A Comprehensive Survey of International

SOYBEAN Research

Genetics, Physiology, Agronomy and Nitrogen Relationships

Edited by James E. Board

A COMPREHENSIVE SURVEY OF INTERNATIONAL SOYBEAN RESEARCH - GENETICS, PHYSIOLOGY, AGRONOMY AND NITROGEN RELATIONSHIPS

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Contents

Section 3 Soybean Agronomy and Physiology 181

Preface

Soybean is the most important oilseed and livestock feed crop in the world, accounting for 58% of total world oilseed production and 69% of protein meal consumption by livestock. These dual uses are attributed to the crop's high protein content (nearly 40% of seed weight) and oil content (approximately 20%); characteristics that are not rivaled by any other agronomic crop. Besides its use as a high-protein livestock and poultry feed, and oilseed crop (used in margarines, cooking oils, and baked and fried food products), soybean has various other industrial uses such as biodiesel, fatty acids, plastics, coatings, lubricants, and hydraulic fluids. In Asian countries such as China, Japan and Indonesia, the whole seed is directly consumed as human food; or it is incorporated into human food items such as tofu, tempeh, soy milk, soy cheese, or other products. Soybean consumption as human food is increasing outside of Asia. Recently, health benefits for soybean have been recognized for heart disease, cancer, osteoporosis, and menopause. The American Heart Association recommends daily human consumption of 25 mg of soybean to help prevent heart and circulatory diseases.

In 2010, 258.4 million metric tons of soybean were produced in the world, having a value of $111 billion. Over 80% of the world's soybeans are produced in three countries: the USA, Brazil, and Argentina. These three countries are also the main exporters of soybean to the world market. Major importing countries are China, Japan, the European Union, and Mexico. A testimony to the increasing importance of soybean on the world agricultural stage is in the stunning growth of production shown by Argentina and Brazil over the last 25 years. Between 1986 and 2010, the production has risen from 17.3 to 70 million metric tons in Brazil (a four-fold increase) and from 7 to 49.5 million metric tons in Argentina (a seven-fold increase). Both countries have demonstrated to the world how an organized effort of research, education and extension can create an entire industry around production and use of an agricultural commodity.

Against the backdrop of soybean's striking ascendancy is the increased research interest in the crop throughout the world. The objective of this book is to provide readers with a view of the high quality of soybean research being conducted in so many different parts of the world. With all the dissension and rancor in the world (wars, terrorism, financial panic, etc.) it is truly heartening to see the efforts being made to create a greater understanding of soybean in so many diverse parts of the world. Such efforts will go a long way to meeting increased demand for soybeans; a demand driven by increased world population and rising living standards. Because expansion of agricultural land to meet this demand is limited, the only way to meet increased world demand for soybean is by greater production per area of currently available land. This is why research, such as that contained in this book, is so vital for future soybean production.

It is in this light that I would like to acknowledge all the authors for their outstanding efforts in composing these chapters. The information presents a comprehensive view of research efforts in genetics, plant physiology, agronomy, agricultural economics, and nitrogen relationships that will benefit soybean stakeholders and scientists throughout the world. We hope you enjoy the book.

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Section 1

Soybean Nitrogen Relationships

A Proteomics Approach to Study Soybean and Its Symbiont *Bradyrhizobium japonicum* –A Review

Sowmyalakshmi Subramanian and Donald L. Smith

Additional information is available at the end of the chapter

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1. Introduction

Soil is a dynamic environment due to fluctuations in climatic conditions that affect pH, temperature, water and nutrient availability. These factors, along with agricultural management practices, affect the soil micro-flora health and the capacity for effective plant-microbe interactions. Despite these constant changes, soil constitutes one of the most productive of earth's ecospheres and is a hub for evolutionary and other adaptive activities.

1.1. Biological nitrogen fixation

Biological nitrogen fixation (BNF) is one of the most important phenomena occurring in nature, only exceeded by photosynthesis [1,2]. One of the most common limiting factors in plant growth is the availability of nitrogen [3]. Although 4/5ths of earth's atmosphere is comprised of nitrogen, the ability to utilize atmospheric nitrogen is restricted to a few groups of prokaryotes that are able to covert atmospheric nitrogen to ammonia and, in the case of the legume symbiosis, make some of this available to plants. Predominantly, members of the plant family Leguminosae have evolved with nitrogen fixing bacteria from the family Rhizobiaceae. In summary, the plants excrete specific chemical signals to attract the nitrogen fixing bacteria towards their roots. They also give the bacteria access to their roots, allowing them to colonize and reside in the root nodules, where the modified bacteria (bacteroids) can perform nitrogen fixation [1,4,5]. This process is of great interest to scientists in general, and agriculture specifically, since this highly complex recognition and elicitation is co-ordinated through gene expression and cellular differentiation, followed by plant growth and development; it has the potential to minimize the use of artificial nitrogen fertilizers and pesticides in crop management. This biological nitrogen fixation process is complex, but has been best examined in some detail in the context of soybean-*Bradyrhizobium* plant-microbe interactions.

1.2. Soybean – The plant

Soybean (*Glycine max* (L.) Merrill) is a globally important commercial crop, grown mainly for its protein, oil and nutraceutical contents. The seeds of this legume are 40% protein and 20% oil. Each year soybean provides more protein and vegetable oil than any other cultivated crop in the world.

Soybean originated in China, where it has been under cultivation for more than 5000 years [6]. The annual wild soybean (*G. soja*) and the current cultivated soybean (*G. max*) can be found growing in China, Japan, Korea and the far east of Russia, with the richest diversity and broadest distribution in China, where extensive germplasms are available. The National Gene Bank at the Institute of Crop Germplasm Resources, part of Chinese Academy of Agriculture Sciences (ICGR-CAAS), Beijing, contains close to 24,000 soybean accessions, including wild soybean types. Soybean was introduced into North America during the 18th century, but intense cultivation started in the 1940s – 1950s and now North America is the world's largest producer of soybean [7,8]. Although grown worldwide for its protein and oil, high value added products such as plant functional nutraceuticals, including phospholipids, saponins, isoflavones, oligosaccharides and edible fibre, have gained importance in the last decade. Interestingly, while genistein and diadzein are signal molecules involved in the root nodulation process, the same compounds can attenuate osteoporosis in post-menopausal women. The other isoflavones have anti-cancer, anti-oxidant, positive cardiovascular and cerebrovascular effects [9]. More recently soybean oil has also been used as an oil source for biodiesel [10-14].

Table 1 provides the latest statistics on soybean cultivation and production as available at FAOSTAT [15]

	World	Africa	Americas	Asia	Europe	Oceania	Canada
Area harvested (Ha)	102,386,923	1,090,708	78,811,779	19,713,738	2,739,398	31,300	1,476,800
Yield (Hg/Ha)	25,548	13,309	28,864	14,100	17,491	19,042	29,424
Production (Tonnes)	261,578,498	1,451,646	227,480,272	27,795,578	4,791,402	59,600	4,345,300
Seeds (Tonnes)	6,983,352	43,283	4,838,633	1,906,313	193,870	1,252	154,300
Soybean oil (Tonnes)	39,761,852	390,660	24,028,558	12,442,496	2,890,760	9,377	241,300

Table 1. Soybean production statistics (FAOSTAT 2010)

Soybean is a well-known nitrogen fixer and has been a model plant for the study of BNF. Its importance in BNF led to the genome sequencing of soybean; details of the soybean genome are available at soybase.org (*G. max* and *G. soja* sequences are available at NCBI as well). Although considerable work has been conducted on other legumes with respect to biological nitrogen fixation, we focus only on soybean for this review.

The efficiency of BNF depends on climatic factors such as temperature and photoperiod [16]; the effectiveness of a given soybean cultivar in fixing atmospheric nitrogen depends on the interaction between the cultivar's genome and conditions such as soil moisture and soil nutrient availability [17,18]; and the competitiveness of the bacterial strains available, relative to indigenous and less effective strains, plus the amount and type of inoculants applied, and interactions with other, possibly antagonistic, agrochemicals that are used in crop protection [19]. The most important criteria, however, is the selection of an appropriate strain of *B. japonicum* since specific strains can be very specific to soybean cultivar, and subject to influence by specific edaphic factors [20,21,22]. Under most conditions, soybean meets 50-60% of its nitrogen demand through BNF, but it can provide 100% from this source [23].

