell culture also need to be considered [9]. Generally, the greatest response is achieved when young tissues are used in vitro because they are easier to surface disinfect. The following factors can decrease contamination and improve response in culture:

a. Healthy plants selected from plants that are not under nutritional or water stress or exhibiting disease symptoms can assist in establishing virus-free plants or plants without internal contaminants.

b. Young tissue explant.

c. Use seedlings of aseptically germinated seeds. Have a low rate of contamination (externally and internally) as compared to other explant source. The choice of explant tissue will vary, depending on what type of a response is desired from the cell culture [55].

Explant infection and co-cultivation: The use of coty-node explants provide the regeneration-competent cells in embryonic axis for *Agrobacterium* infection to improve regeneration competency of the tissues. Efficacy of explant infection by *Agrobacterium* does not rely on the regeneration process alone, but, also depends on the bacterial strain used. Hyper-virulent strains which constitutively express *vir* genes responsible for the transfer and integration of T-DNA are required. Rejuvenation of the bacterial culture before use in the transformation process is also a prerequisite. The re-initiation step allows bacteria to grow from a lag phase to reach growth acceleration or exponential growth state. In the course of this period, the bacterial cells will repair macromolecular damages that accumulated during stationary phase and the synthesis of cellular components necessary for growth [30]. *A. tumefaciens* with pTF 101 vector was used in our study [38] for in vitro transformation of soybean due to its better re-initiation capacity, compared to Ω PKY vector. In part, the infection of explants can be further enhanced by supplementation of the co-cultivation culture with organic additives such as acetosyringone to induce expression of these *vir* genes [49]. Nevertheless, numerous reports indicate that the host and tissue specificity associated with vectors carrying genes of interest present a major challenge [49, 56, 69]. The cited problem is one of the major reasons why there is no routine protocol currently applied in genetic transformation of soybean without showing genotype specificity.

Shoot induction: This stage is more reliant on the culture media composition, type of explant used and the efficient recovery of transformed shoots. The selection of effective antibiotics is also very crucial to the success of shoot induction in vitro. Antibiotics are important in removing residual *A. tumefaciens* in the culture. Resistance of the transforming bacteria to the antibiotics could cause contamination problems during co-cultivation and shoot induction stages. A study by Maheswaran et al. [37] emphasised the importance of selecting a good strain of *Agrobacterium* which shows no antibiotic resistance. The report suggests the suitability of strain LBA 4404 for apple transformation since it can be effectively eliminated from culture using considerably lower concentration (100 μg mL^{-1}) of carbenicillin and mefoxin. This was in contrast to other findings where strains such as pTF 102/Ω PKY derived from EHA 101 were used for the transformation of soybean [48, 70]. The expensive β-lactam antibiotics such as cefotaxime and vancomycin are commonly used for elimination of *A. tumefaciens* in plant transformation. Our preliminary study on the efficiency of aminoglycoside (rifampicin, tetracycline and hygromycin) antibiotics at 500 mg/l concentration against *Agrobacterium*, pTF 101 and Ω PKY, showed effective elimination of the two strains. The recovered adventitious shoots grew to maturity and survived the continuous application of glufosinate-ammonium used as a selective agent for identification of transformed plants [38]. The trend observed in the study and other reports [59, 63] suggest an emerging problem of antibiotic-strain relationship that specific antibiotics could be required for a specific strain of *A. tumefaciens* used during transformation. Aminoglycoside antibiotics are mostly used in the transformation process as selectable markers.

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Shoot elongation and rooting: In our study, more than 50% of transformed shoots elongated and rooted within two weeks of culture in each stage [38]. Vigorous elongation and root growth was mostly observed on plant growth regulator (PGR)-free MS basal medium containing antibiotics. Furthermore, our observations show that elongation of transformed shoots can be rapidly achieved when the induced shoots and clumps are subcultured on the elongation medium while still attached to their cotyledonary explants. Young shoots excised-off the explants, subcultured for elongation showed high sensitivity to the media composition, suffering immediate marginal chlorotic and necrotic symptoms. There were no shoot abnormalities as a result of the media or any unusual differences in terms of the morphology between all elongated shoots. On the other hand, there were no notable morphological differences in the adventitious root phenotypes developed on PGR-free MS medium supplemented with 6.0 mg/l glufosinate. The adventitious root formation occurred on all shoots without the presence of auxins such as indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) in the culture medium as observed by Polisetty et al. [52]. The short adventitious roots without lateral roots were accompanied by a light green callus at the base of the explant's cut surfaces. This is a normal response as the injured tissues mitotically divide as a response to the tissue damage which occurred on the explants (**Figure 1E**). The reduced root morphology in contrast to normal root development in the control without hormones indicates the role played by exogenous growth regulators in influencing the levels of endogenous hormones. In our case, it was BA (2.0 mg/l) exogenously applied during shoot induction cultures resulting in the lack of vigorous root development. Success of transient expression during transformation is usually demonstrated using β-glucuronidase (Gus) activity or glufosinate resistance. Various plant parts (roots, pollen grains, stamens and seeds) of primary transformants could be used [27] for GUS assay. Techniques such as Southern blot analysis can also be used in further probing for stable integration of the gene of interest in glufosinate resistant/Gus positive plants.

Ex vitro acclimatisation and care for surviving plants: It is necessary to have a growth room with well-regulated light and temperature to achieve acclimatisation of transformed plants. Good insulation and a proper day-night period ratio should be properly determined, especially when working with different species in the same growth room. A substantial number of in vitro produced plants do not survive during acclimatisation. For efficient acclimatisation of tissue culture derived plants, rooted shoots should be first transferred in culture vessels half-filled with sterile vermiculite and covered with transparent plastic bag. The size of the vessels to be used can also be determined by looking at the height of your rooted plantlets. This allows plantlets to develop fully functional shoot and root systems. Cultures should therefore, be maintained in a tissue culture growth room under 16 h photoperiod of 50–60 μmol $m^{-2}s^{-1}$ light at 24 ± 2°C. When plantlets grow to second trifoliate (V2) stage, they can then be transferred in 15-cm plastic pots containing sterile vermiculite and then, taken to a growth room at same temperature but, with 150–200 μmol $m^{-2}s^{-1}$ light intensity and 16-h photoperiod. Plantlets are kept under this condition until they reach R1 reproductive stage to increase growth and reduce mortality. The plantlets are supplied daily with distilled water and once a week with half strength Hoagland nutrient solution [18]. This process minimises the effect of environmental stresses that plants endure when they are subsequently exposed from their unique microenvironment. The physiological and anatomical characteristics of in vitro developed plants highly necessitate their gradual acclimatisation to the environment outside tissue culture conditions.

4. In vivo-based genetic transformation

In vivo transformation is also a process in which foreign genes can be integrated and expressed in genomes of plants, with which tissue culture systems do not yield desired results. In analogy with in vitro transformation of soybean, in vivo transformation also allows for the use of *A. tumefaciens* bacteria for transformation. Even if this method does not entirely guarantee elimination of the hurdles faced during transformation, its adoption guides future efforts on improving genetic transformation of recalcitrant legume crops. A number of reports have indicated that challenges encountered in soybean transformation are predominantly caused by the difficulties that exist in plant regeneration and low transgene expression in tissue culture [4, 46]. This method eliminates the restrictions of culture contamination as a result of ineffective antibiotics and tissue culture derived genetic variations. Furthermore, it could result in higher transformation frequencies and enable massive reduction in the number of infertile transgenic plants regenerated during in vitro culture [35].

4.1. Seedling development and *A. tumefaciens* injection

The generation of in vivo genetically modified plants carrying the DNA of interest requires appropriate choice of plant material to be used in transformation, in addition to the physical factors that include humidity, temperature and light. Like in in vitro culture, this method also targets embryogenic tissues that would ultimately induce organogenesis of transformed adventitious shoots. Birch [6] reviewed the protocols targeting young apical meristems for genetic transformation in soybean, corn, wheat and rice. The report indicated the advantage of using excised or partially disrupted meristems which have a high capacity to regenerate transformed shoots and roots when they are infected with *Agrobacterium*. Some of the reports that used non-tissue culture-based approaches in plant transformation include Chee and Slighton [10] and Hu and Wang [26]. In our study, soybean seedlings were established by first imbibing the seeds in sterile distilled water containing 2.0 mg/l BA for 12 h. This was carried out in order to produce strong seedlings with thicker hypocotyls that are directly used as a reliable plant material for *Agrobacterium* injection. Furthermore, the seeds were imbibed to increase the rate of germination. Moist sterile vermiculite was used as a supporting medium. The procedure adds to the emphasis by McDonald et al. [40] that seed imbibition is the most critical stage in successful soybean plant establishment. Absorption of water by the seed parts (seed coat, embryonic axis and cotyledons) and the whole seed triggers enzyme-catalysed metabolic processes in the tissues of the germinating seeds. Our results proved that higher seed germination rates can be achieved from seeds imbibed in BA than the control without BA, leading to the production of stout seedlings with increased stem diameters and broad well-developed leaf areas similar to seedlings developed in tissue culture. The observed seedling morphology is mostly attributed to the role of BA in seedling development. Similar observations were made by Patil et al. [47] with *D. purpurea* L. seeds using 10.0 μM BA. The growth parameters such as shoot and root lengths were shown to be significantly reduced in length but, increased in width as a result of the variety of growth and morphogenetic responses [57]. Although BA could induce multiple shoot growth, it further indicates that not all responses are stimulatory, as seen in the suppression of the development of roots and shoots.

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4.2. Infection of seedlings with *Agrobacterium*

When BA pretreated seedlings are injected with *Agrobacterium* carrying Ω PKY vector construct suspension at their cotyledonary junctions, infected seedlings' health was not severely affected by the wounding caused [38]. The wounded tissues could appear necrotic which may result due to tissue damage and the release of phenolic compounds causing oxidative browning and subsequent death of some cells. Reports show that less oxidised tissues could improve the transient integration and expression of transferred genetic materials in plant cells [8, 45]. However, studies such as those of Paz et al. [48] on in vitro transformation indicated that wounded tissue browning can be prevented by the application of antioxidants such as L-cysteine and dithiothreitol (DTT). These are the predominantly used antioxidants during co-cultivation of in vitro infected explants. In in vivo transformation, such compounds are added in the osmoticum solution (prepared by adding 1.0 M NaCl and 200 μM acetosyringone in sterile distilled water) applied subsequently to Agro-injection of the seedling explant. No deaths of infected seedlings were observed as a result of infectious wounding in our study [38]. Observations come from the morphology of the pretreated seedlings and the effect of BA in delaying tissue senescence. Laloue et al. [31] demonstrated that cytokinins can play a role of retarding senescence and chlorophyll degradation, particularly in aging organs.

4.3. Proliferation of transformed axillary shoots

Adventitious shoots induction is considerably easy in vivo than in vitro. The use of cotyledonary regions on developed seedlings facilitated high competency of multiple buds and shoots proliferation and plant regeneration. The use of cotyledonary regions is predominantly practised in in vitro tissue culture, with the aid of solid media-containing cytokinins. Since the method is well-known for its competency in shoots proliferation, it was tested for in vivo shoot regeneration. As previously mentioned, Agro-injection on the seedlings' cotyledonary junction made embryogenic tissues at that axis accessible for genetic transformation. It should be noted that transgenic soybean shoots have been successfully produced via *Agrobacterium*-mediated genetic transformation in vitro using mature or immature cotyledonary explants from this regions [49, 69]. However, this is the first report on the use of soybean cotyledonary embryogenic axis from mature seedlings for the development of a regeneration protocol in vivo, without the use of tissue culture. As shown in **Figure 2**, both axillary meristems on each seedling can be exploited for the induction of transformed axillary shoots. The adventitious shoots were initiated by simply excising off the epicotyls at the junctions. Later, the regenerated shoots can also be excised from the junctions and transferred on sterile vermiculite for simultaneous growth and rooting. The data are summarised in **Table 1**. Juvenile plants derived from BA pretreated seedlings exhibit thicker stems (3–5 mm), high number of axillary branches (3–4) obtained within a period of 3 weeks and a larger number of leaves (3–4 trifoliate leaves) as compared to plants with lesser number of axillary branches and leaves in the control. Growth and morphogenetic features of the regenerated plantlets clearly indicated a positive influence by pretreatment of seeds with BA (2.0 mg/l). According to Dybing and Reese [17], pretreatment of soybean seeds with hormones (2 mM BA) leads to vigorous growth and subsequent pleiotropic effects of flowering, fruiting and increasing seed yield with more than 80% pod set. A considerable

Figure 2. Examples of steps for *in vivo* transformation using seedling explants of soybean: A—shoot formation on infected seedling, B—acclimatisation of regenerated shoots maintained under controlled growth conditions and C—an 11-week-old acclimatised plant transplanted into plastic pot.

Culture		Soybean seed germination		Soybean shoots regeneration			
		PGR (mg/l)	Germination (%)	Culture medium	PGR (mg/l)	Mean shoot no.	Regeneration (%)
In vitro	MS	2.0	95[a]	MS-SIM 1	2.0	4.86[b]	76.6[a]
	Control	–	77[b]	MS-SIM 2	2.0	7.27[a]	63.3[b]
				MS-SIM 3	2.0	3.80[c]	60.0[c]
				MS-Control	–	1.3[d]	0[d]
In vivo	–	2.0	97[a]	–	–	1.7[a]	85[a]
	Control	–	87[b]	–	–	1.2[b]	0[b]

Note: Data were analyzed using ANOVA and values within columns followed by the same letters are not significantly different at the 5% confidence level. Regeneration percentage=(no. of explants with two or more shoots/total no. of explants) × 100.MS, Murashige and Skoog; SIM, shoot induction medium; PGR, plant growth regulator [38].

Table 1. Summary table showing the germination percentage of soybean seeds and efficiency of shoot regeneration on soybean explants infected with *Agrobacterium tumefaciens* carrying the p TF 101 vector construct.

difference in root morphology of the initiated transformed shoots in contrast with the control soybean plants was also observed.

The control plants were characterised by the vigorous root growth of the primary roots with many branching or lateral roots, whereas transformed plants had stunted root growth without distinct main roots and fewer lateral roots. This may be a drawback when attempting to ensure that sufficient numbers of transformed plants are grown in the outside soil environment. Poor root growth also limits nutrient and water uptake adequately required for growth, especially, when growth reaches reproductive stages. However, the cytokinin compound used mainly regulates shoot proliferation. Cho et al. [12] observed similar root morphology after transformation with *Agrobacterium rhizogenes* and linked this to the integration and expression of the DNA in soybean genome. The reference stated that infected plants showed stunted root growth with reduction in both root initiation and root development. The observed root phenotype was physiologically attributed to the effect of plant regulatory

factors (phytohormones) that were produced by plant cells responding to infection by *A. rhizogenes*, which harboured one of the pBINm-gfp5-ER or pBI121 binary vectors.

4.4. Growth and screening of transformed plants

Although the induced soybean shoots showed a positive and significant growth in a growth room, one of the most important aspects of in vivo transformation is to maintain their growth and conduct proper transgenic screening procedures. According to Tian-fu and Jin-ling [60], soybean plants require relatively short day-light period (usually, 8–10 h) and continuous dark period of about 14–16 h to reach and achieve reproductive growth. This is mainly because soybeans are highly susceptible to photoperiods and flower abortion can be easily caused by long day photoperiod. Production of flowers, fruit pods and seeds that were observed on all transformed plants were affected by photoperiod. Regarding the part of screening, Hinchee et al. reported soybean genetic transformation using *Agrobacterium* strain pTiT37-SE harbouring plasmid vector MON894 conferring kanamycin and glyphosate tolerance. The successfully regenerated transgenic plants managed to survive and continue their growth with kanamycin and glyphosate supplemented medium. In our case, the *bar* gene conferring tolerance against glufosinate-ammonium was used. A 6.0 mg/l of glufosinate-ammonium ($C_5H_{12}NO_4P$) was added as a selective agent in a Hoagland nutrient solution [18] used to water the regenerated shoots on a daily basis. Besides that, spraying leaf surfaces of matured soybean plants with glufosinate was also carried out on a weekly basis. A total of 153 infected plants survived continuous application of glufosinate. Data of regeneration percentage following employment of the herbicide are shown in **Table 1**. It is advantageous to use glufosinate as a selection pressure to segregate transformants from non-transformed plants because it minimises the effect of chimerism [70]. Severe chlorosis and necrosis, subsequently leading to the death of plants were observed in 1–2 week in non-transformed plants. The transformed plants were able to withstand the heavy application of the herbicide and showed smooth recovery from abrasions observed 3–5 days after surface spray application. Successful selection of transformed plants using glufosinate application was also reported by Murugananthan et al. [43], Montaque et al. [41] and others who clearly indicated that it can be reliably used as a selection regime to get rid of non-transformed plants, particularly without fasciation. In contrast, kanamycin selection system used in tissue culture has been found to produce unsatisfactory results illustrating phenotypic abnormalities in regenerated plants by Bean et al. [2] and Montaque et al. [41].

4.5. Acclimatisation of in vivo transformed soybean plants

Hardening of plants and transfer to plastic pots containing soil vermiculite are challenging factors as well for the survival of in vivo regenerated plants. The greenhouse environment poses many challenges including lower relative humidities, higher light levels and septic conditions. It is important to know and understand the effects of these factors on further growth and development of the plants. For example, the longer light period can affect flower formation, as previously mentioned. Plant survival rate of 70% on average was achieved in our study, which was even higher than the survival rate of 60% on average in tissue culture-

derived plants [38]. Minor phenotypic setbacks were observed. Regenerated plants produced new young leaves at the shoot tips to continue growth but the young leaves died and fell off before any further development. This ceased the growth and resulted in the stunted growth of the regenerated plants. Zia et al. [68] reported similar morphological characteristics during in vivo Agro-injection of soybean pods in transformation of soybean seed embryos. Bermnier and Claire [3] reported retarded growth of transformed plants. The plants showed early flowering, which later resulted in flower abortion.

5. Other factors affecting soybean transformation

It has been already documented that in vitro and in vivo plant genetic transformations are the key modern plant biotechnology techniques in the possible improvement of recalcitrant crops. The methods allow regeneration to occur under controlled microenvironments provided that balanced nutritional requirements are met. They serve as efficient alternatives to conventional breeding in producing new cultivars. However, the development of a reliable and a more efficient genetic transformation system intensely slows progress in new cultivar outputs. The challenges faced in many soybean line, continue being irrepressible and create recalcitrance of this crop to genetic transformation. Most reports recommended condition standardization for T-DNA transfer and expression in host plant cells. However, the effect of intrinsic factors such as the genotypes of *Agrobacterium* strain (modified supervirulent strains) and elite soybean genotypes considerably influence the process. Conditions that include growth medium, plant growth regulators, temperature and the type of explants used highly influence production of transgenic plants. The use of minimum explant sterilisation time, co-cultivation time and explant source vigour are among factors reported by Paz et al. [48]. All factors mentioned above set precedence to the success of genetic transformation in soybean, and if found not well-optimised, then the intricate interplay between plant host cells and bacterial genetic elements may be negatively affected.

6. Plants transfer to a natural environment

Ultimate success of in vivo or in vitro regeneration of transformed plants lies upon transfer into soil and reestablishment of vigorous growth under natural conditions (**Figure 3**). If these stages are achieved, plant growth can be easily dramatically accelerated minimising the poor survival rates that are frequently encountered. Normally, in vitro regenerated plants are difficult to acclimatise into soil because of their heterotrophic mode of nutrition provided with sucrose and mineral nutrients and the mode placed under conditions of limited light and low gaseous exchange [61]. During acclimatisation, the transition from a heterotrophic to photoautotrophic state is highly required. Plants experience a brief period of stress due to the incapability to adapt under lower relative humidity and high light intensity and the failure to immediately regulate water losses. A problem concerning the major challenges is that the transfer of plants into soil increases plant intolerance to water stress. Extensive water deficit that may occur could severely injure the plant [29]. It normally takes place when the loss

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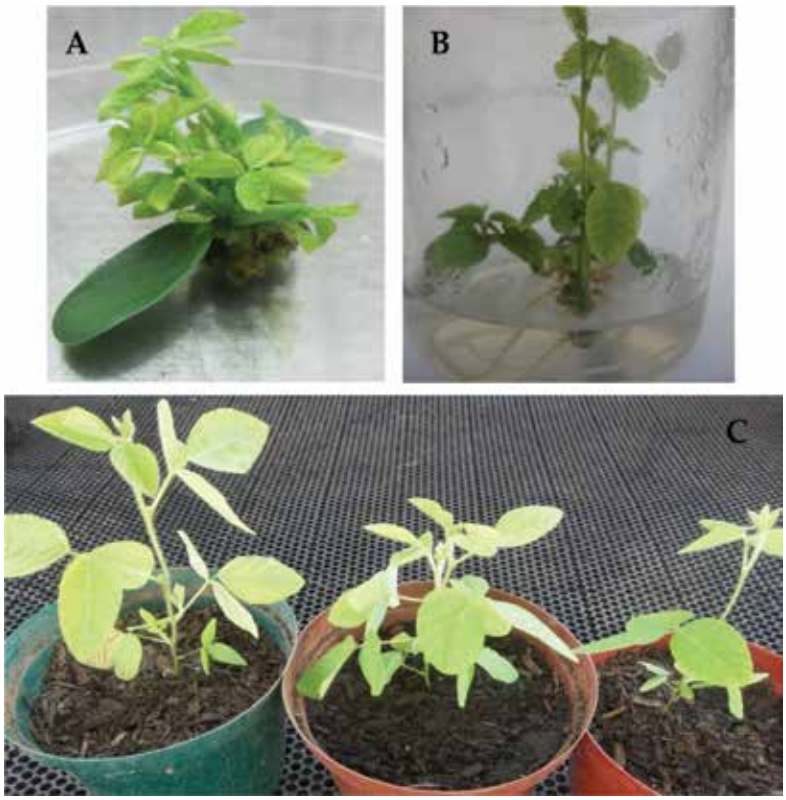

Figure 3. Stages involved during shoot regeneration in *in vitro* culture. Depending on the viability of explant and explant source, vigorous axillary shoot clusters can be obtained rapidly: A—induced multiple shoot clusters, B—rooting of elongated shoots and C—plants transplanted in soil.

of water in the tissues exceeds the ability of the roots in absorbing water. In this case, plant water content will decrease and the plant will not be able to sustain its normal processes. The decrease in water content will not support plant cell and tissue development [67].

6.1. Effect of water deficit on soybean growth

Inefficient water supply to plant tissue could be a result of the inability of roots (undeveloped and non-functional roots) to absorb enough water or due to the lack of rainfall or irrigation for a period of time sufficient to deplete soil moisture. This phenomenon is referred to as drought. Drought conditions that are constantly occurring in most parts of the world necessitate the development of transgenic plants that can grow during increasing environmental fluctuations [5]. Drought has been found to be a major limitation to soybean growth as the most important environmental factor influencing major yield losses for this crop [14, 50]. Drought affects production in soybean by: (a) interfering with symbiotic fixation of atmospheric nitrogen (N_2) by *Rhizobia* bacteria, (b) decrease in CO_2 assimilation and leaf area development resulting in poor nodulation, (c) increase in soybean susceptibility to weeds, insects and diseases and (d) increase

in flower and fruit abortion [25, 36, 42]. To overcome these limitations, soybean transformation should be improved to enable stable transfer and expression of gene such as *Oryza* cystatin-1 (*oc-1*) gene taken from rice (*Oryza sativa*), which confers tolerance to drought stress. The gene codes for proteolytic enzyme inhibitor that inhibits or suppresses protease enzyme activity normally induced in response to stresses such as wounding, cold and drought [66]. Proteases (like cysteine protease enzymes) are decoded in the host cells' cytoplasm following drought stress to cause degradation of essential proteins, thus resulting in death of tissues. The cysteine protease production can be inhibited by the *oc-1* gene coded cysteine protease inhibitor. The successful in vitro or in vivo soybean transformation incorporating the *oc-1* gene in host plant's genome may have a profound effect of inhibiting the role of the enzyme during water deficit, thus producing drought tolerant soybean plants.

7. Future research and development

Globally, transgenic soybean development and production are currently led by multinational companies such as Aventis, Crop Science, Monsanto and Syngenta. These companies are well-acknowledged for their supply of mostly transgenic and a few non-transgenic soybean seeds used for both commercial scale farming and industrial processing. Their cooperative controls emanating from developed countries are currently resulting in a slow shifting of research to crop management practices or innovations that save labour costs (such as herbicide tolerance) rather than those that create employment and produce drought tolerant crops. However, to make genetic engineering beneficial to the greater masses of poor people, particularly in Africa, development of genetically modified organisms (GMOs) including soybean should be aimed for enhancing plant growth, nutritional quality of seeds and properties increasing yields. *A. tumefaciens*-mediated genetic transformation system has proved to be a superior soybean transformation method. This is based on the fact that the technique offers significant advantages over other transformation systems. Those are easy manipulation, stable gene integration and expression, and lower transgene copy number [28]. Therefore, research must continue focussing on optimising the currently used genetic transformation protocols since (a) lower transformation rates are still obtained and (b) there is a need for an efficient protocol that will enable transformation of many elite soybeans since many lines are insusceptible to *Agrobacterium* infection. The lengthy transformation processes with complicated steps also need to be modified. These steps require long tissue culture periods, which need or consume large amounts of chemicals. More focuses also need to be directed to in vivo transformation of soybean. The procedure showed higher potential of success since many problems encountered during tissue culture can be less of concern. For example, in vivo transformation minimises chances of generating chimeras predominantly found in tissue (callus) cultures. Furthermore, the problem of contamination may be a thing of the past, since strong suppression of *Agrobacterium* would be no longer a prerequisite for successful transformation. Future studies will be focussed on testing other soybean cultivars using the modified protocol to check if they have similar trend of response thereby increasing the regeneration rates of transformed shoots. Given the nature of genetic transformation in soybean, optimisation of assays such as GUS assay should be considered in the strengthening of positive identification of transgenic soybean plants.

http://dx.doi.org/10.5772/66708

8. Conclusions

Although soybean is classified as a recalcitrant crop to *Agrobacterium*-mediated genetic transformation, considerable progress has been made in the optimisation of this technique. The development of in vitro and in vivo procedures for transformation of this crop will make possible the establishment of a routinely used genotype non-specific protocol. With findings of certain aminoglycoside antibiotics being effective against *Agrobacterium* and non-toxic to soybean plant tissues, these suggest progress and possible consideration in the application of the microbicides for *Agrobacterium*-mediated genetic transformation. The production of glufosinate resistant soybean plants by both the in vivo Agro-injection method and the in vitro tissue culture transformation appeared to be valuable complementary tools since in vitro system alone may not be sufficient.

Acknowledgements

This work was supported by the National Research Foundation, Republic of South Africa.

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Chapter 6

Nematodes Affecting Soybean and Sustainable Practices for Their Management

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/67030

Abstract

Plant-parasitic nematodes are one of the limiting factors for soybean production worldwide. Overall, plant-parasitic nematodes alone cause an estimated annual crop loss of $78 billion worldwide and an average crop yield loss of 10–15%. This imposes a challenge to sustainable production of food worldwide, since there has been increasing demand for food supply and food security. Unsustainable cropping production systems with monocultures, intensive use of soils and expansion of crops to newly opened areas have intensified problems associated with new pests and diseases. Thus, finding and applying sustainable methods to control diseases associated with soybean are in current need. Over hundred nematode species, comprising fifty genera, have been reported in association with soybean. Of these, the root-knot nematode *Meloidogyne* spp., cyst nematode *Heterodera glycines*, lesion nematode *Pratylenchus brachyurus* and the reniform nematode *Rotylenchulus reniformis* are major nematode species limiting soybean production. Here, we report an up-to-date literature review on the biology, symptoms, damage and control methods used for these nematodes species. Additionally, unusual and emergent nematode species affecting soybean are discussed.

Keywords: control, damage, lesion nematode, plant parasitic nematodes, RKN, yield loss

1. Introduction

Soybean (*Glycine max L.* Merr) is the most important oilseed crop worldwide. Over the past 20 years, soybean production worldwide has doubled reaching ca. 210 million tons. In addition, consumption has increased faster than its production. It is estimated that this trend will continue in the near future and that the increased demand for soybeans will reach 300 million tons by the year 2020. Soy is used for food and feed, production of raw materials for the industry

of vegetable oil and other purposes such as the manufacture of plastics, lubricants, candles, varnishes, soaps, biodiesel and lecithin [1].

According to the United States Department of Agriculture (USDA), in the 2013/2014 cropping year, USA, Brazil and Argentina accounted for 81.40% of the total world production of soybeans, and China by 64.26% of all world imports. Brazil accounted for the production of 86.27 million tons of soybeans, that is, 44.50% of the Brazilian production of grains with Brazilian average productivity of 3000 kg/ha, which is the second largest producer and processor of grains into meal and oil [1].

Currently, the rationality of production and the use of alternative fuels derived from biomass, especially bio-ethanol and vegetable oils, are being increasingly recommended to complement or improve the energy matrices worldwide. Among the producing crops for energy biomass used for biodiesel production features the soybean that is being currently studied as a promising crop for the production of biodiesel. However, in addition to economic feasibility studies including energy efficiency, organization of production system and crop adaptation, it is necessary to take into account policy studies related to diseases and pests in agricultural systems where crops are or will be implemented in order to decrease losses due to pathogen attack.

There has been increasing demand for food supply and food security. Unsustainable cropping production system with monocultures, intensive planting and expansion of crops to newly opened areas has increased problems associated with new pests and diseases. Among these problems, plant parasitic nematodes are one of the limiting factors for soybean production worldwide. Plant parasitic nematodes alone cause an estimated annual crop loss of $78 billion worldwide and an average crop yield loss of 10–15%. Nonetheless, soybean yield loss due to nematode parasitism is quite variable and mostly depends on factors such as nematode species, their population levels, susceptibility of the cultivars, cropping systems, temperatures, time of the year, region and soil factors including soil texture, pH and fertility. The yield loss can reach up to 30–100% in some reported cases [2, 3].

More than 100 nematode species, comprising 50 genera, have been reported in association with soybeans. In Brazil, the species that cause the most damage to soybean are *Meloidogyne javanica, Meloidogyne incognita, Heterodera glycines* [4, 5], *Pratylenchus brachyurus*, the reniformis nematode *Rotylenchulus reniformis* [6] and *Tubixaba tuxaua* [7].

Since pathogens such as plant parasitic nematodes represent major losses in agricultural systems, especially when the systems are not managed sustainably, the searches for information on the occurrence of nematodes in the production system, population density, species, levels of damage, and monitoring and management of these populations are essential in regions where crops will be implemented.

The goal of this chapter is to report a literature review of main nematode species affecting soybean worldwide and the methods used for their sustainable management in the field. Although there are numerous nematode species associated with soybean, few of them have been continuously reported as major constraint to soybean production worldwide. These include: (i) *Meloidogyne* spp.; (ii) *H. glycines*; (iii) *Pratylenchus brachyurys* and (iv) *R. reniformis* (**Table 1**).

Common name	Species name
Root-knot nematodes	*Meloidogyne* spp.
	M. incognita
	M. javanica[*]
Soybean cyst nematode	*Heterodera glycines*[*]
Root lesion nematode	*Pratylenchus brachyurus*[*]
Reniform nematode	*Rotylenchulus reniformis*[*]
Lance nematodes	*Hoplolaimus* spp.
Spiral nematodes	*Helicotylenhus* spp.
	Helicotylenchus dihystera
Sting nematodes	*Belonolaimus* spp.
Other emerging nematode species	*Aphelenchoides* sp.
	Scutellonema brachyurus
	Tubixaba tuxaua

[*]Indicate the most damaging nematodes on soybean.

Table 1. Most common plant parasitic nematodes associated with soybean worldwide.

2. Major nematode species affecting soybean

2.1. Root-knot nematodes (*Meloidogyne* spp.)

Root-knot nematodes (RKN), *Meloidogyne* spp., are the most economically important group of plant parasitic nematodes worldwide. Currently, there are more than 90 described RKN species [8] parasitizing more than 2000 plant species, which represent a real threat to the agriculture worldwide [9]. RKN species that are associated with soybean in Brazil include *M. incognita, M. javanica* and *M. arenaria* [10–12]. *M. javanica* and *M. incognita* have wide distribution in soybean-growing areas in Brazil, whereas *M. javanica* is the most aggressive species with broad geographic distribution, due to favorable conditions for its multiplication in susceptible hosts [11].

In several field surveys for RKN nematodes in main regions of soybean production in Brazil, *M. javanica* was reported as the most prevalent species (64%) [13]. In Ref. [4], the authors found that *M. javanica* (77%) and *M. incognita* (31%) were the most prevalent two RKN species on soybean fields. Other RKN species have also been reported in soybean fields with lesser extents, including *M. morocciensis* [13], *M. paranaensis* [14] and *M. ethiopica* [15].

In a survey of more than hundreds of soybean fields in USA, it was found [16] that *M. incognita* was the most prevalent species (70%), followed by *M. javanica* (24%) and *M. arenaria* (6%). In a study carried out in South Africa [17], the study found that 91% of soil and root samples were infested by *Meloidogyne* spp. including *M. incognita*, *M. javanica*, *M. arenaria, M. hapla* and *M. ethiopica*. Studies carried out in Argentina [18] reporting the distribution and frequency of

RKN nematodes in soybean fields found that the *Meloidogyne* spp. were widely distributed in the country and that *M. incognita* and *M. javanica* were the most species frequently detected. Other species were *M. arenaria*, *M. cruciani*, *M. decalineata*, *M. hapla* and *M. ottersoni.*

Symptoms in the field include yellowing and sub-development of infested plants. RKN nematodes induce hypertrophy and hyperplasia of infected cells leading to swelling of tissues commonly known as galls. The number and sizes of galls vary depending on the susceptibility of the cultivar, population density and favorable temperatures [19]. RKN-infected roots change their nutrient and water uptake, leading to decreased yield. Commonly, there are high levels of intraspecific variation within *Meloidogyne* genome, and this variability may play an important role in changes in morphology and cytogenetics and ultimately their capacities to reproduce in certain hosts [20].

RKN are endo-sedentary parasitic nematodes. The second-stage juvenile (J2) is the infective stage. After RKN hatch from eggs, the J2 migrates through the soil toward suitable root and uses special enzymes and the stylets to force penetration into the vascular cylinder where RKN establish their feeding site by inducing hypertrophy and hyperplasia of a group of cells leading to swelling and formation of giant cells. On this site, nematode goes through three more molting to become a swollen young female. Mature females begin laying eggs in the root, forming mass eggs wrapped in a gelatinous matrix. Each egg mass contains 400–500 eggs on average, and it is formed in the midst of cortical parenchyma or on the surface of the roots. The embryonic development of the nematode results in the first stage (J1) passing through an ecdysis in the egg, followed by the second stage (J2). Adult males do not feed on soybean roots; they leave the root and move freely in the soil until they die [20].

To control RKN is extremely difficult. Currently, the most effective and environmentally sound way to control RKN is the use of resistant cultivars that stand good yield and have been tested in a particular region where soybeans are planted [11], the use of tolerant genetic materials and rotation/succession with non- and poor host crops [10, 12]. The use of nematicides at planting or via seed treatment is an option. However, they are costly, not very effective, and have side effects to human and to the environment [21].

Currently, several soybean genotypes have been described as resistant or moderately resistant to *M. javanica* and *M. incognita*, even though the levels of resistance are not very high [11]. Almost all soybean cultivars are descended from a single source of resistance from the cultivar 'Bragg' from USA. In the cultivar Bragg, there are other sources of resistance that is used in breeding programs, such as Hartwig, Kirby, Cordell and Leflore cultivars that exhibit resistance to the soybean cyst nematode *H. glycines* [11] in addition to the resistance to *Meloidogyne*.

For the 2014/2015 cropping year, the following soybean cultivars were released by Embrapa with reported resistance to *M. javanica* (BRS Corisco), *M. incognita* and *M. javanica* (BRS 7980), *M. incognita* (BRS 8180RR), *M. javanica*, *M. incognita* and the cyst nematode *H. glycines* (BRS73800RR) [22, 23].

Prior to using resistant soybean cultivar in a certain area, grower should consider the nematode species present in the field, because, although there may be predominance of one species of nematode over another, the presence of mixed populations is very common, which may

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limit the use of resistant varieties. In addition, in the choice of soybean cultivar, grower must take into consideration the adaptation and yield potential of the cultivar.

However, other control methods for RKN should also be considered. For instance, the use of antagonistic non-host plants such as *Crotalaria spectabilis, C. grantiana, C. mucronata, C. paulinea, Stizolobiun aterrinnum, S. cinereum* or *Raphanus sativus* has been shown to reduce *M. javanica* and *M. incognita* nematode population density and improve soil quality [24]. Therefore, the use of these combined management methods may be effective to reduce initial nematode population in soybean-growing areas and enhance soybean yield.

2.2. Soybean cyst nematode (*H. glycines*)

The soybean cist nematode, *H. glycines,* is one of the most yield-limiting nematode species, which affects soybeans, *Phaseolus vulgaris*, *Vigna angulares* and *Vigna radiata* in several regions worldwide [11, 25, 26]. This nematode was first reported in 1915 in Japan, since then it has been disseminated to most areas where soybean is cultivated. In Brazil, soybean cyst nematode was first reported in 1991, and nowadays, it is present in most areas where soybean are planted covering an area of ca. 2–3 million hectares [11, 26, 27]. Yield losses associated with this nematode can be as high as 30% depending on the region, soybean system and genetic background of the cultivar [11, 28].

Mature females of soybean cyst nematode retain their eggs inside their bodies after their deaths. The body is formed by a cuticle embedded with polyphenol tanning, resulting in a hard protective structure name cyst which is viable overwintering in the soil for up to 6–8 years. Thus, once the nematode is introduced in soybean fields, it is almost impossible to eliminate it completely. Females can also lay eggs in egg sacs. With stimuli of host root exudate, moisture and temperature, juvenile nematode (J2) hatches from eggs or emerges from cyst and moves freely in the soil. This is the only life stage that is able to infect plant. Following the stimulus of root exudate, J2 can use its stylet and degrading enzymes to penetrate the root and gain access into vascular tissue. There, J2 injects special enzymes that modify and transform a group of cells into specialized feeding sites (nurse cells). Female nematode then becomes lemon shape and eventually breaks through the root tissue and becomes exposed on the root surface. Mature female produces eggs (200–400) either in egg sac or inside the body (cyst) [24, 28]. The cyst then persists in the soil for several years. The entire cycle is completed in 24–30 days depending on optimal conditions, such as moisture and temperature (23–28°C) [26, 28, 29].

Usually, the nematodes can complete three to six generations a year. The cyst nematode reproduces by sexual reproduction (amphimixis), and its genome is characterized by an extremely high diversity, leading to several races of the pathogen. Soybean cyst nematodes can be spread to long distances effectively by means of infested soil particles, farm machinery, vehicles, tools, wind, water and animals among others [29, 30].

Symptoms of soybean cyst nematode can be mistaken for other disease symptoms as well as for management-associated problems, including iron and other nutrient deficiencies, herbicide toxicity and drought stress [29]. The appearance of cyst nematode symptoms attributed to other causes may lead to non-detection of the nematode for several years until its population

builds up. Soybean infected with cyst nematode appears in the field as irregular patches of stunted, yellowed, less developed plants. Usually, symptoms are more severe in light sandy soils, but it also occurs in heavy soil. The root system infected with soybean cyst nematode is smaller and stunted, and the infection affects nodule formation and decreases nitrogen fixation. In addition, nematode-infected roots are more prone to soilborne fungi and bacterial pathogen secondary infection. The presence of adult females and cysts attached to soybean roots is typical of soybean cyst nematode infection [25, 26, 29].

Since soybean cyst nematodes can survive inside cysts for several years, once the nematodes have been introduced into soybean field, they are not likely to be easily eradicated. Nonetheless, there are recommended cropping management practices that minimize the problem. For instance, the use of soybean resistant varieties is the most effective way to control this nematode and have been used successfully. Several soybean cultivars have been released, and their sources of resistance come from the parental cultivars PI88788 or the Peking. To avoid breakdown of resistance, it is recommended to use cultivars alternately [11, 29]. It is recommended to use alternately among susceptible and resistant cultivars. For instance, use one susceptible cultivar after two to four years of cultivation with resistant cultivars [29]. In Brazil, most resistant soybean cultivars are specific to races 1 and 3 and are not well adapted to every soybean-growing region. Besides, due to soybean rust disease, growers are choosing early maturity varieties which are more susceptible to cyst nematode [11].

Another effective method in controlling soybean cyst nematode is the use of rotation with non-host crops. Soybean cyst nematode has a narrow host range which facilitates rotation with other non-host crops. The pathogen levels in the soil significantly decrease, once there is no suitable host for infection. Good examples of non-host crops are maize, sorghum, oat, alfalfa, rice, cotton, sunflower and castor bean. Rotation for 1 year with one of these crops significantly decreased the population level of cyst nematode, allowing planting of susceptible soybean cultivar in the following cycle [11].

Although the use of soybean resistant cultivars and crop rotation has worked well, growers should be careful to provide good management practices in soybean areas, including good pH levels and soil fertility in order to maintain the effects of these disease control methods [11, 25, 29]. In summary, the scheme using crop rotation and resistant and susceptible varieties is the best way to manage soybean cyst nematode. For example, the use of maize, resistant soybean cultivar and susceptible soybean cultivar has been one of the best approaches to manage the nematode in the field [11].

2.3. Root lesion nematode (*P. brachyurus*)

Root lesion nematode, *P. brachyurus,* has become an increasingly important parasite of soybean in the tropics and subtropics [31]. It has a broad host range and is widely distributed in tropical and subtropical regions, especially in Brazil [32], Southern United States and Africa [32, 33]. Its occurrence in soybean fields has been increasing lately due to expansion and cultivation of soybean in newly opened area of native savannas in Brazil contributing to the overall losses observed in soybean field due to nematode attack [10, 34, 35].

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More than 50% of soybean in Brazil are produced in the Cerrado region (vegetation like savanna), with an increase in production over 100% in the central and northeast region [35]. The expansion in areas planted with soybean in the Cerrado region has contributed to intensive agriculture, leading to agronomic challenges, including nematode infection [35]. Among plant parasitic nematodes that infect soybean, *P. brachyurus* is one of the most important species, especially in these newly opened areas in the Cerrado region. Recently, its incidence has been rising with overall losses of soybean estimated at ca. 10–30%, especially under sandy soil and irregular rainfall [6, 35].

Pratylenchus species is commonly referred to as the root lesion nematode due to the typical symptoms of necrosis it causes in the roots. The species is considered a migratory endoparasitic nematode, normally found within the roots and between the roots and soil particles [31]. *Pratylenchus* species is smaller than 1 mm length. Males and females are wormlike, differing only in the sexual characters. Females have one ovary (monovarial) and reproduce by sexual reproduction called amphimixis or by mitotic and meiotic parthenogenesis. They are easily recognized by the sclerotized labial region and ventral overlapping esophageal glands and usually by dark intestinal contents. The stylet is well developed with broad basal bulbs. Most species are polyphagous, showing the ability to parasitize cultivated plants—perennials, semi-perennials, annuals as well as weeds [31].

Root lesion nematodes are more common on sandy soils and regions with high temperatures. Eggs are deposited in the roots (cortex) or in the soil. The incubation period ranges from six to eight days at a temperature of 28–30°C. The first molt takes place inside the egg and the other three occur out of the egg. Males and females emerge in 29–32 days. However, at low temperatures the life cycle may be longer. Usually, this nematode occurs in low population in the soil and at high population inside root tissue [33].

P. brachyurus population levels usually decrease in dry season and increase at optimum soil moisture. However, under greenhouse this nematode was able to survive 21 months in soil without any host or moisture, indicating its increased ability to persist in the soil [31]. In the evaluation for the survival of *P. braqhyurus* at different substrates with low moisture content, it was found [36] that even after 90 days in dry soil, there were a high number of viable nematodes in the substrates. This finding indicated that the nematode was able to survive in the off season in the absence of host, contributing to new source of inoculum for the following crop.

All *P. brachyurus* adult and juvenile stages are infective and migratory and move freely within the roots and between the roots and soil. The nematode uses its stylet to penetrate by means of mechanical force or using degrading enzymes. However, the nematode easily leaves the root system when conditions become unfavorable, switching to the soil [31]. Therefore, when the nematode parasitizes soybean roots, it causes lesions in the roots, causing damage to the cortical parenchyma and forming galleries in the tissue due to intense feeding and movement of the nematode, and these lesions lead to secondary infection by fungi and bacterial pathogens [37].

Symptoms of *P. brachyurus* infection in soybean are nonspecific and can easily be overlooked or confused with symptoms caused by other pathogens, nutritional deficiencies or water

stress. Infected soybean plants are less developed and less bulky, with root necrosis and discolored roots (reddish-brown to dark-brown), due to the coalescence of many necrotic lesions internally caused by the nematode. Such symptoms limit water and nutrient uptake in the roots, leading to aerial symptoms that can be seen in stunted plants with chlorosis, wilting and overall yield losses [38].

Pathogenicity studies suggested that *P. brachyurus* is well adapted to parasitism. For instance, even at an extremely high population levels in the soil, it usually is not enough to kill host plant. Moreover, damage threshold varies greatly depending on environmental factors and combination of *Pratylenchus* species and host plant, ranging from 0.05 to 30 individuals per cm^3 of soil [37].

P. brachyurus is common in soybean fields in Central and Northern Brazil and is associated with soybean yield losses in the fields. Studies on the soybean yield losses under the field conditions are difficult to carry out, since several factors may occur simultaneously masking the real effect of the nematode. For instance, factors such as weeds, low soil fertility, uneven distribution of rainfall as well as other diseases may result in low soybean yield [38]. Nonetheless, surveys in this area have shown a positive correlation of increased nematode incidence with a drop in soybean yield. For example, in Central Brazil, soybean yield was normally around 2600 kg per ha, while during cropping season of 2008/2009 it dropped to 2400 kg per ha and to 1850 kg per ha during 2009/2010 [39]. In this region, yield losses of up to 30% due to the nematode have been reported.

In Northern Brazil, *P. brachyurus* has commonly been found in soybean fields, especially in the Cerrado region of Tocantins State. Nematode infestation in this region has been increasing and certainly has interfered with soybean yield due to high population densities mainly due to monoculture systems and use of newly opened areas. The population densities of *P. brachyurus* observed in different soybean areas located in Tocantins State ranged from 23 to 8482 individuals per 10 g of roots and were negatively correlated with soybean yield losses [35].

