INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

MINERAL NITROGEN INHIBITION AND SIGNAL PRODUCTION IN SOYBEAN - *B. JAPONICUM* SYMBIOSIS

By

Pan, Bo

A thesis submitted to the Faculty of Graduate Studied and Research
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Department of Plant Science
Macdonald Campus of McGill University,
Montreal, Quebec

August, 1999

Pan, Bo

Short title:

Isoflavonoids and nitrogen inhibition in soybean-*B. japonicum* symbiosis

ABSTRACT

Bo Pan

In the N_2 fixing legume symbiosis, mineral nitrogen (N) not only decreases N_2 fixation, but also delays and inhibits the formation and development of nodules. The purposes of this thesis were to elucidate the role of signaling in the mineral N effects on nodulation and nitrogen fixation in soybean [*Glycine max* (L.) Merr.] and to attempt to find ways to overcome this inhibition. The responses of soybean plants, in terms of daidzein and genistein synthesis and exudation, to different mineral N levels were studied. Daidzein and genistein distribution patterns varied with plant organs, mineral N levels, and plant development stages. Mineral N inhibited daidzein and genistein contents and concentrations in soybean root and shoot extracts, but did not affect root daidzein and genistein excretion in the same way. In both synthesis and excretion, daidzein and genistein were not affected equally by mineral N treatments. Variability existed among soybean cultivars in the responses of root daidzein and genistein contents and concentrations to mineral N levels. The amount of daidzein and genistein excreted by soybean roots did not always correspond to the daidzein and genistein contents and concentrations inside the roots. On the *Bradyrhizobium japonicum* side, *nod* gene expression was inhibited by mineral nitrogen. Genistein was used to pre-incubate *B. japonicum* cells or was applied directly into the plant growing medium. The results showed that genistein manipulation increased nodule weight and nodule nitrogen fixation under greenhouse conditions, but interactions existed among soybean cultivars, genistein concentrations and nitrate levels. Similar results were found under field conditions. Soybean yield was increased on sandy-loam soil by preincubation of *B. japonicum* cells with genistein. Addition of genistein beginning at the onset of nitrogen fixation also improved soybean nodulation and nitrogen fixation. Soybean cultivars had different sensitivities to genistein addition. Addition of genistein partially overcame the inhibitory effect of mineral N on *nod* gene expression.

Other studies also show that temperature affected genistein and daidzein content and concentration in soybean roots. The effect of temperature varied among soybean cultivars.

Some PGPR strains can mitigate the negative effects of nitrate on soybean nodulation and nitrogen fixation, however, this is influenced by soybean genotype. Applying PGPR together with genistein preincubation of *B. japonicum* cells improved soybean nodualtion and increased yield. The level of improvement varied among soybean cultivars and PGPR strains. Preincubation of *B. japonicum* cells with genistein improved strain competitiveness under greenhouse, but not field conditions.

Overall, these findings suggested that both plant-to-*Bradyrhizobium* and *Bradyrhizobium*-to-plant signals play important roles in the effects of mineral N on nodulation and N fixation. Signal manipulation could partially overcome the inhibitory effects of mineral N on soybean-*B. japonicum* N fixation symbiosis.

RÉSUMÉ

Bo Pan

Chezles plantes fixatrices d'azote (N), l' N minéral inhibe la formation et le développement des nodules. Le but de cette thèse était d'élucider le rôle deomolecules signales das la repression de la nodulation tede la fixation por le N, chezle soja [*Glycine max* (L.) Merr.] et de trouver un moyen pour vaincre cette inhibition. On a étudié la synthèse et l'exsudation de la daidzeine et de la génisteine parle soja en réponse à différents niveaux d'N. La distribution de la daidzeine et de la génisteine dépendait du stade de développement et de l'organe végétal et du niveaux d'N. L'N minéral réduit la concentration de la daidzeine et de la génisteine dans les extraits de tiges et de racines mais n'a pas affecté l'excrétion de ces deux produits de la même façon. Les différents niveaux d'N n'ont pas affecté la synthèse et l'excrétion de la daidzeine et de la génisteine de la même façon. Les concentrations de la daidzeine et de la génisteine dans les racines des différents cultivars de soja en réponse à l'N étaient variables. La quantité de daidzeine et de génisteine excrétée par les racines ne correspondait pas toujours à la quantité synthetisée dans les racines. L'N a inhibé l'expression du gène *nod* de *Bradyrhizobium japonicum.* La génisteine a été utilisée pour la pré-incubation de *B. japonicum* ou en application directe dans le milieu de croissance des plantes. Dans un environnement contrôlé, la manipulation de la génisteine a causé une augmentation de la masse des nodules et de la fixation azotée, mais il y avait une interaction entre les concentrations de génisteine, les cultivars de soja et l'N. Des résultats similaires ont été trouvé aux champs. La pré-incubation de *B. japonicum* avec de la génisteine, a augmenté le rendement du soja sur un loam sableux. L'addition de la génisteine en début de fixation a amélioré la nodulation et la fixation d' N par le soja. L'addition de la génisteine a partiellement surmonté l'effet inhibiteur de l'N sur l'expression du gène *nod.*

D'autres études montrent que la température affecte la concentration de la daidzeine et la génisteine dans les racines du soja. L'effet de la température était différent selon les cultivars. Quelques souches de rhizobactéries qui favorisent la croissance des plantes (RFCP) ont pu atténuer les effets négatifs du nitrate sur la nodulation et la fixation du soja. Cependant ceci fut influencé par le gêno type de soja.

L'application de RFCp au moment de la pré-incubation de *B. japonicum* avec la génisteine a amélioré la nodulation du soja et a augmenté le rendement. L'amélioration a varié avec le cultivar de soja et la souche de RFCP utilisés. La pré-incubation de *B. japonicum* avec la génisteine a amélioré la compétitivité des souches dans les serres mais non aux champs.

En somme, ces résultats suggèrent que le signal de la plante à *B. japonicum* et de *B. japonicum* à la plante joue un rôle important dans l'effet de l'N sur la nodulation et la fixation de l'azote. La manipulation du signal pourrait partiellement surmonter l'effet inhibiteur de l'N sur la symbiose soja-*B. japonicum*.

ACKNOWLEDGMENTS

Many people helped me during my studies. Their names are too numerous to mention here, but my thanks go out to each and every one of them. They know who they are. I want to single out a few people for special thanks.

I would like to express my sincere appreciation to my supervisor, Dr. Donald L. Smith, for his support and encouragement throughout my study. His conscientious review of each version of every manuscript is appreciated.

I must also express my gratitude to Dr. T Charles, Dr. B Driscoll, Dr. S. Sparace, Dr. T. Paulitz for their valuable suggestions or permitting me to use their laboratory facilities whenever needed.

I am thankful for the expert technical support of Dr F. Zhang and Mr. S. Leibovitch, their help made my project easier to pursue. Many thanks are also addressed to Caroline, Roslyn and Louise, for their guidance and always being available to answer my questions.

My appreciation goes to my friends and colleagues Yuming, Xiaomin, Yinghai, Hao, Liqun, Chunquan, Xiben, Carlos, Mario, Sultan, Ali, Muhammad, Casper for their help and the memorable times. I also want to express my appreciation to Micheline for translating the abstract to French.

I would like to dedicate this thesis to my wife Baoling and our son Zhendan. They played a major role in encouraging me and pushing me to attaining my goal when times were tough. Finally, I cannot forget my family in China and my colleagues and teachers in Laiyang Agricultural University and Beijing Agricultural University.

TABLE OF CONTENTS

LIST OF FIGURES

Figure

LIST OF TABLES

Table

CONTRIBUTIONS OF CO-AUTHORS TO MANUSCRIPTS FOR PUBLICATION

This thesis has been written in the form of manuscripts to be submitted to scientific journals. This format has been approved by the Faculty of Graduate Studies and Research as outlined in the "Guidelines Concerning Thesis Preparation", B. 2 **Manuscripts and Authorship**, which states:

Candidates have the option, subject to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that thesis copies are bound as an integral part of the thesis.

If this option is chosen, **connecting texts, providing logical bridges between the different papers, are mandatory.**

The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. **The thesis must include, as separate chapters or sections:** (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary.

Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (e. g. in appendices) to show a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent;** supervisors must attest to the accuracy of such claims at the Ph. D. Oral Defense. Since the task of the examiners is made more difficult in thesis cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers.

Thus, the contents of sections 3 to 12 inclusively are drawn, respectively, from the manuscripts for publication. The manuscripts from which sections 3, and 5 to 12 are taken were co-authored by D. L. Smith and myself. Dr. D. L. Smith, my supervisor at the Macdonald Campus of McGill University, provided funds, assistance and supervisory guidance from the outset of the research to the reviewing of the manuscripts before submission for publication. He was also responsible for arranging laboratory, growth bench, experimental field space, the use of computers, and providing technical assistance.

The manuscript from which section 4 is drawn was co-authored by Dr. Zhang F. Dr. D.L. Smith and myself. Dr. Zhang, a former student in Dr. Smith's laboratory, helped me to design this experiment. The contribution of Dr. Smith to this manuscript was as described above.

Section 1. GENERAL INTRODUCTION

1.1 Introduction

Nitrogen (N) is an essential element for the growth and development of all organisms. However, most life forms are unable to use N_2 directly. The forms of N usable by plants are present in limited supply in the soil and are easily lost by leaching, denitrification and volatilisation. Because crops generally require relatively large amounts of N for growth, it frequently becomes the most limiting soil nutrient. N fertilizer ranks first among the external inputs required to maximize outputs from agriculture. It is also one of the most important factors in fostering the human population expansion of the 20th century (Smil, 1997). As a result of massive introduction of N fertilizer, many deleterious environmental consequences have been created. Problems range from local health to global changes, from deep underground to high in the stratosphere.

Soybean is one of the most important oilseed crops and high protein feed supplements for humans and livestock in the world. Soybean production has dominated world oilseed production during this century. Soybean production in Quebec has risen sharply, from less than 3,000 ha in 1986 to about 120,000 ha in 1998.

As a nodulting legume, soybean [*Glycine max* (L.) Merr.] forms an N_2 fixing symbiosis with *Bradyrhizobium japonicum* and *Sinorhizobium fredii*. Soybean N fixation can provide the plant with a source of N additional to that in soil, and reduce the farmer's dependence on fertilizer N. Soybean has been a valuable rotation and intercropping crop and has contributed enormously to the sustaining of agriculture since ancient times. The N fixation ability, nutrient value and environmental adaptation ensure that soybean will continue to be an important world food crop in an era of increasing food demand and concerns for the sustainability of agricultural production systems (Keyser and Li, 1992). With limited land on which to expand and the emphasis on sustainable systems, increases in soybean production will come mostly from increases in yield per unit area. Improvements in biological N fixation can help achieve this goal.

Legumes, including soybean, frequently fail to achieve maximum N_2 fixation under field conditions (Atkins, 1994). Any one of many factors, which simply limit plant

growth, could be responsible for yield depression (Lie, 1974). There are also a number of limitations intrinsic to legumes, which are imposed by the nature of the symbiosis itself (Quispel, 1974). Mineral N is a unique factor in that it promotes plant growth, but inhibits legume nodulation and N fixation in N-rich soils or following application of N fertilizer.

Mineral N affects every stage of symbiotic development and both partners in the symbiosis (for reviews see Streeter, 1988; Carroll and Mathews, 1990). There is, as yet, no convincing evidence to explain the mechanism of these effects, although several explanations have been postulated. Signal production and exchanges of rhizobia (nodulation factors) and host plants (*nod* gene inducers, such as isoflavonoids) have been studied in recent year (Promé and Demont, 1996; Verma, 1992). The intention of this study was to investigate the effects of mineral N on signal production of host plants and rhizobia. It is hoped that such an investigation will contribute to the understanding of the mechanisms of mineral N inhibition of nodulation and N_2 fixation, help to overcome the inhibitory effect of mineral N, increase N accumulation, and reduce the reliance of both soybean and the following crops on expensive and potentially polluting N fertilizer.

1.2 Hypotheses

General hypothesis:

B. japonicum and soybean signal production and exchange control nodulation (nodule formation process including root hair curling, infection thread formation and nodule initiation) and nitrogen fixation (function of nodule, that is nitrogenase activity), such that environmental factors, which affect nodulation and nitrogen fixation, interrupt signal production and exchange. Thus, nodulation and nitrogen fixation can be improved through signal manipulation.

Specific hypotheses:

1. Mineral nitrogen affects the synthesis and excretion of isoflavonoids by soybean plants.
2. Mineral nitrogen affects *B. japonicum nod* gene expression and Nod factor production.
3. Variation exists among soybean cultivars for flavonoid synthesis and exudation.

4. Manipulation of signalling, by addition of genistein, can partially overcome the inhibitory effects of mineral nitrogen on nodulation and nitrogen fixation.
5. Plant growth promoting rhizobacteria can improve soybean nodulation.
6. *B. japonicum* strain competitiveness is related to signal production and exchange.

Based on the hypotheses, the following objectives were formulated:

1.3 Objectives

General objective:

To determine the effects of environmental factors (mainly mineral N) on the signal production and exchange during the establishment of the soybean N_2 fixing symbiosis.

Specific objectives:

1. To determine temperature effects on daidzein and genistein synthesis in three soybean cultivars.
2. To determine the effect of genistein addition to the rooting medium at the onset of nitrogen fixation on nodulation and N fixation.
3. To determine the level of variation among soybean cultivars for daidzein and genistein synthesis and exudation at different nitrate levels.
4. To determine the degree of correlation of daidzein and genistein production by soybean roots with soybean nodulation.
5. To determine the effects of mineral nitrogen levels on genistein and daidzein concentrations and contents in soybean tissues and exudates.
6. To determine the relationships of daidzein and genistein concentrations in various plant parts.
7. To determine the effects of root growth environment on daidzein and genistein synthesis and exudation.
8. To determine the effect of incubating *B. japonicum* cells with genistein on strain competition.
9. To determine PGPR effects on mineral N inhibition of soybean nodulation and N fixation.
10. To determine the combined effects of PGPR and genistein on nodulation and N fixation.

Section 2

GENERAL LITERATURE REVIEW

2.1 Soybean

Soybean [*Glycine max* (L.) Merr.] is one of the most important oilseed crops and protein sources for humans and livestock in the world. It has been a valuable rotation and intercropping crop, usually with corn (Johntson et al., 1975). Soybean production has dominated world oilseed production during this century.

Soybean is a subtropical legume. The symbiotic association of *B. japonicum* with roots of soybean provides an N input system to the plant. Alternatively, nitrate is readily taken up from the soil by soybean roots and provides a second N input system. Both symbiotic N_2 fixation and NO_3^- utilization may be essential for maximum yield (Harper, 1974).

The cultivated soybean is diploid and is part of the genus *Glycine* Wild that belongs to the subfamily Papilionoideae of the family Leguminosae (Hymowitz and Newell, 1981). The root system of soybean is diffuse and consists of a taproot, which usually cannot be distinguished from other roots of similar diameter, with a large number of secondary roots that in turn support roots of higher order. In addition, multi-branched adventitious roots emerge from the lower portion of the hypocotyl. At maturity the root system may be extensively nodulated.

The soybean-*Bradyrhizobium* symbiosis can fix about 300kg N ha^{-1} under good conditions (Keyser and Li, 1992). Nitrogen fixation generally reaches a peak at early pod-fill and declines during late reproductive phases (Imsande, 1989). The plant moblizes a large quantity of N from vegetative tissue to meet the demand for seed N, whereas the net rate of NO_3^- uptake gradually declines throughout pod fill. Therefore nodulation on lateral and deep roots may be essential for maximum N_2 fixation, in order to match the high N demand during pod fill (Zapata et al., 1987).

Research on the bacterial partner, which is more easily manipulated by genetic methods, but difficult to handle in ecological terms, has shown that most processes in nodulation are governed by overriding plant control systems. These deal with the initial

blueprint for the construction of the nodule, the strong plant defence systems that control bacterial occupancy, the plant's control of carbohydrate as well as oxygen supply to the encased bacteroid, and finally the plant control of nodule senescence (Gresshoff, 1993). Early cross-inoculation studies showed that the plant genome and not the bacterium control nodule structure. The entire developmental pathway needed for nodule formation is plant encoded and requires the presence of a particular plant genetic background (Gresshoff, 1993). The number of nodules per plant is genetically limited (Fujita et al., 1991).

2.2 *(Brady)Rhizobium*

The ability to reduce N_2 is limited to prokaryotes. Legumes and a few other plant species have the ability to fix atmospheric N_2 through symbiotic relationships with *Rhizobium* (Sprent and Sprent, 1990). The bacterial component can live in soil but does not fix N_2 without the host (except at low pO_2 under laboratory conditions).

Rhizobia (i.e., nodule-forming bacteria) are a heterogeneous group, which contain at least four distinct genera: *Rhizobium, Bradyrhizobium, Sinorhizobium* and *Azorhizobium* (Young, 1996). The slow-growing nodulation bacteria, which have specific associations with soybean, are referred to as *Bradyrhizobium.* Some soybean varieties can also nodulate with the fast growing *Sinorhizobium fredii.*

Bacterial cells exhibit various types of surfaces. Often they are surrounded by capsules and/or layers of slime, chiefly composed of acidic and neutral polysaccharides (or sometimes acidic polypeptides). Internal to such layers is the outer most permanent surfaces of the bacterial cell, the outer membrane of Gram-negative bacteria. The membrane has a lipid bilayer, whose outer leaflet is composed mainly of abundant lipopolysaccharides (LPSs). The outer membrane also contains abundant integral proteins, but the polysaccharide portion of the LPS molecules restricts access to these proteins.

Ecological research into the root-nodule bacteria has lagged behind genetic and physiological work (Barnet, 1991). It requires rapid and reliable methods for strain

identification. It is still not possible to define which aspects of the physical environment are important in controlling the distribution and growth of *(Brady)Rhizobium*. In recent years, a major development was a revival of *Rhizobium* microbiology, recognizing the rhizosphere as a determining factor for efficiency of symbiosis and the bacterium as a living soil organism rather as a Nod factory (IS-MPMI meeting report, 1997). There are marked differences between strains in both the maximum temperature at which they can survive and the range over which they will grow (Hartel and Alexander, 1984)

At this time, we know little about the distribution of the root-nodule bacteria at either the global or microsite level, but the importance of such data is being recognized. The distribution of host legumes is another factor controlling the distribution of *(Brady)Rhizobium* in soil populations. There is abundant evidence that the availability of the specific host is not necessary for a strain's continued presence in the short term (Brunel et al., 1988), but is required to maintain them in the longer term. There is substantial variation within local populations of nodule bacteria (Broughton et al., 1987), supporting the concept that each site provides a range of niches which can maintain a diverse population. The rhizoplane population and the proportion of nodules formed by introduced strains has been shown to decline with distance from the site of inoculation (McDermott and Graham, 1989) possibly indicating that their movement is minimal. Approximately 90 percent of cells of indigenous *B. japonicum* in an agricultural field soil were attached to soil particles (Ozawa and Yamaguchi, 1986), although the remaining ten percent of free cells may well be sufficient for continued migration. The effects of lowered soil water are also likely to be important and the most likely explanation is that, as soil water levels decrease, thinning of water films, emptying of soil pores and reduction in the extent of continuous water-filled pathways will reduce bacterial movement and nutrient accessibility.

Our knowledge of the ecology of the nodule bacteria is still insufficient for it to be of practical use in the establishment of inoculant strains. Current research has only emphasized the complexity of the problem.

2.3 Nitrogen (N) Fixation

2.3.1 N and N_2 fixation

N, a necessary element in the composition of proteins, nucleic acids and other major cellular components, is an essential molecular building block for the growth and all organisms. However, most life forms, including all plants and animals, are unable to use N_2 directly. The usable forms of N by plants (most notable, nitrate and ammonia) are present in limited supply in the soil and are easily lost by both leaching and by denitrification. Because crops generally require relatively large amounts of N for growth, it frequently becomes the limiting soil nutrient. N fertilizer has been one of the most important factors fostering the human population expansion of the 20^{th} century (Smil, 1997). N fertilizer ranks first among the external inputs required to maximize outputs in agriculture and as a result contributes substantially to environmental pollution (Bohlool et al., 1992).

Symbiotic nitrogen fixation is defined as the reduction of atmospheric N_2 to ammonia through the symbiosis of host plants and nitrogen fixing bacteria. Biological nitrogen fixation is estimated to convert 175×10^6 t of N from N_2 to ammonia every year, whereas global industrial processes fix about 60×10^6 t.

2.3.2 Nodule Formation

The development of nitrogen-fixing nodules has been characterized as occurring in a series of stages, each of which may be influenced by one or more genes in each symbiotic partner. These stages are listed as follows*:

1. Root (rhizoplane) chemotaxis/colonization
2. Multiplication and colonization of the root surface
3. Marked root hair curling
4. Infection thread formation
5. Nodule initiation (meristem formation, differentiation)
6. Bacterial release into plant cells
7. Bacteroid development and multiplication

8. Nitrogen fixation (nitrogenase activity)

9. Nodule persistence, senescence

These stages can be roughly divided into three phases: recognition and infection (invasion), nodule formation, and N_2 fixation.

2.3.2.1 Signal Exchange and Recognition

Recognition is the initial step in a multi-step sequence leading to formation of root nodules. The recognition process involves a specific or selective response between bacterial cells and the legume host root-hair cell. The molecular mechanisms of recognition can be considered as a form of cell-to-cell interorganismal communication.

2.3.2.1.1 Plant Signals to *(Brady)Rhizobium*

The first exchange of signals involves the secretion of phenolic compounds, isoflavonoids by host plants (Peters and Verma, 1990). Isoflavonoids have been reported in root exudates for many years (Rao, 1990). Isoflavonoids appear to have multiple roles during several stages of nodule development. Early in the symbiosis, the host releases flavonoid signals that are perceived by the bacteria and trigger the co-ordinated expression of a series of nodulation (*nod*) genes (Long, 1989). Although *nodD* (*nodD1*) genes in *Rhizobium spp.* are constitutively expressed (Rossen et al., 1985), *nodD1* expression in *B. japonicum* is inducible by isoflavonoids or soybean seed extract (Banfalvi et al., 1988). Eight isoflavones capable of inducing nod gene expression were purified from soybean seed extract and their structures determined (Smit et al., 1992). Daidzein and genistein are the major components of soybean root exudates, which induce the *nod* genes of *B. japonicum* (Kosslak et al., 1987). Daidzein has less *nod* gene inducing ability than does genistein (Sutherland et al., 1990). These two compounds are synthesized constitutively and stored in the conjugated form (Graham et al., 1990). These signal compounds are often excreted by the portion of the root with emerging root hairs, the region that is most susceptible to infection by *(Brady)rhizobia* (Verma, 1992). Detailed studies with soybean roots showed that cotton string placed directly on the root

tip collected more daidzein than string touching tissue 1 cm behind the tip (Graham, 1991). A study with 3-day-old alfalfa seedling showed that ß-galactosidase activity was present only in bacteria (*nod::laz* gene fusion) surrounding the root tip and in the region of young root hairs (Peters and Long, 1988).

Isoflavonoids originate from the phenylpropanoid pathway. They occur in legumes grown under sterile conditions without exposure to any apparent biological and environmental stress factors (Tiller et al., 1994). Their levels can also be influenced by the inoculation of Rhizobia (Pueppke et al., 1998). Eleven enzymes, such as phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), are involved in the biosynthesis of genistein in soybean roots (Barz and Welle, 1992). Structural variations in isoflavonoids released from different legumes affect rhizobial *nod* gene transcription and determine their host-plant specificity through interactions with NodD proteins (Spaink et al., 1987).

As soon as it was established that some isoflavonoids are natural *nod*-gene inducers, the molecular specificity of rhizobia for isoflavonoids was tested by supplying numerous commercially available compounds. Those studies also showed that a C-7 hydroxylation (flavone numbering system) contributed to activity in most systems (Rolfe, 1988), but the availability of different NodD proteins and varying levels of inducing activity permitted no global conclusions about structure-activity relationships in isoflavonoids. One concept from those studies, which has been supported by later work with compounds actually released from legume plants, is that a weak *nod*-gene inducer can attenuate the activity of a stronger inducer (Djordjevic et al., 1987). It is important to understand the synthesis and exudation of both inducing and inhibiting compounds in the developing legume root.

A *nodC-nodZ* fusion facilitated this work by detecting active molecules at far lower concentrations than would have been possible with normal analytical methods. These isoflavonoids occur predominantly in the form of 7-O-glucoside-6"-O-malonate conjugates (Graham et al., 1990). The flavonoid conjugates are located in vacuoles (Stafford, 1992). Seeds and roots of common bean and alfalfa release different

isoflavonoids. It is not known, for example, whether isoflavonoids extracted from legume seeds with 2-butanone, also are released into aqueous solution during germination. While luteolin activates the NodD1, but not the NodD2, protein in *R. meliloti*, the bean isoflavonoids activate all the three *nodD* gene products in *R. leguminosarum*. bv. *phaseoli*. Naturally occurring mutations in *nodD* genes could dramatically alter flavonoid-NodD interactions just as laboratory mutations do (Burn et al., 1987; Spaink et al., 1989).

Because release of any compound from plants involves at least an energy cost associated with synthesis and possibly a cost for storage and/or exudation, it is reasonable to expect that plants regulate production of *nod*-gene-inducing isoflavonoids. Such controls relating to flavonoid synthesis, storage, exudation, or modification after exudation may operate at the genetic and/or biochemical level. On the other hand, stimulation of isoflavonoid biosynthesis in the plants is a common feature of many infecting micro-organisms irrespective of whether they are bacteria, fungi, viruses or nematodes (for review see Dakora and Phillips, 1996). In soybean roots, the formation and exudation of daidzein, genistein and coumestrol can be elicited by pure Nod factors from *B. japonicum* (Schmidt et al., 1994). Works in Phillips's laboratory showed that isoflavonoids are released as aglycones or glycosidic conjugates. The latter are less active but have a higher solubility in water, and they can be converted to active forms by bacterial glycosidases (Hungria et al., 1991; Maxwell and Phillips, 1990). *Rhizobium* strains were found having different abilities to converted the conjugated forms into genistein and daidzein (Pueppke et al., 1998).

Recourt et al. (1991) has determined that some of the enzymes of the phenylpropanoid pathway increase in activity soon after inoculation. There are several possible roles for isoflavonoids in the root after or besides infection. One is that new *nod* gene-inducing isoflavonoids are secreted into the root environment (van Brussel et al., 1990). These additional inducers may be required to induce *nod* gene expression in rhizosphere or after infection; *nod* genes are expressed even after *Rhizobium* cells have invaded the nodule (Sharma and Signer, 1990). Another possibility is that the root

responds to *Rhizobium* inoculation as an infected plant to an invading pathogen. Estabrook and Sengupta-Gopalan (1991) found that subsets of the PAL and CHS gene families are specifically induced in soybean roots after infection with *B. japonicum*, and the symbiosis-specific PAL and CHS genes in soybean were not induced by stress or pathogen interactions. Preculturing *B. japonicum* cells with 10μM genistein or daidzein induced resistance against the phytoalexin glyceollin suggesting that transcriptional regulation may be involved in this phenomenon (Parniske et al., 1991). A third possibility is that isoflavonoids disrupt the normal endogenous hormone levels of the root by functioning as endogenous auxin transport inhibitors.

The spectrum of secreted isoflavonoids may change with conditions, which may have ecological and developmental consequences for the free-living rhiozbia (Lawson et al., 1995). The presence of rhizobial cells around the roots can alter how plants synthesize and/or release *nod*-gene-inducing isoflavonoids. Studies with soybean showed that roots inoculated with *B. japonicum* contained additional daidzein and genistein (Cho and Harper, 1991a). Inoculation with an infective rhizobial symbiont causes a change in the internal flavonoid pool of the root. This second wave of isoflavonoids leads to an increased *nod* gene-inducing activity (Schmidt et al., 1994). Experiments with vetch indicate the presence of *R. leguminosarum.* bv. *viceae* bacteria strongly affects the release of *nod*-gene-inducing isoflavonoids into root exudate (Recourt et al., 1991; van Brussel et al., 1990), and some new *nod*-gene inducers were released. Whether the new *nod*-gene inducers released in the presence of rhizobia also regulate other, presently unknown, bacterial genes remains to be determined. Another point that should be established in these types of experiments is whether additional isoflavonoids that do not induce *nod* genes are released by the plant in response to rhizobia. If such isoflavonoids are released from legume roots in response to rhizobia, they could be part of the well-known plant response to microbes and other elicitors which results in the release of isoflavonoid phytoalexins (Dixon and Lamb, 1990).

The concept that legume plants may control the level of *nod*-gene-inducing activity in the rhizosphere by releasing inhibitors (Firmin et al., 1986; Djordjevic, 1987)

was supported by the demonstration that compounds released naturally from alfalfa roots interact to affect *nodC-nodZ* transcription in vitro (Peters and Long, 1988). Those data indicate that the weak inducers, 7,4'-dihydroxyflavone and liquiritigentin, present in root exudate can decrease the effectiveness of the very strong *nod*-gene inducer 4,4'-dihydroxy-2'-methoxychalcone. Thus, it is conceivable that legumes can alter the effectiveness of a strong inducer in the rhizosphere by releasing weaker inducers which would compete for binding NodD proteins. Flavonoid inducers of nodulation genes stimulate *Rhizobium fredii* USDA257 to export proteins into the surrounding environment (Krishnan and Pueppke, 1993). The *nod* gene inducer genistein can alter the composition and molecular mass distribution of extracellar polysaccharides produced by *Rhizobium fredii* USDA193 (Dunn et al., 1992).

Low concentrations of isoflavonoids can affect the growth rate of *(Brady)rhizobia*. In the case of *B. japonicum*, one of two strains studied grew faster with 1μM daidzein, but not 10 μM (D' Arcy-lameta and Jay, 1987). The measured increases in optical density, viable cell counts and total protein for *R. meliloti* were reproducible when cells were growing slowly on the defined medium, but no flavonoid effect on growth rate was detected when cells were growing more rapidly on a complex medium. One important feature of these data lies in the fact that most microbes are generally believed to be either resting or growing at less than optimum rates in soil. Thus growth factors from plants could favour development of certain bacterial populations.

The discovery that isoflavonoids are plant signals to microbes has important implications for understanding and managing soil ecosystems. A broad view of isoflavonoids as plant signals to soil microbes recognizes that because plant genes are responsible for producing isoflavonoids, all the agronomic tools available for altering those genes and for managing plant species that release certain isoflavonoids from living or dead roots are available for modifying the flavonoid profile of soils.

The effectiveness of isoflavonoids is found to vary between cultivars (Horvath et al., 1986; Zaat et al., 1988). Cho and Harper (199lb) reported that there is a strong positive relationship between root isoflavone concentrations and soybean nodule

numbers. Exogenous abscisic acid application into the soybean root medium resulted in a decrease in nodule number and weight in both hypernodulating and wild-type soybean plants, and isoflavonoid concentrations also markedly decreased in response to abscisic acid application (Cho and Harper, 1993).

It has been reported that adding isoflavonoids to the rhizosphere of alfalfa increased nodulation and N_2 fixation by *R. meliloti* under controlled environment conditions. In one example, 10 μM luteolin had a positive effect on Hairy Peruvian alfalfa (Kapulnik et al., 1987), in the other case 10 μM naringenin produced a similar response in Rijka T9 alfalfa (Jain et al., 1990). Presumably those results indicate that, under the conditions of this experiments, the natural isoflavonoids released from seeds and roots of those alfalfa cultivars limited nodulation. Thus adding more isoflavonoids may have enhanced nodulation by promoting growth of *R. meliloti* and by inducing *nod* genes in a larger proportion of the rhizobial population. However, attempts to extend these observations to other alfalfa cultivars by adding luteolin or 4', 4-dihydroxy-2' - methoychalcone to rhizosphere have not increased nodulation (for a review see Phillips, 1992). Addition of genistein directly into soil or incubation of *B. japonicum* with genistein increased nitrogen fixation under cool spring environment conditions (Zhang and Smith, 1995). In addition to our present understanding as to which isoflavonoids are released by legumes and how those compounds affect *(Brady)Rhizobium*, more detailed studies to determine when and under what conditions isoflavonoids limit nodulation are needed to clarify the basis of these apparently conflicting results.

Data available for *B. japonicum* show that not all strains nodulating soybean respond identically to isoflavonoids. The *nod* genes in *B. japonicum* USDA110, for example, were induced by daidzein in the presence of chrysin and naringenin, while the daidzein effect on other strains was greatly reduced by those compounds (Kosslak et al., 1990). Other workers, who tested approximately 1,000 compounds for inhibition of genistein-induced transcription of *nodY-nodZ* in *B. japonicum*, identified 7-hydroxy-5-methylflavone as a very strong inhibitor (Cuningham et al., 1991). Both studies concluded that natural variation among *B. japonicum* probably will prevent chemical

inhibitors of *nod*-gene induction from being commercially useful (Kosslak et al., 1990; Cuningham et al., 1991). An alternative method for favouring nodulation of soybean by certain strains of *B. japonicum* may involve the isoflavonoid-inducible *nol*A gene, which allows *Rhizobia* to nodulate certain soybean genotypes (Sadowsky et al., 1991). Whether that genotype-specific nodulation is based on the presence of unique isoflavonoids has not been reported.

Studies have shown that more than 30 genes in *Sinorhizobium meliloti* have now been found that are regulated by betaines, isoflavonoids, or water-soluble vitamins (IS-MPMI meeting report, 1997). The initial signal from the plant root is only capable of inducing Nod factor synthesis in the appropriate bacterial symbiont (Cardenas et al., 1995). Thus, colonization specificity is in some cases directed by the plant signal (Long, 1996). Based on the study of *nod* gene induction, it is possible to modify commercial inoculant preparation to include nodulation gene-inducing compounds (Hungria and Stacey, 1997). Alternatively, it may be possible to select for legume host varieties that produce large amounts or certain amounts of nodulation gene-inducing compounds.

Lectins (carbohydrate binding proteins or glycoproteins) are found in seeds, roots, leaves, and stems of legumes. Those found on the root are often concentrated in the area where nodule initiation occurs. Bohlool and Schmidt (1974) have proposed the involvement of plant lectins in the recognition process. The biochemical role of the lectins, and the symbiotic events, in which they participate, remain subject to debate.

Nodule morphogenisis appears to be elicited by at least two distinct signals: one from *(Brady)Rhizobium*, a product of the *nod* genes (Nod factor), and a second signal, which is generated within the plant tissues after treatment with Nod factor. Changes in the balance of endogenous plant hormones or the sensitivity of plant tissues to these hormones are likely to be involved. These hormonal changes may be triggered by endogenous isoflavonoids produced by the root in response to inoculation with *Rhizobium* (Hirsch, 1992). Long and Cooper (1988) reported that a plasmid causing constitutive synthesis of the cytokinin and zeatin, allowed Nod$^-$ (*nodABC*) melilotin mutants to cause partial nodule formation on alfalfa. Jacobs and Rubery (1988) reported

that isoflavonoids are also natural ligands of the naphthylphthalamic acid receptor and act as anti-auxins. Rhizobia can produce auxins, gibberellins, and cytokinins (see references in Torrey, 1986). IAA production by *R. meliloti* is stimulated by the addition of isoflavonoids (Prinsen et al., 1991). Taller and Sturtevant (1991) found that there was a difference in the type of cytokinins produced by *R. meliloti* after treatment with *nod*-gene inducing isoflavonoids, implying that cytokinin production may be NodD-regulated. Hirsch et al. (1989) proposed that treating alfalfa roots with auxin transport inhibitors could lead to an endogenous hormone imbalance which is manifested by cell divisions, the formation of pseudonodules, and the expression of early nodulin genes. But even if such a block in auxin transport occurs in roots, it is not known whether the block results in higher or lower concentrations of auxin relative to the levels of the other endogenous hormones in the pseudonodule-forming root segment. These findings have led to the development of a secondary signal model (Hirsch, 1992; Cooper and Long, 1994; Hirsch and Fang, 1994) in which the initial bacterial signal is transducted by a series of steps that lead to changes in the activities of endogenous hormones (Long, 1996).

2.3.2.1.2 *(Brady)Rhizobium* Signals to the Legume Plant

The variety and range of plants that form nodules when infected by any one rhizobial strain can vary enormously. *Rhizobium* species NGR234 can nodulate a very wide range of legumes in over 70 genera. The range of plants nodulated is determined principally by the bacterial nodulation (*nod*) genes, several of which encode proteins that are involved in the biosynthesis of nodulation signalling molecules (Nod factors) (Dénarié et al., 1992). These substituted lipo-chitin oligosaccharides (LCOs, e.g. Nod factor), which function as plant morphogens, consist of a chito-oligosaccharide backbone (ß-1,4-linked N-acetylglucosamine residues) and carry an N-linked acyl group at the terminal nonreducing glucosamine residue.

(Brady)rhizobial cells appear to produce complex mixtures of LCO species. *B. japonicum* strains make a variety of Nod factors, with one strain making more than ten different structures (Carlson et al., 1993). The acyl groups may be $C_{18:1}$, $C_{16:0}$ or $C_{16:1}$

depending on the particular strain, with $C_{18:1}$ being the most common type mixtures of Nod factors from *B. japonicum* elicited the transcription of early nodulin genes in soybean more efficiently than did the signal molecule species (Minami et al., 1996), which could explain the diversity observed in Nod factor synthesised by a signal rhizobial strain: different Nod factors could differentially activate plant genes involved in the symbiotic process. Demont-Caulet et al. (1999) also reported that a mixture of Nod factors was slightly more active than the synthetic analogue of the major natural product.

Nod factors can induce various root responses, e.g. root hair deformation, depolarisation of the root hair membrane potential, induction of nodulin gene expression, formation of nodule primordia, induction of flavonoid synthesis genes and induction of mitosis (for reviews see Heidstra and Bisseling, 1996; Spaink, 1996).

Nod factor structure does not correlate with rhizobial taxonomy and phylogeny as derived from molecular studies of rRNA, but it correlates instead with the rhizobial host range, indicating that a given plant has defined Nod factor structural requirements for the triggering of symbiotic responses (Dénarié et al., 1996). Two genetically distant soybean symbionts, *B. japonicum* and *Sinorhiozbium fredii*, both produce similar Nod factors containing, on the reducing N-acetyl-glucosamine, an O-fucosyl substitution which may thus be involved in recognition by their plant host (Sanjuan et al., 1992)

The *nodA*, *nodB* and *nodC* genes are conserved in all nodulating rhizobial strains. Mutation of any of these genes blocks nodulation completely and their products are essential for the synthesis of *nod* factors. NodC is similar in sequence to chitin synthase and is involved in the biosynthesis of the oligosaccharide backbone (Geremia et al., 1994). NodB is a chitin oligosaccharide deacetylase that removes the N-acetyl group from the terminal glucosamine residue (John et al., 1993), and NodA (an acyl transferase) is involved in the attachment of the fatty acyl chain to the deacetylated amino group (Rohrig et al., 1994). NodM and NodN are also involved in the formation of Nod factors.

Many other *nod*-gene products contribute to the specificity of Nod factors by modifying their structures. These modification may be for protection against degradation by the host plant or optimization of binding affinities for a specific plant receptor

(Spaink, 1996). Studies have suggested that the synthesis of the Nod factor core and the modifying side groups can be differently regulated (Cren et al., 1995).

Modifications of LCOs are believed to mediate host range in rhizobia-legume symbioses (Schultze and Kondorosi, 1995). Plant responses demonstrate an interdependence of backbone length and the presence of substitutions on the reducing end. The plant recognizes lipo-chitiooligosaccharides without reducing end substitutions despite the importance of these modifications for host range (Stokkermans et al., 1995). These data give further support to the hypothesis that the modifications of the chitin backbone function to protect the molecule from hydrolytic enzymes rather than as a part of a species-specific lock and key-binding mechanism required for signal transduction.

The *nodI* and *nodJ* genes are often in the same operon as *nodABC*. NodI is related to a large family of ATP-dependent membrane transporters and NodJ is a very hydrophobic membrane protein (Evans and Dowinie, 1986). Mutants of *R. leguminosarum* bv. *trifolii* lacking these genes secrete significantly lower amounts of Nod factors than normal and accumulate the Nod factors in the cells, showing that NodI and NodJ are involved in secretion of Nod factors (Mckay and Djordjevic, 1993). *R. leguminosarum* bv. *viciae nodI* or *nodJ* mutants secrete normal levels of Nod factors and the Nod factors do not accumulate in the cell (Spaink et al., 1992), presumably because an alternative secretion system is present.

The production, and in particular the excretion, of Nod metabolites of *R. leguminosarum* bv, *trifolii* was restricted by a range of environmental conditions which are associated with poorer nodulation in the field. Lowered phosphate level, lowered pH, and lowered temperature all reduce production and excretion of Nod factors, with certain combinations of these treatments causing the greatest reduction. The excretion rather than production of Nod factors is more sensitive to certain environmental stresses, such as low phosphate levels and high nitrogen concentrations (Mckay and Djordievic, 1993). Dusha et al. (1989) found that high levels of KNO_3 (70 mM) do not affect the expression of *nod*ABC in *R. meliloti* (this does not necessarily indicate that Nod factors were produced or excreted). In Mckay and Djordievic's study (1993), high levels of KNO_3 or

$(NH_4)_2SO_4$ were shown to substantially reduce the excretion of Nod factors by ANU845m (pRI4003). Environmental conditions that permit the expression of a gene do not necessarily allow the functioning of a metabolic pathway and the formation of end products. Measurement of the physiologically active products (Nod factors) may well have more value when we try to relate field-based observations to precise molecular events.

Changes in nodule occupancy by different rhizobial serogroups as a result of environmental change provide a practical insight into plant-microbe interactions that can be related to the production and excretion of Nod factors. Previous studies (Almendras et al., 1987; Mullen et al., 1988) have shown how nodule occupancy by different serogroups of rhizobia can be dramatically changed as a result of changes in the phosphate concentration, growth temperature, or pH. These observed changes in nodule occupancy as a result of environmental change may well relate to differences in the abilities of strains to produce and excrete Nod factors and hence to be competitive. The ability of different strains to produce and release Nod factors is likely to be a major determinant of nodule occupancy, strain competition in the soil and should be considered when screening strains suitable for adverse environments.

Lipo-chitin oligosaccharide Nod factors can induce plant responses such as the deformation of root hairs at concentrations as low as 10^{-12} M, implying that there is a very high affinity receptor for these morphogens. At high concentration (such as 10^{-8} M), they can induce nodule morphogenesis (Truchet et al., 1991; Stokkermans and Peters, 1994). This is particularly clear with alfalfa (Truchet et al., 1991) and *Glycine soja* (Stokkermans and Peters, 1994). In some legumes, including alfalfa and vetch, nodule morphogenesis is initiated in inner cortical cells and the induction of nodule morphogenesis indicated that these cells could recognize Nod factors. However, this could also have resulted from a secondary signal synthesized by, for example, root hair cells in response to Nod factors. As well as inducing nodule morphogenesis, Nod factors also stimulate the formation of cytoplasmic bridges in cortical cells (van Brussel et al., 1992), and it is these that form the pathway along which infection threads develop. Thus,

the plant (rather than the bacteria) defines where infection threads grow and so where the bacteria enter the developing nodule.