1.3. *Bradyrhizobium japonicum*

B. japonicum, is a gram negative, rod shaped nitrogen fixing member of the rhizobia and is an N_2-fixing symbiont of soybean. *B. japonicum* strain USDA110, was originally isolated from soybean nodules in Florida, USA, in 1957 and has been widely used for the purpose of molecular genetics, physiology, and ecology, owing to its superior symbiotic nitrogen fixation activity with soybean, relative to other evaluated strains. The genome sequence of this strain has been determined; the bacterial genome is circular, 9.11 Million bp long and contains approximately 8373 predicted genes, with an average GC content of 64.1% [24,25].

Initially attached to the root-hair tips of soybean plants, rhizobia colonize within the roots and are eventually localized within symbiosomes, surrounded by plant membrane. This symbiotic relationship provides a safe niche and a constant carbon source for the bacteria while the plant derives the benefits of bacterial nitrogen fixation, which allows for the use of readily available nitrogen for plant growth. Inoculation of soybean with *B. japonicum* often increases seed yield [eg. 26].

B. japonicum synthesize a wide array of carbohydrates, such as lipopolysaccharides, capsular polysaccharides, exopolysaccharides (EPS), nodule polysaccharides, lipo-chitin oligosaccharides, and cyclic glucans, all of which play a role in the BNF symbiosis. Bacteria produce polysaccharide degrading enzymes, such as polygalacturonase and carboxymethylcellulase, cleave glycosidic bonds of the host cell wall at areas where bacteria are concentrated, creating erosion pits in the epidermal layer of the roots, allowing the bacteria gain entry to the roots [27]. The energy source for *B. japonicum* is the sugar trehalose, which is taken up readily and converted to CO_2 [28,29,30,31]. On the other hand UDP-glucose is taken up in large quantities but metabolized slowly, like sucrose and glucose. Promotion of plant growth causes more O_2 to be released and more CO_2 to be taken up [24,27].

1.4. Lipo-chitooligosaccharide (LCO) from *Bradyrhizobium japonicum*

As mentioned earlier in this review, the process of nodulation in legumes begins with a complex signal exchange between host plants and rhizobia. The first step in rhizobial establishment in plant roots is production of isoflavonoids as plant-to-bacterial signals; the most common in the soybean-*B. japonicum* symbiosis being genestin and diadzein [32], which trigger the *nod* genes in the bacteria which, in turn, produce LCOs, or Nod factors, that act as return signals to the plants and start the process of root hair curling, leading to nodule formation. Some recent literature has also shown that jasmonates can also cause *nod* gene activation in *B. japonicum* although the strain specificities are very different from those of isoflavonoids such as genistein [33-36]. LCOs are oligosaccharides of β-1,4-linked N-acetyl-D-glucosamine coded for by a series of *nod* genes and are rhizobia specific [37,38]. The nodDABCIJ genes, conserved in all nodulating rhizobia [37,39,40] are organized as a transcriptional unit and regulated by plant-to-rhizobia signals such isoflavanoids [41-43].

Nodulation and subsequent nitrogen fixation are affected by environmental factors. It has been observed that, under sub-optimal root zone temperatures (for soybean 15-17 ºC), pH stress and in the presence of nitrogen, isoflavanoid signal levels are reduced; while high temperature (39 ºC) increases non-specific isoflavanoid production and reduces *nod* gene activation, thereby affecting nodulation [44]). Our laboratory has isolated and identified the major LCO molecule produced by *B. japonicum* 532C as Nod Bj V (C18:1;MeFuc) [45]. This Nod factor contains a methyl-fucose group at the reducing end that is encoded by the host-specific *nodZ* gene [46], which is an essential component for soybean-rhizobia interactions.

LCOs also positively and directly affect plant growth and development in legumes and non-legumes. The potential role of LCOs in plant growth regulation was first reported by Denarie and Cullimore [47]). Nod genes A and B from *R. meliloti*, when introduced into tobacco, altered the phenotype by producing bifurcated leaves and stems, suggesting a role for *nod* genes in plant morphogenesis [48]. The development of somatic embryos of Norway spruce is enhanced by treatment with purified Nod factor from *Rhizobium* sp. NGR234. It has been suggested that these Nod factors can substitute for auxin and cytokinin like activities in promoting embryo development, and that the chitin core of the nod factor is an essential component for regulation of plant development [49,50]. Some of the LCO induced enod genes in non-legumes seem to encode for defence related responses, such as chitinase and PR proteins [42,43], peroxidase [51] and enzymes of phenylpropanoid pathway, such as L-phenylalanine ammonia-lyase (PAL) [52]. Seed gemination and seedling establishment is enhanced in soybean, common bean, maize, rice, canola, apple and grapes, accompanied by increased photosynthetic rates [53]. Hydroponically grown maize showed an increase in root growth when LCO was applied to the hydroponic solution [54,55] and foliar application to greenhouse grown maize resulted in increases in photosynthetic rate, leaf area and dry matter [56]. Foliar application to tomato, during early and late flowering stages, increased flowering and fruiting and also fruit yield [57]. An increase in mycorrhizal colonization (*Gigaspora margarita*) was observed in *Pinus abies* treated with LCO [50,58]. Recent research in our laboratory, on soybean leaves treated with LCOs under sub-optimal growth conditions, revealed the up-regulation of over 600 genes, many of which are defense and stress response related, or transcription factors; microarray results show that the transcriptome of the leaves is highly responsive to LCO treatment at 48 h post treatment [59]. These results suggest the need to investigate more carefully the mechanisms by which microbe-to-plant signals help plants accommodate abiotic and biotic stress conditions.

Since the protein quality of soybean plays an important role in overall agricultural and in nutraceuticals production, it is imperative that we study the proteomics of soybean and its symbiont *B. japonicum*, not only for better understanding of the crop, but also for the betterment of agriculture practices and production of better high value added food products for human consumption.

1.5. Proteomics as a part of integrative systems biology

The "omics" approach to knowledge gain in biology has advanced considerably in the recent years. The triangulation approach of integrating transcriptomics, proteomics and metabolomics is being used currently to study interconnectivity of molecular level responses of crop plants to various conditions of stress tolerance and adaptation of plants, thus improving systems level understanding of plant biology [60, 61].

While transcriptomics is an important tool for studying gene expression, proteomics actually portrays the functionality of the genes expressed. Several techniques are available for studying differential expression of protein profiles, and can be broadly classified as gel-based and MS (mass spectrometry)-based quantification methods. The gel based approach uses conventional, two-dimensional (2-D) gel electrophoresis, and 2-D fluorescence difference gel electrophoresis (2D-DIGE), both based on separation of proteins according to isoelectric point, followed by separation by molecular mass. The separated protein spots are then isolated and subjected to MS analysis for identification. Major drawbacks of these techniques are laborious sample preparation and inability to identify low abundance, hydrophobic and basic proteins.

The MS based approach can be a label-based quantitation, where the plants or cells are grown in media containing ^{15}N metabolite label or using ^{15}N as the nitrogen source. Label-free quantitation, however, is easier and allows analysis of multiple and unlimited samples. This technique, also referred to as MudPIT (multidimensional protein identification technology), is a method used to study proteins from whole-cell lysate and/or a purified complex of proteins [62,63]. The total set of proteins or proteins from designated target sites are isolated and subjected to standard protease digestions (eg. such as tryptic digestion). In brief, flash frozen leaf samples are ground in liquid nitrogen and polyphenols; tannins and other interfering substances such as chlorophyll are removed. The processed tissue is resuspended in a chaotropic reagent to extract proteins in the upper phase, and the plant debris is discarded [64-70]. The total protein set, in the resulting solution, is further quantified using the Lowry method [71]. The protein samples (2 μg of total protein each), once digested with trypsin, can then be loaded onto a microcapillary column packed with reverse phase and strong cation exchange resins. The peptides get separated in the column, based on their charge and hydrophobicity. The columns are connected to a quarternary high-performance liquid chromatography pump and coupled with an ion trap mass spectrometer, to ionize the samples within the column and spray them directly into a tandem mass spectrometer. This allows for a very effective and high level of peptide separation within the mixture, and detects the eluting peptides to produce a mass spectrum. The detected peptide ions, at measured mass-to-charge (m/z) ratios with sufficient intensity, are selected for collision-induced dissociation (CID). This procedure allows for the fragmenting of the peptides to produce a product ion spectrum, the MS/MS spectrum. In addition, the fragmentation occurs preferentially at the amide bonds, to generate N-terminal fragments (b ions) and C-terminal fragments (y ions) at specific m/z ratios, providing structural information about the amino acid sequence and sites of modification. The b ion and y ion patterns are matched to a peptide sequence in a translated genomic database to help identify the proteins present in the sample [72-75]. A variety of database searching and compiling algorithms are used to interpret the data obtained for structure and function of the identified proteins.