To assess the effect of *P. brachyurus* on soybean variety MONSOY 9144 under field conditions in an area located in the Cerrado region with sandy soil, Lima et al. [35] determined the nematode population level inside and outside infestation pattern and correlated it to soybean production parameters (number of internodes, stem diameter, leaf number, root dry weight, shoot dry weight, pod number and plant height). They observed that the higher the population density of the nematode, the less the performance of soybean, thus showing that the nematode had negative effect on soybean. They also reported that the impact of *P. brachyurus* was severe in fields with sandy soil causing reductions in plant height (ca. 82%), dry weight of seeds (85%), dry weight of stems (81%) and pod number (39.7%) in nematode infestation foci compared with plants outside the foci. Results of a study carried out in 2011/2012 in Vera, Mato Grosso state by Franchini et al. [40] indicated that there was a highly negative correlation between soybean yield and nematode population, with 50 kg loss in yield for every 82 nematodes per gram of soybean roots. Overall, yield losses ranged from 50 to 1400 kg/ha, with an average of 600 kg/ha (21%) yield reduction.

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P. brachyurus is highly polyphagous, of which control is difficult. According to Santana Gomes et al. [24], it is difficult to control this nematode in soybean field, especially when using soybean in rotation or succession with other economically important agronomic crops that may also reproduce the nematode. These crops usually are cotton, pasture, corn, bean, sorghum, peanut, potato, tobacco, eucalyptus, rubber, pigeon pea, pineapple, some vegetables, sugarcane, coffee and rice [41].

Among these economically important crops, maize is the main one used in succession or rotation especially with soybeans. However, this management strategy enables increase in *P. brachyurus* population, since most maize hybrids tested have controversial results for resistance to this nematode. Furthermore, only a fraction of the commercial genetic material currently available has been evaluated, and generally the most productive hybrids multiply this nematode [41].

The use of soybean resistant varieties is probably the best way to control nematode infection, because it is easy, cheap, effective and environmentally safe. Host resistance has been explored. However, no *P. brachyurus*-resistant soybean varieties have been identified so far [42]. Although several soybean genotypes have been studied regarding their resistance against this nematode [43], breeding resistant cultivars against *P. brachyurus* is difficult due to the fact that this nematode is polyphagous and lacks a close interaction with their hosts [41]. Hence, current management recommendations for reducing *P. brachyurus* in soybean include crop rotation or succession with non-host, as well as fallow [35].

Seed treatment with nematicides has also been suggested to control *P. brachyurus*. According to Bessi et al. [44], seed treatment with nematicides can be an effective control method for this nematode, since it avoids damage in early stages of plant development, which stimulates the development of roots and minimizes the effect in case of drought stress. Several studies reported the effect of the seed treatment in reducing *P. brachyurus* population level in soybean [45]. Nonetheless, studies reporting the effectiveness of the seed treatment in controlling this nematode are still quite rare, and its use is questionable due to high cost of nematicides and low effectiveness due to short period of time in which plants are protected.

2.4. Reniform nematode (*R. reniformis*)

The reniform nematode, *R. reniformis,* was first described in 1940 infecting cowpea roots (*Vigna sinensis* Endl.) in Hawaii [46]. As a plant parasitic nematode, it was first reported in cotton in Georgia and in tomato in Florida [47]. This nematode belongs to the phylum of Nematoda, class of Chromadorea, subclass of Secernentia, order of Rhabditida, superfamily of Tylenchoidea, family of Hoplolaimidae, genera of *Rotylenchulus* and species of *R. reniformis,* reviewed in Ref. [48].

R. reniformis has a broad host range and parasitizes more than 140 plant species within 46 plant families. It parasitizes ca. 57 plant species with economic importance. Among the plants, cotton, pineapple, sweet potato and soybean are the most affected ones [49]. Other crops have been rated as good hosts to *R. reniformis*, including tea, tomato, kidney bean, cowpea, pigeon

pea, castor bean, passion fruit (purple/yellow), melon, papaya, potato, okra, citrus, coffee, banana and a significant number of weeds. The nematode *R. reniformis*, therefore, is considered a cosmopolitan species and is currently widely distributed in tropical, subtropical and temperate areas of South America, North America, Caribbean, Africa, southern Europe, the Middle East, Asia, Australia and the Pacific [50].

Sikora et al. [51] studied *R. reniformis* survival in soybean fields in Alabama/USA and found that the nematode showed a 32% survival rate, being the most common plant parasitic nematode present in the area and that the nematode may significantly reduce soybean yield and cause economic losses as well. The authors also hypothesized that the high incidence of the nematode was probably due to a change from cotton to soybean.

R. reniformis is widely distributed in Brazil and is found to parasitize soybean, pineapple, banana, coffee, castor bean, passion fruit, tomato and cotton [48]. From the end of the 1990s, the reniform nematode has increased its importance in soybean, especially in the west of Brazil where soybean is planted following cotton crop. It is estimated that currently, the nematode occurs at high population densities in about 29% of areas planted with soybeans in Mato Grosso State, one of the main soybean-producing states in Brazil [11]. *R. reniformis* has also been reported in other soybean-producing regions in Brazil. However, information about yield losses and economic damage is scarce [19].

The genera *Rotylenchulus* have 10 species, and they are considered semi-sedentary endoparasitic nematodes whose life cycle comprises the following sequence of events: worm shape sexually immature females are the infective stage. Females migrate in the soil searching for soybean roots or another suitable host and penetrate them until reaching the anterior region of the parenchyma pericycle. There, the nematodes inject special proteins to induce specialized cells (nurse cells) to feed them until the end of their life cycle. Once nematodes begin to feed, they become sedentary and have gradually swollen bodies, until they reach sexual maturity. The portion of the female body that stays out of the root acquires a shape similar to a kidney, from which the nematode name 'reniform' comes. Females lay eggs in egg masses (50–120 per mass) on the surface of rootlets. The optimum temperature is around 30°C. The proportion of males and females is approximately 1:1, and they reproduce by sexual reproduction called amphimixis. However, some populations can reproduce by parthenogenesis, and in this case, the males are rare or absent. *R. reniformis* males do not parasitize roots of soybean, and their stylets and esophaguses are much less developed compared to females, reviewed in Ref. [48].

Symptoms caused by *R. reniformis* in soybean somewhat differ from those caused by other nematodes. Soybean crops grown in infested soils are characterized by uneven growth, with large areas of undeveloped plants, which resembles mineral deficiency or soil compaction. There is also no occurrence of typical yellowing spots. Roots parasitized with *R. reniformis* do not show hypertrophy and hyperplasia of cells and do not form galls. The root system shows poorer development and in some points of the root can be seen a layer of soil adhered to the egg masses of the nematode, which are produced outside the root tissue. Yet, unlike other nematode species occurring in soybeans, the reniformis nematode does not seem to have its occurrence limited by soil texture, occurring in sandy soils as well as in clay soils. *R. reniformis* usually is the predominant species in clay soils [11].

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The wide host range of *R. reniformis* is a limiting factor for the use of crop rotation to manage this nematode species. An alternative is the use of cover crop such as *C. spectabilis* or its use in consortium with maize which has shown good results in decreasing nematode population level. In fact, this consortium was the most effective way to reduce this nematode population [31]. The reniform nematode is one of the major nematode species infecting cotton, maize and soybean in central Brazil. Considering its high aggressiveness to cotton and the increase in areas planted in rotation/succession with cotton, soybean and maize in central Brazil, it is very important to know which cultivars are indicated for planting in these areas [52].

A promising alternative control to *R. reniformis* is the use of crops such as rice and peanut and the *Brachiaria* grass at an integration scheme with crop/animal husbandry or in rotation with soybean or cotton. In addition, crops grown in autumn/winter that are used as cover crop in tillage systems and are resistant to *R. reniformis*, including *Brachiaria*, turnip, sorghum, black oat, millet and the Indian goose grass are good option as well. Growers should avoid the cultivation of amaranth and quinoa, which are both susceptible. Due to variation observed within varieties/hybrids of different plant species, it is recommended to test for host suitability prior to planting these varieties. The reniform nematode is well persistent in the soil, and depending on its population density, there may be a need for at least two years of cultivation with non-host species in order to decrease its population level [11].

Considering the host resistance, some soybean varieties are recommended against *R. reniformis*. For example, soybean cultivar BRS 399RR is tolerant to glyphosate and resistant to multiple nematode species, including the root-knot nematodes *M. incognita* and *M. javanica*, the reniform nematode *R. reniformis* and races 3 and 14 of the cist nematode *H. glycines*, and shows tolerance to the lesion nematode *P. brachyurus* [22]. Soybean cultivars BRS 359RR and BRS 360RR have also been reported as resistant to *R. reniformis*. These are short-cycle cultivars allowing planting of second maize cropping within a planting year besides being resistant to this nematode [23].

Additionally, other sources of resistance have been assessed against *R. reniformis*. For instance, Asmus [52] reported some soybean cultivars as resistant to this nematode, which had similar performance in comparison with the resistant control cultivar Custer. Except for the PI88788, soybean cultivars that have been rated as resistant to the cyst nematode also conferred resistance to *R. reniformis* [52]. The soybean cultivars 'M-SOY 8001' and 'CD 201' have also been reported as resistant to *R. reniformis* [53].

3. Other emerging nematode species threatening soybean production

Lately in Brazil cases of unusual, emergent nematode species affecting soybean fields have been reported. For instance, the nematodes *Aphelenchoides* sp. [54], *T. tuxaua* [7], *Scutellonema brachyurus* [55] and *Helicotylenchus dihystera* have been found in high population densities with fast dissemination in soybean fields, in which infected plants have shown pronounced symptoms typical of nematode infection.

A new soybean disease named 'crazy soybean' is caused by *Aphelenchoides* sp. [55] and characterized by reduced yield. Due to the disease, abortions of flowers and pods and distorted

leaves have been seen in some regions of soybean-producing areas. The intensified land use with major monoculture crops and unsustainable management practices has resulted in new problems associated with nematode infection. In future studies, it is important to determine the real distribution of nematode in soybean fields, the mechanism by which nematode decreases soybean yield and the ways to manage nematode [56].

4. Concluding remarks

This review has shown that there are a significant number of plant parasitic nematodes negatively impacting soybean yield worldwide. Due to intensified land use, monocultures and the use of unsustainable management practices, uncommon nematode species are becoming a new threat to soybean production. This imposes a real challenge to researches to search promptly for new approaches to manage these nematode diseases. Researches and growers should recognize that in fact, nematode diseases negatively impact soybean yield and that sustainable management practices should be considered in order to decrease nematode population level and have a good yield performance. When deciding the management approaches, it is essential to consider them holistically.

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Chapter 7

Application and Conversion of Soybean Hulls

Hua-Min Liu and Hao-Yang Li

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66744

Abstract

Soybean is one of the most cultivated crops in the world, with a global production of approximately 240 million tons, generating about 18–20 million tons of hulls, the major by-product of soy industry. The chemical composition of soybean hulls depends on the efficiency of the dehulling process, and so, the soybean hulls may contain variable amounts of cellulose (29–51%), hemicelluloses (10–25%), lignin (1–4%), pectins (4–8%), proteins (11–15%), and minor extractives. This chapter provides a review on the composition and structure of soybean hulls, especially in regard to the application and conversion of the compositions. Current applications of soybean hulls are utilizations to animal feed, treatment of wastewater, dietary fiber, and herbal medicine. The conversion of soybean hulls is concerned with ethanol production, bio-oil, polysaccharides, microfibrils, peroxidase, and oligopeptides. On the basis of the relevant findings, we recommend the use of soybean hulls as important source on environment, energy, animal breeding, materials, chemicals, medicine, and food.

Keywords: soybean hulls, application, conversion, dietary fiber, polysaccharides

1. Introduction

Soybeans are one of the most worthy crops in the world because of their high protein and oil content, which provides a wide variety of uses [1]. Soybean protein has been used in livestock and aquaculture feeds and is highly digestible, along with many human foods [2–4]. Soybean oil is used as a food and feed ingredient as well as in biodiesel production and cosmetics [5, 6]. Soybean hulls, accounting for a substantial fraction (7–8%) of the total mass of soybean, are the largest amount of by-products in the soybean process industry. In contrast to the oil and proteins, there is a fairly common perception that hull is a "waste" product of soybean processing [7]. It is predicted that the total world soybean production will be 371.3 million tons by 2030 and there will be 29.7–37.1 million tons of soybean hulls available [8].

The chemical composition of soybean hulls depends on the efficiency of the dehulling process, and so, the soybean hulls may contain variable amounts of cellulose (29–51%), hemicelluloses (10–25%), lignin (1–4%), pectins (4–8%), proteins (11–15%), and minor extractives [9–11]. Therefore, soybean hulls are primarily lignocellulose material. However, unlike many other lignocellulosic material such as hardwood or switchgrass, soybean hulls are easy degradable [9, 11]. Chemically, cellulose is a linear polymer of 250 to over 10,000 glucose units linked by β-1,4 glycosidic bonds. Pectin is a polysaccharide consisting of a backbone of α-1,4 linked galacturonic acid residues usually up to 100 residues in length. The galacturonic acid residues are commonly methylesterified or acetylated, and the backbone may include substitutions of rhamnose and/or branching chains consisting of arabinose and galactose [12]. Hemicellulose is a group of wall polysaccharide that is characterized by being neither cellulose nor pectin and by having β-1,4-linked backbone of glucose, mannose, or xylose [13]. The backbone is frequently decorated with a variety of sugar side chains or acetyl ester groups [14]. The average degree of polymerization of hemicellulose is in the range of 80–200. Lignin is a heterogeneous biopolymer in lignocellulose formed by radical-mediated oxidative coupling of phenyl-propane unit linked together through various types of ether and carbon-carbon bonds [15].

The low lignin content in soybean hulls makes the residues have a very wide variety of application (**Figure 1**). Due to this biomass composition, soybean hulls are widely used as animal feed [16]. In addition, soybean hull is lignocellulosic material containing a small proportion of lignin, as compared with other agro-residues, and has a good potential for saccharification, because lignin is a major hindrance for enzymatic hydrolysis of biomass [17]. Soybean hulls also contain a large amount of dietary fibers (DFs), and have been used as a batter ingredient to decrease the fat contents in cakes and cookies [18]. Moreover, soybean hulls have also been identified as a rich source of peroxidases and as an agro-industrial residue; they are a low-cost alternative for resulting in biocatalyst production [19]. This review summarizes the present knowledge on the composition, application, and conversion of soybean hulls.

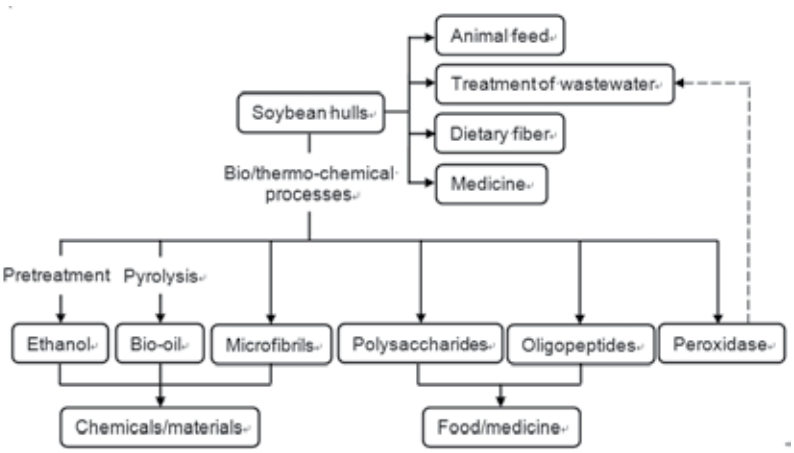

Figure 1. Application and conversion of soybean hulls.

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2. Compositions and structure of soybean hulls

2.1. Cellulose

Cellulose derived most frequently from wood is widely used in a range of applications including composites, papermaking, food additives, textile, and pharmaceutical industries [20]. More importantly, cellulose is also useful for bio-ethanol production after enzymatic hydrolysis. Cellulose is a linear polymer of anhydroglucose unit linked at the one and four carbon atoms by a β-glycoside bond [21]. This is confirmed by the presence of three hydroxyl groups with various acidity/reactivity, secondary OH at the C-2, secondary OH at the C-3, and primary OH at the C-6 position, and accordingly, by the formation of different strong intermolecular hydrogen bonds [22]. Based on carbon nuclear magnetic resonance (^{13}C NMR) spectra and X-ray diffraction patterns, four major polymorphs of cellulose have been reported and named cellulose I, II, III, and IV [23]. Cellulose I is the most abundant native crystalline form and can be converted into the other polymorphs through a variety of treatments. Cellulose I consists of two phases, I_α and I_β. Cellulose I_α has one-chain triclinic structure and cellulose I_β has two-chain monoclinic structure and they differ in hydrogen bonding [24]. The chemical, physical, and biological properties of cellulose depend on its shape properties such as its ease of deformability and its intrinsic form [23]. The noncrystalline cellulose is also important because of higher chemical reactivity of noncrystalline (or amorphous) cellulose.

2.2. Hemicellulose

Hemicellulose, next to cellulose, refers to a large group of complex polysaccharide in cell wall of plants [25]. Unlike cellulose, it is a low-molecular-weight polysaccharide, associated in plant cell wall with lignin and cellulose. It forms covalent bonds (mainly α-benzyl ether linkages) with lignin, hydrogen bonds with cellulose, and ester linkages with hydroxycinnamic acids and acetyl units, which restrict the liberation of hemicellulosic polymers from the cell wall matrix [26]. Large variations in hemicellulose content and chemical structure can occur between various lignocellulosic materials. Many methods have been used to isolate hemicellulosic polymers from plant materials, which include extraction with alkaline, alkali, organic solvent, or twin-screw extrusion and ultrasonication treatments, as well as steam or microwave treatment [26]. For higher lignin content materials, they must be delignified and/or pretreated in some way prior to extraction of hemicelluloses, such as pretreatment by sodium chlorite in acetic acid solution. For soybean hulls, they do not require delignification prior to isolation of hemicelluloses, as compared with other lignocellulosic biomass, because of low content of lignin. The major hemicelluloses in soybean hulls are composed of α-L-arabinofuranosyl, L-arabino-4-O-methyl-D-glucurono-D-xylan, 4-O-methyl-glucuronic acid and α-D-galactose units attached with substituted sugars [27, 28]. These hemicelluloses have the potential to be integrated in a wide variety of applications, including thickeners, film-former substances, emulsifiers, binders, and stabilizers in the food, cosmetic, and pharmaceutical industries [29]. In addition, they can be easily hydrolyzed into hexose (mannose, glucose, and galactose) and pentose (arabinose and xylose), and can be transformed into fuel ethanol and other value-added chemicals, including furfural, 5-hydroxymethylfurfural (HMF), xylitol, and levulinic acid (**Figure 2**) [30].

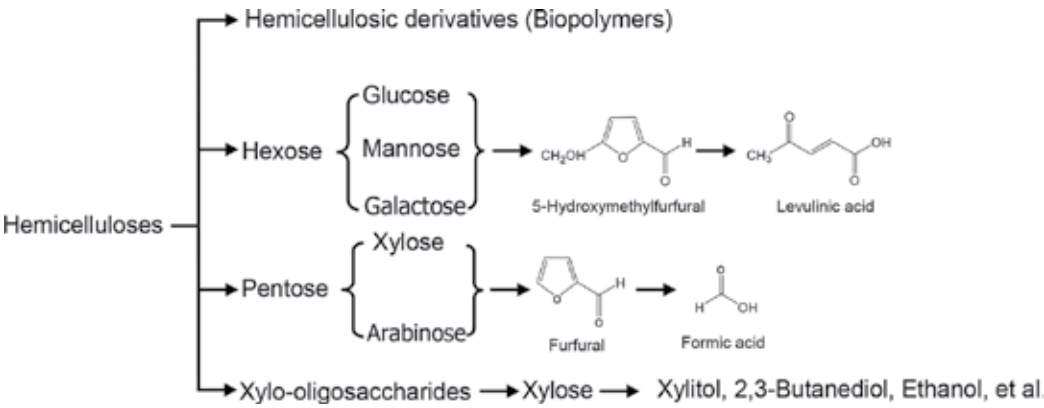

Figure 2. The potential products from hemicelluloses [13].

2.3. Pectin

Pectin is a complex polysaccharide consisting of D-galacturonic acid linked by α-1,4 glycosidic linkages [31]. The molecular weight of pectin varies from 50,000 to 150,000 Da depending on the source materials and extraction procedure. Pectin is a highly valuable functional food ingredient and is very important in creating or modifying the texture of jellies, jams, and confectionery, and in low-fat dairy products. Soybean hulls were potentially inexpensive commercial sources of pectin. Soybean hull pectin (SHP) mainly contains galactose, xylose, galacturonic acid, arabinose, glucose, and rhamnose. The chemical composition of the extracted soybean hull pectin has been comparatively investigated with that of commercially soybean hull pectin (CSHP) and citrus pectin (CP) by Yamaguchi et al. (**Table 1**) [32]. The results showed that SHP had a molecular weight similar to the CSHP and CP. Glucose content in SHP was higher as compared with CSHP and CP, but other sugar contents were

Composition	SHP	CSHP	CP
Galacturonate, % Dry material basis	33.0	18.5	85.8
(% Esterified galacturonate, % Dry material basis)	18.1	0	73.7
Neutral sugar composition, % Dry material basis			
Rhamnose + Fucose	8.0	8.0	25.1
Arabinose	24.2	26.3	15.6
Xylose	2.7	2.5	1.8
Mannose	0	0	0
Galactose	49.8	59.5	49.5
Glucose	15.3	3.7	8

Table 1. Composition of soybean hull pectin (SHP), commercially available soluble soybean hulls pectin (CSHP), and citrus pectin (CP) [32].

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similar between SHP and CSHP. The SHP had a similar galacturonan structure to that of CP, but SHP contains more arabinose and glucose, less rhamnose and fucose, and more xylose as compared with CP. The SHP extracted by Yamaguchi et al. [32] showed the degree of esterification of 18.1%, belonging to low methoxyl pectin.

2.4. Lignin

Lignin is a three-dimensional amorphous biopolymer formed by three major monolignols, that is, p-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S) of various ratios,

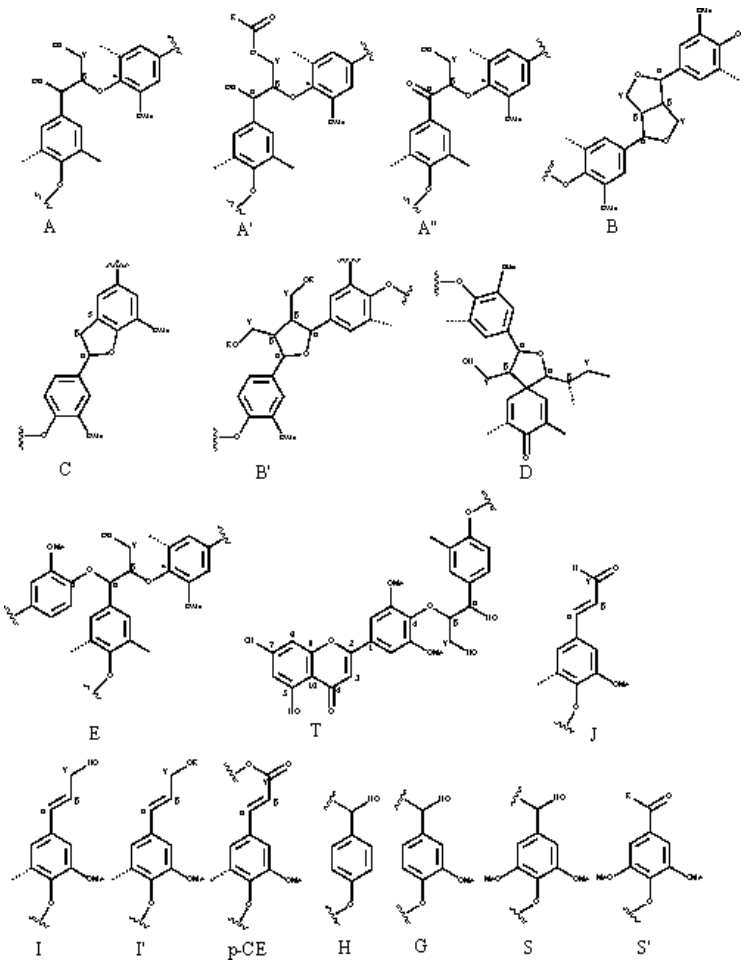

Figure 3. Main lignin structures present in bamboo lignin: (A) β-O-4 alkyl-aryl ethers; (A′) β-O-4 alkyl-aryl ethers with acylated γ-OH with p-coumaric acid; (A″) Cα-oxidized β-O-4 structures; (B) resinols; (B′) tetrahydrofuran; (C) phenylcoumarans; (D) spirodienones; (E) α, β-diaryl ethers; (T) a likely incorporation of tricin into the lignin polymer through a G-type β-O-4 linkage; (I) p-hydroxycinnamyl alcohol end groups; (I′) p-hydroxycinnamyl alcohol end groups acylated at the γ-OH; (J) cinnamyl aldehyde end groups; (p-CE) p-coumarates; (H) p-hydroxyphenyl units; (G) guaiacyl units; (S) syringyl units. (S′) oxidized syringyl units bearing a carbonyl at Cα [33].

linked together by different types of ether (β-O-4′) and carbon-carbon (β′-β′ and β′-5′) linkages (**Figure 3**). Besides, lignin is covalently linked to hemicellulosic polysaccharides, forming a lignin-hemicellulose network made up of phenyl-glycoside, benzyl-ether, and benzyl-ester bonds [34]. Despite the extensive investigations of lignin, the complex and irregular structure of lignin has not been completely understood up to now. Lignin is considered as the most abundant sources of aromatic compound in nature and can be utilized for adhesives or chemical reagents to replace those derived from oil. For the lignin of soybean hulls, it is not usually utilized as a major value product, due to its lower content. However, the soybean hull is a good resource for lipid production due to its low lignin content and it has been proven in the bioconversion process that soybean hulls can be utilized without any pretreatment [9].

2.5. Protein

The chemical composition of soybean hulls depends on the efficiency of the dehulling process. If soybean meal with high protein content is required, the dehulling process is more intense in order to avoid contamination of the meal with pieces of hulls [10]. In general, the soybean hulls may contain 11–15% of proteins. Soybean proteins are commercially and extensively used in food products due to their functional properties, low cost, and high nutritional value. Soybean proteins are composed almost exclusively of two globular protein fractions called 11S (glycinin) and 7S (β-conglycinin) [35].

3. Application of soybean hulls

3.1. Animal feed

The by-products of agro-industrial may become an economical alternative to corn grain in ruminant diets, especially when the price of corn is high due to the increase of demand from the ethanol industry [36]. Soybean hulls are by-product from the soybean-processing industry, where the soybean is de-hulled leaving a highly digestible, fibrous feed [37]. Due to their compositions, the biomass is widely used as animal feed. Many investigations have demonstrated that there are advantages of using soybean hulls as an energy source for ruminants in replacement of corn, as long as they are supplied together with effective fiber sources to reduce the rate of passage and enable ruminant fermentation [38–40]. For example, the excessive use of starch in equine diets can lead to fermentation of the ingested material by amylolytic bacteria in the large intestine resulting in an increase in lactic acid production and increased production of short-chain fatty acids, which can cause intestinal disorders such as laminitis or colic [41]. However, studies on the inclusion of soybean hulls in equine diets have shown promising a decrease in starch level without compromising the caloric density of the feed [42]. It was suggested that diets with up to 28% soybean hulls can be used as equine feed without negatively affecting digestibility, the selected microbiota or short-chain fatty acids concentrations, and physicochemical characteristics in the feces [38]. Soybean hulls can also be a resource in maintaining sheep meat production without compromising product quality. Investigation has been carried out for the improvement of sheep diets by soybean hulls, which leads to the

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improvement of the fatty acid composition of meat and the production of meat with adequate levels of fat which reduce the levels of saturated fatty acids [43]. The investigation found that the inclusion of soybean hulls in the sheep diet increased the total lipid content, conjugated linoleic acid, and omega 3 fatty acids. The increase of unsaturated and polyunsaturated fatty acids ensured greater consumer satisfaction, since the population was increasingly attentive to health. Soybean hulls can also be used replacing ground corn in diets of goats in the early lactation, because they improve the digestibility of the diet and nutrients, do not change the physical and chemical quality or productive performance of the milk, and increase the content of omega 3 fatty acids in the milk [44]. Soybean hulls can replace corn grain to supply about 30% of the dry matter in high-grain content diets without negatively affecting either the digestion of nutrients or fermentation in gastrointestinal tract or the performance of dairy cows [45]. Vinay Kumar [46] investigated the effect of soybean hulls on the physicochemical characteristics, color, texture, and storage stability of chicken meat nuggets. The results showed that the addition of soybean hulls to chicken nuggets improved nutritional value, sustained the desired cooking yield and emulsion stability, and helped in improving instrumental textural and color values. In addition, the inclusion of soybean hulls in the chicken diet increased the storage times of meat.

3.2. Treatment of wastewater

Fresh water is a limited and essential natural resource for the development of a series of living organisms in aquatic environments as well as for humans, all of which require its preservation [46]. The quality of the water is being negatively affected by the world's population growth along with accelerated industrial development that generally involves processes requiring a huge consumption of water and the release of wastewaters back into water bodies [46]. Current methods used to treat wastewater include chemical precipitation, oxidation and chemical reduction, filtration, electrochemical treatment, ion exchange, reverse osmosis, evaporation, and adsorption [47, 48]. Among these techniques, adsorption is an economic and efficient method, based on flexible and simple operating conceptions and the use of regenerative adsorbents, for the removal of inorganic or organic pollutants with high efficiency in many cases [48]. Biosorption is the binding of radionuclides and metal ions onto the cellular structure of biological materials, which contain their functional groups and ligands [49]. Biosorbent materials that are lignocellulosic, containing cellulose, hemicelluloses, and lignin, have high adsorption properties due to the ion exchange capabilities [50]. Biosorbent materials have some advantages. For example, they can be regenerated for reuse, can recover the biosorbent material, do not require much energy input, and do not produce a toxic sludge [49, 51]. Much attention has been given to the use of soybean hulls in the remediation of heavy metals [46, 49, 52]. Soybean hulls without the soluble dietary fiber (SDF) present good metal-binding property and can be used as novel biosorbent [53]. The preparation of soybean hulls including pretreatment, drying, modification, activation, and so on was presented to make the preparation process feasible and economical [46, 54, 55]. Generally, adsorption of inorganic or organic pollutants in wastewater by soybean hulls has been limited and the hull modification is desirable to enhance adsorption especially of metal ions. Aparecido N. Módenes [46] investigated the absorption characteristic of the soybean hulls absorbent by

various modification methods for the removal of Cd2+ and Pb2+. The results showed that an increase in the sorption capacity of Pb2+ ions of around 20% was achieved as compared with the unmodified material and that an insignificant improvement in the sorption capacity of Cd2+ ions was obtained when the soybean hulls were modified by treating them with strong base (0.1–1.0 M NaOH). Functional groups such as phosphoryl, hydroxyl, and carboxyl could be the activated sites on soybean hulls sorbent, with metal ion uptake on a neutral sorbent surface occurring via an ion exchange process [46]. The addition of surface functional groups by chemical reaction with NaOH could be responsible for the increase in the biosorbent surface area and consequently greater metal sorption capacity as compared with the untreated material [56]. Investigation has demonstrated that soybean hulls work well at removing textile dyes from contaminated water [52]. Results of the investigation indicated that the soybean hulls and rice hulls worked well at removing the Safranin T and Direct Violet 51 dyes from solution. The soybean hull samples were more effective at removing the Remazol Brilliant Blue R dye as compared with rice hull samples.

3.3. Dietary fiber

Dietary fiber can be defined as "the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine" [57]. Dietary fiber is a complex component of natural carbohydrate polymer which consists of a variety of nonstarch polysaccharides such as hemicellulose, cellulose, lignin, and pectin [58]. The beneficial role of dietary fiber in health and nutrition has been demonstrated in normal gastrointestinal and physiological functions, including carbohydrate and lipid metabolism, and in the reduction of chronic ailments such as coronary heart disease, diabetes, obesity, and some cancers [59]. Dietary fiber can typically be divided into soluble dietary fiber and insoluble dietary fiber (IDF). SDF includes pectins and some hemicelluloses. Cellulose, lignin, and some hemicelluloses are examples of dietary fiber classified as IDF. Soybean hulls contain the majority of the fibers with a higher level of IDF. Acid-base hydrolysis and autoclaving significantly affect the SDF, IDF, and total DF distribution in soybean hulls [60]. Kumar et al. [61] and Goldnon and Brown [62] reported that 4% addition of soy hull flours had no impact on the cooking yield and texture of chicken nuggets and pork patties, respectively. Investigation by Kumar et al. [63] indicated that 3–5% addition of soybean hull flours slightly improved emulsion stability and water-holding capacity of chicken nuggets. Kim et al. [64] indicated that insoluble fiber from soybean hulls through acid and alkali hydrolysis influenced positive effects on reduction in cooking loss and increase in hardness of meat without any adverse effect on springiness and cohesiveness, and minimized color alteration. The investigation also indicated that acid-base hydrolysis and autoclaving processes in soybean hulls could significantly boost total dietary fiber content, showing the great potential in various food applications due to the functional properties [60].

3.4. Medicine

Soybean with black, brown, yellow, and green seed coats possesses antioxidant capacity varying with color because of differences in phenolic levels and composition which is anthocyanins, phenolic acids (chlorogenic and caffeic acids), isoflavones, and proanthocyanidins

[65–67]. Black soybean has been used as an herbal medicine to treat edema and jaundice. It has also been used to treat enuresis by affecting the functions of the spleen and kidney [68]. The hull of the black soybean has been used for the treatment of headache and vertigo, as well as for detoxification and diuresis [68]. Black soybeans were reported to contain anthocyanins and only brown and black soybeans contain proanthocyanins [66, 67]. Investigation showed that black soybean had the highest antioxidant activity as compared with other colored seed coat soybeans [65]. The antioxidant activity of back soybeans is related to their phenolic pigments in the seed coats [69, 70]. In vitro anticancer investigation reported that polysaccharides from black soybean may induce differentiation and inhibit proliferation in human leukemic U937 cells [71]. Anthocyanins isolated from black soybean hulls display growth inhibitory effects and strong apoptosis induction effect against human leukemia Molt 4B cells [72]. Animal experiments indicated that the intake of extract from black soybean hulls effectively enhanced memory and learning ability in rats [73]. The extract of the black soybean hulls has also been used as dietary ingredient including pigments and nutraceuticals [68].

4. Conversion of soybean hulls

4.1. Ethanol production

The National Biofuels Action Plan released in October 2008 states that expanding annual biofuels production to 36 billion gallons by 2022 would be a key component in America's movement toward clean, affordable, and secure energy sources [11]. The interest for ethanol production from renewable resources has increased in the last decade, directly related to environmental and economic concerns over fossil fuels [74]. Currently, ethanol is mainly produced from sugarcane and corn (in the Brazil and USA, respectively), accounting for 66% of worldwide production [75]. However, recently, there has been increasing interest in cellulosic ethanol production, because biomass is an abundant feedstock that is inexpensive and has a high cellulosic content [10, 76]. Lignocellulosic biomass needs to be decomposed into its monomers in order to release fermentable sugars, and which is achieved by using diluted acids or enzymes. The cellulose in the biomass is scarcely affected by the diluted acid hydrolysis, requiring other physicochemical hydrolyses at higher temperatures to result in sugar decomposition, which may lead to metabolic inhibition during fermentation [77]. Lignocellulosic biomass mainly consists of lignin, cellulose, hemicelluloses, and small amounts of extractives. Cellulose structure allows the formation of intermolecular and intramolecular hydrogen bonds, generating organized rigid crystalline regions. The biological role of hemicellulose is the cross-linked interaction with lignin and cellulose, which strengthens the cell wall and embedding of the crystalline cellulose elementary fibrils [78]. The close association between hemicellulose and lignin impedes enzyme access to hemicellulose, which in turn affects accessibility to cellulose [79]. Thus, pretreatment by various technologies is a crucial prerequisite to break down the rigidity of the biomass prior to enzyme hydrolysis process. Soybean hulls are an agricultural residue produced during the processing of soybeans, and the lignocellulosic material contains a small proportion of lignin (1.4–2%) when compared to other biomass. Therefore, soybean hulls are an attractive source of fermentable sugars for cellulosic ethanol

production. Hickert et al. [74] investigated the conversion of pentoses and hexoses liberated from high osmotic pressure soybean hull hydrolysate into ethanol by various immobilized cerevisiae. The soybean hulls were hydrolyzed in a two-step sulfuric acid-enzyme pretreatment, resulting in more than 72% of saccharification. The yields of bioconversion of soybean hulls into ethanol were 38–47%. Physicochemical pretreatments of soybean hulls for hemicellulose removal were essential in order to improve the material digestibility at the enzymatic hydrolysis stage. Cassales et al. [80] investigated various acid concentrations in order to achieve high sugar release and low generation of toxic compounds. Yoo et al. [11] studied the pretreatment of soybean hulls by thermomechanical extrusion. Mielenz et al. [9] reported high yields of ethanol by simultaneous saccharification and fermentation of soybean hulls without pretreatment, because of the low lignin content. However, the time of fermentation was very long (about 9 days). Rojas et al. [10] reported a process for the recovery of proteins from soybean hulls, mainly as oligopeptides, and the production of ethanol from the remaining lignocellulosic fraction. In addition to ethanol production from soybean hulls, Zhang and Hu [81] studied a new application of soybean hulls to be converted to fungal lipids for biodiesel production through solid-state fermentation. The results showed that the total final lipid reached 47.9-mg lipid from a 1-g soybean hull after the conversion, which is 3.3-fold higher as compared with initial lipid reserve in the soybean hulls. The solid-state fermentation is a more cost-effective process because of low-energy expenditure, its low capital cost, less expensive downstream processing, high volumetric productivity, low wastewater output, and less fermentation space needed [82].

4.2. Bio-oil

Lignocellulosic biomass can be converted into useful form of energy using biochemical and thermochemical processes, but thermochemical conversion technology finds its dominance due to high efficient conversion to gas, liquid, and solid products under thermal conditions [83]. The liquid product called bio-oil is a complex mixture of water and organic chemicals, which are alcohols, aldehydes, acids, ketones, esters, heterocyclic derivatives, and phenolic compounds [84]. There are two typical thermochemical processes to produce liquid product with high yield: pyrolysis and liquefaction. During the pyrolysis processes, the biomass feedstock is heated in the absence of air to a high temperature (400–1000°C), resulting in the formation of bio-oils and gaseous products. Another important method to convert the biomass into liquid fuel is liquefaction in solvents (such as acetone, ethanol, water, or their mixtures) by heat. By the method, biomass can be decomposed into liquid at a mild temperature and a high pressure as compared with the pyrolysis process [85]. Oliveira et al. [86] studied soybean hull bio-oil produced by fast pyrolysis. The main components of the bio-oil were analyzed by gas chromatography/mass spectrometry (GC/MS). The results indicated that the soybean hull bio-oil can be used as an alternative source of chemical products with higher added value. As a result of the decomposition of cellulose, hemicellulose, and lignin, the soybean hull can be transformed into products having various molecular structures. The soybean hull bio-oil was proved to be a complex mixture of a variety of organic compounds (more than 60 compounds were identified) [86]. For the aqueous phase of the soybean hull bio-oil (acid extraction), the main compounds were pyridine (17.06%), acetic acid (9.12%), phenol (16.94%), pyrole (5.14%),

and acetamide (5.73%). The high acidity presented in the aqueous phase of soybean hull bio-oil is probably because of the thermal degradation of hemicelluloses, which produces acids as the final product of reactions involving the removal of acetyl groups [87]. Cellulose can be decomposed into levoglucosan at first, and then the levoglucosan can be generated by depolymerization reactions, which produce small quantities of acids, such as propionic acid and acetic acid, as well as furans (furfural, furfuraldehydes, and pyrans) [88]. In the organic phase, the main compounds identified in soybean hull bio-oil were phenol (14.88%), 4-methylphenol (12.55%), and 2-methylphenol (7.59%) [86]. The phenol compounds and derivatives were obviously due to the decomposition products from lignin (and maybe hemicellulose and cellulose). Those phenolic compounds can be separated from soybean hull bio-oil by using vapor distillation, reverse osmosis membranes, and solvent extraction [89–91].

4.3. Polysaccharides

Polysaccharides are species of macromolecular substance existing widely in organisms. It has been reported that plant polysaccharides or their derivatives have strong antioxidant activities and can be explored as novel potential antioxidants [92]. Some of the polysaccharides have been targeted as important candidates for the development of effective and nontoxic medicines with strong free radical-scavenging and antioxidant activities [93]. The insoluble carbohydrate fraction in soybean hulls contains 50% hemicelluloses, 30% pectins, and 20% celluloses [94]. Therefore, the soybean hulls are potentially commercial source of polysaccharides. Liu et al. [27] studied the extraction of soybean hull polysaccharides by hot-compressed water in a batch system. The results showed that a moderate temperature (160°C) and short extraction time (60 min) were suitable for the preparation of soybean hull polysaccharides. In the sugar composition of the polysaccharide products, arabinose constituted 35.6–46.9%. Nagata et al. [95] investigated the effects of soybean hull polysaccharides on serum immunoglobulin concentration and production of NO and interleukin-1β from peritoneal macrophages. The soybean hull polysaccharides consisted of arabinose, galactose, xylose, glucose, and rhamnose, and the molecular weight was 500,000. The investigation demonstrated that soybean hull polysaccharides enhanced humoral immunity and activation of macrophages, thereby leading to the augmentation of immune responses in rats.

4.4. Microfibrils

Microfibrillated cellulose developed for the first time in the early 1980s by Turbak and coauthors can be obtained through mechanical treatments such as refining and high-pressure homogenization [96]. Microfibrillar cellulose is a bio-based material with interesting intrinsic properties that make it attractive in many applications. It is characterized by a high specific surface area, flexibility, and crystallinity, and contains a large amount of hydroxyl groups [97], all of which influence its interactions in liquid dispersions or in solid films. Merci et al. [98] produced the microfibrillar cellulose from soybean hulls by using a simple method based on reactive extrusion. The reported microfibrillar cellulose produced from soybean hulls was composed of short and rod-shaped fibers, and had a cellulose content of 83.79% and crystallinity index of 70%. Miranda et al. [99] studied the kinetics of degradation process of cellulose extracted from soybean hulls and compared its behavior to commercial microcrystalline

cellulose under inert environment. The results indicated that kinetic degradation behavior of soybean hull cellulose was more similar to commercial microcrystalline cellulose. However, the activation energy value of commercial microcrystalline cellulose was higher as compared with soybean hull cellulose. Ferrer et al. [7] isolated cellulosic microfibrils (SMF) and brick-like microparticles (SMP) from soybean hulls by combining mechanical and chemical pretreatments. The SMF and SMP chemical compositions included residual polysaccharides and lignin that endow such biologically derived materials with properties typical of nanocellulosics. As compared with those of micro- and nanofibrillated cellulose obtained from fully bleached wood fibers, the SMF and SMP exhibited enhanced crystallinity and thermal stability. In addition, a strong shear-thinning behavior was observed for aqueous dispersions of SMF and SMP, revealing that cellulose microstructures are of interest for rheology modification, coatings, and films. These SMF and SMP extracted from soybean hulls have been used in films and also combined with wood-based micro- and nanofibrillar cellulose in hybrid systems [100]. The hybrid films displayed similar strength and barrier performance to those of neat nanofibrillar cellulose films, thus offering an option for reduced cost while keeping a performance from synergistic contributions of the components. Furthermore, dense films with low porosity, a characteristic essential for barrier properties, can be easily produced by replacing up to 75% of micro- and nanofibrillar cellulose with SMF or SMP.

4.5. Peroxidase

The extraction of enzymes from agro-industrial residues is an alternative for reducing costs in biocatalyst production. Soybean hull peroxidase (SHP, E.C. 1.11.1.7) is a glycoprotein that belongs to plant peroxidase superfamily that also includes horseradish (HRP), peanut, and barley peroxidases [101]. Because of the high thermostability, broad pH stability, and cheap source for production from soybean hulls [102], SHP is a more promising biocatalyst for industrial use as compared with the widely used HRP. SHP was previously used for the removal of aqueous phenols from wastewaters in stirred membrane reactor, as a bromination catalyst, for luminal oxidation, for the synthesis of polyaniline, and in organic solvents [103–106]. Then, higher-value commodities such as diagnosis tests and therapeutics would require more costly alternatives such as purified or recombinant peroxidases. Soybean hull peroxidase has a ferriprotoporphyrin IX prosthetic group located at the active site. The catalytic mechanism follows a peroxidase ping-pong mechanism involving the two-electron transfer from hydrogen peroxide to the heme, creating an oxidized form of the enzyme, "compound I." Successive one-electron reductions return the enzyme to its native or reduced state via an intermediate oxidized form of the enzyme, "compound II" [107]. As compared with free enzymes, immobilized enzymes offer more advantages, such as enhanced stability against various denaturing conditions, easier product and enzyme recovery, higher catalytic activity, continuous operation of enzymatic processes, reusability, and reduced susceptibility to microbial contamination [108, 109]. Chagas et al. [110] extracted peroxidase from soybean hulls and immobilized the enzymes on chitosan beads cross-linked with glutaraldehyde. The immobilized enzyme showed a potential of 50% in the oxidation of caffeic acid after four consecutive cycles.

4.6. Oligopeptides

Soybean oligopeptides produced by proteolysis or microbial fermentation techniques followed by purification protocols are widely used in the food industry. The soybean hulls

may contain 11–15% of proteins, and the proteins can be transferred into oligopeptides by various techniques. Most commercial productions of oligopeptides use batch hydrolysis, which depends on various factors such as protein denaturation, hydrolysis temperature, and protease specificity [111]. The hydrolysate of protein is a complex mixture of peptides with various lengths. Molecular size of the peptides has a major effect on functional properties. In general, smaller peptides with less than six amino acids have the greatest impact on cell growth and production [112]. Rojas et al. [10] published the results concerning the recovery of proteins from soybean hulls by hydrolysis, mainly as oligopeptides, and subsequent ethanol production from the remaining lignocellulosic fraction. The results indicated that soybean hulls might be a promising feedstock for the production of a high-value protein hydrolysate composed mainly of low-molecular-weight oligopeptides.