The first response observed after adding Nod factors is a depolarization of the plasma membrane of root hair cells of alfalfa (Ehrhardt et al., 1992), thus providing a rapid assay to evaluate early responses of root hair cells and to use pharmacological approaches to explore the transduction pathways. This depolarization starts within 1 min of adding the Nod factor and occurs with concentrations of 10^{-9}-10^{-11} M. NodO has been postulated to amplify the effect of Nod factors, and this may occur by facilitating the uptake of Nod factors or by stimulation of an ion flux across the plant plasma membrane (Sutton et al., 1994). Depolarization of plant cell membranes is often seen with bacterial pathogens and elicitors of plant-defence responses.

Nod factors could potentially be transformed before they interact with the plant. In the presence of roots, Nod factors are rapidly degraded by plant chitinases and this process is likely to play an important role in the fate of Nod factors both in the rhizosphere and in the plant tissues (Staehelin et al., 1995). It was shown that structural modifications in the Nod factors influence their stability against hydrolysis by purified chitinases of various plant species (Staehelin et al., 1994).

Certain oligosaccharides prepared by degradation of microbial and plant cell walls have been shown to be involved in the control of plant gene expression, and the term oligosaccharins has been proposed to designate such molecules with regulatory activity (Ryan and Farmer, 1991). Rhizobial Nod factors represent a novel class of physiologically-significant oligosaccharins, characterized by a chitinoligomer backbone, mono N-acylated at the non-reducing end. Several lines of circumstantial evidence raise the possibility that these rhizobial signals are mimicking endogenous plant molecules involved in the control of development. First, *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium* strains, despite being genetically very distantly related, produce similar Nod factors. Second, the fact that the *nodABC* genes are required for nodulation of the non-legume *Parasponia*, strongly indicates that rhizobial lipo-chitooligosaccharidic Nod factors are also able to elicit developmental responses in plants other than legumes.

Third, Nod factors from *R. leguminosarum* bv. *viciae* can rescue embryo formation at an unpermissive temperature with a mutant carrot cell line. The ability of this culture to generate somatic embryos is temperature sensitive (De Jong et al., 1993). Purified Nod factors also enhance significantly the expression of several cell-cycle marker genes in microcallus suspension cultures of *Medicago sativa*. The Nod factors thus appear to stimulate the progression of the cell cycle (Savoure et al, 1994).

Duelli and Noel (1997) reported that seed exudate (anthocyanins) and root exudate of *Phaseolus vulgaris* induce modifications of rhizobial lipopolysaccharide. It has become clear now that regulation of the synthesis of rhizobial surface polysaccharides is an important factor in establishment of an effective symbiosis, and that *nod* genes encode only a part of the nodulation story (IS-MPMI meeting report, 1997; Eggleston et al., 1996).

Mellor and Collings (1995) presented some excellent hypotheses regarding the relationships among Nod factors, plant defence reactions and nodulation.

2.3.2.2 Infection and nodule development

Root infection by *(Brady)rhizobia* is a multistep process that is initiated by preinfection events in the rhizosphere. *Bradyrhizobia* respond by positive chemotaxis to plant root exudates and move toward localized sites on the legume roots (Barbour et al., 1991). Nodulation begins when *(Brady)rhizobia* attach themselves to epidermal cells. Once near the root surface, *(Brady)rhizobia* cause alterations in the growth of emerging and growing root hairs, leading to root hair curling. Epidermal cells with immature or as yet unformed root hairs are the usual sites for bacterial penetration (Bhuvaneswari et al., 1980). Elongated root hairs are sometimes infected, but this is rather unusual (Rao and Keister, 1978). Infected hairs are always shorter than mature intact hairs, and they are markedly curled. *Bradyrhizobia* enter the roots at the sites where roots hair cell walls are hydrolyzed. The penetration occurs by invagination of the plasma membrane. Growth of the infection thread is co-ordinated by the plant cytoskeleton in association with the nucleus. Infection threads may branch within a root hair (Turgeon and Bauer, 1982). An

infection thread, including its dividing bacteria, grows 60 to 70 μm to the base of the root hair cell. The host plant reacts by depositing new cell wall material around the lesion in the form of an inwardly growing tube. The bacteria travel from host cell to host cell via the infection thread and its branches. Infection thread formation and penetration are relatively rare events.

Concomitantly with formation of the infection thread, cortical cell divisions take place several cells distant from the advancing infection thread to form a nodule primordium, and the infection thread grows toward this primordium. In *Glycine max*, the first divisions occur in the hypodermis and spread to the outer layers of the cortex (Calevert et al., 1984). These mitoses increase cell numbers in the cortical layer, which then becomes the main area of infected cells (Newcomb et al., 1979). The combination of multiple threads and branching of threads in the cortex results in penetration of many, but not all of these cells. The peripheral uninfected area becomes the nodule cortex, which includes a scleroid layer and several vascular bundles. Cell division ceases early during nodule development and the final form of the nodule results from cell enlargement rather than cell division.

At some time during or following mitotic activity, rhizobia are released into the cytoplasm of individual target cell through a small thin area on the surface of the infection threads. The bacteria are enveloped by a plasma membrane of plant origin, called the "peribacteroid membrane". The bacteria then undergo rapid multiplication, accompanied by matched peribacteroid membrane synthesis, until the host cell becomes densely filled with packets of bacteroids (Werner and Morschel, 1978).

The route of infection is characteristic for the host. A given legume is infected by the same type of mechanism regardless of the infecting strain (Rolfe and Gresshoff, 1988). Similarily, the structural and developmental characteristics of an efficient nodule are specified by the host which possesses the genetic information for symbiotic infection and nodulation; the role of the bacteria is to switch on this plant developmental program (for a review see Dénarie et al., 1992).

As the nodule matures, oxygen-rich leghemoglobin develops gradually in the host

tissue and the nodule becomes pink, remaining so until it begins to senesce. As leghemoglobin forms and bacteria cease dividing, N_2 fixation begins (Lersten and Garlson, 1987).

The problem of nodule senescence has received little attention. The initial crop of nodules is frequently senescent by early pod fill (Zapata et al., 1987), and subsequent nodulation, apparently extremely critical for enhanced seed yield (Imsande, 1989), is due to less or ineffective indigenous strains (Hardason et al., 1989). One way of improving overall fixation is to prolong the period of nodule activity. The longevity of nodules is related to the growth habit of the host plant. *(Brady)Rhizobium* strain effects on nodule longevity have not been adequately characterized. There is a distinction between the senescence of whole nodules and the senescence of zones within the nodule tissue. The first obvious signs of nodule senescence are a brown colour, wrinkled surface, and soft texture. A change in colour may occur in the central zone, from pink or red to brown, grey, or green. Environmental changes (light intensity, day length, water supply, oxygen concentration, CO_2 concentration, soil pH and nutrient levels, pollution, and infection by other organisms) detrimental to plant growth may result in premature nodule senescence. The controlling effects of photosynthetic rate on nodule nitrogenase activity have been reviewed (Vance and Helchel, 1991). Increased levels of carbohydrate may delay but not prevent nodule senescence. Low root temperatures delay senescence, while high root temperature has inhibitory effects on nodule development and accelerates nodule senescence.

2.3.2.3 Chemistry and Biochemistry of Nitrogen Fixation

The mature nodules carry out at least three major functions. The first of these is glucose catabolism giving energy, reducing-power, and carbon skeletons for the plant cell cytoplasm and organelles. The second is the metabolism of nitrogen fixation in the bacteroid. The third is ammonia assimilation in the plant cytoplasm, giving a net conversion of carbon skeletons and ammonia to amino acids and/or ureides, which are then exported from the nodule.

Nitrogenase, which comprises 30% of the total protein in infected root nodule cells, has been purified from all known types of N_2-fixing organisms, with the exception of archaebacteria (Sprent and Sprent, 1990). Nitrogenases are known to be made up of two distinct parts, dinitrogenase reductase (Fe-protein), which is an electron carrier and is used to reduce the other enzyme, dinitrogenase (Mo-Fe protein), which is the enzyme responsible for the reduction of N_2.

The symbiotic reduction of nitrogen gas to NH_4^+ and its subsequent assimilation into other nitrogenous compounds in legume root nodules is an energy demanding process that involves both the plant host and the bacterial symbiont. ATP synthesis is provided by oxidative phosphorylation, resulting in the need for high rates of O_2 consumption in legume nodules. However, O_2 is a potent inhibitor of nitrogenase. To maintain a very low free O_2 concentration conducive to N_2 fixation, legume nodules vary their permeability to O_2 diffusion (Hunt et al., 1987). A nodule gaseous diffusion barrier, composed of water-filled intercellular pores located within the nodule cortex regulates oxygen flux from the rhizosphere into the nodule and prevents nitrogenase inhibition by O_2. To achieve high rates of respiratory O_2 consumption at low infected cell O_2 concentration, O_2 binds to a hemo-protein (leghemoglobin), which facilitates O_2 diffusion throughout the cytosol (Hunt and layzell, 1993).

Studies have shown that a gradual increase in the external partial pressure of O_2 stimulate nodule metabolism and N_2 fixation (Hunt et al., 1989; Diaz del Castillo et al., 1992). Weisz and Sinclair (1988) showed that soybean nodule activity and nodule gas permeability were both closely correlated to, and reduced by, low soil temperature, probably due to temperature mediated changes in the turgor of cortex cells. Also, treatments such as NO_3^- fertilization, phloem sap deprivation, or prolonged exposure to an $Ar:O_2$ atmosphere have all been shown to inhibit nitrogenase activity by decreasing nodule permeability to O_2 diffusion (Hartwig et al., 1987; Vessey et al., 1988; Diaz del Castillo et al., 1992), but the mechanism by which cortical permeability is altered is controversial and a hypothesis of "solute-transport mediated control of nodule function" was put forward (for a review see Walsh, 1995). Under these conditions, the reduced

flux of oxygen into the nodules limited nodule respiration, and resulted in inhibition of both host carbohydrate conversion to ATP and production of reductant required for nitrogenase activity (Weisz and Sinclair, 1988).

Soybean nodules are dependent on reduced C from the leaf--either C fixed over the preceding hours through photosynthesis or C remoblized from leaf starch. Current photosynthate is transported to the nodule in the form of sucrose and is stored in the plant fraction of nodules as starch. The leaf starch reserve is the largest pool of C available to support nodule respiration, although the nodule polyhydroxybutyrate (PHB) reserve is large. The published evidence convincingly indicates that legumes and other N_2-fixing species respond to an increased supply of photosynthetic products over the long term by coordinated increases in the mass of all plant organs, including nodules (Vance and Heichel, 1991).

Ammonia, fixed by nitrogenase, is excreted from bacteroids, moving freely across membranes. Thus, it is assumed to flow out of the bacteroid by diffusion, down the concentration gradient caused by its continual removal from the host cytoplasm (Millor and Werner, 1990). Glutamine may be further used for asparagine or purine synthesis, depending on whether the plant is an amide-transporting legume (most of this group are temperate legumes) or a ureide-transporting legume (most of this group are tropical legumes). The amide exporters transport asparagine and glutamine while ureide exporters transport either allantoin and allantoic acid or citrulline. Soybean is a ureide exporting legume, in which glutamine, glycine, and aspartate are used for purine synthesis in plastids of infected cells. Purine nucleotide is transformed to xanthine and uric acid, which is transformed by peroxisomes in neighbouring, uninfected cells to allantoin. This is further transformed on the smooth endoplasmic reticulum of uninfected cells to allantoic acid (Millor and Werner, 1990).

During biological N_2 fixation, H^+ and N_2 are simultaneously reduced by the bacterial enzyme nitrogenase by the following reaction:

$$N_2 + 8H^+ + 8e^- + 16\ MgATP \rightarrow 2NH_3 + H_2 + 16\ MgADP + 16Pi$$

The H_2 produced during N_2 fixation may by partially or entirely reoxidized by a

bacterial uptake hydrogenase (Hup^+) enzyme that is expressed in many legume symbioses. In Hup^- symbioses, H_2 evaluation can be measured and used to monitor either relative nitrogenase activity during N_2 fixation (Hunt and Layzell, 1993) or total nitrogenase activity when, in the absence of the substrate N_2 and other nonphysiological substrates, H_2 is the only product of nitrogenase (Moloney et al., 1994). Obviously, H_2 production wastes ATP energy involved in the transfer of electrons used to reduce $2H^+$ to make H_2. The relative efficiency can be calculated as

1 - (H_2 evolved in air) / (H_2 evolved in $Ar:O_2$).

Under conditions where rhizosphere pO_2 and pN_2 are close to atmospheric levels, electron allocation coefficient values for nitrogenase range from 0.4 to 0.9 (Layzell et al., 1984; Diaz del Castillo et al., 1992). Relative efficiency values for soybean have been measured at 0.6 - 0.8. The action of hydrogenase does not alter the N_2 fixing reaction, but can result in recapture of some energy that would otherwise be lost from the complex as H_2.

2.3.3 General genetic characteristics

As N_2 fixation is a symbiosis, the genetic aspects of both organisms, those of the bacteria and those of the higher plant, are involved. In recent years, advances have occurred in several major areas: the identification of new nodulation genes; the use of genetically modified *(Brady)Rhizobium* to study mechanisms and the analysis of *nod* gene regulation

2.3.3.1 Nodulin genes and Nodulins

Nodulin genes are plant genes exclusively expressed during the development of the N_2 fixing symbiosis. They can be divided into early and late nodulin genes, according to the times of their expression during nodule development (Govers et al., 1987). The proteins encoded by these genes are called nodulins.

Early nodulins can be involved in root hair deformation, infection, or nodule morphogenesis. Genes encoding early nodulins are induced during rhizobial infection,

growth of the infection thread, and stimulation of cortical cell divisions (Kouchi and Hata, 1993).

Later nodulins, associated with nodule function, are detectable after the nodule has developed and bacterial release has taken place. They function in oxygen transport, cell wall architecture, possibly plant defense, sugar and nitrogen metabolism as well as membrane transport (Gresshoff, 1993). Late nodulin genes are activated in the developing and mature nodule concomitantly with the onset of nitrogen fixation activity. Leghemoglobin (Lb) transcript detection was used as a marker for the expression of late nodulin genes. Leghemoglobin is detectable just before nitrogenase activity can be measured (Verma et al., 1979).

2.3.3.2 *(Brady)Rhizobium* genes and their functions

Over 50 different nodulation genes have been identified (Stacey, 1995). The *(Brady)Rhizobium* genes essential for infection and nodule formation can be divided into two classes. One class includes several sets of genes involved in the formation of the bacterial cell surface, such as genes determining the synthesis of exopolysaccharides (*exo* genes), lipopolysaccharides (*lps* genes), capsular polysaccharides or K-antigens, and ß-1,2-glucans (*ndv* genes). Mutations in these genes disturb the infection process to various degrees (Noel, 1992).

The second class consists of the nodulation genes. They can be categorised as: (a) the *nod* genes (i.e. the 55 known *nod, nol, noe* genes), essential for the induction of plant response, and subdivided into the common *nod* genes [*nodA*, B, C, I, and J, functionally interchangeable among different species of *(Brady)Rhizobium*] (Appelbaum, 1990) and host specific *nod* genes (*nod*E, F, D, and H), (b) the *fix* genes (those that have been shown to be required for symbiotic nitrogen fixation, but whose function is not known to be analogous to a free-living function, are referred to as *fix* genes), which are involved in the process of N fixation, (c) the *nif* genes (those with homologs in free-living nitrogen fixation systems are referred to as *nif* genes), about 20 in number, which are really a subset *fix* genes and code for nitrogenase and related proteins.

In the *Rhizobium* species the *nod, fix,* and *nif* genes are all contained on a symplasmid, while in *Bradyrhizobium* species these genes are on the chromosome (Appelbaum et al, 1990).

The *nod* DABC genes have been shown to be essential for root hair curling, formation of the infection thread, and the induction of cortical cell division, thus for the earliest steps in the developmental program of the root nodule in which no nodulin gene expression has been identified yet. Mutations in *nodABC* prevent marked root hair curling (Hac^-) and formation of infection threads (Inf^-). The *nodABC* genes are also involved in morphogenesis and encode functions that stimulate cell division without direct contact between the bacteria and the stimulated plant cells. Overproduction of the *nod*ABC gene products either by increased gene copy number or from strong promoters proved deleterious to nodulation (Knight et al., 1986). Thus, the concentration of the *nod*ABC gene products is critical for the proper development of the symbiosis. Host-specific *nod* genes influence root hair curling, infection thread formation, and nodule initiation in various ways.

B. japonicum possesses two *nodD* genes (i.e. $nodD_1$ and $nodD_2$) arranged tandemly (Gottfert et al., 1989). The inability to induce gene transcription in a $nodD_1nodD_2$ deletion mutant can only be complemented by $nodD_1$, and not by $nodD_2$ (Gottfert et al., 1992), which is dependent on the presence of isoflavones (Smit et al., 1992). Eight isoflavones capable of inducing *nod* gene expression were purified from soybean seed extracts and their structure determined (Smit et al., 1992). These same eight compounds are present in root exudates. The combined presence of genistein, daidzein and their glucosides apparently results in enhanced *nod* gene expression.

In *B. japonicum*, the *nolA* gene is located downstream of $nodD_2$ and transfer of *nolA* to wild-type cells and various mutants results in a marked reduction in *nodY* and $nodD_1$ expression (Dockendorff et al., 1994). This means, in addition to NodD, NolA is also involved in *nod* gene regulation.

Mutation in $nodD_1$ dramatically reduces *nod* gene expression, but has no effect on soybean nodulation. Moreover, a $nodD_1$-$nodD_2$ deletion mutant is still Nod^+. In addition

to $NodD_1$, NodV may also be involved in recognizing and responding to isoflavone inducers (Stacey, 1995). Recent evidence has suggested that, in the absence of NodD, NodW is essential for nodulation of soybean (Sanjuan et al., 1994).

In some cases, such as *R. leguminosarum*, *nodD* also autogenously regulates its own expression (Rossen et al., 1985). In several cases, it has been shown that different *nodD* genes confer distinctive patterns of *nod* gene response to a variety of pure isoflavonoids or plant exudates (Spaink et al., 1987; Bassam et al., 1988). NodD is a 33 kd protein, which binds the promoters of inducible *nod* genes. These inducible promoters are characterized by a long, highly conserved sequence, the '*nod* box'. The NodD protein displays a footprint on *nod* gene promoters of about 55-60bp, which corresponds very closely to the extent of the *nod* box. These biochemical studies thus support the proposal that NodD is a DNA binding transcriptional activator.

The *fix* and *nif* genes contribute to the late stages of nodule development and nodule function. They appear to influence the level of expression of late nodulin genes. In nodules formed on pea by *nif* and *fix* mutants the amount of mRNA of the late nodulin genes is 10 to 40% of the amount found in wild-type nodules (Govers et al., 1985). In *Klebsiella pneumoniae* about 20 contiguous *nif* genes are required for nitrogenase activity and growth on a N-free medium (Appelbaum, 1990). The functions of the *fix* genes are less clear. It is established, at least in *R. meliloti*, that the *fix*L gene product is a protein that spans the membrane of the bacteroid and senses the oxygen concentration inside the nodule (Earl et al., 1987).

Many genetic loci that influence the symbiosis have been identified in a variety of *(Brady)Rhizobium* strains. In many cases, the genes have been cloned and sequenced, and the products have been associated with a particular stage of infection, nodule development, or nitrogen fixation. Spaink et al. (1989) has constructed a hybrid *nodD* gene that does not require a flavonoid for *nod* gene expression. A *Rhizobium* strain with this hybrid *nodD* gene expresses increased nitrogenase activity in the nodule. This and other examples show that genetic alterations can increase the ability of rhizobia to fix nitrogen.

However, the biochemical activities of the proteins encoded by symbiotic genes have not been defined, except in the case of some *nif* genes (i.e., *nif*HDK, encoding the nitrogenase polypeptides). The possibility that other symbiotic genes remain to be discovered should not be overlooked. The identification of genes that may affect the survival and competitiveness of *(Brady)Rhizobium* in nature is just beginning.

Fisher (1993) recently reviewed genetic regulation of nitrogen fixation in rhizobia. Yuen and Stacey (1996) showed that organic acids (i.e., acetate, fumarate, L-malate) significantly inhibit *nod* gene expression in *B. japonicum* and may be important in suppressing *nod* gene expression in nodules.

2.4 Environmental Effects on Nitrogen Fixation

The consequences of enhancing N_2 fixation cannot be assessed in isolation because it is only one of several primary components of cropping systems which are under the control of human managers. Other factors, such as the amount of fertilizer, water supply, phenotypes, numbers of plants and bacteria present, can modify the effects of increased N_2 fixation (Brockwell et al., 1988).

Inadequate nodulation of legumes is not always manifested in poor yield because the plant compensates by increased uptake of soil N (Herridge, 1984). Such exploitation of the reserves of the soil N is not sustainable. Nodulation failure resulting from unfavourable environmental conditions has been attributed to a breakdown of the early events of nodulation, such as stimulation of root hair curling and formation of infection threads, and can occur even when the growth of the individual partners is not unduly affected by the limiting condition.

2.4.1 Soil Mineral Nitrogen

Many legume species have direct access to biologically fixed nitrogen, but preferentially utilize soil mineral N rather than develop a root nodule symbiosis with *(Brady)Rhizobium*. All the stages of symbiotic development that have been investigated are inhibited by mineral N, including isoflavonoid production (Cho and Harper, 1991a;

1991b), root hair deformation, attachment of rhizobia to the root hairs, root hair curling, infection thread formation, the level of immunologically detectable lectin on the root surface, and other infection events (for reviews see Streeter, 1988; Carroll and Mathews, 1990). Additionally, nitrate delays the appearance of nodules (Darbyshire, 1966; Gibson and Harper, 1985), inhibits the number of nodules that are formed, slows nodule development, reduces specific nitrogenase activity (Eardly et al., 1984), and it induces premature nodule senescence (Schuller et al., 1986). It is generally accepted that nitrate is particularly inhibitory to nodule growth and nitrogenase activity, and somewhat less inhibitory to the infection process.

Infection is inhibited only at relatively high mineral N concentrations. Although high nitrate (18 mM) will completely inhibit nodule formation, a delay of only 5 days in the nitrate treatment after inoculation allows some infections to generate functional nodules (Streeter, 1988). IAA added with nitrate will partially offset the negative effect of nitrate on infection (Dixon, 1969). The localized effect of nitrate on nodulation has been demonstrated in several legume species using split-root systems (Kosslak and Bohlool, 1984). Nodule development is affected on the nitrate-free portion of the root, only if the level of nitrate is very high on the other half of the root.

With long-term exposure of plants to 4 to 8 mM NO_3^-, effects of NO_3^- on nodule growth were greater than effects of NO_3^- on specific nitrogenase activity (Streeter, 1988). Studies with field-grown plants also indicate much greater effects on nodule growth than on specific nodule activity (Miller et al., 1982).

A few studies have compared both *(Brady)Rhizobium* and legume genotypes and their response to combined N and these studies seem to indicate that differences among legume genotypes are probably greater than differences among *(Brady)Rhzobium* strains (Ralston and Imsande, 1983). Some strain variation in the sensitivity of soybean symbioses to nitrate does exist; however, the presently known variation seems insufficient to lead to a substantial field benefit (McNeil, 1982). While variation does exist at low or medium levels of nitrate, no striking differences have been reported at higher levels of nitrate. A slight, but significant, bacterial effect on the regulation of

nodulation by nitrate is the observation that a high dose inoculation partially alleviates the degree of inhibition in soybean.

Malik et al. (1987) demonstrated that if exposure to nitrate was delayed for 18 hours after the time of inoculation, inhibition was much reduced. Exposure to nitrate for periods of 4 to 24 hours after inoculation, followed by transfer to no-nitrate conditions for the remainder of the time, did not result in substantial inhibition of nodule number. This indicated that the effects of nitrate on infection initiation could be almost entirely reversible.

Although the inhibition of nodulation by NO_3^- has been known for over 100 years, the mechanisms involved are still poorly understood. A number of hypotheses have been proposed for the mechanism of nitrate inhibition including 1) inhibition of nitrognase activity (Roberts and Bill, 1981; Vessey and Waterer, 1992), 2) carbohydrate deprivation (Latimore et al., 1977), and 3) nitrite effects on nitrogenase (Rigaud and Puppo, 1977), 4) decrease in isoflavonoids (Cho and Harper, 1991).

Some studies suggest a pivotal role of O_2 supply to bacteriods as a common theme in the mechanism of inhibition (Hunt et al., 1988). Vessey et al (1988) demonstrated that with NO_3^- application phloem supply to nodules is sharply reduced. Regardless of whether the casual factor is a decline in carbohydrate supply to nodules or a more direct effect of nitrate on the nodule, a decrease in O_2 diffusion into the infected zone and consequently an O_2 limitation of nitrogenase activity is indicated.

There are some other possible mechanisms for nitrate inhibition, including enzyme activities, leghemoglobin/oxygen balance, pH and ion balance, ammonium concentration and metabolism in bacteriods, all of them still need to be tested (Ligero et al., 1991). Our present knowledge of the *Rhizobium*-legume symbiosis would suggest that there are many, many more similarities than differences among species. Regardless of one's position on this issue, there is a practical point, namely, it is dangerous to draw conclusions regarding mechanisms based on studies with a single symbiotic system. It is highly important to compare the response of at least a few different legumes and *Rhizobium* strains before drawing conclusions about mechanism. A second issue is

whether it is likely that mechanisms for the inhibition of infection, of nodule growth, and inhibition of nitrogenase are likely to be the same. (The three inhibitory effects represent three very different processes).

Further research on nitrate effects will continue to contribute to the knowledge of nodulation in general. It will be useful in differentiating between the basic host and microbial requirements for nodulation and the interaction of these symbiotic partners.

2.4. 2 Temperature

As a subtropical legume, soybean requires temperatures in the range of 25 to 30°C for optimal plant growth and development (Jones and Tisdate, 1921). When soil temperature drops below this range, legume nodulation and N fixation are negatively affected. Matthews and Hayes (1982) have shown that decreasing RZT below 25°C results in decreased nodule growth and total N fixation per plant, while at a RZT of 15°C, the plant net nitrogenase activity is reduced by 25% (Walsh and Layzell, 1986), and nodulation ceases in plants at 10°C RZT (Matthews and Hayes, 1982). Lynch and Smith (1993a) reported that a RZT of 15°C delays the onset of N fixation until approximately 4 to 6 weeks after inoculation, while for plants grown at 25°C the onset of N fixation is at 2 weeks after inoculation.

Lie (1981) noted that all stages of nodule formation and functioning are affected by suboptimal RZT. Experiments involving the transfer of legume plants, at various stages of their life cycle, between optimal and suboptimal RZTs have generally indicated that infection and early development processes are the most sensitive to low RZT (Lindemann and Ham, 1979; Matthews and Hayes, 1982). Gibson (1971), in a review of the data on environmental effects on the legume *Rhizobium* symbiosis, suggested that low temperatures retard root hair infection more than nodule initiation, nodule development, or N assimilation. With temperate legumes such as *Trifolium parviflorum* and *T. glomeratum*, Kumarasinghe and Nutman (1979) found that the onset of infection and rate of infection thread development in root hairs varied greatly with soil temperature. They found that at optimal soil temperatures (from 18 to 30°C) infections were initiated earlier

and in larger numbers than at low (6 to 12°C) or moderately high (36°C) temperatures. Studies of the effects of suboptimal RZTs on soybean concluded that these conditions decrease N fixation activity by the nitrogenase enzyme complex (Layzell et al., 1984a) and suppress and/or delay root infection and nodulation (Walsh and Layzell, 1986).

The effects of low temperature on the function of N_2-fixing nodules may be due, in part, to the changes in nodule oxygen permeability (Walsh and Layzell, 1986). Low temperature is known to increase O_2 solubility and decrease its diffusion coefficient. Leghemoglobin is also likely to be affected by low temperatures as a result of both a lower diffusion coefficient for the oxygenated form of leghemoglobin (Moll, 1968) and a change in the affinity of leghemoglobin for O_2 and for other substrates (Stevens, 1982). The respiratory capacity of a nodule is also likely to be lower at low temperatures (Earnshaw, 1981).

Soybean plants export the fixed N in the nodule in the form of ureide. The solubility of ureide is low and decreases sharply as temperature declines, therefore low temperature may also limit the rate of export of fixed N from the nodule (Sprent, 1982). In turn, higher N concentrations inside the nodule may inhibit N_2 fixation.

Decreases in temperature result in progressively less bacteroid tissue (Lie, 1981) and delay its rate of formation (Fyson and Sprent, 1982). Roughley (1970) reported that infection is delayed at low temperatures, but once infected, nodule development is very rapid. However, nodules formed at low temperatures contain low amounts of bacteroid-containing tissue, almost none at 7°C (Roughley, 1970; Roughley et al., 1981).

The effect of low temperature on soybean nodulation, and N fixation and N assimilation may also be mediated via effects on plant physiology and growth. For soybean, decreased aerial temperature resulted in decreased relative growth rate, stomatal conductance, net CO_2 exchange rate, leaf assimilate export rates, and leaf elongation rate (Duke et al., 1979; Musser et al., 1983). Many adverse effects of low RZT on chilling-sensitive plants can be attributed to low temperature-induced membrane phase transitions which decrease the activity of membrane-bound enzymes (Duke et al., 1977; Duck et al., 1978). The effect of low temperature on N_2 fixation and NO_3^- N assimilation

may also be mediated via effects on photosynthesis or translocation, as has been demonstrated in the case of limitations to nitrogenase activity. Decreased shoot and root respiration and increased carbon levels (partly as starch) in mature leaves and stems at low RZTs (Walsh and Layzell, 1986) reduced transportation of energy to nodules and decreased nodule function.

All legumes are C_3 plants with optimum temperatures for photosynthesis between 15°C to 25°C (Lie, 1981). At leaf temperatures greater than 30°C net photosynthesis is reduced. This effects the rate of N_2 fixation. Again the degree and duration of the stress are both important. At higher temperatures a rapid degeneration of nodules takes place, resulting in a shortening of the period for nitrogen fixation (Lie, 1981). There are differences among the bacterial strains in the response to high temperatures (Lie, 1974), and selection of rhizobial strains for high temperatures is feasible. High temperatures reduce the numbers of lateral roots and root hairs. The few root hairs formed have an abnormal form, bulbous and very short (Lie, 1974).

2.4.3 Water

The water use efficiency of legumes is generally low and they are, as a group, quite sensitive to drought stress. Water supply has a major effect on nodulation and N_2 fixation. In dry soil infection is restricted because of the absence of normal root hairs; instead, short, stubby root hairs appear, which are inadequate for infection by rhizobia (Lie, 1981). Water stress significantly decreases the number of infection threads formed and inhibits nodulation. This effect on infection is reversible, when the plants are re-watered immature hairs resume normal growth and become infected. Following successful infection, reduced water supply can retard nodule development (Sprent and Sprent, 1990). Lower water potentials in the nodules may reduce N_2-fixing activity (Pankhurst and Sprent, 1976), with an accompanying reduction in nodule respiration, reduction in transport of fixed N out of nodules, and impairment of photosynthate supply from a stressed shoot system (Huang et al., 1975). N_2 fixation activity reduces as water potential decreases and ceases irreversibly in detached nodules when the fresh weight

drops below about 80% of the fully turgid weight (Raper and Kramer, 1987). The effects of drought stress on N_2 fixation usually have been perceived as a consequence of straight forward physiological responses acting on nitrogenase activity and involving exclusively one of three mechanisms: carbon shortage, oxygen limitation, or feedback regulation by nitrogen accumulation (for a review see Serraj et al., 1999).

Strain difference exists in drought resistance and survival (Athar and Jhonson, 1996; 1997). Several indigenous strains of *B. japonicum* were superior as inocula over commercial strains when soybean was grown under drought conditions (Hunt et al., 1988). Genetic variation in N_2 fixation response to water deficits also exists among legume cultivars (Sinclair and Serraj, 1995; Purcell et al., 1998).

2.4.4 Salt and Soil pH

Legumes have long been recognized as either sensitive or only moderately resistant to salinity (for review see Zahran, 1991). It is very difficult to separate salt effects on the legume symbiosis into those acting on the symbiotic relationship directly and those affecting the plant and, therefore, affecting the relationship through the plant (Brignoli and Lauteri, 1991). Salt may directly affect infection and nodule development (Zahran and Sprent, 1984). Under severe stress both the reduction in leghemoglobin content and inhibition of bacteroid respiration may be salt-induced and contribute to inhibition of nitrogen fixation (Delgado et al., 1994).

Under high salty concentrations nodule water activity is negatively affected by osmotic withdrawal from nodules. Bacterial colonization and root hair curling of plants maintained at 100 mol m^{-3} NaCl are both reduced when compared to those plants grown at 50 mol m^{-3} and the proportion of root hairs containing infection threads is reduced by about 30% (Zahran and Sprent, 1984). Three weeks after treatment nodule numbers are reduced by about 50% in the saline treatment, and nodule weight by more than 60%.

Genotypic differences were observed in growth and nitrogen fixation in sixteen soybean cultivars grown under NaCl (80 mM) stress, and the root played a primary role in the regulation of ion uptake, as well as growth and nitrogen fixation, under salt stress

(Velagaleti et al., 1990)

In most species nodulation will not occur below pH 4.5 to 4.8. Low pH generally reduces nodulation. This may be due to reduced growth and multiplication of rhizobia in the soil (Rice et al., 1977), increased numbers of ineffective rhizobia, or sensitivity of the infection process (Date, 1988). Some strains of *(Brady)Rhizobium* isolated from low pH soils will only grow at pH around 4.5. Acid soil may also be low in available calcium, magnesium, phosphorus, and molybdenum and may contain levels of aluminium and manganese toxic to the host plant (Sprent and Sprent, 1990). Lowering the medium pH from 7.0 to 5.0 affected the number and relative concentration of the Nod metabolites made (Mckay and Djordjevic, 1993).

The exudation from subclover roots of isoflavonoids required for nod gene induction in *R. leguminosarum* bv. *trifolii* was reduced when the plants were grown at a pH of < 5 (Richardson et al., 1988a). Low pH and increased aluminium concentrations were also shown to restrict *nod* gene induction in *R. leguminosarum* bv, *trifolii*, although this effect could be partially reversed by addition of calcium (Richardson et al., 1988b).

2.4.5 Biological Factors

2.4.5.1 Inoculation and Strain Competition

The difficulty in establishing large numbers of nodule bacteria in the presence of other soil microorganisms has led to suggestions that nodule bacteria are poor competitors for soil nutrients (Alexander, 1985). Adequate populations of *B. japonicum* must be present to produce a well-nodulated soybean crop. Smith et al. (1981) determined that an inoculum level above 1×10^5 rhizobia per centimetre of row was necessary to establish effective nodulation. High soybean yields also require adequate levels of phosphorous and potassium (Johntson, 1987). Liming acid soils to a pH of 6.0 to 6.5 is an important prerequisite for profitable soybean production (Scott and Aldrich, 1983).

Pure culture inoculation of legumes was demonstrated as early as 1896. However, legume inoculants do not demonstrably affect yields in many soils where

indigenous strains are present. One limitation is the problem of competition. Most of the data for soybean indicate that between 5 and 20% of nodules will be formed by the inoculant strains in the first year following introduction of the strain (Streeter, 1994). Superior nitrogen-fixing inoculum strains are often unable to form a significant proportion of the nodules in a field situation because of competition from indigenous strains. Thus, improvements in the competitiveness as well as the nitrogen-fixation efficiency of inoculants would be desirable (Appelbaum, 1990). The prospects of developing strains of legume nodule bacteria that provide higher productivity for the leguminous plant was described recently by Robert and Triplett (1996).

Methods for measuring nodulation competitiveness have been discussed (Triplett and Sadowsky, 1992). Three factors (a prokaryotic genome, an eukaryotic genome, and the environment) interact to influence the outcome of field inoculations. McDermott and Graham (1990) showed that speed of nodulation is crucial to nodulation competitiveness.

Used judiciously where needed and performed properly, legume inoculation is a significant agent for improving crop productivity and soil fertility. Although rhizobia are as widely distributed as legumes themselves, there are many soils devoid of strains suitable for an introduced legume and some soils where rhizobia are few after a prolonged period without production of the legume. Inoculants are prepared by adding fermenter-grown broth containing a large population of rhizobia to powdered carrier followed by a period of incubation. Graham-Weiss et al. (1987) proposed direct fermentation on nutrient-supplemented vermiculite for production of bacterial inoculants. Griffith and Roughley (1992) have suggested that some advance might be made through the control of moisture contents in peat culture and correct use of packaging films. The oxygen and nutrient requirements of rhizobia in peat culture remain to be properly addressed. The stage of growth of the fermenter-grown rhizobia when it is added to the peat carrier may be of significant factor determining the final population of the peat inoculant (Brockwell and Bottomley, 1995).

A prime aim of legume inoculation is to maximize survival of inoculant during the period between its introduction to the soil and the development of a legume

rhizosphere which it can colonize. Application of inoculant to the seed surface prior to sowing is the traditional, most commonly used and easiest means of inoculation, although viability of the rhizobia is subject to the hazards of drying, fertilizer contact and seed coat toxicity. Tenacity is an important characteristic of adhesives used to ensure that inoculant is not lost from machinery.

Many factors influence the success of an inoculant per se. Perhaps the most important are the inoculum rate and the abundance in the soil of indigenous rhizobia capable of nodulating host. Weaver and Frederick (1974a) found in greenhouse experiments that the number of nodules on *Glycine max* increased with increasing rates of *B. japonicum* inoculant. These increases were not observed, however, in soils containing more than 1000 rhizobia g^{-1} soil. Results of field studies were similar (Weaver and Frederick, 1974b), and the authors suggested that in order for the inoculant to form 50% of the nodules, the inoculum should be supplied at 1000 times the level of indigenous rhizobium population.

The ability of a *(Brady)Rhizobium* strain to adapt to prevailing soil conditions and to persist into subsequent growing seasons is an important consideration in choosing inocula. Mcloughlin et al. (1984) used a dominant field isolate of *R. trifolii* as an inoculant in the field in which it originated. It was much more competitive than two non-indigenous strains used in the study, forming the majority of nodules and persisting well into the second growing season. Fast-growing soybean rhizobia (*S. fredii*) of Chinese origin are more effective for Chinese lines of soybean, including *Glycine soja*, than for North American cultivars (Dowling and Bohlool, 1985).

(Brady)Rhizobium strains vary tremendously in their response to the environment. Nutritional response, soil, temperature, phage resistance, mineral composition, nutrient availability (as well as toxicity), and previous land usage affect rhizobia in different ways (Trinick, 1985; Schmidt et al., 1985; Hodgson et al., 1986; Hartel et al., 1984). In addition, there is strong circumstantial evidence that genetic exchange occurs among rhizobia in soil (Broughton et al., 1984).

Environmental factors affect the growth of both plants and rhizobia. As plants

must develop a rhizosphere to support rhizobial growth as well as build a morphologically developed nodule to house the invading rhizobia, it is probable that any factor that adversely affects plant growth will also profoundly affect competition for nodulation. McNeil (1982) found that one strain of *B. japonicum* out-competed another for nodulation sites in the presence of nitrate. Growth of *Rhizobium* in pure culture under low phosphate levels has been found to vary among strains (Beck and Munns, 1984). Phosphorous limitation is exacerbated by low pH and the combination of pH and phosphorous levels can have a strong influence on competition for nodulation (Almendras and Bottomley, 1985). Extreme soil acidity is usually linked with high aluminum levels and these inhibit growth of the legume host (Franco and Munns, 1982), but this has little effect on the survival of rhizobia in the soil (Hartel and Alexander, 1983). Low pH (in liquid medium) inhibits nodulation by *Bradyrhizobium* (Evans et al., 1980). Changes in soil pH may also alter the relative proportion of nodules formed by competing strains of *R. meliloti* (Dughri and Bottomley, 1983).

Soil temperature is a major factor in the ability of *Rhizobium* to persist and compete. Strains differ in their ability to survive and grow over a range of temperatures (Hartel and Alexander, 1984). Weber and Miller (1972) planted soybean plants in pots containing soil with a large population of indigenous rhizobia and found different serogroups predominated in the nodules of plants that were incubated at different temperatures. Two strains of *R. trifolii* were inoculated onto clover plants; one strain formed the majority of nodules at 12°C, but the other strain was more competitive at 25°C, and the bacterial strain x temperature x variety interaction was highly significant (Hardarson and Jones, 1979). So, even though temperature cannot be controlled under field conditions, *(Brady)Rhizobium* can be selected for optimum nitrogen fixation or competitive abilities under the prevailing conditions so that the strain's optimum temperature for nodulation and competitiveness are similar to the soil temperature at the time of inoculation. Furthermore, rhizobia with a metabolism adapted to a particular temperature may compete better at that temperature.

Drought conditions, which are often found in conjunction with salt stress, also

affect *(Brady)Rhizobium* survival and competition (Osa-Afiana and Alexader, 1982). An isolate from Tanzania (where drought is frequent) was much more competitive under extreme drought stress than two European isolates, suggesting that the ability to compete in dry soil may also be heritable

A study on carbon distribution in the *Vicia faba* symbiosis revealed that nodules on plants infected with VAM receive twice as much fixed carbon as those on uninfected plants (Kucey and Paul, 1982). As yet, there is no evidence that mycorrhizal fungi influence competition among *(Brady)Rhizobium* strains (Kosslak and Bohlool, 1984) or compete for *(Brady)Rhizobium* infection sites (Subba Rao, 1984).

Bacteriocins are functionally defined as specific, non-self-propagating agents produced by one bacterium and antagonistic toward other similar bacteria. Bacteriocin-producing strains are common among the Rhizobiaceae. *B. japonicum* bacteriocin-producing strains did not have an advantage over sensitive strains in competition for nodulation of *G. max* grown on perlite (Gross and Vidaver, 1978). Bacteriocins can provide a selective advantage against undesirable rhizobia. In competition tests this ineffective strain formed nodules more successfully than an effective strain. These examples point to an important role for bacteriocin-like substances in competition among *(Brady)Rhizobium* strains; bacteriocins presumably affect competition by altering the population of sensitive rhizobia in the rhizosphere.

Many compounds have been identified that inhibit flavonoid induction of *nod* genes in *(Brady)Rhizobium* spp., and the formation of soybean nodules can be prevented by the use of such inhibitors (Cunningham et al., 1991; Kosslak et al., 1990). Altered EPS production may enhance the competitiveness of strains of *B. japonicum* (Bhagwat et al., 1991)

The intrinsic diversity of rhizobia suggests great potential for competition among strains for attachment and nodule initiation. Some strains might have higher-affinity receptors, e.g. for lectins, than other strains, or greater competence for attachment or nodule formation. Host-specific genes have also been implicated in competition between *R. trifoii* strains (Djordjevic et al., 1985). It is also possible that rhizobia that

can initiate infection and nodulation quickly and efficiently will have a competitive advantage over those that are slower. Initial nodules are known to apically suppress further nodulation in legumes, and the delayed inoculation of a second strain has been shown to influence competition (Kosslak et al., 1983). When less competitive strains were inoculated before competitive strains, the nodule occupancy of the less competitive strains was increased. Diatloff and Brockwell (1976) observed that a strain of *B. japonicum* that was unsuccessful in forming nodules on one *G. max* cultivar could prevent other strains of *B. japonicum* in mixed inocula from nodulating this line.