2. Analyses of soybean proteomics

2.1. Physiological and biological changes in the soybean proteome

2.1.1. Whole plant organs

The various tissues of soybean have specific groups of associated proteins at each developmental stage. While leaves at various developmental stages showed 26 differentially expressed proteins, the first trifoliate stage manifested the greatest increase in protein types of the outer/inner envelope of choloroplast membrane and also of the protein transport machineries. Young leaves showed abundant chaperonin-60, while HSP 70 and TP-synthase b were present in all the tissues analyzed. Age dependent correlation was observed in net photosynthesis rate, chlorophyll content and carbon assimilation. During the flowering stage, flower tissue expressed 29 proteins that were exclusively involved in protein transport and assembly of mitochondria, secondary metabolism and pollen tube growth (Ahsan and Komatsu., 2009 [76]. Soybean peroxisomal adenine nucleotide carrier (GmPNC1) is associated with the peroxisomal membrane and facilitates ATP and ADP importing activities. The proteins At PNC1 and At PNC2 are arabidopsis orthologs of Gm PNC1. Under constant darkness, Gm PNC1 increased in cotyledons up to 5 days post germination and the levels were rapidly reduced when the seedlings were exposed to light. RNA interference studies on arabidopsis At PNC1 and At PNC2 suggests that PNC1 assists with transport of ATP/ADP in the peroxisomal fatty acid-b oxidation pathway post germination (Arai et al., 2008 [77]. This probably helps the seedling establish vigour for future growth.

In order to establish if xylem proteins and the apoplast conduit are involved in long distance signalling in autoregulation of nodulation (AON) in the soybean-*B. japonicum* symbiosis, xylem and apoplast fluids were collected from hypocotyl, epicotyl and stem tissues. In addition, proteins from imbibing seeds were evaluated to determine possible relationships of these proteins with the xylem and apoplast proteins, especially during the seed to seedling stage transition. The proteins secreted from imbibing seeds were different from the set of xylem-related proteins. Hypocotyl, epicotyl and stem xylem proteins were generally similar. Comparison of wild type and nts1007 plants showed no difference in xylem protein profiles, suggesting that xylem proteins were not involved in AON. However, a lipid transfer protein and Kunitz trypsin inhibitor, both known to have roles in plant signalling, were identified within the xylem proteins [78].

Proteomic studies on chasmogamous (CH) CH cv. Toyosuzu and cleistogamous (CL) CL cv. Karafuto-1 flowerbuds using 2D gel revealed differential protein levels of β-galactosidase and protein disulfide isomerase. Cleistogamy occurs in plants under diverse stress conditions, such as drought and cold, and can also vary with temperature and light [79]. Soybean cv Maverick was used to study proteomics during seed filling stages, at 2, 3, 4, 5 and 6 weeks after flowering, using 2D and MALDI-TOF-MS. Storage proteins, proteins involved in metabolism and metabolite transport and defense related proteins were the most abundant, along with cysteine and methionine biosynthesis proteins, lipoxygenases and 14-3-3-like proteins [80,81].

Based on these findings, it is clear that the plant partitions its proteomics based on ontogeny and this specificity probably plays a crucial role in organ maturation and transition from one stage to another in the plants life cycle. Understanding this is of fundamental importance in agriculture, global food production, biofuel production and issues such as plant responses to climate change.

2.1.2. Seeds

Both 2D gel and peptide mass fingerprinting techniques (MALDI-TOF-MS) were used to study the proteins of mature and dry soybean (cv. Jefferson) seeds. Sucrose binding proteins, alcohol dehydrogenase and seed maturation proteins were some of the key proteins identified (Mooney and Thelen 2004 [82]. A comparison of four methods for protein isolation and purification from soybean seed was one of the first reports on soybean proteomics; thiourea/urea and TCA protocols were found to be the best. Proteins extracted with these two methods and further characterized by MALDI-TOF-MS and LC-MS helped identify proteins such as β-conglycinin, glycinin, Kunitz trypsin inhibitor, alcohol dehydrogenase, Gm Bd 28K allergen and sugar binding proteins in seeds [83]. The two major soybean storage proteins are α-conglycinin and glycinin. While the α-conglycinin subunits separated well in the pH range 3.0-10.0, glycinin polypeptides could be separated in pH ranges 4.0-7.0 and 6.0-11.0. Apart from these major storage proteins, this combined proteomic approach (2D-PAGE and immobilized pH gradient strips) also identified 44 storage proteins in wild soybean (*G. soja*) and 34 additional storage proteins in its cultivated counterpart (*G. max*) [84]. A comparative proteome analysis of soybean seed and seedling tissue suggested that there were dramatic changes in the protein profiles during seed germination and during seedling growth. The seed storage proteins β-conglycinin and glycinin were seen to degrade rapidly and their degradation products were either accumulated or degraded further as the seeds germinated. This degradation of the storage proteins indicates that the proteolysis process provides amino acids and energy for the growing seedlings, and gives access to new detail regarding these processes [85].

Synthesis of soybean glycinin and conglycinin, was suppressed by RNA interference. The storage protein knockdown (SP2) seeds were very similar to the wild type during development and at maturity. Proteomic analysis of the SP2 soybean genotypes and next-generation transcript sequencing (RNA-Seq) suggested that the seeds could rebalance their transcriptome and metabolome in the face of at least some alterations. GFP quantification for glycinin allele mimics further revealed that glycinin was not involved in proteome rebalance and that seeds are capable of compensating through increases in other storage proteins, to maintain normal protein content, even if the major storage proteins were not available [86].

Transgenic soybean seeds have higher amounts of malondialdehyde, ascorbate peroxidase, glutathione reductase, and catalase (29.8, 30.6, 71.4, and 35.3%, respectively) than non-transgenic seeds. Precursors of glycinin, allergen Gly m Bd 28k, actin and sucrose binding proteins were the other proteins identified [87,88]. High protein accessions of soybean (with 45 % or more protein in seeds) were compared with soybean cultivar Williams 82. 2-DE-MALDI-TOF-MS followed by Delta2D image analysis showed huge differences in 11S storage globulins amongst the accessions. In addition, the trait for high protein from PI407788A was moved to experimental line LG99-469 and was stable upon transformation [89,90].

2.1.3. Roots, root hairs and nodules

Since the root apical meristem (RAM) is responsible for the growth of the plant root system and root architecture plays and important role in determining the performance of crop plants, a proteome reference map of the soybean root apex and the differentiated root zone was established. The root apex samples comprised of 1 mm of the root apex, encasing the RAM, the quiescent center and the root cap. The predominant proteins in the root belonged to those of stress response, glycolysis, redox homeostasis and protein processing machinery. The root apex contained key proteins, such as those involved in redox homeostasis and flavonoid biosynthesis, but was underrepresented in glycolysis, stress response and TCA cycle related proteins [91]. Analysis of the proteome of isolated soybean root hair cells using 2-D gel and shotgun proteomics approaches identified proteins involved in basic cell metabolism, those whose functions are specific to root hair cell activities, including water and nutrient uptake, vesicle trafficking, and hormone and secondary metabolism [92, 93]. Proteomic studies of soybean roots and root hairs after *B. japonicum* inoculation explains the importance of initial plant-bacteria symbiotic interaction. A 2-D, MALDI-TOF, MS based approach shows that enzymes such as chitinase and phosphoenolpyruvate carboxylase are differentially expressed in root hairs. As well as peroxidase and phenylalanine-ammonia lyase, found to be expressed during rhizhobial inoculation, other novel proteins such as phospholipase D and phosphoglucomutase were found to be expressed [94]. Nodule cytosol proteins from soybean cv. Williams 82 were found to be 28% related to carbon metabolism, 12% related to nitrogen metabolism, 12% related to reactive oxygen metabolism and 11% related to vesicular trafficking proteins. The vesicular trafficking proteins could be involved in the exchange of micro- and macro-molecules during the process of nodulation, while carbon, nitrogen and reactive oxygen species are related to physiological functions during nitrogen fixation [95]. The peribacteroid membrane (PBM) of the soybean symbiosome contains chaperonins such as HSP60, BiP (HSP70) and PDI, and serine and thiol protease, all of which are involved in protein translocation, folding, maturation and degradation of proteins related to the symbiosomes. Nodulin proteins 53b and 26B, associated with the PBM, were also present, although their function is not clear [96].