5. Conclusion

Soybean hulls are a major by-product in the soybean-processing industry, and have a variable chemical composition of cellulose (29–51%), hemicellulose (10–25%), lignin (1–4%), pectin (4–8%), proteins (11–15%), and minor extractives. The low lignin content in soybean hulls makes the residues have a very wide variety of applications. Due to their compositions, the soybean hulls are widely used as animal feed and have demonstrated the advantages of using as an energy source for ruminants in replacement of corn. Adsorption of inorganic or organic pollutants in wastewater by soybean hulls has been limited and the hull modification is desirable to enhance adsorption, especially of metal ions. The soybean hulls are potentially commercial source of ethanol production, dietary fiber, microfibrils, polysaccharides, and pectin. Soybean hulls can be converted into useful form of energy such as bio-oil by thermochemical processes. The extraction of peroxidase from soybean hulls is an alternative for reducing costs in biocatalyst production. The peroxidase has been used for the removal of aqueous phenols from wastewaters in stirred membrane reactor, as a bromination catalyst, for luminal oxidation, for the synthesis of polyaniline, and in organic solvents. The protein content in soybean hulls has produced a high-value protein hydrolysate composed mainly of low-molecular-weight oligopeptides.

6. Acknowledgment

We sincerely acknowledge the financial support by the Fundamental Research Funds for the Henan Provincial Colleges and Universities (2014YWQN01).

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Chapter 8

Effects of Drought and Elevated Atmospheric Carbon Dioxide on Seed Nutrition and ^{15}N and ^{13}C Natural Abundance Isotopes in Soybean Under Controlled Environments

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/67511

Abstract

The objective of the current research was to evaluate the effects of drought and elevated CO_2 on seed production and seed nutrition under controlled conditions in soybean. Soybean plants were subjected to ambient and elevated CO_2 and under irrigated and drought conditions. The results showed that drought or drought with elevated CO_2 resulted in high protein and oleic acid, but low in oil and linoleic and linolenic acids. Significant decrease of sucrose, glucose, and fructose concentrations was noticed, but high content of raffinose and stachyose was observed. Nutrients such as N, P, K, and some micro-nutrients were reduced under drought or drought with normal or elevated CO_2 concentrations. Seed δ ^{15}N ($^{15}N/^{14}N$ ratio) and δ ^{13}C ($^{13}C/^{12}C$ ratio) natural abundance isotopes were also altered under drought or drought with ambient or elevated CO_2 concentrations, reflecting nitrogen and carbon metabolism changes. The current research demonstrated that global climate changes may lead to changes in seed nutrition, and nitrogen and carbon metabolism. Efforts of breeders to select for these traits will sustain food source and food security for humans and livestock as soybean is a major source for protein and oil for human consumption and soymeal for animals.

Keywords: elevated carbon dioxide, climate change, seed composition, seed nutrition, drought, soybean

1. Introduction

Global climate changes due to elevated CO_2 are expected to lead to high heat and drought in some regions, affecting crop production and seed nutrition [1]. Climate change, whether is caused naturally or human-made, threatens crop production and food security. It was reported that food systems that include food availability, food access, and food utilization will suffer when food systems are stressed [1]. It is predicted that climate change will alter the food systems through its effects on crop production and rainfall, leading to drought or flooding or warmer or cooler temperatures [1]. Global warming resulted from climate change leads to high risk of drought and warmer temperatures, resulting in an increase of water demands and evaporation (http://www.c2es.org/science-impacts/extreme-weather/drought) [2]. The USA historically has suffered major droughts, and the living memories of the Dust Bowl of the 1930s or the drought of the 1950s and recently the droughts of 2011 in Texas and 2012 in the USA are examples, highlighting our vulnerabilities to drought as we move forward to warmer and drier climate (http://www.c2es.org/science-impacts/extreme-weather/drought) [2]. It was also reported that a doubling of CO_2 concentration could result in changes in global climate, including precipitation patterns, resulting in drought conditions in some areas of the world [3]. Generally, elevated CO_2 concentration during crop growth enhances CO_2 exchange rate and final yield [4] as growth at elevated CO_2 stimulates photosynthesis and increases carbon supply in C_3 crops and enhances nutrient supply to match the increase in C acquisition [4]. However, drought stress results in a decrease in CO_2 exchange rate. The mechanisms involved in the reduction of CO_2 exchange rate are still not well understood, although part of the reduction was attributed to stomatal closure [5], leaf water potentials [6], decreases in activities of Photosystem I and Photosystem II [7–9], effects on assimilation products, and photosynthetic enzyme activities [6].

The effects of climate changes on yield were previously reported [1], and it was shown that severe drought stress can lead to crop losses of up to 90% [10–12]. While there has been considerable progress in understanding the sensitivities of crop yield to climate change, evaluation of climate change factors such as CO_2, elevated temperature, or drought on seed nutrition remains rather limited [13]. Drought resulted from climate change is expected to alter the biochemistry and physiology of crops, impacting the nutritional value of the crop grains. Alteration of carbohydrates including starch and soluble sugars such as glucose, fructose, and sucrose occurs under stress conditions [14]. It is well known that carbohydrates are the major product of photosynthesis, which are transported to stems and leaves (important reserve of photo-assimilates for grain filling) and consequently to the sink (grains) during grain-filling stage [15]. The sugars, sucrose and raffinose, are also known to protect cells against oxidative damage and accumulate later during responses to stress [16, 17]. It was reported that the high levels of raffinose and stachyose were thought to play a role in the acquisition of desiccation tolerance due to over expression of galactinol synthase and accumulation of galactinol and raffinose improving drought tolerance [18, 19]. The mechanisms of involvement of these sugars in drought tolerance are still not fully understood [19–21]. The accumulation of proline, amino acids, and organic acid such as malic acid, fumaric acid, and citric acid is also a common biochemical indicator occurring at high level under drought stress [12]. The accumulation of

http://dx.doi.org/10.5772/67511

these compounds is a part of the mechanism of osmotic adjustment to maintain water potential gradient under drought conditions and to protect subcellular structure from the damaging effects of drought stress [22]. The high level of proline accumulation under drought stress was previously identified as a mechanism for the protection of cytosolic proteins and organelles [12]. Therefore, these biochemical compounds are important to understand the relationship between drought stress and drought tolerance and for decision-making when breeding for drought tolerance [23]. Since drought stress results in limiting the growth and development of the plant and consequently the supply of the photosynthate to grain during grain filling [24], the chemical composition of the grain and its quality can be affected by drought stress [12, 25]. The effect of drought on seed quality could be due to the decrease of total soluble sugars especially in the grain under drought stress due to decreases in the levels of sucrose, stachyose, and verbascose. Sucrose plays an important role in the development of the grain and is sensitive to drought stress and involved in the synthesis of raffinose oligosaccharides including stachyose and verbascose, and these sugars play an important role in seed tolerance to desiccation and against oxidative effects of drought stress [18, 26]. Some sugars, for example, maltose, accumulate in the grain under drought stress, maybe due to starch degradation [12]. For example, during the remobilization phase, starch remobilized was significantly lower under drought stress, but the total amounts of soluble sugars and amino acids remobilized were greater [12], contrasting previous studies that showed higher carbohydrate accumulation under drought stress [27]. Therefore, the role of carbohydrates in drought tolerance is still not well understood, and further investigations are needed to reach the conclusive results.

Research on the possible effects of elevated CO_2 and drought resulted from climate changes on biochemical and chemical composition and mineral nutrition of major crops is limited. We chose soybean as a subject of our research because soybean is a major crop in the world; it is a major source of human nutrition because it contains protein (40%), oil (20%), fatty acids, amino acids, carbohydrates (30%), crude fiber (5%), and ash (5%) and it contains minerals (such as P, K, Ca, Mg, Fe, Cu, Mn, Zn, and Mo), vitamins (B1, B2, and B6), phytoestrogen, such as isoflavones, and phenolics. The objective of the current research was to investigate the possible effects of elevated CO_2 and drought on seed chemical composition (protein, oil, fatty acids, sugars, and minerals). A special attention was given to possible alteration of seed $\delta^{15}N$ ($^{15}N/^{14}N$ ratio) and $\delta\ ^{13}C$ ($^{13}C/^{12}C$ ratio) natural abundance isotopes as they reflect nitrogen and carbon metabolism.

2. Materials and methods

2.1. Growth conditions

The experiments were conducted under controlled environments of growth chambers. Soybean seeds were planted in vermiculite and grown under greenhouse conditions until V1 stage where similar plants were transported to pots filled with field soil. The soil texture was 8% sand, 31.6% silt, and 60.4% clay. Soil nutrient concentrations of macro- and

micronutrients were adequate to support the growth and development of plants till maturity. When plants reached R5 (beginning of seed-fill stage) [28], they were transferred to the growth chambers until full maturity. Two soybean cultivars, Freedom and Hutcheson of maturity group V, were used. The plants were subjected to the following four treatments (T): T1 = plants were grown irrigated and subjected to 360 μmol mol^{-1} CO_2 concentration; T2 = plants were grown irrigated and subjected to 700 μmol mol^{-1} CO_2 concentration; T3 = plants were subjected to drought and to 360 μmol mol^{-1} CO_2 concentration; and T4 = plants were subjected to drought and to 700 μmol mol^{-1} CO_2 concentration. Drought stress treatment was imposed by growing the plants at soil water potential of about −199 kPa. For irrigated plants, soil water potential was kept at about −15 to −20 kPa, which was considered to give the field water holding capacity. Soil water potential was monitored by using soil water potential sensors and read by Soil Moisture Meter (Watermark Company, Inc., Wisconsin, USA). For irrigated experiment, the plants were watered as needed. For CO_2 treatment, CO_2 concentration was supplied by CO_2 cylinder located outside the growth chamber, and the rate of CO_2 was controlled by a regulator, monitoring the CO_2 concentration flow. The CO_2 flowed through the tube to the growth chamber. The growth chamber was equipped with CO_2 sensor to read the concentration of CO_2 inside the growth chamber. The plants were grown under growth chamber conditions supplied with light source of photon flux density of about 1000 μmol m^{-2} s^{-1} and provided with a combination of 10,400 W high-pressure sodium and metal halide lights. The experiments were conducted under constant normal temperature of 26/16°C, day/night.

2.2. Determination of seed minerals, N, S, and C

Mature seeds were ground using a Laboratory Mill 3600 (Perten, Springfield, IL, USA), and the ground samples were analyzed for minerals, N, S, and C, by digesting 0.6 g of the ground-dried plant materials in HNO_3 in a microwave digestion system as detailed elsewhere [29, 30]. Potassium was determined by inductively coupled plasma spectrometry [29, 30]. Each content of N, C, and S was measured in a 0.25 g ground-dried sample which was combusted in atmospheric oxygen of 1350°C, and then the elements N, S, and C were converted to N_2, SO_2, and CO_2, respectively. The contents of N, S, and C in seed were measured by an elemental analyzer using thermal conductivity cells (LECOCNS-2000 elemental analyzer, LECO Corporation, St. Joseph, MI, USA) [29, 30].

2.3. Determinations of seed protein, oil and fatty acids

The mature seed samples of 25 g were ground using the Laboratory Mill 3600, and seed protein, oil, and fatty acids in the samples were analyzed by near-infrared reflectance [29–31] using a diode array feed analyzer AD 7200 (Perten, Springfield, IL USA). The calibration equation was developed using Preteen's Thermo Galactic Grams PLS IQ software, and the calibration curve was established using Association Official Analytical Chemists (AOAC) methods. The contents of protein and oil were determined based on a seed dry matter [29, 31]. The contents of palmitic, stearic, oleic, linoleic, and linolenic fatty acids were determined based on a total oil basis [29].

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2.4. Determinations of sucrose, raffinose, stachyose, glucose, and fructose

Seed sucrose, raffinose, and stachyose were determined as detailed elsewhere (Bellaloui et al. [29]) by near-infrared reflectance using the AD 7200 array feed analyzer. Sugars were determined based on a seed dry matter basis [29–31]. The concentration of glucose in mature seed was determined by an enzymatic reaction using a Glucose (HK) Assay Kit, Product Code GAHK-20 (Sigma-Aldrich Co., St Louis, MO, USA) as detailed elsewhere [29]. The concentrations were determined spectrophotometrically by reading the samples at absorbance of 340 nm using the Beckman Coulter DU 800 spectrophotometer. The concentration was expressed as mg g^{-1} dry weight. Fructose concentration in mature seed was determined based on an enzymatic reaction using a Fructose Assay Kit, Product Code FA-20 (Sigma-Aldrich Co., St. Louis, MO, USA) as detailed by Bellaloui et al. elsewhere [29]. Fructose concentration was determined spectrophotometrically by the Beckman Coulter DU 800 spectrophotometer by reading the samples at absorbance of 340 nm. Fructose concentration in seed was expressed as mg g^{-1} dry weight.

2.5. Boron determination

Boron concentrations in mature seeds were determined using the Azomethine-H method [32, 33] as reported elsewhere [29]. Briefly, 1.0 g of ground sample was ashed at 500°C, extracted with 20 ml of 2 M HCl at 90°C for 10 min, and then 4 ml of a buffer solution (containing 25% ammonium acetate, 1.5% EDTA, and 12.5% acetic acid) was added to a filtered 2-ml sample. An amount of 4 ml of fresh azomethine-H solution (0.45% azomethine-H and 1% of ascorbic acid) [34] was added. Boron concentration was determined spectrophotometrically by reading the samples at absorbance of 420 nm using a Beckman Coulter DU 800 spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA). The concentration was expressed as mg kg^{-1} dry weight.

2.6. Iron determination

Iron concentration in mature seed was determined according to the method described elsewhere [35, 36]. Briefly, 2 g of dry ground samples were acid wet digested, extracted, and reacted with reduced ferrous Fe with 1,10-phenanthroline and as detailed elsewhere [29]. After the extraction, the soluble constituents were dissolved in 2 M HCl, and the phenanthroline solution of 0.25% (w/v) in 25% (v/v) ethanol and quinol solution (1% w/v) was used as a reagent. Standard curve of Fe was created by preparing a range of concentrations from 0.0 to 4 μg ml^{-1} of Fe in 0.4 M HCl. Iron concentration was determined spectrophotometrically by reading the samples at absorbance of 510 nm using the Beckman Coulter DU 800 spectrophotometer. The concentration was expressed as mg kg^{-1} dry weight.

2.7. Phosphorus determination

Phosphorus content in mature seeds was measured according to [37]. Phosphorus determination was based on the yellow phosphor-vanado-molybdate complex as detailed elsewhere [29]. Briefly, dry ground seed samples of 2 g were ashed. Then, 10 ml of 6MHCl was added,

and phosphorus was extracted using 2 ml of 36% v/v HCl under heat and filtration and 5 ml of 5 M HCl. A volume of 5 ml of reagent (ammonium molybdate-ammonium metavanadate) was added to 5 ml of the filtrate. The reagent ammonium molybdate-ammonium metavanadate was prepared by dissolving 25 g of ammonium molybdate and 1.25 g of ammonium metavanadate in distilled water. The standard curve solutions of P were prepared by using a range of concentrations from 0 to 50 μg ml^{-1} using dihydrogen orthophosphates. The concentration of P was determined spectrophotometrically by reading the absorbance at 400 nm using the Beckman Coulter DU 800 spectrophotometer.

2.8. Experimental design and statistical analysis

Two experiments were carried out in a split plot design with arrangement of treatments in a randomized complete block design (RCBD) with four replicates. Main plot was drought/CO_2 treatment and subplot was cultivar. Each experiment was considered as a replicate for the main plot. One growth chamber was used for each drought/CO_2 treatment. Treatments in each experiment were the following: T1 = plants were grown irrigated and subjected to 360 μmol mol^{-1} CO_2 concentration; T2 = plants were grown irrigated and subjected to 700 μmol mol^{-1} CO_2 concentration; T3 = plants were grown under drought and subjected to 360 μmol mol^{-1} CO_2 concentration; and T4 = plants were grown under drought and subjected to 700 μmol mol^{-1} CO_2 concentration. Two soybean cultivars were used. The analysis of variance of data was conducted using PROC MIXED in SAS [38]. Experiment (EXP), drought, CO_2 concentration (CO_2), and their interactions were considered fixed effects, and replication within EXP and replication × drought × CO_2 × CV within EXP were considered random effects. The level of significance was $P \leq 0.05$. Detailed design of the current experiments was similar to that previously reported by Bellaloui et al. [39].

3. Results and discussion

Analysis of variance showed that the main effects of experiment (EXP), drought, CO_2, and cultivar (CV) were the most significant factors affecting variability of seed protein, oil, fatty acids, and sugars (**Table 1**). There were no significant effects of the interactions between EXP and other factors, which were expected. Interactions between CO_2, CV, and drought showed significant effects for some seed constituents such as protein, oleic, glucose, fructose, and sucrose, indicating the different responses of cultivars to drought and CO_2 (**Table 1**). Since there were no significant interaction effects between EXP and the others factors, the results were combined across the two experiments. Analysis of variance showed that drought, CO_2, and CV were the major factors affecting the variability for macro- and micronutrients in seed (**Table 2**). There were no effects of EXP on nutrients, as expected, because the experiments were conducted under controlled environmental conditions of growth chambers. Both CO_2 and CV significantly interacted with drought, indicating that the effect of CO_2 was influenced by drought and the cultivars responded differently due to genotype and genetic background effect (**Table 2**).

Treatments	Protein	Oil	Oleic	Linolenic	Glucose	Fructose	Sucrose	Raffinose	Stachyose
EXP	ns[1]	ns	ns	ns	ns	ns	ns	ns	ns
Drought[2]	*	*	**	**	***	**	**	*	*
CO_2	**	*	*	*	***	**	***	*	*
CV	*	ns	*	ns	ns	*	*	ns	ns
EXP × drought	ns	ns	ns	ns	ns	ns	ns	ns	ns
EXP × CO_2	ns	ns	ns	ns	ns	ns	ns	ns	ns
EXP × CV	ns	ns	ns	ns	ns	ns	ns	ns	ns
Drought × CO_2	ns	ns	ns	ns	ns	ns	ns	ns	ns
Drought × CV	ns	ns	*	ns	*	*	***	ns	ns
CO_2 × CV	*	ns	ns	ns	ns	*	*	ns	ns
EXP × drought × CO_2 × CV	ns	ns	ns	ns	ns	ns	ns	ns	ns

Treatments used were four: T1 = plants were grown irrigated and subjected to 360 μmol mol^{-1} CO_2 concentration; T2 = plants were grown irrigated and subjected to 700 μmol mol^{-1} CO_2 concentration; T3 = plants were grown under drought and subjected to 360 μmol mol^{-1} CO_2 concentration; and T4 = plants were grown under drought and subjected to 700 μmol mol^{-1} CO_2 concentration. Plants were grown in the greenhouse, and they were transferred to growth chambers at the beginning of seed-fill stage (R5) until full maturity (R8). The experiments were conducted under constant normal temperature of 26/16°C, day/night.

[1] * = significance at $P \leq 0.05$; ** = significance at $P \leq 0.01$; *** = significance at $P \leq 0.001$; ns = not significant.

[2] Drought treatment was imposed by growing the plants at soil water potential of about −199 kPa. For irrigated plant, soil water potential was kept at about −15 to −20 kPa.

Table 1. Analysis of variance for soybean cultivars (Freedom and Hutcheson, Maturity group V) for seed protein, oil, fatty acids (%), and sugars (glucose, fructose, sucrose, raffinose, and stachyose, mg g^{-1}) and their responses to the main factors of experiment (EXP, two experiments were conducted), drought, carbon dioxide (CO_2), cultivar (CV), and their interactions.

Treatments	N	P	K	Mg	Fe	B	Cu	Zn	Mn
EXP	ns[1]	ns	ns	ns	ns	ns	ns	ns	ns
Drought[2]	***	***	**	*	*	***	*	*	*
CO_2	*	*	*	*	*	*	*	**	**
CV	*	**	*	*	***	*	*	*	*
EXP × drought	ns	ns	ns	ns	ns	ns	ns	ns	ns
EXP × CO_2	ns	ns	ns	ns	ns	ns	ns	ns	ns
EXP × CV	ns	ns	ns	ns	ns	ns	ns	ns	ns
Drought × CO_2	*	*	*	*	*	***	*	**	*
Drought × CV	**	*	*	*	**	*	*	*	*
CO_2 × CV	**	ns	*	ns	ns	*	ns	*	ns
EXP × drought × CO_2 × CV	ns	ns	ns	ns	ns	ns	ns	ns	ns

Treatments used were four: T1 = plants were grown irrigated and subjected to 360 μmol mol^{-1} CO_2 concentration; T2 = plants were grown irrigated and subjected to 700 μmol mol^{-1} CO_2 concentration; T3 = plants were grown under drought and subjected to 360 μmol mol^{-1} CO_2 concentration; and T4 = plants were grown under drought and subjected to 700 μmol mol^{-1} CO_2 concentration. Plants were grown in the greenhouse, and they were transferred to growth chambers at the beginning of seed-fill stage until maturation (R8). The experiments were conducted under constant normal temperature of 26/16°C, day/night.

* = significance at $P \leq 0.05$; ** = significance at $P \leq 0.01$; *** = significance at $P \leq 0.001$; ns = not significant

Drought treatment was imposed by growing the plants at soil water potential of about −199 kPa. For irrigated plant, soil water potential was kept at about −15 to −20 kPa.

Table 2. Analysis of variance for soybean cultivars (Freedom and Hutcheson, maturity group V) for seed macro- and micronutrients (N, P, K, and Mg: %; Fe, B, Cu, Zn, and Mn: mg kg^{-1}) as affected by the main effect factors of experiment (EXP, two experiments were conducted), carbon dioxide (CO_2), cultivar (CV), and their interactions.

http://dx.doi.org/10.5772/67511

Mean values of seed constituents showed that seed protein, oleic acid, raffinose, and stachyose were higher, and oil, linolenic acid, glucose, fructose, and sucrose were lower under drought or drought with elevated CO_2 conditions (**Table 3**). Seed N was higher under drought or drought with elevated CO_2. However, seed contents of P, K, Mg, Fe, B, Cu, Zn, and Mn were generally lower under drought or drought with elevated CO_2. Comparing with ambient CO_2, elevated CO_2 showed higher contents of seed protein, oil, glucose, sucrose, raffinose, and stachyose and lower contents of seed N, P, B, and Cu in Freedom cultivar only. Cultivar Hutcheson responded differently to ambient CO_2 or elevated CO_2. The different responses of cultivars to ambient CO_2 or elevated CO_2 are suggested to be due to genotype and/or genetic background (**Tables 1–4**). What is clear is that seed oleic acid was higher and linolenic acid was lower and most nutrients were lower under elevated CO_2 (**Tables 3–6**). $\delta\ ^{15}N$ ($^{15}N/^{14}N$ Ratio) and $\delta\ ^{13}C$ ($^{13}C/^{12}C$ Ratio) natural abundance isotopes showed alterations under drought and drought with elevated CO_2, indicating that there were changes of nitrogen and carbon metabolism under drought stress (**Figures 1** and **2**).

The higher contents of seed protein, oleic acid, raffinose, and stachyose under drought or drought with elevated CO_2 conditions could be due to drought effects and physiological and biochemical responses to stress. It was reported that drought resulted from climate change is predicted to alter the biochemistry and physiology of crops, impacting the nutritional value of the crop grains [12]. The effects of elevated CO_2 and drought on seed composition (protein, oil, fatty acids, and sugars) and minerals were previously reported. However, results of the effects of elevated CO_2 on seed composition are still conflicting. For example, small effects of elevated CO_2 on seed composition were found [40], whereas others found a significant effect of elevated CO_2 on soybean seed oil in cultivars Essex, Holladay, and NK6955 [41]. The authors also found that oleic fatty acid concentration was positively affected and protein concentration was not affected by CO_2. Recently, it was found that elevated CO_2 resulted in decreased content of protein and increased contents of oil and oleic acid [39].

It was reported that alteration of carbohydrates such as starch and soluble sugars such as glucose, fructose, and sucrose occurred under stress conditions [14] and sugars of sucrose and raffinose may play a role in protecting cells against oxidative damage and accumulate as a response to stress [16, 17]. Other researchers suggested that high levels of raffinose and stachyose contribute to the acquisition of desiccation tolerance due to overexpression of galactinol synthase and accumulation of galactinol and raffinose improving drought tolerance [18, 19]. Sucrose was reported to be involved in the synthesis of raffinose oligosaccharides including stachyose and verbascose, and these sugars play an important role in seed tolerance to desiccation and drought stress [18, 26]. Some sugars, for example, maltose, accumulate in the grain under drought stress, maybe due to starch degradation [12]. The mechanisms of the involvement of these sugars in drought tolerance are still not fully understood [19–21]. It was concluded that these biochemical compounds can be used as drought indicators and can be used in breeding program to select for drought tolerance [12, 23]. CO_2 elevation resulted in a decrease of seed Na, Ca, Mg, S, Fe, Zn, and Mn [42, 43]. The percentage decrease of nutrients by elevated CO_2 ranged from 0.7 to 19.5%, except for K and P [44]. The decrease of macro- and micronutrients by elevated CO_2 was due to the dilution effect induced by the increase of carbohydrates in seeds [12, 39, 42–44].

Treatments[1]	Protein	Oil	Oleic	Linolenic	Glucose	Fructose	Sucrose	Raffinose	Stachyose
T1	38.5 c	21.3 b	22.2 c	9.6 a	1.4 b	1.01 a	61 b	8.7 d	33.6 d
T2	39.0 c	22.1 a	27.5 b	8.7 b	2.1 a	0.98 a	67 a	9.6 c	34.9 c
T3	41.2 a	19.4 c	29.7 a	6.3 c	0.8 c	0.54 b	32 d	11.5 a	45.3 a
T4	40.3 b	19.6 c	29.5 a	6.8 c	0.9 c	0.52 b	39 c	10.3 b	40.7 b

Treatments used were four: T1 = plants were grown irrigated and subjected to 360 μmol mol^{-1} CO_2 concentration; T2 = plants were grown irrigated and subjected to 700 μmol mol^{-1} CO_2 concentration; T3 = plants were grown under drought and subjected to 360 μmol mol^{-1} CO_2 concentration; and T4 = plants were grown under drought and subjected to 700 μmol mol^{-1} CO_2 concentration. Plants were grown in the greenhouse, and they were transferred to growth chambers at the beginning of seed-fill stage (R5) until full maturity (R8). The experiments were conducted under constant normal temperature of 26/16°C, day/night.

[1]Means within a column of each water treatment followed by the same letter are not significantly different at the 5% level as determined by Fishers' least significant difference (LSD) test. Values are means of four replicates. Drought treatment was imposed by growing the plants at soil water potential of about −199 kPa. For irrigated plant, soil water potential was kept at about −15 to −20 kPa.

Table 3. Drought and elevated carbon dioxide effects on soybean (Freedom cultivar, maturity group V) seed protein, oil, fatty acids (%), and sugars (glucose, fructose, sucrose, raffinose, and stachyose, mg g^{-1}).

Treatments[1]	N	P	K	Mg	Fe	B	Cu	Zn	Mn
T1	6.5 a	0.64 a	1.9 a	0.34 a	75 a	64 a	12.4 a	51.2 a	42.5 a
T2	5.1 c	0.53 b	1.5 b	0.33 a	72 a	57 b	9.3 b	52.3 a	41.2 a
T3	6.7 a	0.47 c	1.1 c	0.23 b	65 b	41 d	8.2 c	46.5 c	37.6 c
T4	6.1 b	0.46 c	1.2 c	0.34 a	63 b	47 c	8.5 c	49.6 b	40.0 b

Treatments used were four: T1 = plants were grown irrigated and subjected to 360 μmol mol^{-1} CO_2 concentration; T2 = plants were grown irrigated and subjected to 700 μmol mol^{-1} CO_2 concentration; T3 = plants were grown under drought and subjected to 360 μmol mol^{-1} CO_2 concentration; and T4 = plants were grown under drought and subjected to 700 μmol mol^{-1} CO_2 concentration. Plants were grown in the greenhouse, and they were transferred to growth chambers at the beginning of seed-fill stage (R5) until full maturity (R8). The experiments were conducted under constant normal temperature of 26/16°C, day/night.

[1]Means within a column of each water treatment followed by the same letter are not significantly different at the 5% level as determined by Fishers' LSD test. Values are means of four replicates. Drought treatment was imposed by growing the plants at soil water potential of about −199 kPa. For irrigated plant, soil water potential was kept at about −15 to −20 kPa.

Table 4. Drought and elevated carbon dioxide effects on soybean (Freedom cultivar, maturity group V) seed macro- and micronutrients (N, P, K, and Mg: %; Fe, B, Cu, Zn, and Mn: mg kg^{-1}).

Treatments[1]	Protein	Oil	Oleic	Linolenic	Glucose	Fructose	Sucrose	Raffinose	Stachyose
T1	39.8 c	21.3 a	21.4 c	10.5 a	2.4 a	1.23 a	68 a	11.2 c	41.4 c
T2	40.1 c	21.4 a	29.7 a	9.7 b	2.6 a	1.21 a	66 a	12.5 b	40.5 d
T3	43.5 b	18.5 b	29.6 a	7.5 c	0.7 c	0.73 c	51 c	13.5 a	47.6 a
T4	44.1 a	18.3 b	28.1 b	7.3 c	1.3 b	0.85 b	60 b	13.7 a	46.8 b

Treatments used were four: T1 = plants were grown irrigated and subjected to 360 μmol mol^{-1} CO_2 concentration; T2 = plants were grown irrigated and subjected to 700 μmol mol^{-1} CO_2 concentration; T3 = plants were grown under drought and subjected to 360 μmol mol^{-1} CO_2 concentration; and T4 = plants were grown under drought and subjected to 700 μmol mol^{-1} CO_2 concentration. Plants were grown in the greenhouse, and they were transferred to growth chambers at the beginning of seed-fill stage (R5) until full maturity (R8). The experiments were conducted under constant normal temperature of 26/16°C, day/night.

[1]Means within a column of each water treatment followed by the same letter are not significantly different at the 5% level as determined by Fishers' LSD test. Values are means of four replicates. Drought treatment was imposed by growing the plants at soil water potential of about −199 kPa. For irrigated plant, soil water potential was kept at about −15 to −20 kPa.

Table 5. Drought and elevated carbon dioxide effects on soybean (Hutcheson cultivar, maturity group V) seed protein, oil, fatty acids (%), and sugars (glucose, fructose, sucrose, raffinose, and stachyose, mg g^{-1}).

Treatments[1]	N	P	K	Mg	Fe	B	Cu	Zn	Mn
T1	6.9 a	0.72 a	2.7 a	0.31 a	76 b	67 a	11.3 a	48 a	43 a
T2	5.3 c	0.59 b	1.7 b	0.26 b	81 a	65 a	10.5 b	43 b	39 b
T3	6.3 b	0.42 c	1.2 c	0.20 c	60 c	51 b	8.1 d	31 d	31 c
T4	6.1 b	0.45 c	1.1 c	0.22 c	63 c	53 b	9.8 c	35 c	33 c

Treatments used were four: T1 = plants were grown irrigated and subjected to 360 μmol mol^{-1} CO_2 concentration; T2 = plants were grown irrigated and subjected to 700 μmol mol^{-1} CO_2 concentration; T3 = plants were grown under drought and subjected to 360 μmol mol^{-1} CO_2 concentration; and T4 = plants were grown under drought and subjected to 700 μmol mol^{-1} CO_2 concentration. Plants were grown in the greenhouse, and they were transferred to growth chambers at the beginning of seed-fill stage (R5) until full maturity (R8). The experiments were conducted under constant normal temperature of 26/16°C, day/night.

[1]Means within a column of each water treatment followed by the same letter are not significantly different at the 5% level as determined by Fishers' LSD test. Values are means of four replicates. Drought treatment was imposed by growing the plants at soil water potential of about −199 kPa. For irrigated plant, soil water potential was kept at about −15 to −20 kPa.

Table 6. Drought and elevated carbon dioxide effects on soybean (Hutcheson cultivar, maturity group V) seed macro- and micronutrients (N, P, K, and Mg: %; Fe, B, Cu, Zn, and Mn: mg kg^{-1}).

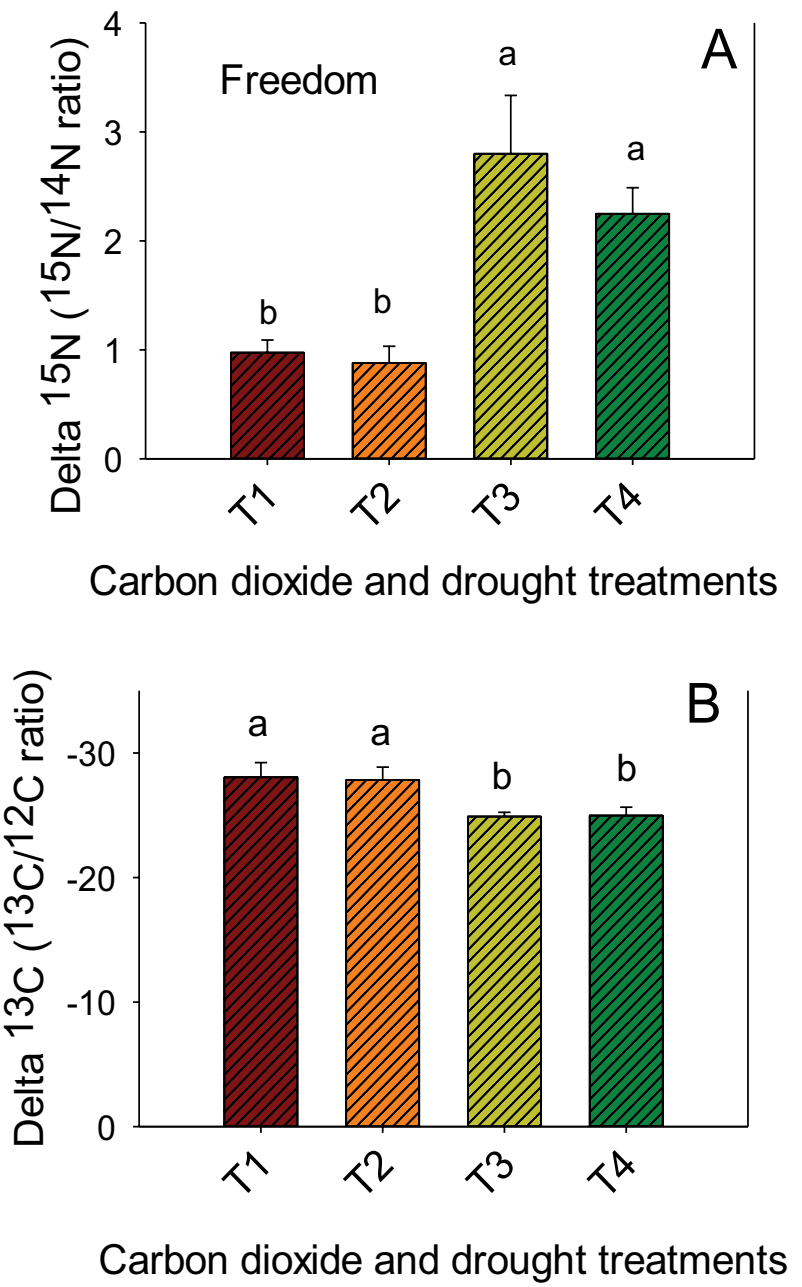

Figure 1. Cultivar Freedom (maturity group V). δ ^{15}N ($^{15}N/^{14}N$ ratio) (A) and δ ^{13}C ($^{13}C/^{12}C$ ratio) (B) natural abundance isotope in soybean seed as influenced by drought and elevated CO_2. Treatments used were four: T1 = plants were grown irrigated and subjected to 360 μmol mol^{-1} CO_2 concentration; T2 = plants were grown irrigated and subjected to 700 μmol mol^{-1} CO_2 concentration; T3 = plants were grown under drought and subjected to 360 μmol mol^{-1} CO_2 concentration; and T4 = plants were grown under drought and subjected to 700 μmol mol^{-1} CO_2 concentration. Plants were grown in the greenhouse, and they were transferred to growth chambers at the beginning of seed-fill stage (R5) until full maturity (R8). The experiments were conducted under constant normal temperature of 26/16°C, day/night.

http://dx.doi.org/10.5772/67511

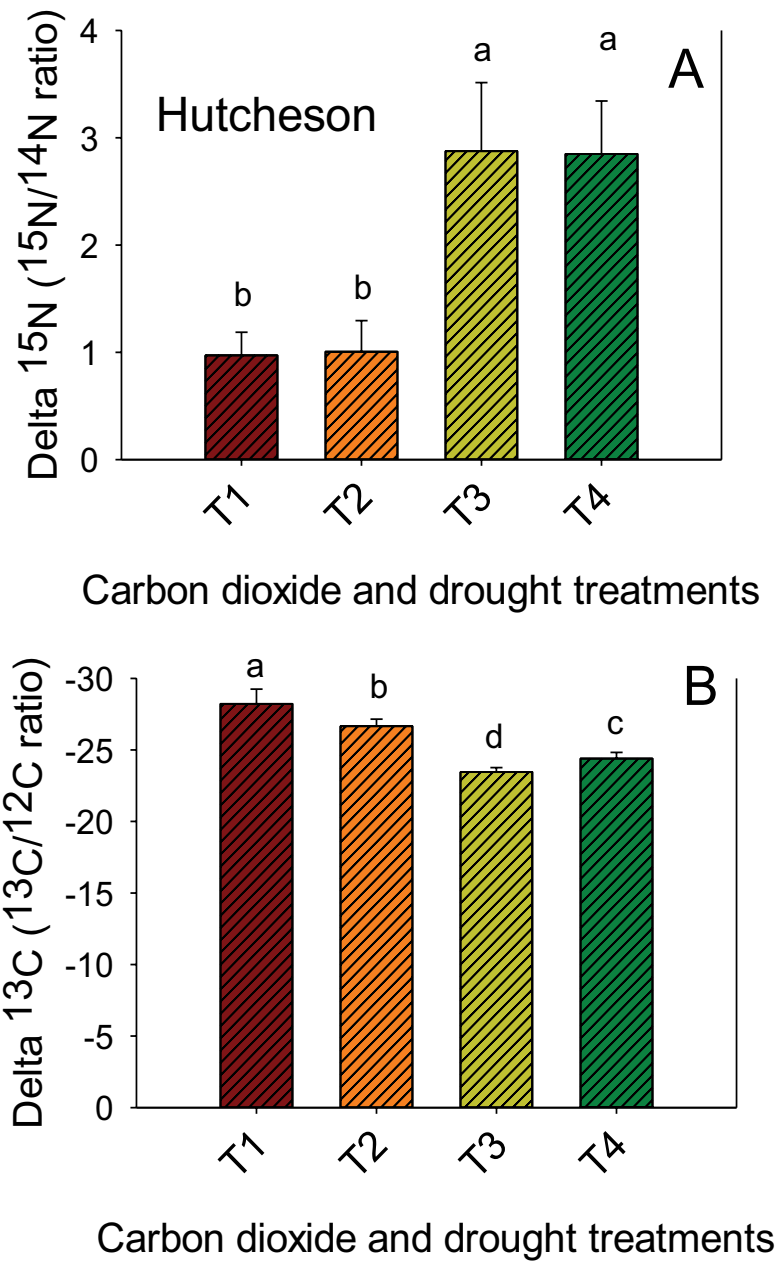

Figure 2. Cultivar Hutcheson (maturity group V). δ ^{15}N ($^{15}N/^{14}N$ ratio) (A) and δ ^{13}C ($^{13}C/^{12}C$ ratio) (B) natural abundance isotope in soybean seed as affected by drought and elevated CO_2. Treatments used were four: T1 = plants were grown irrigated and subjected to 360 μmol mol^{-1} CO_2 concentration; T2 = plants were grown irrigated and subjected to 700 μmol mol^{-1} CO_2 concentration; T3 = plants were grown under drought and subjected to 360 μmol mol^{-1} CO_2 concentration; and T4 = plants were grown under drought and subjected to 700 μmol mol^{-1} CO_2 concentration. Plants were grown in the greenhouse, and they were transferred to growth chambers at the beginning of seed-fill stage (R5) until full maturity (R8). The experiments were conducted under constant normal temperature of 26/16°C, day/night.

Our results showed that elevated CO_2 concentration enhanced oleic acid and carbohydrates (mainly sucrose and glucose) and reduced NPK and some micronutrients compared with ambient CO_2. The increase of carbohydrates due to elevated CO_2 could result from enhanced photosynthesis resulting in higher total carbohydrates [4]. The decrease of NPK and some micronutrients was due to the dilution of concentrations of the nutrients by higher levels of carbohydrates as supported by previous research [12, 39, 42–44]. Higher seed protein, oleic acid, raffinose and stachyose, and lower oil, linolenic acid, glucose, fructose, and sucrose under drought or drought with elevated CO_2 conditions can be used as biochemical indicators/markers to be used for breeding to select for drought tolerance.

Therefore, research is needed to quantify the negative impact of elevated CO_2 and its interactions with biotic and abiotic stresses, including drought, on seed quality to breed for higher seed nutritional qualities and develop appropriate crop management systems [43, 45]. It was reported that the physiological processes influenced by drought can be identified by the biochemical characterization of plant tissues under stress conditions [46]. Limited research was conducted to characterize the biochemical compounds such as protein, amino acids, carbohydrates, and phenolics in the breeding program as a tool for selection, but this tool has promise in selecting for abiotic stress tolerance [24, 47]. The changes of $\delta\ ^{15}N$ ($^{15}N/^{14}N$ Ratio) and $\delta\ ^{13}C$ ($^{13}C/^{12}C$ Ratio) natural abundance isotopes under drought and drought with elevated CO_2 indicated the increase of ^{15}N derived from plant gas exchange through stomatal conductance and CO_2 fixation [48, 49]. The alteration of $^{13}C/^{12}C$ ratio indicated a possible shift of carbon metabolism leading to less discrimination against $\delta\ ^{13}C$ [50].

4. Conclusions

The current research demonstrated that elevated CO_2, drought, and drought combined with elevated CO_2 altered seed biochemical compounds, including protein, carbohydrates, and minerals. This research increases our knowledge of the interaction between elevated CO_2 and drought resulting from climate changes for seed chemical composition and mineral nutrition.

The high level of oleic acid and low level of linolenic acid are desirable, and they contribute to oil stability and long shelf life of the oil. Sugars of both raffinose and stachyose may play a possible role in drought stress response. Alterations of $\delta\ ^{15}N$ and $\delta\ ^{13}C$ natural abundance isotopes indicated changes in nitrogen and carbon metabolism. The characterization of these seed biochemical compounds under elevated CO_2 or drought stress can be used as biomarkers in breeding program to select for crop drought tolerance and high seed nutritional qualities, as these traits are related to seed production, quality, and food security. Since limited research was conducted on the effects of climate change on seed quality and mineral nutrition, further research is needed.

Acknowledgements

We thank Sandra Mosley for lab analysis and greenhouse and growth chamber technical assistance. This work was supported by the US Department of Agriculture, Agricultural Research

Service Project 6402-21220-012-00D. Mentioning trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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Chapter 9

Role of Nitrogen on Growth and Seed Yield of Soybean and a New Fertilization Technique to Promote Nitrogen Fixation and Seed Yield

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66743

Abstract

Soybean is an important crop for human food and feed for livestock. World soybean production is increasing especially in North and South America. Soybean seeds contain a high percentage of protein about 35–40%, and they require a large amount of nitrogen compared with other crops. Soybean plants make root nodules with rhizobia, and rhizobia can fix atmospheric N_2 and give the fixed N to the host soybean plants. Also, soybean can absorb nitrogen usually nitrate from soil or fertilizers. The amount of total assimilated nitrogen in shoot is proportional to the soybean seed yield either from nitrogen fixation or from nitrogen absorption, and the nitrogen availability is very important for soybean cultivation. Maintenance of a high and long-term nitrogen fixation activity is very important for a high production of soybean. However, application of chemical nitrogen fertilizers usually depresses nodule formation and nitrogen fixation. Nitrate in direct contact with a nodulated part of roots causes severe inhibition of nodule growth and nitrogen fixation, although a distant part of nodules from nitrate application gives no or little effect. Deep placement of slow-release nitrogen fertilizers, coated urea, or lime nitrogen promoted the growth and seed yield and quality of soybean without depressing nitrogen fixation.

Keywords: nitrogen fixation, soybean, deep placement, coated urea, lime nitrogen

1. Introduction

1.1. World soybean production and seed yield

Soybean (*Glycine max* (L.) Merr.) is originated in East Asia, but now it is widely cultivated in tropical, subtropical, and temperate climatic regions with an optimum mean temperature of 20–30°C. Soybean seed is one of the most important protein sources for human and livestock all over the world. In addition, soybean is a major oilseed crop in the world providing 58% of world oil seed production [1]. Soybean is cultivated over a wide range of latitudes, from the equator to high latitudes of at least 50° N [2], although each cultivar adapts to the narrow range of latitudes. Soybean is a short-day plant, and flowering is induced when the day length is shorter than a critical length. This sensitivity to photoperiod is weak or absent in soybean cultivars adapted to high latitudes, which should initiate flowering in early summer to mature in frost-free seasons [2].

Annual production of soybean is 276 M t (million tons) in 2013, and be the fourth of the major grain crops, after maize (1017 M t), paddy rice (746 M t), and wheat (713 M t) [3]. The soybean cultivation area is about 111 M ha in 2013. Recently, world soybean production has been increasing (79 M t in 1983, 115 M t in 1993, 191 M t in 2003, and 276 M t in 2013) [3]. The main soybean production countries (annual production in 2013) are the USA (89.5 M t), Brazil (81.7 M t), Argentina (49.3 M t), China (12.5 M t), and India (12.0 M t). Soybean production in Japan was only 200,000 t and it accounted for 8% of the total consumption in Japan. The annual production of soybean in the USA increased about twice during recent 30 years, and it was 5.6 times and 12.3 times in Brazil and Argentina (**Figures 1** and **2**). The annual production

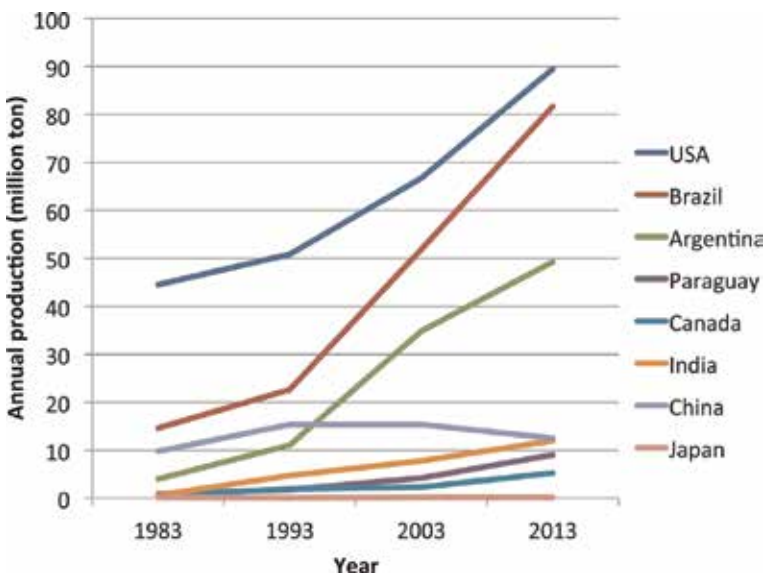

Figure 1. Changes in annual production of soybean in each country from 1983 to 2013. (Data from FAOSTAT (FAO Statistical Database (United Nations))).