Streeter (1994) pointed out that strain versus plant genotype interactions observed in artificial environments such as growth pouches are likely to be significantly different from those in soil. Strain comparisons should involve multiple plant genotypes and, if not done in the field, should involve the use of soil and some control of root temperature.

That the host exerts a specific selective effect on a mixed soil population has been known for over thirty years (Vincent and Waters, 1953; Cregan and Keyser, 1988), and the geognostic basis of the selection has been studied (Chandra and Pareek, 1985; Jones and Hardarson, 1979). In *G. max* the inheritance of preference for a particular *Rhizobium* strain it may be related to a single gene (Materon and Vincent, 1980). Thus it may be possible to breed legumes that can differentiate between various indigenous rhizobial strains. Presumably, it will be possible to manipulate both the host and the rhizobial genes in such a way that nodulation will only be possible with specific legume-*Rhizobium* combinations. To achieve any of these goals, however, it is vital that research on competition for nodulation goes hand in hand with legume breeding program based on a thorough understanding the molecular biology of nitrogen fixation. Studies will continue on strain competition and the ability of inoculants to compete successfully with naturalized rhizobia for nodulation sites. The ability to persist as a permanent of soil microflora (Date, 1991) and to maintain symbiotic characteristics (Brunel et al., 1988) will remain objectives in inoculation strain selection.

2.4.5.2 Mycorrhizal Fungi

It has long been known that most legumes are also normally colonized by arbuscular mycorrhizal (AM) fungi. In all cases examined, the legume/*Rhizobium*/mycorrhiza association is more efficient than the association of any two of these (Barea and Azcon-Aguilar 1983). Improved phosphorous uptake by mycorrhizae is recognized as the major cause of plant growth enhancement. An N_2-fixing legume will have a larger P requirement than that a non-fixing one and if the soil is P limiting, a mycorrhizal legume will fix more N than a non-mycorrhizal one.

AM fungi can also facilitate the transfer of nutrients between adjacent plants. Hamel et al. (1991), using the ^{15}N dilution method, demonstrated that N_2 fixed by soybean plants can be transferred to its intercropped cereal partners by mycorrhizal fungi. They found that the presence of *Glomus versiforme* increased the efficiency of indirect ^{15}N transfer from the labelled soybean plants to corn by 45%. However, negative results were found in alfalfa-timothy and alfalfa-bromegrass mixtures (Hamel et al., 1991).

Both *(Brady)Rhizobium* and AM fungi produce plant hormones or plant hormone-like substances, such as auxins, gibberellins and cytokinins. As well, AM fungi enhance the production of some these compounds in the roots of plants. Some plant-to-bacterium signal compounds (phenolic) have recently been shown to stimulate the germination of mycorrhizal spores (Gianinazzi-Pearson et al., 1989). Thus the possibility exists that the presence of the appropriate *(Brady)Rhizobium* may enhance the likelihood of mycorrhizal infection. The rate of *Glycine max* mycorrhizal infection has been shown to increase under drought stress, and mycorrhizal inoculation has alleviated drought stress under arid conditions with *Acacia* and *Leucaena* (Robson and Bottomly, 1991). As with simple N_2-fixing and mycorrhizal symbioses there are genotype interactions that determine the effectiveness of the tripartite association. Presumably some combination(s) of legume, mycorrhizal fungi and N_2-fixing bacterial genomes would be optimal, while others would promote plant growth less well. It is on soils low in both N and P that dually infected legumes have a large potential acting as pioneer species.

2.4.5.3 Plant Growth Promoting Rhizobacteria

In recent years there has been renewed interest in the use of soil bacteria which, when applied to seeds, tubers or roots, are able to colonize plant roots and stimulate plant growth and crop yield. These have been termed plant growth promoting rhizobacteria (PGPR) (Kloepper et al., 1980) and have been shown to increase plant growth, development, and yields in such non-legume crops as potato, radish, sugar beet, wheat and canola (Gaskins et al., 1985; Polonenko et al., 1987). Some reports suggest positive effects of some PGPR on the legume N_2-fixing symbiosis; the bacteria responsible for this effect are termed nodule promoting rhizobacteria (NPR). Inoculation with PGPR, often pseudomonads, and *(Brady)Rhizobium* has been demonstrated to positively affect symbiotic N fixation by enhancing root nodule number or mass (Grimes and Mount, 1984; Polonenko et al., 1987; Yahalom et al., 1987) and increasing nitrogenase activity (Iruthayathas et al., 1983). Zhang and Smith (1996) concluded that inoculation of soybean plants with PGPR strains produced a wide range of effects, which varied among strains of PGPR and over root zone temperatures.

Work in our laboratory has shown that PGPR increase early season nodulation and total seasonal nitrogen fixation of soybean grown in an area with cool spring soils. The ability of PGPR to stimulate soybean nodulation and growth is related to their ability to colonize soybean roots, and this largely depends on root zone temperature. All steps in early nodulation were stimulated by the presence of PGPR.

2.4.5.4 Pathology and predation

When N_2-fixing organisms are afflicted with pests or pathogens the fixation process is usually affected. Conversely, the N_2-fixing symbiosis may affect the plant disease either by its effect on plant nutrition status or directly (Sprent and Sprent, 1990). Plant viruses affect the structure, physiology and biology of both root nodules and host plants. Nodules may have levels of virus such that nodule activity is directly affected (Sprent and Sprent, 1990). Root nodules may be eaten by some insects or larvae (Wolfson, 1987).

(Brady)Rhizobium have been shown to be sensitive to low concentrations of fungicides, and the fungicide treatment of seeds often leads to poor nodulation (Curley and Burton, 1975; Graham et al., 1980). As different levels of resistance to fungicides have been observed among strains of *Rhizobium* (Faizah et al., 1980), it should be possible to use resistant isolates as inoculants.

Both bacterial and protozoan predators in soil are known to multiply at the expense of *(Brady)Rhizobium*. The practical importance of protozoa in limiting populations of *(Brady)Rhizobium* is still uncertain. The most significant effect of bacteriophages is likely to be the production of genetic change in host populations. *Bdellovibrio*, an intracellular bacterial parasite of *Rhizobium* first isolated from plant pathogenic bacteria, is widespread in the soil and in the laboratory, and is capable of infecting and lysing large numbers of rhizobia.

Preface to section 3

Section 3 is composed of a manuscript by Pan B and Smith DL published in *Journal of Agronomy & Crop Science*, 1998, 180:77-82. The literature cited in this and the following sections is listed together in the reference section at the end of the thesis. Figures and tables in each section are always presented at the end of each section.

Previous work in our laboratory showed that low temperature delayed soybean nodulation and nitrogen fixation. I started with this experiment to measure signal compound production in soybean roots at three root zone temperatures using three soybean cultivars. This experiment also introduced me to the concepts associated with signal compounds and the methodologies for measuring them.

Section 3

Genistein and Daidzein Concentrations and Contents in Seedling Roots of Three Soybean Cultivars Grown under Three Root Zone Temperatures

3.1 Abstract

Daidzein and genistein are plant-to-bacterium signal compounds involved in soybean nodule formation. They can induce *nod* gene expression in *Bradyrhizobium japonicum*. The objective of this study was to determine whether the production of signal molecules was affected by low root zone temperatures (RZTs) in a manner that varied among soybean cultivars. Daidzein and genistein concentrations of soybean seedling roots were measured at three RZTs by high performance liquid chromatography (HPLC). The results indicated that daidzein content and concentration per plant were higher at 15 and 17.5°C than those at 25°C. AC Bravor had higher daidzein contents and concentrations than did Maple Glen and KG20. At 17.5°C, KG20 had higher genistein content and concentration levels than Maple Glen, and no difference existed for the two cultivars at 15 and 25°C. Daidzein contents and concentrations of Maple Glen and AC Bravor increased with harvest time. However, for cultivar KG20, the content and concentration decreased at 19 days after inoculation. Genistein contents and concentrations of the 3 cultivars increased under each RZT up to the last harvest. There was an interaction between soybean cultivar and RZT for root genistein and daidzein contents and concentrations. The content and concentration of daidzein in soybean seedling roots were much higher (more than 5 times) than those of genistein.

Key words: *Glycine max* [L.] Merr., Root, Temperature, Genistein, Daidzein.

3.2 Introduction

The establishment of an effective N_2-fixing nodule requires expression of the bacterial (*nod*) genes and the resulting production of Nod factors (Stacey et al., 1992). Prior to this

process, an exchange of molecular signals between the host plant and *(Brady)Rhizobium* over space and time is essential (Long, 1989). The first apparent exchange of signals involves the secretion of phenolic compounds, flavones and isoflavones, by legumes (Peters and Verma, 1990). The plant flavones and isoflavones represent an important class of plant-to-bacterium signal molecules active during nodulation. They appear to have multiple roles and act during several stages of nodule development, including triggering the co-ordinated expression of a series of bacterial *nod* genes (Long, 1989), inhibiting the induction of *nod* genes (Kosslak et al., 1990), acting as chemo-attractants (Barbour et al., 1991), stimulating or inhibiting the growth of specific *(Brady)Rhizobium* strains (Hartiwig et al., 1991; Kape et al., 1992), playing a role inside the root during nodule meristem formation (Schmidt et al., 1992), and functioning as modulators of polar auxin transport (Jacobs and Rubery, 1988).

The isoflavones genistein and daidzein are the major compounds in soybean (*Glycine max* [L.] Merr.) root extracts responsible for induction of *nod* genes of *Bradyrhizobium japonicum* (Kosslak et al., 1987). Cho and Harper (1991) reported that a hypernodulating soybean mutant, derived from the cultivar Williams, had higher root concentrations of isoflavonoid compounds (genistein and daidzein) than did Williams at 9 to 12 days after inoculation. Matthews et al. (1989) showed that root extract from soybean cultivar Williams had three to six times more ability to induce *Bradyrhizobium nod* genes than did 3-day-old cultivar Bragg seedlings. Kapulnik et al. (1987) reported that the super-nodulating alfalfa HP32 had more root nodules and fixed more N_2 than the parental HP population. Flavonoid extracts of HP32 seedling roots were shown to contain a 60% higher concentration of compounds that induce the transcription of a *nodABC-lacZ* fusion in *R. meliloti* than comparable extracts of HP roots.

If genetic variance for the ability to produce signal molecules at low RZTs exists, plant breeders might be able to select for improved soybean nodulation at low RZTs by selecting for genotypes with high isoflavonoid concentrations. In this study, we used three soybean cultivars of different maturities to test for variance among cultivars for genistein

and daidzein root concentrations under low RZTs.

3.3 Materials and Methods

Soybean cultivars KG20 (short maturity, 113 days in Quebec), Maple Glen (middle maturity, 121 days in Quebec) and AC Bravor (long maturity, 128 days in Quebec) were used. They have all been developed for production under the short season conditions of eastern Canada. The experiment was carried out on a controlled environment growth bench (Model GB48, Controlled Environments Ltd., Winnipeg, MB, Canada) at an irradiance of 300 $\mu mol\ m^{-2}\ s^{-1}$ for a 16:8 h (day:night) photoperiod and a constant air temperature of 25°C. The plastic plant pots were sealed to the bottom of tanks. A hole through the bottom of the tanks allowed each pot to drain when it was watered. The root zone temperatures were controlled (± 0.5°C) by circulating cooled water in the tanks around the pots. Soybean was grown in a mixture of Turface (Applied Industrial Materials Corp., Deerfield, IL):sand (1:1, v/v). Plants were watered with a modified full strength Hoagland's solution (Zhang and Smith, 1995).

The experiment tested three RZTs, 15, 17.5 and 25°C, and was arranged in a split plot design with three replications. The main plot factor was RZT and cultivar was the subplot. Six soybean seeds were directly sown into each pot. Plants were inoculated at the VC stage (Fehr et al., 1971). Under different RZTs, the times for the plants to reach the VC stage were different, so the plants were inoculated at different times for each RZT.

The inoculum was produced by culturing *B. japonicum* strain USDA110 in yeast extract mannitol broth in 250 mL flasks shaken at 125 rpm at 25°C. When the sub-culture reached mid log phase, pure medium was used to dilute the inoculum to an OD_{620} of 0.08 (equivalent to 10^8 cells mL^{-1}). Two mL of inoculum were added to 20 mL of distilled water which was then divided evenly among the pots. After inoculation, a layer of perlite (1 cm depth) was placed on the top of each pot to provide an insulating layer between the rooting medium surface and the air.

Plants were harvested at 2, 6, 10, 15 and 19 days after the inoculation. The roots

were washed in distilled water and carefully dried with paper towels before being weighed. Harvested soybean roots were placed in vacuum flasks with 10 times the root fresh weight of 80% methanol-dH_2O. Root tissue was placed under vacuum for 20 minutes. The pH was adjusted to 5.3 with 0.1 N HCL. Samples were transferred into 250 mL flasks at 4°C and shaken for 48 h at 150 rpm. Plant roots and extracts were separated by filtering through Whatman No. 1 filter papers. The resulting extracts were rotary evaporated to remove the methanol fraction. The aqueous fraction was freeze-dried, and resuspended to equal 10 mg of extract dry weight per mL of distilled water. The resuspended extracts were phase-partitioned three times against equal volumes of ethyl acetate, retaining each organic fraction. Dry sodium sulphate was added to absorb the remaining water. The clear ethyl acetate fraction was decanted and rotary evaporated at 30°C until dry. The sample was then redissolved in 1 mL of HPLC grade pure methanol and centrifuged for 15 min before being run through an HPLC.

The genistein and daidzein concentrations were determined using a Waters HPLC system (Waters Associates Inc., Milford, MA) consisting of a model 712 WISP, two 510 pumps and a 441 UV detector operating at 254 nm. Separation was achieved by a 3.9 x 300mm μBondapak C18 column (Waters Associates Inc. Milford, MA) using a mobile phase consisting of a 60:40 ratio of methanol:distilled water. The run time for separation was 15 minutes with an isocratic flow rate of 1.0 mL min^{-1}. Commercial genistein (4, 5, 7-trihydroxyisoflavone, purity of 98%, Sigma) and daidzein (4', 7-Dihydroxyisoflavone, purity > 98%, ICN) were used as standards.

Results were analyzed statistically by analysis of variance using the Statistical Analysis System computer procedure (SAS Institute Inc., 1988). The LSD test was applied to make comparisons among the means at the 0.05 level of significance, when analysis of variance showed significant treatment effects.

3.4 Results

Daidzein Concentration and Content over Time

Daidzein concentrations of the three soybean cultivars increased with successive harvest time (Fig. 3.1). The concentrations were lower for the first two harvests, and then increased strongly at later harvest. At 17.5 and 25°C, daidzein concentration and content were generally lower than at 15°C RZT.

At 15°C, the daidzein content of all the three cultivars increased with harvest time (Fig. 3.2). There were large increases in daidzein content at the last harvest for all three cultivars. At 17.5°C, daidzein content increased with harvest time, the only exception being at the 5th harvest; at that time daidzein content of KG20 decreased (Fig. 3.2). At 25°C, the daidzein content of soybean cultivar KG20 decreased at the 4th and 5th harvests. For Maple Glen and AC Bravor, daidzein content increased from the first to the fifth harvest (Fig. 3.2).

Genistein Concentration and Content over Time

Genistein concentrations and contents increased with harvest time (Fig. 3.3 and 3.4). Genistein concentrations of all three cultivars increased strongly from the third harvest on. Genistein concentrations and contents were much lower (less than one fifth) than those of daidzein (Fig. 3.1, 3.2, 3.3 and 3.4).

Daidzein and Genistein Contents and Concentrations over Temperature and Cultivars

Daidzein concentrations and contents of soybean roots were higher under 15 and 17.5°C RZTs than under the 25°C RZT (Fig. 3.1 and 3.2). There was little difference in daidzein content and concentration between 15 and 17.5 °C (results not shown). At each RZT, AC Bravor had higher daidzein contents and concentrations than did Maple Glen and KG20.

At 15 and 25°C RZT, there were no differences among the three cultivars for genistein concentrations and contents (Fig. 3.3 and 3. 4). At 17.5°C RZT, KG20 and AC

Bravor had higher genistein concentrations and contents than Maple Glen.

3.5 Discussion

RZTs and Isoflavonoid Concentration and Content

In soybean root systems, daidzein and genistein concentration and content can be affected by the activities of enzymes leading to their biosynthesis and/or excretion. Eleven enzymes including phenylalanine ammonia lyase (PAL) and chalcone synthase, are involved in the biosynthesis of genistein and daidzein in soybean plants (Barz and Welle, 1992). Isoflavonoid biosynthesis and excretion are different processes, and RZT could have differential effects on them. For many plants, the extractable amount of PAL increases as temperature declines. This is because the rate of synthesis is less affected by the temperature than the rate of degration (Graham and Paterson, 1982). Graham (1991a) pointed out several lines of evidence which suggest that the release of isoflavones may be a specific and regulated phenomenon, and that soybean seeds may possess a feedback mechanism to regulate the exudation of their very large stores of isoflavone signal molecules. This excretion process may also be inhibited by low temperature. The balance between the synthesis and excretion will determine the concentrations and contents of genistein and daidzein in soybean roots. If this balance is affected by RZT, the concentration will be affected. This could account for the RZT induced changes in genistein and daidzein concentrations we observed in soybean roots.

The results of this experiment are in general agreement with the findings of Zhang and Smith (1996). They found that when soybean plants were germinated and maintained at RZTs ranging from 13°C to 17°C, root genistein concentration and content per plant were lower than those of plants germinated and maintained at RZTs above 17°C. Their explanation was that under lower RZTs the biosynthesis of genistein in plant tissues may have been reduced by declining relative activity of enzymes in the synthesis path way. Our results show no significant change and a trend toward a decrease with decreasing RZT in root genistein concentration over RZT for the cultivar Maple Glen, which was the only

cultivar tested by Zhang and Smith (1996). This pattern is different from that of the other two cultivars tested here. If suboptimal (below 17°C) RZTs reduced genistein biosynthesis, then the increased genistein concentration and content in their experiment could only be explained by the decrease in genistein excretion with more genistein retained inside the root instead of being excreted into the growth medium. In this respect, their findings also agree with our results.

Cultivars and Changes in Signal Molecule Levels over Time

Daidzein and genistein concentrations and contents of the three soybean cultivars used in this experiment responded differently (to different degrees) to RZTs, but the overall pattern of response was the same. The increases in daidzein and genistein concentrations and contents up to 19 days after inoculation are in agreement with the results of Cho and Harper (1991). When alfalfa plants were grown under controlled environment conditions, isoflavonoid conjugates continued to accumulate as the plants matured, over a 57-day period (Tiller et al., 1994). Interactions existed between cultivar and RZT, for daidzein and genistein concentrations. Daidzein and genistein contents and concentrations of the late maturing soybean AC Bravor tended to be higher, while the levels of those variables were not different between the early and middle maturing soybean cultivars. The possibility that late maturity cultivars are more affected than early maturity ones has to be further investigated as only one cultivar of each soybean maturity group was used in this experiment.

Conjugates of the isoflavones daidzein and genistein are major metabolites in all embryonic organs within the dry seed and seedling root, hypocotyl, and cotyledon tissues at all times after germination (Graham, 1991). It has been reported that following inoculation with *Rhizobium meliloti* alfalfa seedlings exude isoflavonoid conjugates and that formononetin 7-O-glucoside-6'-O-malonate is a selective activator of *nod*-genes in the nodulating bacteria (Dakora et al., 1993). This observation suggests that isoflavonoid conjugates are also important in signalling between root and rhizosphere. Both the

formation and hydrolysis of these isoflavone conjugates are subject to an intricate regulation program, and they may play a direct role in the responses of soybean tissues to elicitor treatments (Graham, 1991). In this study, we only measured daidzein and genistein concentration and content. The quantity, regulation and function of the conjugate forms of isoflavones among soybean cultivars may be important and needs to be studied.

Since the effects of RZT on root genistein concentration varied among cultivars, presumably due to differential effects on the balance between synthesis and excretion, a practical aspect of this work is that simple selection of root genistein concentration in a plant breeding program, could not be used to develop soybean cultivars which nodulate better at lower RZTs. It would appear that a plant breeder would have to measure and attempt to increase actual signal molecule excretion from soybean roots at lower RZT. This is likely to be a more difficult task.

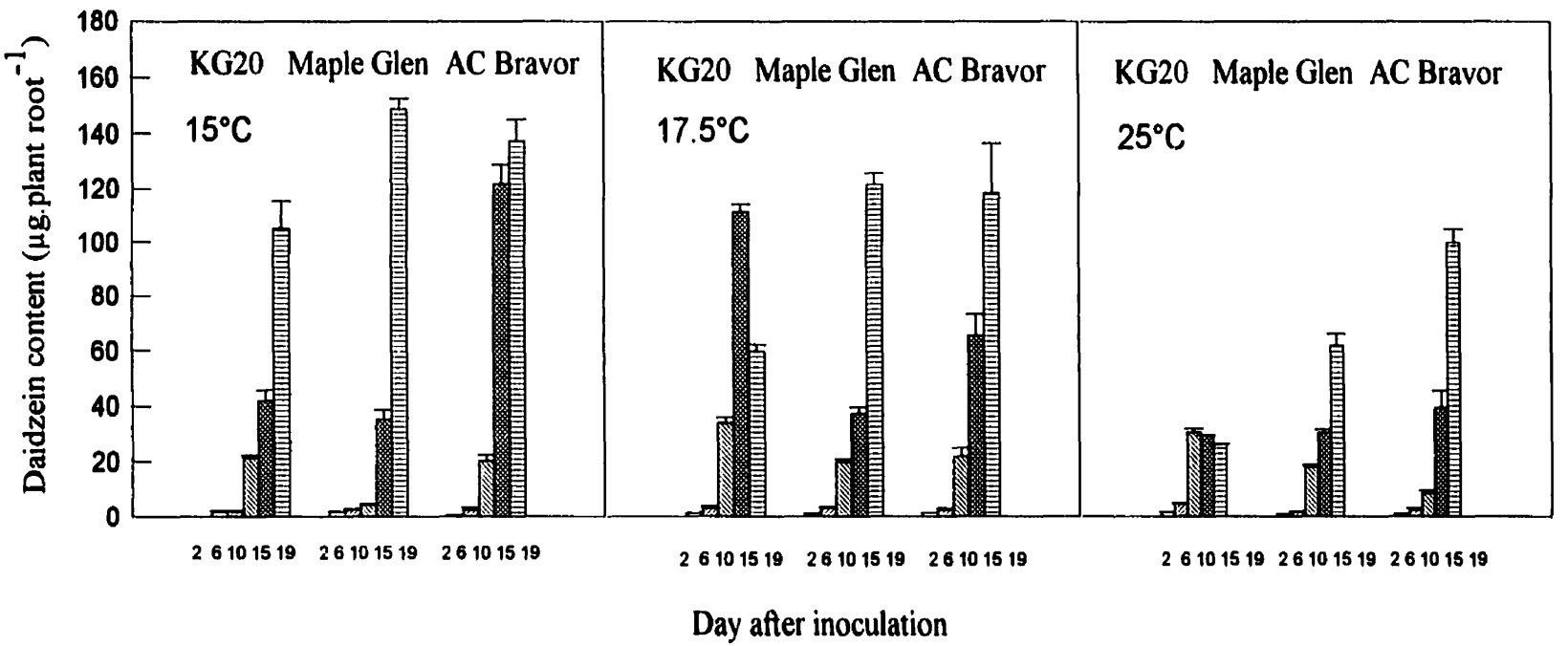

Fig. 3.1 Daidzein contents of soybean seedling roots under three root zone temperatures. Bars indicate one standard error unit.

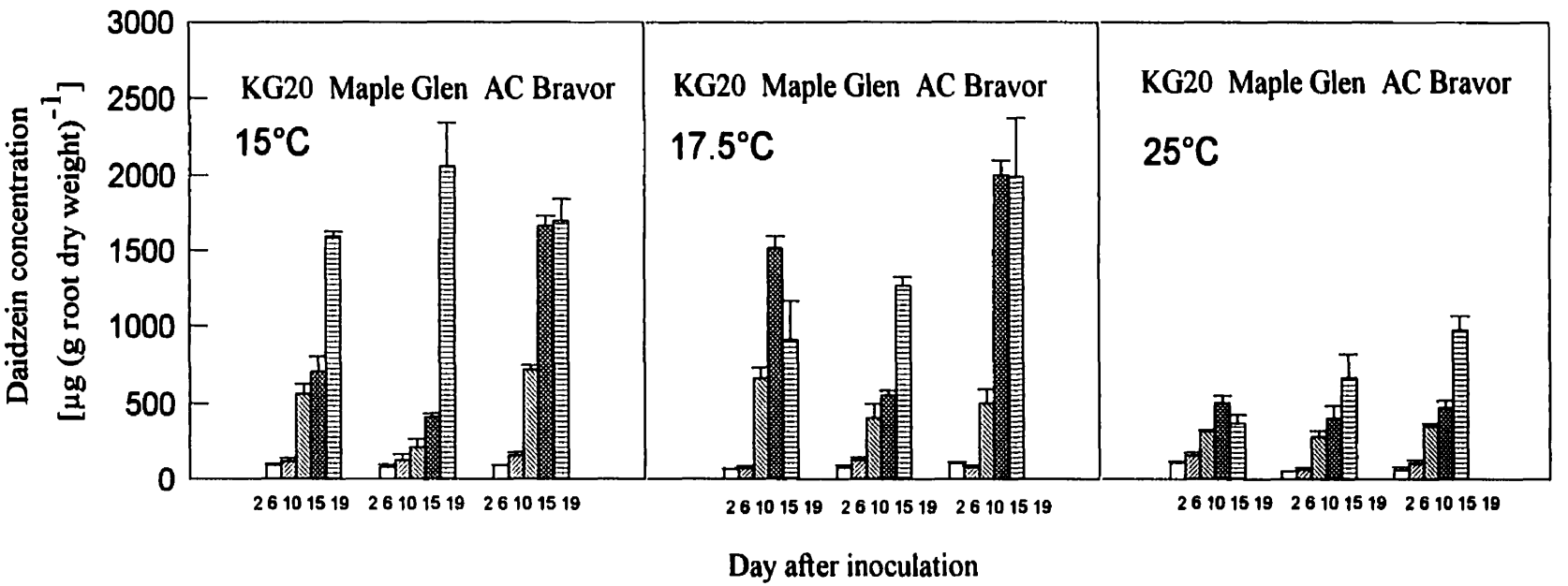

Fig. 3.2 Daidzein concentrations of soybean seedling roots under three root zone temperatures. Bars indicate one standard error unit.

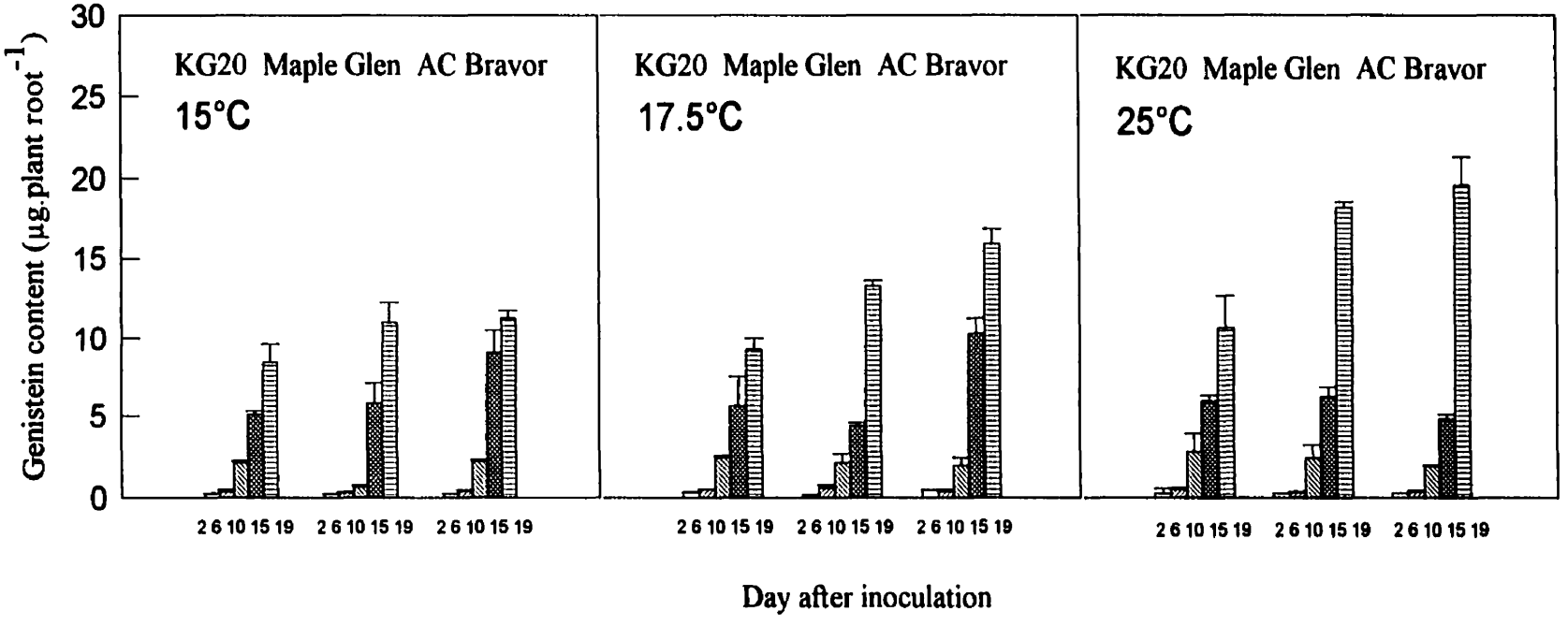

Fig. 3.3 Genistein contents of soybean seedling roots under three root zone temperatures. Bars indicate one standard error unit.

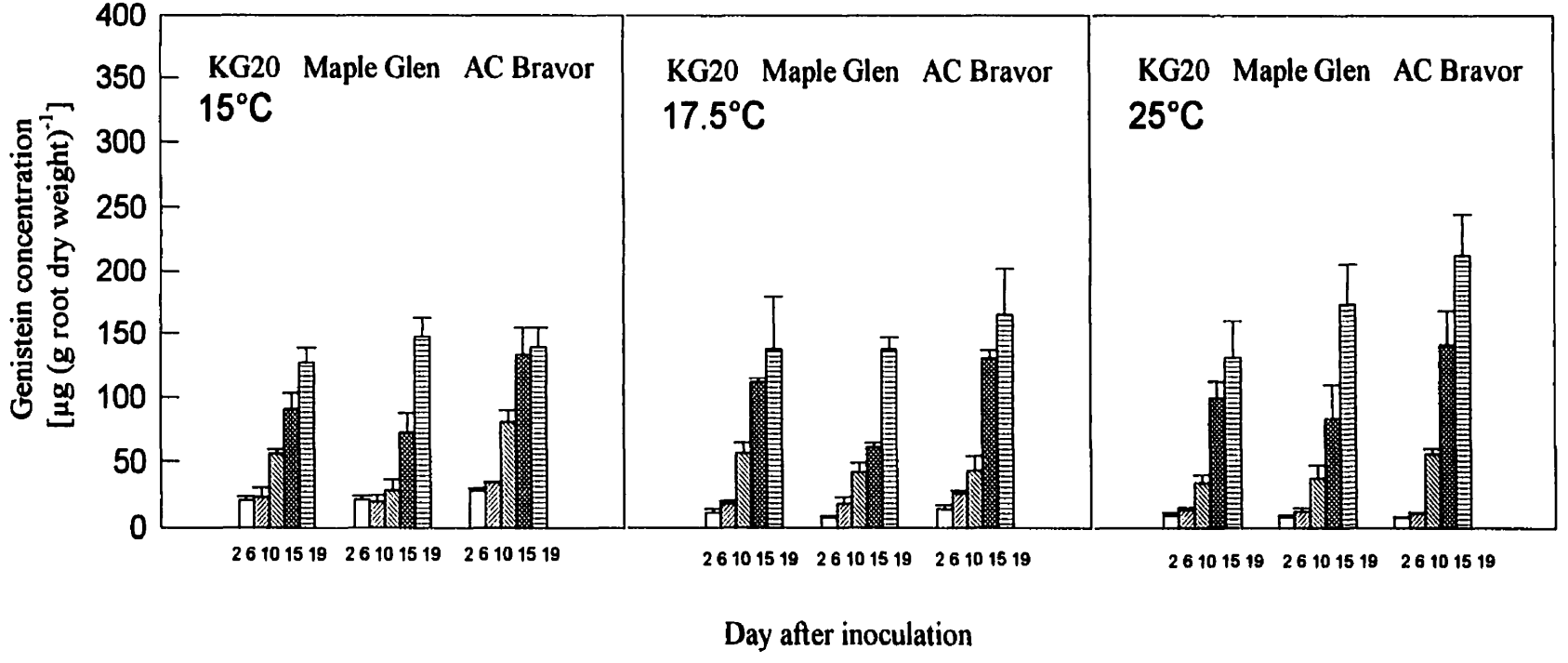

Fig. 3.4 Genistein concentrations of soybean seedling roots under three root zone temperatures. Bars indicate one standard error unit.

Preface to section 4

Section 4 is composed of a manuscript by Pan B, Zhang F and Smith DL published in *Journal of Plant Nutrition*, 1998, 21:1631-1639.

Previous work in our laboratory showed that genistein concentration in soybean root system increases with seedling development, and decreases after the onset of N_2 fixation. This experiment was conducted to determine whether addition of genistein to the rooting medium at the onset of N_2 fixation would increase nodulation thereafter. We have manipulated soybean nodulation and nitrogen fixation with signal compound additions. This experiment also introduced me the concepts of soybean nodulation and N_2 fixation.

Section 4

Genistein Addition to the Rhizosphere of Soybean [*Glycine max* (L.) Merr.] Increase Nodulation

4.1 Abstract

In the legume-*(Brady)Rhizobium* symbiosis, signal exchange between the host-plant and the symbiotic bacterium is an essential step in nodule formation. Genistein is the most effective plant-to-bacterium signal in the soybean [*Glycine max* (L.) Merr.] N_2-fixing symbiosis. Its concentration in soybean root system increases with seedling development, and decreases immediately after the onset of N_2 fixation. This study was conducted to determine whether addition of genistein to the soil rhizosphere at the onset of N_2 fixation would increase nodulation thereafter. The results indicated that watering soybean plants with a solution containing genistein beginning at the onset of N_2-fixation increased nodule size, nodule number and nodule weight per plant. Shoot nitrogen concentration was also increased. Soybean cultivar AC Bravor was more sensitive to genistein addition than Maple Glen.

Key Words: *Glycine max*, genistein, nodulation

4.2 Introduction

Nitrogen availability is an important factor governing yields of agricultural crops. The legume-*(Brady)Rhizobium* symbiosis channels large quantities of atmospheric nitrogen into both agricultural and non-agricultural systems, and thus is of fundamental economic and ecological significance.

Development of N_2-fixing legume root nodules involves a complex series of interactions between the infecting bacteria and the host root (Stacey 1995). Leguminous plants are able to synthesize specific phenolic compounds that are sensed by rhizobacteria (Peters and Verma 1990). These low molecular weight compounds activate the *nodD* gene(s) of *(Brady)Rhizobium*, which control(s) the expression of the common *nod* genes

(Kosslak et al. 1987). As a result of *nod*-gene induction, lipo-chitooligosaccharide nodulation factors (Nod factors) are produced by the bacterial symbiont, which in turn elicit root hair deformation, initiation of cortical cell division, and induction of specific changes in cell morphology; that is, these compounds trigger a plant organogenic program leading to nodule formation (Dénarié and Cullimore 1993). A precise exchange of molecular signals between *(Brady)Rhizobium* and host plants is essential to the development of an effective legume root nodule (Verma 1992).

Because isoflavonoids can cause *nod*-gene induction at concentrations in the nanomolar and low micromolar range, agronomists could manipulate the effect of these compounds through direct application of the natural product, selection of the host-strain combinations that stimulate increased synthesis and release of active *nod*-gene inducers, or through plant breeding. As a result of those manipulations, flavonoid-induced transcription of *nod* genes and sustained gene expression could enhance nodulation, promote N_2 fixation and increase grain yields (Dakora 1995). Some legume cultivars apparently supply limiting amounts of the isoflavonoids (Hungria and Phillips 1993; Kapulnik et al. 1987). The effectiveness of isoflavones was found to vary among soybean cultivars (Horvath et al. 1986).

The isoflavones daidzein and genistein are the major components of soybean root extracts responsible for inducing the *nod* gene(s) of *B. japonicum* (Kosslak et al. 1987). Daidzein has less *nod* gene-inducing ability than does genistein (Sutherland et al. 1990). Preincubation of *B. japonicum* with genistein increased nodule number and hastened the onset of N_2 fixation at suboptimal root zone temperatures (Zhang and Smith 1995). Those increases lead to an increase in nitrogen-fixation ability and a reduction in nitrogen limitation of soybean grown in short season areas. Genistein concentration increases with early soybean root development, and then decreases after the onset of N_2-fixation (Zhang and Smith 1996 b). To date, there have been no investigations of whether or how genistein addition to the rhizosphere, beginning at the onset of nitrogen fixation, alters plant nodulation and growth. In this study, we test the hypothesis that adding genistein to

the rhizosphere by watering soybean plants with a genistein solution beginning at the onset of N_2 fixation increases soybean nodulation, and total N_2-fixing ability, leading to increased nitrogen and dry matter accumulation.

4.3 Material and Methods

Experimental design

The experiment was carried out under greenhouse conditions. The air temperature regime was 30/20°C day/night, with the relative humidity being 60 to 70%. The highest daylight intensity was 900 μmol m^{-2} s^{-1}. Turface (Applied Industrial Material Corp., Deerfield, IL):sand (1:1, v/v) was used as a growing medium. Two factors were included in the experiment, genistein concentrations (0 and 5 μM) and soybean cultivars (AC Bravor and Maple Glen). The experiments were arranged in a random completely block design with four replications. Genistein applications were started at the date of onset of N_2 fixation [14 days after inoculation (DAI), indicated by sharply increased photosynthetic rates; Zhang and Smith 1994], and continued throughout the experiment. When the plants needed to be watered, a 10 mM genistein stock solution was added to Hoagland's solution (Hoagland and Arnon 1950), in an amount sufficient to make the 5μM genistein solution, and 250 mL of Hoagland's solution containing either 0 or 5 μM genistein were added to each of the pots at each watering (2 to 7 times per week depending on the plant size). That amount of solution is enough to wet the whole growth medium and whole root system was evenly watered. A total of 12 mg of genistein was added to each pot. In general, molecules of this type are not available as a carbon source for bacteria (Peters and Verma 1990). AC Bravor and Maple Glen were selected as they have been shown to respond differently to application of genistein under cool soil conditions (Zhang and Smith 1996 a).

Plant materials and inoculation

Seeds of the soybean cultivars AC Bravor and Maple Glen were surface-sterilized in 4%

sodium hypochlorite for 7 min, and then rinsed several times with distilled water. Five seeds were directly sown in each of the pots. When the seedlings reached the vegetative-cotyledon stage (VC, unifoliolate leaves unrolled sufficiently that the edges were not touching, Fehr et al., 1971), seedlings were thinned to 2 plants per pot. The Hoagland's solution used in these studies was modified to provide a nitrogen-free solution: the $Ca(NO_3)_2$ and KNO_3 were replaced with 1 mM $CaCl_2$, 1 mM K_2HPO_4, and 1 mM KH_2PO_4.

The inocula were produced by culturing *B. japonicum* stain USDA110 in yeast extract-mannitol broth in 250-mL flasks shaken at 120 rpm at 25°C. A five day old sub-culture (OD≈0.4) was diluted with distilled water to an A_{620} of 0.08 (approximately 10^8 cells mL^{-1}). After thinning the seedlings, 1 mL of inoculum was applied by pipette onto the rooting medium at the base of the plant.

Data collection

Plants were harvested five times. The first harvest was at 14 DAI, and subsequent harvests were made every 10 days. For each harvest small nodule (diameter < 1.5 mm) number, large nodule (diameter ≥ 1.5 mm) number, nodule weights, shoot and root weights, and shoot and root nitrogen concentrations were measured. The photosynthetic rates and chlorophyll fluorescence levels of the central uppermost fully expanded leaflet were measured prior to each harvest. Photosynthetic measurements were taken with a LI-6200 portable photosynthesis system (Li-Cor Inc., Lincoln, NE) between 10:00 and 12:00 in the morning. Chlorophyll fluorescence was measured with a CF-1000 chlorophyll fluorescence measurement system (Morgan Scientific Inc., Andover, MA) at the same time. Nitrogen content was measured by Kjeldahl analysis (Kjeltec system, Tecator AB, Hoganas, Sweden).

Statistical Analysis

Results were statistically analyzed by analysis of variance using the Statistical

Analysis System computer procedure (SAS Institute Inc. 1988).

4.4 Results

Genistein effects on nodulation

Genistein increased large nodule numbers of AC Bravor plants at the third, fourth and fifth harvest (Fig. 4.1A). The greatest increase occurred at the fifth harvest, plants watered with genistein produced 10-32% more nodules than non-genistein watered control plants. There were no changes in large nodule numbers for Maple Glen.

Genistein application decreased small nodule numbers at the second and third harvests (Fig. 4.1B). By the fourth and fifth harvests, genistein increased small nodule numbers. Both the decreases and increases in small nodule numbers were larger for AC Bravor than Maple Glen.

Genistein irrigation increased nodule weights per plant at the 3rd and 4th harvests (Fig. 4.2A).

Genistein addition increased individual nodule weight for both AC Bravor and Maple Glen plants, with the largest proportional increases being 83% for Maple Glen, and 31% for AC Bravor, both at the third harvest (Fig. 4.2B).

Genistein effects on plant nitrogen content and plant growth

Genistein increased shoot (Fig. 4.3), but not root (data not shown), nitrogen content at most harvests.

Leaf photosynthetic rate and chlorophyll fluorescence were not affected by genistein treatment (data not shown).

4.5 Discussion

Adding 5 μM genistein to the rooting medium beginning at the onset of N_2 fixation altered nodulation, and to some extent, N_2 fixation. Genistein addition may promote *nod* gene expression (Kosslak et al. 1987) of the *B. japonicum* rooting medium leading higher

levels of Nod factor production by *B. japonicum* cells than would otherwise have been the case. Kosslak et al. (1987) showed that 5 μM genistein is sufficient to maximally induce the nodulation genes. The expression of bradyrhizobial *nod* genes have been shown to stimulate production of the bacterial Nod factor, able to induce many of the early events in nodule development and regulate plant morphogenesis (Spaink 1996).