2.2. Soybean proteomics under stress conditions

Like all plants, soybean also encounters various stressors during its life cycle. Work related to flooding, drought, salt, heat, biotic stressors, metal toxicity, ozone, phosphorous deficiency and seed protein allergens are reviewed here.

2.2.1. Flooding stress

Plasma membrane proteins from the root and hypocotyl of soybean seedlings were purified and subjected to 2-D gel electrophoresis, followed by MS and protein sequencing, and also using nanoliquid chromatography followed by nano-LC-MS/MS based proteomics. The two techniques were used to compare the proteins present, and this indicated that during flooding stress proteins typically found in the cell wall were up-regulated in the plasma membrane. Also, the anti-oxidative proteins were up-regulated to protect the cells from oxidative damage, heat shock proteins to protect protein degradation and signaling proteins to regulate ion homeostasis [97]. MS based proteomics applied to root tips of two-day-old seedlings flooded for 1 day showed increased levels of proteins involved in energy production. Proteins involved in cell structure maintenance and protein folding were negatively affected, as was their phosphorylation status [98].

Two-day-old germinated soybean seeds were subjected to water logging for 12 h and total RNA and proteins were analyzed from the root and hypocotyl. At the transcriptional level, the expression of genes for alcohol fermentation, ethylene biosynthesis, pathogen defense, and cell wall loosening were all significantly up-regulated, while scavengers and chaperons of reactive oxygen species were seen to change only at the translational level. Transcriptional and translational level changes were observed for hemoglobin, acid phosphatase, and Kunitz trypsin protease inhibitors. This adaptive strategy might be for both hypoxia and more direct damage of cells by excessive water [99]). Proteins from 2-day-old soybean seedlings flooded for 12 h were analyzed using 2-D gel MS, 2-D fluorescence difference gel electrophoresis, and nanoliquid chromatography. Early responses to flooding involved proteins related to glycolysis and fermentation, and inducers of heat shock proteins. Glucose degradation and sucrose accumulation increased due to activation of glycolysis and down-regulation of sucrose degrading enzymes, in addition the methylglyoxal pathway, a detoxification system linked to glycolysis, was up-regulated. 2-D gel based phosphoproteomic analysis showed that proteins involved in protein synthesis and folding were dephosphorylated under flooding conditions [100]. Water logging stress imposed on very early soybean seedlings (V2 stage) resulted in a gradual increase of lipid peroxidation and *in vivo* H_2O_2 production. Proteomic studies of the roots using 2-D gel, MALDI-TOF-MS or electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis, identified 14 up-regulated and 5 down-regulated proteins. Five newly discovered proteins were associated with water logging, a known anaerobic stress. The proteins included those associated with signal transduction, programmed cell death, RNA processing, redox homeostasis and energy metabolism.

Increases in glycolysis and fermentation pathway associated proteins were indicative of adaptation of the plant to this alternate energy provision pathway. Other novel proteins, such as a translation initiation factor, apyrase, auxin-amidohydrolase and coproporphyrinogen oxidase, were also identified [101]. Mitochondrial proteomics from 2-day-flooded 4-day-old soybean seedlings identified increases in the levels of proteins and metabolites associated with TCA cycle and the γ-amino butyrate shunt. Increases in NADH and NAD and a decrease in ATP during the stress suggest that the electron transport chain is disrupted, although NADH production increases through TCA cycle activity [102].

Soybean seeds germinated for 48 h were subjected to water logging stress for 6-48 h. In addition to general stress responses due to increases in reactive oxygen species scavengers, several glycolytic enzymes were up-regulated, suggesting changes in energy generation [103].

2.2.2. Water stress – Drought

Soybean root activities are affected during water stress. The root can be partitioned into zones 1 (apical 4 mm zone) and 2 (4-8 mm zone), based on maximum elongation during well watered conditions. Soluble proteins from these regions, studied under both well-watered and water deficit stress conditions, revealed region-specific regulation of the phenylpropanoid pathway. Zone 1 of roots manifested increases in isoflavanoid biosynthesis related enzymes and proteins that contribute to growth and maintenance of the roots under water stress conditions. However, zone 2 of water stressed roots manifested up-regulation of caffeoyl-CoA *O*-methyltransferase (a protein involved in lignin biosynthesis), protective proteins related to oxidative damage, ferritin proteins that sequester iron, and 20S proteasome α-subunit A. Increases in lignin accumulation and ferritin proteins preventing availability of free iron in this zone were suggested to be the factors affecting root growth during water stress [104]. An investigation of the soybean plasma membrane proteome, under osmotic stress, was conducted using 2-day-old seedlings subjected to 10% PEG for 2 days; both gel- and nano-LC MS/MS-based proteomics methods were utilized to analyze the samples. Out of the 86 proteins identified by nano-LC MS/MS approach, 11 were up-regulated and 75 proteins down-regulated under PEG mediated stress. Three homologues of plasma membrane transporter proteins H1-ATPase and calnexin were prominent [105]. Similarly, 3-day-old soybean seedlings were subjected to 10% PEG treatment or water withdrawal and samples collected from roots, hypocotyl and leaves, 4-days after treatment, for proteome analysis. The root was the most responsive and affected organ for both drought stress induction methods. The leaves showed increases in metabolism-related proteins, while the energy production and protein synthesis machineries were negatively affected. HSP70, actin isoform B and ascorbate peroxidase were up-regulated in all the tissues analyzed. Importantly, methionine synthase, a drought response protein, decreased, suggesting negative effects of drought stress on these seedlings [106].

2.2.3. High temperature stress

Tissue specific proteomics under high temperature stress revealed 54, 35 and 61 differentially expressed proteins in the leaves, stems and roots, respectively. Heat shock proteins and those involved in antioxidant defense were up-regulated while proteins for photosynthesis, amino acid and protein synthesis and secondary metabolism were down- regulated. HSP70 and other low molecular weight HSPs were seen in all the tissues analyzed. ChsHSP and CPN-60 were tissue specific and the sHSPs were found only in tissues under heat stress, and were not induced by other stresses such as cold or hydrogen peroxide exposure [107].

2.2.4. Salt stress

Salt stress is also an important abiotic stressor that affects crop growth and productivity. Of the 20% of agricultural land available globally, 50% of the cropland is estimated by the United Nations Environment Program (The UNEP) to be salt-stressed [108]. As the plant grows under salt stresses conditions, depending on the severity of the stress, the plants can experience reduced photosynthesis, protein and energy production, and changes in lipid metabolism [109,110]. As soil salinity increase, the effects on seed germination and germinating seedlings are profound. Responses to salinity and drought stress are similar; they affect the osmotic activity of the root system, thereby affecting the movement of water and nutrients into the plants. In Canadian soils, salinity varies between spring and fall and the most saline conditions are seen at the soil surface just after spring thaw. In the Canadian prairies, the dominant salts of saline seeps include calcium (Ca), magnesium (Mg) and sodium (Na) cations, and sulphate (SO_4^-) anions [111]. Soybean is very sensitive to Cl^-, but not greatly affected by Na^+, because of its ability to restrict movement of Na^+ to leaves [112].