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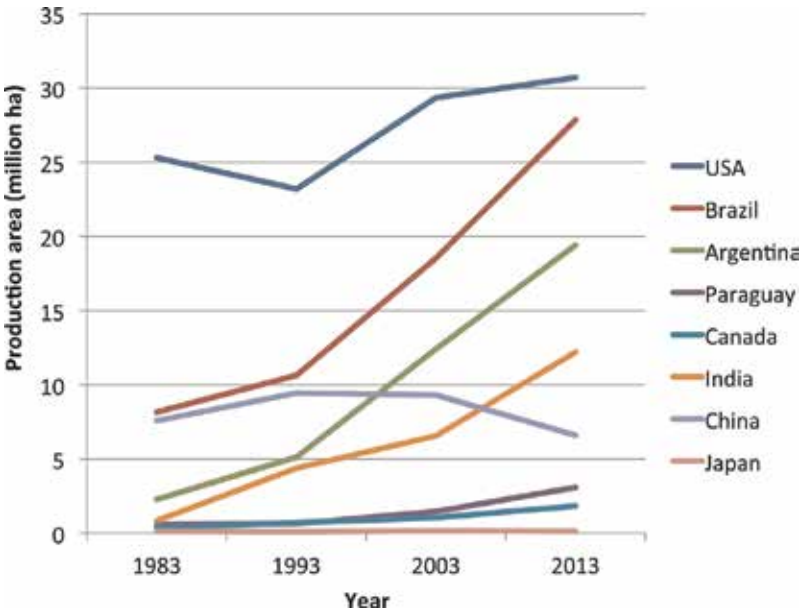

Figure 2. Changes in the production area of soybean in each country from 1983 to 2013. (Data from FAOSTAT).

	Yield in 2013 (ton ha^{-1})	Yield in 1983 (ton ha^{-1})	Ratio (2013/1983)	Yield increase (kg ha^{-1} year^{-1})
USA	2.91	1.76	1.65	38
Brazil	2.93	1.79	1.64	38
Argentina	2.54	1.75	1.45	26
Paraguay	2.95	1.47	2.01	49
Canada	2.86	2.02	1.42	28
India	0.98	0.73	1.34	8
China	1.89	1.29	1.47	20
Japan	1.55	1.51	1.03	1
World total	2.48	1.62	1.53	29

Data from FAOSTAT.

Table 1. Changes in soybean seed yield from 1983 to 2013.

and production area in India increased during the recent 30 years, but these decreased from 2003 to 2013 in China. The world average seed yield is 2.48 t ha^{-1} in 2013, and high in the USA (2.91 t ha^{-1}), Brazil (2.93 t ha^{-1}), Argentina (2.54 t ha^{-1}), Paraguay (2.95 t ha^{-1}), and Canada (2.86 t ha^{-1}) compared with China (1.89 t ha^{-1}), Japan (1.55 t ha^{-1}), and India (0.98 t ha^{-1}) (**Table 1**). The average annual increase of seed yield from 1983 to 2013 was high in the USA

(38 kg ha^{-1} $year^{-1}$), Brazil (38 kg ha^{-1} $year^{-1}$), Argentina (26 kg ha^{-1} $year^{-1}$), Paraguay (49 kg ha^{-1} $year^{-1}$), and Canada (28 kg ha^{-1} $year^{-1}$) compared with China (20 kg ha^{-1} $year^{-1}$), India (8 kg ha^{-1} $year^{-1}$), and Japan (1 kg ha^{-1} $year^{-1}$) (**Table 1**). Board and Kahlon (2011) [4] suggested that recent yield gains in the USA are 50% due to cultivar genetic improvement and 50% to improve cultural practices. Potential gains from improved cultural practices for any given locate are usually determined by comparing farmer yields with those done using recommended practice [4]. In the USA, the improvement of cultural practices can be expected to increase yield anywhere from 25 to 66% [4]. They suggested that the yield increase for Asian countries would be even greater, since their yield levels are substantially low due to many biotic and abiotic stresses [4].

1.2. Soybean seed yield potential

Soybean seed yield can be obtained at 4–6 t ha^{-1} with well-managed fields under good climate and soil conditions [5]. Recently, a high yield over 10 t ha^{-1} was recorded in 2008 and 2010 by an innovative farmer Mr. Kip Cullers in Missouri, the USA [6]. This fact indicates that the potential soybean productivity is much higher than we thought. Soybean yield potential has been defined as the maximum yield of a crop cultivar grown in an environment to which it is adapted, with nutrients and water non-limiting, and pests and diseases effectively controlled [7, 8]. In the USA corn-belt region, soybean yield potential has been estimated to be in the range of 6–8 t ha^{-1} [7, 9, 10].

Table 2 shows the composition of water, macronutrients, and mineral elements in seeds of soybeans, beans, rice, wheat, and maize [11]. The nutrient composition of soybean seeds (per 100 g) produced in Japan is as follows [11]: water 12.5 g, protein 35.3 g,

	Soybean	Bean	Rice	Wheat	Maize
Macronutrients (g/100 g)					
Water	12.5	16.5	15.5	12.5	14.5
Protein	35.3	19.9	6.8	10.6	8.6
Lipid	19	2.2	2.7	3.1	5
Carbohydrate	28.2	57.8	73.8	72.2	70.6
Ash	5	3.6	1.2	1.6	1.3
Minerals (mg/100 g)					
Potassium	1900	1500	230	470	290
Phosphorous	580	400	290	350	270
Calcium	240	130	9	26	5
Magnesium	220	150	110	80	75
Iron	9.4	6	21	3.2	1.9

Data from 2009 All Guide Standard Tables of Food Composition in Japan [11].

Table 2. Comparison of concentration of nutrients in various crop seeds.

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lipids 19.0 g, carbohydrates 28.2 g, and minerals 5 g. The composition is quite different from the other grain crop seeds such as rice, wheat, and maize. The protein concentration in soybean seeds is about four to five times higher than that of rice, wheat, and maize, but carbohydrate concentration is lower. Bean seeds contain less protein (19.9%) and much less lipid (2.2%) than those in soybean seeds. Mineral contents in soybean seeds are also much higher than those in rice, wheat, and maize, especially potassium and calcium.

Sinclair and De Wit reported that different compositions of macronutrients in crop seeds affect biomass productivity of seeds (g of seed per g of photosynthate) [12], and the bioproductivity of soybean seed is 0.5 and rice grain is 0.75. This means 4 t of soybean seed yield is energetically equivalent to 6 t of rice grain production, which is almost the average of Japanese rice production (6.73 t ha^{-1} in 2013). It was also reported that 1 g of carbohydrate, protein, and lipid requires 1.21, 2.48, and 3.03 g of glucose, respectively [13]. When these values are applied to the compositions of various seeds in **Table 2**, 1.79, 1.26, 1.14, 1.23, and 1.22 g of glucose are theoretically required for the production of 1 g of soybean, bean, rice, wheat, and maize seeds, respectively. Based on this calculation, 1 g of soybean seeds needs 57% more glucose, as compared with 1 g of rice grain. Therefore, over 4 t ha^{-1} of soybean yield can be expected by the good agricultural practices under good soil and climatic conditions, although the average soybean yields remain at 1.5 t ha^{-1} in Japan.

1.3. Nutrient acquisition by soybean plants

Figure 3 shows the fundamental physiological processes of nutrient acquisition by soybean plants related to the growth and seed yield. In order to achieve high-yield potential, soybean must sustain high photosynthesis rates and accumulate large amounts of N.

Soybean seeds are large and store nutrients in the cotyledons to support the initial growth of about 7–10 days after planting. Soybean development is separated into the vegetative development period and reproductive development period at the initial flowering stage, and the developmental stages of soybean are expressed by the descriptions by Fehr and Caviness [14]. In the case of soybean, the vegetative growth of leaves, stems, and nodes overlaps with the reproductive growth until seed initiation (R5) stage. The period of vegetative growth and reproductive growth varies by cultivars and cultivated sites, but the vegetative growth period is about 2 months and reproductive development period is about 3 months in typical Japanese soybean cultivation.

For high soybean seed yield, both the optimum vegetative growth and reproductive growth are necessary. Photosynthesis by leaves and sufficient but not excess water and nutrients absorption from roots are very important to support vigorous plant growth (**Figure 3**). In addition, soybean can fix atmospheric dinitrogen (N_2) by root nodules, which are symbiotic organ with soil bacteria called rhizobia.

Ohlrogge and Kamprath [15] calculated nutrient requirement of high-yielding soybeans, which have 8.96 t ha^{-1} total dry matter including 3.36 t ha^{-1} of grain and 5.60 t ha^{-1} of vegetative parts (**Table 3**). For 1 kg of soybean seed production, about 1024 g of C, 963 g of O, and 131 g of H are required through photosynthesis from CO_2 in the air and H_2O from soil. Of the soil-derived nutrients, N, K, Ca, Mg, P, and S are required about 93, 32, 23, 10, 9, and 7 g,

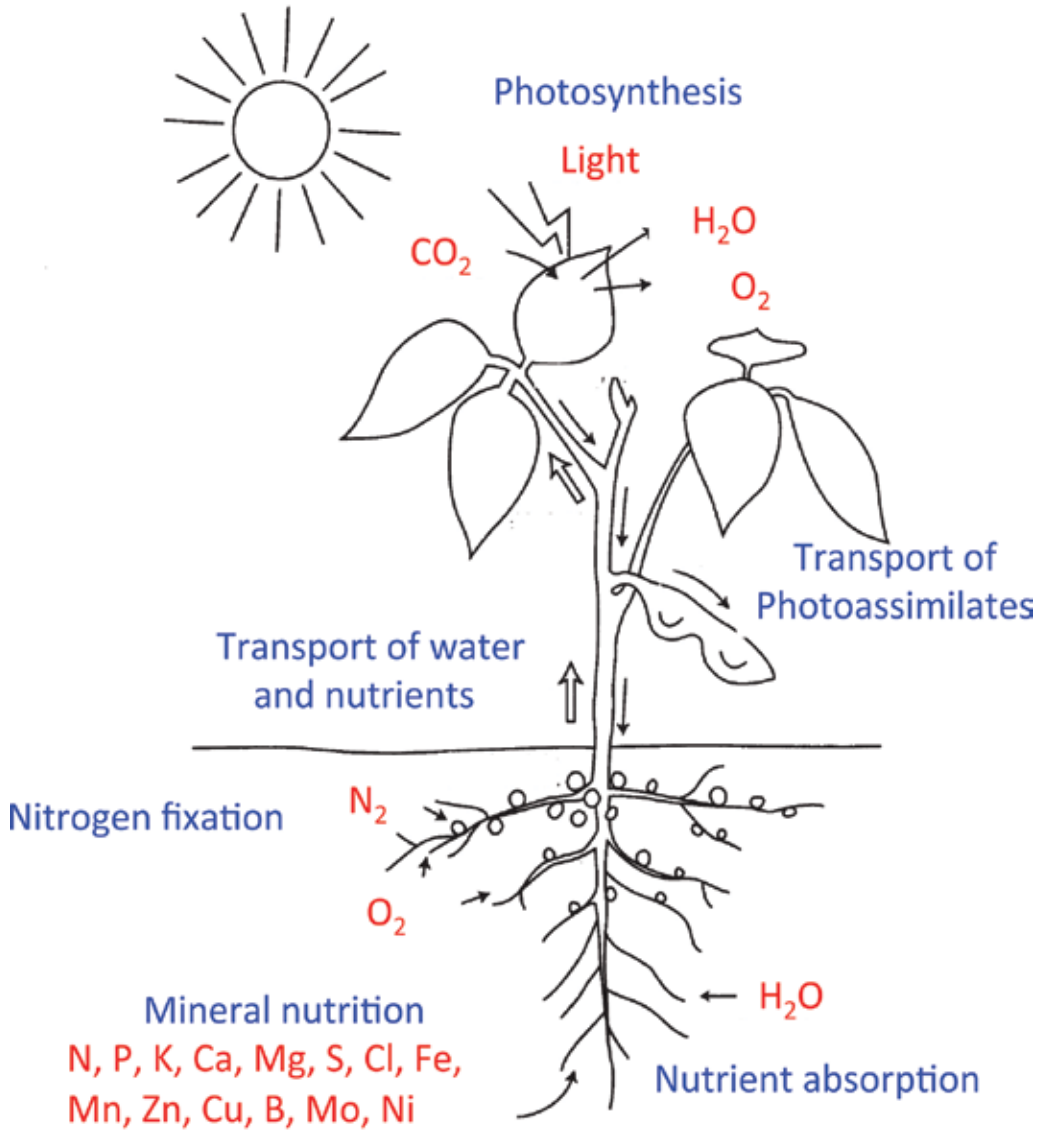

Figure 3. Physiological processes of nutrient acquisition by soybean.

respectively. Although the amounts of requirement for micronutrients, Cl, Fe, Mn, Zn, Cu, B, and Mo, are very low, these are essential for soybean growth.

1.4. Biotic and abiotic constraints to reduce soybean growth and seed yield

Among the factors inherent in agricultural production, the climatic conditions are the most difficult to control and they are of greater limiting factors in the maximum yield [16]. Abiotic stresses such as drought, excessive rain, extreme temperature, and low light can significantly

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Element	Symbol	g kg^{-1} of seed DW	g kg^{-1} of seed*
Carbon	C	1170	1024
Oxygen	O	1100	963
Hydrogen	H	150	131
Nitrogen	N	107	93
Potassium	K	37	32
Calcium	Ca	27	23
Magnesium	Mg	12	10
Phosphorous	P	10	9
Sulfur	S	8.3	7.3
Chlorine	Cl	3.3	2.9
Iron	Fe	0.57	0.496
Manganese	Mn	0.20	0.175
Zinc	Zn	0.07	0.058
Copper	Cu	0.03	0.029
Boron	B	0.03	0.029
Molybdenum	Mo	0.00	0.003
Cobalt	Co	0.00	0.001

Data are calculated from Ohlrogge and Kamprath [15].
The amount of nutrient requirement is in total plant dry matter including grains, stems, leaves, and roots.
*Values are calculated using seed water content of 12.5%.

Table 3. Requirement of nutrients for soybean seed production.

reduce yields of crops [16]. Next to the climatic factors, adverse soil conditions are major constraints for soybean production. In Japan, soybeans are cultivated mainly in rotated paddy rice fields and the bad drainage of water restricts nitrogen fixation and root growth and results in low productivity of soybean. In these fields, it is recommended to make open-channel drainage and under-drainage to accelerate the water drainage after a heavy rain. In addition to such physical conditions of soil, chemical conditions such as pH, contents of nutrients, and the availabilities limit soybean yield. Soil fertility is also important to support soybean growth. Among biotic constraints, weeds, insects, and diseases are serious problems to reduce plant growth and seed productivity.

2. Role of nitrogen on growth and seed yield and quality of soybean

2.1. Nitrogen requirement for seed production

A high yield of soybean requires a large amount of N, and soybean plants should continue to assimilate nitrogen during both vegetative and reproductive stages. Many field data showed that the total amount of N assimilated in a plant shoot is highly correlated with

the soybean seed yield. In **Figure 4**, the relationship between total amount of N in soybean shoot at R7 stage and seed yield is shown in a rotated paddy field in Niigata [17–21]. A linear correlation (r = 0.855) between seed yield and the amount of nitrogen accumulation in the shoot was observed. Salvagiotti et al. [7] reviewed the relationships among seed yield, nitrogen uptake, nitrogen fixation, and nitrogen fertilizer based on 637 published data sets [7]. A mean increase of 13-kg soybean seed yield per kg N increase in above-ground part was obtained from these data, which is equivalent to the 77 kg N required for 1 ton of seed production.

Rice crops assimilate about 80% of N until flowering, while soybean plants assimilate only about 20% of total N until initial flowering stage (R1 stage) (**Figure 5**). Therefore, continuous assimilation of nitrogen after initial flowering stage is essential for good growth and high seed yield of soybean plants.

To obtain high seed yield of soybean, good nodulation and high and long-lasting nitrogen fixation activity are very important, because the availability of soil N is generally insufficient to support soybean growth and seed N and chemical starter fertilizer N is lost in a few weeks after planting (**Figure 6**). Soybean nodule formation and nodule growth are influenced by various soil conditions such as water content, pH, nutrition, and climatic conditions such as solar radiation, temperature, rainfall, and so on. Soybean forms root nodules associated with soil bacteria, bradyrhizobia, and can fix atmospheric N_2. Soybean can absorb and utilize inorganic nitrogen such as nitrate and ammonia from soil or fertilizer. Usually, a high yield of soybean has been obtained in a field with high soil fertility or with the application of organic manure. Supply of low and constant concentration of nitrogen from soil or organic manure

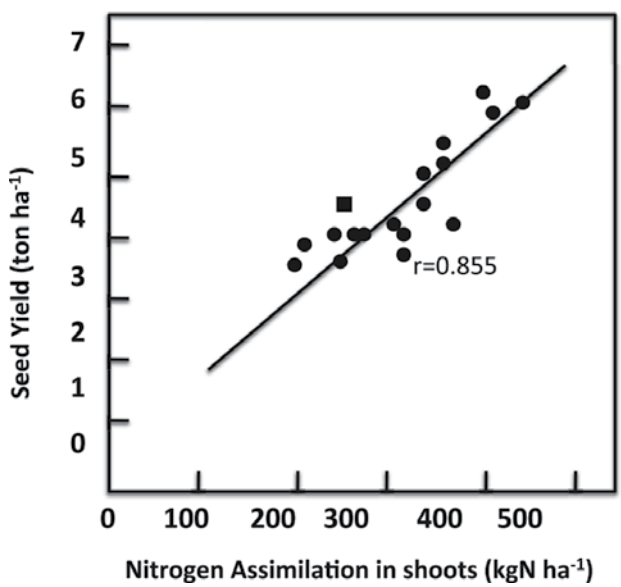

Figure 4. Relationship between the total amount of nitrogen assimilation and seed yield in soybean shoot at R7 stage (Takahashi et al. [5]).

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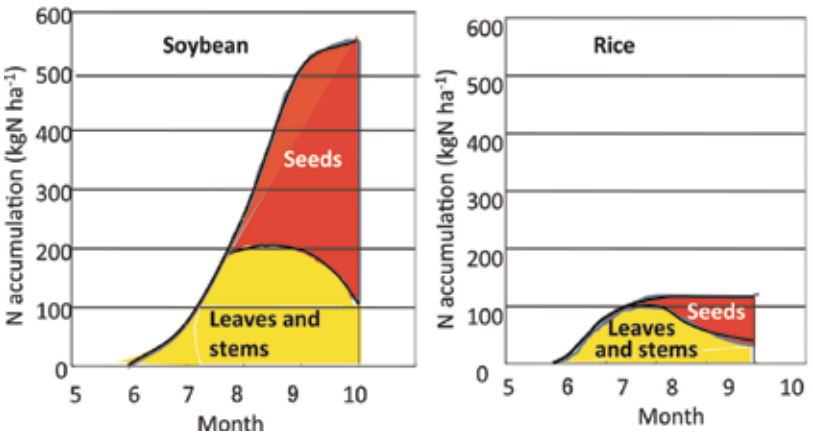

Figure 5. Comparison of N accumulation in soybean and rice plant during vegetative and reproductive stages (Ohyama et al. [38]).

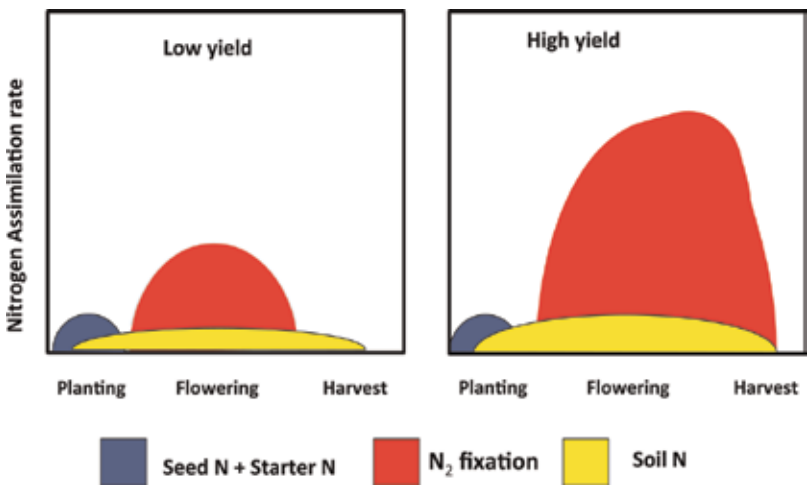

Figure 6. Comparison of the nitrogen assimilation patterns coming from seed N + starter N, soil N, and N_2 fixation between low-yield and high-yield model [38].

may support the soybean growth without depressing nodulation and nitrogen fixation activity. Nevertheless, a high concentration of mineral N depresses nodule formation and nitrogen fixation activity, especially nitrate, the most abundant inorganic nitrogen in upland fields, severely inhibits nodulation and nitrogen fixation [22–24].

Soybean plants assimilate the N from three sources, N derived from symbiotic N_2 fixation by root nodules (Ndfa), N absorbed from soil mineralized N (Ndfs), and N derived from fertilizer when applied (Ndff) (**Figure 7**). One ton of soybean seed requires about 70–90-kg N assimilation, which is about four times more than in the case of rice [25]. It is necessary to use both N_2 fixation by root nodules and absorbed N from roots for the maximum seed yield of soybean [26, 27]. Sole

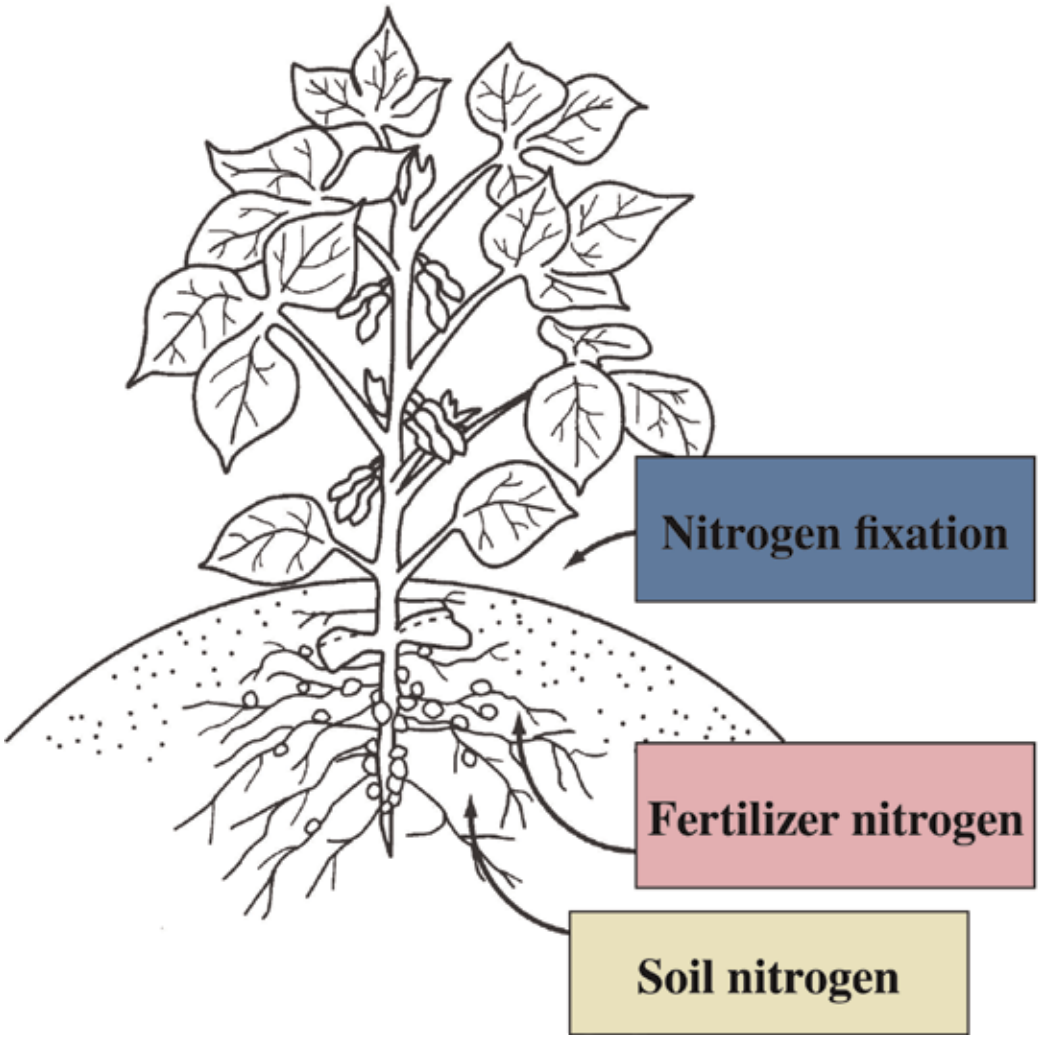

Figure 7. Three sources of N assimilated in soybean plants.

N_2 fixation is often insufficient to support vigorous vegetative growth, which results in the reduction of seed yield. On the other hand, a heavy supply of N often depresses nodule development and N_2 fixation activity, and accelerates nodule senescence, which also results in the reduction of seed yield. Moreover, a heavy supply of N from fertilizer or from soil causes the over-luxuriant growth of shoot, resulting in lodging and poor pod formation. Therefore, no nitrogen fertilizer is applied or only a small amount of N fertilizer is applied as a "starter N."

Initial nodulation mainly occurs at the basal part of the main roots, but these nodules are broken down earlier, and many nodules are formed at the lateral roots during reproductive stage (**Figure 8**) [28].

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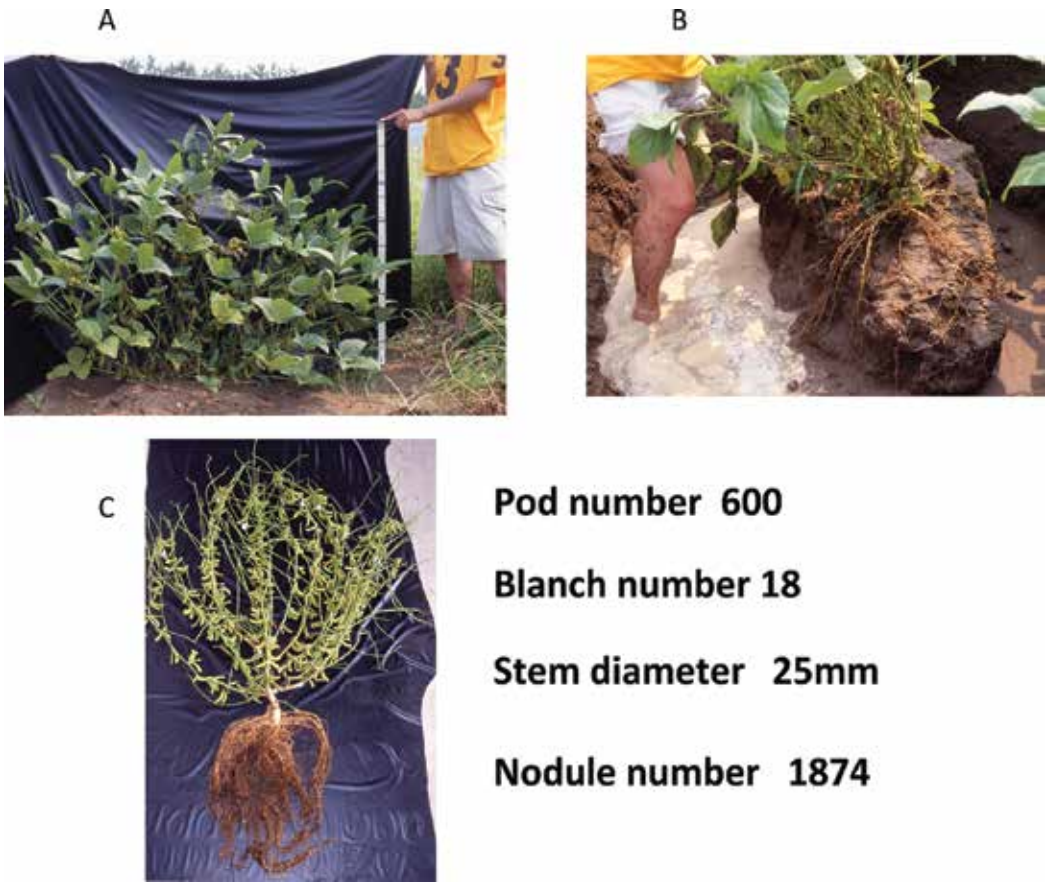

Figure 8. A giant soybean (cultivar Williams) plant cultivated at planting density of 2 plants m^{-2} (from Suganuma et al. [28]). A: One big plant. B: Roots are recovered from soil. C: Whole body of giant soybean.

2.2. Field assessment of nitrogen fixation and nitrogen absorption by simple relative ureide method

There are several methods for field assessment of nitrogen fixation by nodules and nitrogen absorption by soybean roots, such as N balance method comparing nodulated and non-nodulated isolines, ^{15}N dilution method, ^{15}N natural abundance method, and relative ureide method [29]. Simple relative ureide method is the most convenient and reliable with a low-cost and inexpensive apparatus.

Kushizaki et al. discovered that nodulated soybean plants contain a large amount of ureides (allantoin and allantoic acid) in stems, while non-nodulating isoline contains much less amount of ureides [30]. The $^{15}N_2$ was exposed to the nodulated roots of soybean plants, and the initial assimilation of ^{15}N was investigated in cytosol (plant cytoplasm) and bacteroid (a symbiotic state of rhizobia in nodules) fractions [31–33]. The result suggested that most of the fixed ^{15}N is immediately exported from bacteroid to plant cytosol and initially assimilated into glutamine and glutamate via GS/GOGAT pathway in cytosol, then metabolized into

Fertilizer application	Year	Cultivar	Stage	Method	%Ndfa	Reference
Control	1984	T202	Seed	$\delta^{15}N$	75	Yoneyama et al. [40]
Control	1990	Enrei	R7	RU	74	Takahashi et al. [19]
Control	1990	T202	R7	RU	75	Takahashi et al. [20]
Control	1990	T201/T202	R7	N-balance	75	Takahashi et al. [20]
Deep placement	1990	Enrei	R7	RU	64	Takahashi et al. [19]
Deep placement	1990	T202	R7	RU	59	Takahashi et al. [20]
Deep placement	1990	T201/T202	R7	N-balance	65	Takahashi et al. [20]
Top dressing	1990	Enrei	R7	RU	65	Takahashi et al. [19]
Top dressing	1990	T202	R7	RU	60	Takahashi et al. [20]
Top dressing	1990	T201/T202	R7	N-balance	62	Takahashi et al. [20]

Data from Takahashi et al. [39].
$\delta^{15}N$: ^{15}N natural abundance method, RU: relative ureide method, N-balance: N-balance method using nodulating and non-nodulating soybean isolines.

Table 4. Estimated percentage of Ndfa in soybean cultivation in the rotated paddy field in Niigata Agricultural Experiment Station.

various amino acids via transamination from glutamate. Then, ureides, allantoin and allantoic acid, are synthesized from amino acids and amides in cytosol.

On the other hand, after adding $^{15}NO_3^-$ in the culture solution of hydroponic soybean, the ^{15}N concentration of asparagine increased markedly, indicating that asparagine is a major assimilatory compound of NO_3^- in soybean roots [34].

Many kinds of tropical grain legumes, such as soybean, common bean, cowpea, pigeon pea, and mung bean that have spherical determinate type of nodules, transport the bulk of fixed N as ureides (allantoin and allantoic acid). On the other hand, nitrate and amino acids (especially asparagine) are the major transport forms of N absorbed by soybean roots [34, 35]. Herridge et al. [36, 37] developed the "relative ureide method" for evaluating the % Ndfa (percentage of nitrogen derived from atmospheric dinitrogen) by analyzing the concentrations of nitrogen compounds in xylem sap obtained from bleeding sap of a cut stump or by vacuum collection from stems. The concentrations of ureide-N, nitrate-N, and α-amino-N can be determined by colorimetry [38].

This method is reliable in the field assessment of % Ndfa of soybeans, without any requirement of reference plants such as non-nodulated isolines. This method is also applicable for experiments with variable N fertilizer application (**Table 4**), and no preparation before sampling is necessary. The modified equation can be adopted for the estimation of % Ndfa in field experiment [38].

2.3. Quantitative estimation of daily N_2 fixation and N absorption rate by modification of xylem-sap sampling and colorimetric methods for relative ureide method

By periodical sampling of soybean shoots and xylem sap, a quantitative estimation of the seasonal changes in N_2 fixation activity and N absorption rate is possible [29, 39, 41]. Soybean

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plants are harvested at R1, R3, R5, and R7 stages, or R1, R3, and R7 stages, and relative ureide-N in xylem sap and plant N is analyzed. **Figure 9** shows the example of the evaluation of Ndfa by simple relative ureide method [42].

McClure and Israel [43] analyzed the transport of nitrogen in the xylem sap of soybean plants, and proposed the percentage of ureide-N in xylem sap may be used as an indicator of the relative contribution of N_2 fixation to the total input of plant N. Herridge and Peoples [36] compared the ureide assay and ^{15}N dilution method and proposed the calibration equations. Regarding the equations, we proposed an equation as 100 × ureide-N/(ureide-N + amide-N + nitrate-N), where amide-N is "2 × α-amino-N," because major form of amino acids and amides is asparagine which has two N atoms in a molecule [43–45]. When plants are periodically harvested, the nitrogen fixation activity and nitrate absorption rate can be estimated from the relative ureide percentage and total N increase in the shoots [19, 21, 29, 39, 41].

For relative ureide analysis, xylem-sap bleeding from the cut surface of the lower part of the main stem is collected in a tube (**Figure 10A**), and the concentrations of ureides, amides, and nitrates in sap are determined by an optical spectrometer. Sometimes, xylem sap cannot be collected in the tube, especially when soil is dry or during late growth stages. Herridge and Peoples [36] used a vacuum-extracted exudate from the lower part of the main stem of the cut shoot or hot-water extraction of the stems [36].

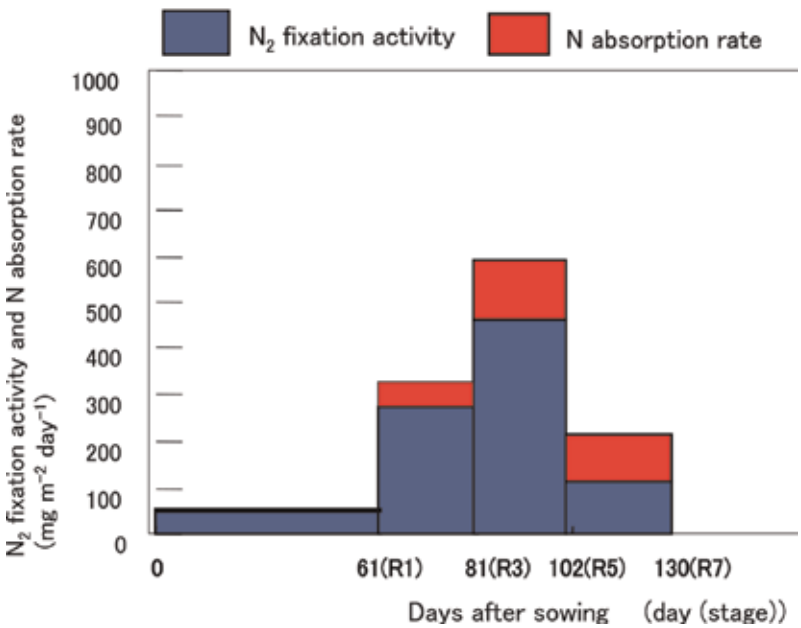

Figure 9. Changes in daily N_2 fixation activity and N absorption rate in soybean plants cultivated in Niigata with paper pot inoculation method (Tewari et al. [42]).

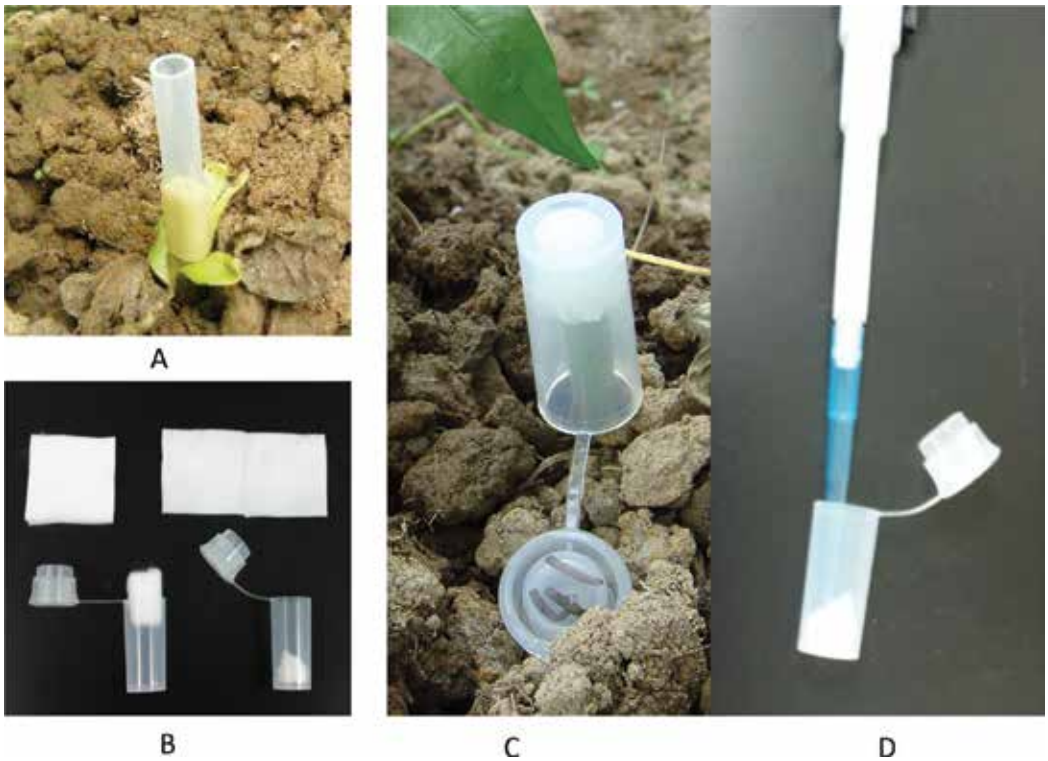

Figure 10. Collection of root-bleeding xylem sap (Sakazume et al. [46]). A: Xylem sap is collected in a tigon tube inserted to the woody part of the stem. B: Preparation of plastic cup with absorbent cotton. C: The plastic cup is put on a cut stem and xylem sap can be absorbed in cotton. D: The sap is recovered from cotton by sucking with an auto-pipette.

Recently, we collected the root-bleeding xylem sap as shown in **Figure 10C** [46]. The lower part of the main stem just below the node with cotyledons was cut by a pair of pruning shears. We used a tigon tube being inserted to the woody part of the stem (**Figure 10A**). Xylem sap started to exude at several minutes after cutting by a root pressure. However, the tube sometimes escaped from the stem or xylem sap leaked when the stem shape was irregular. Recently, we used a 6-mL plastic cup with absorbent cotton (**Figure 10B**). The plastic cup was put on the cut stem and xylem sap could be absorbed in the cotton (**Figure 10C**), and the sap was recovered by sucking with an auto-pipette (**Figure 10D**).

For each standard analysis of ureides, amides, and nitrate, 50 μL of sample solution was necessary for estimating relative ureide-N percentage. In addition, it was time-consuming to analyze each component by using glass test tubes. We developed a micro-scale analysis of these components in xylem sap using 2.5 μL of xylem sap instead of 50 μL for standard assay. The colorimetric reaction was carried out in a 1.5-mL Eppendorf centrifuge tube, and the 200 μL of reaction mixture was put into the well of a 96-well microplate, and the optical absorbance was measured by a microplate reader (**Figure 11**) [46].

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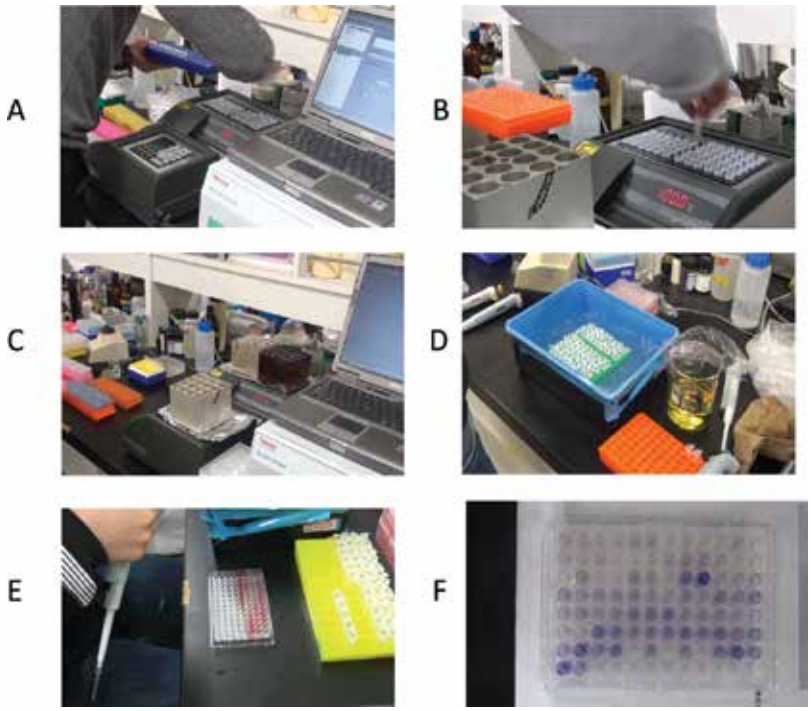

Figure 11. A micro-scale analysis of components in xylem sap of 2.5 μL (Sakazume et al. [46]). A–D: The colorimetric reaction was carried out in a 1.5-mL Eppendorf centrifuge tube. E, F: 200 μL of reaction mixture was put into the well of a 96-well microplate, and the optical absorbance was measured by a microplate reader.

3. Deep placement of slow-release nitrogen fertilizer promotes nitrogen fixation, and increases soybean seed yields

3.1. Deep placement of coated urea

Efficient fertilizer application is critical to crop production, economical benefit, and ecological advantages. The chemical formula, amount, size, as well as timing and placement of fertilizer affect the fertilizer use efficiency and consequently crop yield. For soybean cultivation in Japan, a basal dressing of compound fertilizers containing ammonium sulfate or urea at the rate of 20–40 kgN ha^{-1} is generally applied as "starter N."

Takahashi et al. [17–21] investigated the effect of deep placement of coated urea (CU) to promote soybean seed yield in a rotated paddy field in Niigata, Japan. The soil is a fine-textured gray lowland soil: texture; CL (clay loam), pH(H_2O); 6.6, CEC (cation exchange capacity); 28.8 (cmol(+) kg^{-1}), total carbon content; 10.9 g kg^{-1}, total N content; 1.02 g kg^{-1}, amount of mineralized N determined by the incubation of air dry soil under upland conditions for 4 weeks at 30°C; 47 mg kg^{-1}. Field experiment was conducted with three fertilizer N treatments. Control was conventional basal dressing of 16 kgN ha^{-1} as ammonium sulfate in

surface layer about 0–10 cm supplemented with 60 kg P_2O_5 ha^{-1}, 80 kg K_2O ha^{-1}, and 1000 kg $Ca(OH)_2$ ha^{-1}. Deep placement was 100 kgN ha^{-1} using 100-day type of coated urea, and top dressing was 100 kgN ha^{-1} by using 70-day type of coated urea at initial flowering stage. The seeds of soybean were grown by single stem training (75 × 15 cm) at the planting density of 8.9 plants m^{-2}.

Hundred-day-type coated urea hyperbolically releases urea and 80% of which is released in 100 days in water at 25°C. The deep placement of nitrogen fertilizer was carried out using the fertilizer injector devised by Shioya (**Figures 12** and **13**). Hundred-day-type-coated urea was applied just under the seed placement lines at a depth of about 20 cm. Top dressing of 70-day

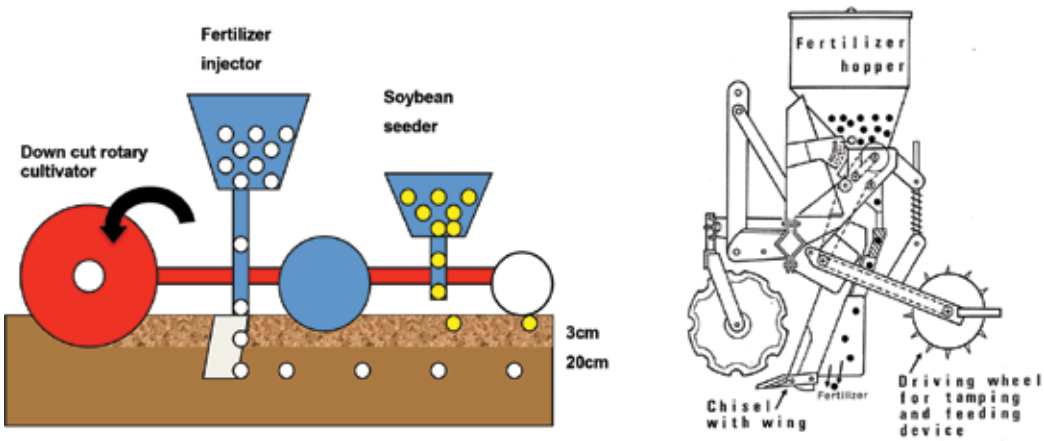

Figure 12. Fertilizer injector used for deep placement of coated urea (Takahashi et al. [39]).