Preincubation of *B. japonicum* with genistein or application of genistein into the plant rhizosphere at the time of seeding accelerate nodulation and the onset of nitrogen fixation in cool spring production areas, leading to increases in total fixed nitrogen, grain and protein yield. Those increases are mainly due to the alteration of the time-course of each nodulation stage (Zhang and Smith 1996 a). In this study, genistein was added only after the beginning of nitrogen fixation and the temperatures of both the shoot and root zone were in the optimal range (25-30°C) for soybean nodulation. The observed increases in nodule size and weight per nodule suggested that soybean nodulation can be partially altered by addition of genistein into the rhizosphere. The increase in nodule size may be related to the increases in nodule cell division or bacterial cell numbers. Since isoflavonoids can function as modulators of polar auxin transport (Jacobs and Rubery 1988), it is tempting to speculate that they could locally disturb the auxin-cytokinin balance, leading to the induction of nodule meristems (Schmidt et al. 1994). D'Arcy-lameta & Jay (1987) reported that low micromolar concentrations of isoflavonoids stimulate the growth rate of rhizobia. Increased nodule size may also be due to the effects of genistein on the bacterial *nod* genes and Nod factor, which trigger the formation of the root nodules by initiating cell divisions at distinct sites, and possibly by affecting regulation of the plant cell cycle (Verma 1992). The increase in weight per nodule could be due to stimulation of cell division in developing nodules.

The nodulation and plant nitrogen concentration of cultivar AC Bravor tended to be more responsive to genistein application either by way of pretreated *B. japonicum* under lower root zone temperatures (Zhang and Smith 1995) or addition of genistein to the root medium after nitrogen fixation had commenced under an optimal temperature

regime (Fig. 4.1 and 4.3). This is in accordance with the results of other workers (Hungaria and Phillips 1993; Kapulnik et al. 1987) who also found differences among legume cultivars for signal molecule activity. This phenomenon could be related to genistein synthesis and/or exudation abilities of the two soybean cultivars. Kapulnik et al. (1987), for example, reported that the superior nodulation and N_2-fixation of HP32 alfalfa compared to HP alfalfa are associated with a 77% increase in the amount of plant tissue luteolin.

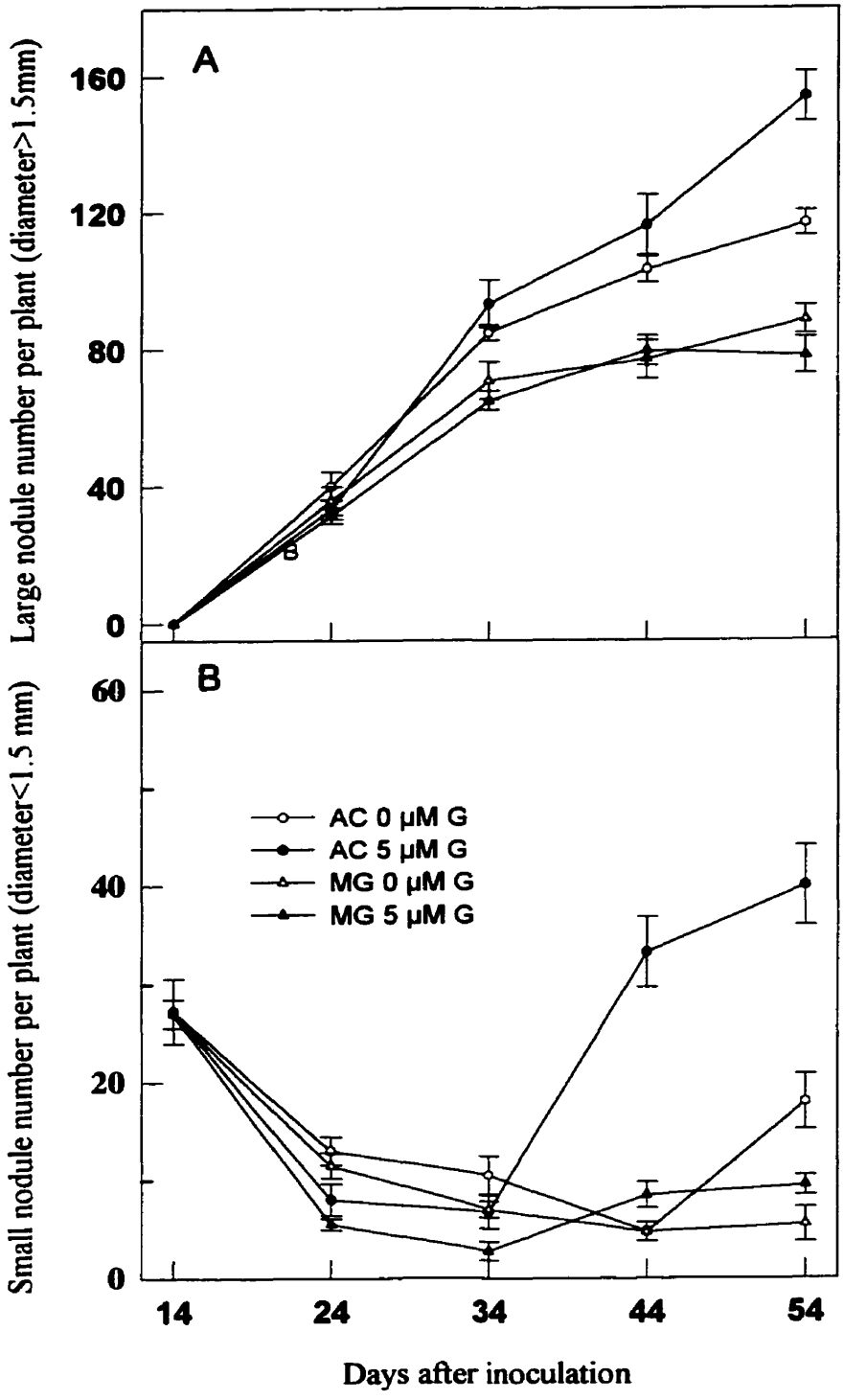

Fig. 4.1 The effects of genistein addition to the rhizosphere at the onset of nitrogen fixation on the number of large (A) and small (B) nodules. AC =AC Bravor. MG = Maple Glen. G = genistein.
Bars indicate one standard error unit.

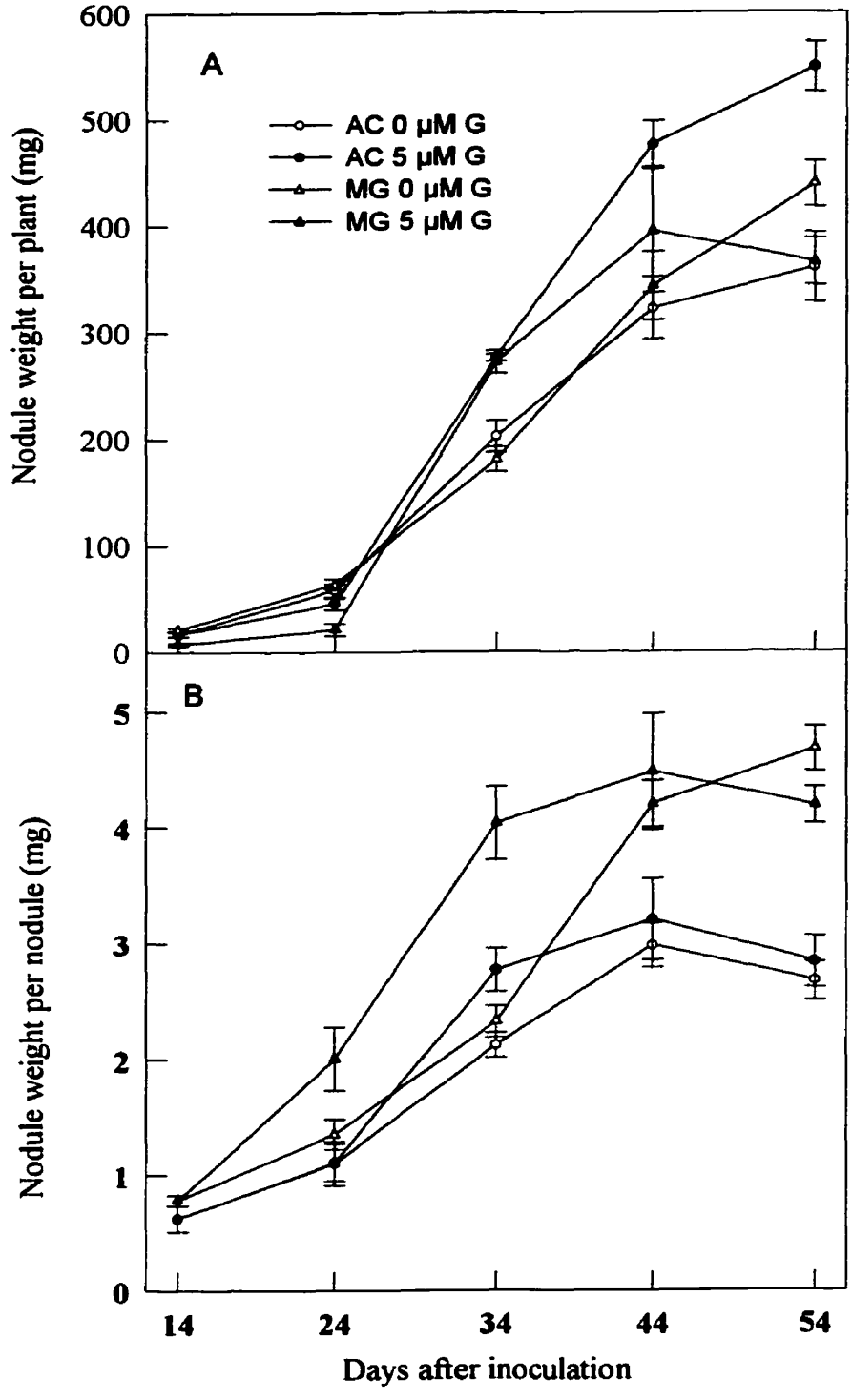

Fig. 4.2 The effects of genistein addition to the rhizosphere at the onset of nitrogen fixation on nodule weigth per plant (A) and nodule weight per nodule (B). AC =AC Bravor. MG = Maple Glen. G = genistein. Bars indicate one standard error unit.

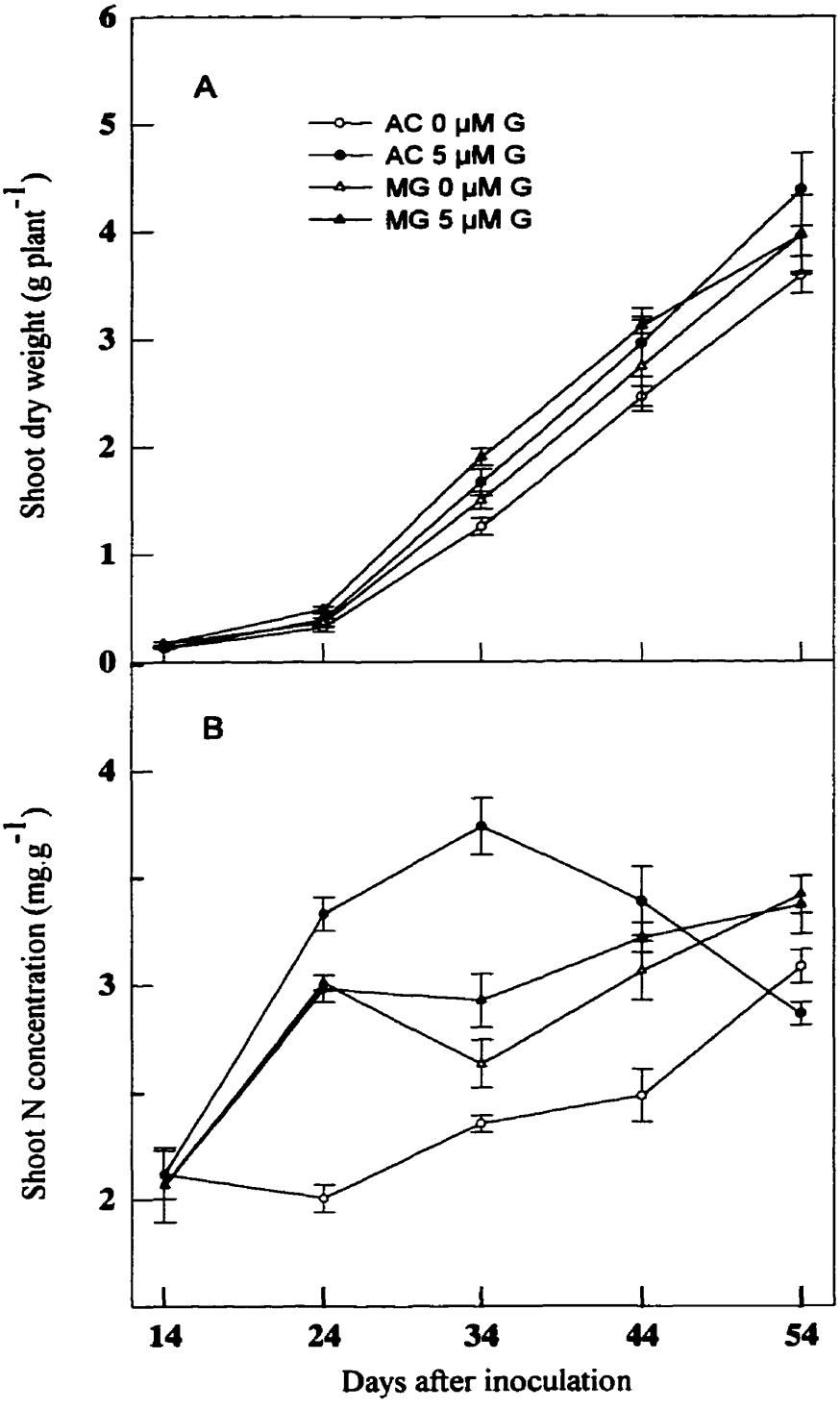

Fig. 4.3 The effects of genistein addition to the rhizosphere at the onset of nitrogen fixation on shoot nitrogen concentration and shoot dry weight. AC =AC Bravor. MG = Maple Glen. G = genistein. Bars indicate one standard error unit.

Preface to section 5

Section 5 is composed of a manuscript by Pan B and Smith DL and will be submitted to *Journal of Experimental Botany*.

It has been known for several decades that mineral N inhibits soybean nodulation and N fixation. However, the mechanism is not well understood, although several explanations have been proposed. The chapter immediately proceeding this one showed that addition of signal molecules could lead to continued modification after the first nodules formed begin to fix N_2. Using signal measurement methodology learned in the first experiment, this experiment investigated signal production from the soybean plants at different mineral N levels under greenhouse condition.

Section 5

Mineral Nitrogen and Daidzein and Genistein Levels in Soybean Tissues and Root Exudates

5.1 Abstract

Isoflavonoids, principally daidzein and genistein, excreted by soybean (*Glycine max* Merr. [L.]) roots activate the transcription of *Bradyrhizobium japonium nod* genes involved in the formation of root nodules. It has been shown that mineral nitrogen (N) reduces the concentration of daidzein and genistein in soybean roots, but this could be due to reduced synthesis or enhanced rates of excretion from the root. The effect of mineral nitrogen on soybean root exudation of isoflavonoids has not been previously determined. The study was conducted under carefully controlled greenhouse conditions. Soybean plants were grown in growth pouches and in pots. The soybean cultivar was Maple Glen. Daidzein and genistein contents and concentrations in soybean roots, shoots and in root exudates were determined using high performance liquid chromatography (HPLC). The results showed that 10 mM ammonium nitrate strongly inhibited root daidzein and genistein contents and concentrations, while 1 mM ammonium nitrate had less inhibitory effect. As the plants grew, daidzein and genistein contents increased in roots, and increased and then decreased in shoots. Daidzein and genistein exudation was not inhibited by the ammonium nitrate treatments. Changes in daidzein and genistein ratios indicated that neither their synthesis nor their exudation was affected equally by ammonium nitrate treatments. Growth methods may have affected soybean tissue daidzein and genistein contents and concentrations.

Key words: *Glycine max* [L.] Merr., genistein, daidzein, ammonium nitrate

5.2 Introduction

An exchange of molecular signals between the host plant and *(Brady)Rhizobium* over space and time is essential for the successful establishment of an effective N_2-fixing nodule (Long, 1989). The first apparent exchange of signals involves host plant secretion of phenolic compounds, flavones and isoflavones, produced through phenylpropanoid

metabolism (Peters and Verma, 1990). The isoflavonoid, daidzein and genistein, are the major compounds in soybean (*Glycine max* [L.] Merr.) root extracts responsible for induction of *nod* genes of *B. japonicum* (Kosslak et al., 1987). Low concentrations of isoflavonoids also have effects on chemotaxis, growth and development of bacteria and fungi (Phillips, 1992).

Legume plants can fix nitrogen biologically through symbiosis, but preferentially utilise mineral nitrogen (including urea, nitrate and ammonium). Soybean nodulation and nitrogen fixation are inhibited by high concentrations of combined N in the rooting media (Streeter, 1988). Researchers have been trying to understand the mechanism(s) that underlies the inhibition effects and/or to find bacterial and plant genotypes that are insensitive to mineral nitrogen in order to overcome this inhibition.

It is still the case that the mechanism by which mineral N inhibits nodulation and nitrogen fixation is not well understood, although several explanations have been proposed (for reviews see Streeter 1988; Carroll and Mathews, 1990). Changes in the isoflavonoid content of host plants have been suggested as a possible explanation (Appelbaum 1990; Cho and Harper, 1991). Cho and Harper (1991) reported that a hypernodulating soybean mutant had higher root concentrations of daidzein and genistein than did the parental cultivar at 9 to 12 days after inoculation. When 2.5 mM $(NH_4)_2SO_4$ was applied to the soybean plants, the concentrations of three isoflavonoids in the roots were decreased (Cho and Harper, 1990). However, root isoflavonoid content is only one part of the story. To induce the expression of *nod* genes in *B. japonicum* cells living in soil daidzein and genistein have to be excreted into the soil environment. It is therefore necessary to elucidate the effect of mineral nitrate on the excretion of daidzein and genistein from soybean roots. Isoflavonoids have been studied largely in seeds and roots (Cho and Harper, 1990). Since plants can regulate isoflavonoid production (Phillips, 1992), daidzein and genistein contents in plant tissues other than roots may affect the rate of excretion from roots.

Previous studies have shown that the responses of legume nodules to combined N are much more pronounced when plants are grown in a solution culture than when plants are grown in a solid medium (Ralston and Imsande, 1983). Alfalfa plants accumulate

higher concentrations of isoflavonoid conjugates in both leaves and roots when grown under field conditions than under controlled environment conditions (Tiller et al., 1994). The objectives of this study were to determine the effects of mineral nitrogen levels on daidzein and genistein contents and concentrations in soybean tissues and exudates, to determine relationships among concentrations in various plant parts and to determine the effects of root growth environment on daidzein and genistein contents and concentrations in soybean tissues.

5.3 Materials and Methods

Plant growth conditions

The experiments were organized following a random completely design with three replications. The soybean cultivar used was Maple Glen. The experiment was carried out in McGill University's Macdonald Campus research greenhouse. Air temperature was controlled at 25 ± 2°C and additional illumination of 300 μmol m^{-2} s^{-1} was supplied by high pressure sodium lights (P.L. Light Systems Canada) for a 16:8 h (day:night) photoperiod.

The experiment was conducted twice, once with plants grown in growth pouches (15 X 16 cm, Mega International, Minneapolis, MN), which allows easy collection of the root exudates, and once in pots, where the roots are able to develop under more normal circumstances. Both pots and plastic growth pouches are often used to study soybean nodulation and nitrogen fixation in indoor experiments. Plastic growth pouches are also used as a standard method in inoculant evaluation (Canada Department of Agriculture, 1991).

For the growth pouch experiment, soybean seeds were germinated in autoclaved vermiculite with the radicals pointing downward. When the radicals had elongated to about 1 cm, two germinated seeds were transferred to each growth pouch and covered with a sterilized, moist cotton pad for three days under dark conditions until the shoots had emerged. For the pot experiment, soybean plants were grown in a mixture of Turface (Applied Industrial Materials Corp., Deerfield, IL) : sand (2:1, v/v). Two seeds were sown in each pot (15 cm in diameter x 18 cm in height).

Soybean plants were grown at 0, 1, and 10 mM ammonium nitrate. One to two mM mineral N is considered the necessary concentration for a healthy plant growth and 10 mM ammonium nitrate causes inhibition of nodulation (Streeter, 1988). The ammonium nitrate solution was applied beginning at sowing, and was added each time the plants needed water. N-free Hoagland's solution (see Chapter 3) was added once a week for the growth pouch experiment and twice a week for the pot experiment, beginning at the time when the cotyledons turned yellow. The correct amount of ammonium nitrate was added to the nutrient solution or water for each treatment immediately prior to watering, and 250 mL of nutrient solution or water were added to each pot (diameter 15 cm and height 20 cm) at each watering. That amount of solution was enough to wet the whole rooting medium and cause some solution to run out the bottom of the pots, so that the pots were flushed at each watering.

Inoculant preparation

The inoculum was produced by culturing *B. japonicum* strain USDA110 in yeast extract mannitol broth in 250 mL flasks shaken at 125 rpm at 25°C. When the sub-culture reached mid log phase, pure medium was used to dilute the inoculum to an OD_{620} of 0.08 (equivalent to 10^8 cells mL^{-1}). Two mL of inoculum was added to 20 mL of distilled water and then watered evenly into the pots. The inoculant was applied one week after germination.

Preparation of samples for HPLC analysis

Plants were harvested at 4 days before inoculation, on the day of inoculation, and 3, 11, and 25 days after inoculation. For measurement of root exudation, roots were carefully removed from the growth pouches and washed with 50 mL 80% methanol. The methanol wash was put back into the growth pouch and shaken for 5 min before collecting the methanol. Another 50 mL of 80% methanol was added to the growth pouches, shaken for 5 min, and collected. The collected methanol samples were rotary evaporated to remove the methanol. The aqueous fraction was phase-partitioned three times against equal volumes of ethyl acetate, retaining each organic fraction. At the end

of the third phase partitioning, the aqueous fraction was removed and dry sodium sulphate was added to absorb any remaining water. The clear ethyl acetate fraction was decanted and rotary evaporated at 30°C until dry. The sample was then redissolved in 1.5 mL of HPLC grade pure methanol and centrifuged for 10 min at 8000 g before being run through an HPLC.

Plants were cut immediately above the uppermost root and separated into shoot and root. The same sample preparation procedure was used for both shoots and roots.

In the pot experiment, roots were washed in distilled water and carefully dried with paper towels before being weighed. Harvested soybean roots were placed in vacuum flasks with 10 times the root fresh weight of 80% methanol-dH_2O. Root tissue was placed under vacuum for 20 minutes. The pH was measured when the vacuum process was finished, and then adjusted to 5.3 with 0.1 N HCL. Samples were transferred into 250 mL flasks at 4°C and shaken for 48 h at 150 rpm. Plant roots and extracts were separated by filtering through Whatman No. 1 filter paper. Roots were collected and weighed after drying at 80 °C for 72 h. The resulting extracts were rotary evaporated to remove the methanol. The aqueous fraction was phase-partitioned and prepared for HPLC as described above.

HPLC measurement

The daidzein and genistein concentrations were determined using a Waters HPLC system (Waters Associates Inc., Milford, MA) consisting of a model 712 WISP, two 510 pumps and a 441 UV detector operating at 254 nm. Separation was achieved by a 3.9 x 300mm μBondapak C18 column (Waters Associates Inc. Milford, MA) using a mobile phase consisting of a 60:40 ratio of methanol : distilled water. The run time for separation was 15 minutes with an isocratic flow rate of 1 mL min^{-1}. Commercial genistein (4, 5, 7-trihydroxyisoflavone, purity of 98%, Sigma) and daidzein (4', 7-Dihydroxyisoflavone, purity > 98%, ICN) were used as standards.

Data analysis

Results were analysed statistically by analysis of variance using the Statistical Analysis System computer procedure (SAS Institute Inc., 1988). The LSD test was applied to make comparisons among the means at the 0.05 level of significance, when analysis of variance showed significant treatment effects.

5.4 Results

Root daidzein and genistein content and concentration

In the growth pouch experiment, differences in root daidzein and genistein contents among the three ammonium nitrate levels was not obvious at the first four harvests (Fig. 5.1 A & B). Twenty-five days after inoculation, 10 mM ammonium nitrate decreased root daidzein content as much as 90.0% and genistein content as much as 85.9% as compared to controls without N. Unlike contents, differences in daidzein and genistein concentrations existed from early harvest (Fig. 5.1 C & D). Ammonium nitrate decreased root daidzein and genistein concentrations, and the decreases were most obvious at the last harvest.

In the pot experiment, differences in daidzein and genistein contents were found at the early harvests (Fig. 5.2 A & B). Twenty-five days after inoculation, 10 mM ammonium nitrate decreased root daidzein content as much as 77.3% and genistein content as much as 64.8 as compared to controls without N. Root daidzein and genistein contents were slightly lower for soybean plants grown in 1 mM than in 0 mM ammonium nitrate. Ammonium nitrate treatments decreased daidzein and genistein concentrations (Fig. 5.2 C & D). Root genistein concentrations were the highest at the first harvest and decreased as the soybean plants grew.

Shoot daidzein and genistein content and concentration

Shoot daidzein and genistein contents and concentrations were reduced by 10 mM ammonium nitrate treatments in both growth pouch and pot experiments.

In the growth pouch experiment, soybean grown in zero ammonium nitrate had the highest shoot daidzein and genistein contents at 11 days after inoculation (Fig. 5.3 A & B). Twenty-five days after inoculation, soybean plants grown in 1 mM ammonium

nitrate had the highest shoot daidzein and genistein contents. Unlike shoot daidzein content, shoot genistein content was decreased at the last harvest. Similar changes were found in shoot daidzein and genistein concentrations (Fig. 5.3 C & D).

In the pot experiment, shoot daidzein and genistein contents were usually higher when soybean was grown in 0 mM ammonium nitrate than in 1 mM ammonium nitrate (Fig. 5.4 A & B). Shoot daidzein and genistein contents had different patterns as soybean plants grew: daidzein increased steadily, genistein increased to the second harvest and then derceased. Shoot daidzein and genistein concentrations increased until 3 days after inoculation and then decreased (Fig. 5.4 C & D). Shoots had lowest daidzein and genistein concentrations when grown at 10 mM ammonium nitrate.

Root daidzein and genistein exudation

Unlike daidzein and genistein contents and concentrations inside the root, total daidzein and genistein exudation were not inhibited by the ammonium treatments.

The patterns of exudation of daidzein and genistein per gram of root dry weight (Fig. 5.5 A & B) were different from the total exudate levels. Daidzein exudation was highest at the first two harvests, decreased thereafter, and was the lowest at the last harvest. No difference was found in genistein exudation at the first harvests. Soybean plants grown at 0 ammonium nitrate always had the lowest daidzein and genistein exudation.

Total daidzein and genistein exudation per plant from soybean roots followed a similar pattern (Fig. 5.5 C & D). Soybean grown in 10 mM ammonium nitrate had a higher level of daidzein and genistein in exudates at the first three harvests and soybean grown in 0 mM ammonium nitrate had lowest. In the last two harvests soybean plants grown in 1 mM ammonium nitrate had greater total daidzein and genistein exudation that previous harvests.

Ratio of genistein and daidzein (GDR) in roots, shoots and exudates

In the growth pouch experiment, ammonium nitrate level affected GDR. In exudates, 1 mM ammonium nitrate usually resulted in the highest GDR (Fig. 5.6A).

GDR was lowest at the last two harvests at 0 mM ammonium nitrate. In roots, 10 mM ammonium nitrate resulted in the lowest GDR at the first two harvests and the highest GDR in the last three harvests (Fig. 5.6B). GDR was lower at the first harvest, increased at the second harvest and then deceased as the soybean plants grew. Shoot GDR was also lower at the last harvest (Fig. 5.6C).

In the pot experiment, both root and shoot GDR decreased as soybean plants developed (Fig. 5.7A & B). Soybean plants grown at 0 mM ammonium nitrate resulted in the lowest GDR from the second harvest to the last. No obvious trend in shoot GDR was found among the three levels of ammonium nitrate.

Shoot GDR was much higher than that of the root in both growth pouch and pot experiments.

5.5 Discussion

Ammonium nitrate inhibited shoot and root daidzein and genistein contents and concentrations. Our results confirm the results observed in soybean roots (Cho and Harper, 1990). The biochemical mechanism underlying this reduction is still not clear. In our experiment, we found a clear relationship between extract pH and ammonium nitrate levels. The pH of shoot and root extracts was always lower as the ammonium nitrate concentration increased (data not shown). The decrease in extract pH relates to tissue pH and hence could affect some enzymatic activities related to isoflavonoid synthesis. Changes in pH could also affect isoflavonoid exduation and the hydrolization of isoflavonoid conjugates. D'Arcy-Lameta (1986) reported that a hydrolysis of polyphenolic conjugates takes place at the time of exudation.

Our data showed that 10 mM ammonium nitrate stimulated total root exudation of daidzein and genistein at the time of inoculation and 1 mM ammonium nitrate resulted in the greatest exudation at 11 and 25 days after inoculation. Ammonium nitrate accumulation in the media may have been one of the causes for the high exudation. Dostanova et al (1979) reported that higher exudation of an isoflavone when pea seedlings were subjected to either sodium chloride or sulphate salinity. Greater total exudation was not the result of root growth due to the higher levels of N. In fact, root dry

weight in the growth pouch experiment was decreased by 21.46% when plants were grown in 10 mM N as compared to the control without N at 25 days after inoculation.

Root isoflavonoid exudation patterns were different from the isoflavonoid content and concentration patterns inside the root and shoot. Such observations suggest that the effect of ammonium nitrate on isoflavonoid exudation processes is different from its effect on the synthesis process. Previous studies indicated that legume seed and root exudates might contain substances that are both stimulatory and inhibitory to nodulation (Siqueira et al., 1991). Plants may control nodulation through intervention of highly active, and weak stimulators and/or inhibitors, or by enzymatic reaction in the rhizosphere (Lynn and Chang, 1990). The effect of high ammonium nitrate levels on the exudation of those inhibitory substances, weaker but competitive inducers, and the ratio of those signals is not clear, but deserves further investigation. On the other hand, although daidzein and genistein in the exudate contact *B. japonicum* cells directly and promote *nod* gene expression, the importance of daidzein and genistein levels inside the roots and the role they might play in the establishment of nodules after the bacterial cells enter into the root has not been elucidated.

Shoot and root daidzein and genistein contents and concentrations changed differently at different sampling time. The difference could be related to the environment they developed under, as isoflavonoid metabolism is light regulated (Stafford, 1990) and light has a pronounced effect on the distribution of isoflavones (Graham, 1991). Tissue specificity of the expression of flavonoid genes has been reported in a few cases (Dooner, 1983). Higher shoot genistein content and concentration could affect root isoflavonoid level, and hence nodulation, as Cho and Harper (1991b) reported control of nodulation by isoflavonoid levels in a soybean hypernodulating mutant.

Differences in daidzein and genistein contents and concentrations were found between the two growth methods used. When soybean was grown in growth pouches, daidzein and genistein contents and concentrations were high, especially at the last harvest. This may have been caused by gradual accumulation of daidzein and genistein inside the growth pouches which could have feed-back effects on levels inside roots. In addition, the growth pouch environment resulted in less root dry weight (0.1 g per plant)

than in pots (1.7 g per plant) at the last harvest. This difference led to a higher concentration with the lower root dry weight. These results suggest that we should be cautious about extrapolating growth pouch experiment results.

Soybean growth is not completely normal when grown in media without a mineral N supply (Streeter, 1988). Many investigations have demonstrated that maximum symbiotic performance is associated with low concentrations of combined N in the rooting media (Nutman, 1956; Harper, 1974). Our data have shown that ammonium nitrate level affected isoflavonoid synthesis and exudation. Given the importance of isoflavonoids in the symbiosis, our results supported the idea that soybean plants (including those used as controls) should be grown with a small amount of combined N (i.e. 1 to 2 mM in sand culture) (Streeter, 1988).

In conclusion, our results show that daidzein and genistein distribution patterns varied with organ, ammonium nitrate levels, plant developmental stages, and growing methods. Ammonium nitrate inhibited daidzein and genistein contents and concentration in root and shoot extract, but did not decrease daidzein and genistein exudation. In both synthesis and excretion, daidzein and genistein were not affected equally by ammonium nitrate treatments.

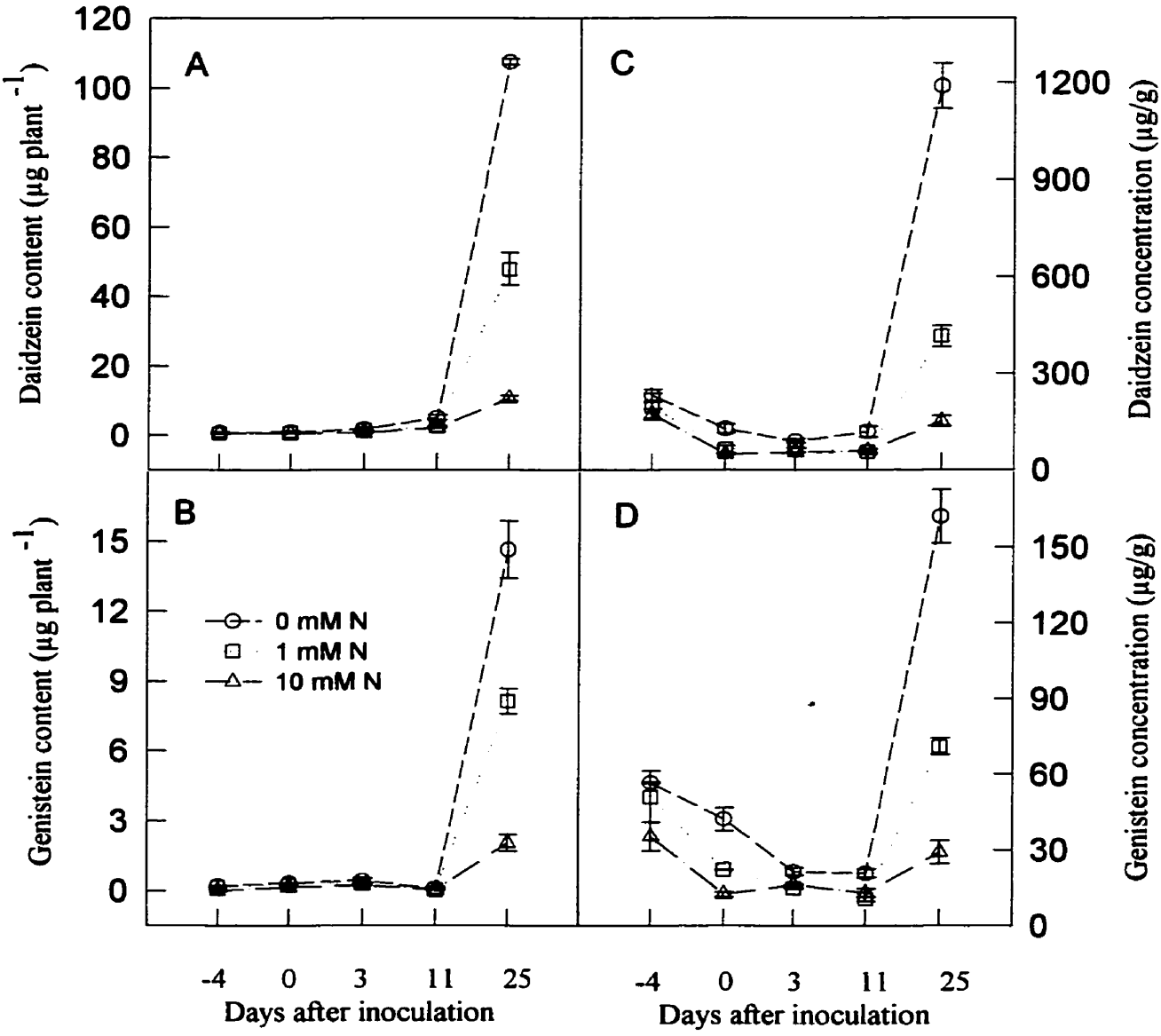

Fig. 5.1 Root daidzein and genistein content (A, B) and concentration (C, D) of soybean plants grown in growth pouches. Vertical bars indicate one stand error unit.

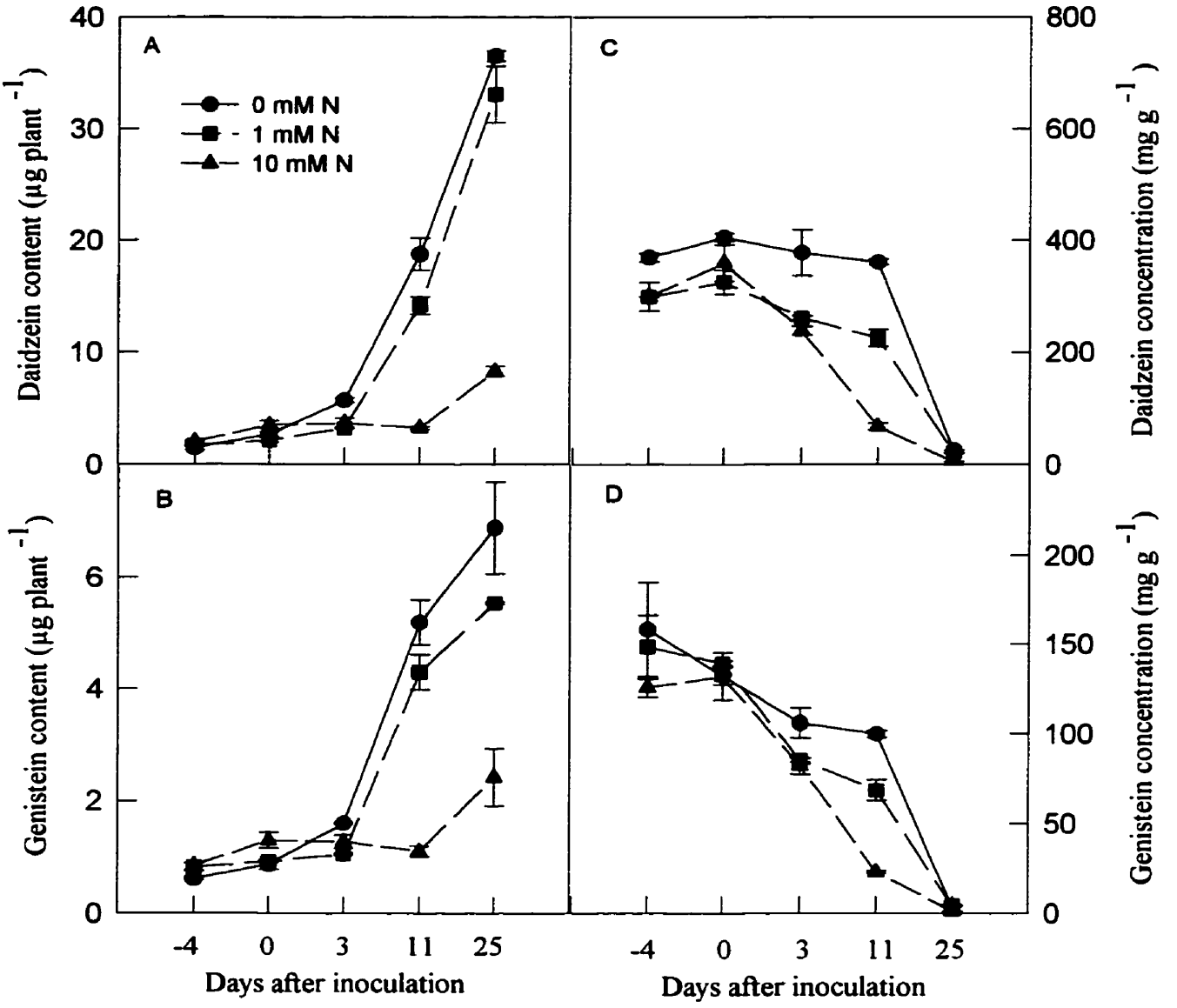

Fig. 5.2 Root daidzein and genistein content and concentration of soybean plants grown in pots. Vertical bars indicate one stand error unit.

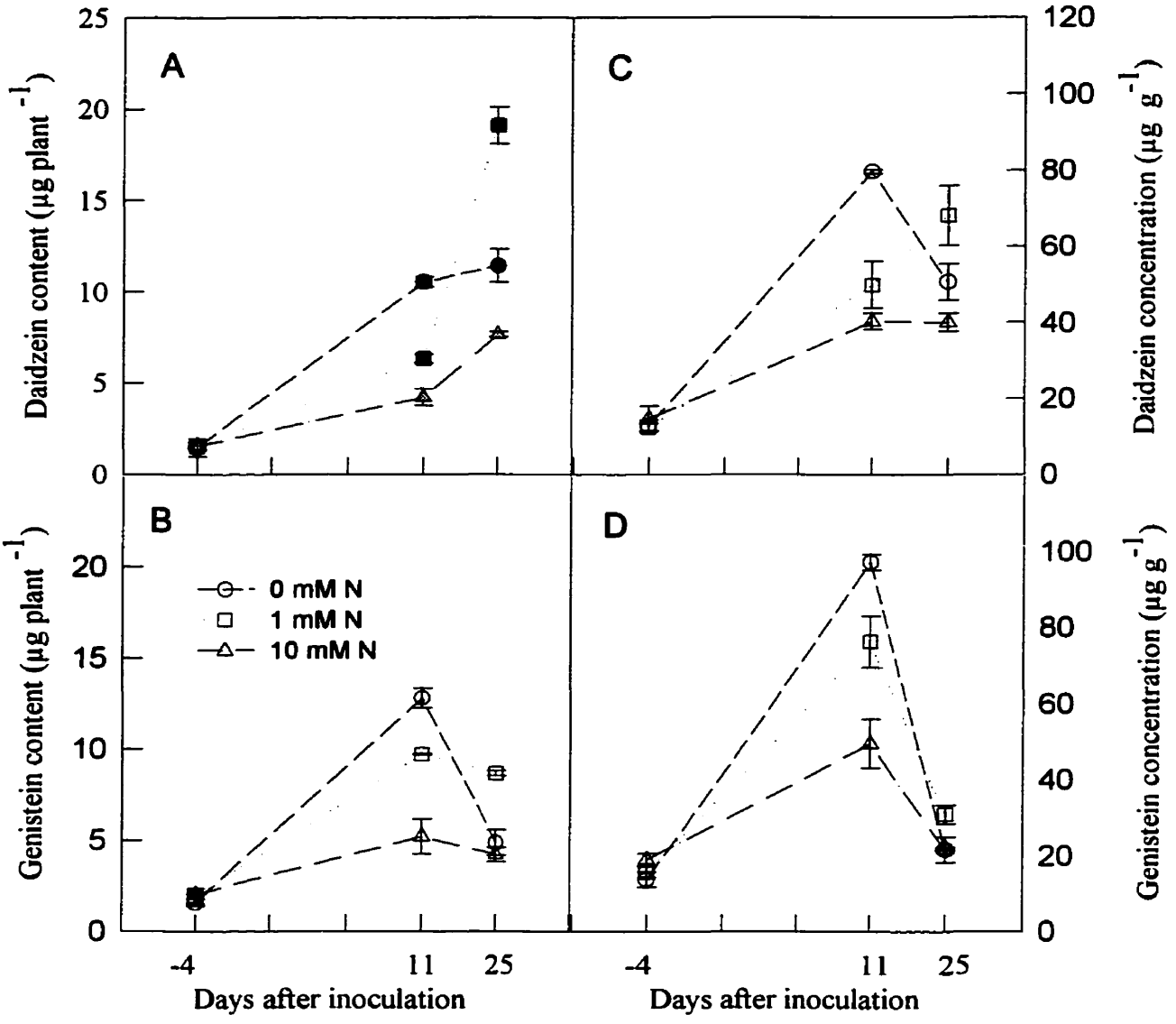

Fig. 5.3 Shoot daidzein and genistein content and concentration of soybean plants grown in growth pouches. Vertical bars indicate one stand error unit.