This first report regarding soybean seedling proteomic responses to salt stress evaluated length and fresh weight of the hypocotyl and roots of soybean exposed to a series of NaCl concentrations. At 200 mM NaCl, the length and fresh weight of hypocotyl and roots were greatly reduced, with a simultaneous increase in proline content, suggesting activation of mechanisms for coping with salt stress. In addition, hypocotyl and root samples from 100 mM NaCl treated seedlings up-regulated seven key proteins, such as late embryogenesis-abundant protein, b-conglycinin, elicitor peptide three precursor, and basic/helix-loop-helix protein. The same treatment caused down-regulation of protease inhibitor, lectin, and stem 31-kDa glycoprotein precursor. This combination of up- and down-regulated proteins indicates a metabolic shift and could represent a strategy used by soybean seedlings to enhance tolerance of, or adapt to, salt stress [113].

Sobhanian et al. [110,114] found that treatment of soybean seedlings with 80 mM NaCl arrests the growth and development of both hypocotyl and roots. This study assessed effects on leaf, hypocotyl and root proteomics of salt treated soybean seedlings and found that reduction of glyceraldehyde-3-phospahte dehydrogenase was indicative of reduction in ATP production, and down-regulation of calreticulin was associated with disruption in the calcium signalling pathway, both of which are associated with decreased plant growth. The levels of other proteins, such as kinesin motor protein, trypsin inhibitor, alcohol dehydrogenase and annexin, were also found to change, suggesting that these proteins might play different roles in soybean salt tolerance and adaptation [110,114].

Soybean cultivars Lee68 and N2899 are salt-tolerant and salt-sensitive, respectively. The percentage germination was not affected when exposed to 100 mmol L^{-1} NaCl, however, the mean germination time for Lee68 (0.3 days) and N2899 (1.0 day) was delayed, compared with control plants. Hormonal responses to salt stress differed between these cultivars. Both cultivars, increased abscisic acid levels and decreased giberrelic acid (GA 1, 3) and isopentyladenosine concentrations; auxin (IAA) increased in Lee68, but remained unchanged in N2899. 2-D gel electrophoresis, followed by MALDI-TOF-MS analysis, of the proteins from germinated seeds suggested increases in ferritin and the 20S proteasome subunit β-6 in both the cultivars. Glyceraldehyde 3-phosphate dehydrogenase, glutathione *S*-transferase (GST) 9, GST 10, and seed maturation protein PM36 were down-regulated in Lee68, but these proteins were naturally present in low concentrations in N2899 and were seen to up-regulate following exposure to salt stress [115].

2.2.5. Biotic stress

The soybean-*Phytophthora soje* plant-oomycete interaction is of agriculture and economic importance, as this oomycete causes soybean root and stem rot, translating to an annual global loss of $1-2 billion US. Twenty-six proteins were significantly affected in a resistant soybean cultivar (Yudou25) and 20 in a sensitive one (NG6255), as determined by 2-D gel analysis, followed by MALDI-TOF-MS. The distribution pattern of the affected proteins were - 26% energy regulation, 15% protein destination and storage, 11% defense against disease, 11% metabolism, 9% protein synthesis, 4% secondary metabolism, and 24% unknown/hypothetical proteins [116].

Soybean mosaic virus (SBMV) causes one of the most serious viral infections of soybean; leaves of infected plants were studied at a series of time points using 2-D gel electrophoresis, followed by MALDI-TOF-MS and tandem TOF/TOF-MS. Proteins expressed in the inoculated leaves were identified and were seen to be involved in protein degradation, defense signalling, coping with changes in the levels of reactive oxygen species, cell wall reinforcement, and energy and metabolism regulation. Quantitative real time PCR was used to focus on gene expression related to some of these proteins. Photosynthesis and metabolism related genes were down-regulated at all the time points, while most of the energy related genes (respiration in this case) were up-regulated for at least five of the six time points studied [117]. At the time of this writing, this report is the only one addressing the proteomic approach to molecular understanding of soybean-SBMV interaction.

2.2.6. Other miscellaneous stress related reports

Aluminium toxicity is often observed in acidic soils and Baxi 10 (BX10) is an Al-resistant cultivar. One-week-old soybean seedlings treated with 50 mM $AlCl_3$ for 24, 48 and 72 h were studied for characterization of root proteins in response to Al; and 2-D gel electrophoresis followed by MS revealed 39 proteins expressed differentially following Al treatment. Of these 21 were up-regulated (such as heat shock proteins, glutathione S-transferase, chalcone related synthetase, GTP-binding protein, ABC transporters and ATP binding proteins). Five proteins were also down-regulated and 15 newly induced proteins were present following AL treatment [118].

The process of nitrogen fixation demands large amounts of phosphorus [119]. When soybean plants are starved of phosphorus, 44 phosphate starvation proteins are expressed in soybean nodules [120].

Label free proteomics, coupled with multiple reaction monitoring (MRM) with synthetic isotope labelled peptides, was used to study 10 allergens from 20 non-genetically modified commercial varieties of soybean. The concentration of these allergens varied between 0.5-5.7 μg mg^{-1} of soybean protein. At the time of this writing, this is the only proteomic report on soybean allergens [121].

The responses of soybean plants exposed to 116 ppb O_3 involved significant changes to carbon metabolism, photosynthesis, amino acid, flavanoid and isoprenoid biosynthesis, signaling, homeostasis, anti-oxidant and redox pathways [122], as indicated by shifts in expression of the relevant proteins.

More information regarding soybean functional genomics and proteomics is available at the publicly accessible Soybean Knowledgebase (SoyKB) http://soykb.org/ [123].

3. *Bradyrhizobium japonicum* and its proteomics/exoproteomics

Culturing bacteria *in vitro* can cause changes in the bacterial physiology and genetics. In order to discriminate between types of these differences, *B. japonicum* cultivated in HM media and those isolated from root nodules were studied for their protein profile using 2-D PAGE and MALDI-TOF. The cultured cells showed greater levels of proteins related to fatty acid, nucleic acid and cell surface synthesis. While carbon metabolism proteins related to global protein synthesis, maturation and degradation and membrane transporters seemed to be similar in both cultured and nodule isolated bacteria, nitrogen metabolism was more pronounced in the bacteroids. Despite the quantitative differences in some proteins in the cultured and nodule isolated bacteria, it was observed that the various proteins in common between them performed similar functions [124]. A high resolution 2-D gel electrophoresis analysis of these bacteroids revealed a number of proteins, of which about 180 spots could be identified using the *B. japonicum* database (http://www.kazusa.or.jp/index.html) [125]. The bacteroids showed a lack of defined fatty acid and nuclei acid metabolic pathways, but were rich in proteins related to protein synthesis, scaffolding and degradation. Other proteins with high expression levels were associated with cellular detoxification, stress regulation and signalling, all of which clearly establishes that differentiation into bacteroids results in a clear shift on metabolism and expression of metabolic pathways required by the bacteroids for their specialized activities [126].

Since competitiveness plays an important role in this symbiotic relationship, 2-D gel electrophoresis, image and data analysis, and in-gel digestion proteomic studies, were conducted on *B. japonicum* 4534, a strain with high competitiveness, and *B. japonicum* 4222, with low competitiveness, for nodulation. When treated with diadzein, both the strains showed upregulation of proteins: 24 in *B. japonicum* 4534 and 10 in *B. japonicum* 4222. Upon treatment with diadzein and other extracellular materials such as extracellular enzymes and polysaccharides involved in nodulation of the strains tested, the numbers increased to 78 (43 up-regulated and 35 down-regulated) and 47 (25 up-regulated and 22 down-regulated) in these two strains. Proteins not related to nodulation were also present, and the higher number of proteins expressed by *B. japonicum* 4534 may be the reason for increased competitiveness during symbiosis [127]. Comparative studies on whole cell extracts of genistein induced and non-induced cultures of a strain used in commercial inoculants in Brazil, *B. japonicum* CPAC 15 (=SEMIA 5079), and of two genetically related strains grown *in vitro* were conducted using 2-D gel electrophoresis followed by mass spectrometry. Some of the noteworthy proteins belonged to the cytoplasmic flagellar component FliG, periplasmic ABC transporters, proteins related to the biosynthesis of exopolysaccharides (ExoN), proteins that maintain redox state and the regulon PhyR$^{-\sigma\mathrm{EcfG}}$, which is known to increase the competitiveness of *B. japonicum* and also help the bacteria under stress conditions, and several other hypothetical proteins [128].