Figure 13. The photograph of deep placement of coated urea taken in 1988.

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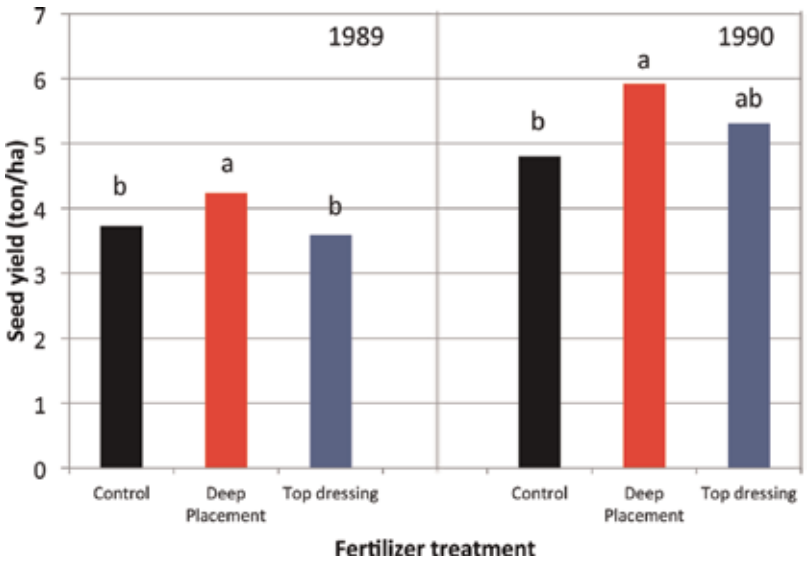

Figure 14. Seed yield of soybean in control, deep placement of 100-day-type-coated urea or top dressing of 70-day-type-coated urea (Takahashi et al. [17, 19]).

type of coated urea was carried out at R1 stage by broadcasting the fertilizer just before the earthing up intertillage.

Figure 14 shows the seed yield in 1989 and 1990. In 1989, seed yield by deep placement was 4.24 t ha^{-1} and 14% higher than the conventional control treatment (3.73 t ha^{-1}). The top-dressing treatment decreased seed yield about 4% and the yield was 3.59 t ha^{-1}. In 1990, seed yield by deep placement was 5.92 t ha^{-1} and 23% higher than the conventional treatment (4.80 t ha^{-1}). The top-dressing treatment increased seed yield about 11% and the yield was 5.31 t ha^{-1}.

Figure 15 shows the seasonal changes in daily N_2 fixation activity and N absorption rate calculated by a simple relative ureide method described before. Control plants mainly used fixed N_2 until R5 stage, and N absorption rate was higher after R5–R7 stage. In deep placement N_2 fixation activity was not depressed and N absorption rate was higher than control plants. As a result, the total amount of assimilated N in deep placement was 39.3 g m^{-2} and higher than control treatment (33.0 g m^{-2}). In the case of top-dressing treatment, N absorption rate after R3 stage became higher, but N_2 fixation activity after R5 stage became lower, and the total N was 36.3 g m^{-2}. The fertilizer use efficiency was evaluated by ^{15}N-labeled fertilizers, and the N recovery rate in the deep placement of 100-day-type-coated urea was 62% and much higher than top-dressed 70-day-type-coated urea (33%) and basal dressing of ammonium sulfate (9%) at R7 stage. The leaf area index (LAI) was higher in deep placement of 100-day-type-coated urea (2.99) than control (1.96) and top dressing of 70-day-type-coated urea (2.28) at R7 stage. The higher LAI might support the nodule activity during the pod-filling stage [19].

Takahashi et al. analyzed the accumulation of ammonium-N, nitrate-N, and urea-N in surface soil of 0–10 cm and deep soil of 15–25 cm in the field at R1, R3, R5, and R7 stages (**Figure 16**)

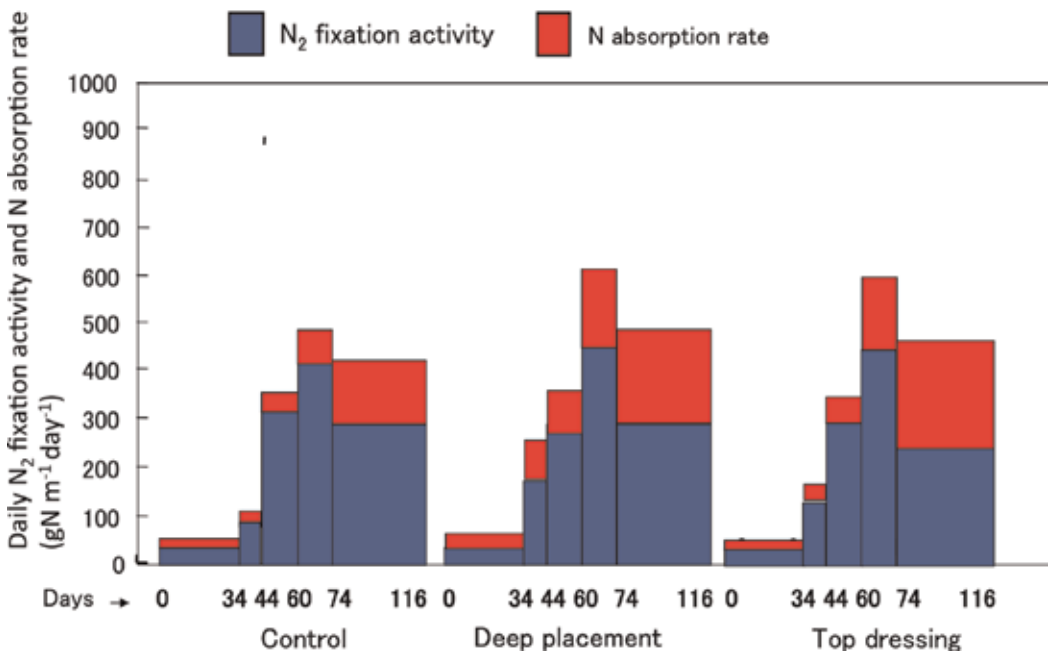

Figure 15. Changes in daily N_2 fixation activity and N absorption rate in control, deep placement of 100-day-type-coated urea or top dressing of 70-day-type-coated urea (Takahashi et al. [19]).

[20]. A high accumulation of ammonium-N was observed in the deep layer (15–25 cm) of soil at R3 and R5 stages of deep placement of 100-day-type-coated urea, although ammonium and nitrate accumulation was not observed in the surface layer at any stage of deep placement of coated urea. The result indicates that deep placement of coated urea slowly released urea and urea inside the particle was rapidly degraded to ammonium and it remained in the deep layer of soil for relatively long time. The slow nitrification rate might be due to low oxygen concentrations and low nitrification activities in deeper layers in rotated paddy fields in Niigata [20]. When 70-day type of coated urea was applied in the surface layer by top dressing at R1 stage, ammonium and nitrate concentration in the surface layer at R3 stage was significantly higher than control treatment. The accumulation of ammonium and nitrate in the surface layer where nodulation occurs might inhibit nodulation and nitrogen fixation.

3.2. Deep placement of lime nitrogen

Coated urea is suitable for deep placement, because it gradually releases urea until reproductive growth stage in accordance with soybean N requirement. However, as the price of coated urea is relatively expensive about five times higher than urea, the cost of it may be a burden for farmers. Tewari et al. compared the effect of deep placement of lime nitrogen (LN) with coated urea [41, 42, 47–51]. Lime nitrogen has been produced by artificial nitrogen fixation, which was done by Frank and Caro in 1901 prior to the establishment of Harber and Bosch process to convert atmospheric N_2 to ammonia in 1906. Lime nitrogen contains 60% of calcium cyanamide ($CaCN_2$) with calcium oxide (CaO) and free carbon (C). The fertilizer-grade lime nitrogen

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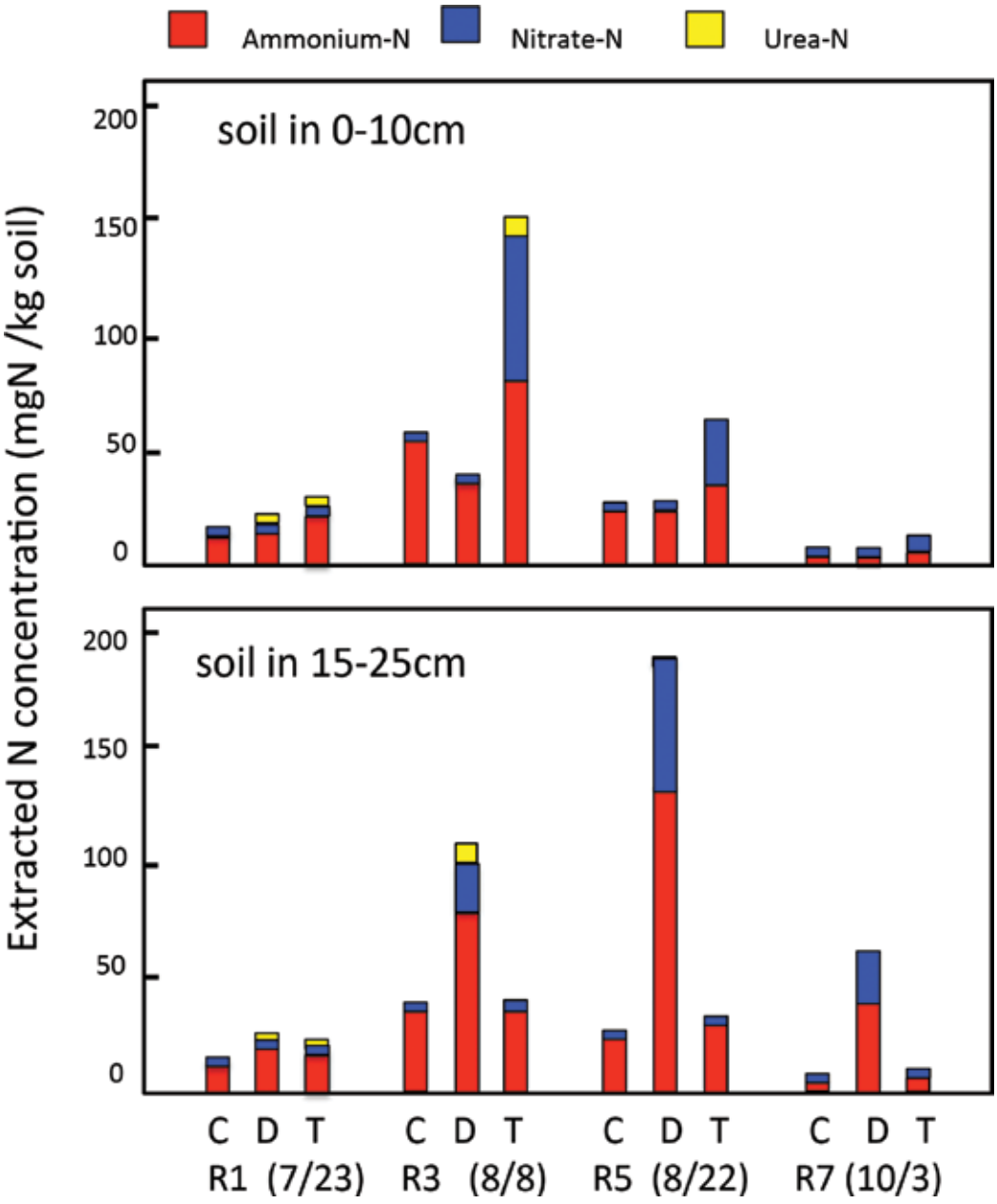

Figure 16. Changes in ammonium, nitrate, and urea concentration in the upper layer (0–10 cm) and deep layer (15–25 cm) of soybean field (Takahashi et al. [20]). C: control. D: Deep placement of 100-day-type-coated urea. T: Top dressing of 70-day-type-coated urea.

contains 21% N, 11% Ca, 11% C, 5% oil, 2–4% water, and oxides of aluminum, iron, and silicon [52]. $CaCN_2$ is converted to urea in soil, then the urea is hydrolyzed to ammonium and carbon dioxide. In the presence of moisture and air, dicyandiamide is formed from cyanamide of

$CaCN_2$, and this is a potent nitrification inhibitor, which inhibits the oxidation of ammonium to nitrate. Therefore, the ammonium produced by $CaCN_2$ decomposition persists for a long period of time and the nitrate concentration remains low in soil.

In 2001, fertilizer experiments were conducted in three sites in Niigata, Japan, of a rotated paddy field [46], a newly reclaimed wet land piled up with about 40-cm depth of surplus soil [48], and a sandy dune field [48]. Four fertilizer treatments of control without deep placement and deep placements of urea, 100-day-type-coated urea, and lime nitrogen were conducted. In addition, three different inoculation methods of *Bradyrhizobia* were carried out. They were non-inoculated paper pot (NIPP), direct inoculation transplanting (DT), and inoculated paper pot (IPP). Paper pot was made of biodegradable paper in soil. The pot was opened at the bottom to allow root expansion below the pot. The paper pot used was filled with vermiculite and inoculated with *B. japonicum* USDA110. Plants were grown in a paper pot for 10 days after planting, and transplanted to the field. Direct inoculation was seed inoculation by suspension of *B. japonicum* without using paper pot. Other plants were germinated in non-inoculated paper pot, and transplanted at 10 days after planting.

Table 5 shows the seed yields of three sites in which fertilizers and inoculation treatments were carried out. In the rotated paddy field, significant higher yield was observed by deep placement of coated urea and lime nitrogen compared with control without deep placement of urea. Among the same fertilizer treatments, the seed yields with IPP and DT inoculation methods tended to exceed that with NIPP.

Inoculation method	Fertilizer treatment	Rotated paddy field	Reclaimed field	Sandy dune field
NIPP	Control	288b	78b	172b
	Urea	453a	286a	246a
	Coated urea	429a	358a	249a
	Lime nitrogen	460a	340a	250a
DT	Control	314b	194b	191b
	Urea	422ab	336a	262a
	Coated urea	535a	397a	271a
	Lime nitrogen	541a	356a	267a
IPP	Control	331b	201c	183b
	Urea	467b	290b	273a
	Coated urea	604a	400a	305a
	Lime nitrogen	612a	419a	332a

NIPP: Non-inoculated paper pot. DT: Direct transplanting of inoculated seedlings. IPP: Inoculated paper pot. Means followed by the same letter are not significantly different at 5% level in the same inoculation methods in the same field.

Table 5. Seed yield of soybean cultivated with deep placement of N fertilizers and different inoculation methods in three fields of Niigata in 2001. Data from Tewari et al. [47–49].

Fertilizer treatment	Total N (g m^{-2})	Ndfa (g m^{-2})	Ndfs + Ndff (g m^{-2})	%Ndfa
Control	22.4b	13.6b	8.8b	61
Urea	28.3b	13.2b	15.1a	47
Coated urea	31.0a	21.3a	9.7b	69
Lime nitrogen	33.4a	21.4a	12.0ab	64

Data from Tewari et al. [47].

Table 6. Nitrogen origin of soybean cultivated with various N fertilizers and inoculated paper pot in rotated paddy field of Niigata (2001).

Similar results were observed in the newly reclaimed field and the sandy dune field, although seed yield levels were lower than those in rotated paddy field, possibly due to lower soil fertility in these fields. Irrespective of field types and inoculation methods, deep placement of lime nitrogen and coated urea gave the promotive effect on seed yield of soybean. The application of lime nitrogen tended to give higher seed yield than that of coated urea, although data were statistically not significant. The results of three field experiments confirmed that deep placement of coated urea can be replaced by lime nitrogen.

Table 6 shows the nitrogen origin of soybean shoots at R7 stage. Total N assimilation was higher in deep placement of coated urea (31.0 g m^{-2}) and lime nitrogen (33.4 g m^{-2}) than in urea (28.3 g m^{-2}) and control (22.4 g m^{-2}) treatment. Based on the analyses of Ndfa and Ndfs + Ndff, it was confirmed that Ndfa from deep placement of coated urea (21.3 g m^{-2}) and lime nitrogen (21.4 g m^{-2}) was higher than that in urea (13.2 g m^{-2}) and control (13.6 g m^{-2}) treatment. This indicates that deep placement of lime nitrogen and coated urea promoted nitrogen fixation without inhibiting it.

3.3. Effect of the depth of placement of lime nitrogen on seed yield and nitrogen assimilation

The effects of different depths of placement of lime nitrogen at 10, 15, and 20 cm were compared in a rotated paddy field at the Niigata Agricultural Research Institute in 2003 [49]. In addition to conventional basal application of mixed chemical fertilizer (ammonium sulfate 16 k kgN ha^{-1}, P_2O_5 60 kg ha^{-1}, K_2O 80 kg ha^{-1}, and $Ca(OH)_2$ 1000 kg ha^{-1} in the plow layer at a depth of 0–10 cm), lime nitrogen (100 kgN ha^{-1}) was placed in 10, 15, or 20 cm depth just under sowing line.

Figure 17 shows the seed yield classified with seed quality. The total seed yield was highest in the deep placement of lime nitrogen at 20 cm (617 g m^{-2}) compared with 15-cm depth (526 g m^{-2}), 10-cm depth (515 g m^{-2}), and control without deep placement (428 g m^{-2}). The yield of good seeds was significantly higher in deep placement of lime nitrogen at 20-cm depth.

The daily N_2 fixation activity and N absorption rate were calculated using the simple relative ureide method (**Figure 18**). Average daily N_2 fixation activity and N absorption rate were relatively low until 71 days after sowing (R2 stage). From 71 days (R1) to 102 days (R5) after sowing, both the N_2 fixation activity and N absorption rate were high in lime nitrogen

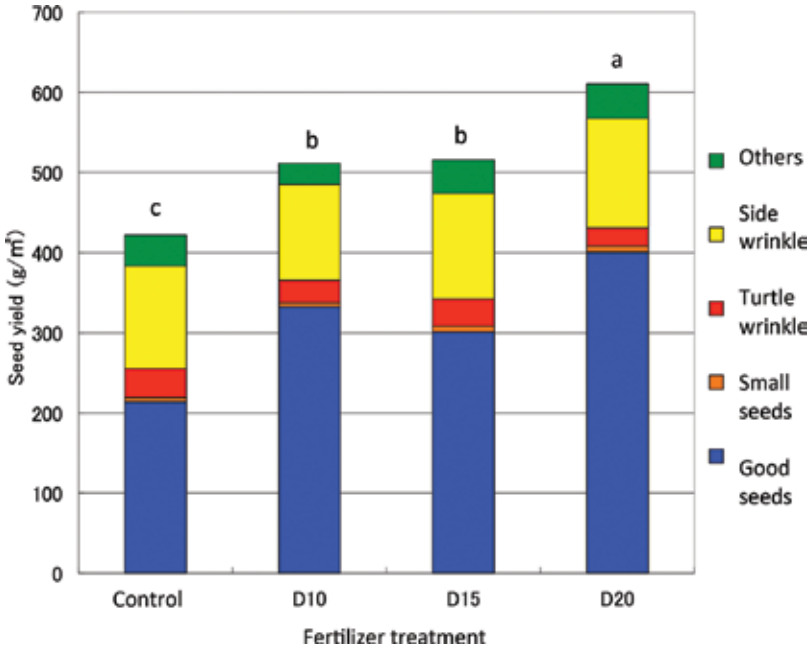

Figure 17. Seed yield classified with seed quality of soybeans cultivated with different depths of lime nitrogen placement (Tewari et al. [50]). Control: lime nitrogen was not applied. D10: lime nitrogen was applied at 10-cm depth. D15: lime nitrogen was applied at 15-cm depth. D20: lime nitrogen was applied at 20-cm depth.

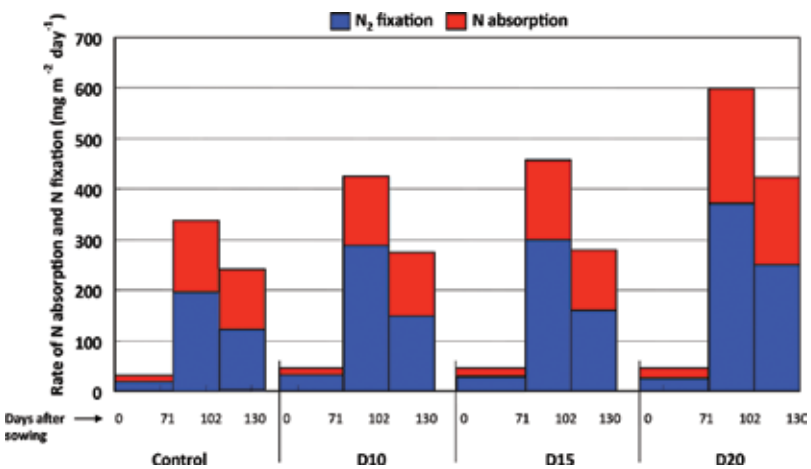

Figure 18. Changes in daily N_2 fixation activity and N absorption rate in soybean plants grown with different depths of lime nitrogen placement (Tewari et al. [50]). Control: lime nitrogen was not applied, D10: lime nitrogen was applied at 10 cm, D15: lime nitrogen was applied at 15 cm, D20: lime nitrogen was applied at 20 cm.

treatment of especially 20-cm depth compared with control plants. From 102 days (R5) to 130 days after sowing (R7), the N_2 fixation activity declined in all fertilizer treatments, but D20 plants (20-cm depth) kept the highest N_2 fixation activity and N absorption rate.

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3.4. Effect of the amount of placement of lime nitrogen on seed yield and nitrogen assimilation

The effect of different amounts of placement of lime nitrogen at 50, 100, or 200 kgN ha^{-1} was compared in a rotated paddy field at the Niigata Agricultural Research Institute in 2003 [51]. In addition to conventional basal application of mixed chemical fertilizers in the plow layer of a depth of 0–10 cm, lime nitrogen (50, 100, or 200 kgN ha^{-1}) was placed in 20-cm depth under soil surface just below sowing line.

Figure 19 shows the seed yield classified with seed quality. The total seed yield was highest in the deep placement of 100 kgN ha^{-1} lime nitrogen (570 g/m^2) compared with 50 kgN ha^{-1} (510 g/m^2), 200 kgN ha^{-1} (500 g/m^2), and control without deep placement (470 g/m^2). The yield of good seeds increased in deep placement of lime nitrogen at 20-cm depth.

The daily N_2 fixation activity and N absorption rate were calculated using the simple relative ureide method (**Figure 20**). The average daily N_2 fixation activity and N absorption rate were relatively low until 71 days after sowing (R2 stage). From 71 to 102 days (R5 stage) after sowing, both the N_2 fixation activity and N absorption rate were higher in LN treatment of especially 100 kgN ha^{-1} compared with control plants.

From 102 to 130 days after sowing (R7 stage), the N_2 fixation activity declined in all fertilizer treatments, but plants of 100 kgN ha^{-1} kept high N_2 fixation activity and N absorption rate during this period. When 200 kgN ha^{-1} was applied, N_2 fixation activity was lower than that at 100 kgN ha^{-1} of lime nitrogen.

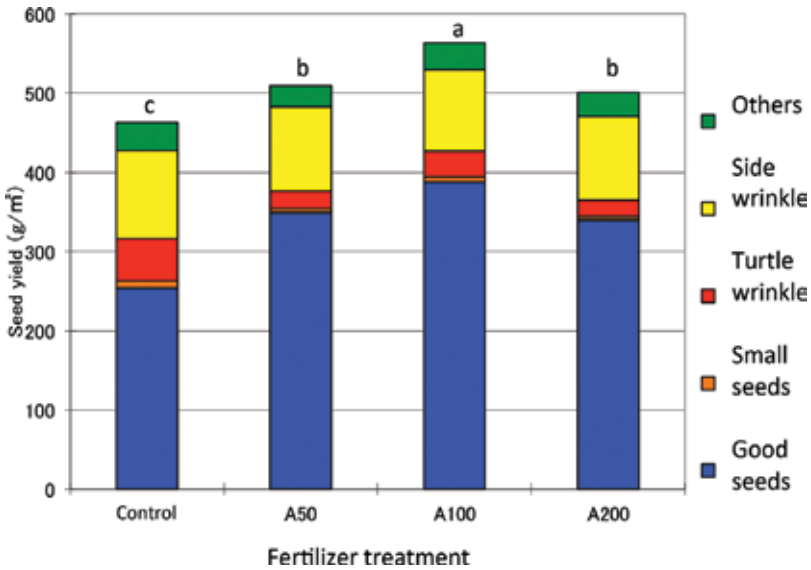

Figure 19. Seed yield classified with seed quality of soybeans cultivated with different rates of lime nitrogen at 20-cm depth (Tewari et al. [51]). Control: lime nitrogen was not applied, A50: 50 kgN ha^{-1} lime nitrogen was applied, A100: 100 kgN ha^{-1} lime nitrogen was applied, A200: 200 kgN ha^{-1} lime nitrogen was applied.

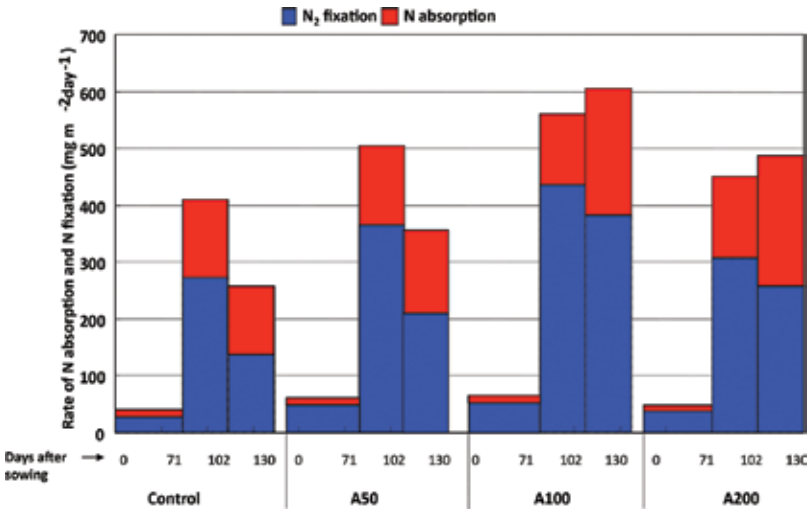

Figure 20. Changes in daily N_2 fixation activity and N absorption rate in soybean plants grown with different rates of lime nitrogen placed at 20-cm depth (Tewari et al. [51]). Control: lime nitrogen was not applied, A50: 50 kgN ha^{-1} lime nitrogen was applied, A100: 100 kgN ha^{-1} lime nitrogen was applied, A200: 200 kgN ha^{-1} lime nitrogen was applied.

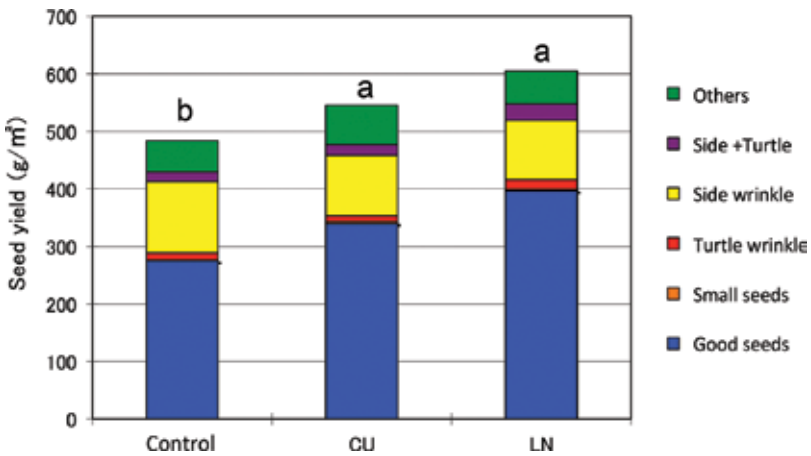

Figure 21. Seed yield classified with seed quality of soybeans grown with deep placement of coated urea and lime nitrogen (Tewari et al. [42]). Control: lime nitrogen was not applied, CU: 100-day-type-coated urea was applied at 20-cm depth, LN: lime nitrogen was applied at 20-cm depth.

3.5. Comparison of seasonal absorption patterns of coated urea and lime nitrogen

The experiment was carried out in a rotated paddy field in Niigata Agricultural Research Institute in 2004, and the utilization of deep placement of ^{15}N-labeled coated urea (CU) and lime nitrogen (LN) was investigated at R1, R3, R5, and R7 stages [42]. In this experiment, seed yield was higher in deep placement of LN or CU than in control treatment (**Figure 21**). **Figure 22** shows changes in daily N_2 fixation activity and N absorption rate. From R1 (61 DAS (days after sowing)) to R5 (102 DAS) stage, both N_2 fixation activity and N absorption rate were higher in deep placement of CU or LN compared with control treatment. From R5 to

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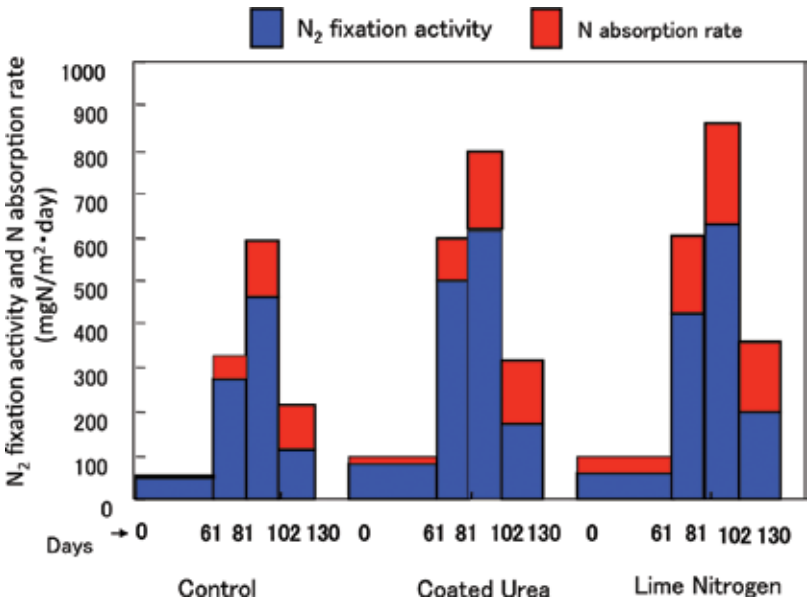

Figure 22. Changes in daily N_2 fixation activity and N absorption rate in soybean plants cultivated in Niigata (Tewari et al. [42]). Control: lime nitrogen was not applied, CU: 100-day-type-coated urea was applied at 20 cm, LN: lime nitrogen was applied at 20-cm depth.

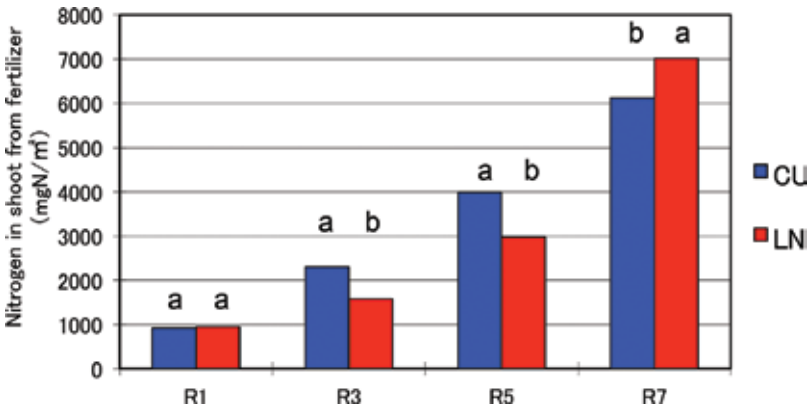

Figure 23. Nitrogen recovered in soybean shoot grown with deep placement of 100-day-type-coated urea or lime nitrogen (Tewari et al. [42]). CU: Deep placement of 100-day-type-coated urea, LN: deep placement of lime nitrogen.

R7, the daily N_2 fixation activity declined in all treatments, but plants grown with deep placement of CU and LN maintained the higher activity than control plants.

Figure 23 shows the changes in the content of labeled N in soybean plants cultivated with ^{15}N-labeled CU or LN. At R1 stage, the absorption rates of N from CU and LN were almost the same, but those were higher with CU than with LN at R3 and R5 stages. However, at the R7 stage the labeled N content increased markedly with LN and exceeded that with CU. At R7, fertilizer use efficiencies were 70% in LN and 61% in CU.

3.6. Effect of deep placement of lime nitrogen on distribution of root nodules

The experiment was conducted in a rotated paddy field of Niigata Agricultural Research Institute, Nagaoka [53]. Conventional basal dressing of chemical fertilizer containing ammonium sulfate (16 kgN ha^{-1}), fused magnesium phosphate (60 kg P_2O_5 ha^{-1}), and potassium chloride (80 kg K_2O ha^{-1}) was plowed in a soil layer at 0–10-cm depth. A soybean plant was planted in the center of the wooden box (length, 45 cm; width, 4.5 cm; depth, 30 cm) filled with the field soil (**Figure 24**). The boxes were embedded in the field. Two fertilizer treatments were conducted. Control was no

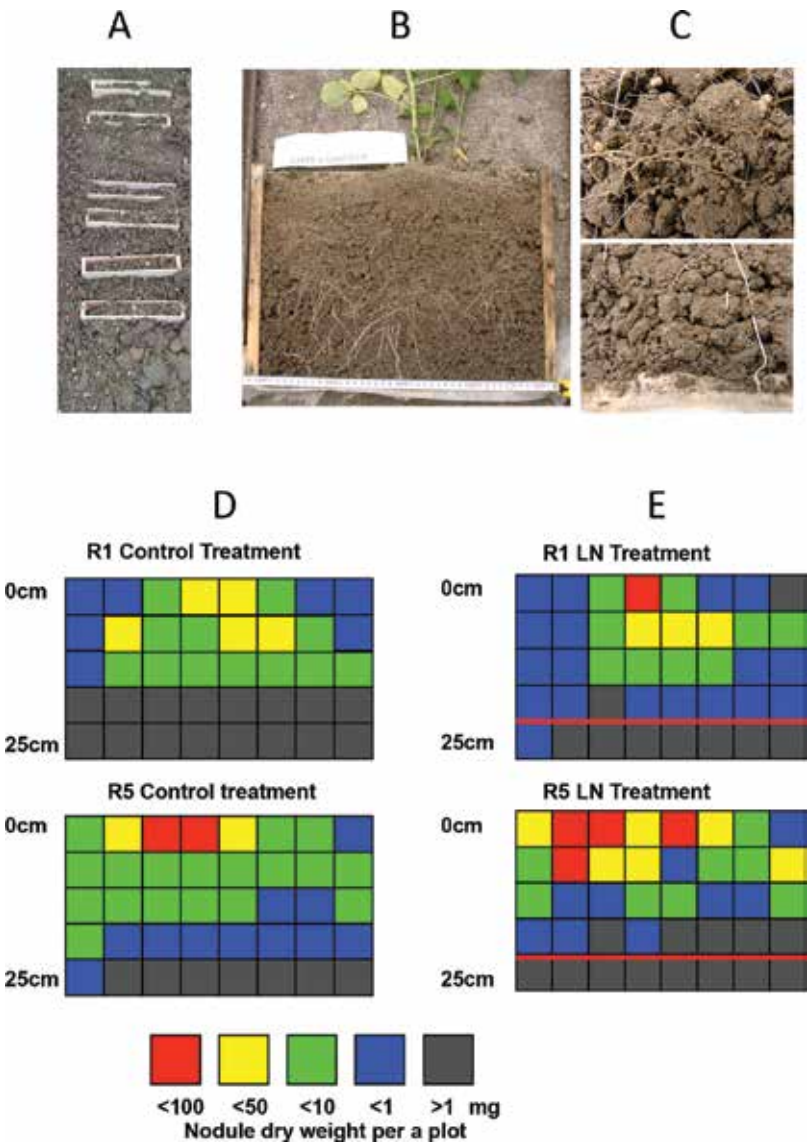

Figure 24. Distribution of nodules in the root box (rhizobox) (Ohyama et al. [53]). A: Placement of root box in soybean field; B: inside of root box; C: nodules and roots; D: control treatment without deep placement; E: deep placement of lime nitrogen at 20-cm depth.

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additional fertilizer, and for deep placement of lime nitrogen, lime nitrogen (1.12 gN plant^{-1}) was applied at 20-cm depth in the box. At R1 (initial flowering) stage and R5 (pod-filling) stage, the rhizoboxes were dug out. The soil in the box was separated into blocks with each profile of 5 × 5 cm, and the soils were washed out. The dry weights of roots and nodules were measured.

Total nodule weight of the plants treated with deep placement of lime nitrogen was 0.57 g plant^{-1} and lower than the control nodule weight of 0.73 g plant^{-1} at initial flowering stage (R1 stage). The nodule distribution was not different between deep placement of lime nitrogen and control treatment at R1 stage. At R5 stage, the total nodule dry weight of deep placement of lime nitrogen was 1.17 g plant^{-1} and much higher than that in control plants (0.73 g plant^{-1}). The nodule weight in the upper layer of soil in lime nitrogen treatment was higher than that in control treatment. This result supported the promotion of nitrogen fixation by deep placement of lime nitrogen estimated by relative ureide method. The dry weight of the shoot was also higher in deep placement of lime nitrogen (37 g plant^{-1}) compared with control plants (28 g plant^{-1}) at R5 stage. These results demonstrated that deep placement of lime nitrogen provides

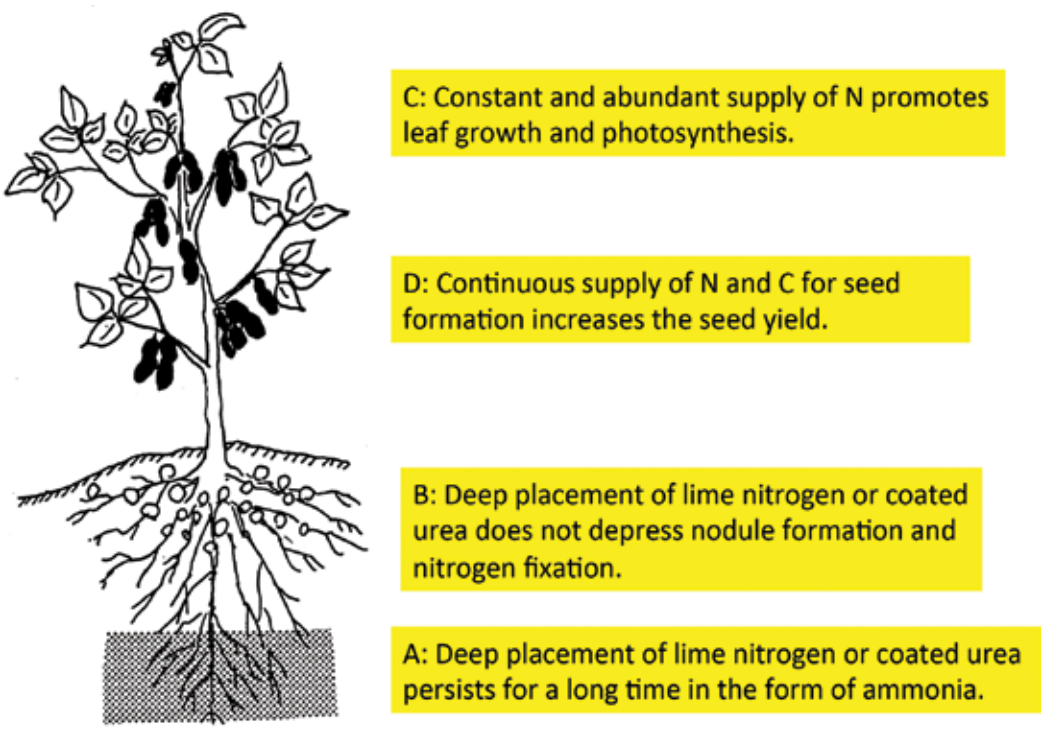

Figure 25. Promotive effects of deep placement of lime nitrogen or coated urea. (A) Basal dressing of deep placement of lime nitrogen or coated urea persists in the form of ammonia for a long time until reproductive stages. Plant roots can absorb N from lower soil layer, and the root growth and water and nutrient absorption may be increased [18]. Lime nitrogen contains Ca, and the Ca application may be beneficial for soybean growth, because soybean requires much Ca. Making a slit in soil under planting may improve water drainage. (B) Deep placement of lime nitrogen or coated urea did not increase the concentration of nitrate or ammonium in the upper layer where nodules are mainly formed. So this method does not inhibit nodulation and nitrogen fixation. (C) Continuous supply of N from lower soil promotes leaf photosynthetic activity until seed-maturing stage. Therefore, abundant photosynthate may be transported to nodules as well as seeds. (D) Continuous supply of N and C for seed growth increases the seed yield and improves the seed quality.

the nitrogen needed in the reproductive stage and vigorous photosynthetic activity of the leaves promotes nodule growth and nitrogen fixation. Takahashi et al. [5] reported that deep placement of coated urea increased the total dry weight of shoots and leaf area index in R7 compared with control treatment without deep placement. The abundant supply of C and N decreased the flower and pod shedding, and the seed number and seed weight increased.

3.7. Summary of the effect of deep placement of coated urea or lime nitrogen

Figure 25 summarizes the effect of deep placement of lime nitrogen or coated urea to promote seed yield of soybean.

4. Conclusions and perspectives

For maintaining agricultural production and protecting the environment, efficient use of N fertilizer is crucial. To optimize the chemical form, rate, timing, and placement of the fertilizer are important for the demand of various crops grown under various conditions. Deep placement of lime nitrogen or coated urea promoted the soybean growth and seed yield through promotion of nitrogen fixation after initial flowering stage. The promotive effect on nodulation by deep placement of lime nitrogen was confirmed by rhizobox experiment.

The effects of deep placement of N-, P-, and K-fertilizers on growth, nodulation, and yield of soybean have been investigated by Groneman [54]. Recently, deep placement of lime nitrogen has been tested in many agricultural fields, and the seed yield and quality are mostly improved. However, this technique has not been used in a large-scale farming. The agricultural machines, which can efficiently put fertilizers in deep place with high speed drive, should be developed. In addition, as the price of coated urea and lime nitrogen is relatively expensive, it is beneficial to use cheap nitrification inhibitors with urea or ammonia fertilizers.

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Chapter 10

Production of Soybean-Derived Feed Material Free from Salmonella Contamination: An Essential Food Safety Challenge

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66332

Abstract

Soybean meal is the world's most important source of protein for poultry and livestock. Due to frequent contamination of Salmonella, soy is since long unfortunately also found to be a high-riskfeed material for the introduction of Salmonella to the animal food production. This chapter focuses on the importance of biosecurity and hygiene in the production of soy-based animal feed. Those strategies and methods found to be effective tools for the production of a Salmonella-safe soy feed material in crushing plants and feed mills are reviewed and presented. It is also shown that the implementation of those methods at a limited cost can prevent animal feed from being the weakest food safety link in the food chain.

Keywords: Salmonella-contaminated feed material, food safety, soybean, crushing plant, feed mill

1. Introduction

This chapter focuses on the importance of biosecurity and hygiene in the production of soy-based animal feed to avoid the spread of Salmonella to the animal food production making animal feed the weakest link in the food chain.

Soybean meal is considered as the world's most important source of protein for poultry and livestock [1]. In a corresponding way, Salmonella is a major food-borne pathogen, which globally is estimated to cause 93 million enteric infections and 155,000 diarrheal deaths each year [2]. Salmonella is most often detected in meat and animal-derived food products, which are the primary source of Salmonella when spread to humans following consumption of poultry,

beef, pork, eggs, and milk. Vegetables and other food products can also be a source if contaminated with feces from Salmonella-infected animals [3–5].

In order to reduce the burden of human salmonellosis, efforts are directed to reduce the prevalence of Salmonella in the animal production including the animal feed. Like in humans, Salmonella is thus an orally acquired infection and the key route of transmission and Salmonella-contaminated feed is in both pigs and poultry found to be a major source of Salmonella [6, 7] which most likely is the case also for other food animal species.

Salmonella-contaminated soybeans and soybean meal are further found to be riskfeed materials of special importance for introducing Salmonella into the animal feed and the subsequent animal food chain [8]. Soy-based feed materials are of special importance because of not only being widely used but also being widely distributed, e.g., from South America to Europe. Possibly occurring Salmonella contamination in soy feed materials can thus be widely transmitted between countries and continents in case HACCP (Hazard Analysis & Critical Control Point)-based program and associated control measures are not in place.

Following an overview of the usage of animal feed, the object of this chapter is to describe the strategies applied for producing Salmonella-safe soybean meal and compound feed based on a review of current knowledge and experiences.

2. Usage of major feed materials

Animal feed includes a variety of different feed materials primarily of vegetable and, to a limited extent, also of animal origin. Cereals and forages are the major feed components. In addition, fast-growing animals such as broiler chickens and fattening pigs, as well as high-producing animals such as dairy cows and laying hens, require more protein- and energy-rich feed components. The feed composition varies by animal species, type of production, and geographic regions. Statistics are available from different sources and in this chapter exemplified through data from Europe (EU28) provided by European Feed Manufacturers Federation [9]. For EU28 about 49% (233 million tons) of the feed consumed by the livestock was grown and used on the farm of origin (mostly roughages). Of the feed material or compound feed purchased by livestock producers to supplement their own feed, compound feed accounted for 83% out of which the protein-rich cakes and meals accounted for 28% [9]. The total global compound feed production 2014 was 967 million tons out of which the major producers were China (19%), USA (18%), EU28 (16%), and Brazil (7%).

2.1. Sources of protein-rich feed cakes and meals

The access to protein-rich feed materials is essential in today's animal food production. The importance of protein-rich feed materials may be further strengthened in the light of the estimated need to increase the global food animal production by >70% within the coming 40 years to feed the global population [10]. The demand for protein-rich feed is primarily met by a variety of products derived from vegetable protein. These products, often called cakes and meals, are generally by- or co-products from the food industry, e.g., from mills

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crushing plants for oilseeds, sugar-producing plants, distilleries, and starch factories. The special importance of the soybean crop also for the global food supply is that soybean oil is the world's most widely produced and widely consumed vegetable oil. Of the feed materials used by the EU/28 feed industry 2014, 28% were cakes and meals and soy meal accounted for 60% [9]. Meat and bone meals from slaughter or rendering plants are currently banned in the EU and elsewhere since the BSE (bovine spongiform encephalopathy) crisis in the 1990s.