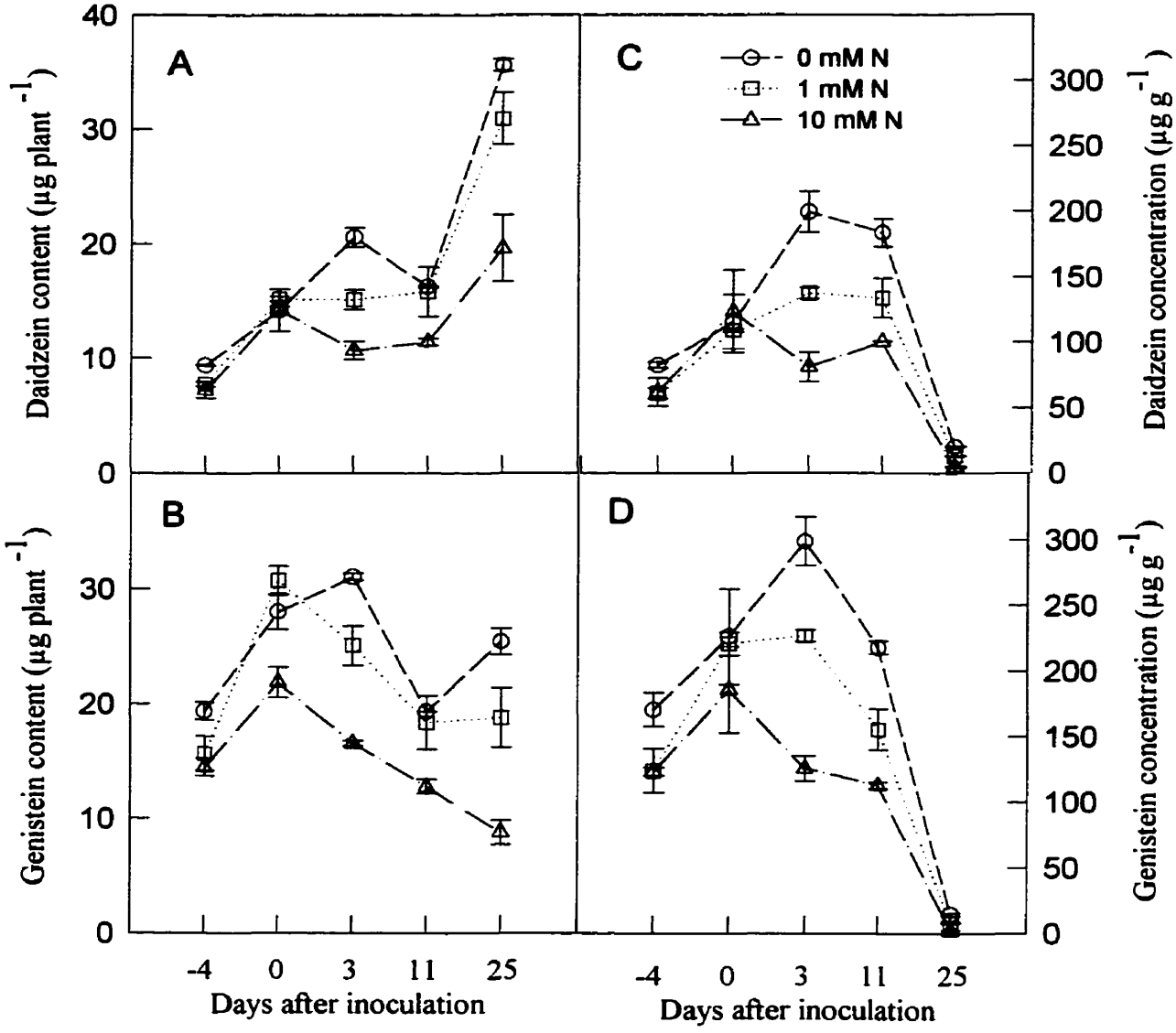

Fig. 5.4 Shoot daidzein and genistein content and concentration of soybean plants grown in pots. Vertical bars indicate one stand error unit.

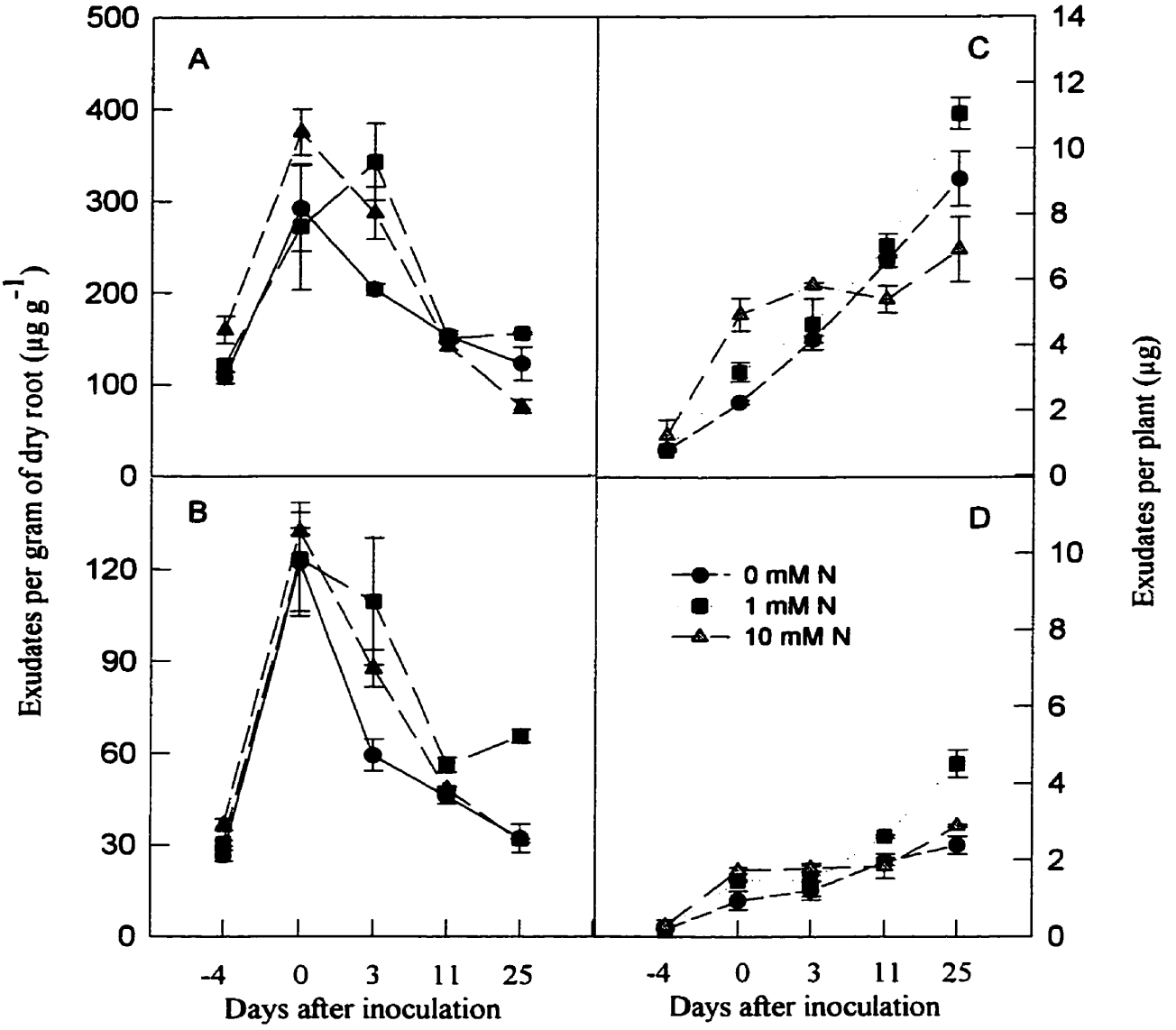

Fig. 5.5 Daidzein (A, C) and genistein (B, D) exudation from soybean root grown in growth pouches. Vertical bars indcate one standard error unit.

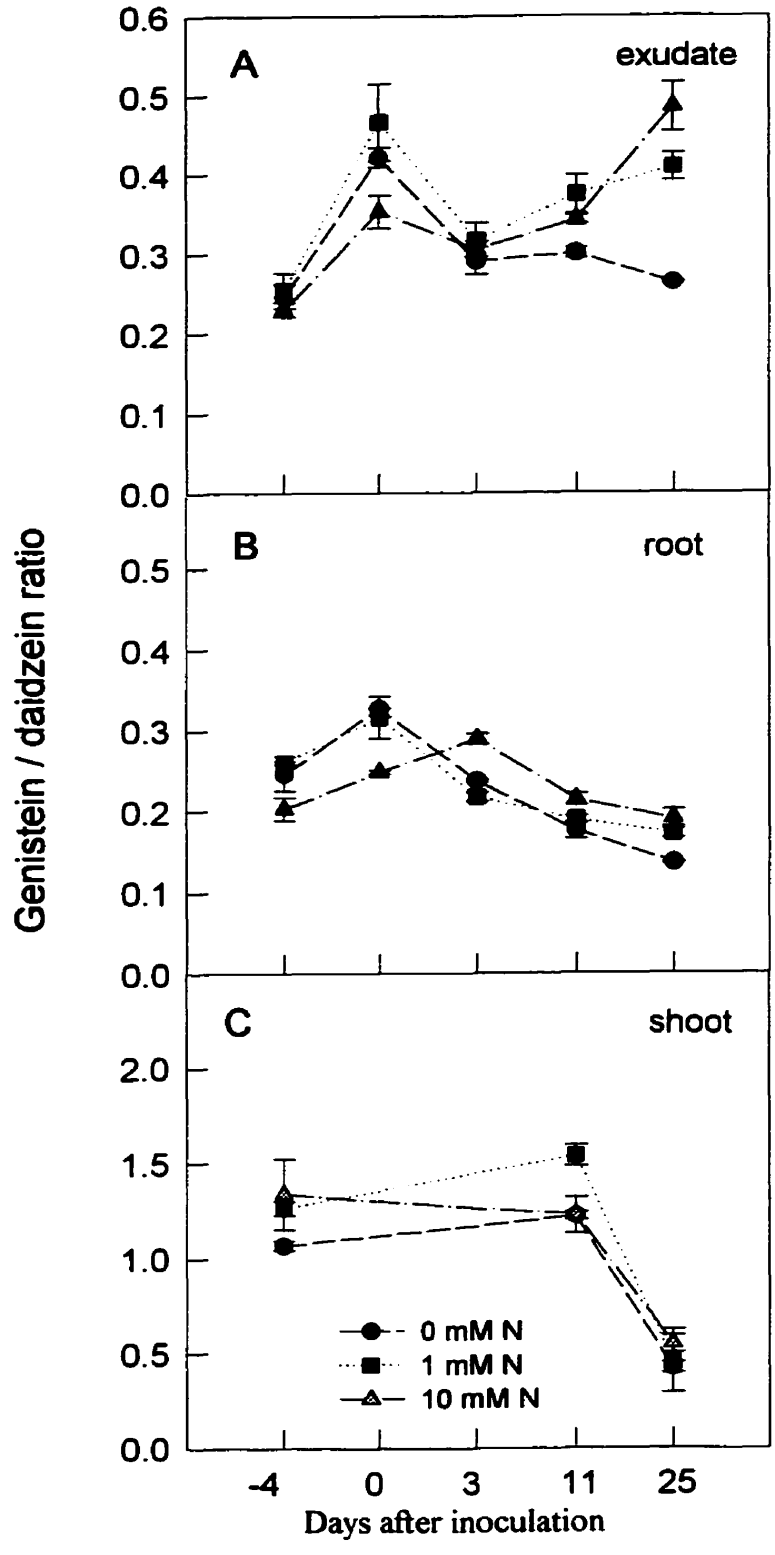

Fig. 5.6 Daidzein and genistein ratio in shoot, root and root exudate of soybean plants grown in growth pouches. Vertical bars indicate one standard error unit.

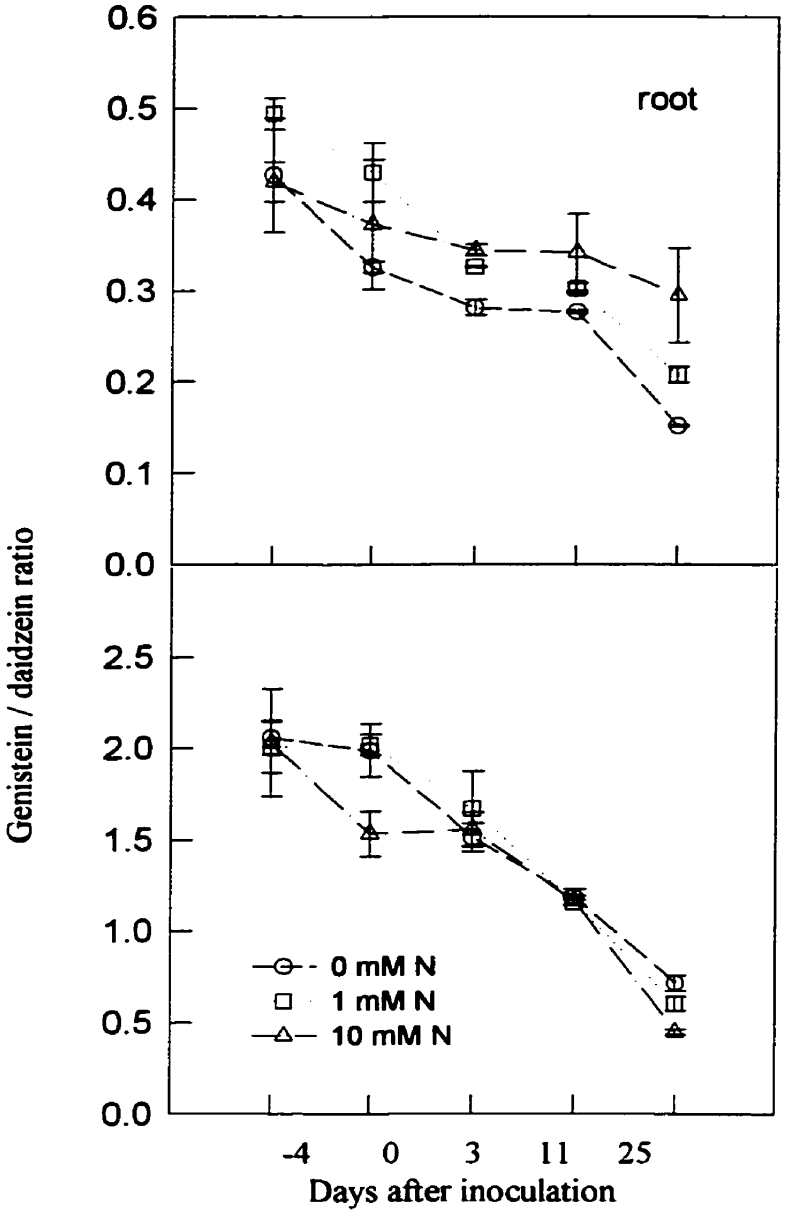

Figure 5.7 Daidzein and genistein ratio in shoot and root of soybean plants grown in pots. Vertical abrs indicate one standard error unit.

Preface to section 6

Section 6 is composed of a manuscript by Pan B and Smith DL and will be submitted to *Journal of Experimental Botany*.

This work extended the same phenomenon as in section 5, but focused on the variability among soybean cultivars for signal (isoflavonoid) synthesis and exudation in the presence or absence of mineral N.

Section 6

Variability among soybean (*Glycine max* [L.] Merr.) cultivars for isoflavonoid synthesis and exudation in the presence of nitrate

6.1 Abstract

The isoflavonds genistein and daidzein are the major compounds in soybean root extracts responsible for induction of *nod* genes of *Bradyrhizobium japonicum*. Nitrate inhibits legume nodulation and nitrogen fixation. The responses of daidzein and genistein synthesis and exudation to nitrate, and the correlation between daidzein and genistein exudation with nodulation were studied, using eight soybean cultivars and three levels of potassium nitrate. The experiment was conducted under greenhouse conditions with three replications. Soybean plants were grown in growth pouches. The results showed that changes in root daidzein and genistein contents of the eight soybean cultivars varied in response to nitrate levels. Changes in the concentrations of root daidzein and genistein were similar among cultivars, although some differences existed. The amount of daidzein and genistein excreted per plant by soybean roots did not correspond to the inside root daidzein and genistein contents and concentrations. Daidzein and genistein contents and concentrations in root extracts were positively correlated with nodule number and weight; daidzein and genistein contents and concentrations in root exudates were negatively correlated with nodule number and weight, although the correlation coefficients were low.

Key words: soybean, genistein, daidzein, potassium nitrate

6.2 Introduction

The isoflavonoid genistein and daidzein are the major compounds in soybean (*Glycine max* [L.] Merr.) root extracts responsible for induction of *nod* genes of *B. japonicum* (Kosslak et al., 1987). Low concentrations of isoflavonoids also have effects on chemotaxis, growth and development of bacteria and fungi (Rao, 1990). Addition of genistein improves soybean nodulation at suboptimal temperatures (Zhang and Smith, 1995). Soybean isoflavonoids have been studied in roots (Cho and Harper, 1991) and

exudates (D'Arcy-Lameya, 1986) of a few cultivars (Graham, 1991). The variability among soybean cultivars for nitrate effects on the accumulation and exudation of daidzein and genistein has received little study.

Legume plants can fix nitrogen biologically through a symbiotic association, but preferentially utilise mineral nitrogen. Nitrate is one of the most potent inhibitors of nitrogen fixation (Streeter, 1988). Changes in isoflavonoid content of the host plants have been suggested as a possible explanation (Appelbaum 1990; Cho and Harper 1991).

Of the two symbiotic partners, *B. japonicum* was less affected by nitrate than the host plant (McNeil, 1982) and it is the plant that is the primary control point for nodulation in the presence of nitrate (Gibson and Harper, 1985). Species differ considerably in their symbiotic tolerance to mineral N and substantial natural variation may exist among legume lines and cultivars (Peoples and Craswell, 1992). Hardarson and Zapata (1984) reported that a great variability existed among soybean germplasms for ability to fix N_2 at different inorganic N levels, and also that the potential exists to breed for nitrogen fixation associated traits. Cho and Harper (1991) reported a hypernodulating soybean mutant that had higher root concentrations of genistein and daidzein than did the parental cultivar at 9-12 days after inoculation.

The number of nodules per plant is genetically limited (Fujita et al, 1991). The challenge of improving the nitrogen fixation capacity of the legumes through selection and breeding is complex because there are two components to consider: the (brady)rhizobia and the host plant. Many of the breeding programs aimed at enhancing nitrogen fixation have chosen to ignore this complication and have focused mainly on manipulation of the host plant (Peoples and Craswell, 1992). The isoflavonoid signal molecules are a bridge between the host plant and the (brady)rhizobia. Selection of isoflavonoid production ability by the host plant root system may offer a new means of selecting symbiotically improved soybean cultivars.

The present study was initiated to determine: (1) soybean cultivar variability in daidzein and genistein synthesis and exudation responses to nitrate levels, (2) the degree of correlation of daidzein and genistein with nodulation. Genistein and daidzein content and concentration in soybean roots and shoots, and genistein and daidzein exudation from

soybean roots were determined. It is hoped that this will increase our understanding and provide the basis for future genetic manipulation of the host plant metabolic pathways to maximize *nod* gene activation and to control the symbiosis for agricultural improvement.

6.3 Materials and methods

Plant growth conditions

The experiments were organized following a random completely design with three replications. Eight soybean cultivars were used. Six cultivars (two from each of the early, middle and late maturity groups in the 1997 recommend list of the Québec Council of Plant Production (CPVQ) plus one non-nodlating and one super-nodulating genotype were selected. The experiment was conducted in McGill University's Macdonald Campus research greenhouse. Air temperature was controlled at 25 ± 2°C and additional illumination of 300 μmol m^{-2} s^{-1} was added by high pressure sodium lamps for a 16:8 h (day:night) photoperiod.

Soybean seeds were germinated in sterilised vermiculite with the seed radicals pointing downward. When the radicals elongated to about 1 cm (two days after sowing), the germinated seeds were transferred to growth pouches (15 X 16 cm, Mega International, Minneapolis, MN), and covered with a sterilized, moist cotton pad for three days under dark conditions, until the shoots had emerged.

Mineral N treatments were 0, 1, and 10 mM potassium nitrate. One mM mineral N is considered the necessary concentration for a healthy plant growth and 10 mM potassium nitrate causes inhibition of nodulation (Streeter, 1988). Exposure to potassium nitrate solution began when the seedlings were transferred to growth pouches, and was added each time that the plants needed water.

Nodule number was determined at 27 days after inoculation. Nodule weight was measured after drying at 80 °C for 72 h.

Inoculant preparation

The inoculum was produced by culturing *B. japonicum* strain USDA110 in yeast extract mannitol broth in 250 mL flasks shaken at 125 rpm at 25°C. When the sub-

culture reached mid log phase, pure medium was used to dilute the inoculum to an OD_{620} of 0.08 (equivalent to 10^8 cells mL^{-1}). One mL of inoculum was added to the base part of each plant root using a pipette. The inoculant was applied nine days after germination.

Preparation of samples for HPLC analysis

Plants were harvested at 13 days after inoculation. For the measurement of root exudation, roots were carefully removed from the growth pouches and washed with 50 mL 80% methanol. The methanol wash was put back into the growth pouch and shaken for 5 min before collecting the methanol. Another 50 mL of 80% methanol was added to the growth pouch, shaken 5 min, and collected. The collected methanol samples were rotary evaporated to remove the methanol. The aqueous fraction was phase-partitioned three times against equal volumes of ethyl acetate, retaining each organic fraction. At the end of the third phase partitioning, the aqueous fraction was removed and dry sodium sulphate was added to absorb the remaining water. The clear ethyl acetate fraction was decanted and rotary evaporated at 30°C until dry. The sample was then re-dissolved in 1.5 mL of HPLC grade methanol and centrifuged for 10 min at 8000 g before being run through an HPLC.

Plant roots were separated from the stems immediately above the uppermost lateral root. Roots were washed in distilled water and carefully dried with paper towels before being weighed. Harvested soybean roots were placed in vacuum flasks with 10 times the root fresh weight of 80% methanol-dH_2O. Root tissue was placed under vacuum for 20 minutes. The pH was measured when the vacuum process was finished, and then adjusted to 5.3 with 0.1 N HCL. Samples were transferred into 250 mL flasks at 4°C and shaken for 48 h at 150 rpm. Plant roots and extracts were separated by filtering through Whatman No. 1 filter paper. Roots were collected and weighed after drying at 80 °C for 72 h. The resulting extracts were rotary evaporated to remove the methanol. The aqueous fraction was phase-partitioned and prepared for HPLC as described above.

HPLC measurement

The genistein and daidzein concentrations were determined using a Waters HPLC system (Waters Associates Inc., Milford, MA) consisting of a model 712 WISP, two 510 pumps and a 441 UV detector operating at 254 nm. Separation was achieved by a 3.9 x 300 mm μBondapak C18 column (Waters Associates Inc., Milford, MA) using a mobile phase consisting of a 60:40 ratio of methanol : distilled water. The run time for separation was 15 minutes with an isocratic flow rate of 1 mL min^{-1}. Commercial genistein (4, 5, 7-trihydroxyisoflavone, purity of 98%, Sigma) and daidzein (4', 7-dihydroxyisoflavone, purity > 98%, ICN) were used as standards.

Data analysis

The experiment was subject to analysis of variance (Steel and Torrie, 1980). Correlation analyses were conducted for the nodule number and weight with isoflavonoid contents and concentrations. All statistical analyses were performed using the Statistical Analysis System computer package (SAS Inst., Cary, NC, 1988).

6.4 Results:

Daidzein and genistein contents and concentrations in soybean roots

The responses of root daidzein and genistein contents per plant to nitrate varied among the eight soybean cultivars (Fig. 6.1). Ten mM nitrate inhibited root daidzein and genistein contents of all eight cultivars. Of the eight soybean cultivars four had highest root daidzein and genistein contents at 0 nitrate and four at 1 mM nitrate. Daidzein and genistein contents did not always follow the same patterns of changes. Maple Glen had the highest daidzein content at 0 nitrate, while KG30 had the highest genistein content at 1 mM nitrate.

Changes in the concentrations of root daidzein and genistein were similar, but not always the same as contents of root daidzein and genistein. (Fig. 6.2). Five cultivars had their highest daidzein concentration at 0 mM nitrate and three at 1 mM nitrate. The non-nodulating genotype had daidzein and genistein contents and concentrations that were among the lowest values at both 1 and 10 mM nitrate levels.

Daidzein and genistein in root exudates

The amount of daidzein and genistein excreted per plant by roots did not correspond to the root daidzein and genistein contents or concentrations (Fig. 6.3). Four out of the eight cultivars had the highest daidzein exudation at 10 mM nitrate. The non-nodulating genotype generally had the highest daidzein exudation of all genotypes. A similar pattern was found for genistein exudation, although some exceptions existed. Cultivar 9007 had the highest genistein exudation at 1 mM nitrate, while cultivar Maple Glen had the highest genistein exudation at 0 mM. Genistein exudation by Nordet varied little within nitrate levels.

For daidzein the effects of nitrate on exudation per gram of root were similar to the effects on total exudation (Fig. 6.4), but for genistein higher levels of nitrate often reduced the amount exuded per gram root. The super-nodulating cultivar had the highest genistein exudation at 10 mM nitrate. The non-nodulating cultivar had low genistein exudation levels at 1 and 10 mM nitrate. The cultivar Bayfield had the highest genistein exudation at 1 mM nitrate.

Nodule number, nodule weight, root dry weight of soybean plants and their relations with daidzein and genistein

Nodule number and especially weight were inhibited at 10 mM nitrate (Fig. 6.5). At 0 and 1 mM nitrate nodule number and weight were similar, although some exceptions existed. Soybean cultivar difference in nodule number and weight also existed in the response to nitrate treatment. Nodule weights of Maple Glen and the super-nodulating genotype were increased at 1 mM nitrate. Root dry weights of five out of the eight cultivars were increased at 1 mM nitrate (Fig. 6.5). Ten mM nitrate generally resulted in less root growth than 1 mM, except for 9007.

Correlations of daidzein and genistein with nodule number, nodule weight and root dry weight varied among the three nitrate levels (Table 6.1). In both root extracts and exudates, daidzein and genistein contents and concentrations usually had high correlation with root dry weight. In root extracts, there were no or low correlation of

daidzein and genistein contents and concentrations with nodule number and weight. In exudates, daidzein contents and concentrations had high negative correlation with nodule number and nodule weight at three nitrate levels. The correlation of daidzein and genistein contents and concentrations in root extracts with those in exudates varied among the three nitrate levels (Table 6.2).

6.5 Discussion

Cultivar differences in isoflavonoid synthesis and exudation

The results presented here indicate that root daidzein and genistein contents per plant of the eight soybean cultivars tested had different responses to nitrate. Salomonsson et al. (1978) reported that root contents of the isoflavonoids formononetin and genistein differ substantially among plant species. Ten mM nitrate inhibited root daidzein and genistein contents and concentrations of all the eight cultivars used on our study, including the supernodulating and non-nodultaing cultivar. These results support the conclusion of Cho and Harper (1991) that differential nitrate inhibition of nodulation may be partially due to changes in isoflavonoid levels. The nodulation control in non-nodulating genotype may be due to factors totally unrelated to isoflavonoids (Cho and Harper, 1991) or indirectly related to isoflavonoids and other derivatives in phenylpropanoid pathway. They perform other functions in plants, including as natural auxin transport inhibitors (Jacobs and Rubery, 1988), and are causative in cytokinin-like activities (Binns et al., 1987). Both auxin and cytokinin were suggested to play roles in nodule formation (Hirsch et al., 1989; Long and Cooper, 1988).

Our study extended the currently published observations by evaluating isoflavonoid levels in the exduation of root. Daidzein and genistein concentrations in exudate differed greatly among the eight cultivars and nitrate treatments. Daidzein and genistein contents and concentrations of the super-nodulting cultivar were high in the exudate at 10 mM nitrate (Fig. 6.2 & 6.4). This suggests high nitrate levels had less inhibitory effect on daidzein and genistein exudation by this super-nodulitng genotype. This could be the reason why nodulation by this genotype is less sensitive to nitrate than others. But, in our study, the nodule number and weight of the super-nodulting cultivar

were not high at 27 days after inoculation. We do not know if the super-nodulaing genotype would form more nodules in the later growth stages, as the nodulation process of this genotype is not well characterized.

The responses of genistein to nitrate were not parallel to the daidzein response to nitrate for all eight soybean cultivars. In a study of nitrate effects on three isoflavonoids Rossiter (1969) found biochanin A less affected than genistein and formononetin in leaves of subteranean clover. In our study, for all eight cultivars, daidzein was the dominant isoflavonoid both inside the root and in exudates and at all nitrate levels. Pueppke et al. (1998) also reported that daidzein was dominant in root exudates of two cultivars, whether or not rhizobia were present.

Relationships between daidzein and genistein, and nodulation

The correlation coefficients for the relationships between daidzein and genistein contents and concentrations, and nodule number and weight were very low. Mathews et al. (1989) showed that 3-day-old seedlings of the soybean cultivar Williams have a 10 times greater ability to induce *B. japonicum nod* genes than do 3-day-old cultivar Bragg seedlings, although mature plants have the same nodulation frequency. Root isoflavonoids can affect biological nitrogen fixation either by influencing root hair formation and root growth and/or by having a direct effect on the microorganisms concerned (Rao, 1990). The final outcome could be a result of an interaction between the two factors. Daidzein and genistein are inducers of *nod* gene expression. However, at concentrations greater than 5 μM, both daidzein and genistein inhibit the growth of *B. japonicum* cells (Kosslak et al., 1987). The observed negative correlation of daidzein and genistein in exudates with nodule number, nodule weight could be due to other current undetermined chemicals exuded by soybean plants. Several flavonoids have also been found to antagonize the induction process (Firmin et al., 1986). Nitrate effect on the synthesis and release of those antagonizing compounds is still not clear. It is important to understand the synthesis and exudation of both inducing and inhibiting compound in developing root (Peters and Long, 1988).

The detected daidzein and genistein could have other functions in plant development and growth. They are the precursor of phytoallexins. The availability of stored isoflavones may facilitate rapid synthesis of more-complex isoflavonoids in response to various biological elicitors. Kaplan et al. (1980) reported that flavonoid accumulation in roots of soybean and lima bean seems to be one of the basic biochemical mechanisms in response to nematode attack.

In conclusion, soybean cultivars responded differently to nitrate treatment in daidzein and genistein synthesis and exudation. Nitrate inhibited root daidzein and genistein contents and concentrations. The conclusion that soybean isoflavonoid level is the primary controlling factor in nodulation can not be reached, and other mechanisms of nitrate inhibition directly or indirectly related to isoflavonoid, should be pursued especially for the non-nodulating cutivar. This study indicated that soybean isoflavonoid content and concentration can not be used as a selection standard to increase nodulation, before the quantitative relationship between isoflavonoids and nodulation is better understood.

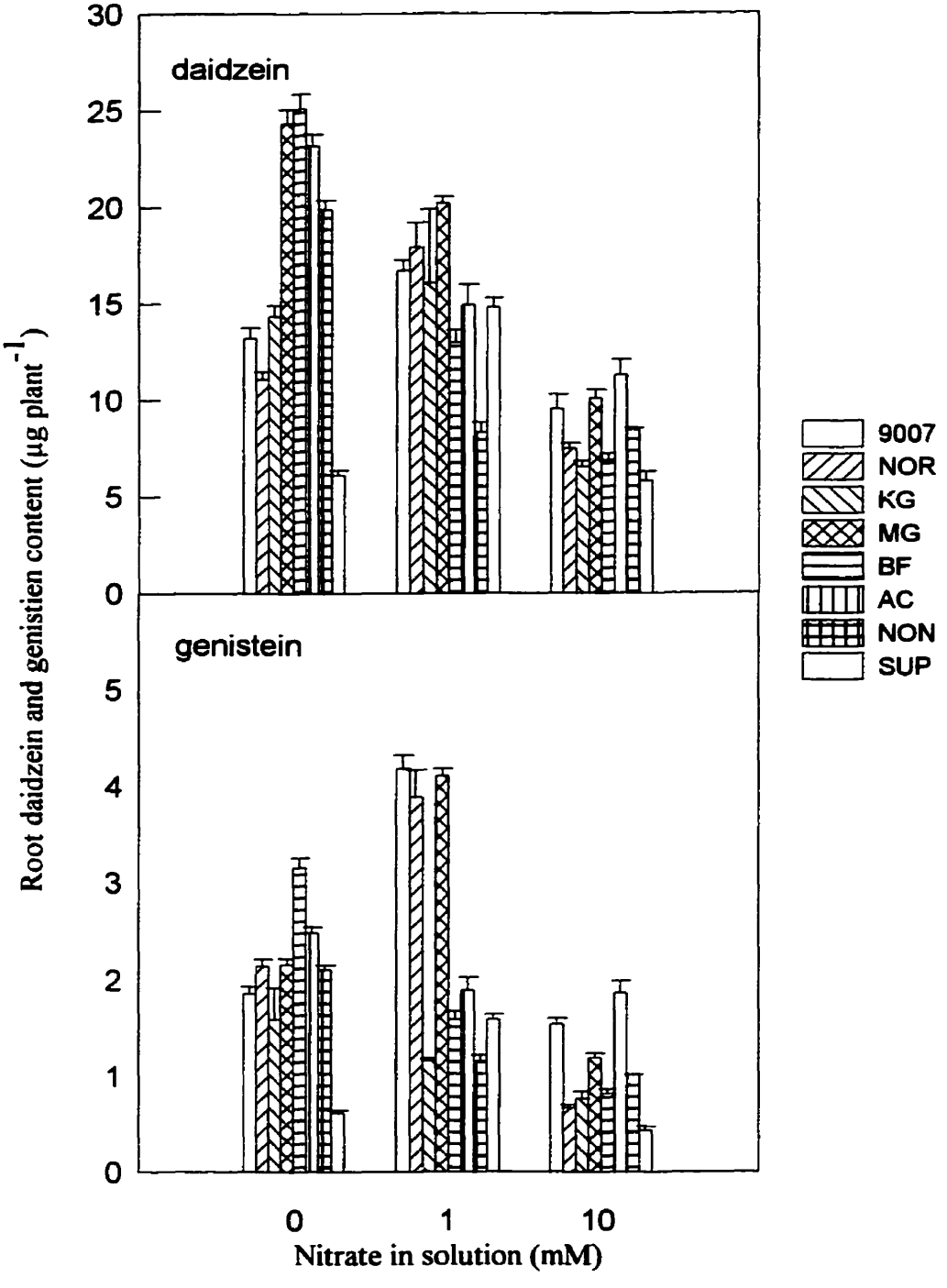

Fig. 6.1 Root daidzein and genistein contents of soybean plants grown at three levels of nitrate. Vertical bars indicate one standard error unit.

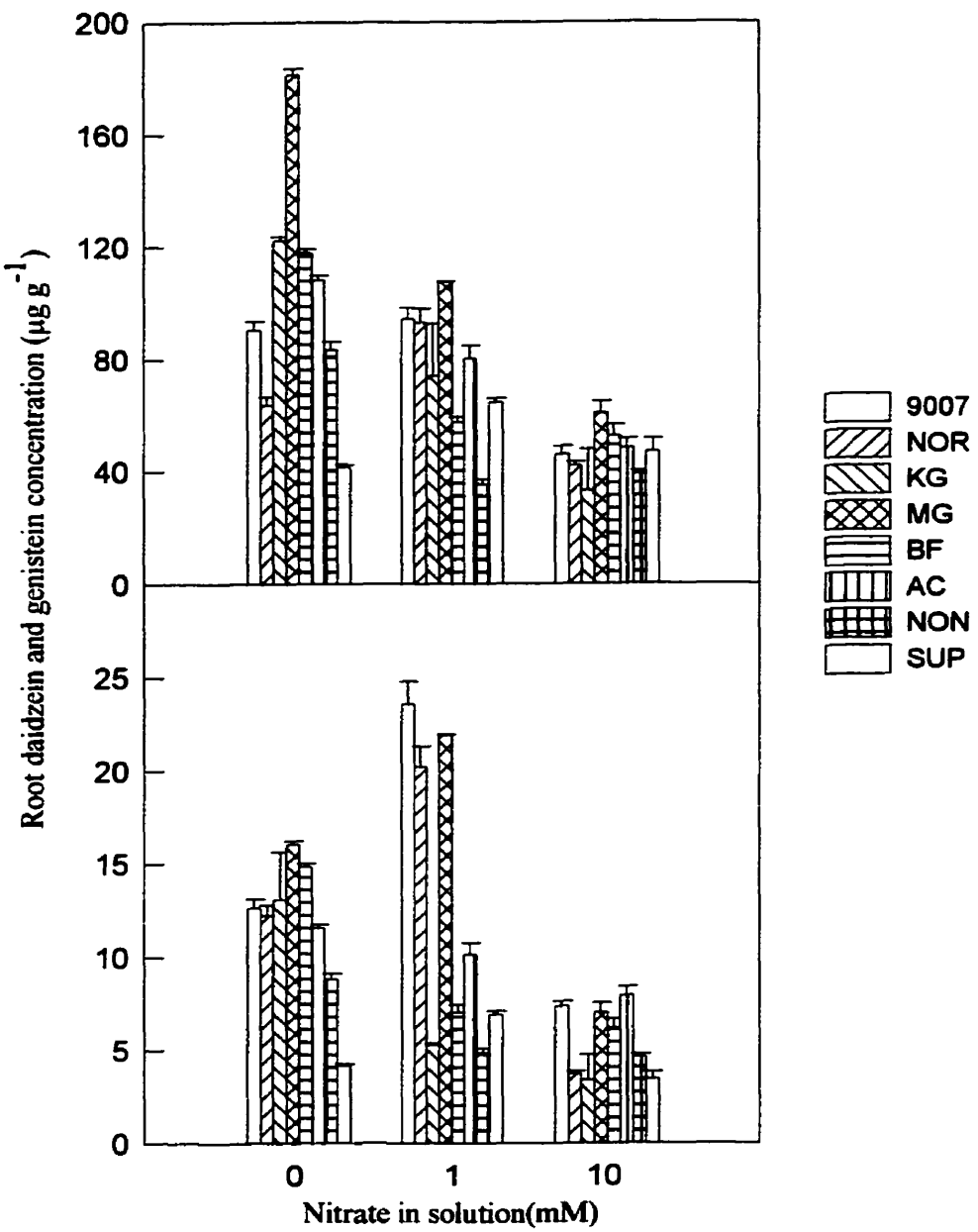

Fig. 6.2 Root daidzein and genistein concentrations of soybean plants grown at three levels of nitrate. Vertical bars indicate one standard error unit.

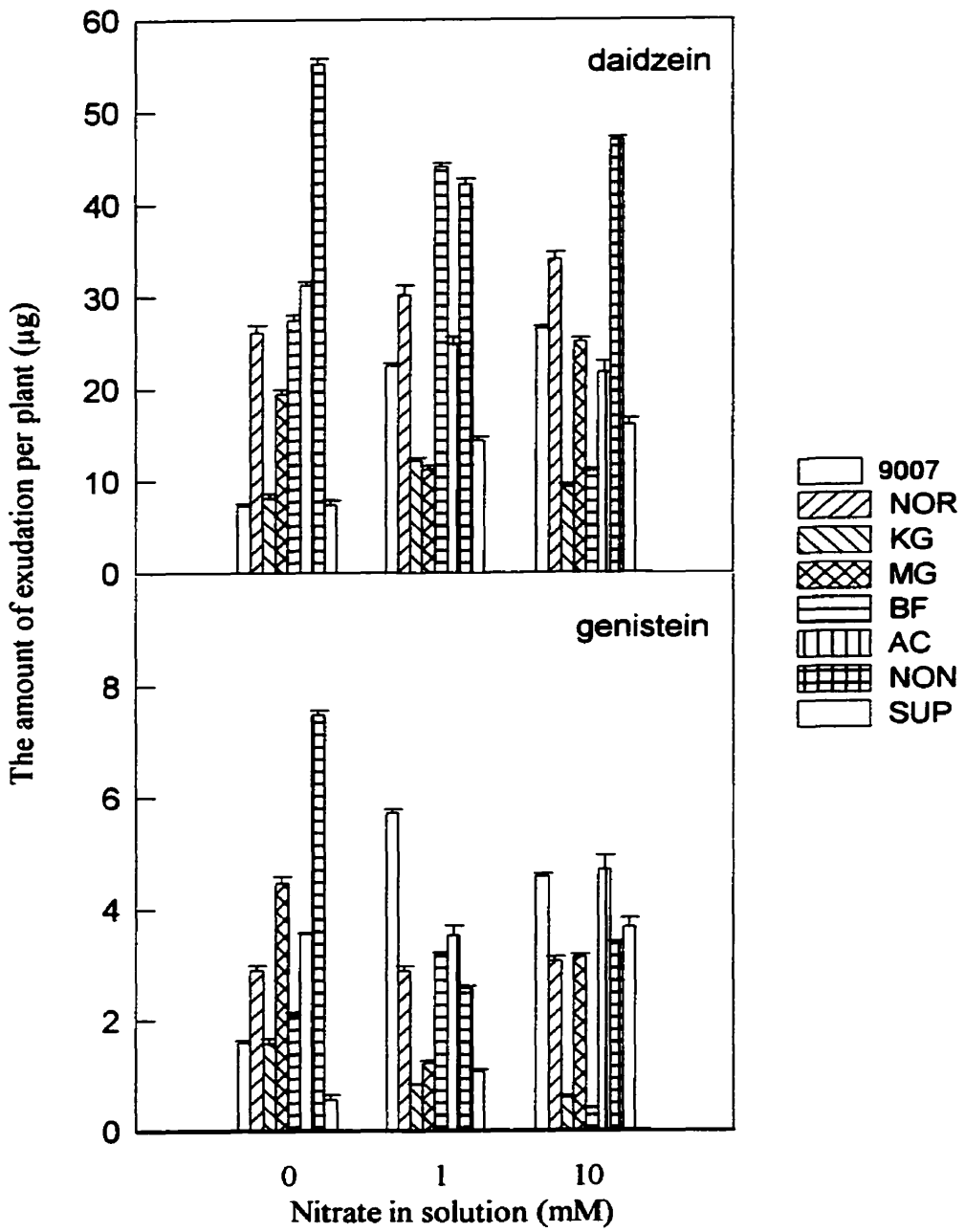

Fig. 6.3 Daidzein and genistein exudation per plant of soybean grown at three levels of nitrate. Vertical abrs indicate one standard error unit.