B. japonicum utilizes the bacterial Type III secretion system (TTSS). In order for TTSS to be effective it requires a flavonoid inducer. The *tts* gene cluster of *B. japonicum* is regulated by the isoflavone genistein. In its presence NodD1 and NodW activate the *ttsI*, which is a two-component response regulator, necessary for expression of other genes in the *tts* cluster. In addition, the operons governing the TtsI regulon have a conserved motif in the *tts* box promotor region, which underscores the importance of regulation of TTSS in *B. japonicum*. Flagellin is a bulk protein synthesized by *B. japonicum* that plays an important role in TTSS. Mutant *B. japonicum* cells created by deleting the flagellin genes *bll6865* and *bll6866* were studied for their exoprotein profiles, in comparison with the non-mutated strains. Upon induction using genistein, it was observed that amongst the identifiable proteins, Blr1752 similar to NopP of *Rhizobium* sp. strain NGR234, Blr1656 (GunA2) having endoglucanase activity and three other proteins having similarity to proteins of the flagellar apparatus were detected. However, none of these proteins were detected in the mutant exoproteome, suggesting that these proteins are the products of a highly conserved *tts* box motif containing genes that encode these secreted proteins [129 and references therein].

A study of 2-D gel electrophoresis combined with MALDI-TOF MS for the identification of *B. japonicum* strains 110, BJDΔ283 and BJD567 exoproteomes revealed a high frequency of substrate-binding proteins of the ABC transporter family. Addition of genistein to the cultures altered the exoproteome; three flagellar proteins and a nodulation outer protein, Pgl, were identified. Further shotgun mass spectrometry of the genistein induced exoproteome revealed the presence of nodulation outer proteins, NopB, NopH, NopT and type III-secreted protein GunA2. Addition of diadzein or coumerstrol, instead of genistein, to the cell culture showed a reduction in the type III-secreted protein GunA2 [130]. *B. japonicum* cell lines derived from strain SEMIA 566 are adapted to stressful environmental conditions in Brazil. They also vary in their capacity for symbiotic nitrogen fixation. A representational difference analysis study was conducted on the strains S 370 and S 516, derived from SEMIA 566. Strain S 370 produces the nodulation outer protein P gene, which is strongly associated with the TTSS, and is also the major determinant of effective nodulation [131].

B. japonicum strain CPAC 15 (5SEMIA 5079) is a strain used in commercial inoculants; it belongs to the same serogroup as strain USDA 123 and is used in Brazil on soybean. Both of these strains are known to be highly competitive and saprophytic. Apart from *B. japonicum* strain USDA 110, which has been sequenced [24,25], CPAC 15 is the only stain that has been partially sequenced in any significant measure [132]. CPAC 15 and two related strains, S 370 and S 516, were studied using whole-cell 2-D protein gel electrophoresis and spot profiles of selected proteins using MS. Cytoplasmic and periplasmic proteins found to occur in diverse metabolic pathways related to the saprophytic properties of CPAC 15; 26 hypothetical proteins were identified [133].

B. japonicum strain USDA 110 from soybean plants cultivated in growth chambers were harvested at 21 days of symbiosis and subjected to transcriptomics studies and proteomics using gelLC-MS/MS. Through this integrated approach 27.8% of the theoretical proteome and 43% of the predicted genes and proteins were detected. Analysis of the biological and functional pathways highlighted proteins involved in carbon and nitrogen metabolism: several enzymes of the TCA cycle, gluconeogenesis and pentose phosphate pathway. Experiments with bacteroids obtained from soybean plants grown under field conditions showed identical results [134 and references therein].

4. Other dimensions to soybean-rhizobacteria interactions

Apart from *B. japonicum*, which produces LCOs, other rhizobacteria, such as *Bacillus thuringiensis* NEB17 reside in the rhizosphere of higher plants [135], forming a phyto-microbiome, much like the human microbiome, now realized to be so important in human health [136]. *Bacillus thuringiensis* NEB17 is symbiotic with *B. japonicum*, produce bacteriocins. *Bacillus* species were first reported to produce bacteriocins in 1976. The low-molecular-weight bacteriocins of gram-positive bacteria have bactericidal activity, mainly against certain other gram-positive bacteria [137]. Bacteriocins are ribosomally produced peptides which affect the growth of related bacterial species. The most studied bacteriocin is colicin, produced by members of the Enterobacteriaceae [138]. Due to their commercial importance as natural preservatives and as therapeutic agents against pathogenic bacteria, these antimicrobial peptides have been a major area of scientific research [137,139].

Bacteriocins are grouped into four distinct classes based on the peptide characteristics such as post translational modifications, side chains, heat stability, N-terminal sequence homology and molecular weight [140]. *Bacillus thuringiensis* NEB17 was isolated from soybean root nodules as putative endophytic bacteria in 1998 in our laboratory. When co-inoculated with *B. japonicum* under nitrogen free conditions this bacterium promoted soybean growth, nodulation and grain yield [141, 142]. Subsequently, the causative agent of plant growth promotion, a bacteriocin, was isolated from *B. thuringiensis* NEB17, and is now referred to as thuricin 17 [143]. Initially, its partial sequence was determined [144], and its full sequence has been more recently reported [145]. Thuricin 17 is a low molecular weight peptide of 3162 Da, stable across a pH range of 1.0–9.25, highly heat resistant and is inactivated by treatment with proteolytic enzymes. Based on its N-terminal sequence homology of thuricin 17 and that of the also newly isolated bacthuricin F4, a new class of bacteriocins, class IId was proposed [143]. The bacteriocins produced by *B. thuringiensis* strain NEB17 (Th17) and *B. thuringiensis* subsp. kurstaki BUPM4 (bacthuricin F4 - 3160.05 Da) have been reported to show functional similarities and anti-microbial activities [146]. In addition, thuricin 17, applied as leaf spray and root drench, has positive effects on soybean and corn growth, which was first reported from our laboratory [145]; this constituted the first report of plant growth stimulation by a bacteriocin.

Proteomic profiling of both these bacteria are underway in our laboratory and we hope to acquire some indications of plant proteomic shifts related to biological nitrogen fixation through these experiments over the next few months.

5. Conclusions and future perspectives

Soybean is an important protein and oil seed crop and BNF is an important source of nitrogen for the crop. Considerable work has been conducted regarding soybean proteomics, facilitated by recent advancements in technology, but a more systematic approach to this method is required in order to understand the intricacies of plant growth and development in the face of interactions with various symbionts. There is wide variation in the ability of *B. japonicum* strains to fix atmospheric nitrogen and screening of the various strains known to us, in the light of specific agro-climatic conditions, will help improve effective BNF at a very low cost. In this regard, the proteomic profile can be of immense help in highlighting the protein-protein interactions that are involved during the process of nodule initiation, formation and sustenance. This in-depth knowledge of the role of proteins in nodulation and plant growth promotion processes will assist in further improvement of soybean cultivars and their associated *B. japonicum* strains, for a better and more sustainable agriculture.

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Chapter 2

The Development and Regulation of Soybean Nodules

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Legumes: Environmentally and agronomically important plant species

Legumes represent the third largest family of angiosperms, with > 18,000 species worldwide (*Leguminosae* or *Fabaceae*). Due to their high nutritional value, they have been cultivated by many cultures for use in agriculture. Indeed, legumes currently represent some of the most important food, feed and fuel crops grown around the world, second only to cereals. They have a global-production value of > $200 billion per year and are cultivated on 12–15% of the world's available, arable land (Graham and Vance 2003; Peoples et al., 2009; Jensen et al., 2012). Collectively, ~247 million tons of legumes are produced each year, and represent > 25% of the world's primary crop production (Graham and Vance 2003; European Association for Grain Legume Research 2009). Some key legume crop species include: soybean, bean, pea, chickpea, cowpea, pigionpea and alfalfa.