2.2. Globalized feed supply

In the same way as for food, the supply of animal feed for most countries is no longer solely based on domestic production. In a country *like* Sweden, only 72% of the non-forage feed materials for both swine and poultry are of domestic origin and for cattle the proportion is even lower (62%). Soybeans are rarely grown in the EU as well as in several countries elsewhere with a similar climate in contrast to, e.g., USA. The EU feed industry is therefore dependent on importing vegetable protein, with soybeans or soya bean meals being the major products. These are imported mainly from South America where Argentina and Brazil are the major producers. According to the European Feed Manufacturers' Federation (FEFAC), the self-sufficiency in EU28 is 2% for soybean [9]. The protein from soybeans is essential because it can only to a limited extent be replaced by, e.g., rapeseed meal because the latter contains higher levels of anti-nutritional factors.

3. Feed production: special reference to soy

3.1. Crushing and crushing industries

The oilseed processing involves the disaggregation of seeds, such as soybeans, palm kernels and rape and sunflower into crude vegetable oil, animal feed, and fiber. The crude oil is refined into food-grade, or may be used as an industrial or fuel feedstock. This process aims to release the oil and involves different methods for pressing or crushing the oilseed, the reason why these industries often are called "crushers." The crushing involves either the use of a screw/expeller or hydraulic press between plates. Due to friction in the screw, the temperature is raised up to 130–140°C, higher than in hydraulic press. Generally, the material in the crushing plants reaches >100°C for 20 min [11]. The product after the crushing is called cakes which usually are ground to a meal. Solvent extraction is also done, typically with hexane, resulting in extraction meal which further is toasted or heat treated. The meals obtained are the typical feed ingredients and used under different names which reflect the different production methods applied that consequently affect their nutritional properties and often also the hygienic quality.

The crushing plants are either located in the seed production area or at the area of animal production or close to harbors. This is the case for soya beans and, e.g., Brazil's export to Europe is either soybean meal, crushed in Brazil or soya beans to be crushed in Europe. These products are transported by ships to the larger harbors of Europe for further transportation by smaller vessels or trucks to crushers or feed mills. To a certain extent, soya bean meal is also directly transported to and mixed into compounded feed at farms.

From the point of risk management of Salmonella contamination of feed, it is interesting to note that the majority of feed and crushing industry is concentrated to a very limited number of large often globally operating companies. In the EU, more than 75% of the European-crushing capacity belongs to a small number of major international groups and the majority (80%) of the oilseed producers ("the crushers") are organized in the EU Oil and Protein meal Industry; FEDIOL [12]. Another major player is COCERAL [13] composed of national feed trade organizations, indicating the international activity of the supply of feed materials to the food animal production industry. In the USA, NOPA [14] represents 12 companies including 57 plants that process soybeans, accounting for approximately 95% of all soybeans that are processed (crushed) in the United States.

3.2. Feed mills-compound feed

Compound feed is produced in feed mills. In contrast to the case of feed ingredients, the trade and distribution of the finished rations are usually regional. The feed is produced in large volumes and even a medium-sized plant can produce around 20 tons of compound feed per hour and usually operates 24 h/day and 5–7 days a week [15].

Although the size, age, and technical construction may vary between feed mills, they have similar functions in common (as illustrated in **Figure 1**). Their basic functions include handling and storage of incoming ingredients, the processing of the ingredients, and the conditioning and heat treatment of feed. These functions include different steps [16, 17].

After mixing, the mixture of feed ingredients is transported and stored as meal or mash feed in the finished-product bins, or for heat treatment in a conditioning and pelleting process which

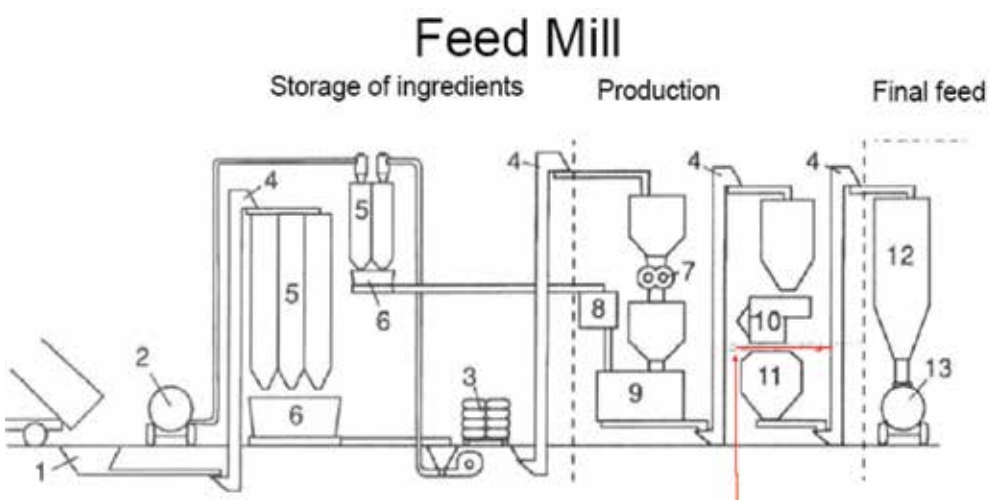

Figure 1. Schematic diagram of a feedmill. Source: Ref. [17]. 1. Intake pit for trucks, 2. Pneumatic intake for liquids, 3. Intake hopper for bagged ingredients, 4. Elevators, 5. Storage bins for ingredients, 6. Scales, 7. Grinder, 8. Pre- mix bins for premixes, vitamins,and so on, 9. Mixer, 10. Conditioner and pellet press, 11. Pellet cooler, 12. Storage bins for compound feeding stuffs, 13. Feed truck being loaded at out-loading gantry. Red arrows show the physical separation between "clean" and "nonclean" parts.

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are of special importance in relation to Salmonella contamination. Conditioning, followed by pelleting in a pellet press or expanding are the usual processing procedures performed in most feed mills when heat-treated feed is manufactured, although meals may also be heat treated, e.g., for poultry-breeding flocks in some countries.

Typically, the meal is introduced into the conditioner where steam is added to raise the temperature to a preset level. Conditioning and pelleting combined result in exposure of the feed to temperatures from around 50–90°C. The moisture content of the feed after pelleting is approximately 15% and is cooled to ambient temperature and dried to approximately 12–13% as rapidly as possible to prevent condensation in the transport equipment and storage containers. After processing, the feed is stored a short period of time in silos or out-loading bins for compound-feeding stuffs before being transported to the farm. It is a feature of modern feed production that there is very limited storage capacity and most feed is dispatched within hours of production, so there is normally no opportunity for test and release programs.

The design and construction of the feed plant will to varying degrees allow effective physical separation of the clean (post-processing) and non-clean (ingredient storage and handling) parts of the production and likewise permit effective cleaning measures. The buildup of dust is a factor inherent in feed manufacturing. Therefore, adequate dust aspiration systems and vacuum line-cleaning equipment in the feed mill are important to keep the mill in a clean condition. It is also important that the aspiration of dust is not done with systems that contribute to the circulation of potentially Salmonella-contaminated particles in the mill.

3.3. Oilseed meal in farm-level production

Although a substantial proportion of animal feed is home-produced on farm, for certain sectors, and in particular for poultry, the majority of the feed is bought in as compound feed. For ruminants, the predominant part of the feed ration is generally home-grown or local forage and cereals. For pigs, around half of the feed is usually homemade, and in the UK the Meat and Livestock Commission has estimated that around 40% of feed produced for pigs is home-produced. Purchased feed is occasionally non-heat-treated meal, but is usually heat-treated-pelleted feed.

Home-mixed feed is rarely pelleted and never heat-treated and should be considered as a risk when farmers as feed ingredient use soymeal of unknown Salmonella status. When available, by-products from the food industry such as whey, brewers, or distiller's grains and bakery waste are therefore included in non-poultry diets. This may involve the use of liquid feed systems.

4. Salmonella contamination of feed

4.1. Evaluation of published date

The result from those numerous studies found in the scientific literature and the gray literature on Salmonella in animal feed requires careful evaluation. As an example, a systematic

review found that only 277 out of 5071 publications in English were considered to be relevant for the assessment of feeding management practice and Salmonella prevalence in live and market-weight finisher swine [18]. The sampling procedures usually differ and typically have a low sensitivity for detecting Salmonella contamination, and may well lead to underestimation of the level of contamination [19]. In the absence of harmonized-sampling procedures, results from testing of feed in different countries or in different studies are difficult to compare. The results of testing should be viewed as representing a minimum level of contamination. The same can be said for the data reported to the European Food Safety Authority (EFSA) from 19 Member States during 2008, in which Salmonella-positive findings ranged from 0 to 3.6% in cattle and pig feed up to 8.3% in poultry feed [20].

Results of various studies also need to be related to the actual Salmonella situation. The EU-harmonized studies have thus revealed significant difference between Member States for the Salmonella status of poultry and swine and associated food products [20]. Similarly, there is a wide variation between the control actions applied in feed and animal production—from non-acceptance of Salmonella contamination to no actions taken.

Documentation of the importance of Salmonella in feed as well as of interventions requires that Salmonella contamination from other sources than feed can be excluded which is most easily achieved and performed in countries with a low level of Salmonella contamination as indicated in this review.

4.2. Detection and sampling

4.2.1. Method for detecting and sub-typing

Authorized methods for the isolation of Salmonella from feed and feed-associated samples follow standard bacteriological procedures, namely ISO 6579 and NMKL-71 [21]. The same methods are principally also applied in the food industry and on samples from different environmental as well as clinical sources. Modified semisolid rappaport vassiliadis (MSRV)-based methods have been shown to be suitable for testing of feed [22]. An official authorization also of their use for feed and foodstuffs as well as for samples from primary food animal production would offer significant benefits in terms of harmonization across the food chain. There may be strain variations and situations when existing Salmonella bacteria may be more difficult to isolate, but the above methods should in a reliable way detect Salmonella contamination in a sample as the level of competing flora is normally low.

Immunological (enzyme immune-linked sorbent assay (ELISA)) and molecular polymerase chain reaction (PCR)-based methods are available for an indirect and potentially faster detection of Salmonella contamination and identification of negative samples as a complement to the standard bacteriological procedures [21, 23]. These are sometimes used to screen ingredients before processing or by primary poultry-breeding companies to test stored feed before placing on breeding farms.

However, direct methods with isolation of the microbe are needed for the sub-typing into serovars which is required for tracing and epidemiological studies. It should be highlighted that there is a risk that not all serovars present in a sample will be detected by these methods,

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if only one colony is selected for the final confirmation. A further sub-typing of isolated serovars, e.g., by pulsed field gel electrophoresis (PFGE) or variable number tandem repeat (VNTR) facilitates tracing and is useful when different strains of the same serovar or phage type occur [24]. A combination of antimicrobial resistance pattern and sub-typing result may add additional discrimination.

4.2.2. Sampling methods

A major challenge is how to design a suitable sampling protocol to substantiate freedom from Salmonella contamination to a defined level of confidence, in particular in large volumes of feed or feed ingredients, e.g., in a truck or a shipload. In contrast to a herd of animals, which is made up of a defined number of discrete sampling units—the individual animals, no corresponding unit exists for feed. Instead, a 25-g sample is often used as a sampling unit and "freedom" from Salmonella defined as absence in the number of specified samples examined. The problem is that the absence of Salmonella in the samples does not verify true Salmonella absence in a feed batch.

The testing of a selected number of samples in these situations should give a representative measure of the Salmonella contamination in the whole lot. The selection can be based on different methods such as simple random sampling, stratified random sampling, and systemic random sampling according to principles described in different ISO—documents and elsewhere. However, uncertainties exist, in particular relating to the uneven distribution of the low concentration of Salmonella contamination that is typically present in feed. Different statistically based models and methods have been worked out to address this challenge as described and reviewed by Biotracer [25]. This is an integrated EU project established in 2007 with one of the objectives to suggest sampling for tracking and tracing contamination of Salmonella along the feed chain. An EFSA opinion on microbial contamination of feed also highlighted the need for harmonized and validated methods [8].

Only a few countries are known to have implemented such sampling protocols, and in the absence of harmonized sampling or harmonized regulations for the control of Salmonella in feed or in feed production, there seems to be no agreed standard methods available for ensuring freedom of Salmonella, e.g., in a feed ingredient before used in feed production or of a compounded feed as a guarantee for a farmer. When a control is applied, testing for "freedom in a 25 gram sample" is often used, but freedom in one 25-g sample of feed gives very unreliable information on the contamination in the situations described above [26]. Foster found that testing around 60 negative 25-g samples was required to conclude with 95% confidence that the contamination level is less than 1cfu per 526-g feed, which indicates the level of sampling that would be required to provide a meaningful result [27].

Testing of the final product alone is thus usually not sufficient to ensure Salmonella freedom. However, it is possible to ensure that a feed in practice is free from significant levels of contamination by Salmonella if instead the whole production process is controlled. A Salmonella-free feed could thus be defined as coming from a "Salmonella safe feed production," since the possible contamination of Salmonella is so low that it is very unlikely that it would result in an infection in animals consuming the feed. The official approval of feed mills could be based

on the existence of an appropriate HACCP-based control and the use of associated strategies for interventions. This approach is successfully applied in Finland, Norway, and Sweden and is also found to be effective when applied for crushing plants for rapeseed [28] and soya beans [29].

If oilseed meal cannot be obtained from crushing plants under appropriate and effective HACCP program for the control of Salmonella, their products should be tested before introduction to the feed mill as applied in Sweden [30]. The surveillance of such high-riskfeed ingredients is based on a sampling procedure which takes into consideration an uneven distribution of Salmonella contamination and is designed to detect contamination in 5% of the batch with 95% probability [31]. The size of the analytical sample is 25 g and usually eight samples are analyzed, each consisting of 10 pooled sub-samples of 2.5 g.

A representative sampling meets large practical difficulties in particular from large volumes of feed or feed ingredients that seemingly are inaccessible. To overcome these problems, it is advised to "sample in a moving stream" meaning that samples are taken when the commodity is circulated. This can be done manually or by the use of an automated inline statistical sampling device.

4.3. Salmonella in different feed materials

4.3.1. Animal-derived protein

Animal-derived protein has historically been found to be contaminated by Salmonella. From USA, several studies describe a high rate of contamination in by-products of animal origin. For example, 43 serovars of Salmonella were isolated from 175 (18%) of 980 samples of such products from 22 states [32], or 28 serovars from 37 (18.5%) of 200 samples of poultry and other animal by-products used in poultry feeds [33]. In a third study, 13% of 5712 samples of bone meal, feather meal, fish meal, and egg products were Salmonella contaminated with 59 serovars [34]. Similar observations were made in Europe. The heavy contamination that could occur in these feed ingredients was demonstrated when up to 12 different serovars were isolated in a single contaminated batch, which in fact in Sweden in 1960 initiated an organized control program for Salmonella in feed, in particular for high-risk products such as animal-derived protein [35].

The source of the contamination appears to be carcasses from Salmonella-infected animal that are subject to an ineffective rendering process. Before the BSE crisis, animals that might have died from different infections, the so-called fallen stock, was also generally rendered, resulting in the risk for feed-borne spread also of other infections, and, e.g., anthrax was found to be spread to swine herds fed contaminated meat and bone meal [36]. The treatment in the rendering process [133°C for at least 20 min at 3 bar pressure to inactivate TSE (transmissible spongiform encephalopathies) agents] should destroy all incoming Salmonella. However, as for crushing plants and feed mills, there is a significant high risk of recontamination from a Salmonella-contaminated environment in the absence of effective hygienic routines [8] and leaking cooker seals can contaminate expellers with fluid from incompletely cooked material. The introduction of Salmonella through fish meal highlights a risk from proteins derived also

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from fish [4, 37], although it is uncertain if the fish themselves are the primary source of such contamination.

4.3.2. Vegetable proteins

Long-term experiences and data from several countries have highlighted and verified that vegetable proteins, cakes and meal, are frequently contaminated by Salmonella. In a comprehensive study from Poland, based on an annual examination of up to 80,000 batches of feed up to 15.0 and 15.4% of imported lots of soya bean and rapeseed meal were, respectively, found to be Salmonella-contaminated in 2005–2007 [38]. Salmonella is frequently also isolated from consignments of vegetable proteins which are tested before being used as feed ingredients in Sweden. During 2004–2005, 5250 pooled samples were analyzed from 795 consignments and 14.6% of the soybean meal and 10.0% of the rapeseed meal samples were contaminated [30]. When the majority of the imported soy was from South America, 20.1% of the consignments were contaminated and even higher levels, up to 30%, were regularly found in previous studies [39]. The frequent isolation of Salmonella from vegetable proteins is in agreement with several observations from different countries [8, 15]. However, a direct comparison of data from different studies is difficult due to differences in sampling and testing procedures [40]. This is illustrated in a Danish study where a low-sensitivity sampling program (i.e., one sample per batch/shipment of imported soybean meal) detected 35 isolates of Salmonella during 1994–2003 compared to 1086 isolates when 22 shipments were investigated during 2004 with a more intensified sampling [41].

Palm kernel and maize gluten are other feed sources of vegetable protein from which Salmonella also frequently is reported. Experiences indicate a lower prevalence and in one study 9% of 67 batches of maize meal and one of 127 batches of palm kernel meal were Salmonella-contaminated during a 2-year period (2004–2005) [30]. The EFSA zoonoses report data also point out oilseeds, e.g., soya bean products, as a risk factor for introducing Salmonella into the feed chain [20].

In summary, oilseed feed ingredients are often contaminated by Salmonella although it is difficult to compare the level of contamination between different studies due to variation in sampling and culture techniques applied. From an epidemiological point of view, there is a need to raise awareness in the industry that raw oilseeds and pulses may already be frequently contaminated before entering the crushing plants. There is also a need to reflect why in particular certain feed ingredients are frequently Salmonella contaminated, often with several serovars of Salmonella. Apart from in-house contamination in crushing plants and feed mills as well as contamination during transport and storage, studies indicate that, e.g., soybeans often are heavily contaminated already when entering the crushing plant. In a Norwegian plant-crushing-imported soybeans, dust samples from the arriving beans are taken upon arrival, as part of the HACCP control. During a 19-year period (1994–2012), Salmonella was on an annual average isolated from approximately 34 (12–62)% of the samples and in total 94 different serovars were identified [29].

Data on Salmonella contamination at the growing cite of soybeans seem to be lacking. However, it is logical to suggest that like for vegetables, soybeans may be contaminated by

Salmonella-contaminated water used for irrigation or through manure used as fertilizer (i.e., "For example, see [42]"). This part of the Salmonella epidemiology requires further studies but indicates an infection cycle of Salmonella including links of fecal contamination from both humans and animals.

4.3.3. Grain and forage

The other major feed ingredients, grain and forage, are not primarily considered as high-risk products for Salmonella contamination of feed. When these feed materials are found to be Salmonella contaminated it is considered as a result of contamination from wildlife and during storage as further described below [43].

5. Methods of prevention and control

The prevention and control of Salmonella contamination of feed requires an integrated approach involving all links of the feed chain. Experiences have shown that there is no silver bullet that can meet all the challenges involved. Instead, a combination of precautions and actions is needed and an overall strategic approach is required to avoid being lost in details. Jones [44] has thus separated the control measures into three major strategies: prevention of contamination, reduction of multiplication, and procedures to kill the pathogen. It is helpful to consider an overall flow chart of the possible sources of contamination throughout the process [45]. A conceptual model is described for the pig feed chain, which can be adapted and applied to formulate a control program for the feed chain in question [15].

It should initially also be emphasized that it is possible to produce Salmonella-free feed under commercial and industrialized conditions as demonstrated by the fact that it is safe, even for broiler chickens. The young broiler is very sensitive to per-oral exposure to Salmonella and it is reported to be possible to become infected from ingestion of even few very Salmonella bacteria [46]. In the Scandinavian countries (Sweden, Norway, and Finland) with a long tradition of control of Salmonella in feed and broiler chickens, the incidence of Salmonella in broiler production is found to be very low when each flock is tested before slaughter [20].

5.1. Feed mills and crushing plants

Although many experiences on the control of Salmonella in feed mills have not been published in scientific literature, the major risks for Salmonella contamination of compounded feed are identified and also ways to minimize those risks, (i.e., "For example, see Ref. [39], several studies by Davies and coworkers [19, 43, 47] as well as [8, 15, 44]"). The results of those studies are a good scientific base for advice in individual situations.

The data and advice in this chapter, which mostly focuses on feed mills, can be used also for crushing plants which work under similar hygienic conditions. This reflects that published data on hygienic routines and Salmonella control in crushing plants are surprisingly rare and contrasted with the importance of oilseed meals as a potential major source for the Salmonella contamination of feed mills and subsequently of food animals and of the food chain.

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5.1.1. Major risk

Long-term experiences have demonstrated that the major primary source for Salmonella contamination of feed mills and the compounded feed is Salmonella-contaminated feed ingredients, e.g., [48]. Monitoring of nine feed mills revealed that the intake pits were the most frequently contaminated sampling site and on average 24% of the samples were positive for Salmonella [47]. This risk is rather similar for all feed productions using vegetable proteins, which is a high-risk product for Salmonella contamination. In the EU, this in particular concerns soybeans or soybean meal out of which 98% is imported from South America [9] and also domestically produced rapeseed meal as well as some animal-derived proteins when used. In all countries, there is thus a continuous risk for introducing Salmonella to the food chain via Salmonella-contaminated feed ingredients [8].

To minimize the risk described above, the most logical approach would be to prevent or eliminate Salmonella contamination as early as possible in the feed chain. In the case of the soybean meal, the focus of this chapter, Salmonella, should ideally have been already eliminated at the crushing or rendering plants. As described above, the process in these industries normally includes heat treatment that should readily eliminate Salmonella contamination and if recontamination is avoided, it would allow the production of Salmonella-free feed ingredients. However, available data indicate that it is generally not the case. Data on Salmonella contamination in crushing plants indicate the frequent occurrence of environmental contamination and cross-contamination, including re-contamination of heat-treated products [44, 49]. However, data from certain crushing plants also demonstrate that in spite of heavy Salmonella contamination of incoming soybeans (mean 30% of dust samples), it is possible to produce soybean meal which, based on testing and epidemiological experiences during several years, is found to be free from Salmonella [29]. Similar experiences exist from a rapeseed-crushing plant using the same control strategies as advised for feed mills [28].

The Salmonella-contaminated feed ingredients including soybean meal put an extra pressure on the feed mills to reduce the risk that incoming Salmonella is transmitted to the compounded feed. A strategy applied in Sweden to minimize this risk is to categorize the feed ingredients according to risk for Salmonella contamination. The high-riskfeed categories have to be tested negative for Salmonella contamination before being used for feed production. They are not allowed to enter the feed mill before a negative test result is at hand. Consignments found to be Salmonella contaminated are decontaminated by organic acids followed by re-testing with negative result before use [30].

As described below, heat treatment can be used to eliminate remaining Salmonella contamination. However, on an EU level, only 30–40% of the industrial feed is estimated to be heat treated [15], which emphasizes the importance of the Salmonella status of the feed ingredients. In the absence of heat treatment, Salmonella from contaminated feed ingredients easily is transmitted to the compounded feed. In such situations, the feed production does not include any specific processes aiming at reducing Salmonella contamination unless subsequently chemical treatment is applied. The observed decrease in the concentration of incoming Salmonella microbes in such production lines is primarily a result of dilution [19].

A second major source for Salmonella contamination at the feed mill is Salmonella-infected animal vectors which can contaminate feed ingredients during storage, at the intake pit as well as of the compounded feed. Salmonella was frequently (10 out of 51 samples) isolated from wild bird droppings from intake pit areas but also from warehouses and out-loading gantries and similar experiences are gained from other countries [47]. *Salmonella typhimurium* from wild bird is a particular concern. When feed materials are stored in open flat silos, rodents and cats may be additional sources for contamination of the feed material. Epidemiological experiences also suggest that birds, through fecal shedding, can infect animals via contaminated feed. The significance of this route was illustrated when virulent Newcastle Disease Virus, PPMV-1, in 1984 caused 22 outbreaks in chickens. It is widely accepted that the source of the PPMV-1 was poultry feed contaminated with feces from infected feral pigeons [50, 51].

Feed mills are thus attractive to birds as well as rodents if feed spillage and dust are not carefully and continuously removed from the environment. Such dust is thus often found to be Salmonella contaminated and wild birds, rodents, as well as humans can serve as vectors for contamination of the feed production [15]. To minimize these risks, the external environment of feed mills should be kept clean and in addition to rodent control, wild birds should be prevented from nesting and perching in roof spaces and gantries to avoid risk for direct and indirect fecal contamination of the feed.

In addition to the previous risks of external contamination, the cooler area is identified as probably the most important risk due to its potential to multiply a Salmonella contamination from incoming organisms that have survived the heat treatment process or have been introduced by the cooling air, if applied. The heat treatment associated with the pelleting process is thus also a potential hazard because steam is added to the feed. It is essential that this humidity as well as the heat is rapidly removed, which is done in the cooler by large volumes of air [44]. If the feed is not properly cooled, the cooling area can act as an incubator, allowing for fast multiplication of existing Salmonella microbes. Such contamination can not only directly contaminate the compounded feed but also establish a contamination in moist and fatty aggregates that can persist and act as a source for recontamination of the feed [47, 52, 53]. Experiences have shown that it is important to avoid the introduction of Salmonella by the cooling air. The air inlet to the cooling should be placed externally so that contamination by dust from, e.g., potentially Salmonella-contaminated feed ingredients, is minimized and the air is passed through a filter that removes dust and particles.

In order to avoid bacterial growth, experiences have demonstrated the need to avoid moisture in the whole-feed production system [43, 44]. Moisture can be caused by water cleaning, leakage, and condensation. In addition to the cooler area (see above), condensation of water may thus also occur in other places inside the feed transport systems as well as during storage [15, 44]. Experience from certain feed mills has also shown the existence of an in-house contamination indicated by the recurrent isolation of certain serovars of Salmonella during a period of many years in spite of various interventions [30]. This indicates that certain strains survive at unidentified spots or may be adapted to survive, e.g., by the help of bio-film formation [54].

There is also a need to develop methods for inspection and dry cleaning of the interior of the production line. Davies and Hinton [43] have highlighted the problems associated with

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inspecting certain coolers and the removing of internal coatings. The mills, in particular those of older age, are not constructed to meet hygienic requirements for cleaning and disinfection and access to critical sites to allow inspection and cleaning of the inside of production lines [43]. When found necessary, new openings for inspections are required as well as in critical situations modifications of the basic building constructions or equipment to overcome recurrent problems [55]. Decontamination of persistent contamination is demanding and requires special competence and in difficult situations dismantling of the whole process line has been found necessary [16, 43].

5.1.2. Heat treatment

Various studies have verified the Salmonella-reducing effect of heat treatment (i.e., "For example, see [11]"). It is reported that heating between 80 and 85°C for 1 min in most cases should eliminate Salmonella [53]. However, the elimination is dependent on the level of initial contamination and the set temperature and time range may not be reached to all parts of the feed [11]. Certain strains of Salmonella may also be more resistant to high temperatures which might explain the occurrence of Salmonella in feed following heat treatment [56, 57]. However, it is also reported that Salmonella contamination following heat treatment is most likely caused by internal contamination of the cooler [19].

The initial period after shutdown of the production before the intended temperature is reached is identified as a risk. Destruction or manual recirculation of the first feed produced before the set temperature is reached or during temperature dips is applied to avoid that contaminating Salmonella may survive due to too low temperature. Processes with automatic recirculation have been applied but found to be a risk for residual Salmonella contaminations. Another approach is therefore to automatically avoid the process to start until designated working temperature is reached.

It is important to note that the purpose of pelleting and the associated heat treatment is primarily to improve feed conversion and the handling qualities and feed intake, and not the hygienic feed quality although its hygienic potential early was recognized [58]. Temperature and time limits for the process are therefore guided to meet also nutritional requirements and exposure of the feed to too high temperature may have negative effects on certain feed ingredients such as amino acids and vitamins. As a complement to experimental data, empirical field data may give a more realistic result of the efficiency of heat treatment for eliminating Salmonella. Treatment at approximately 80–82°C for 30 s as generally applied by the feed industry in Sweden is found to result in Salmonella-safe poultry feed as described above. In summary, it is not possible to specify a minimum temperature and time range that under all conditions would be sufficient to eliminate contaminations of Salmonella in the industrial feed production [59]. Instead, as is applied in the food industry, monitoring of Salmonella and enterobacteriaceae contamination is used to ensure the efficiency of the process.

5.1.3. Dry storage

In the same way as it is important to prevent and reduce contamination of Salmonella at all steps of the feed production, it is equally important to prevent multiplication of possibly

contaminating Salmonella microbes which can survive for considerable time in various materials [44]. Keeping all feed ingredients and the compounded feed under dry condition is therefore an essential requirement (i.e., "For example, see Ref. [44]"). In order to avoid microbial multiplication, grains should be dried to approximately 13–14% and oilseeds to 7–9% moisture content corresponding to a water activity of around 0.4–0.65 [60].

5.1.4. Chemical treatment

Chemical treatment, mostly by organic acids, has been used to control Salmonella in feed production [8]. Such treatment seems to be used as a way to reduce or eliminate a Salmonella contamination from a batch of feed ingredients or as a general treatment of the feed fed to animals. The former use is applied, e.g., on feed ingredients found to be Salmonella contaminated before its use in feed production as described elsewhere in this document [30]. The latter use may have various reasons and in terms of Salmonella control it merely seems to be used in herds as a way to prevent intestinal colonization of Salmonella not only from feed but also from the environment [16].

Formaldehyde, which is found to be efficient for microbial decontamination of equipment and animal houses, is found to be effective with higher activity than acids also for decontamination of feed (i.e., "For example, see [61, 62]"). Formaldehyde has been used in combination with organic acids in order to achieve a synergistic effect allowing lower levels of formaldehyde and acids which, e.g., minimizes operator and possible food safety hazard, which is a reason why the use of formaldehyde in the EU so far in feed industry largely is limited to equipment and feeding systems. According to the feed-additive legislation, formaldehyde is only authorized at community level as preservative for skimmed milk for pigs up to the age of 6 months and for all species or categories of animals as silage additive [8].

5.1.5. Dust removal and cross-contamination

Dust and spillages are often found to be contaminated by Salmonella and therefore suitable for sampling (i.e., "For example, see Ref. [19]"). Programs for avoiding and removing dust and spillage also inside feed mill operations are found to be another essential requirement for avoiding the buildup of a Salmonella contamination that easily can be spread by cross-contamination.

The odds of contamination increase each time feed is handled [44] and it is reported that some ingredients may be handled up to 15 times before transport to the user [63]. Trucks used for the transport of manure on farms must certainly, e.g., not be used for transport of feed ingredients to crushing plants and feed mills. The logistics and management should thus be designed to avoid cross-contamination and separate storage of ingredients and the compounded feed, thus separating the so-called clean and unclean parts of the mill. This also applies to workers and visitors and in-house routines and the use of, e.g., protective clothes. It should be ensured that equipment and tools for service and repair are kept separate for clean and potentially contaminated areas. In some feed mills, external service people are not allowed to bring in their own equipment in order to avoid external contamination in critical places and accordingly, e.g., installation of second-hand-processing equipment from other mills is a major risk.

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5.1.6. HACCP including monitoring

The control of Salmonella in feed operation faces a continuously moving target. The risk for contamination varies over time but can never be excluded. An appropriate implementation of the control methods described above requires a careful planning as well as the design of a monitoring of Salmonella contamination. The monitoring should be based on the bacteriological examination for Salmonella of samples from dust and spillage [15, 19, 44]. This process should follow the same principles as in food safety programs and be based on hazard analysis and critical control point principles, HACCP [8]. This should also include strategies for interventions when Salmonella contamination occurs and most important their implementation when required. Due to the risk for contamination of the compounded feed and the built-up of in-house contamination, it is important to endeavor to control Salmonella contamination whenever it is found [44]. The challenges to avoid Salmonella contamination in feed mills also apply for crushing plants, and the outline of a HACCP program for a crushing plant is described [28]. The HACCP program has to be adapted to each feed operation.

For reasons described above, sampling only of the compounded feed is inappropriate. This was highlighted in Sweden in 1993 when sampling of the compounded feed was unable to detect a contamination by Salmonella Livingstone in a feed mill in spite that serovars during a 7-month period was spread by the feed from the mill and repeatedly infected flocks of broiler chickens at 15 producers [64]. Based on this experience and following a poultry producer demand, the feed control was changed from sampling the end product to sampling at critical control points. The aim was to detect contamination of Salmonella as early as possible in the production process starting at the intake. The following control points were identified: top of bin for final feed (compound feed), room for pellet coolers, top of pellet cooler, dust from the production line dust aspiration system (filter), and from intake pit/bottom part of elevator for feed materials [48].This event also demonstrates that trace back investigations from Salmonella-infected herds can be used as part of a monitoring to detect the spread of Salmonella by feed [65].

The proof of an effective HACCP program in a crushing plant or a feed mill is that Salmonella contamination actually over time is identified in the unclean part of the production chain but not or rarely in the "clean areas" and final products [29, **Figure 2**].

Usually, most attention is paid to poultry feed because poultry is considered to be more sensitive to Salmonella exposure than, e.g., ruminants. Accordingly, heat treatment of feed is in some countries a legal demand only for poultry feed. However, feed for poultry and for other food animal species is often produced in the same mill. Although separate production lines including the transport to farms then should be applied, experiences have shown that cross-contamination easily occurs. In addition to experiences from cross-contamination with Salmonella, the risk for cross-contamination was typically visualized during the initial phase of the BSE crisis when feed intended for poultry and swine could end up in cattle feed. Other examples of cross-contamination are when medicals mixed into feed to one species have been observed in another animal species. A way to avoid such problems is to apply the same standard for the control of Salmonella in feed to all animal species. A risk assessment by EFSA has also stated that an overall requirement should be the final feed to all food animals is free

from Salmonella [8]. When this is not the case, experience has shown that cross-contamination, e.g., between transports from feed mills to farms may occur if no through cleaning and disinfection procedures are applied. The importance of letting transport wagons as well as other equipment dry after cleaning and disinfection [43] is also emphasized.

5.2. Farm production

There appears to be very little literature concerning the risk for introduction of Salmonella to livestock as a result of home-mixing of feed although many studies are done in herds already being Salmonella infected [16]. In such studies, the possible effect of Salmonella-contaminated feed is usually measured only through the prevalence of Salmonella infections of animals being fed and are usually reported from countries and herds with a medium or high prevalence of Salmonella. Possible sources of the Salmonella infection in animals under such conditions are thus not only the feed but most likely to a much larger extent the result of direct and indirect transmission from neighboring animals although the initial source of introduction can have been the feed. The evaluation of the importance of Salmonella-contaminated feed in such herds and in particular when they are contaminated by several serovars of Salmonella (i.e., "For example, see Ref. [66]") requires comprehensive epidemiological studies. These need to be based on adequate monitoring for Salmonella and if possible the use of molecular-genotyping methods that allows for tracing of a Salmonella infection [67].

In a similar way as Salmonella-contaminating feed ingredients can be transmitted to the compounded feed in feed mills, this can also occur when such ingredients are used in farms. However, due to the difficulties to detect a Salmonella contamination as described above,

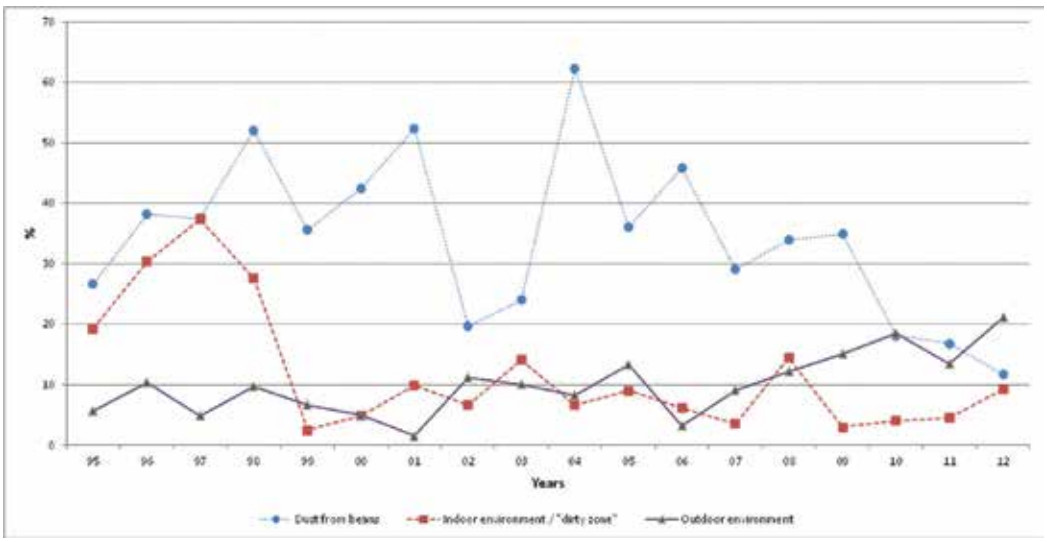

Figure 2. Isolation of *Salmonella* during 1994–2012 from a Norwegian plant-crushing-imported soybean (% of total samples). During the whole period, *Salmonella* (0%) was not isolated from the end products—not visible in the diagram. Source: Ref. [29].

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there are so far in practice no available simple methods for farmers to ensure that bought-in feed ingredients or compounded feed is free from Salmonella. Such a guarantee can only be obtained when the feed products are produced in mill or crushing plant operating effective control of Salmonella. A quality control to ensure freedom of Salmonella contamination of feed ingredients and compounded feed on the market would enable farmers to safe-sourcing procedures to minimize the risk of introducing Salmonella through the feed and this is of high priority for high-risk products such as soybean meal.

6. Significance of Salmonella in feed

6.1. Epidemiological aspects

A striking example emphasizing the potential of contaminated animal feed to act as a source of Salmonella infections in humans occurred when *Salmonella agona* emerged as a public health problem in several countries due to the widespread use of contaminated fish meal that was imported as feed material. In the period 1968–1972, a rapid increase of human infections with *S. agona* occurred in the United States as well as in Europe [4]. Since then, *S. agona* is among the most prevalent serotypes in humans. It is estimated that the serotype up to 2001 caused less than one million human illnesses in the United States alone since it was introduced in animal feed in 1968 [4].

In older literature, Salmonella was sometimes referred to as a ubiquitous bacterium which, like, e.g., *Escherichia coli*, normally exists in the intestinal flora of animals and in the environment. This is a gross simplification and is not normally the case, in particular not for the major food animal species, and the occurrence of Salmonella in their environment and wildlife is usually a spillover effect from Salmonella-infected animals [68–70]. The epidemiology of Salmonella is characterized by a great potential to adapt to survival in certain animal species and in the environment. Although Salmonella under certain conditions also may multiply in environmental niches such as in the feed, their reservoir and major place for multiplication are infected animals (including wildlife) and humans following per oral ingestion. Salmonella is then excreted in feces during varying periods, often in large numbers in particular during the acute stage of the infection, e.g., 10^{6-7}cfu per gram feces (i.e., "For example, see [71]").

As highlighted above, one of the major risk products for Salmonella contamination of the feed, the soybeans, is often heavily contaminated with a variety of serovars already when entering the crushing plant, which indicates links to fecal contamination from both humans and animals. Irrespectively of the primary source of this contamination, it is obvious that in the absence of effective Salmonella control of the feed probably for several decades, generations of food animals have been continuously exposed to Salmonella through their feed. This is likely to be the primary source of most of the Salmonella infection that is now resident in food animal breeding and production and in their environment in most countries.

Interestingly, the annual incidence of salmonellosis in humans in the industrialized world steadily increased from around the 1950s until it reached alarming proportions during the *S. enteritidis* pandemic in the late 1980s and early 1990s and annually in Germany alone was

estimated to have caused two million human food-borne infections [72]. At that time, WHO (World Health Organization) concluded that the industrialization of food animal production had opened the door to the food chain for Salmonella.

The meat inspection procedures that were designed to ensure food safety by detecting lesions due to tuberculosis and zoonotic parasites such as Trichinella could not detect and eliminate carcasses contaminated with Salmonella. Apart from heat treatment, decontamination, and irradiation, there is still no effective means of ensuring Salmonella-safe food animal products for humans at the slaughter stage, or in the marketing of shell eggs, without effective control in primary production.

Following assessment of risk-mitigation options of Salmonella in swine and pork production [6] and in feed [8], a quantitative risk assessment concluded that in both breeder and slaughter pigs, infected incoming pigs and Salmonella-contaminated feed are the major sources of Salmonella [6]. A similar situation is the case for poultry. The importance of feed is further emphasized in that Salmonella-free feed is required to maintain the breeding animals free from Salmonella.

Data that document the importance of Salmonella in feed as well as of interventions are most easily achieved and performed in countries with a low level of Salmonella contamination. In other cases as described above, in-depth epidemiological studies are usually required. In such studies, it is also essential to consider the time factor for accessing consequences of introduction of Salmonella as well as of interventions [8]. The *S. agona* introduced to USA and elsewhere by fishmeal in the late 1960s as described above is today not considered to be being feed borne though it is still regularly found in vegetable proteins and finished feed in many countries. The lack of targeted studies is probably a major reason why feed as a source of introduction of new serovars of Salmonella in animal herds often is underestimated.

Very few data are available on the possible human health impact of feed as a source of contamination, which also should vary with the preventive measures applied in the whole feed and food chain. In one study from Denmark, it was estimated that 2.1% of domestically acquired human Salmonella infections during 1999–2003 could be attributed to feed-borne serotypes acquired through the consumption of domestic pork and beef and the dominating source of Salmonella was contaminated by imported soybean products [73]. However, apart from less intensive sampling of the feed material, major human pathogenic serovars (*S. typhimurium* and *S. enteritidis*) with a special ability to establish themselves in food animal populations were not included in the estimation.

The time factor also has to be considered when assessing interventions against Salmonella contamination in feed. In regions or farms with a high prevalence of Salmonella in animals, isolated interventions against Salmonella contamination on feed cannot directly be expected to result in a lower prevalence in animals or on carcasses after slaughter because other sources for the infection are relatively much larger and need to be addressed simultaneously [6, 74, 75].

6.2. Serovars involved

The Salmonella isolated from feed or feed ingredients includes a wide range of serovars and to some extent also those serovars frequently causing disease in humans, e.g., in Sweden 38

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serovars of Salmonella were isolated from feed-associated sources during a 2-year period. Four (10.5%) of the serovars isolated were among the 10 most common isolates of human cases of salmonellosis in the EU, and 30 (78.9%) had also been isolated from human cases of salmonellosis diagnosed in Sweden during a 10-year period [30]. Salmonella serovars that frequently are causing infections in both animals and humans, e.g., *S. enteritidis* and *S. typhimurium*, are regularly isolated from feed [8]. When animals are exposed to such strains, they have a potential to multiply and spread on the farms and by trade to other farms as well as to humans following slaughter.

In another study of soybeans, 94 serovars of Salmonella were during a 19-year period isolated from soybeans dust samples during unloading of all shiploads from South America to a Norwegian-crushing plant. Those serovars included nine (90%) of the EU 2012 top 10 serovars isolated from clinical cases of salmonellosis in humans, including major animal pathogenic serovars such as spp. *Typhimurium* and *Enteritidis* [29].

Only a limited number of the serovars normally cause disease in animals and the majority appears to be transient colonizers of the intestine. However, some of them can become adapted to certain animal species which facilitate their survival and spread. From the beginning, around 1980s, this was observed in Europe when a sharp increase in the prevalence of *S. derby* in swine occurred in several countries [76], probably because that serovar had become adapted to swine [77]. Another important example is *S. enteritidis* phage type 4 which during the 1980s appears to have adapted to poultry and infection of their eggs leading to a rapid spread in the world poultry production and a pandemic spread also in humans. *S. enteritidis* is since then the major Salmonella serovar-causing food-borne illness in humans [20, 78]. Such events cannot be foreseen and because all serovars are considered to be potentially pathogenic for humans [8], there is little scientific support for a Salmonella-control policy in feed that is limited only to certain serovars. The importance of control of Salmonella in the feed and of the breeding animals is indicated by the fact that the existence of such controls probably was the reason why the Swedish poultry production was not involved in the worldwide spread of *S. enteritidis* in the late 1980s [79].

The relatively low occurrence in animal feed of serovars most frequently causing food-borne illness in humans has often been used as an argument against the potential importance of feed as a source of such infections. However, that argument is fallacious and seems to neglect that even an uncommon Salmonella following a per oral ingestion of an animal causes an active infection, often with a rapid multiplication of the microbe in contrast to being a passive contamination in the environment, feed, and in the subsequent food chain [44]. In fact, the same message was given already in 1969 by a United States Department of Agriculture (USDA) Committee on Salmonella, which concluded that this discrepancy in the assessment probably has contributed more than anything else to the fact that effective action has not been taken to control contamination of feed [80].

6.3. Cost for control of feed

In contrast to available data on how to prevent and control Salmonella contamination of feed, there is a considerable gap of published data on the actual cost of those actions [81]. It is

currently also important to fill out that gap when considering that the costs, although unspecified, are sometimes used as an argument against implementing a control [73].

In a recent study, the total extra cost achieving a Salmonella-safe compound feed, when such a control is established, was estimated at 1.8–2.3 € per ton of feed [82]. Of that cost, 25% relates to the prevention of Salmonella-contaminated high-risk vegetable feed materials (mainly soybean meal and rapeseed meal) from entering feed mills, and 75% for measures within the feed mills. Based on the feed formulations applied, those costs in relation to the farmers' 2012 price for compound feed were almost equal for broilers and dairy cows (0.7%). Due to less use of protein concentrate to fatten pigs, the costs were lower (0.6%). These costs interestingly also include costly events to decontaminate a whole feed mill in case of serious contamination. However, because the cost is split on the large volumes produced the relative cost in relation to the feed price is surprisingly low. These estimations should be of general value since the feed production generally includes the same technical approach in most countries and the price for feed materials and compound feed follows the global prices on feed commodities. These limited costs suggest that recommendations by FDA (Food and Drug Administration) already in 1991 [83] as well as later [83] to enforce a Salmonella-negative policy for animal feed are realistic and economically feasible to prevent a dissemination of the pathogen to animal herds, their environment, and potentially to human food products.