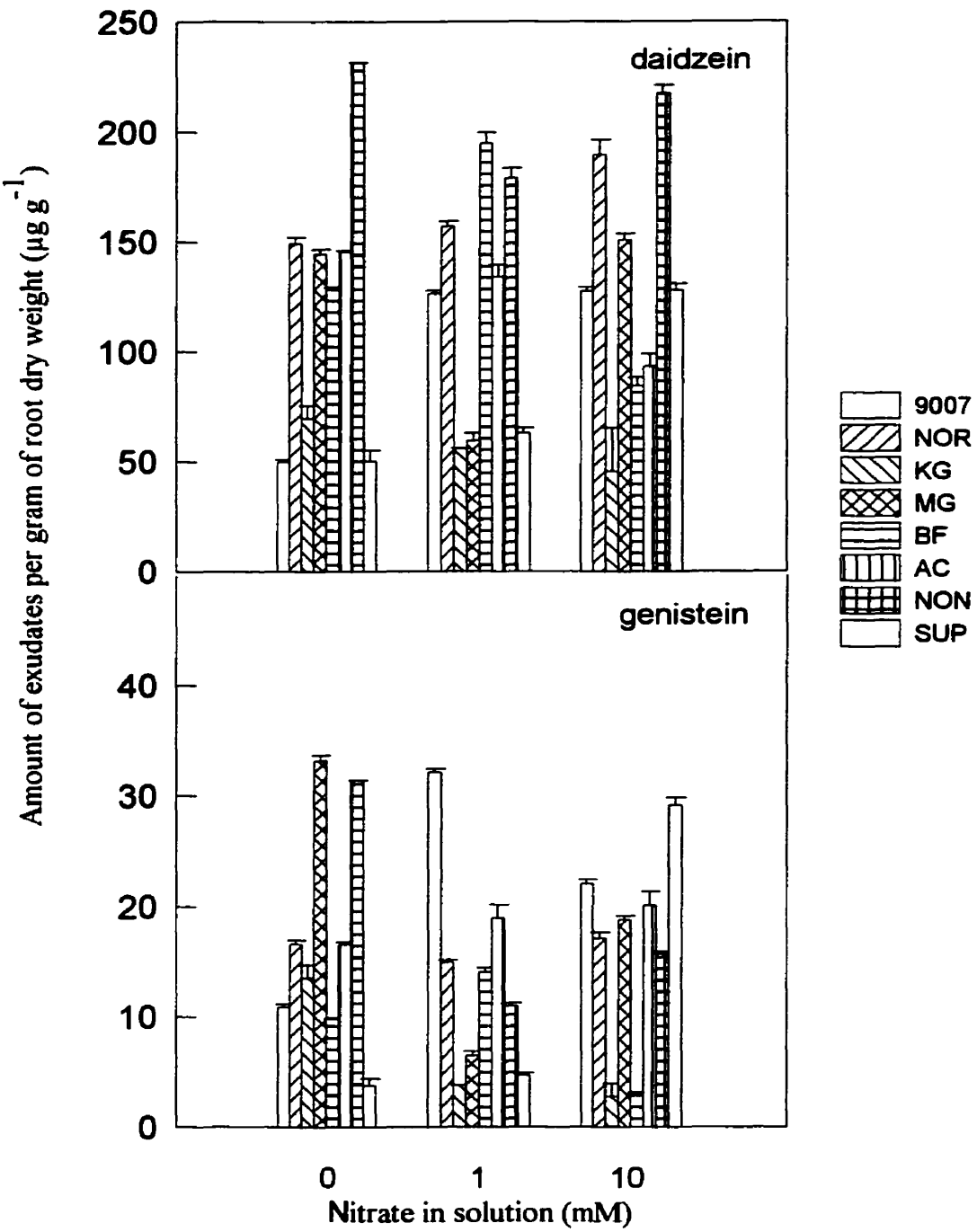

Fig. 6.4 Daidzein and genistien exudation per gram of root dry weight Vertical bars indicate one standard error unit.

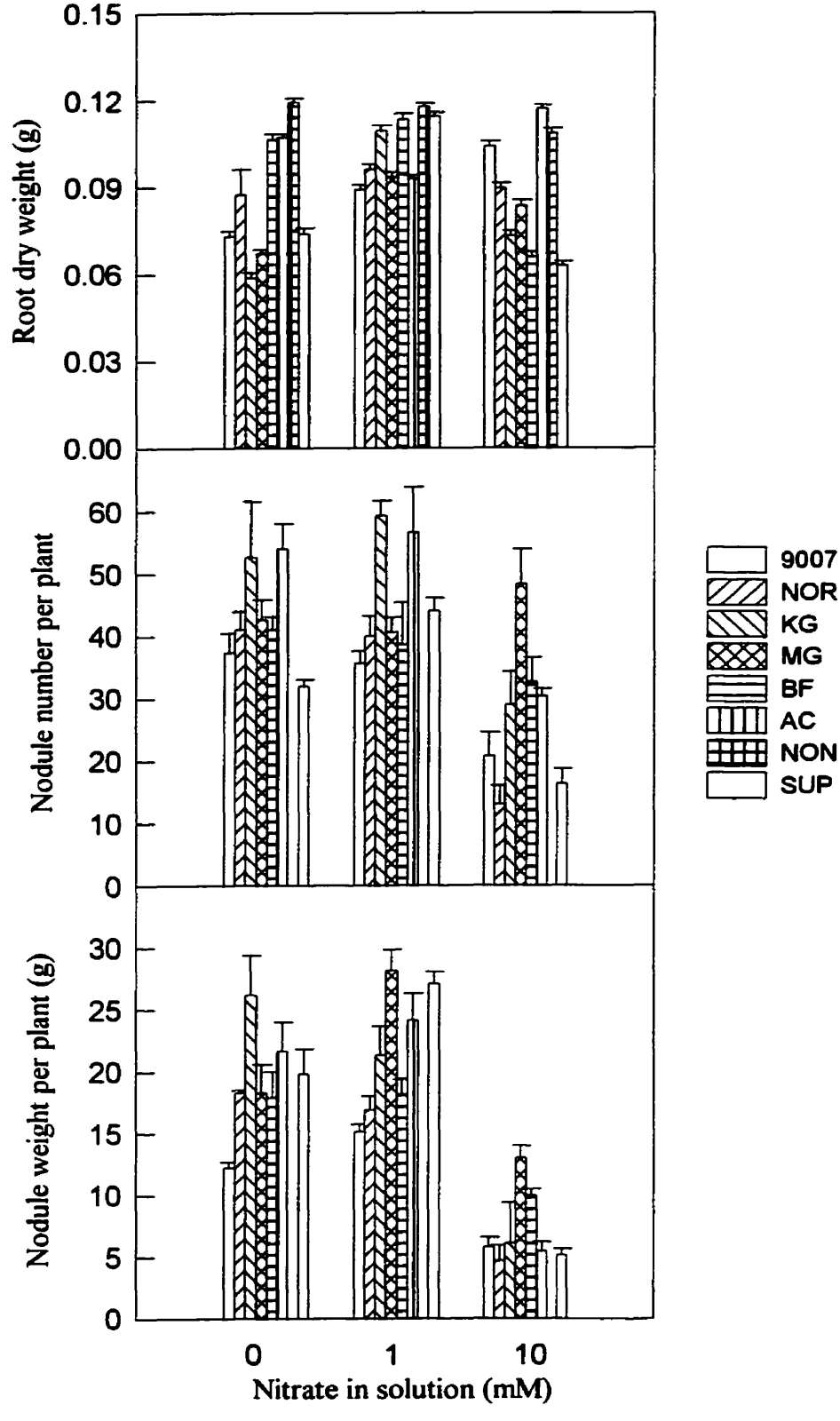

Fig. 6.5 Nodule number, nodule weight and root dry weight of soybean plants grown at three levels of nitrate. Vertical bars indicate one standard error unit.

Table 6.1 Correlation of daidzein and genistein contents and concentration with nodule number, nodule weight and root dry weight (RDW) in roots and in exudates

	0 mM nitrate			1 mM nitrate			10 mM nitrate		
	Nodule number	Nodule weight	Root dry weight	Nodule number	Nodule weight	Root dry weight	Nodule number	Nodule weight	Root dry weight
				Root					
Daidzein content	0.045 ns	-0.167 ns	0.409 *	0.530 **	0.535 **	-0.589 **	0.248 ns	0.074 ns	0.742 **
Genistein content	0.176 ns	0.002 ns	0.538 **	0.064 ns	0.217 ns	-0.809 **	0.248 ns	0.103 ns	0.799 **
Daidzein concentration	0.304 ns	0.150 ns	-0.251 ns	0.473 *	0.489 *	-0.786 **	0.294 ns	0.172 ns	-0.116 ns
Genistein concentration	0.474 *	0.348 *	-0.174 ns	0.075 ns	0.214 ns	-0.848 **	0.352 *	0.204 ns	0.387 *
				Exudates					
Daidzein content	-0.589 **	-0.643 **	0.876 **	-0.576 **	-0.703 **	0.345 *	-0.563 **	-0.488 *	0.646 **
Genistein content	-0.590 **	-0.669 **	0.592 **	-0.181 ns	-0.354 *	-0.497 *	-0.215 ns	-0.265 ns	0.693 **
Daidzein concentration	-0.506 *	-0.560 **	0.738 **	-0.503 *	-0.657 **	0.121 ns	-0.543 **	-0.431 *	0.345 *
Genistein concentration	-0.360 *	-0.451 *	0.210 ns	-0.015 ns	-0.261 ns	-0.607 **	-0.217 ns	-0.196 ns	0.272 ns

ns means not significant at 0.05 probability level. * and ** indicate significant at 0.05 and 0.01 probability levels.

Table 6.2 Correlation of daidzein and genistein contents and concentrations, between root extracts and root exudates

	Daidzein content	Genistein content	Daidzein concentration	Genistein concentration
0 mM nitrate	0.491 *	0.364 *	0.154 ns	0.353 *
1 mM nitrate	-0.597 **	0.387 *	0.414 *	0.519 **
10 mM nitrate	0.315 ns	0.557 **	0.151 ns	0.142 ns

* and ** indicate significant at 0.05 and 0.01 probability levels.
ns means not significant at 0.05 probability level.

Preface to section 7

Section 7 is composed of a manuscript by Pan B and Smith DL and will be submitted to *Plant and Soil*.

After studying host plant signal production, the bradyrhizobial partner was studied. This experiment looked at potential signaling back from the bacterium to the plant. *B. japonicum nod* gene expression was investigated at different mineral N levels. Genistein was then added to the bacterial culture to see if it could overcome the inhibitory effects of mineral N on *nod* gene expression.

Section 7

Nitrate Inhibits *Bradyrhizobium Japonicum NodABC* gene Expression and Genistein Overcomes the Inhibition

7.1 Abstract

In the soybean-*Bradyrhizobium japonicum* symbiosis, the isoflavonoid genistein has been identified as one of the major compounds in soybean seed and root extract and plays a role in inducing the expression of the *B. japonicum nodYABC* operon. As a result of the *nod* gene induction the bacteria-to-plant signal, lipo chito-oligasaccharide nodulation factors, is produced by the bacterial symbiont. These molecules elicit root hair deformation, cortical cell division and nodulin gene expression in plant roots. Soybean nodulation and nitrogen fixation are inhibited by combined nitrogen. Using a *nodY:lacZ* fusion of *B. japonicum*, *nod* gene expression was studied over time, with different nitrate sources and with different concentrations of genistein. The objective of this work was to determine whether genistein addition would overcome the nitrogen inhibition of *nod* gene expression in *B. japonicum*. A factorial experiment with three N sources (potassium nitrate, ammonium sulfate and urea), three levels of each N source (0, 1 and 5 mM), and two levels of genistein (0 and 5 μM) was conducted. The results showed that the relative ß-galactosidase activities (RGA) increased from 6 hours after genistein addition, reached peaks at 48 h, then decreased to a plateau level. *Nod* gene expression was inhibited by all the three N sources, although ammonium sulfate at 1mM was not inhibitory. Potassium nitrate was the inhibitoriest of the three N sources used, while ammonium sulfate was the least. The combination of 1 mM ammonium sulfate and 5μM genistein resulted in the highest RGA. Genistein addition overcame the inhibitory effects of ammonium sulfate on *nod* gene expression.

7.2 Introduction

The specific interaction between the soil bacterium *Bradyrhizobium japonicum* and its host plant soybean (*Glycine max*) results in the formation of root nodules. The formation of nitrogen fixing nodules is a complex, multi-step process requiring a number of plant and bacterial genes. The common *nodDABC* genes have been found in all *Rhizobium* and *Bradyrhizobium* species examined, and are essential for nodulation and nitrogen fixation (Long, 1989). In *Bradyrhizobium* species the *nodY* gene is present upstream of *nodABC* within the same operon and *nodY* is cotranscribed with *nodABC* (Banfalvi et al., 1988). The common *nod* genes of *B. japonicum* can be induced by host-produced flavonoid compounds (Peters et al., 1986). In the soybean-bacteria symbiosis, the isoflavonoid genistein has been identified as one of the major compounds in soybean seed and root extracts responsible for the inducing expression of the *B. japonicum nodYABC* operon (Kosslak et al., 1987). As a result of *nod* gene induction, lipo-chito-oligasaccharide nodulation factors are produced by the bacterial symbiont (Lerouge et al., 1990), which in turn elicits root hair deformation, cortical cell division and nodulin gene expression in soybean plant roots (for a review see Spaink, 1996).

Many legume species have direct access to biologically fixed nitrogen, but preferentially utilize nitrate rather than develop a root nodule symbiosis with *(Brady)Rhizobium*. Soybean nodulation and nitrogen fixation can be inhibited by nitrate, urea and ammonium (for a review see Streeter, 1988). However, a small amount of combined nitrogen in the soil is beneficial to the yield of some legumes, such as soybean (Harper, 1974). Another problem in the inhibition of nodulation and nitrogen fixation is that the effects of combined N vary depending on the type of combined N (Vigue et al., 1977; Yoshida and Yatazawa 1967). Nodulation processes are generally more sensitive to nitrate than to ammonium and urea is only slightly inhibitory at least with certain legumes (Ryo and Phung, 1984; Dart and Wildon, 1970).

There are several mechanisms of nitrate inhibition on nodulation and nitrogen fixation (Streeter, 1988). However, most of the early studies were concentrated on the

plant side (Carroll and Mathews, 1990). Some *B. japonicum* strain variation in the sensitivity of soybean symbioses to nitrate does exist; however, the presently known variation seems insufficient to lead to a substantial field benefit (McNeil, 1982). Using a *nod::lacZ* fusion, Dusha et al. (1989) reported that expression of *Rhizobium meliloti nod* ABC and *nodD3* operons is ammonia repressible. Wang and Stacey (1990) also reported that expression of *nodD1* and *nodYABC* operons of *B. japonicum* is also repressed by ammonia. Using the same approach, Baev et al. (1992) reported that the induction of the *Rhizobium leguminosarum* bv. *viciae nodD* and *nodABC* genes by the flavanone naringenin is not affected in response to different nitrate and/or ammonium salt concentrations.

Flavonoid contents of the symbiotic host plants have been shown to affect legume nodulation and nitrogen fixation (Appelbaum, 1990). A hypernodulating soybean mutant had higher root concentrations of isoflavonoid compounds than did the parental cultivar at 9-12 days after inoculation (Cho and Harper, 1991). When 2.5 mM $(NH_4)_2SO_4$ was applied to the soybean plants, the concentration of three isoflavonoids in the root were decreased (Cho and Harper, 1990). Kapulnik et al. (1987) reported that the superior nodulation and N_2 fixation of HP32 alfalfa compared to HP alfalfa were associated with a 77% increase in the amount of plant tissue luteolin (the preferred inducer signal for *Rhizobium meliloti nod* genes). When genistein was applied to either *B. japonicum* inoculum (Zhang and Smith, 1995) or soil (Zhang and Smith, 1996) soybean nodulation and nitrogen fixation ability were increased at low root zone temperatures. Soybean nodulation and nitrogen fixation inhibitions by nitrate were partially overcome when applying genistein to different nitrate growing media (Pan and Smith, unpub. data).

The research described below was designed to determine: (1) the time course *nod* gene expression in the presence of ammonium sulfate, (2) the *nod* gene expression inhibiting ability of different N sources and (3) the ability of genistein to overcome mineral N inhibition of *nod* gene expression.

7.3 Material and Methods

Bacterial strain:

Bradyrhizobium japonicum strain ZB977 (USDA110 containing the plasmid pZB32) was used in this experiment (from Dr G. Stacey, University of Tennessee, Knoxville, Tennessee). In pZB32 there is a translational fusion between the *B. japonicum nodY* open-reading frame and *lacZ* of *Escherichia coli*. The amount of ß-galactosidase activity is used to reflect the expression of the isoflavonoid-inducible *nod* genes.

ß-Galactosidase assays:

ß-Galactosidase was measured by the methods of Miller (1972), in which toluene was used for opening cells. Background ß-galactosidase activity was determined by non-induced ZB977. The calculation of ß-galactosidase specific activity (GSA) was based on the formula: activity = OD_{420}*1000/(OD_{600}*time*volume) (Miller, 1972). OD_{420} value was used to indicate the total ß-galactosidase specific activity (TGA).

Experiment one:

Bacteria were grown in yeast extract mannitol medium (YEM). Tetracycline was added to a concentration of 100 $\mu g\ mL^{-1}$ to maintain pZB32. Fifty mL cultures in 250 mL flasks were shaken at 250 rpm at 25°C for 6 days. For the subculture, 2 mL of culture (OD_{600}=0.243) was added into 50 mL of fresh YEM medium in 250 mL flasks, and shaken under the same conditions as the initial culture. The appropriate amount of ammonium sulfate stock solution (in distilled water) and genistein (in pure methanol) were added to the subculture medium before bacterial culture inoculum were added. The final genistein and ammonium sulfate concentrations of the subculture were 0 and 10 mM. Growth inhibition of the *Rhizobium* strains by low levels of methanol is known to be slight (Pankhurst and Biggs, 1980), so no methanol control was used in this experiment. As the *nodD* or *nodY* fusion activity is little affected by increasing salt concentration (Wang and Stacey, 1990), no salt control was used in this experiment. Samples were taken at 6, 12,

24, 48, 72, 96 and 148 h after subculture. All flasks were returned to the shaker immediately after sampling. Following each sampling time, ß-galactosidase activity was measured.

Experiment two:

Bacteria and cultural conditions were the same as in experiment one. Based on the results of experiment 1, 48 and 96 h after subculture was fixed as harvest times in this experiment. Ammonium sulfate concentrations were at 0, 1, 5, 10 and 20 mM. Genistein concentrations were 0, 5, 20, 40 and 60 µM.

Experiment three:

The general conditions of this experiment were the same as experiments one and two. In this experiment, genistein was fixed at 0 and 5 µM. Three N source, potassium nitrate, ammonium sulfate and urea, were used. Samples were taken at 48 and 96 h after subculture.

Experimental design and statistical analysis:

Random completely block designs were used in each experiment. When the required space exceeded that available on one shaker, two blocks were placed on a second shaker. Each experiment was repeated at least two times.

Results were statistically analysed by analysis of variance using the Statistical Analysis System computer package (SAS Institute Inc., 1988).

7.4 Results

Time course expression of nod genes and inhibition of nod gene expression by ammonium

To monitor the time course *nod* gene expression after genistein addition, ß-galactosidase activity was assayed at regular intervals. There were no differences among

the treatments at 6 h after genistein addition (Fig 7.1B). RGA increased from 6 h after genistein addition, reached peaks at 48 h, then decreased. At 148 h after genistein addition, RGA were lower than those at 96 h at 10 mM ammonium sulfate, while the total ß-galactosidase activities (TGA) were greater at 148 h than those at 96 h at 0 ammonium sulfate (Fig 7.1A). At 0 ammonium sulfate the TGA was higher than those at 10 mM ammonium sulfate. Thus *nod* gene expression was inhibited by ammonium sulfate.

Genistein effects on nod gene expression and cell growth

There were interactions between genistein and ammonium sulfate for RGA. Without ammonium sulfate RGA was lower at the lower concentration of genistein (Fig 7.2C). At 1 mM ammonium sulfate 5μM genistein resulted in the highest RGA. As ammonium sulfate concentration increased, higher levels of genistein were required to increase the RGA. Thus genistein was able to overcome the inhibition of *nod* genes by ammonium sulfate. The pattern in TGA was almost the same as RGA, except that 5 μM genistein showed high levels of TGA at all ammonium sulfate levels (Fig 7.2B). Genistein inhibited *B. japonicum* cell growth (Fig 7.2A).

Nod gene expression with different N-sources

Nod gene expression was inhibited by all three N sources (Fig 7.3C). Potassium nitrate was the most inhibitory among the three N sources used, while ammonium sulfate was the least inhibitory. The differences in TGA among the three N sources were much less than the differences in RGA (Fig 7.3B). Bacterial growth was promoted by nitrate potassium and ammonium sulfate (Fig 7.3A). Urea decreased *B. japonicum* cell number at the concentrations used in this experiment. The reason for the decrease is not clear. The decreases in *B. japonicum* cell density could partially explain the high value of RGA, although TGA was also high with urea treatments (Fig 7.3B). The highest value of TGA was found with 1 mM ammonium sulfate. Inhibition of *B. japonicum* cell growth by genistein was also observed in this experiment (Fig 7.3A).

7.5 Discussion

The inhibition of *nod* gene expression by ammonium is in agreement with the results of Wang and Stacey (1990) and Dusha et al. (1989). The inhibition of *nod* gene expression could lead to decreased Nod factor production, which may partially explain the inhibition of nodulation and nitrogen fixation at high levels of ammonium (Streeter, 1988). The selection of 5 and 20 μM genistein concentrations were based on previous experiments (Zhang and Smith, 1995). In their experiment they reported that at lower root zone temperatures 20 μM genistein and at 25°C 5 μM genistein were the most effective concentrations for stimulating nodulation. This experiment was conducted at room temperature and 5 μM genistein produced the highest level of *nod* gene induction in the absence of ammonium sulfate.

ß-Galactosidase activity calculated according to Miller's methods (Miller, 1972), takes cell density into account. The problem here is that unlike other inducers, such as daidzein and coumestrol which have been shown to promote the growth of *B. japonicum* (D'Arcy-Lameta and Jay, 1987), genistein was found to inhibit *B. japonicum* growth and the inhibition was concentration dependent (see Fig. 7.2A). Comparing the RGA and TGA we found that at 6 h after genistein addition, the 0 N treatment had higher TGA than the 10 mM N treatment, although there was no difference in RGA (Fig 7.1A). TGA were lower with 20 μM genistein and 10 mM ammonium sulfate from 48 to 148 h after genistein addition. There was an increase in TGA for all treatments at the last sampling. Ten mM ammonium sulfate decreased RGA, while 0 N showed increases in RGA at the last sampling. This may be explained by the increases in cell density with 10 mM ammonium sulfate. Genistein effects on *B. japonicum* growth could explain the differences between RGA and TGA. From a practical point of view, TGA might be more important than RGA. The relationship between ß-galactosidase activity (either RGA or TGA) and nodulation is not clear at this time and should be studied.

Genistein had significant detrimental effects on *B. japonicum* cell growth (Fig 7.2A). This result is contrary to the study of Pankhurst and Biggs (1980). They reported

little or no inhibitory activity of the *B. japonicum* growth by genistein. Negative effects of genistein on growth were also found in another studied. When 20 μM genistein was watered to a soybean rooting medium for four successive day, soybean roots turned brown (Pan and Smith, unpub. data).

Previous work with soybean plants found that nitrate was the form of combined N most inhibitory to soybean nodulation and nitrogen fixation (Yoshida and Yatazawa, 1967), and that urea had much less inhibitory effect on nodulation than nitrate (Vigue et al., 1977). The results reported here suggest that nitrate, as well as ammonium sulfate and urea, exert their effects early in the nodulation process and at least partially, through the bacterial cells. Calvert et al. (1984) reported that, for soybean nodule formation, the initial stages in the infection process appear to be the most sensitive steps. Inhibition is substantially alleviated by delaying exposure to nitrate until 18 h after inoculation (Malik et al., 1987). This indicated that most of the nitrate-sensitive events of infection were functionally complete within less than 18 h. This sensitive event may include plant isoflavonoid production, bacterial reception of those isoflavonoids and bacteria-to-plant signal production by the common and specific *nod* genes. With increasing combined N concentrations, RGAs were decreased for all three forms of N. Urea treatment resulted in higher RGA values than ammonium sulfate.

The results of this work partially explain the inhibition of nodulation and nitrogen fixation by mineral nitrogen and indicate an important role on the bacterial side. Stacey et al., (1997) reported that *nod* gene expression in *B. japonicum* is a much more complex system and involves more regulatory proteins than just NodD, NodV and NodW comprise a second isoflavone recognition system (the first being NodD) in *B. japonicum* (Sanjuan et al., 1994). The effect of nitrate on the expression of the second isoflavone recognition system (or perhaps on currently unknown systems) is not clear at this time. Organic acids can also inhibit the *nod* gene expression in *B. japonicum* (Yuen and Stacey, 1996), suggesting that other regulation systems exist.

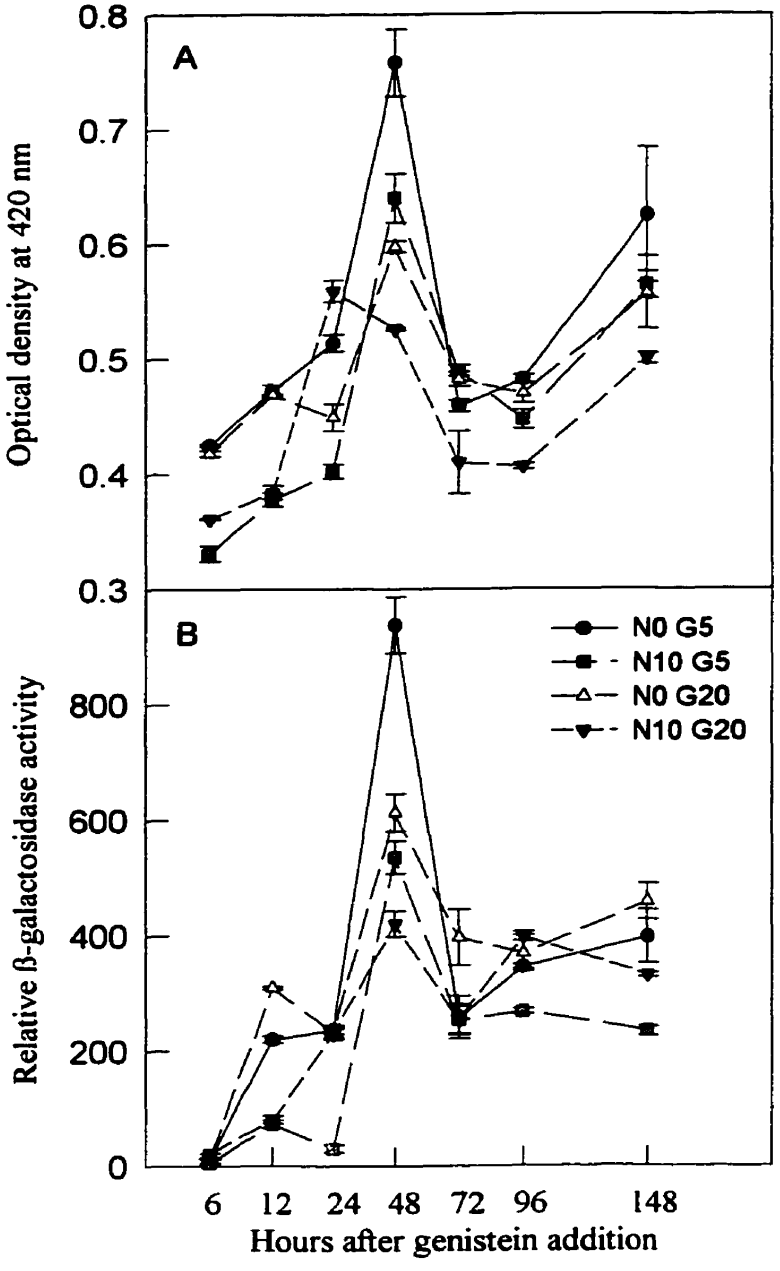

Fig. 7.1 ß-galactosidase activities of *B. japonicum* at different sampling times. N0 and N10 mean 0 and 10 mM ammonium sulphate respectively. G5 and G20 mean 5 and 20 µM genistein respectively. The vertical bars indicate one standard error unit.

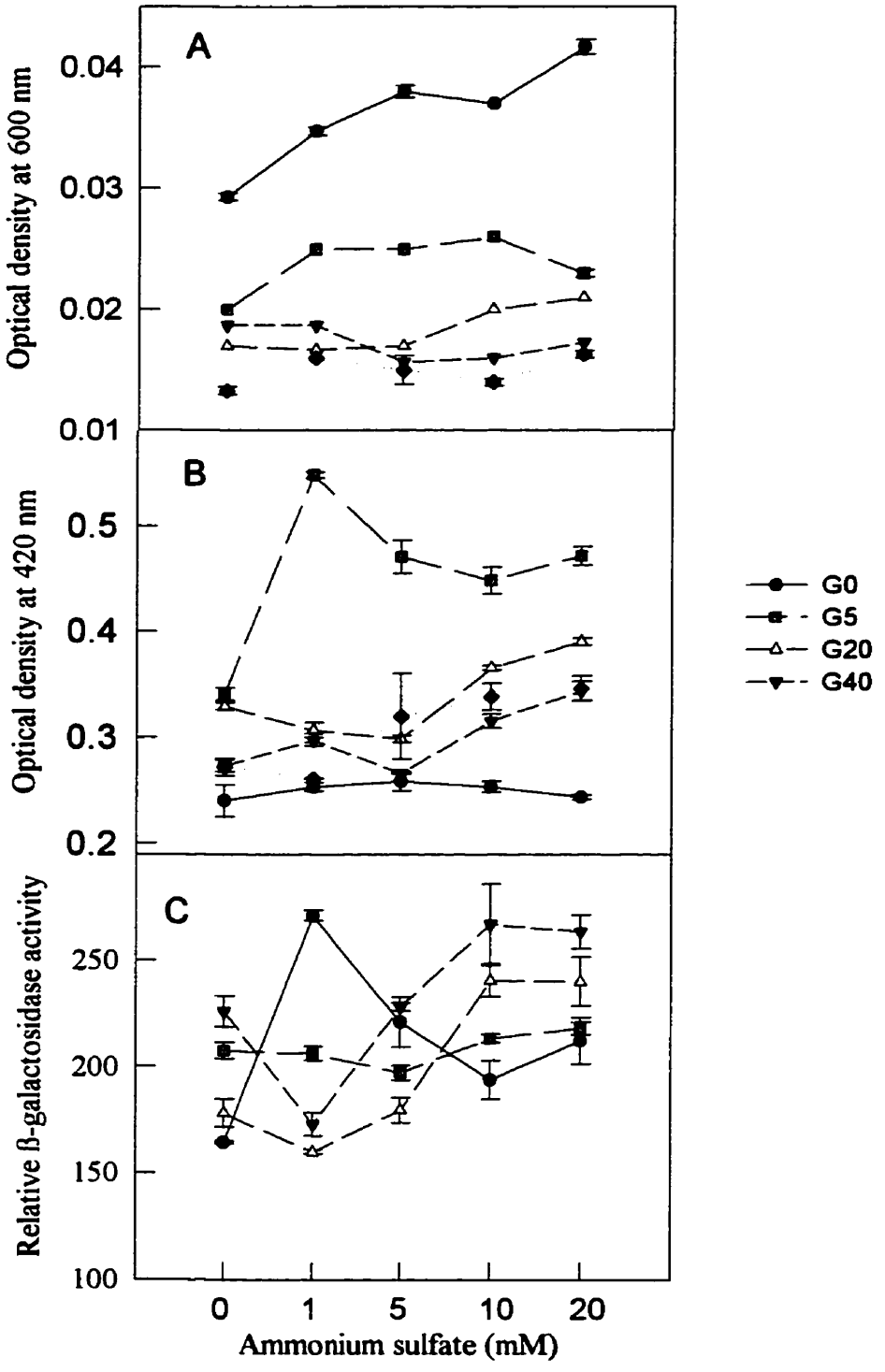

Fig. 7.2 -galactosidase activities of *B. japonicum* grown at different concentrations of ammonium sulphate and genistein. N0 and N10 mean 0 and 10 mM ammonium sulphate respectively. G5 and G20 mean 5 and 20 µM genistein respectively. The vertical bars indicate one standard error unit.

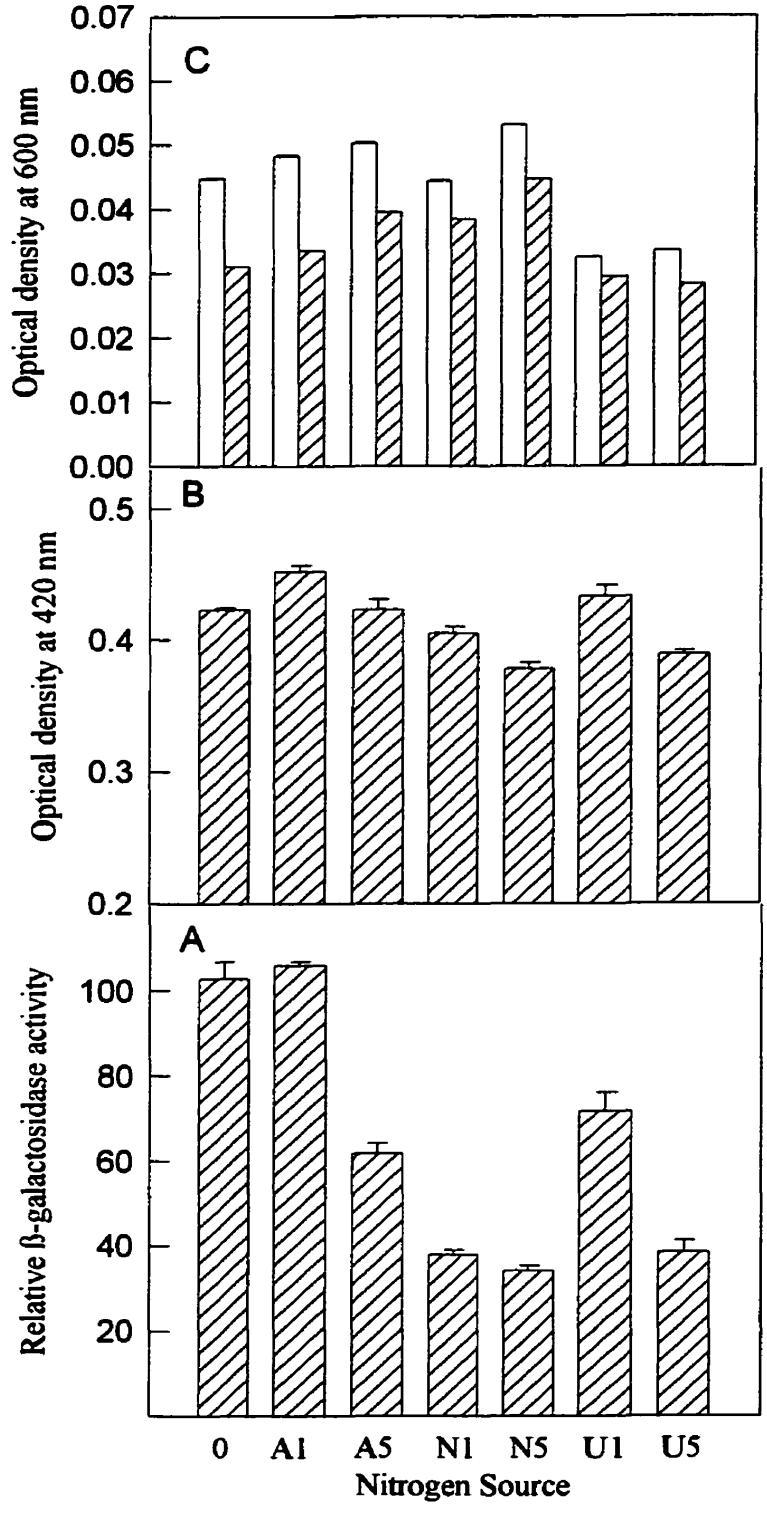

Fig. 7.3 Comparision of -galactosidase activities when *B. japonicum* cells were grown in potasium nitrate (N), ammonium sulphate (A) and urea (U). 0, 1 and 5 associated with nitrogen source mean nitrogen concentrations in mM. Genistein concentration was 5 µM in B and C. The vertical bars indicate one standard error unit.

Preface to section 8

Section 8 is composed of a manuscript by Pan B and Smith DL accepted by *Journal of Agricultural Science*, 1999

Knowing that mineral N could inhibit isoflavonoid synthesis and exudation (Sections 5 and 6), genistein was added to the rooting medium or was used to incubate bradyrhizobial cells, hoping this could compensate for the decrease in inside isoflavonoid concentration of host plants and possible need for more signal by the bacteria and overcome the negative effects of mineral N.

Section 8

The effect of application of genistein to *Bradyrhizobium japonicum* culture and its rooting medium on soybean growth, nodulation and nitrogen assimilation in the presence of nitrate

8.1 Abstract

In the soybean [*Glycine max* (L.) Merr.]-*B. japonicum* symbiosis, genistein has been identified as one of the major compounds in soybean seed and root extracts responsible for inducing the expression of the *B. japonicum nod* genes. High combined nitrogen in the growth medium inhibits nodulation and nitrogen assimilation. Experiments were conducted to test the possibility of overcoming this inhibition by adding genistein to the rooting medium or incubation of *B. japonicum* cells with genistein. The experiments were conducted in a glasshouse using a completely randomized design with two soybean cultivars, three rooting medium nitrate concentrations (0, 5 and 10 mM) and four genistein treatments. The genistein treatments were 0 (control), incubation of *B. japonicum* cells with 5 μM genistein, and regular watering with 5 μM or 20 μM genistein. Two and three way interactions among the three experimental factors existed. Root growth was inhibited by repeated watering with 20 μM genistein. Weight per nodule was greater at 5 mM than at 0 mM nitrate. At 10 mM nitrate watering with genistein resulted in increases of nodule dry weight per plant. Shoot nitrogen contents were increased at 5 mM nitrate by genistein incubation and watering with 20 μM genistein. Watering with 5 μM genistein increased nodule nitrogen concentrations at both 5 and 10 mM nitrates. The two soybean cultivars used had different responses to the genistein and nitrate treatments. Genistein could, at least partially, overcome the inhibition of soybean nodulation and nitrogen assimilation by nitrate.

8.2 Introduction

The interaction between the soil bacterium *B. japonicum* and its host plant, soybean,

results in the formation of nitrogen-fixing root nodules. Nodule formation is a complex, multi-step process requiring the activity and regulation of both plant and bacterial genes, which control the signal exchange between plants and nitrogen-fixing bacteria. In the soybean-*B. japonicum* symbiosis, genistein has been identified as one of the major isoflavonoid compounds in soybean seed and root extracts responsible for inducing the expression of the *B. japonicum nodYABC* operon (Kosslak et al., 1987). As a result of *nod* gene induction, lipo-chito oligosaccharide nodulation factors (Nod factors) are produced by the bacterial symbiont (Lerouge et al., 1990), which in turn elicit root hair deformation, cortical cell division and nodulin gene expression in soybean roots (for a review see Spaink (1996)).

Legume plants have access to biologically fixed nitrogen, but preferentially utilize combined nitrogen (including nitrate and ammonium). Soybean nodulation and nitrogen fixation are inhibited by high concentrations of combined nitrogen in the rooting media (for a review see Streeter, 1988). Nodulation processes are generally more sensitive to nitrate than to ammonium (Ryo and Phung 1984). Many investigations have demonstrated that maximum symbiotic performance is associated with low concentration of combined nitrogen in the rooting media (Nutman 1956; Harper 1974). Studies of biomass (Imsande 1998) and leaf fluorescence (Maury et al., 1993) also showed that, in the presence of non-inhibitory concentration of fertilizer-N, the photosynthetic efficiency of a soybean plant is increased by rapid N_2 fixation during pod fill. The inhibition of soybean nodulation can be alleviated by high inoculum doses (Lawson et al., 1988) and overcome by the ethylene inhibitor aminoethoxyvinylglycine (Ligero et al., 1991).

The mechanism of the combined nitrogen inhibition on nodulation and nitrogen assimilation is not well understand, although several explanations have been proposed (Streeter, 1988; Carroll and Mathews, 1990). Changes in isoflavonoid content of the symbiotic host plants has recently been suggested as a possible explanation (Appelbaum, 1990; Cho and Harper, 1991). A hypernodulating soybean mutant had higher root concentrations of isoflavonoid compounds than did the parental cultivar at 9-12 days after

inoculation (Cho and Harper, 1991). When 2.5 mM $(NH_4)_2SO_4$ was applied to the soybean plants, the concentrations of three isoflavonoids in the roots were decreased (Cho and Harper, 1990). When genistein was applied to either *B. japonicum* inoculant (Zhang and Smith, 1995) or soil (Zhang and Smith, 1996), soybean nodulation and nitrogen assimilation were increased at low root zone temperatures, but soil nitrate concentration was not investigated in those experiments. The expression of the common *nod* genes is repressed by ammonia (Dusha et al., 1989; Wang and Stacey, 1990).

In this experiment, we incubated *B. japonicum* with genistein or applied genistein to the rooting medium under three nitrate concentrations to determine: (1) the effects of genistein on soybean growth, nodulation and nitrogen assimilation, and (2) if genistein could overcome the inhibition of nitrate on nodulation and nitrogen assimilation.

8.3 Materials and Methods

Experimental condition and design

The experiments were done in a glasshouse conditions. The air temperature regime was 25/18°C (day/night), and the relative humidity was 65%. The day : night cycle was 14:10 hours. On cloudless days the light intensity reached 900 μmol m^{-2} s^{-1}. Turface (Applied Industrial Material Corp, Deerfield, IL) : sand (1:1, v/v) was used as a rooting medium.