Legumes play a significant role in both agriculture and in natural ecosystems by having a unique influence on the nitrogen cycle. They do so via a highly-specialized symbiotic relationship with soil bacteria commonly called rhizobia. Via a sophisticated signaling exchange, the bacteria infect the plant root and induce the formation of novel organs, termed nodules (Ferguson et al., 2010). The nodule is colonized by the bacteria and provides them with an ideal habitat to convert or 'fix' atmospheric di-nitrogen into other nitrogen-based compounds, such as ammonia, which can be used by the plant. This benefits the plant directly, as nitrogen is essential for growth. It also benefits the rhizosphere as the soil nitrogen content is replenished once the plant dies and subsequently decomposes (referred to as 'green manure'). As a result, legumes are often used in crop rotations in agriculture. In addition, some legumes are being exploited as a source of sustainable biofuel. Species such as soybean, and the tree legume *Pongamia pinnata*, offer the most promise due to their high seed oil content (Scott et al., 2008).

2. Nitrogen fertilizer: An unsustainable reliance in modern agriculture

Current agriculture practices are heavily reliant on nitrogen-based fertilizers to achieve high yields (Peoples *et al.,* 2009; Jensen et al., 2012). Indeed, it is estimated that half of the world's population is fed as a direct result of nitrogen fertilizer use (Erisman et al., 2008). Although this approach works well to maximize crop production, it is a very risky dependence that is not sustainable, and is inefficient, expensive, and often pollutes. Hence, there are a number of very sound reasons to lessen these nitrogen inputs and alleviate the current reliance.

The production of industrial nitrogen fertilizers requires a great deal of fossil fuel, involving what is known as the Haber-Bosch process. Once manufactured, additional fossil fuel is needed to transport the fertilizer to its place of sale, and then even more fossil fuel is needed to apply it in the field. Collectively, this accounts for a staggering 50% of fossil fuel use in modern agriculture and 5% of the world's annual consumption of natural gas (Crutzen et al., 2007; Canfield et al., 2010). As a direct result, the purchase and application of nitrogen fertilizer can be quite expensive, preventing many farmers in developing regions of the world from being able to use it. Moreover, as the cost of fossil fuel continues to rise, so too does the cost of nitrogen fertilizer. This can considerably cut into farmer profits and often leads to costs being passed on to the consumer.

In addition to the monetary costs associated with nitrogen fertilizer use, there are often considerable environmental costs. It is reported that industrial manipulation of the nitrogen cycle has already exceeded safe boundaries for global environmental change (Rockstrøm et al., 2009; Canfield et al., 2010; Charpentier and Oldroyd 2010; Beatty and Good 2011). Due to the combustion of fossil fuel, the production and application of nitrogen fertilizer results in large quantities of carbon dioxide (CO_2) being released into the atmosphere. This contributes to the greenhouse effect, as does the release of nitrous oxide (N_2O), which is produced during the decomposition of nitrogen fertilizer in the soil and is estimated to be 292 times more active as a greenhouse gas than CO_2 (Crutzen et al., 2007).

Each year >100 million tonnes of nitrogen are applied to crops worldwide (Glass 2003). This excessive use of nitrogen reduces the levels of trace nutrients in the soil and increases soil acidity. Moreover, the application of nitrogen-based fertilizers is a largely inefficient process, with as much as 30 - 50% lost to leaching. This nitrogen run-off can cause significant environmental damage, including the eutrophication of waterways resulting from associated algal blooms, *etc*. (Vance 2001). Recent reports indicate that the cost to fix this environmental damage will significantly outweigh the economic benefit gained from nitrogen fertilizer use (Sutton et al., 2011).

The excess runoff from nitrogen fertilizer can also contaminate drinking water. High levels of nitrogen in drinking water can lead to methemoglobinaemia, commonly referred to as

Blue-baby syndrome because of the blue-grey skin colour displayed by affected infants (Murphy 1991; Knobeloch et al., 2000). Blue-baby syndrome is potentially fatal and occurs when the hemoglobin of an infant's red blood cells is oxidized to methemoglobin, which is unable to properly transport oxygen.

3. Biological nitrogen fixation: A sound alternative to nitrogen fertilizer

Attempts to reduce the use of nitrogen fertilizers need to incorporate responsible, cost effective and environmentally sound options; an improved use of legumes and an increased understanding of legume nodulation represent such options. Indeed, this symbiotic relationship between legumes and rhizobia represents the most important nitrogen-fixation association in the world, with an annual production of approximately 200 million tons of nitrogen (Peoples et al., 2009; Jensen *et al.,* 2012). Optimising this symbiosis can increase crop yields and enhance soil fertility, whilst reducing the negative monetary costs and environmental impacts associated with nitrogen fertilizer use (Hirel et al., 2007; Peoples et al., 2009; Canfield et al., 2010). Hence, increasing our use of legume crops and identifying critical factors required to control nodulation are seen as pivotal steps towards reducing our reliance on nitrogen fertilizers and improving agricultural sustainability (*e. g.* , Giller and Cadisch 1995; Vance 2001; Peoples et al., 2009; Rockstrøm *et al.,* 2009; Canfield et al., 2010; Jensen et al., 2012).

Soybean alone is estimated to produce up to 200 kg N ha^{-1} in aboveground biomass in a single growing season. Of the soybean nitrogen content, 58-68% is estimated to be derived from symbiotic nitrogen fixation (Salvagiotti et al., 2008; Peoples et al., 2009; Jensen et al., 2012). Following harvesting, the remaining portions of the plant, including roots and nodules which represent 30-60% of the nitrogen content, are left to replenish the nitrogen content of the surrounding soil (Mahieu et al., 2007; McNeill and Fillery 2008).

4. Legume nodules: The perfect environment for rhizobia nitrogen fixation

Nodulation is a complex process orchestrated by a multitude of bacteria and plant signals (reviewed in Ferguson and Mathesius 2003; Ferguson et al., 2010). The process is initiated by plant roots secreting flavonoid molecules into the soil. This attracts compatible rhizobia and concomitantly stimulates them to synthesize a highly specific signal molecule called Nod factor. The rhizobia strain that is compatible with soybean is *Bradyrhizobium japonicum*. The plant perceives Nod factor via LysM receptors on the root. In soybean, these receptors are called GmNFR1 and GmNFR5 (Indrasumunar et al., 2010, 2011). Nod factor perception triggers a subsequent signaling cascade that is required for proper nodule establishment. The known legume and rhizobia genes/signals that are involved in this signaling cascade have recently been thoroughly reviewed in Ferguson (2012).

The presence of the rhizobia together with their Nod factor signal molecule initiates the nodulation infection process. Root hair penetration is the most common form of rhizobia invasion. The bacteria attach to emerging root hairs, which begin to deform and eventually encapsulate some of the bacteria, which are continuously dividing (Callaham and Torrey 1981; Turgeon and Bauer 1985). This process happens in as little as 6 – 8 h post-inoculation (Yao and Vincent 1969; Bhuvaneswari et al., 1981; Bhuvaneswari and Solheim 1985; Turgeon and Bauer 1982, 1985). Specialized structures, called infection threads, begin to form and provide a passage way for the bacteria to enter the root (reviewed by Gage 2004). These infection threads are predominately comprised of plant cell wall components and they permit the bacteria to continue proliferating within the host plant.

As the process of rhizobia infection occurs, cortical cells in the root begin to divide and eventually give rise to the nodule primordium (Calvert et al., 1984; Mathews et al., 1989). The position of the nodule primordium is typically adjacent to the radial cells of the xylem, and away from the phloem. This positioning is thought to be largely dependent on plant hormone levels, namely gradients of the gaseous hormone, ethylene (Heidstra et al., 1997; Gresshoff et al., 2009; Lohar *et al.,* 2009). Additional tissues, including vascular tissues and central nodule tissues that are composed of both invaded and non-invaded cells, also develop to form the nodule structure (Newcomb et al., 1979; Calvert et al., 1984; Ferguson and Reid 2005).

Infection threads initiating in the root hair eventually grow and extend towards the dividing nodule primordium located in the root cortex. Once there, rhizobia located at the tip of the infection threads are released into an infection droplet that separates and is released into the cytoplasm of the host cell. Within the cytoplasm, the rhizobia are encapsulated by a specialized plant-derived membrane, known as the peribacteroid membrane, making what is commonly referred to as the symbiosome (Udvardi and Day 1997).