7. Conclusion and future perspective

The initial strategy to prevent human food-borne salmonellosis at the consumer levels by good hygiene and safe cooking was insufficient [84]. Instead, the annual increase in the incidence of human food-borne salmonellosis that was generally observed since around 1950–1960 continued until the *S. enteritidis* pandemic in the end of 1980s initiated more active control of Salmonella in poultry in the EU and elsewhere [20, 78, 85]. In the EU, that control which focused on actions at the preharvest level is found to be the reason to the significant shift to a decreasing trend of the human incidence of salmonellosis [20]. However, due to less effective interventions in USA food-borne salmonellosis in 2011 is still one of the few food-borne pathogens for which illnesses have not significantly declined over the past 10 years [86]. In the EU, the control of Salmonella will be extended to other food animals, starting with pigs. In addition, the traditional meat inspection procedures are under review in the EU guided by the need to improve food safety against today's major food-borne zoonotic agents such as Salmonella, with addition of preharvest controls.

Substantial efforts will be required to reduce the Salmonella prevalence in herds of pigs and cattle. It would therefore be logical and probably most cost-effective to start this process by efforts to prevent animals from becoming infected through their feed, as well as through the breeding pyramid, and so apply the top-down approach that has been a successful concept for the control of Salmonella in poultry [7, 40]. Because important feed ingredients often are contaminated, stringent efforts to control Salmonella have to be implemented at the crushing plants and feed mills. A HACCP-based quality control system to ensure freedom of Salmonella contamination of feed ingredients and compound feed on the market would enable farmers

http://dx.doi.org/10.5772/66332

to minimize the risk of introducing Salmonella through the feed. That approach would also be necessary to avoid Salmonella-contaminated feed jeopardizing their efforts to eliminate Salmonella at the farms.

As described in this review, long-term experience has shown that it is possible to produce feed that is free from Salmonella. In many countries, monitoring of Salmonella in feed and animals has also provided substantial experience on the epidemiology relating to the occurrence of Salmonella in feed. The cost for the production of Salmonella-safe feed materials and compound feed has also been estimated to be <1% of the farmers' feed cost when such a control is established. The missing point seems to be the lack of stringent regulatory or economic incentives needed to combine improved monitoring with appropriate interventions. A proactive policy would help avoid urgent actions that might be imposed if new highly virulent serovars such as *S. enteritidis* should occur as an emerging threat to animal and public health in the future.

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Chapter 11

Soybean: For Textile Applications and Its Printing

Ozan Avinc and Arzu Yavas

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66725

Abstract

It is vital to colorize sustainable, renewable, ecologic natural-based soybean fiber properly via printing for the textile and fashion industry. Optimum steaming-fixation conditions in respect of colorimetric values and color fastness properties should be determined for dye class in order to obtain the best possible print quality on soybean fiber fabric. This study exhibits that acid and 1:2 metal-complex dyes (originally used for printing of natural protein fibers such as wool and silk) and special reactive dyes (used for wool and polyamide fibers printing) can be used for regenerated soybean fiber printing leading to high color strength with adequate color fastness performance. Steaming at 102°C for 40 and 45 minutes are the optimum fixation conditions for acid and 1:2 metal-complex dyes on soybean fiber fabrics, respectively. On the other hand, steamings at 102°C for 20 minutes and 30 minutes are the optimum fixation conditions for wool-type reactive dyes and polyamide-type reactive dyes on soybean fiber fabrics, respectively. These optimum steam-fixation durations for each dye class led to the highest light fastness levels. Optimum steam fixation durations for 1:2 metal-complex and reactive dye classes (for both wool and polyamide) on printed soybean fibers displayed quite high and commercially acceptable wash fastness and good and commercially acceptable dry rub fastness and moderate to good wet rub fastness levels performance.

Keywords: soybean, soybean fiber, printing, dye, reactive dye, metal complex dye, acid dye, fixation, fastness, color

1. Introduction

Rising world population needs more amount of textile fibers from year to year leading to necessity for higher world fiber production to meet increasing world fiber demand [1]. This surplus fiber demand has been recently met by the increase of manmade fiber production from petrochemicals which are processed using highly toxic chemical methods and will not decompose naturally [2]. Moreover, increasing oil prices, descending petroleum reserves and

rising concerns regarding ecology set off alarm bells. Therefore, researchers and textile manufacturers are seeking biodegradable, sustainable and renewable textile fiber alternatives as an effective tool for compensating the world fiber demand while reducing the influence of the textile industry on the environment due to rising consumer awareness and demands about eco-friendly and organic products [2, 3]. Soybean plant is the source for one of those promising renewable, sustainable and biodegradable fibers for more sustainable world textile production. Soybean plant is a species of legume native to East Asia and its bean is not only edible but also has many uses [4]. One of those uses is in the textile industry. The soybean plant can be used for both cellulosic- and protein-based textile fiber production [5]. The first attempts to produce textile fibers from soybean protein were carried out during the mid-twentieth century [1, 6–8]. However, there were noteworthy challenges on its production in economic quantities and on fiber performance such as fiber strength that led to a decreasing interest for soybean protein fiber at that time [5, 9]. Nonetheless, as aforementioned, at the end of the twentieth century, there was a growing attention on eco-friendly natural-based sustainable biodegradable fibers due to ecological concerns, which leads to the awakening of promising soybean protein fiber. Key technological developments also provide opportunity for soybean protein fiber production with an ecologically friendly route leading to renewed interest [10–13]. Soybean cultivation has recently become much more cost-effective and soybean is one of the most abundant agricultural crops [14]. Therefore, soybean is cheap and abundant [6]. Furthermore, recent technical performance enhancement of soybean protein fiber via genetic-engineering techniques extends the commercial scope of this fiber [10, 11]. Therefore, in the 2000s, new soybean protein fiber, made from soybean protein and polyvinyl alcohol, was developed and a new soybean fibers' production process commercially promoted, standardized and launched to the textile markets [1]. The previous tenacity-related problems were also overcome with the inclusion of polyvinyl alcohol. Modern techniques for soybean fiber production make use of cutting-edge bioengineering principles by means of usable protein that is extracted from waste materials: the leftover dregs from soybean oil, tofu and soymilk production [15]. On the other hand, in the case of cellulosic-based textile fiber from soybean plant, natural cellulose fibers were produced from soybean straw by a simple alkaline extraction in 2009 and the researchers reported that these fibers exhibit similar properties and structure to natural cellulose fibers from conventional sources and natural cellulose fibers derived from soybean straws could be suitable for textile, composite and other industrial uses [2, 16].

Soybean fiber is the only protein-based botanic fiber and derived from renewable plant sources and a man-made fiber and manufactured in China in vast amount [2, 17]. Soybean fiber is manufactured from soybean protein that can be manufactured in massive quantities and at a low cost [17]. Soybean fiber is a kind of regenerative plant fiber that is created from regenerated soya *GlycineMax* soybean proteins along with polyvinyl alcohol (PVA) as a predominant component [1]. So in other way of saying, soybean fiber (SPF) is natural plant-based man-made regenerated protein fiber that is produced from a blend of soybean protein and polyvinyl alcohol [9, 18]. The soybean plant, its seeds and soybean protein fiber are shown in **Figure 1**.

Soybeans contain great quantity of proteins, approximately 37–42%, compared to peanut (25%), milk (3.2%) and corn (10%) proteins [1, 21]. Soybean proteins can be used as food, feed,

http://dx.doi.org/10.5772/66725

Figure 1. Soybean plant, soybean seeds and soybean fiber (SPF) [9, 19, 20].

textile fiber, pharmaceutical, ink, adhesive, emulsion, cleansing material and plastic [1, 6, 7]. Soybean proteins are globular proteins and they are composed of varied individual proteins and a large variety of molecular-sized protein aggregates [9, 18]. The most important proteins of soybean are globulins and soybean proteins have two storage proteins: glycinin (30% of the total soybean seed protein) and β-conglycinin (predominant and 30–50% of the total soybean seed) [1, 9, 18]. Soybean proteins comprise 18 amino acids and the predominant amino acid of soybean protein is glutamic acid with 18.2% share [1, 18]. In more detail, soybean proteins consist of glycine (8.8%), alanine (7.5%), phenylalanine (4.4%), valine (6.3%), leucine (9.8%), isoleucine (4.8%), serine (6.4%), threonine (4.3%), tyrosine, aspartic acid (12.8%), glutamic acid (18.2%), histidine (5.5%), arginine (0.8%), lysine (3.9%), tryptophan and proline (5.6%) [1, 18]. Moreover, soybean protein also includes little amount of sulfur containing amino acids such as cysteine (1%) and methionine (0.35%) [1]. Globular proteins comprise polypeptide segments that are linked by hydrogen and disulfide bonds and electrostatic and hydrophobic interactions [1, 18].

Liquefied soybean protein is extruded from soybean after the extraction of soybean oil and mechanically processed to manufacture soybean protein fiber by utilizing new bioengineering technology [2, 22]. The manufacturers of soybean protein fiber declared that the soybean fiber production is ecofriendly and does not impart any damage to atmosphere, environment, human body and water [9, 18]. Soybean fiber production steps are displayed in **Figure 2**. But initially, oil is extracted from soybean and residual cake from the extraction is kept aside [15]. Soybean protein is not suitable for fiber spinning owing to its globular structure and for this reason denaturation and degradation processes, which are important processes for fiber formation, are applied to soybean protein to convert the protein solution into a spinnable fiber spinning dope [18]. The denaturation process of soybean protein could be carried out with alkalis, heat, or enzymes using bioengineering techniques [18, 23, 24]. There are only conformational changes occurring in denaturation stage and in this step, the protein molecule unfolds to result in linear protein chains retaining its primary structure [18]. Subsequently protein spinning solution is prepared with polyvinyl alcohol (PVA) and protein

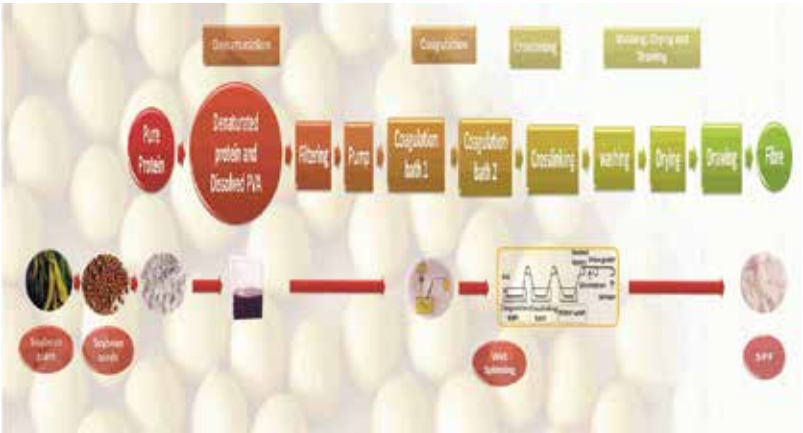

Figure 2. Production steps of soybean fiber [9, 13, 18, 20].

that is extracted from this residual protein cake [15] (**Figure 2**). Then, fiber spinning solution is spun using the wet spinning method. In this part, the fiber spinning dope solution, which comprises soybean and polyvinyl alcohol, is filtered and then forced through the spinneret. In the spinnerets, molecular chains are oriented and arranged into a structure involving crystalline and amorphous regions [18]. This orientation is greatly maintained in two sequential coagulation baths and after the coagulation steps, the cross-linking process is applied to soybean fibers in order to improve their mechanical properties [18] (**Figure 2**). Coagulated fibers are passed into a cross-linking bath after winding and it is reported that cross-linking step with glutaraldehyde could improve the mechanical properties of soybean protein fiber [18]. The last stages of the soybean protein fiber manufacture are washing, drying, followed by the drawing process in order to enhance the tensile strength properties of soybean fibers. Then the fiber can undergo winding, heat setting and cutting processes. Finally, soybean fibers with various specifications and varied lengths can be produced [15].

Soybean protein fiber is under the classification of Azlon group and it is also known as "vegetable cashmere," "artificial cashmere," and "soy silk" due to its cashmere feel [5, 9, 25]. The natural color of soybean protein fibers is pale yellow or cream [5, 15]. Soybean fiber merges environmental advantages with satisfactory textile performance. As aforementioned, soybean fiber is eco-friendly, sustainable and biodegradable fiber [23]. Actually, this fiber can exhibit not only numerous aesthetic qualities in association with natural fibers, but also physical features which are more akin to those of the synthetic fibers [5]. Soybean fiber is soft, smooth, light and has natural luster like silk fiber, which contributes a luxurious appearance to its fabric [17, 25]. Soybean fiber exhibits perfect draping ability leading to elegant appearance and feeling with comfortable wearing conditions [17]. Moreover, soybean fiber displays excellent moisture absorption performances like those of cotton fiber but superior ventilation and moisture transmission properties leading to perfect moisture management ability [12, 17, 25, 26]. Soybean fiber fabrics are warm and comfortable with high heat of wetting [17].

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Soybean fibers possess good mechanical properties such as single soybean fiber tenacity of 3.0 cN/dtex that is higher than that of silk, wool and cotton fibers [17, 25]. Nonetheless, the wet strength of soybean fiber is 35–50% of its dry strength [17]. What is more, this fiber also displays splendid easy wash, fast-dry and crease-resistance performance [25, 27, 28]. Soybean protein fiber exhibits antibacterial resistance for *Styphalococcus aureuses*, *Coli bacillus* and *Candica albicans* [1, 25]. They also have beneficial effect on human skin and human health due to their amino acid content [15]. The amino acids of soybean protein fiber could activate the collagen protein in the human skin, resist tickling and evaporate the skin [25]. Moreover, ultraviolet radiation absorption performance is better than that of cotton, viscose and silk fibers and can reach up to 99.7% [1, 22, 25].

After all, soybean protein fiber can satisfy the performance, comfort and functional demands of conventional and technical textile goods [22]. Therefore, soybean protein fiber has many various end-use application areas in the textile industry such as nonwovens, infant clothes, apparel, t-shirt, skirt, bed linen, undergarments, sleepwear, sportswear, bed sheets, towels, blankets, etc. [18, 25]. In addition, soybean fiber can be used alone and/or in blends with cashmere, wool, cotton, silk, elastic and synthetic fibers.

There are quite few studies about the coloration, limited to dyeing process, of soybean fibers in the literature, which are dyeing with 1:2 metal-complex, acid, direct, reactive dyes and natural dyes [22, 29–34]. Choi et al. [29] investigated the performance of three acid dyes and some reactive dyes containing different reactive groups on soybean protein fiber in terms of exhaustion, fixation and build-up. In this study, monochlorotriazine (MCT), monofluorotriazine (MFT), difluorochloropyrimidine (DFCP) and vinyl sulfone (VS) reactive groups-based dyes were studied. Soybean protein fiber exhibits good dyeing brilliance and good color fastness to light and perspiration [15].

Moreover, Chongling and Zan-min [35] studied the dyeability of soybean fibers with reactive disperse dyes in the supercritical carbon dioxide environment. However, coloration is not only limited to dyeing process for textile surfaces, textile printing is also an important coloration process of applying color to the textile substrate in certain patterns and/or designs in the textile industry in order to decorate the fabric. Textile printing enables creating patterns, which could be impossible to compose with any other techniques, such as weaving and/or dyeing. It is also right spot to mention that printing is not only an important way of coloration but also a way of self-expressing styles and an important fashion tool. Therefore, it is also important to colorize this sustainable, renewable ecologic natural-based soybean protein fiber via the printing process using available commercial dyes.

In this study, coloration via printing of soybean fiber with commercial chemical dyes [acid dyes, metal-complex dyes and reactive dyes (for polyamide and wool fibers)] and the effect of different steaming durations on colorimetric and color fastness (wash, rub, light fastness, etc.) properties of printed soybean fiber were examined and discussed. The optimum conditions for printing soybean protein fiber have not been studied within the literature reviewed. Therefore, the most appropriate dye class for soybean printing and the optimum fixation durations for soybean fiber printed with each dye type were examined and determined.

Printed soybean fabric samples were fixed with different steaming times (such as 10, 15, 20, 25, 30, 40, 45 minutes) at 102°C. Color fastness (wash, rub and light fastness) and colorimetric (*K*/*S*, CIE *L**, *a**, *b**, *C** and h^o co-ordinates, reflectance spectra and CIE Chromaticity Diagram) properties were investigated and compared.

2. Materials and methods

In this study, 100% soybean fiber single-jersey knitted fabric (fabric weight of 110 g/m^2 and yarn count of 30/1) was utilized for coloration via printing. In order to determine the most suitable dye type for soybean printing, commercial acid dyes, 1:2 metal-complex dyes and reactive dyes (for polyamide and for wool fibers) were applied to soybean fiber via the printing process. It is known that acid, metal complex, reactive and chrome dyes can be used for protein fiber (wool and silk) printing processes. However, 1:1 metal complex dyes and chrome dyes have recently lost their significance in textile printing. Therefore, acid dyes, 1:2 metal complex dyes and reactive dyes have generally been preferred for textile printing purposes. Indeed, printing of wool and silk fibers is carried out using acid, 1:2 metal complex and reactive dyes in the textile industry. Silk fibers can be printed under similar conditions to wool fibers using acid and 1:2 metal-complex dyes. In the case of the printing process with reactive dyes, wool-fiber printing can be carried out under acidic conditions; however, silk fibers can be printed under alkaline conditions due to their higher stability under alkaline conditions in comparison with wool fibers. In this study, soybean fibers were printed with different dye classes, which are recommended for silk, wool and polyamide fibers. Both blue and red dyes were used for each dye class and all dyes were supplied from Huntsman (Huntsman Corporation, USA). Dyestuff information and fixation periods (steaming at 102°C) are shown in **Table 1**. Printing processes on soybean fabrics were carried out using printing paste recipes shown in **Table 2**.

Commercial names of selected dyes	Dye types	Fixation (steaming at 102°C) time (min)
Acid dyes Erionyl® Blue A-4G Erionyl® Red A-3G	Monosulfonic/disulfonic acid dyes	15, 30, 40, 45
1:2 Metal-complex dyes Lanacron Blue 3GL Lanacron Red 5B	Monosulfonated asymmetric 1:2 metal complex dyes	15, 25, 30, 40, 45
Reactive dyes for wool Lanasol Blue 3R Lanasol Red 5B	Bromo acrylamide reactive group reactive dyes	10, 15, 20, 25, 30
Reactive dyes for polyamide Eriofast Blue 3R Eriofast Red B	Novel sulfo group containing reactive dyes	10, 15, 20, 25, 30

Table 1. Dye types and fixation (steaming) time after printing.

Printing paste ingredients	Acid dyes (Erionyl)[a]	Metal-complex dyes (Lanacron)[a]	Reactive dyes (Lanasol)	Reactive dyes (Eriofast)	
Dye	20	20	20	20	g
Thickener (8%, sodium alginate, low viscosity)	–	–	500	500	g
Thickener (guar-based thickener)	500	500	–	–	g
Urea	50	50	50	50	g
Hot water	y	y	–	–	g
Butyldiglycol	40	40	–	40	
Sodium chlorate	5	5	10	10	g
Sodium acetate	–	–	50	–	g
Citric acid			10	10	g
Ammonium sulfate 1:2[b]	60	–	–	–	
Water or thickener	~	~	~	~	
Total	**1000**	**1000**	**1000**	**1000**	**g**

[a] y: Water solubility of neutral dyeing acid dyes and metal complex dyes for printing is generally low, therefore necessary amount of hot water is carefully added to the printing pastes in order to ease the solubility of these dyes
[b] 1:2 (ammonium sulfate solution in water; 1 part of ammonium sulfate and 2 parts of water)

Table 2. Print paste recipes of each dye classes for soybean fiber.

Viscosity degree of printing paste (**Table 2**) was measured with a No. 5 spindle using a Brookfield DV-I Prime Viscometer (20 Rpm) (DV-I PRIME, Brookfield Engineering Laboratories, USA) and measuring viscosity degree 40 poise as a base. Soybean fiber fabrics were printed at 8 m/minutes at press 6 on using Atac laboratory-type printing machine (RGK 40, Atac, Turkey) with 70 Nr PES gauze and a doctor blade 8 mm in diameter under the laboratory conditions.

Printed soybean fiber fabrics were dried in a laboratory-type Atac drying machine (FT-200, Atac, Turkey) at 100°C for 3 minutes. Then, printed soybean fiber fabrics were steamed at 102°C for various fixation steaming durations (**Table 1**) with a laboratory-type steamer (ATC-HB350G, Atac, Turkey) for the dye fixation. It was earlier reported that optimum yields would only be acquired under humid steaming conditions when steaming prints on wool-protein-fiber fabric [36]. Moreover, it is also stated that the most brilliant and color-fast prints can only be acquired in saturated steam fixation at 100–102°C [37]. Therefore, fixation of soybean regenerated protein fiber fabrics following the printing process was carried out with steaming method at 102°C. Various steaming times (**Table 1**) were applied to printed soybean fiber fabrics in order to investigate optimum fixation conditions for soybean fiber printed with each dye class. After the fixation, printed soybean fiber fabrics were washed and dried at room temperature. After printing, the effects of different printing dye types and different steaming fixation times on colorimetric and color fastness properties of printed soybean fiber fabrics were evaluated and compared.

2.1. Colorimetric measurements

The CIE L^*, a^*, b^*, C^* and h^o color coordinates were measured and the K/S (color strength) values calculated from the reflectance values at the appropriate wavelength of maximum absorbance (λ_{max}) for each fabrics using a DataColor SpectraFlash 600 (DataColor SpectraFlash 600, Datacolor International, USA) spectrophotometer (D65 day light, 10° standard observer). CIE color space is a color assessment technique, which compares the sample to be tested to a standard (white). Numerical data were acquired and recorded using a reflectance spectrophotometer (DataColor SpectraFlash 600, Datacolor International, USA) to obtain CIE $L^*a^*b^*$ values as follows:

$$L^*(\text{Lightness/Darkness});\ \text{Black} = 0\ \text{and White} = 100 \tag{1}$$

On the a^* axis (red to green), positive values specify amounts of red while negative values specify amounts of green [a^*; Red = Positive Value ($+a^*$) and Green = Negative Value ($-a^*$)]. In the case of b^* axis (yellow to blue), positive numbers demonstrating increased yellowness and negative numbers demonstrating blueness [b^*; Yellow = Positive Value ($+b^*$) and Blue = Negative Value ($-b^*$)].

The K/S values of the fibers were determined through Kubelka-Munk equation as given below:

$$K/S = (1 - R)^2/2R \tag{2}$$

where R is the reflectance at complete opacity, K is the absorption coefficient and S is the scattering coefficient. Moreover, reflectance spectra, CIE chromaticity diagrams (CIE chromaticity diagram exhibits the mapping of human perception with regard to x and y values. Here, color is stated in regard to these two CIE parameter color coordinates; x and y.), K/S-C^*, a^*-b^* and L^*-C^* colorimetric graphs of printed soybean fabrics were measured and presented. h° (hue angle) is expressed in degrees. The starting point of the hue angle is at the $+a^*$ axis (redness) where the hue angle is 0°. The hue angle is 90° for the $+b^*$ axis (yellowness), 180° for the $-a^*$ axis (greenness) and 270° for the $-b^*$ axis (blueness). Saturation (C^*: Chroma) and h° can be calculated according to below equations:

$$C^* = [(a^*)^2 + (b^*)^2]^{1/2} \tag{3}$$

$$h^o = \arctan(b^*/a^*) \tag{4}$$

2.2. Color fastness determination

Wash, rub (dry and wet) and light fastness properties were determined according to ISO 105:C06 A2S (40°C in a M228 Rotawash machine, SDL ATLAS, UK), ISO 105: X12 and ISO 105: B02 (color fastness to artificial light: Xenon arc lamp) standards, respectively. ISO grey scale was used for the estimation of color fastness of the printed soybean fiber fabrics to washing and to dry and wet rubbing. Color fastness to light was determined using the blue-wool scale.

3. Results and discussion

Data obtained from the assessments of printed soybean fabric colorimetric properties appear in **Tables 3–6** and **Figures 3–26**, while the results of the color fastness properties of printed soybean fabrics appear in **Tables 7** and **8**.

3.1. Colorimetric properties of soybean fiber fabric printed with acid dyes (Erionyl dyes)

Soybean fiber fabrics were printed with the acid dyes that are generally used for wool and silk printing. Colorimetric data of soybean fiber fabrics after printing with acid dyes and following fixation via steaming are shown in **Table 3** and **Figures 3–8**. It can be easily seen that the reflectance spectra of soybean fabrics printed with Erionyl Blue A4G and Erionyl Red A3G dyes (acid dyes) and then fixed via steaming at various steaming periods were very close to

Printed soybean fabrics [dye, fixation (steaming) time]	L^*	a^*	b^*	C^*	h^0	K/S
Erionyl Blue A-4G, 15 min	36.93	−21.88	−21.19	30.46	224.07	15.25
Erionyl Blue A-4G, 30 min	36.36	−21.96	−20.84	30.27	223.50	15.74
Erionyl Blue A-4G, 40 min	34.32	−21.38	−20.75	29.79	224.15	17.33
Erionyl Blue A-4G, 45 min	36.39	−22.05	−20.87	30.36	223.42	15.85
Erionyl Red A-3G, 15 min	39.44	54.54	29.08	61.81	28.07	21.23
Erionyl Red A-3G, 30 min	38.03	54.05	29.09	61.38	28.29	23.16
Erionyl Red A-3G, 40 min	38.40	55.32	30.84	63.33	29.14	24.26
Erionyl Red A-3G 45 min	38.15	53.85	29.03	61.18	28.33	23.05

Table 3. Color coordinates of soybean fabrics printed with acid dyes (Erionyl dyes).

Printed soybean fabrics [dye, fixation (steaming) time]	L^*	a^*	b^*	C^*	h^0	K/S
Lanacron Blue 3 GL, 15 min	21,50	−1,77	−11,04	11,19	260,89	22.05
Lanacron Blue 3GL, 25 min	20.85	−1.66	−10.31	10.94	261.27	22.93
Lanacron Blue 3GL, 30 min	20.32	−1.57	−10.74	1086	261.68	24.01
Lanacron Blue 3GL, 40 min	19.64	−1.22	−10.20	10.27	263.20	24.26
Lanacron Blue 3GL, 45 min	1965	−1.50	−10.22	10.33	261.66	24.65
Lanacron Red 2GL, 15 min	32.23	43.37	17.61	46.81	22.10	20.11
Lanacron Red 2GL, 25 min	32.37	44.57	18.57	4823	22.62	20.94
Lanacron Red 2GL, 30 min	3231	44.35	18.32	47.93	22.44	21.14
Lanacron Red 2GL, 40 min	3198	44.11	18.36	47.73	22.60	21.64
Lanacron Red 2GL, 45 min	30.81	43.40	18.58	47.21	23.18	23.05

Table 4. Color coordinates of soybean fabrics printed with 1:2 metal complex dyes (Lanacron).

Printed soybean fabrics [dye, fixation (steaming) time]	L^*	a^*	b^*	C^*	h^0	K/S
Lanasol Blue 3R, 10 min	3570	−2.31	−23.46	23.57	264.39	9.09
Lanasol Blue 3R, 15 min	34.63	−2.05	−23.69	23.78	265.05	9.85
Lanasol Blue 3R, 20 min	34.50	−2.20	−23.31	23.41	264.60	9.89
Lanasol Blue 3R, 25 min	35.54	−2.23	−24.23	24.33	264.75	9.35
Lanasol Blue 3R, 30 min	35.08	−2.26	−23.61	23.72	264.53	9.55
Lanasol Red 5B, 10 min	28.66	40.15	−7.87	40.91	348.91	20.29
Lanasol Red 5B, 15 min	23.13	40.33	−7.94	41.10	348.86	21.43
Lanasol Red 5B, 20 min	25.87	38.83	−5.57	39.23	351.83	24.78
Lanasol Red 5B, 25 min	23.09	39.77	−7.69	40.51	349.06	21.04
Lanasol Red 5B, 30 min	23.43	39.54	−7.43	40.23	349.36	20.02

Table 5. Color coordinates of soybean fabrics printed with reactive dyes for wool (Lanasol dyes).

Printed soybean fabrics [dye, fixation (steaming) time]	L^*	a^*	b^*	C^*	h^0	K/S
Eriofast Blue 3R, 10 min	36.55	4.43	−39.01	39.26	276.48	10.52
Eriofast Blue 3R, 15 min	35.27	5.49	−39.57	39.95	277.90	11.52
Eriofast Blue 3R, 20 min	34.70	5.24	−39.00	39.36	277.65	11.97
Eriofast Blue 3R, 25 min	34.99	5.12	−38.83	39.16	277.51	11.61
Eriofast Blue 3R, 30 min	32.47	6.76	−39.83	40.40	279.63	14.35
Eriofast Red B, 10 min	43.59	55.63	5.64	55.91	5.79	13.10
Eriofast Red B, 15 min	41.86	55.76	6.46	56.13	6.61	15.41
Eriofast Red B, 20 min	42.37	58.65	8.78	59.30	8.51	16.81
Eriofast Red B, 25 min	41.06	56.19	7.51	56.69	7.61	16.81
Eriofast Red B, 30 min	40.73	56.42	8.05	57.00	8.12	17.20

Table 6. Color coordinates of soybean fabrics printed with reactive dyes for polyamide (Eriofast dyes).

each other and even overlapped for some cases (**Figure 3**). Therefore, soybean fabrics printed with studied acid dyes and then fixed with steaming at different periods exhibited close colorimetric values without drastic changes (**Table 3** and **Figures 5**, **7**). Moreover, the shade differences of the visual appearances of fabrics printed with red (Erionyl Red A3G) and blue (Erionyl Blue A4G) acid dyes were also detected on reflectance spectra, a^*, b^* and h^o values [**Figures 3**, **5** (CIE chromaticity diagram), 7 (a^*-b^* plot) and **Table 3**]. Especially, CIE chromaticity diagram shows the exact shades of printed soybean fabric samples with their measured chromaticity coordinates on two-dimensional (x-y) color diagram (**Figure 5**).

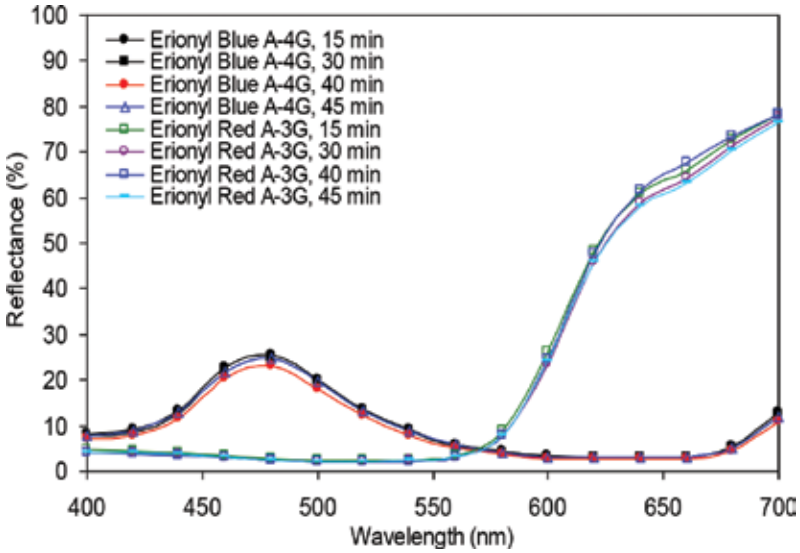

Figure 3. Reflectance (%)-wavelength (nm) spectra of soybean fabrics printed with acid dyes (Erionyl dyes).

There was no big difference between the color strength values (*K*/*S*) of soybean fabrics printed and steamed for 15 and 30 minutes (**Table 3** and **Figure 4**). Increasing steaming time to 40 minutes on soybean fabrics led to a slight increase on color strength. However, it seems that longer steaming period such as 45 minutes was not necessary since such prolonged steaming application resulted in a slight decrease in color strength (**Table 3** and **Figure 4**). The highest color strength values for both Erionyl Blue A 4G (*K*/*S* with 17.33) and Erionyl Red A 3G (*K*/*S* with 24.26) dyes were attained after 40 minutes of steaming for fixation. It is in parallel with the literature which states that relatively long steaming times of 30–60 minutes are generally required to fix acid dyes on other protein fibers; wool or silk [37].

As it can be observed from **Figure 6**, chroma values (*C**) of soybean fabrics printed with Erionyl Blue A-4G and fixed with varying times exhibited close values. As earlier mentioned,

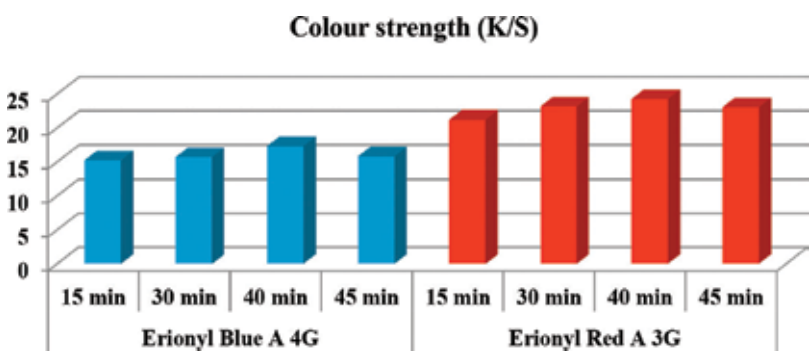

Figure 4. Color strength degrees of soybean fabrics printed with Erionyl acid dyes according to various fixation steaming duration.

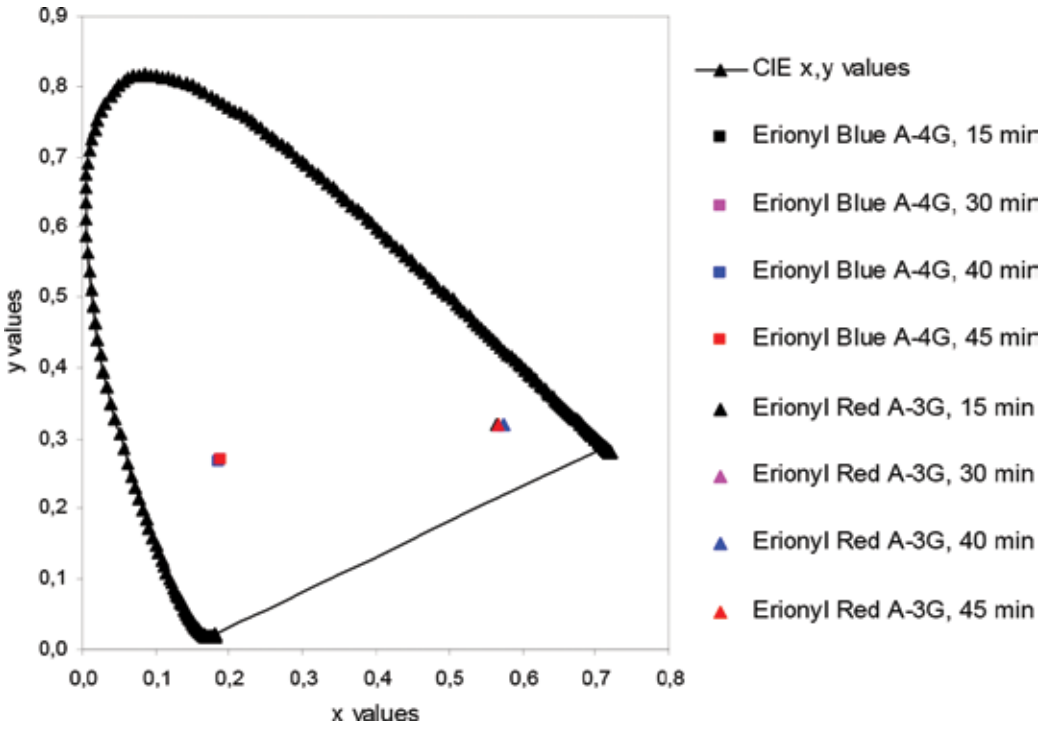

Figure 5. CIE chromaticity diagram showing the chromaticity coordinates of soybean samples printed with the Erionyl acid dyes.

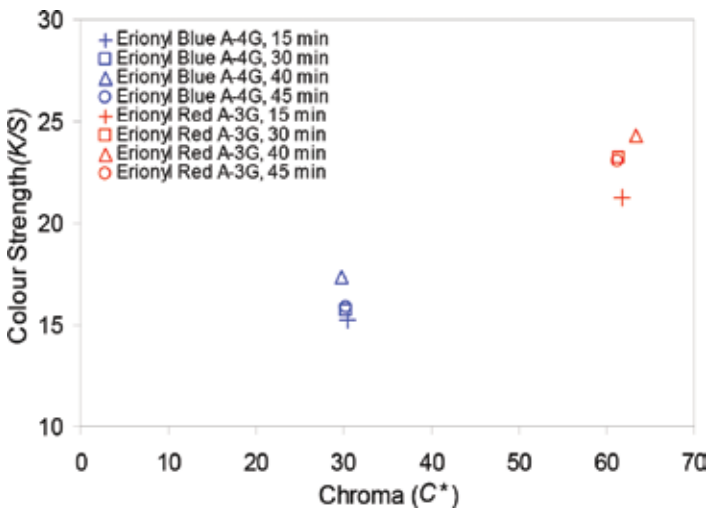

Figure 6. *K/S-C** (color strength versus chroma) diagram of soybean fabrics printed with Erionyl acid dyes and fixed with different steaming periods.

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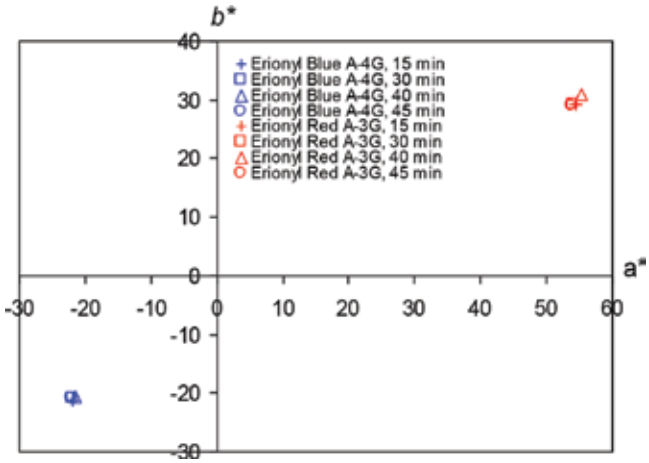

Figure 7. *a*-b** (redness-greenness versus yellowness-blueness) diagram of soybean samples printed with Erionyl acid dyes.

40-minute steaming resulted in the highest color strength (*K/S* of 17.33) for fabrics printed with Erionyl Blue A-4G. On the other hand, in the case of Erionyl Red A-3G, 40 minutes of steaming led to the highest color strength (24.26) and the highest chroma (63.33) on printed soybean fabrics (**Figure 6**). It is clear that both color strength and chroma values of soybean protein fiber fabrics printed with Erionyl Red A-3G were significantly higher than those of soybean printed with Erionyl Blue A-4G (**Figure 6**).

*a** and *b** values of soybean samples printed with Erionyl Blue A-4G and fixed with varied steaming periods were very close to each other (**Figure 7**). On the other hand, in the case of Erionyl Red A-3G, 40-minute steaming resulted in redder and yellower appearance with a

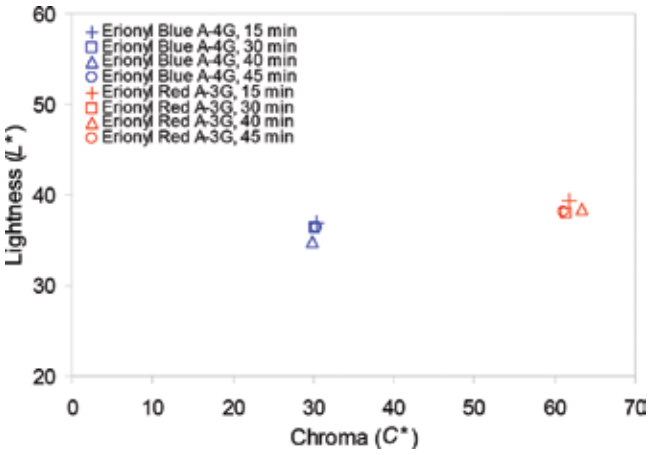

Figure 8. *L*-C** (Lightness versus chroma) diagram of soybean fabrics printed with Erionyl acid dyes and fixed with different steaming periods.

slightly higher *a** value and a slightly higher *b** value in comparison to other steamed samples (**Table 3** and **Figure 7**). Lightness (*L**) and chroma (*C**) degrees of soybean fabrics printed with Erionyl Blue A-4G and fixed with varying times exhibited close values (**Figure 8**). The 40-minute steamed sample exhibited the lowest lightness value of 34.82 leading to the highest color strength of 17.33, as expected (**Figure 8** and **Table 3**). The higher color strength (*K*/*S*) led to the lower lightness values (*L**). In the case of Erionyl Red A-3G dye, as earlier mentioned, the 40-minute steamed sample displayed the highest chroma value (**Figure 8**).

3.2. Colorimetric properties of soybean fiber fabric printed with 1:2 metal complex dyes (Lanacron dyes)

Colorimetric data of soybean fiber fabrics after printing with acid dyes followed by fixation via steaming are shown in **Table 4** and **Figure 9–14**.

It is clear that the reflectance spectra of soybean fabrics printed with Lanacron Blue 3GL and Lanacron Red 2GL dyes (1:2 metal complex dyes) and then fixed via steaming at various steaming periods were very close to each other (**Figure 9**). Prolonged steaming time on soybean fabrics printed with 1:2 metal complex dyes (Lanacron Blue 3GL and Lanacron Red 2GL dyes) resulted in an increase in color strength for both dyes (**Table 4** and **Figure 10**). The highest color strength values were observed for 45-minute steamed soybean samples printed with both Lanacron Blue 3GL (*K*/*S* of 24.65) and Lanacron Red 2GL (*K*/*S* of 23.05) dyes (**Table 4**). It is known that the steam, used after printing, provides the moisture and rapid heating, which gives rise to the transfer of dye molecules from the thickener film (guar-based thickener in our case) to the fiber within a reasonable time [37]. It seems that prolonged steaming time resulted in better fixation and higher attachment rates of the 1:2 metal complex dyes on the soybean fiber leading to higher color strength in general.

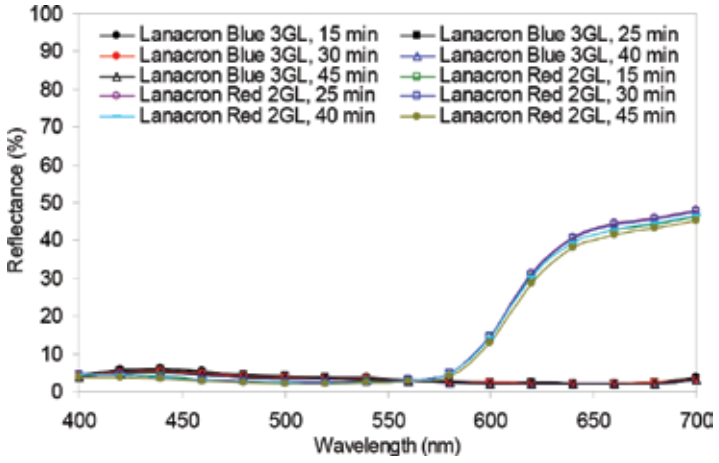

Figure 9. Reflectance (%)-wavelength (nm) spectra of soybean fabrics printed with 1:2 metal complex dyes (Lanacron dyes).

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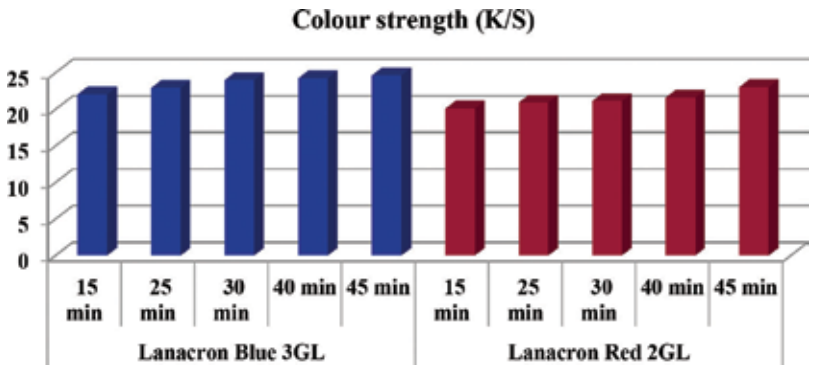

Figure 10. Color strength degrees of soybean fabrics printed with 1:2 metal complex dyes (Lanacron dyes) according to various fixation steaming durations.

The color shade differences of the visual appearances of soybean protein fiber fabrics printed with Lanacron Blue 3GL and Lanacron Red 2GL dyes (1:2 metal complex dyes) were also detected on reflectance spectra, CIE chromaticity diagram, a^*-b^* plot and hue angle (h^o) values (**Figures 9**, **11**, **12** and **Table 4**). Particularly CIE chromaticity diagram displayed the exact shades (red and blue colors) of printed soybean fabric samples with their measured chromaticity coordinates on two-dimensional (x-y) color diagram (**Figure 11**). Soybean samples printed with 1:2 metal complex dyes and fixed with varied steaming periods (15, 25, 30, 40, 45 minutes) exhibited very close a^* and b^* values (**Figure 12**).

Lightness (L^*) and chroma (C^*) degrees of soybean fabrics printed with 1:2 metal complex dyes (Lanacron dyes) and fixed with varying times exhibited close values (**Figure 13**). Printing with metal complex dyes generally results in duller color prints with less brightness [37]. For instance, soybean fabrics printed with Lanacron Red 2GL were brighter than samples printed

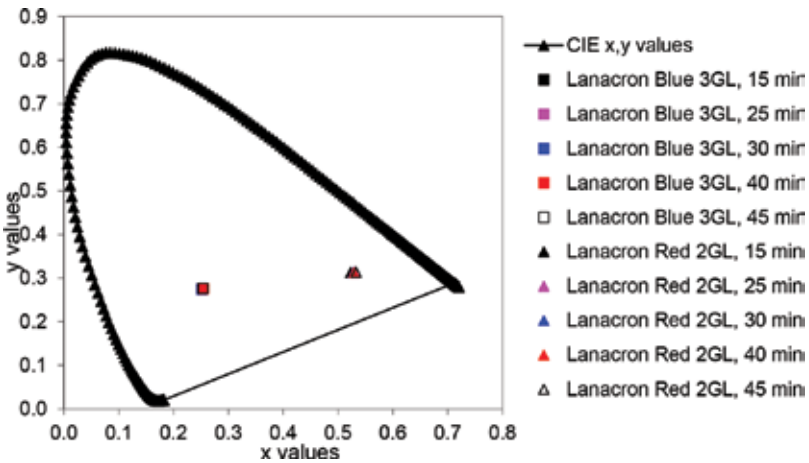

Figure 11. CIE chromaticity diagram showing the chromaticity coordinates of soybean samples printed with 1:2 metal complex dyes (Lanacron dyes).