The genistein treatments were 0 (control), 5 μM genistein incubation of *B. japonicum*, and watering with 5 or 20 μM genistein solutions four times (3 days before inoculation, the day of inoculation, 3 and 6 days after inoculation [DAI]). Nitrate concentrations were 0, 5 and 10 mM KNO_3. The experiment was a random completely design with three replications. Nitrate solution was applied beginning at sowing, and was added each time the plants need water. Hoagland's solution (Hoagland and Arnon, 1950) was added twice a week beginning at the time when the cotyledons turned yellow. The Hoagland's solution used in these studies was modified to provide a nitrogen-free solution: the $Ca(NO_3)_2$ and KNO_3 were replaced with $CaCL_2$, K_2HPO_4, and KH_2PO_4. The correct

amount of nitrate or genstein stock solution were added each time to the nutrient solution for each treatment and 250 ml of nutrient solution were added to each pot (diameter 15 cm and height 20 cm) at each watering. That amount of solution was enough to wet the whole rooting medium and cause some solution to run out the bottom of the pots, so that the pots were flushed at each watering.

Plant materials and inoculation

Seeds of the soybean cultivars AC Bravor and Maple Glen were surface-sterilized in 4% sodium hypochlorite for 7 min, and then rinsed 10 times with distilled water. Five seeds were sown in each pot. When the seedlings reached the vegetative-cotyledon stage (VC, unifoliolate leaves unrolled sufficiently that the edges were not touching, Fehr et al., 1971), the seedlings were thinned to 2 plants per pot.

The inoculant were produced by culturing *B. japonicum* stain 532C in yeast extract-mannitol broth in 250-mL flasks shaken at 150 rpm at 25°C. A 5-day-old sub-culture (OD≈0.4) was diluted with distilled water to an A_{620} of 0.1 (10^8 cells mL^{-1}). Genistein stock solution was added to the diluted bacteria to make the final genistein concentration of 5 μM, and kept at 25 °C for 24 hour. One millilitre of inoculant per plant was applied by pipette onto the rooting medium at the base of the plant at the VC stage.

Data collection

The experiment was conducted twice under the same conditions. Plants were harvested at 20 and 40 DAI. Leaf area and number, small (diameter < 1.5 mm) and large (diameter ≥ 1.5 mm) nodule number, nodule weight, shoot and root weight were measured. Shoot and root nitrogen concentrations were collected in the second experiment and determined by Kjeldahl analysis (Kjeltec system, Tecator AB, Hoganas, Sweden).

Statistical analysis

Data were analysed by analysis of variance using the Statistical Analysis System computer package (SAS Institute Inc., 1988). When the data did not meet the assumptions of ANOVA, appropriate data transformations were conducted. A protected least significant difference test was applied to make comparisons among means. Statements about significant effects in the text related to P value of 0.05 unless otherwise stated.

8.4 Results

Genistein main effects

In the first experiment, plant growth was promoted by genstein incubation at 20 DAI, and by watering with 5 μM genistein at 40 DAI (Table 8.1). Root weight was decreased by watering with 20 μM genistein at both 20 and 40 DAI.

In the second experiment, incubation of *B. japonicum* with genistein increased root dry weight at 20 days after inoculation (Table 8.2), while root growth was inhibited by watering with 20 μM genistein. At 40 DAI shoot dry weights were increased by genistein incubation of *B. japonicum*. Weight per nodule was increased by all genistein treatments.

Genistein and nitrate interactions

In the first experiment, nodule number and weight were increased by genistein incubation of *B. japonicum* cells at 0 nitrate at both 20 and 40 DAI (Table 8.3). At 5 mM nitrate, incubation of *B. japonicum* cells and watering with 5 μM genistein increased nodule number and weight 20 DAI. At 10 mM nitrate nodule number and weight were increased by watering with 5 μM genistein at 40 DAI. Nodule weights were decreased by watering with 20 μM genistein at 0 and 10 mM nitrate at 20 DAI, but not at 40 DAI.

In the second experiment, at 20 DAI genistein had no effect on leaf area at the 0 and 10 mM nitrate (Table 8.4). At 5 mM nitrate, incubation of *B. japonicum* cells with genistein increased leaf area and shoot dry weight. Shoot dry weights were decreased by

incubation *B. japonicum* cells with genistein and watering with 20 μM genistein at 10 mM nitrate. At 40 DAI nodule dry weights were not increased by the genistein treatments at 0 and 5 mM nitrate. At 10 mM nitrate watering with 5 and 20 μM genistein resulted in increases of nodule dry weight. Shoot nitrogen content was increased at 5 mM nitrate by genistein incubation of *B. japonicum* cells and watering with 5 μM genistein. At 10 mM nitrate shoot nitrogen content were increased by watering with 5 μM genistein. Watering with 5 μM genistein increased nodule nitrogen concentration at both 5 and 10 mM nitrates.

Cultivar response to genistein application

At 20 DAI, watering with 5 μM genistein decreased large nodule number of AC Bravor, but had no effect on small nodule number (Table 8.5). For Maple Glen incubation of *B. japonicum* cells with genistein and watering with 5 μM genistein increased large nodule numbers; small nodule number was increased by watering with 5 μM genistein.

Forty DAI leaf nitrogen concentration was increased by inoculation with genistein incubated *B. japonicum* cells for AC Bravor and by watering with 20 μM genistein for Maple Glen. No effects were found for the other treatments.

Cultivar × genistein × nitrate interaction

At 20 DAI, the nodule dry weight of AC Bravor was decreased by watering with 5 μM genistein at the 0 and 10 mM nitrate (Table 8.4). There were numerical, but not statistical, increases in nodule dry weight at 5 mM nitrate for AC Bravor by all genistein treatments. For Maple Glen genistein treatments increased nodule dry weight and weight per nodule at 5 mM nitrate, while at 10 mM nitrate, genistein treatment decreased the nodule dry weight and weight per nodule. Weight per nodule for AC Bravor at 5 mM nitrate was increased by watering with 20 μM genistein.

Forty DAI the nodule nitrogen content of AC Bravor was increased by the incubation treatment at 5 mM nitrate and by watering with genistein at 10 mM nitrate. At

10 mM nitrate, nodule N content for the control was much lower than for plants watered with genistein, suggesting that the inhibition of nitrogen assimilation by nitrate was overcome. There were no effects of genistein on nodule nitrogen content at 0 mM nitrate for AC Bravor. Increases in nodule nitrogen content were also found for Maple Glen, its nodule nitrogen contents were increased by the incubation treatment at 0 mM nitrate, watering with 5 μM genistein at 5 mM nitrate and watering with 20 μM genistein at 10 mM nitrate.

8.5 Discussion

Genistein treatments increased nodule dry weight at 5 mM nitrate. Nodule nitrogen contents were also increased for most of the treatments (Table 8.6). This indicates that genistein overcame the nitrate inhibition of nodulation and nitrogen assimilation, for at least some treatments. Three isoflavonoid compounds, including genstein, were reported to decrease when 2.5 mM $(NH_4)_2SO_4$ was applied to the soybean plants (Cho and Harper, 1990). Nitrate was also shown to inhibit the exudation of both genistein and daidzein (another soybean-to-bacteria signal) (B. Pan and D. L. Smith unpublished). On the other hand, microorganisms in the rhizosphere are very active at degrading the isoflavonoids (Barz, 1970). Thus the addition of genistein to the rooting medium could compensate the decreases of genistein concentration inside the root and outside in the rooting medium (exudation), aiding *B. japonicum* in overcoming nitrate inhibition of *nodABC* gene expression (Wang and Stacey, 1990). It could also have been due to the effect of genistein on the composition and molecular mass distribution of exocellular polysaccharides produced by *B. japonicum* (Dunn et al., 1992).

Differences in soybean cultivar nodulation and nitrogen assimilation responses to genistein treatments were also found by Zhang and Smith (1996). These might be partially explained by differences in root genistein content or concentration. A lucerne population (HP32) has more root nodules and fixes more N_2 than the parental HP population. Further study show that HP32 seedling roots contain 60% higher concentrations of

compounds that induce transcription of a *nodABC-lacZ* fusion in *R. meliloti* than comparable extracts of HP roots (Kapulnik et al., 1987). Soybean cultivars also vary in genistein content and concentration (Pan and Smith, 1998; Cho and Harper, 1990).

When soybean plants were watered with 20 μM genistein, the roots turned brown, indicating that genistein had negative effects on them, and root dry weight was deceased (Table 8.1 and 8.2). Weight per nodule was increased by all three genistein treatments (Table 8.2) and nodule weights per plant were also increased by some of the genistein treatments at 40 DAI (Tables 8.3 and 8. 4). A similar result was found in a previous experiment with watering genistein from the onset of nitrogen fixation (Pan et al., 1998). The increasing effects could be due to the role of isoflavonoids as inhibitors of polar auxin transport (Jacobs and Rubery, 1988), increased Nod factor production (Kosslak et al., 1987) and subsequent regulation of cell cycle (Verma, 1992). In one study we found higher genistein concentrations (10 - 40 μM) had significant detrimental effects on *B. japonicum* cell growth (B. Pan and D. L. Smith, unpublished). The inhibiting effects of genistein may be related to its concentration, as watering soybean seedlings with 5 μM genistein did not affect root colour or root dry weight (Table 8.2). Incubating *B. japonicum* with genistein actually increased root dry weight at 20 DAI. This could be due to the Nod factor production by the inocula. Preincubating *B. japonicum* promotes expression of the common *nod* gene and, thus, the production of Nod factor, which can regulate plant morphogenesis at very low concentrations (Spaink, 1996).

In summary, the genistein treatments used in this experiment increased nodule weight and nodule nitrogen assimilation. Plant growth was promoted by incubation of *B. japonicum* cells with genistein, but watering with 20 μM genistein decreased root weight. Incubation of *B. japonicum* cells with genistein and watering with 5 μM genistein could partially overcome the inhibition of nitrate on nodulation.

Table 8.1 Genistein effects on plant growth in the first experiment

	20 DAI				40 DAI			
Genistein	Plant high (cm)	Leaf number	Shoot weight (g)	Root weight (g)	Plant high (cm)	Leaf number	Shoot weight (g)	Root weight (g)
0	23.89	24.11	1.544	0.561	27.66	42.4	2.83	0.892
5I	27.83	25.87	1.733	0.586	29.44	44.3	3.25	0.955
5W	24.06	23.67	1.552	0.489	30.00	47.6	3.56	0.992
20W	25.28	23.22	1.442	0.470	25.44	41.6	2.52	0.727
S.E. (D.F. = 24)	0.569	0.409	0.0470	0.0175	0.544	1.15	0.142	0.0401

DAI = days after inoculation. I = incubation *B. japonicum* with 5 μM genistein.
W5 and W20 = watering with 5 and 20 μM genistein, respectively.

Table 8.2 Genistein effects on shoot dry weight, root dry weight and nodule weight per nodule in the second experiment

	20 DAI	40 DAI	
Genistein	Root dry weight (g)	Shoot dry weight (g)	Weight per nodule (mg)
0	0.435	2.26	1.790
I	0.473	2.43	1.942
W5	0.432	2.21	2.048
W20	0.396	2.32	2.086
S.E. (D.F. = 48)	0.0155	0.132	0.0563

DAI = days after inoculation.
I = incubation *B. japonicum* with 5 μM genistein.
W5 and W20 = watering with 5 and 20 μM genistein, respectively.

Table 8.3 Genistein and nitrate interaction on nodulation in the first experiment

Nitrate (mM)	Genistein	20 DAI Total nodule No.	20 DAI Nodule weight (mg)	40 DAI Large nodule No.	40 DAI Nodule weight (mg)
0	0	97.3	56.6	98.7	188.8
0	5I	128.7	76.1	120.7	204.2
0	5W	96.3	64.3	90.3	198.3
0	20W	76.7	49.0	88 .0	185.3
S.E. (D.F. = 24)		5.98	3.49	4.39	3.04
5	0	68.0	40.1	77.0	205.4
5	5I	75.7	48.3	80.0	210.3
5	5W	83.3	50.3	82.7	221.2
5	20W	80.3	43.4	73.3	192.1
S.E. (D.F. = 24)		2.65	1.33	1.84	4.15
10	0	41.7	19.1	47.3	173.3
10	5I	46.7	17.2	60.7	180.0
10	5W	47.3	17.2	68.3	186.9
10	20W	39.7	15.3	48.0	168.2
S.E. (D.F. = 24)		1.70	0.64	2.90	3.63

W5 and W20 = watering with 5 and 20 μM genistein, respectively.
I = incubation *B. japonicum* with 5 μM genistein.
DAI = days after inoculation.

Table 8.4 Genistein and nitrate interaction on plant growth, nodulation and nitrogen fixation in the second experiment

		18 DAI		40 DAI			
Nitrate (mM)	Genistein	Leaf area (cm^2)	Shoot dry weight (g)	Nodule dry weight (mg)	Shoot N content (mg)	Nodule N concentration (%)	Plant N content (mg)
0	0	151.2	0.683	168.4	13.2	4.491	59.5
0	I	141.0	0.678	202.6	16.8	4.575	72.7
0	W5	156.0	0.603	189.1	14.0	4.100	62.2
0	W20	131.5	0.683	205.7	18.2	4.140	78.3
S.E. (D.F. = 16)		5.44	0.662	7.02	0.72	0.0795	2.75
5	0	305.2	1.202	254.2	32.5	4.068	147.2
5	I	416.3	1.637	273.9	35.1	4.267	149.4
5	W5	338.2	1.349	297.4	34.1	4.556	158.3
5	W20	310.8	1.307	265.4	32.1	3.948	139.9
S.E. (D.F. = 16)		12.78	0.0580	6.62	1.73	0.0687	4.65
10	0	412.5	1.787	211.0	41.3	3.714	189.1
10	I	383.8	1.560	201.8	41.9	4.182	194.8
10	W5	359.2	1.605	242.3	49.4	4.490	205.8
10	W20	398.2	1.435	255.2	48.8	4.115	194.8
S.E. (D.F. = 16)		11.88	0.0563	5.19	1.51	0.1210	6.44

W5 and W20 = watering with 5 and 20 μM genistein, respectively.
I = incubation *B. japonicum* with 5 μM genistein.
DAI = days after inoculation.

Table 8.5 Cultivar and genistein interaction on nodulation and nitrogen fixation in the second experiment

		20 DAI		40 DAI
Cultivar	Genistein	Large nodule number	Small nodule number	Leaf N concentration (%)
AC Bravor	0	54.3	40.8	3.375
Ac Bravor	I	60.4	47.1	3.650
AC Bravor	W5	40.8	41.7	3.380
AC Bravor	W20	56.1	34.2	3.397
S.E. (D.F. = 24)		4.30	3.30	0.0502
Maple Glen	0	47.3	24.8	3.759
Maple Glen	I	58.8	23.7	3.659
Maple Glen	W5	57.8	36.0	3.893
Maple Glen	W20	56.2	22.2	4.010
S.E. (D.F. = 24)		2.45	2.32	0.0340

W5 and W20 = watering with 5 and 20 μM genistein, respectively.
I = incubation *B. japonicum* with 5 μM genistein.
DAI = days after inoculation.

Table 8.6 Interaction among cultivar, genistein and nitrate on nodulation and nitrogen fixation in the second experiment

		20 DAI				40 DAI	
		Nodule dry weight (mg)		Weight per nodule (mg)		Nodule N content (mg)	
Nitrate (mM)	Genistein	AC Bravor	Maple Glen	AC Bravor	Maple Glen	AC Bravor	Maple Glen
0	0	49.4	42.2	0.484	0.578	7.48	7.568
0	I	54.2	39.1	0.398	0.485	8.013	10.428
0	W5	42.4	49.2	0.492	0.513	7.382	8.155
0	W20	55.2	45.8	0.421	0.548	7.662	9.294
S.E. (D.F. = 8)		2.08	1.57	0.0146	0.0294	0.354	0.433
5	0	36.2	35.5	0.342	0.397	9.404	11.77
5	I	39.3	67.4	0.325	0.643	11.40	11.71
5	W5	43.6	44.0	0.419	0.417	11.07	12.83
5	W20	39.0	50.5	0.434	0.571	10.71	12.73
S.E. (D.F. = 8)		1.11	3.69	0.0241	0.0313	0.448	0.765
10	0	16.4	20.2	0.228	0.397	5.07	10.43
10	I	15.3	14.4	0.299	0.238	6.21	10.40
10	W5	10.7	15.1	0.203	0.177	8.37	10.95
10	W20	11.5	13.8	0.234	0.242	9.28	13.07
S.E. (D.F. = 8)		0.85	0.90	0.0191	0.0252	0.565	0.503

W5 and W20 = watering with 5 and 20 μM genistein, respectively.
I = incubation *B. japonicum* with 5 μM genistein.
DAI = days after inoculation.

Preface to section 9

Section 9 is composed of a manuscript by Pan B and Smith DL and will be submitted to *Crop Science*.

Following work described in Section 8, the same possibility that plant-to-bacterium signal molecules could be used to overcome mineral N inhibition of N_2 fixation was tested under field conditions with different soil types. Genistein was used to incubate bradyrhizobial cells or added to the rooting medium, hoping that this could compensate the decrease in inside isoflavonoid content of host plants and overcome the negative effects of mineral N under field conditions.

Section 9

Preincubation of *B. japonicum* Cells with Genistein Reduces the Inhibitory Effects of Mineral Nitrogen on Soybean Nodulation and Nitrogen Fixation under Field Conditions

9.1 Abstract

Genistein is the major isoflavonoid inducer of *nod* genes in the symbiotic *B. japonicum* cells produced in soybean roots. Reduction in the isoflavonoid content of the host plants has recently been suggested as a possible explanation for the inhibition of mineral nitrogen on the establishment of the symbiosis. In order to determine whether genistein addition could overcome this inhibition, we incubated *B. japonicum* cells (strain 532C) with genistein or added genistein directly into the soil. In addition, nitrogen (in the form of NH_4NO_3) was applied at 0, 20 and 100 kg ha^{-1}. The experiments were conducted on both a sandy-loam soil and a clay-loam soil. Preincubation of *B. japonicum* cells with genistein increased soybean nodule number and nodule weight, especially in the low-N-containing sandy-loam soil and the low N fertilizer treatment. Plant growth and yield were less affected by genistein preincubation than nitrogen assimilation. Total plant nitrogen content was increased by the two genistein preincubation treatments at the early flowering stage. At maturity, shoot and total plant nitrogen contents were increased by the 40 μM genistein preincubation treatment in the sandy-loam soil. Total nitrogen contents were increased by the 20μM genistein treatment only at the 0 and 20 kg ha^{-1} nitrate levels in clay-loam soil. Forty μM genistein preincubation increased soybean yield on sandy-loam soil. No difference was found in 100-seed weight. The results suggest that preincubation of *B. japonicum* cells with genistein could improve soybean nodulation and nitrogen fixation and at least partially overcome the inhibition of mineral nitrogen on soybean nodulation and nitrogen fixation.

Key words: soybean, *Bradyrhizobium japonicum*, nodulation, mineral nitrogen, genistein

9.2 Introduction

The interaction between the soil bacterium *B. japonicum* and its host plant, soybean, results in the formation of nitrogen fixing root nodules. Nodule formation is a complex, multi-step process requiring the activity and regulation of both plant and bacterial genes, which control signal exchange between plants and nitrogen fixing bacteria. In the soybean-*B. japonicum* symbiosis, genistein has been identified as one of the major isoflavonoid compounds in soybean seed and root extracts responsible for inducing the expression of the *B. japonicum nodYABC* operon (Kosslak et al., 1987). As a result of *nod* gene induction, lipo-chito-oligasaccharide nodulation factors (Nod factors) are produced by the bacterial symbiont (Lerouge et al., 1990), which in turn elicits root hair deformation, cortical cell division and nodulin gene expression in soybean roots (reviewed by Spaink, 1996).

Soybean plants have access to atmospheric N_2 through biological nitrogen fixation, but preferentially utilize mineral nitrogen. Nodulation of soybean is delayed or prevented completely and N_2 fixation inhibited in N-rich soils or following application of nitrogenous fertilizer, thus reducing the potential benefit from N_2 fixation (reviewed by Streeter, 1988; Carroll and Mathews, 1990). Many investigations have demonstrated that maximum symbiotic performance is associated with low levels of mineral nitrogen in rooting media (Nutman, 1956; Harper, 1974).

The inhibitions of nodulation and N_2 fixation by mineral N vary with legume species or cultivar, rhizobial strain, plant age, nitrogen form, site of application of mineral nitrogen and with environmental conditions (Pate and Atkins, 1983). The mechanism of the mineral nitrogen inhibition on nodulation and nitrogen assimilation is not well understood, although several explanations have been proposed (Streeter, 1988; Carroll and Mathews, 1990). The inhibition of soybean nodulation can be partially alleviated by high inoculum doses (Lawson et al., 1988) and overcome by the ethylene inhibitor aminoethoxyvinylglycine (Ligero et al., 1991).

Changes in isoflavonoid content of the symbiotic host plants have recently been

suggested as a possible explanation for the inhibition of mineral N (Appelbaum, 1990; Cho and Harper, 1991; Zhang et al., 1999). A hypernodulating soybean mutant had higher root concentrations of isoflavonoid compounds at 9-12 days after inoculation than did the parental cultivar (Cho and Harper, 1991). When 2.5 mM $(NH_4)_2SO_4$ was applied to the soybean plants, the concentrations of three isoflavonoids in the roots were decreased (Cho and Harper, 1990). In *Lupinus albus*, the presence of nitrate reduced the amount of flavonoids present in the root exudate or in the root extract (Wojtaszek et al., 1993). When genistein was applied to either *B. japonicum* inoculant (Zhang and Smith, 1995) or soil (Zhang and Smith, 1996), soybean nodulation and nitrogen assimilation were increased at low root zone temperatures. Nodulation and nitrogen fixation was shown to be improved in alfalfa after addition of luteolin to the plant growth medium (Kapulnik et al., 1987). But soil and growth medium mineral nitrogen levels were not investigated in those experiments. The expression of the common *nod* genes of *B. japonocum* is repressed by ammonia (Dusha et al., 1989; Wang and Stacey 1990), and genistein could partially overcome the inhibition of mineral nitrogen on the expression of *nod* genes (Pan and Smith, unpub. data).

In the present study, we further examined the effect of genistein preincubation on soybean nodulation, nitrogen assimilation and growth under field conditions at three levels of applied N fertilizer.

9.3 Materials and Methods

Experimental Design:

The experiment was organized as a random completely block. The genistein treatments were (1) *B. japonicum* cells without genistein preincubation, (2) preincubation of *B. japonicum* cells with 20 μM genistein, (3) preincubation of *B. japonicum* cells with 40 μM genistein, and (4) addition of 20 μM genistein solution directly into soil without inoculation. The *B. japonicum* strain used was 532C (Hume and Shelp, 1990). In all cases the *B. japonicum* cell density was 10^8 cell mL^{-1}. The three N fertilizer treatments

were 0, 20 and 100 kg ha^{-1} N (in the form of NH_4NO_3) added immediately prior to seedling and incorporated by hand working.

Experimental conditions and plant material:

The experiment was conducted in Emile A. Lods Research Station of McGill University. The experiment was repeated on two soil types. One site was a sandy-loam soil (fine-silty, mixed, nonacid, frigid Humaquept). The crop produced on this site in the previous year was spring barley. The second site was a clay-loam soil (fine, mixed, nonacid, frigid Humaquept). The crop produced on this site in the previous year was corn. The soybean cultivar Bayfield was used at both sites. Soil indigenous *B. japonicum* populations (according to the method of the most probable number, Vincent, 1970) were 2400 and 2500 per gram of soil in the sandy-loam and the clay-loam soils respectively. Row length was 5 m and width was 0.28 m with 8 rows in each plot. To avoid low temperature effects on soybean nodulation (Lynch and Smith, 1993), the planting date was postponed until 15 days after the normal planting date. Soybean seeds were hand planted at a population of 450,000 seeds ha^{-1}.

Inoculant preparation and inoculation:

B. japonicum strain 532C was cultured for 7 days and subcultured for 5 days in 4 L flasks at 25 °C while being shaken at 150 rpm. Filter sterilized genistein stock solution in methyl sulfoxide was added to the subculture to a final genistein concentration of 20 or 40μM under axenic conditions. The subculture was shaken for another 24 hour. The subculture was diluted to an OD of 0.1 (10^8 cells/mL). After soybean seeds were sown into the rows, 50 mL of inoculant was applied evenly into the open rows with a syringe and then the rows were closed. The control was prepared in the same way, except without adding genistein.

Data collection:

Plants were harvested three times: (1) 40 days after planting (V3 stage [Fehr et al.,

1971], June 25), when nodule number and weight, leaf area, root (0-20cm depth) and shoot weight, and plant height were measured, (2) the early flowering stage (July 30), when the same parameters were measured as well as nodule, shoot and root nitrogen concentrations (Kjeltec system, Tecator AB, Hoganas, Sweden), (3) harvest maturity (Sept. 28), when yield (combining the whole plot), 100-seed weight, shoot weight, branch and pod number per plant, seed and shoot (without leaf) N concentration were determined. SPAD readings (Chlorophyll meter SPAD-502, 1989 Minolta Camera Co., Ltd) were used to monitor leaf chlorophyll content (Earl and Tollenaar, 1997), and readings were taken on July 30 and August 12. Photosynthetic rates (Li-6400, LI-COR, Inc., Lincoln, USA) were monitored on Aug. 11.

Statistical Analysis:

Data were analysed by analysis of variance using the Statistical Analysis System computer package (SAS Institute Inc., 1988). A protected least significant difference test (LSD) was applied to make comparisons among means (Steel and Torrie, 1980).

9.4 Results:

Nodulation and plant growth at the early development stage (V3)

Nodule number and weight were increased by 40 μM genistein preincubation in the sandy-loam soil at all three N levels (Table 9.1). Weight per nodule was higher with genistein preincubation than without it. Uninoculated plants had fewer nodules, but had more weight per nodule.

In the clay-loam soil, nodule numbers were increased by 20 μM genistein preincubation at zero N and by 20 and 40 μM genistein preincubation at 20 kg ha^{-1} N (Figure 9.1A). Nodule weight per plant was increased by the two genistein preincubation treatments at zero and 20 kg ha^{-1} N (Figure 9.1B). No difference in nodule number and nodule weight existed at 100 kg ha^{-1} N.

Soybean plant height, leaf area, root and shoot weight were not affected by the

applied treatments at either site at this harvest.

Nodulation, nitrogen assimilation and plant growth at the early flowering stage:

In the sandy-loam soil, nodule number and weight were increased by genistein preincubation treatments (Table 9.2). Shoot weight was greater with genistein preincubation. Plant height and root weight were not affected by the genistein treatments. In the clay-loam soil, nodule number and weight were increased by 20 μM genistein (Table 9.2). No difference existed between the control and 40 μM genistein preincubation for these two parameters. The two genistein treatments increased shoot weight. Forty μM genistein preincubation also increased root weight. At both sites, soybean nodulation and growth was slow in the absence of inoculation.

In the sandy-loam soil (Table 9.3), shoot nitrogen concentration was higher with 40 μM genistein preincubation. Shoot nitrogen content was increased by the two genistein preincubation treatments. Root nitrogen concentration and content were not affected by genistein preincubation treatments. Nodule and total plant nitrogen contents were increased by genistein preincubation treatments. In the clay-loam soil (Table 9.4), shoot nitrogen contents were increased by genistein preincubation treatments. Root nitrogen contents were higher for genistein preincubation treatments than adding genistein directly into soil treatment or uninoculated control. Nodule nitrogen concentration of the 40 μM genistein preincubation and uninoculated treatments were lower than the other two treatments. Nodule nitrogen content was increased by 20 μM genistein preincubation. Total plant nitrogen content was higher for the two genistein preincubation treatments than adding genistein directly into soil treatment or uninoculated control.

Yield and nitrogen contents of seed and shoot at maturity:

In the sandy-loam soil, no difference in 100-seed weight existed between the genistein preincubation treatments and the uninoculated control (Table 9.5). Incubating *B. japonicum* with 40, but not 20, μM genistein increased shoot weight and yield relative to

the adding genistein directly into soil treatment or uninoculated control. Branch number per plant was not affected by the genistein treatments. In the clay-loam soil, no difference in yield existed among the four treatments.

In the sandy-loam soil, shoot and total plant nitrogen contents were increased by 40 μM genistein preincubation (Table 9.6). Interactions existed for seed nitrogen concentration and content between N treatments and genistein treatments. Forty μM genistein increased seed nitrogen concentration and content at the zero N fertilizer level (Figure 9.2). At the 20 kg ha^{-1} N level, both 20 and 40 μM genistein increased seed nitrogen concentrations and contents. In the clay-loam soil, shoot and seed nitrogen concentrations were increased by genistein preincubation treatments (Table 9.6). Seed nitrogen content was lower without inoculation than with inoculation. Total nitrogen content was increased by the 20 μM genistein treatment only at the 0 and 20 kg ha^{-1} N levels (Figure 9.2).

SPAD readings and photosynthetic rates:

Forty μM genistein preincubation resulted in the highest uppermost fully expanded leaf SPAD readings. The uninoculated control had the lowest SPAD readings, observed on both July 30 and August 12 (Table 9.7). The photosynthetic rate of the uppermost fully expanded leaf was higher with the 40 μM genistein treatment than the uninoculated control ($P = 0.1$, data not shown). In the clay-loam soil, no difference was observed for SPAD readings or photosynthetic rate among the four treatments.

9.5 Discussion:

In this report we show that soybean plants inoculated with genistein preincubated *B. japonicum* cells had more nodules, and greater nodule weight per plant and per nodule than the no genistein treatment. Soybean has an autoregulation mechanism for infection thread formation, nodule initiation, final nodule number and nodule weight per plant (Carroll and Mathews, 1990). Usually the more the nodules per plant, the smaller the

nodule size (or weight) per nodule. Preincubation of *B. japonicum* 532C with 40 μM genistein increased nodule number and total nodule weight per plant. Our observations concerning the improvement in nodulation at different N levels are in agreement with previous data obtained at low temperature (Zhang and Smith, 1995). They attributed the increases at low temperature to the alteration of the time-course of each nodulation stage. Preincubation of *B. japonicum* with genistein promotes expression of the common *nod* genes (Kosslak et al., 1987), leading to higher levels of Nod factor production by *B. japonicum* cells than would otherwise have been the case. Those bacteria preincubated with genistein could form nodules faster than the non-incubated inoculant. Bhuvaneswari et al. (1980) reported the infectivity of given host cells is a transient property that appears and then is lost within a few hours. Host responses leading to infection and nodulation are triggered or initiated in less than two hours after inoculation. After nodule formation, autoregulation of nodule growth may reduce the weight per nodule in genistein preincubation treated plots less than the no genistein treated plots. Increased nodule weight following genistein treatment was also found in a previous study (Pan et al., 1998). The mechanisms responsible for this apparent increase in nodule weight are unknown but may include: (i) flavonoids functioning as modulators of polar auxin transport (Jacobs and Rubery 1988) and locally disturbing the auxin-cytokinin balance, leading to the induction of nodule meristems (Schmidt et al., 1994); (ii) flavonoids stimulating the growth rate of rhizobia at low micromolar concentrations (D'arcy-lameta & Jay, 1987); (iii) Nod factors produced due to the effect of genistein on the bacterial *nod* genes, which trigger the formation of the root nodules by initiating cell divisions at distinct sites (Verma 1992); (iv) an increase in isoflavonoid secreted by the host root after Nod factor was applied (van Brussel et al., 1990).

When genistein was watered directly into the soil, soybean nodulation, nitrogen assimilation and seed yield were lower than that of the inoculated control. Those results are different from Zhang and Smith (1996). A low soil indigenous *B. japonicum* population could be the cause, but this was not reported in their study.

Due to the increased nodule number and total weight per plant, plant total nitrogen content was higher at the second and final harvest for the genistein preincubation treatments. But soybean yield was increased only in sandy-loam soil with 40 μM genistein preincubation, even though the total N fixed was higher at the clay-loam site.

The increases in nodule number and weight were more obvious for the sandy-loam soil site and for lower levels of N application than in clay-loam soil and higher levels of N application. At higher levels of applied N fertilizer and in a soil with higher N contents, genistein preincubation treatment still increased nodule number, nodule weight and nitrogen assimilated. Thus the inhibition of nodulation by N could be partially overcome in this soil. Soybean is grown in rotation with other crops, usually corn, which requires and leaves a lot of mineral N in the soil. Thus partially overcoming the N inhibition of nodulation has practical application in soybean production and further study needs to be done to improve this overcoming effect.

In conclusion, genistein preincubation of *B. japonicum* improved soybean nodulation and appears to have increased the total amount of N assimilated. The improvement by genistein is concentration dependent. Additional work needs to be done to fully develop the use of signal molecules such as genistein for use in crop production system.

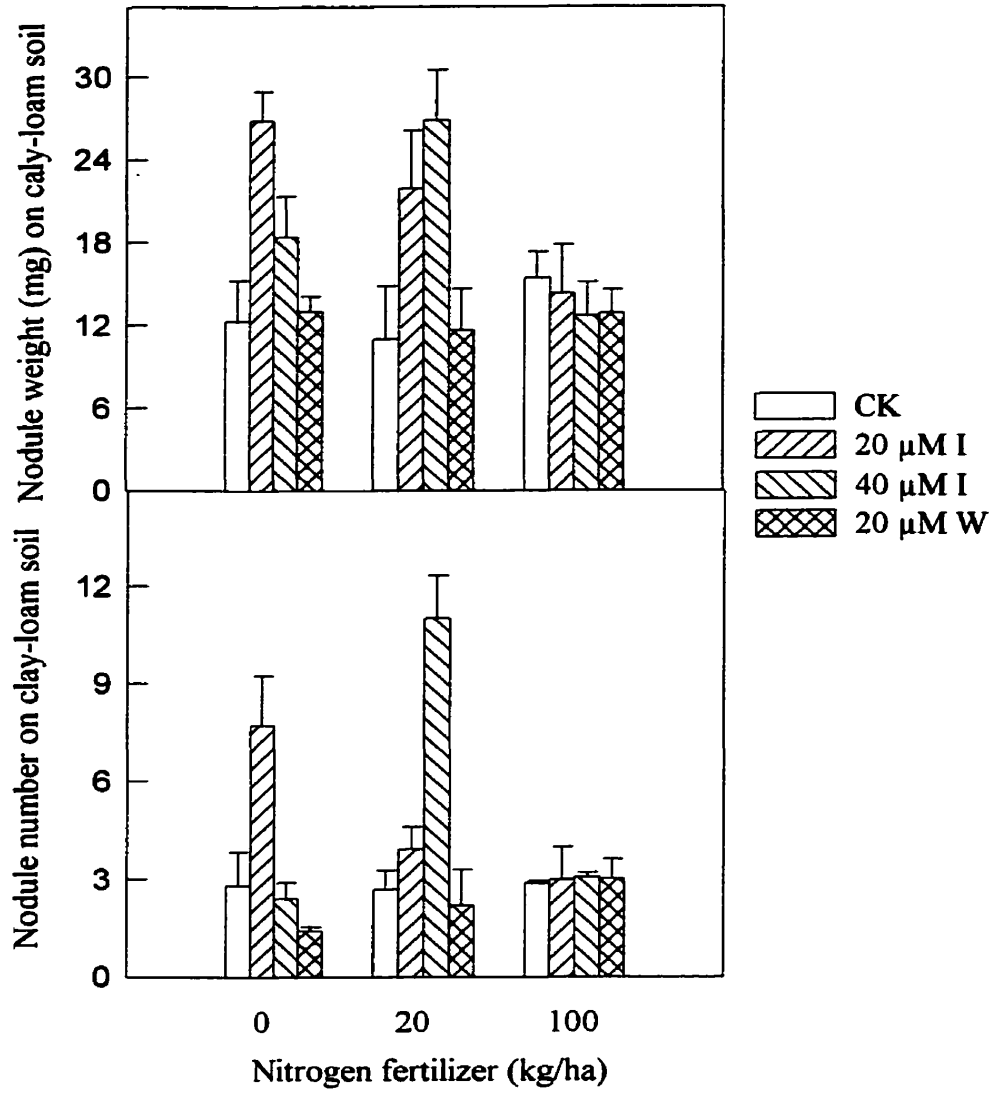

Fig. 9.1 Nodule number (A) and weight (B) in clay-loam soil at an early developmental stage (V3). Bars indicates one standard error unit.
CK = inoculant not treated with genistein.
I20 and I40 = incubation *B. japonicum* cell with 20 and 40 μM genistien.
W20 = 20 μM genistien directly into the rooting medium.

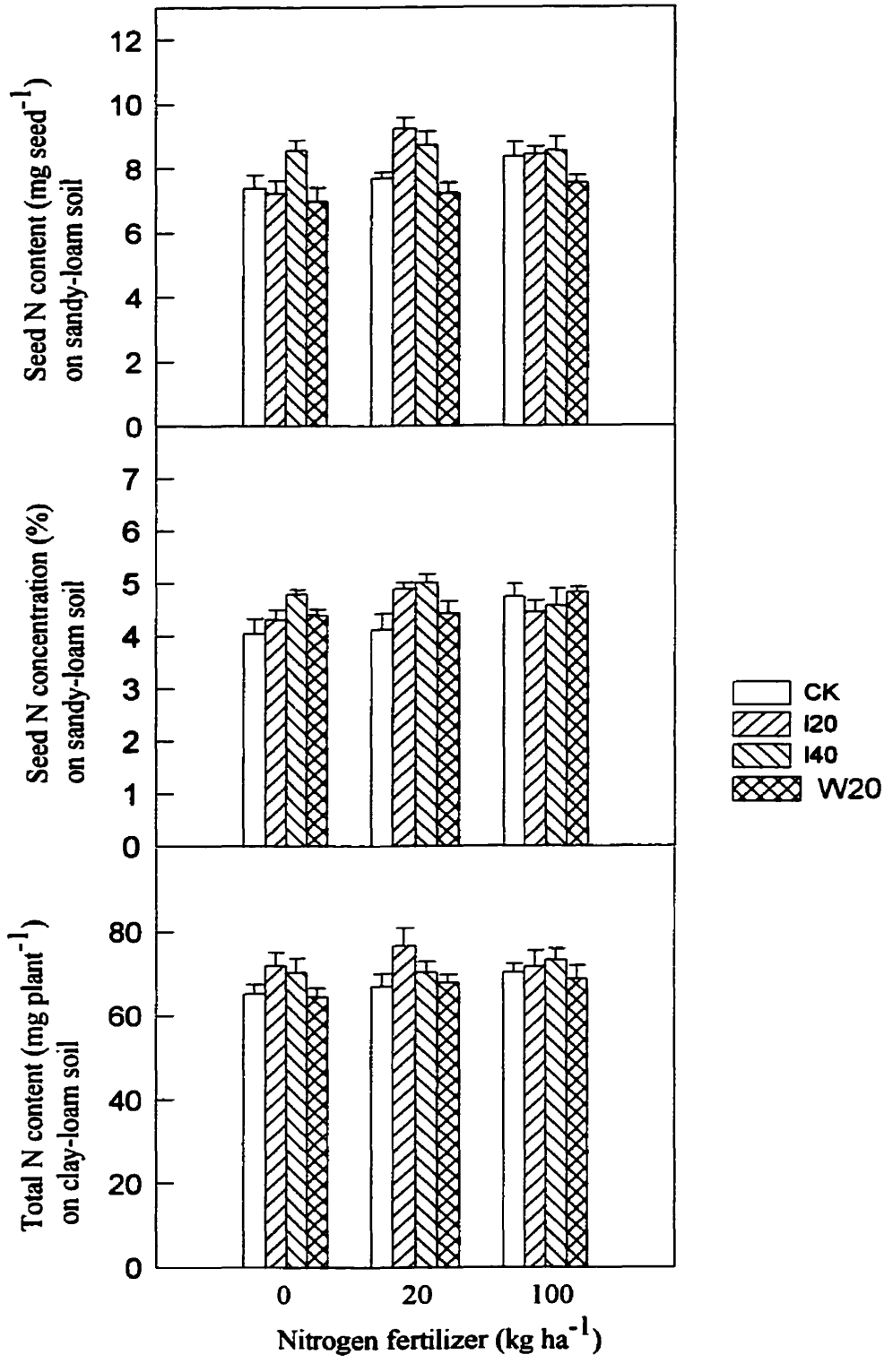

Fig. 9.2 Seed nitrogen content (A) and concentration (B) on sandy-loam soil and total nitrogen content (C) on clay-loam soil at maturity.
Bars indicate on e standard error unit. CK = inoculant not treated by genistein. I20 and I40 = incubation B. japonicum cells with 20 and 40 μM gennistien. W20 20 μM genistien directly into the rooting medium.

Table 9.1 Nodule number and total weight per plant on June 26 in sandy-loam soil

	Nodule number	Nodule weight (mg)
CK	5.07 b	13.22 bc
I20	5.91 b	25.24 b
I40	8.38 a	40.18 a
W20	0.71 c	4.23 c

Means within the same column followed by the same letter are not different by Fisher's protected LSD test at p=0.05. I20, I40 = 532C incubated with 20 or 40 μM genistein, respectively. W20 = water containing 20 μM genistein added directly into the soil. CK= inoculated with *B. japonicum* 532C.

Table 9.2 Nodule number and weight, shoot and root weight per plant at the beginning of flowering

	Sandy-loam soil			Clay-loam soil			
	Nodule number	Nodule weight (mg)	Shoot weight (g)	Nodule number	Nodule weight (mg)	Shoot weight (g)	Root weight (g)
CK	10.2 c	118.4 c	13.8 a	14.7 b	156.6 b	10.3 b	1.1 bc
I20	16.6 b	212.7 b	14.1 a	17.4 a	229.9 a	12.4 a	1.2 ab
I40	36.8 a	356.2 a	12.9 ab	13.4 b	176.5 b	12.1 a	1.3 a
W20	4.2 d	109.1 c	11.7 b	7.7 c	119.1 c	9.3 b	1.0 c

Means within the same column followed by the same letter are not different by Fisher's protected LSD test at p=0.05.
I20, I40 = 532C incubated with 20 or 40 μM genistein, respectively
W20 = water containing 20 μM genistein added directly into the soil.
CK= inoculated with *B. japonicum* 532C.

Table 9.3 Soybean nitrogen fixation related variables on July 30 at the sandy- loam soil site

	SHNC (%)	SHNT (mg)	RNC (%)	RNT (mg)	NNC (%)	NNT (mg)	TNT (mg)
CK	2.25 b	304.3 b	0.81 a	8.37 a	5.30 a	6.27 c	318.9 b
I20	2.33 b	328.6 a	0.78 a	9.01 a	4.96 ab	10.63 b	348.3 a
I40	2.52 a	323.2 a	0.79 a	8.03 a	4.83 ab	15.35 a	346.5 a
W20	1.80 c	178.5 c	0.66 b	6.07 b	4.42 b	5.32 c	210.7 c

SHNC, RNC and NNC are shoot, root and nodule nitrogen concentration, respectively.
SHNT, RNT and TNT are shoot, root and nodule nitrogen content, respectively.
Means within the same column followed by the same letter are not different by Fisher's protected LSD test at p=0.05. CK= inoculated with *B. japonicum* 532C.
I20, I40 = 532C incubated with 20 or 40 μM genistein, respectively
W20 = water containing 20 μM genistein added directly into the soil.