Ultimately, the dividing bacteria differentiate into what are known as bacteroids, which are highly specialized and whose main purpose is to fix atmospheric di-nitrogen gas. Inside the mature nodule, the bacteroids use a nitrogenase enzyme complex to fix the di-nitrogen into forms of nitrogen that the plant can use, such as ammonia. The ammonia, which is toxic to the plant, is then quickly converted into compounds such as glutamate or ureides that are non-toxic and are safely transported throughout the plant. Legume nodules provide the ideal setting for this process as they establish a peripheral oxygen barrier, via physical and metabolic barriers, to create a low-oxygen environment that is essential for nitrogenase activity to occur.

The nodules formed on the roots of soybean plants are referred to as ‘determinate’ nodules. They are spherical and lack a persistent meristem, unlike indeterminate nodule structures that can form on other legume species, particularly those from temperate growing regions (Ferguson et al., 2010). The life-span of a soybean nodule is typically a few weeks, after which they senesce and are replaced by new nodule structures developing on the growing root system. Following nodule senescence, the bacteroids can re-differentiate and become new inoculum for the soil (Gresshoff and Rolfe 1978).

5. Autoregulation of nodulation: Too much of a good thing can be bad

A number of genes that are required for proper nodule formation have been elucidated (reviewed in Caetano-Anollés and Gresshoff 1991; Ferguson et al., 2010; Ferguson, 2012). The loss of any of these genes typically results in a reduced, or a complete lack of, nodule development. In addition to these positive regulators of nodule formation, there are also a number of external and internal factors that act as negative regulators of nodulation. Mutants unable to synthesise or perceive these factors exhibit increased nodule numbers, often referred to as a hyper- or super-nodulation phenotype (Figure 1). Many of these factors function in the Autoregulation of Nodulation (AON) pathway, which is a mechanism used by the host plant to regulate its nodule numbers (reviewed in Reid et al., 2011a). Indeed, less than 10% of all rhizobia infection events result in the establishment of a fully functional nodule, largely due to AON. By controlling nodule development in this way, the host plant can balance its need to acquire nitrogen against its ability to expend energy establishing and maintaining nodules. Supernodulating plants lacking AON are typically developmentally-stunted (when inoculated with a compatible rhizobia strain) and yield poorly as a result of this balance being disrupted (Figure 2).

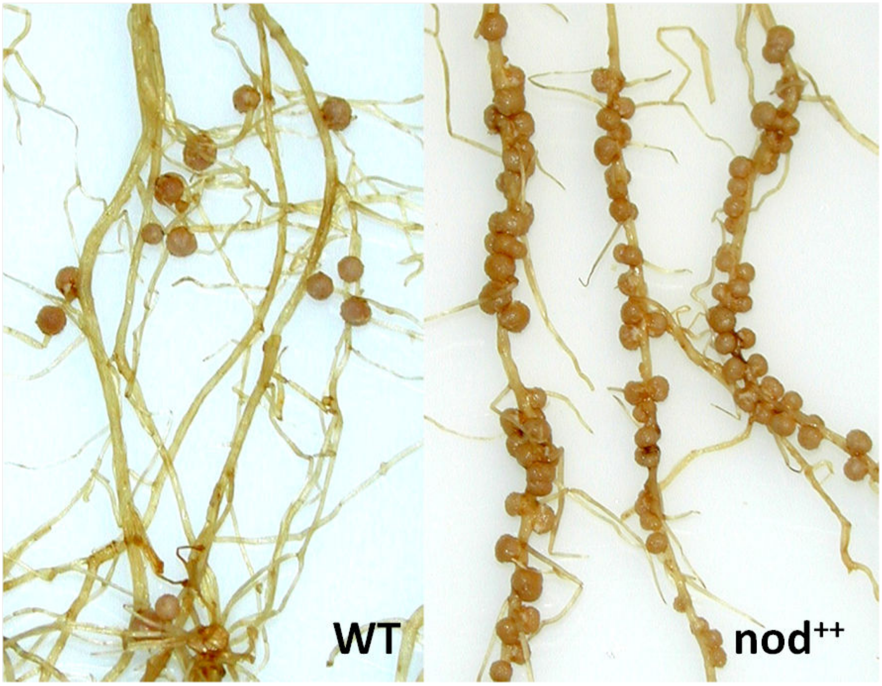

Figure 1. Roots of wild-type (WT) and supernodulating mutant (nod^{++}) soybean plants exhibiting mature nodule structures as a result of a symbiotic interaction with *Bradyrhizobium japonicum*.

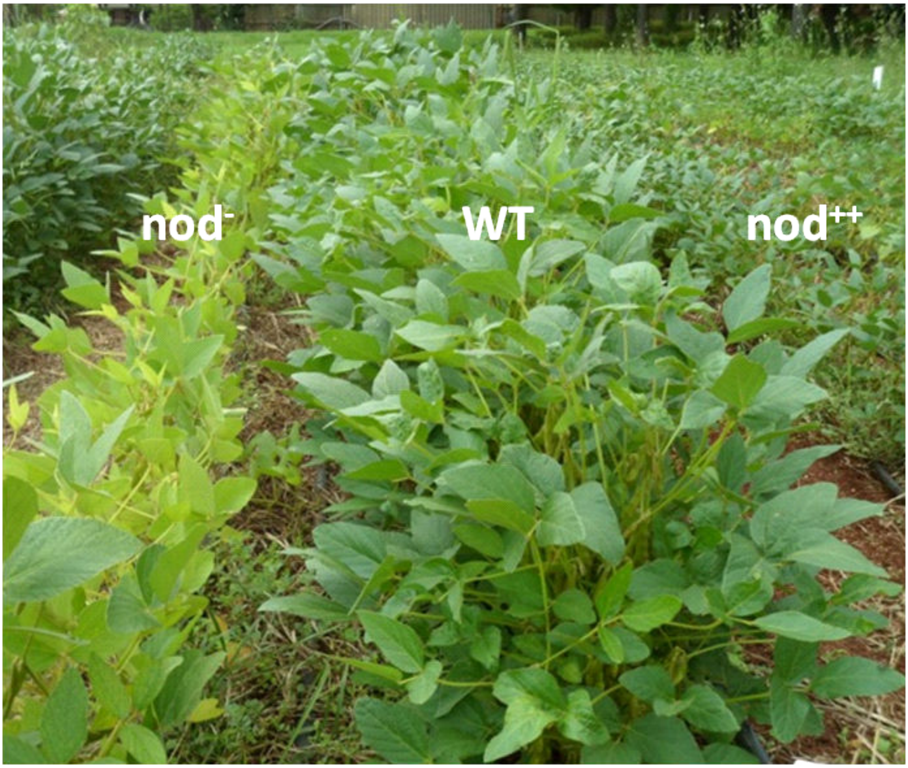

Figure 2. Soybean plants growing in a field in Toowoomba, Queensland, Australia. Mutants unable to form nodules (nod^{-}) are stunted and pale compared with wild-type (WT) plants due to their inability to establish a symbiotic interaction with nitrogen-fixing *Bradyrhizobium japonicum*. Supernodulating mutants (nod^{++}) are significantly stunted in stature as a result of investing too much energy into forming nodule structures.

The AON pathway involves long-distance root-shoot signaling initiated during nodule development by the synthesis of a root-derived signal (Gresshoff and Delves, 1986; Delves et al, 1986; Reid et al., 2011a). Recent work has indicated that this signal is likely a CLV3/ESR-related (CLE) peptide(s) hormone (Okamoto et al., 2009; Mortier et al., 2010; Reid et al., 2011b; Lim et al., 2011). In soybean, these CLE peptides are called Rhizobia Induced CLE1 (RIC1) and RIC2 (Reid et al 2011b; Lim et al., 2011). Grafting and over-expression experiments have shown that these signals travel to the shoot (Delves et al., 1986; Reid et al., 2011b), likely via the xylem, where they, or a product of their action, are perceived by a LRR receptor kinase, called the Nodulation Autoregulation Receptor Kinase (NARK) in soybean (*e. g.* , Searle et al., 2003). NARK may act in a complex with other receptors, such as CLAVATA2 and KLAVIER (Miyazawa et al., 2010; Krusell et al., 2011). This perception results in the production of a novel Shoot-Derived Inhibitor (SDI). The SDI signal subsequently travels from the shoot back down to the roots, likely via the phloem, where it acts to inhibit further nodulation events (Reid et al 2011a). It has recently been established in soybean that SDI is