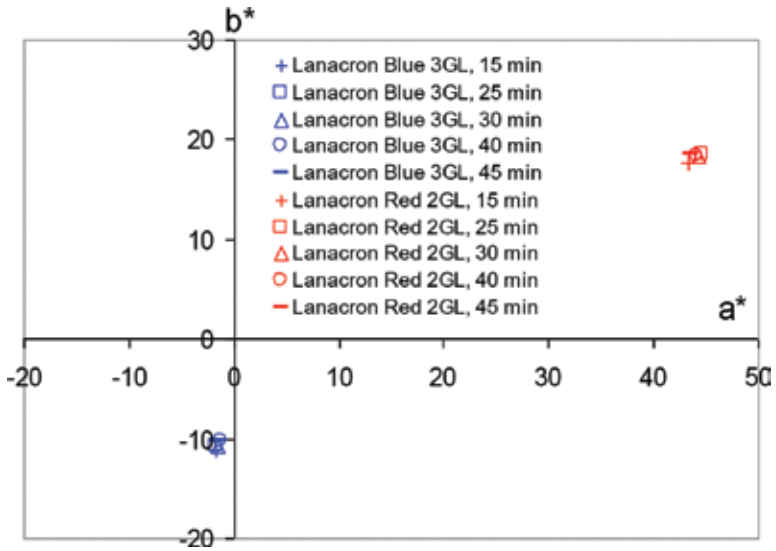

Figure 12. *a*-b** (redness-greenness versus yellowness-blueness) diagram of soybean samples printed with 1:2 metal complex dyes (Lanacron dyes).

with Lanacron Blue 3GL (**Figure 13**). It is known that color brightness increases while *C** and *L** values are both rising at the same time [38]. Acid dyes (Erionyl dyes) led to brighter appearance on soybean fabric in comparison with 1:2 metal complex dyes (Lanacron dyes). Indeed, higher lightness (*L**) and higher chroma (*C**) values were measured in the case of acid dyes (Erionyl Blue A 4G and Erionyl Red A 3G) when compared to 1:2 metal complex dyes

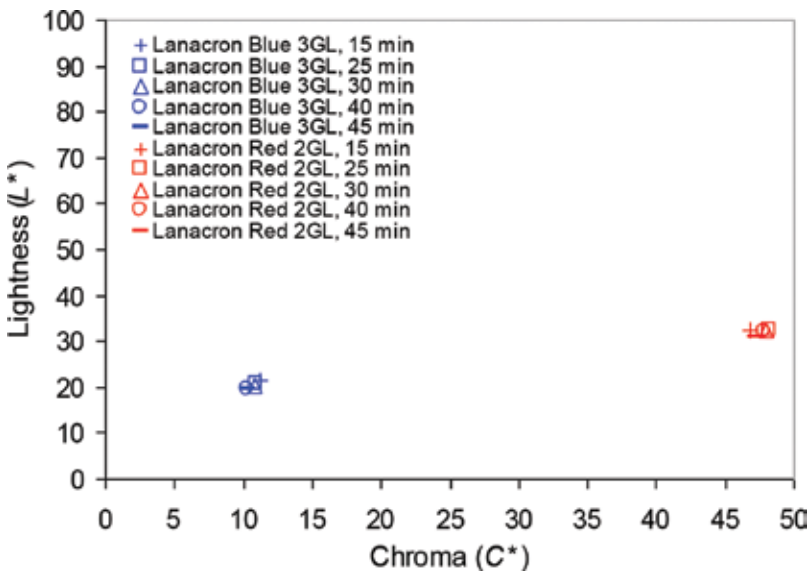

Figure 13. *L*-C** (Lightness versus chroma) diagram of soybean fabrics printed with 1:2 metal complex dyes (Lanacron dyes) and fixed with different steaming periods.

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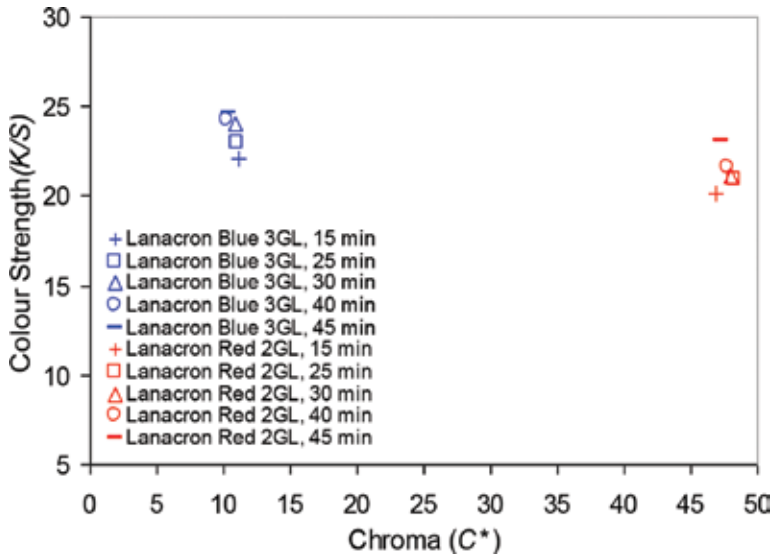

Figure 14. *K*/*S*-*C** (color strength versus chroma) diagram of soybean fabrics printed with 1:2 metal complex dyes (Lanacron dyes) and fixed with different steaming periods.

(Lanacron Blue 3GL and Lanacron Red 2GL) (Tables 3, 4 and **Figures 8** and **13**). Chroma values (*C**) of soybean fabrics printed with Lanacron Blue 3GL and Lanacron Red 2GL and fixed with varying times displayed close values (**Figure 14**). As aforementioned, color yield (*K*/*S*) of printed soybean samples increased with increased fixation periods. It seems that the proper diffusion of large 1:2 metal complex dye molecules into the soybean fiber needs time and the diffusion increases with the increased steaming fixation times leading to a high color yield.

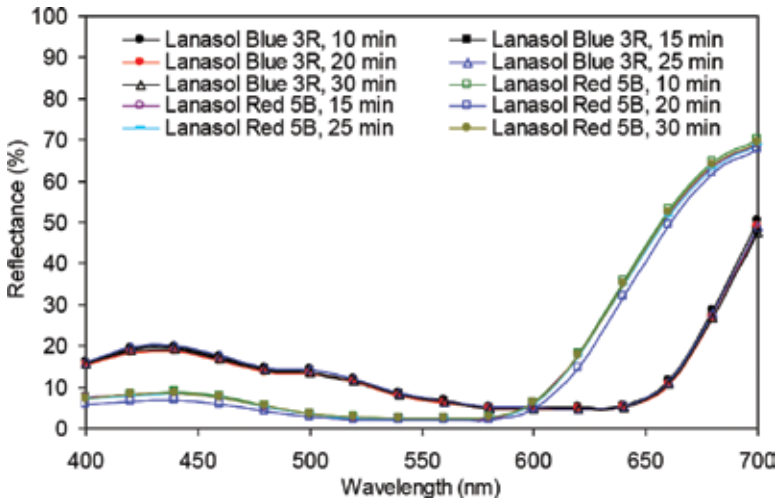

Figure 15. Reflectance (%)-wavelength (nm) spectra of soybean fabrics printed with reactive dyes for wool (Lanasol dyes).

3.3. Colorimetric properties of soybean fiber fabric printed with reactive dyes for wool (Lanasol dyes)

Colorimetric data of soybean fiber fabrics after printing with reactive dyes (for wool) followed by fixation via steaming are shown in **Table 5** and **Figure 15–20**. It is clearly observable that the reflectance spectra of soybean fabrics printed with Lanasol Blue 3R reactive dye and then fixed via steaming at various steaming periods were very close to each other and even overlapped for some cases (**Figure 15**). Soybean fabrics printed with Lanasol Blue 3R and then fixed with steaming at different periods exhibited close colorimetric values without drastic changes (**Table 5** and **Figure 16**, **18–20**). On the other hand, the reflectance spectra of soybean fabrics printed with Lanasol Red 5B reactive dye and then fixed via steaming at various steaming periods were slightly different leading to slightly different color properties (**Table 5** and **Figure 16**, **18–20**).

There was no big difference between the color strength values (*K*/*S*) of soybean fabrics printed with Lanasol Blue 3R dye and then fixed via steaming at various steaming periods (**Figure 16** and **Table 5**). There were differences between the color strength values (*K*/*S*) of printed with Lanasol Red 5B dye and steamed soybean fabrics (**Figure 16** and **Table 5**). The highest color strength values for both Lanasol Red 5B (*K*/*S* with 24.78) and Lanasol Blue 3R (*K*/*S* with 9.89) dyes were obtained after 20 minutes steaming for fixation. Longer steaming periods such as 25 or 30 minutes slightly decreased color strength (**Figure 16** and **Table 5**).

The color shade differences of the visual appearances of soybean protein fiber fabrics printed with Lanasol Blue 3R and Lanasol Red 5B dyes (reactive dyes for wool) were also detected on CIE chromaticity diagram, a^*-b^* plot and hue angle (h^o) values (**Figure 17**, **18** and **Table 5**). It is known that reactive dyes constitute true chemical bonds with the SH, NH, or NH_2 groups in the polypeptide chains in acid media (pH 3–5) at 80–100°C and these dyes can provide brilliant color in prints [37]. Particularly CIE chromaticity diagram displayed the exact shades (maroon and dark blue colors) of printed soybean fabric samples with their measured chromaticity coordinates on two-dimensional (*x*-*y*) color diagram (**Figure 11**). Soybean samples

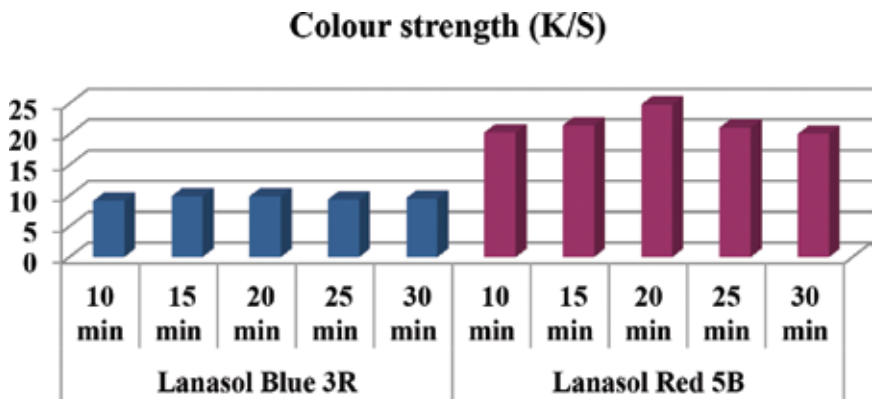

Figure 16. Color strength degrees of soybean fabrics printed with reactive dyes for wool (Lanasol dyes) according to various fixation steaming durations.

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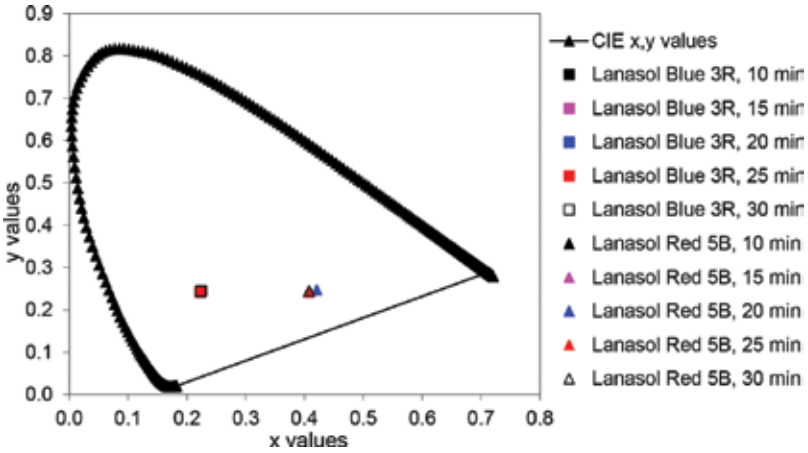

Figure 17. CIE chromaticity diagram showing the chromaticity coordinates of soybean samples printed with reactive dyes for wool (Lanasol dyes).

printed with Lanasol Blue 3R and fixed with varied steaming periods (10, 15, 20, 25, 30 minutes) exhibited very close a^* and b^* values (**Figure 18**). In the case of Lanasol Red 5B dye, 10-, 15-, 25- and 30-minute steamed soybean samples were slightly redder and bluer in comparison with 20-minute steamed soybean sample according to a^* and b^* values (**Figure 18**).

Lightness (L^*) and chroma (C^*) degrees of soybean fabrics printed with Lanasol Blue 3R and fixed with varying times exhibited close values (**Figure 19**). In the case of Lanasol Red 5B dye, 10-, 15-, 25- and 30- minute steamed soybean samples, when compared to 20-minute steamed soybean sample, exhibited slightly higher chroma and higher lightness leading to slightly brighter appearance (**Figure 19**). As aforementioned chroma values (C^*) and color yields (K/S)

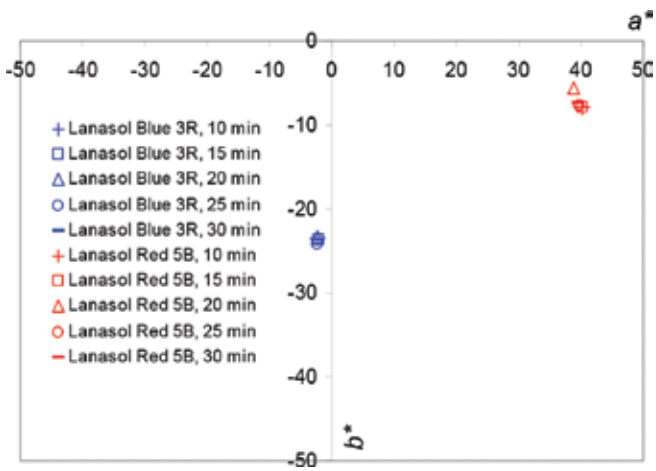

Figure 18. a^*-b^* (redness-greenness versus yellowness-blueness) diagram of soybean samples printed with reactive dyes for wool (Lanasol dyes).

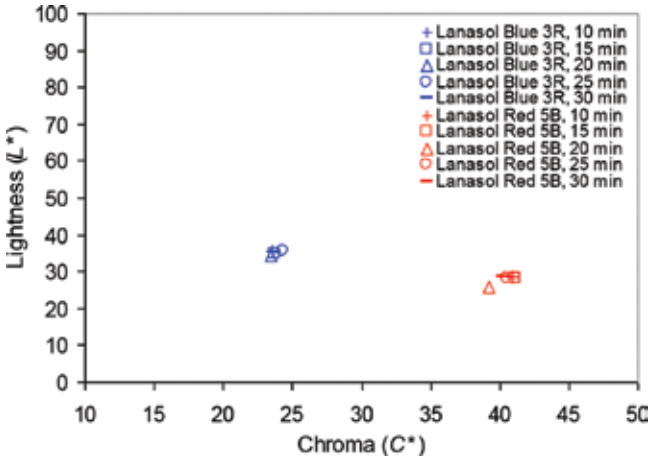

Figure 19. *L*-C** (lightness versus chroma) diagram of soybean fabrics printed with reactive dyes for wool (Lanasol dyes) and fixed with different steaming periods.

of soybean fabrics printed with Lanacron Blue 3GL and fixed with varying times displayed very close values (**Figure 14**). Overall, the highest color strength values (*K*/*S*) for both Lanasol reactive dyes were obtained after 20 minutes of steaming. It is known that reactive dyes for printing wool possess better solubility than acid dyes and can be usually sprinkled directly into the print paste as solids without the use of dye solvents and that they need shorter steaming times which are a clear benefit in continuous steaming [39]. This is clearly in line with the results of soybean fabrics printed with reactive dyes for wool (Lanasol dyes), since, in this case, short steaming time as 20 minutes was enough for satisfying print quality from the color point of view. However, one should be careful while working with reactive dyes in printing, since unlevelness problem in large blotches can occur in some shade areas [39].

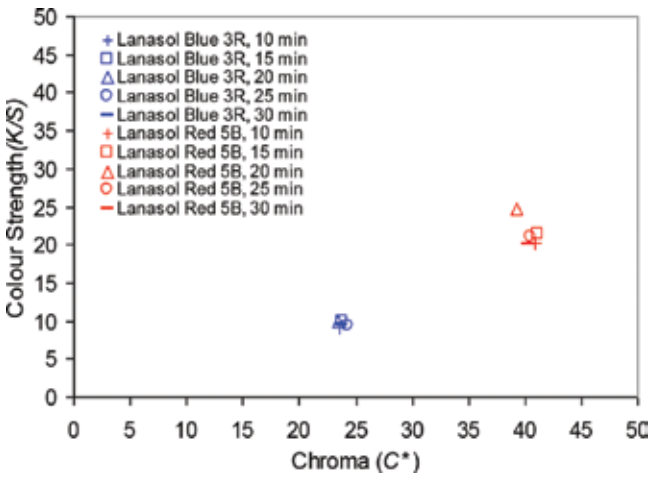

Figure 20. *K*/*S*-*C** (color strength versus chroma) diagram of soybean fabrics printed with reactive dyes for wool (Lanasol dyes) and fixed with different steaming periods.

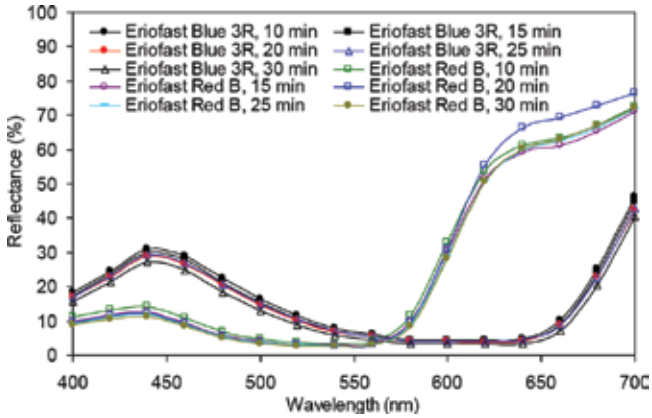

Figure 21. Reflectance (%)-wavelength (nm) spectra of soybean fabrics printed with reactive dyes for polyamide (Eriofast dyes).

3.4. Colorimetric properties of soybean fiber fabric printed with reactive dyes (Eriofast dyes)

Soybean fiber fabrics were printed with the reactive dyes (Eriofast dyes), which are generally recommended for polyamide printing. Colorimetric data of soybean fiber fabrics after printing with reactive dyes (Eriofast dyes) followed by fixation via steaming are shown in **Table 6** and **Figures 21–26**. It can be easily seen that the reflectance spectra of soybean fabrics printed with Eriofast Red B reactive dyes and then fixed via steaming at various steaming periods were slightly different leading to slightly different color properties (**Table 6** and **Figures 21**, **23–26**).

On the other hand, Eriofast Blue 3R printed and fixed with various steaming periods soybean samples exhibited closer reflectance spectra leading to close color properties (**Table 6** and **Figures 21**, **23–26**). Prolonged steaming time in soybean fabrics printed with Eriofast dyes (reactive dyes for polyamide) led to an increase in color strength for both dyes (**Table 6** and **Figure 22**). A similar case was also observed for 1:2 metal complex dyes. The highest color strength values were observed for 30-minute steamed soybean samples printed with both

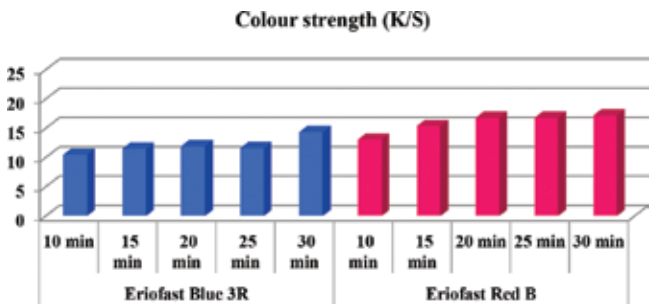

Figure 22. Color strength degrees of soybean fabrics printed with reactive dyes for polyamide (Eriofast dyes) according to various fixation steaming durations.

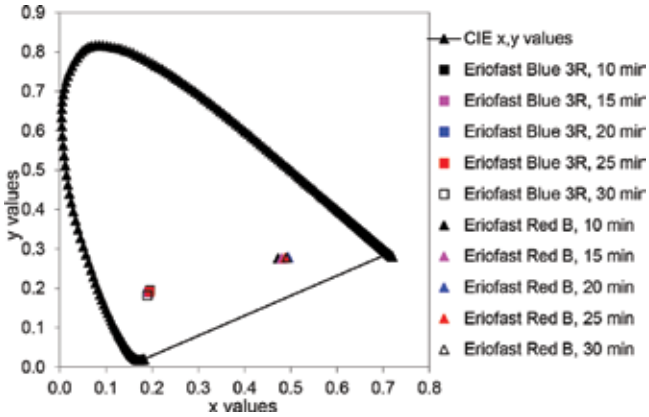

Figure 23. CIE chromaticity diagram showing the chromaticity coordinates of soybean samples printed with reactive dyes for polyamide (Eriofast dyes).

Eriofast Blue 3R (*K*/*S* of 14.35) and Eriofast Red B (*K*/*S* of 17.20) dyes (**Table 6**). It could be said that prolonged steaming time caused better fixation and higher attachment rates of Eriofast reactive dyes in the soybean fiber leading to higher color strength.

The color shade differences of the visual appearances of soybean protein fiber fabrics printed with Eriofast Blue 3R and Eriofast Red B dyes (reactive dyes for polyamide) were also detected on reflectance spectra, CIE chromaticity diagram, *a**-*b** plot and hue angle (h^o) values (**Figures 21**, **23**, **24** and **Table 6**). Particularly CIE chromaticity diagram displayed the exact shades (red and blue colors) of printed soybean fabric samples with their measured chroma-

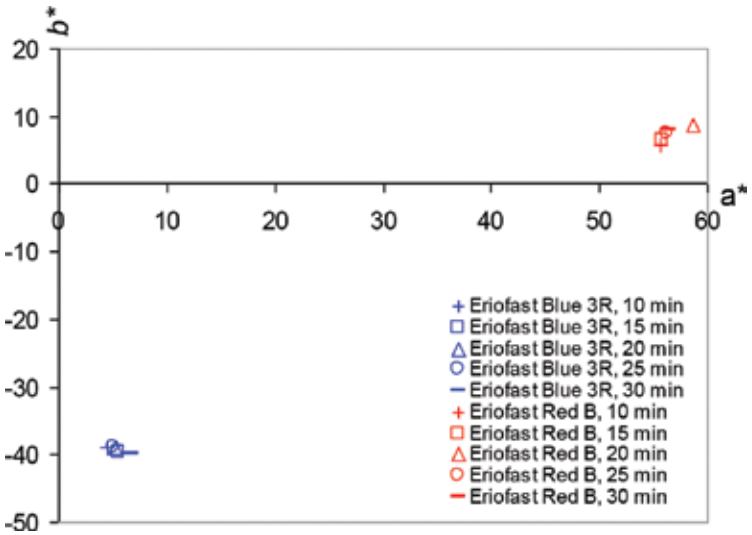

Figure 24. *a**-*b** (redness-greenness versus yellowness-blueness) diagram of soybean samples printed with reactive dyes for polyamide (Eriofast dyes).

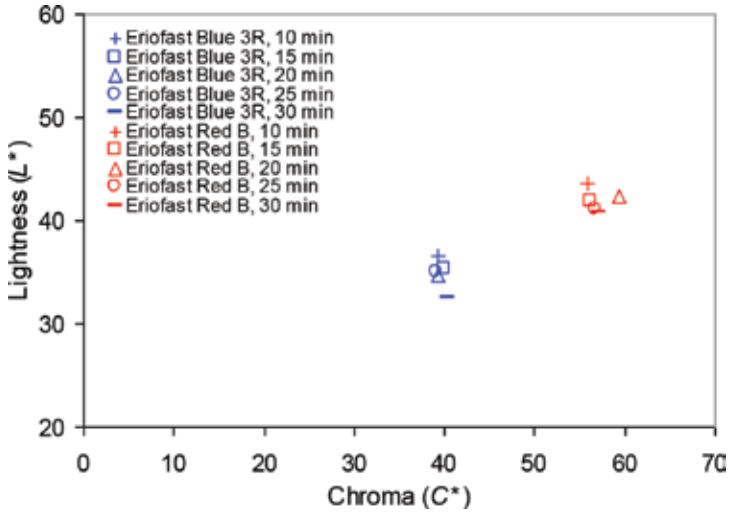

Figure 25. *L*-C** (lightness versus chroma) diagram of soybean fabrics printed with reactive dyes for polyamide (Eriofast dyes) and fixed with different steaming periods.

ticity coordinates (**Figure 23**). Soybean samples printed with Eriofast Blue 3R reactive dye and fixed with varied steaming periods (10, 15, 20, 25 and 30 minutes) displayed close *a** and *b** values (**Figure 24**). A similar observation could be made for Eriofast Red B reactive dye with one exception. Only 20-minute steamed soybean fabric printed with Eriofast Red B dye was slightly redder and yellower due to higher *a** and *b** values (**Figure 24** and **Table 6**).

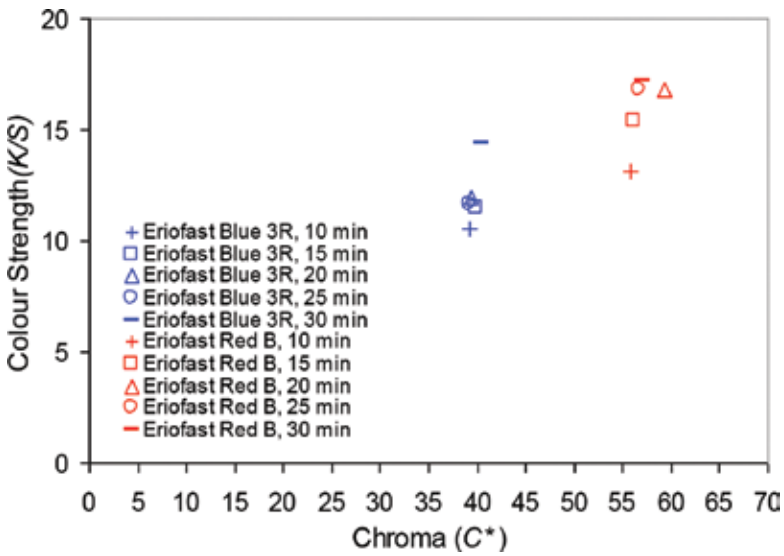

Figure 26. *K/S-C** (color strength versus chroma) diagram of soybean fabrics printed with reactive dyes for polyamide (Eriofast dyes) and fixed with different steaming periods.

Soybean fabrics which are printed with Eriofast reactive dyes and fixed with varying times exhibited close lightness (L^*) and chroma (C^*) values with slight differences (**Figure 25**). 20-minute and 30-minute steamed samples exhibited the highest chroma values for Eriofast Red B (59.3) and Eriofast Blue 3R (40.4), respectively (**Figure 25**). 30-minute steamed samples exhibited the lowest lightness values leading to the highest color strength, as expected (**Figure 25** and **Table 6**). Soybean fabrics printed with Eriofast Red B were brighter than soybean fabrics printed with Eriofast Blue 3R with higher lightness and chroma levels (**Figure 25**). Soybean fabric printed with Eriofast Red B and fixed with 30-minute steaming displayed the highest color strength and chroma value (**Figure 26**). For Eriofast Red B, 30 minutes of steaming resulted in the highest color strength value.

3.5. Color fastness properties of printed soybean fabrics

Color fastness of colored material is a very important factor for buyers' demand [40, 41]. Color fastness is the resistance of color to fade or bleed of colored textile substrates occurring due to various types of influences such as water, light, rubbing, washing, perspiration, etc., which normally occur in textile manufacturing and in our daily use [41, 42]. Washing and light fastness properties are the most important parameters to evaluate the performance of the textile material and to decide its end-use application type [43]. In addition, dry and wet rub fastness properties are also an important for apparel applications [17]. The effects of dye-class type and fixation time by steaming on the color fastness properties of soybean fiber fabrics printed with commercial dyes are discussed below. Wash, rub (dry and wet) and light fastness properties of printed soybean samples are shown in **Tables 7** and **8**.

3.5.1. Light fastness

It seems that increase in steaming time resulted in very slight light fastness performance improvement in some cases (**Table 7**). This observation is quite visible in the case of Eriofast dyes (reactive dyes for polyamide). In this case, prolonged steaming fixation times resulted in up to three quarter point improvement on light fastness values. This is most probably due to their higher color strength leading to higher dye content in the fiber. Although acid dyes resulted in vibrant colors on soybean fibers, their related light fastness values were not so high and in the range of 4–4/5 and 3–3/4 for Erionyl Blue A-4G and Erionyl Red A-3G dyes, respectively (**Table 7**). A 1:2 metal complex dyes (Lanacron dyes) led to the highest light fastness performance of seven rating with only very slight fading on soybean fabrics according to the blue-wool scale (**Table 7**). These quite high light fastness levels are not surprising, since metal complex dyes are known to impart higher fastness properties in comparison with acid dyes [37]. However, on the other hand, metal complex dyes may result in duller colors [37]. Indeed, both measured brightness and light fastness differences between soybean fabrics printed with acid and 1:2 metal complex dyes were in line with this previous experience. Reactive dyes which are recommended for wool fibers (Lanasol dyes) resulted in moderate to good light fastness values on soybean fibers with 4/5–5/6 which are higher than the light fastness levels of acid dyes (Eriofast dyes). Other studied reactive dyes which are recommended for polyamide fibers (Eriofast dyes) caused slightly higher light fastness levels on soybean fibers with 5–5/6 in comparison with reactive dyes for wool fibers (Lanasol dyes) (**Table 7**). These good light fastness

Printed soybean fabrics		K/S	Light fastness	Rub fastness (X12)	
[dye class, dye name, fixation (steaming) time]			(Xenon) (1–8)	Dry	Wet
Acid dyes	**Erionyl Blue A-4G, 15 min**	15.25	4	3–4	2–3
	Erionyl Blue A-4G, 30 min	15.74	4–5	3–4	2–3
	Erionyl Blue A-4G, 40 min	**17.33**	4–5	3–4	2–3
	Erionyl Blue A-4G, 45 min	15.85	4–5	3–4	2–3
	Erionyl Red A-3G, 15 min	21.23	3	4–5	3–4
	Erionyl Red A-3G, 30 min	23.16	3–4	4–5	3–4
	Erionyl Red A-3G, 40 min	**24.26**	3–4	4–5	3–4
	Erionyl Red A-3G, 45 min	23.05	3–4	4–5	3–4
1:2 Metal complex dyes	**Lanacron Blue 3GL, 15 min**	22.05	7	4–5	3–4
	Lanacron Blue 3GL, 25 min	22.93	7	4–5	3–4
	Lanacron Blue 3GL, 30 min	24.01	7	4–5	3–4
	Lanacron Blue 3GL, 40 min	24.26	7	4–5	3–4
	Lanacron Blue 3GL, 45 min	**2.65**	7	4–5	3–4
	Lanacron Red 2GL, 15 min	20.11	7	4	3–4
	Lanacron Red 2GL, 25 min	20.94	7	4	3–4
	Lanacron Red 2GL, 30 min	21.14	7	4	3–4
	Lanacron Red 2GL, 40 min	21.64	7	4–5	3–4
	Lanacron Red 2GL, 45 min	**23.05**	7	4–5	3–4
Reactive dyes for wool	**Lanasol Blue 3R, 10 min**	9.09	4–5	4–5	3–4
	Lanasol Blue 3R, 15 min	9.85	4–5	4–5	3–4
	Lanasol Blue 3R, 20 min	**9.89**	4–5	4–5	3–4
	Lanasol Blue 3R, 25 min	9.35	4–5	4–5	3–4
	Lanasol Blue 3R, 30 min	9.55	4–5	4–5	4
	Lanasol Red 5B, 10 min	20.29	5	4–5	3–4
	Lanasol Red 5B, 15 min	21.43	5	4–5	3–4
	Lanasol Red 5B, 20 min	**24.78**	5–6	4–5	3–4
	Lanasol Red 5B, 25 min	21.04	5	4–5	3–4
	Lanasol Red 5B, 30 min	20.02	5	4–5	3–4
Reactive dyes for polyamide	**Eriofast Blue 3R, 10 min**	10.52	5	4–5	3–4
	Eriofast Blue 3R, 15 min	11.52	5–6	4–5	3–4
	Eriofast Blue 3R, 20 min	11.97	5–6	4–5	3–4
	Eriofast Blue 3R, 25 min	11.61	5–6	4–5	3–4

Printed soybean fabrics	K/S	Light fastness	Rub fastness (X12)	
[dye class, dye name, fixation (steaming) time]		(Xenon) (1–8)	Dry	Wet
Eriofast Blue 3R, 30 min	**14.35**	5–*6*	4–*5*	4
Eriofast Red B, 10 min	13.10	5	4–*5*	3–*4*
Eriofast Red B, 15 min	15.41	5–6	4–*5*	3–*4*
Eriofast Red B, 20 min	16.81	5–6	4–*5*	3–*4*
Eriofast Red B, 25 min	16.81	5–6	4–*5*	3–*4*
Eriofast Red B, 30 min	**17.20**	5–*6*	4–*5*	4

Table 7. Light and rub fastness properties of printed soybean fabrics.

results were not surprising since reactive dyes generate true chemical bonds with the SH, NH, or NH_2 groups in the polypeptide chains of the protein fiber leading to good fastness levels and brilliant colors [37]. Optimum steam fixation durations, which were reported and discussed in the color properties section for each dye class led to the highest light fastness levels. This is most probably owing to the higher color strength (*K*/*S*) with higher dye content in the fiber.

3.5.2. Rub fastness

In analogy with the light fastness performance, the lowest rub fastness levels were obtained for acid dyes, as expected (**Table 7**). The dry and wet rub fastness levels of soybean printed with Erionyl Blue A-4G acid dyes were in the range of 3–4 and 2–3, respectively. Erionyl Red A-3G dyes resulted in up to 1 point improvement for both dry (*4*/5–4/5) and wet (3–4) rub fastness when compared to Erionyl Blue A-4G (**Table 7**). It is known that wool and silk protein fibers printed with acid dyes exhibit very vivid print colors with moderate fastness levels. Therefore, acid dyes must be selected to achieve acceptable light and wet fastness for each end-use, along with the preferred brilliance of hue [37].

Soybean fabrics printed with 1:2 metal complex dyes (Lanacron dyes) exhibited 3–4 gray scale rating for wet rub fastness. In the case of dry rub fastness, blue dye (Lanacron Blue 3GL) resulted in commercially acceptable fastness levels of 4–5 gray scale rating which was about half point higher than those of red dye (Lanacron Red 2GL) (**Table 7**). Reactive dyes, which are recommended for wool fibers (Lanasol dyes), led to moderate to good rub fastness levels on soybean fibers with 3/4–4 for dry rub and 4–*5* for wet rub fastness (**Table 7**). Other studied reactive dyes, which are recommended for polyamide fibers (Eriofast dyes) resulted in similar rub fastness levels on soybean fibers with 3/*4*–4 for dry rub and 4–*5* for wet rub fastness (**Table 7**). It is expected that reactive dyes for printing wool protein fiber exhibit good wet fastness properties [39]. A 1:2 metal complex dyes and reactive dyes (for both wool and polyamide) resulted in quite good and commercially acceptable dry rub fastness and moderate to good wet rub fastness levels. The different steaming times did not result in significant differences on rub fastness level. Prolonged steaming fixation times sometimes resulted in only up to a quarter point improvement on wet rub fastness value.

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Printed soybean fabrics	***K/S***	**Wash fastness staining (C06-A2S)**					
[dye, fixation (steaming) time]		**Diacetate**	**Cotton**	**Polyamide**	**Polyester**	**Acrylic**	**Wool**
Erionyl Blue A-4G, 15 min	15.25	5	4–5	4	4–*5*	5	4–5
Erionyl Blue A-4G, 30 min	15.74	5	4–5	4	4–*5*	5	4–5
Erionyl Blue A-4G, 40 min	**17.33**	5	4–5	4	4–*5*	5	4–5
Erionyl Blue A-4G, 45 min	15.85	5	4–5	4	4–*5*	5	4–5
Erionyl Red A-3G, 15 min	21.23	4–*5*	4	4–5	4–5	4–5	4–*5*
Erionyl Red A-3G, 30 min	23.16	4–*5*	4	4–5	4–5	4–5	4–*5*
Erionyl Red A-3G, 40 min	**24.26**	4–*5*	4	4–5	4–*5*	4–*5*	4–*5*
Erionyl Red A-3G, 45 min	23.05	4–*5*	4	4–5	4–5	4–5	4–*5*
Lanacron Blue 3GL, 15 min	22.05	5	5	4–5	5	5	5
Lanacron Blue 3GL, 25 min	22.93	5	5	4–5	5	5	5
Lanacron Blue 3GL, 30 min	24.01	5	5	4–5	5	5	5
Lanacron Blue 3GL, 40 min	24.26	5	5	4–5	5	5	5
Lanacron Blue 3GL, 45 min	**24.65**	5	5	4–5	5	5	5
Lanacron Red 2GL, 15 min	20.11	5	5	4–5	5	5	5
Lanacron Red 2GL, 25 min	20.94	5	5	4–5	5	5	5
Lanacron Red 2GL, 30 min	21.14	5	5	4–5	5	5	5
Lanacron Red 2GL, 40 min	21.64	5	5	4–5	5	5	5
Lanacron Red 2GL, 45 min	**23.05**	5	5	4–5	5	5	5
Lanasol Blue 3R, 10 min	9.09	5	5	5	5	5	5
Lanasol Blue 3R, 15 min	9.85	5	5	5	5	5	5
Lanasol Blue 3R, 20 min	**9.89**	5	5	5	5	5	5
Lanasol Blue 3R, 25 min	9.35	5	5	5	5	5	5
Lanasol Blue 3R, 30 min	9.55	5	5	5	5	5	5
Lanasol Red 5B, 10 min	20.29	5	4–5	5	5	5	5
Lanasol Red 5B, 15 min	21.43	5	4–5	5	5	5	5
Lanasol Red 5B, 20 min	**24.78**	5	4–5	5	5	5	5
Lanasol Red 5B, 25 min	21.04	5	4–5	5	5	5	5
Lanasol Red 5B, 30 min	20.02	5	4–5	5	5	5	5
Eriofast Blue 3R, 10 min	10.52	5	5	4–*5*	5	5	5
Eriofast Blue 3R, 15 min	11.52	5	5	4–*5*	5	5	5
Eriofast Blue 3R, 20 min	11.97	5	5	4–*5*	5	5	5
Eriofast Blue 3R, 25 min	11.61	5	5	4–*5*	5	5	5
Eriofast Blue 3R, 30 min	**14.35**	5	5	4–5	5	5	5

Printed soybean fabrics [dye, fixation (steaming) time]	*K/S*	Wash fastness staining (C06-A2S)					
		Diacetate	Cotton	Polyamide	Polyester	Acrylic	Wool
Eriofast Red B, 10 min	13.10	5	5	4–5	5	5	5
Eriofast Red B, 15 min	15.41	5	5	4–5	5	5	5
Eriofast Red B, 20 min	16.81	5	5	4–5	5	5	5
Eriofast Red B, 25 min	16.81	5	5	4–5	5	5	5
Eriofast Red B, 30 min	**17.20**	5	5	4–5	5	5	5

Table 8. Wash fastness properties of printed soybean fabrics.

3.5.3. Wash fastness

Printed soybean samples for all dye classes and all steaming times exhibited commercially acceptable wash fastness levels, which are equal to or above 4 gray scale rating (**Table 8**). Most of them were gray scale rating of 5 with no staining at all. The rests exhibited only one point lower wash fastness levels than the maximum available (**Table 8**). Although acid dyes resulted in slightly lower wash fastness levels than other three dye classes, wash fastness levels of soybean fabrics printed with acid dyes are still good and in the commercially acceptable range. A 1:2 metal complex dyes and reactive dyes (for both wool and polyamide) led to quite good and commercially acceptable wash fastness levels. As mentioned earlier, reactive dyes can form covalent bonds with –NH, $–NH_2$, –SH and –OH groups of protein fibers leading to high fastness levels. There were no significant differences between the wash fastness levels due to different dye class, different dye and different fixation steaming time. The different steaming times did not result in significant differences on wash fastness level. Prolonged steaming fixation times sometimes resulted in only up to a quarter point difference on wash fastness value.

4. Conclusions

It is important to colorize sustainable, renewable ecologic natural-based soybean fiber properly via printing for the textile and fashion industry. Dye selection and fixation conditions after printing affect the color yield and quality of the print. Optimum fixation conditions in respect of colorimetric values and color fastness properties should be determined for dye class in order to obtain the best possible print quality on soybean fiber fabric. In the case of soybean protein fabrics printed with acid dyes (Erionyl dyes), the highest color strength values for Erionyl Blue A 4G (*K/S* = 17.33) and Erionyl Red A 3G (*K/S* = 24.26) dyes were obtained after 40 minutes of steaming for fixation. In the case of 1:2 metal complex dyes (Lanacron dyes), the highest color strength values were observed for 45-minute steamed soybean samples with both Lanacron Blue 3GL (*K/S* = 24.65) and Lanacron Red 2GL (*K/S* = 23.05) dyes. These two observations are in parallel with the literature where it was stated that relatively long steaming times of 30–60 minutes are generally required to fix acid and metal complex dyes on wool and silk protein fibers. It is known that the steam used after printing provides

http://dx.doi.org/10.5772/66725

the moisture and rapid heating which gives rise to the transfer of dye molecules from the thickener film to the fiber within a reasonable time. It seems that the proper diffusion of the large 1:2 metal complex dye molecules into the soybean fiber needs a little more time and the diffusion increases with increasing steaming fixation time leading to a high color yield. Acid dyes (Erionyl dyes) led to brighter appearance on soybean fabric in comparison with 1:2 metal complex dyes (Lanacron dyes). The highest color strength values for Lanasol Red 5B (K/S = 24.78) and Lanasol Blue 3R (K/S = 9.89) dyes (Bromo acrylamide reactive group reactive dyes which are generally recommended for wool printing) on soybean were obtained after 20-minute steaming fixation. In the case of novel sulfo group containing Eriofast reactive dyes which are generally recommended for polyamide printing, the highest color strength values were observed for 30-minute steamed soybean samples with Eriofast Blue 3R (K/S = 14.35) and Eriofast Red B (K/S = 17.20) dyes. It is known for printing wool protein fiber that reactive dyes possess better solubility than acid dyes and can be usually sprinkled directly into the print paste as solids without the use of dye solvents and that they need shorter steaming times which is a clear benefit in continuous steaming.

Light fastness values of soybean printed with acid dyes were not so high and in the range of 4–4/5 and 3–3/4 for Erionyl Blue A-4G and Erionyl Red A-3G dyes, respectively. A 1:2 metal complex dyes (Lanacron dyes) led to the highest light fastness performance of 7 rating with only very slight fading on soybean fabrics. Reactive dyes which are recommended for wool and polyamide fibers (Lanasol and Eriofast dyes) resulted in moderate to good light fastness values on soybean fibers with 4/5–5/6 and 5–5/6, respectively, which were higher than the light fastness levels of acid dyes (Eriofast dyes). In analogy with the light fastness performance, the lowest rub fastness levels were obtained for acid dyes. A 1:2 metal complex dyes and reactive dyes (for both wool and polyamide) on soybean printing resulted in quite good and commercially acceptable dry rub fastness and moderate to good wet rub fastness levels. The different steaming times did not result in significant differences on rub fastness level. Prolonged steaming fixation times sometimes resulted in only up to a quarter point improvement on wet rub fastness value. Printed soybean samples for all dye classes and all steaming times exhibited commercially acceptable wash fastness levels, which are equal to or above 4 gray scale rating. Acid dyes resulted in slightly lower wash fastness levels than other three dye classes. A 1:2 metal complex dyes and reactive dyes (for both wool and polyamide) on soybean printing led to quite good and commercially acceptable wash fastness levels. Reactive dyes can form covalent bonds with –NH, $–NH_2$, –SH and –OH groups in the polypeptide chains of protein fibers leading to high fastness levels. The different steaming times did not result in significant differences on wash fastness level. Prolonged steaming fixation times sometimes resulted in only up to a quarter point difference on wash fastness value.

This study exhibits that acid and 1:2 metal-complex dyes (originally used for printing of natural protein fibers such as wool and silk fibers) and special reactive dyes (used for printing of wool and polyamide fibers) can be used for the printing process of regenerated soybean fiber leading to high color strength with adequate color fastness performance. Steaming at 102°C for 40 and 45 minutes are the optimum fixation conditions for acid and 1:2 metal-complex dyes on soybean fiber fabrics, respectively. On the other hand, steamings at 102°C for 20 minutes and 30 minutes are the optimum fixation conditions for wool-type reactive

dyes and polyamide-type reactive dyes on soybean fiber fabrics, respectively. These optimum steam-fixation durations for each dye class led to the highest light fastness levels. This is most probably owing to their higher color strengths (*K*/*S*) with higher dye content in the fiber. Overall, optimum steam fixation durations for 1:2 metal complex and reactive dye classes (for both wool and polyamide) on printed soybean fibers displayed quite high and commercially acceptable wash fastness and good and commercially acceptable dry rub fastness and moderate to good wet rub fastness levels performance.

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Edited by Minobu Kasai

Soybean is one of the organisms largely contributing to our life. Therefore, it is important to know soybean from various aspects. The knowledge and soybean itself will be greatly useful, if they are soundly used. The chapters constituting this book present reviews and researches especially concerning the basis of yield, biomass, and productivity in soybean. Yield, biomass, and productivity in plants are some of the bases for maintaining or improving our ecosystem which includes our life and surrounding environments. Therefore, this book is expected to be useful for many people. Of course, more researches and investigations are important to further gain the knowledge concerning the basis of yield, biomass, and productivity and make them useful for our ecosystem.

ISBN 978-953-51-4850-0

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