Table 9.4 Soybean nitrogen fixation related variables on July 30 at the clay-loam soil site

	SHNT (mg)	RNT (mg)	NNC (%)	NNT (mg)	TNT (mg)
CK	224.1 b	8.26 b	5.18 a	8.08 b	246.8 b
I20	282.9 a	11.23 a	5.02 ab	11.38 a	305.5 a
I40	288.0 a	10.77 a	4.51 c	8.18 b	306.9 a
W20	230.7 b	9.46 ab	4.64 bc	6.59 c	240.5 b

SHNT, RNT, NNT and TNT are shoot, root, nodule and total plant nitorgen content, respectively. NNC is nodule nitrogen concentration.
Means within the same column followed by the same letter are not different by Fisher's protected LSD test at p=0.05.
I20, I40 = 532C incubated with 20 or 40 μM genistein, respectively
W20 = water containing 20 μM genistein added directly into the soil.
CK= inoculated with *B. japonicum* 532C.

Table 9.5 One hundred seed weight and yield at the final harvest on sandy-loam soil

	100-seed weight (g)	Yield (kg ha^{-1})	Shoot weight (g $plant^{-1}$)
CK	17.8 a	2142.2 b	9.9 bc
I20	18.5 a	2228.3 b	10.6 ab
I40	18.9 a	2242.0 a	11.1 a
W20	16.3 b	2003.4 c	8.6 c

Means within the same column followed by the same letter are not different by Fisher's protected LSD test at p=0.05. 20, I40 = 532C incubated with 20 or 40 μM genistein, respectively. W20 = water containing 20 μM genistein added directly into the soil. CK= inoculated with *B. japonicum* 532C.

Table 9.6 Nitrogen concentration and content at maturity

	Sandy-loam soil			Clay-loam soil		
	SHNC (%)	SHNT (mg)	TNT (mg)	SHNC (%)	SENC (%)	SENT (mg)
CK	0.496 a	45.98 b	53.57 b	0.511 b	5.26 b	11.0 a
I20	0.484 a	46.08 b	53.25 b	0.559 a	5.92 a	12.3 a
I40	0.507 a	49.41 a	59.72 a	0.584 a	5.90 a	12.1 a
W20	0.422 b	39.03 c	46.03 c	0.525 b	5.49 b	10.5 b

SHNT, SENT and TONT are shoot, seed and nodule nitrogen content, respectively.
SHNC and SENC are shoot and seed nitrogen concentration, respectively.
Means within the same column followed by the same letter are not different by Fisher's protected LSD test at p=0.05.
I20, I40 = 532C incubated with 20 or 40 μM genistein, respectively.
W20 = water containing 20 μM genistein added directly into the soil.
CK= inoculated with *B. japonicum* 532C.

Table 9.7 SPAD reading from soybean leaves on sandy-loam soil

	July 30	Aug. 12
CK	36.72 b	39.6 b
I20	37.77 ab	40.0 ab
I40	38.51 a	41.4 a
W20	33.81 c	36.9 c

Means within the same column followed by the same letter are not different by Fisher's protected LSD test at p=0.05.
CK= inoculated with *B. japonicum* 532C.
I20, I40 = 532C incubated with 20 or 40 μM genistein, respectively. W20 = water containing 20 μM genistein added directly into the soil.

Preface to section 10

Section 10 is composed of a manuscript by Pan B and Smith DL and will be submitted to *Journal of Plant Nutrition*.

As previous work in our laboratory has shown that both PGPR and genistein addition can overcome low root zone temperature inhibition of soybean nitrogen fixation, and my work had shown that genistein can be helpful in overcoming the inhibition of soybean nodulation by mineral N. In this chapter I tested the ability of PGPR in overcoming the inhibition of soybean nodulation by mineral N.

Section 10

Plant Growth Promoting Rhizobacteria (PGPR) Promotion of Soybean Nodulation, Nitrogen Fixation and Growth at Three Nitrate Levels

10.1 Abstract

PGPR have been shown to improve legume nodulation, but their ability to do so in the presence of different levels of mineral nitrogen, a situation more closely approximating field conditions, has never been investigated. Effects of two PGPR strains on soybean plant growth, nodulation and nitrogen fixation were tested at three levels of nitrate (0, 1 and 5 mM) in a randomized complete block design under greenhouse conditions. Plant growth was promoted by PGPR. PGPR strain 1-102 had better effect than strain 2-68 for most of the variables measured. PGPR strain 1-102 increased shoot, nodule and total plant nitrogen contents. Nodule number and weight were inhibited at 5 mM nitrate. PGPR strains used partially overcame the inhibiting effects of nitrate on nodule number, weight and nitrogen assimilation.

Key words: nitrate, nodulation, plant growth promoting rhizobacteria, soybean

10.2 Introduction

Certain rhizosphere microorganisms, collectively referred to as PGPR, have been shown to increase plant growth, development and yield in crops such as potato, radish, sugar beet and wheat (Gaskin et al., 1985). These increases have been related to plant hormone production (De Freitas and Germida, 1990), nutritional interactions between the rhizobactera and the plant (Chanway et al., 1989), biological control of soil-borne plant pathogens (Sutton and Peng, 1993) and siderophore production by PGPR (Gaskin et al., 1985). There has been renewed interest in the use of soil bacteria which, when applied to seeds, tubers or roots, are able to colonize plant roots and stimulate plant growth and crop yield (Chanway et al., 1989).

When applied to legumes, PGPR have also been shown to affect the development of the nitrogen fixing symbiosis. Co-inoculation studies with PGPR and *Bradyrhizobium japonicum* have demonstrated increased plant vigour, root and shoot weight, seed yield, nodulation and nitrogen fixation (Li and Alexander, 1988; Dashti et al., 1997). Interactions existed between PGPR application and soybean cultivar (Dashti et al., 1998). Soybean seed yield increased following inoculation with a mixed culture of *B. japonicum* and certain PGPR strains, as compared to *B. japonicum* alone (Yahalom et al., 1987). Grimes and Mount (1984) found that a *Pseudomonas putida* strain (M17) markedly increased *Rhizobium* nodulation of bean in field soils. Polonenko et al. (1987) reported that certain rhizobacteria (primarily fluorescent pseudomonades) enhanced root nodule number or mass. Suslow (1982) reported a *Pseudomonas* strain had different effectiveness in different agricultural regions and during different periods of crop growth.

Mineral N has multiple effects on legume nodulation (Streeter, 1988). High levels of mineral N can inhibit nodulation and induce premature nodule senescence (Chen and Phillips, 1977; Carroll and Mathews, 1990). The mechanism of the inhibition is not well understood. While it has been demonstrated that PGPR can improve legume nodulation in the absence of mineral nitrogen, their ability to do so in the presence of mineral nitrogen, a situation more closely approximating field conditions, has not been investigated. Part of the way in which PGPR increase soybean nodulation under field conditions (Dashti et al., 1998) may be by overcoming the suppression of nodulation by nitrate present in the soil. At this time there is no published information regarding this possible mechanism. The objective of this study was to determine PGPR effect on soybean growth at three levels of nitrate, and more importantly, whether PGPR could overcome the negative effects of nitrate on soybean nodulation and nitrogen assimilation.

10.3 Materials and Methods

Experimental design

The experiments were carried out under greenhouse conditions. The air temperature regime was 25/18°C (day/night), and the relative humidity was 65%. On cloudless days the light intensity reached 900 μmol m^{-2} s^{-1}. Turface (Applied Industrial Material Corp., Deerfield, IL):sand (1:1, v/v) was used as a rooting medium.

Soybean cultivar Maple Glen was used in both experiments. Two PGPR strains (*Pseudomonas putida* 1-102 from Yellowknife, NWT; *Serratia liquefaciens* 2-68 from James Bay Soil, NWT) and three nitrate levels (0, 1, 5 mM KNO_3) were organized in a factorial arrangement of a random completely block design. The two PGPR strains were selected according to their best performance in preliminary experiments (data not shown). For both experiments, nitrate solution was applied beginning at sowing, and was added each time the plants required water. Hoagland's solution (Hoagland and Arnon, 1950) was added twice a week beginning when the cotyledons turned yellow. The Hoagland's solution used in these studies was modified to provide a nitrogen-free solution: the $Ca(NO_3)_2$ and KNO_3 were replaced with $CaCl_2$, K_2HPO_4, and KH_2PO_4. The correct amount of nitrate (as KNO_3) was added to the nutrient solution for each treatment concentration and 250 mL of nutrient solution were added to each pot at each watering time. That amount of solution was enough to wet the whole growth medium and cause some solution to run out the bottom of the pots so that the pots were flushed at each watering.

Plant materials and inoculation

Seeds of Maple Glen were surface-sterilized in 4% sodium hypochlorite for 7 min, and then rinsed 10 times with distilled water. Five seeds were sown in each pot. The seedlings were thinned to 2 plants per pot at the VC stage (unifoliolate leaves unrolled sufficiently that the edges were not touching, Fehr et al., 1971).

The inoculant was produced by culturing *B. japonicum* stain 532C in yeast extract mannitol broth in 250-mL flasks shaken at 150 rpm at 25°C. A five day old sub-culture (OD≈0.4) was diluted with distilled water to an A_{620} of 0.16 (approximately 2×10^8 cells mL^{-1}). All the PGPR strains were cultured in *Pseudomonas* medium (Polonenko et al., 1987) in 250 mL flasks shaken at 250 rpm at 25°C. One-day-old PGPR subculture was adjusted with sterilized distilled water to an OD_{420} value of 0.2. Prior to inoculation, the *B. japonicum* 532C broth culture was added to the same amount of a culture of one PGPR strain and the resulting mixture was allowed to stand for half an hour at 25 °C without shaking. One mL of inoculant per plant was applied by pipette onto the rooting medium at the base of the plant at the VC stage.

Data collection

Plants were harvested 22 days after inoculation (DAI) for the first experiment, 34 DAI for the second experiment. Leaf area (Area Measurement System, Delta-T Devices Ltd, England), shoot and root weight, nodule number, nodule weight were measured. Shoot, root and nodule nitrogen content were measured in the second experiment. Nitrogen concentration was measured by Kjeldahl analysis (Kjeltec system, Tecator AB, Hoganas, Sweden).

Statistical Analysis

The two experiments were not harvested at the same date after inoculation, so the results were analysed separately. The data were analysed by analysis of variance using the Statistical Analysis System computer package (SAS Institute Inc., 1988). When analysis of variance showed significant treatment effects, the least significant difference (LSD, preplanned comparison) or multiple comparison test was applied to make comparisons among means at the 0.05 level of significance.

10.4 Results

Experiment 1

Plant growth

Shoot dry weight was increased by PGPR strain 1-102 at three levels of nitrate (Table 10.1). Root dry weight and leaf area were not affected by PGPR. Plant height was increased by strain 1-102 at 0 mM nitrate.

Nodulation

Nodule number was increased by strain 1-102 at 1 mM nitrate. Strain 2-68 had no effect on nodule number. Nodule number was highest at 1 mM nitrate (Table 10.1). No difference in nodule number was found between 0 and 5 mM nitrate. Nodule weight per plant was highest at 1 mM nitrate (Table 10.1). Five mM nitrate decreased nodule weight per plant. At 0 nitrate PGPR strain 2-68 decreased nodule weight per plant. At 1 mM nitrate nodule weight per plant was increased by both strains. Nodule weight per plant was increased by strain 1-102 at 5 mM nitrate.

Experiment 2

Plant growth

Strain 1-102 increased plant height at 0 and 1 mM nitrate (Table 10.2). At 5 mM nitrate strain 2-68 increased plant height. Strains 1-102 and 2-68 increased shoot dry weight. Strain 1-102 increased root dry weight and leaf area at all three nitrate levels.

Nodulation

Thirty four days after inoculation, strain 1-102 increased nodule number at all three nitrate levels (Table 10.2). Strain 2-68 increased nodule number at 1 mM nitrate. Nodule number was highest at 1 mM nitrate and lowest at 5 mM nitrate.

Nodule weight per plant was increased by strain 1-102 at three nitrate levels (Table 10. 2). There were also some positive effects of strain 2-68 on nodule weight. Nodule weight was highest at 1 mM nitrate. Five mM nitrate inhibited nodule weight.

Nitrogen Assimilation

PGPR strain 1-102 increased shoot, root and nodule nitrogen contents (Table 10.3). Strain 2-68 increased total plant nitrogen content at the 0 nitrate level. Nodule nitrogen content was decreased at 5 mM nitrate, but strain 1-102 increased nodule nitrogen content at this level of nitrate.

10.5 Discussion

Inoculation of soybean plants with a mixture of PGPR and *B. japonicum* at three levels of nitrate produced beneficial effects on soybean plant growth, nodulation and nitrogen assimilation. Differences were found between the two strains tested. Thus, the proper PGPR strain should be selected in order to get the most beneficial effects on soybean growth and nodulation at different soil nitrate levels. The increases in plant growth and nodulation are in agreement with the previous report of Li and Alexander (1988).

The 1 mM nitrate treatment increased plant growth and nodulation, and did not inhibit any of the variables measured in this study, including nodulation. Streeter (1988) also reported positive effect of low levels of nitrate on nodulation. PGPR had greater positive effects at this level of nitrate. Five mM nitrate inhibit soybean nodulation and nodule weight. At 5 mM nitrate PGPR 1-102 continued to increase nodule weight over the control, although not as much as at 1 mM nitrate. Thus the PGPR tested could only partially overcome the nitrate inhibition of nodulation.

In the second experiment, strain 1-102 caused the largest increase in soybean growth. In general, the pattern of promotion by PGPR was the same as in experiment I, although fewer of the numerical increases achieved statistical significant at $\alpha = 0.05$. This

may have been due to the difference in sampling times, as plants were harvested 34 DAI for the second experiment and 20 DAI for the first experiment. The improvements in nodulation and nitrogen fixation were in agreement with the increases in shoot, root and nodule nitrogen content (Table 10.3). Zhang et al. (1996) reported that the PGPR strain tested increases soybean photosynthetic rate prior to the onset of nitrogen fixation, which suggested that some aspect of general plant physiology was stimulated by PGPR. It may have been that early photosynthetic rates were increased and that this extra fixed carbon was used to form larger and more numerous nodules and more nitrogen was fixed.

PGPR can increase the growth and yield of non-legume crops (Gaskins et al., 1985) through the production of plant growth hormones (De Freitas and Germida, 1990). Soybean nodulation and nitrogen fixation processes involve several phytohormones (for a review see Arshad and Frankenberger, 1998). Change in the phytohormone balance is a necessary requirement for elicitation of nodule formation (Hirsch and Fang, 1994). Nitrate inhibition of nodulation can be overcome by the ethylene inhibitor aminoethoxyvinylglycine (Ligero et al., 1991). So the effects PGPR on soybean nodulation could be related to changes in the production, exudation and balance those plant phytohormones in the rooting medium and inside the nodules.

The action of PGPR through antibiotic toward certain soil-borne microorganisms (Sutton and Peng 1993) is not a possible mode of action in these experiments, as there was no evidence of pathogen presence.

The application of our results to field soil conditions remains to be tested, as PGPR may influence other soil factors limiting plant growth, nodulation and nitrogen fixation, and the effect of a particular bacterium may vary as a consequence of soil conditions (Lynch, 1990). Other combinations of soybean cultivars, PGPR strains and nitrate levels should also be tested. PGPR effects on soybean yield and final nitrogen assimilation also need to be studied.

Table 10.1 Nodulation, shoot and root weight, leaf area and plant height of soybean cultivar Maple Glen in experiment 1.

nitrate (mM)	PGPR	plant height (cm)	Leaf Area (cm^2)	shoot weight (g)	root weight (g)	nodule number	nodule weight (mg)
0	None	17.6[a]	81	0.49	0.37	60	24
0	1-102	19.4	83	0.65	0.40	56	25
0	2-68	17.3	79	0.51	0.33	52	21
1	None	21.1	132	0.84	0.50	69	29
1	1-102	22.7	147	0.95	0.52	83	45
1	2-68	21.0	134	0.88	0.54	69	32
5	None	23.9	211	1.39	0.81	50	18
5	1-102	23.5	220	1.52	0.79	63	24
5	2-68	24.4	208	1.42	0.89	54	20
nitrate		**	**	**	**	**	**
PGPR		NS	NS	**	NS	NS	**
nitrate*PGPR		NS	NS	NS	NS	NS	NS
LSDnitrate		2.45	33.7	0.21	0.02	12	6.3
LSD_{PGPR}		----	----	0.22	----	----	5.6
$LSD_{nitrate*PGPR}$		----	----	----	----	----	----

** and NS indicate significant or not at the 0.05 probability level, respectively.

[a] Value represents the average of 6 plants.

Plants were harvested 22 days after inoculation.

Table 10.2 Nodule, shoot and root weight nodule number, leaf area and plant height of soybean plants harvested 34 days after inoculation in experiment 2.

nitrate	PGPR	plant height (cm)	leaf area (cm^2)	shoot weight (g)	root weight (g)	nodule number	nodule weight (mg)
0	none	25.4[a]	215	0.95	0.38	95	129
0	1-102	30.3	310	1.58	0.56	113	148
0	2-68	26.2	302	1.19	0.46	98	136
1	none	27.4	412	1.68	0.71	90	152
1	1-102	33.6	457	2.33	0.82	125	197
1	2-68	29.9	408	1.93	0.73	94	166
5	none	38.3	579	2.64	0.95	71	110
5	1-102	39.0	613	3.05	1.08	89	131
5	2-68	42.5	575	2.86	0.99	70	117
nitrate		**	**	**	**	**	**
PGPR		**	NS	**	**	**	**
nitrate*PGPR		**	NS	NS	NS	NS	NS
LSDnitrate		2.24	48.1	0.17	0.06	11.5	24
LSD_{PGPR}		2.24	----	0.20	0.07	9.4	19
$LSDnitrate_{*PGPR}$		3.68	----	----	----	----	----

** and NS indicate significant or not at the 0.05 probability level, respectively.

[a] Value represents the average of 6 plants.

Plants were harvested 34 days after inoculation.

Table 10.3 Shoot, root and nodule nitrogen content of soybean harvested 34 days after inoculation in experiment 2.

Nitrate	PGPR	shoot N (mg)	root N (mg)	nodule N (mg)	total N [a] (mg)
0	None	30.1[b]	10.7	4.3	41.6
0	1-102	51.6	16.8	6.8	74.1
0	2-68	39.1	14.4	5.2	56.3
1	None	56.1	16.1	6.8	75.2
1	1-102	75.4	20.8	8.7	102.4
1	2-68	59.1	18.1	7.9	80.2
5	None	72.2	22.8	3.2	98.8
5	1-102	83.5	27.8	4.6	118.3
5	2-68	77.8	24.2	3.8	107.4
Nitrate		**	**	**	**
PGPR		**	**	**	**
nitrate*PGPR		NS	NS	NS	NS
LSDnitrate		8.88	2.37	1.25	10.2
LSD_{PGPR}		10.2	2.73	1.44	11.7
$LSD_{nitrate*PGPR}$		----	----	----	----

[a] total N = plant total nitrogen content minus seed nitrogen content.
[b] Value represents the average of 6 plants.
** and NS indicate significant or not at the 0.05 probability level, respectively.

Preface to section 11

Section 11 is composed of a manuscript by Pan B and Smith DL and will be submitted to *Crop Science*.

Following work with PGPR and genistein separately, the combining effects of PGPR and genistein on soybean nodulation and nitrogen fixation were tested under field condition in a short growth season area.

Section 11

The Combined Effects of Plant Growth Promoting Rhizobacterium and Genistein on Soybean Growth, Nodulation and Nitrogen Fixation under Field Conditions

11.1 Abstract

PGPR and genistein have been used separately to improve soybean nodulation, nitrogen fixation and yield. Field experiments were conducted to evaluate the combined effect of PGPR and genistein in 1996 and 1997. Plants were harvested at the V3, early flowering and harvest maturity stages. The experiment was structured following a randomised complete block design with 3 replicates. Three factors were tested: two genistein concentrations (0 and 20μM), two PGPR strains (2-68 and 1-102), and two soybean cultivars (AC Bravor and Maple Glen). The results indicated that genistein increased nodule number and nodule weight. The two PGPR strains used affected the measured variables differently. PGPR had no or negative effect on nodule number and nodule weight at the V3 stage, but improved nodulation and plant growth at the early flowering stage. Shoot dry weight was increased by PGPR strain 1-102 at the second harvest in 1996. Root dry weight of AC Bravor at the second harvest was also increased by PGPR strain 1-102 in 1997. At maturity, the shoot nitrogen content of AC Bravor in 1996, seed nitrogen content and total nitrogen yield in 1997 were increased by genistein. Total nitrogen yield of Maple Glen was also increase by PGPR in 1997. Soybean yield was not affected by the treatments in 1996. In 1997, genistein treatment and PGPR strain 1-102 increased soybean yield.

Key words: Soybean, genistein, plant growth promoting rhizobacteria

11.2 Introduction

Soybean (*Glycine max*. (L.) Merr.) is a subtropical legume which requires temperatures in the range of 25 to 30 °C for optimal growth (Jones and Tisdale, 1921). When the temperature is below this, soybean nodulation, nitrogen fixation, growth and yield are negatively affected (Roughley and Date, 1986; Lynch and Smith, 1994). In

short growing season areas, such as the Canadian soybean production region, temperature is considered the major factor limiting soybean yield (Whigham and Minor, 1978). To overcome the negative effects of temperature, several methods have been tried, including using plant growth promoting rhizobacteria (PGPR) (Zhang et al., 1996; Dashti et al., 1997; Dashti et al., 1998) and genistein (Zhang and Smith, 1995).

PGPR are free-living soil bacteria beneficial to plant growth (Kloepper et al., 1989). PGPR can affect plant growth by preventing the deleterious effects of one or more phytopathogenic organisms, providing a compound that acts as a plant growth regulator, or facilitating the uptake of certain nutrients from the environment (Glick, 1995). Later work on soybean and canola showed that PGPR increased seedling emergence when soil temperatures were below 20°C, and they termed such bacteria as emergence-promoting rhizobacteria (Kloepper et al., 1986). Biocoating of seeds, such as radish, with PGPR could also improve plant establishment (Schippers et al., 1995). Co-inoculation of PGPR with *Bradyrhizobium japonicum* has been shown to increase soybean nodulation and nitrogen fixation at suboptimal root zone temperatures. The increases in soybean nodule number due to PGPR were largely due to improved overall physiological performance and growth of the plants (Zhang et al., 1996).

The interaction between the soil bacterium *B. japonicum* and its host plant, soybean, results in the formation of nitrogen fixing root nodules. Nodule formation is a complex, multi-step process requiring the activity and regulation of both plant and bacterial genes which control signal exchange between plants and nitrogen-fixing bacteria. In soybean-*B. japonicum* symbiosis, genistein has been identified as one of the major isoflavonoid compounds in soybean seed and root extracts responsible for inducing the expression of the *B. japonicum nodYABC* operon (Kosslak et al., 1987). As a result of *nod* gene induction, lipo-chitin oligosaccharide nodulation factors (Nod factors) are produced by the bacterial symbiont (Lerouge et al., 1990), which in turn elicit root hair deformation, cortical cell division and nodulin gene expression in soybean roots (for a review see Spaink (1996)). Genistein has been used to improve soybean nodulation and nitrogen fixation under low root zone temperature conditions (Zhang and Smith, 1995).

In this study, we tested the effects of co-inoculation of *B. japonicum* cells preincubated with genistein and PGPR on soybean nodulation, nitrogen fixation, growth and yield under field conditions, to see if PGPR together with genistein can cause larger effects than either alone.

11.3 Materials and Methods

Experimental Design

The experimental structure was a 2x3x2 factorial organised in a random completely block design. The three factors were two genistein levels (0 and 20 μM), three PGPR levels (no PGPR (control), *Serratia proteamaculans* 1-102 (Yellowknife, NWT) and *Serratia liquefaciens* 2-68 (James Bay soil, NWT)) and two soybean cultivars (AC Bravor and Maple Glen). Genistein concentration, PGPR strain and soybean cultivar were selected according to previous experimental results (Zhang and Smith, 1995; Zhang et al., 1996).

Experimental conditions and plant material

The experiments were conducted at the Emile A. Lods Research Station of McGill University in 1996 and 1997, both on Chicot light sandy-loam soil. Row width was 30 cm, length 5 m. There were 8 rows in each plot. The space between plots was 60 cm, and between replicates 1 m. Barley was grown in previous year at the 1996 site and corn the previous year at the 1997 site. In each year, potassium and phosphate were provided by spring application of 300 kg ha^{-1} of 0-8.4-15.8 (N-P-K). Soybean seeds were sown by hand on May 19, 1996 and May 15, 1997. Following emergence, seedlings were thinned to achieve a stand of 450,000 plants ha^{-1}.

Inoculant preparation and inoculation

The inoculants were produced by culturing *B. japonicum* stains in yeast extract-mannitol broth (YEM, Vincent, 1970) in 250-mL flasks shaken at 150 rpm at 25°C for 7 days. To produce inoculant preincubated with genistein (4', 5, 7-Trihydroxyisoflavone, Sigma, Mississauga, Canada), 100 mL of 4-day-old sub-culture (OD≈0.35) was

aseptically added to 500 mL of sterile genistein solution (24 μM, providing a final genistein solution concentration of 20 μM) in a flask and incubated at 30 °C without shaking for 48 h. Following incubation, the cell suspensions were pelleted in sterile centrifuge tubes at 10,000 g for 10 min, washed once with distilled water, and resuspended to an A_{620} of 0.1 (approximately 10^8 cells mL^{-1}). After soybean seeds were sown into the soil by hand, 50 mL of inoculant was applied evenly into the open row with a 60 mL syringe and then covered.

PGPR were cultured in *Pseudomonas* medium (Polonenko et al., 1987) in 250 mL flasks shaken at 250 rpm at 25 °C for 1.5 days. A-one-day old PGPR subculture was diluted to an OD_{420} of 0.1 (cell density approximately 10^8 per mL) and used as inoculant. PGPR inoculant was applied evenly along the row at a rate of one mL per seed using a 50-mL syringe.

Data collection

Air temperature and precipitation were recorded at the Macdonald Campus weather station, McGill University, Ste-Anne-de-Bellevue, Quebec, Canada (Fig. 11.1). Plant samples were harvested three times: (1) V3 stage (Fehr et al., 1971) for nodule number and weight, plant height, root (0-20cm depth) and shoot weight were measured, (2) the early flowering stage for the same parameters were measured as at the first harvest plus nodule, shoot and root nitrogen concentrations (Kjeltec system, Tecator AB, Hoganas, Sweden), (3) harvest maturity for soybean yield (combine harvested from the half of the plot where samples had not been removed), 100-seed weight, shoot weight, branch and pod number per plant, seed and shoot (without leaf) N concentration were determined. SPAD readings (Chlorophyll meter SPAD-502, 1989 Minolta Co., Ltd) were taken to monitor leaf chlorophyll content at V3 stage (Earl and Tollenaar, 1997).

Statistical Analysis

Means of the five plants, randomly selected at each harvest, were used for analysis. Data were analysed by analysis of variance using the Statistical Analysis System computer package (SAS Institute Inc., 1988). An ANOVA protected least

significant difference test (LSD) was applied to make comparisons among means at the 0.05 level of significance. Contrasts were used to make planned specific comparisons (Steel and Torrie, 1980).

11.4 Results

Plant growth and nodulation at V3 stage

Neither PGPR nor genistein affected plant height, leaf area, shoot and root dry weight or SPAD reading in both years in the first harvest time. Interactions existed among soybean cultivar, genistein and PGPR for nodule number and weight. Genistein preincubation of *B. japonicum* increased nodule weight in both years for both soybean cultivars (Table 11.1). Nodule numbers were increased by genistein for Maple Glen in both years and for AC Bravor in 1997.

PGPR strain 2-68 reduced the nodule weight of AC Bravor, but not Maple Glen in both years (Table 11.1). In 1997, PGPR 1-102 plus genistein resulted in the largest nodule weight and nodule number values for Maple Glen. Genistein alone provided the largest nodule weight and number values for AC Bravor.

Nodulation and plant growth at the early flowering stage

At this harvest, nodule number for plants that had received genistein treated inoculant was still higher than the controls (Table 11.2). Nodule weight was increased by genistein incubation for both cultivars. PGPR strain 2-68 decreased the nodule number of AC Bravor in 1997 and nodule weight of AC Bravor in 1996. PGPR strain 1-102 increased both nodule weight and nodule number for Maple Glen in 1997.

Genistein, PGPR 1-102 or genistein plus 1-102 had positive effects on the shoot and root dry weight of AC Bravor (Table 11.2). Generally, AC Bravor responded more to the applied treatments than Maple Glen. No effect was found for root dry weight of Maple Glen in 1997 and for shoot dry weight of Maple Glen in 1996.

Nitrogen fixation at early flowering stage

The two soybean cultivars were not different in terms of accumulated nitrogen responses, and an interaction existed between genistein and PGPR. Genistein alone increased shoot, root and nodule nitrogen content in 1996. In 1996 PGPR and genistein did not affect shoot nitrogen concentration and content (Table 11.3). PGPR strains 2-68 and 1-102 had negative effects on root nitrogen content (Table 11.3). PGPR strain 1-102 plus genistein increased shoot and nodule nitrogen content and nodule nitrogen concentration in 1996 and nodule nitrogen content in 1997.

Shoot and seed nitrogen content and soybean yield

There were interactions between genistein and PGPR at the final harvest for the measured variables. The two soybean cultivars responded differently. Shoot nitrogen content of AC Bravor was increased by genistein in 1996 (Table 11.4). The two PGPR strains increased the shoot nitrogen concentration of Maple Glen in 1996. Neither genistein nor PGPR affected total nitrogen yield in 1996. In 1997 seed nitrogen content and total nitrogen yield of Maple Glen were increased by PGPR strain 1-102. Genistein increased total nitrogen yield of AC Bravor in 1997.

Pod number per plant and branch number per plant were not affected by genistein or PGPR treatments. Genistein and PGPR did not affect soybean yield and one hundred seed weight in 1996 (Fig. 11.2). In 1997 genistein increased soybean yield and one hundred seed weight. PGPR strain 1-102 also increased yield and one hundred seed weight, but to a less extent ($p = 0.1$) than genistein. PGPR strain 2-68 had no effect on these two variables.

11.5 Discussion

Inoculation of soybean plants with PGPR strains and *B. japonicum* preincubated with genistein produced a wide range of effects on nodulation, nitrogen fixation and plant growth and yield. Similar results were found in a greenhouse experiment (Dashti et al., 1997; Dashti et al., 1998). Interactions existed among soybean cultivar, PGPR strain and genistein for the measured variables.

PGPR had both positive and negative effects on soybean nodulation and nitrogen fixation. This may have been due to the different PGPR strains and the effects of environmental conditions on strain performance. Zhang et al (1996) reported one PGPR strain improved soybean nodulation and nitrogen fixation at an optimal root zone temperature, while another caused improvements at low root zone temperatures. Suslow (1982) reported a variable pattern of *Pseudomonas* strain effectiveness in different agricultural regions and during different stages of plant growth. Sometimes the effect of a particular bacterium may vary as a consequence of soil conditions (Lynch, 1990). Those results suggested that in order to get the promotion effect of PGPR on soybean nodulation and growth, proper PGPR strains have to be selected.

A greenhouse experiment has shown that soybean growth was promoted by PGPR at an early growth stage (Dashti, 1996); however, in the present study, the same PGPR strains had no effect on soybean growth in the early stage, but did at the early flowering stage. Part of the reason could be the growing medium. Grimes and Mount (1984) reported different responses of plants to PGPR grown in different media. They showed that a soil peudomonad inhibited *R. phaseoli* on agar media, but stimulated its ability to form nodules on *P. vulgaris* grown in the field. A delayed response of plant growth to inoculation has been reported in other studies (Chanway et al., 1991). Parker and Dangerfield (1975) reported that five weeks after inoculation treated Douglas-fir plants were slightly smaller and weakly chorotic but 13 weeks after treatment inoculated seedlings were visibly larger.

Our data indicate that soybean plant growth promotion by PGPR is not always correlated with increased nodulation and nitrogen fixation (Table 11.2). A similar result was found in a previous study (Zhang, 1996). Soybean nodulation and nitrogen fixation might not be specifically stimulated by the PGPR, and PGPR often enhance overall plant physiology and growth (Li and Alexander, 1988) through phytohormonal or nutritional interactions between the rhizobia and plant (Chanway et al., 1989).

The most consistent effect of genistein on soybean nodulation in our study was an increase in nodule number and nodule weight over the untreated control. Those improvements are in agreement with previous controlled environment data at low root

zone temperature (Zhang and Smith, 1995). They attributed the increases at low temperature to the alteration of the time-course of each nodulation stage. Preincubation of *B. japonicum* with genistein promotes expression of the common *nod* genes (Kosslak et al., 1987), leading to higher levels of Nod factor production by *B. japonicum* cells than would otherwise have been the case. Those bacteria preincubated with genistein could form nodules faster than the non-incubated inoculant, perhaps prior the autoregulation of infection and nodule number. Bhuvaneswari et al. (1980) reported that the infectivity of given host cells is a transient property that appears and then is lost within a few hours. Host responses leading to infection and nodulation are triggered or initiated in less than two hours after inoculation. High nodule weight following genistein treatment was also found in a previous study (Pan et al., 1998).

Due to the increased nodule number and total weight per plant, plant total nitrogen content were higher at the second and final harvest for the genistein preincubation treatments in 1997. Genistein and PGPR also increased yield in 1997. However, soybean yield was not increased, even though the total nitrogen fixed was higher in 1996. Yield formation is a complicated process and is affected by a large number of environmental factors. The lack of yield response to the treatments in 1996 may have been due to a period of drought during the pod filling period in August (Fig. 11.1), although there were increases in nodulation and plant growth at the early flowering stage.

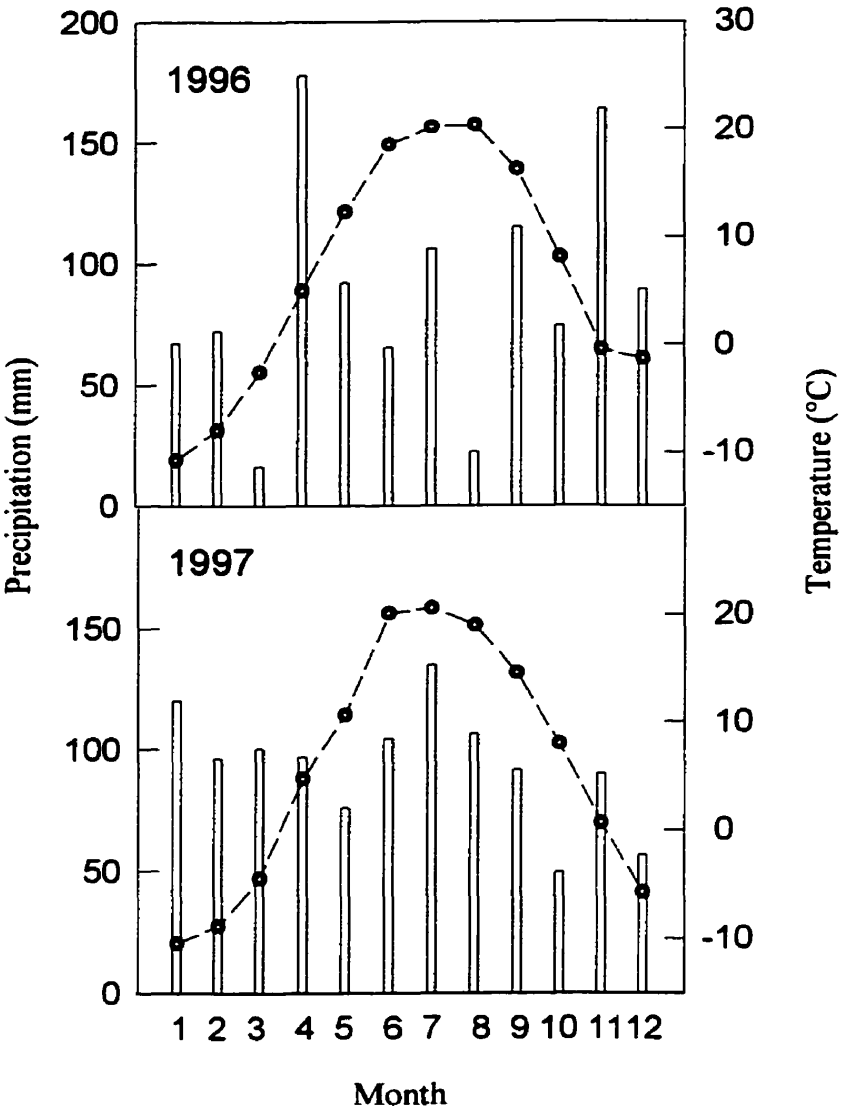

Fig. 11.1 The average monthly air temperature (line) and precipitation (bar) in 1996 and 1997 (Ste-Anne-de-Bellevue, Quebec, Canada)

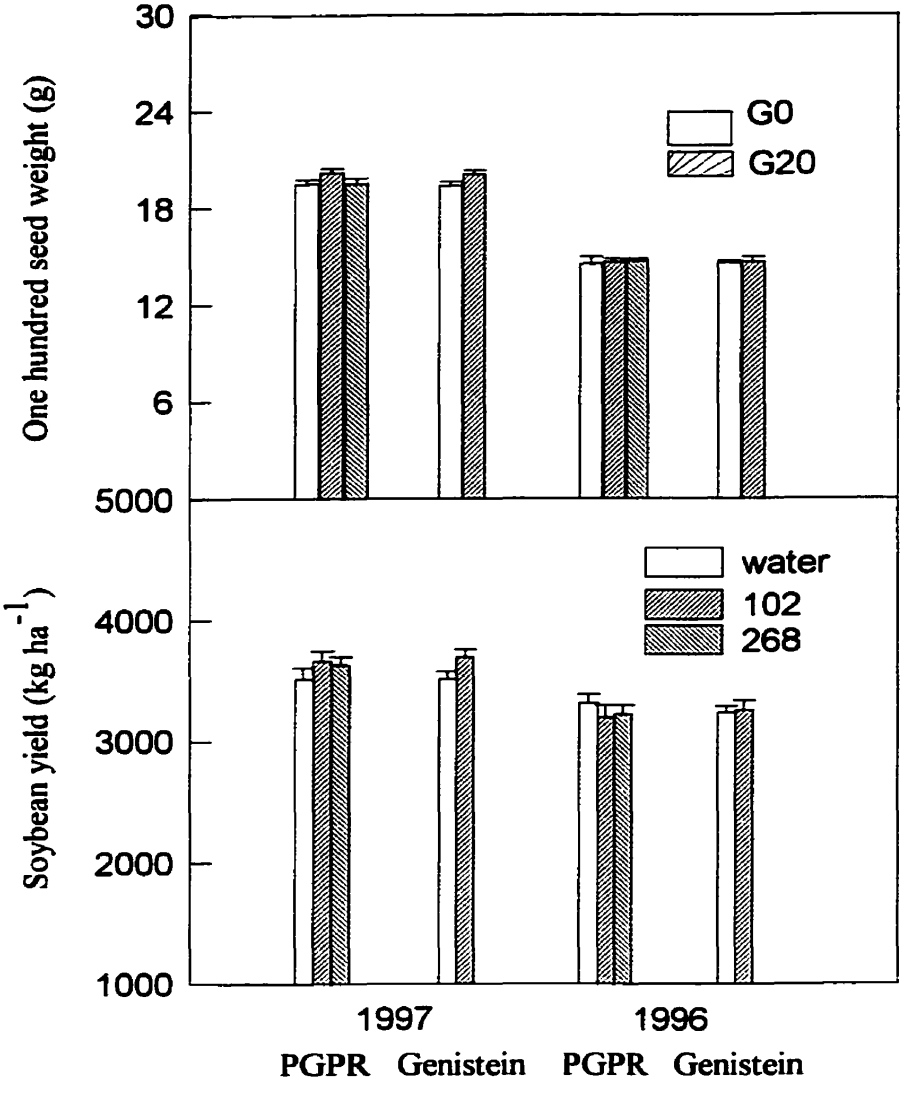

Fig. 11.2 Soybean yield and one hundred seed weight in 1996 and 1997
Bar = one standard error unit

Table 11.1 Nodulation at the early growing stage (V3)

PGPR	Genistein (μM)	AC Bravor Nodule number	AC Bravor Nodule weight (mg)	Maple Glen Nodule number	Maple Glen Nodule weight (mg)
			1996 (40 days after sowing)		
0	0	16.9 b	16.9 b	17.6 b	17.8 a
0	20	18.9 b	23.6 a	18.1 b	17.7 a
102	0	17.9 b	16.2 b	17.1 b	17.4 a
102	20	17.3 b	17.4 a	17.7 b	19.3 b
268	0	18.8 b	15.8 b	16.3 b	15.6 c
268	20	19.7 a	16.7 b	19.9 a	19.2 a
Contrast					
Genistein	20 vs 0	NS	*	**	*
PGPR	102 vs 0	NS	NS	NS	NS
PGPR	268 vs 0	NS	*	NS	NS
			1997 (30 days after sowing)		
0	0	9.75 b	10.52 b	7.60 b	7.88 b
0	20	14.85 a	14.94 a	9.75 ab	8.55 b
102	0	8.75 b	8.01 b	7.42 b	7.30 b
102	20	8.55 b	9.00 b	12.25 a	11.45 a
268	0	9.55 b	9.11 b	8.95 b	9.27 ab
268	20	9.05 b	9.39 b	8.88 b	8.57 b
Contrast					
Genistein	20 vs 0	***	***	***	*
PGPR	102 vs 0	***	***	NS	NS
PGPR	268 vs 0	***	***	NS	NS

NS, *, ** and *** no significant difference and significant at 0.1, 0.05 and 0.001 probability levels, respectively.

Table 11.2 Nodulation and plant growth at the early flowering stage

		AC Bravor				Maple Glen			
PGPR	Genistein (μM)	Nodule number	Nodule weight (mg)	Shoot weight (g)	Root weight (g)	Nodule number	Nodule weight (mg)	Shoot weight (g)	Root weight (g)
				August 16, 1996					
0	0	57.8	255.4	18.78	1.11	58.9	235.1	21.0	1.17
0	20	62.7	290.5 *	24.50 ***	1.30 **	66.9 **	253.1	21.5	1.32 **
102	0	62.2	237.4	21.28 *	1.40 ***	56.2	210.4	23.2	1.22
102	20	58.4	262.0	23.31 **	1.28 **	67.6 **	244.1	22.6	1.29 *
268	0	57.4	234.7	20.94 *	1.24 *	60.2	233.8	22.2	1.25
268	20	63.2	234.6	22.28 *	1.25 *	59.15	218.4	22.5	1.18
Contrast									
Genistein	20 vs 0	NS	*	***	NS	***	NS	NS	NS
PGPR	102 vs 0	NS	NS	NS	**	NS	NS	*	NS
PGPR	268 vs 0	NS	**	NS	NS	NS	NS	NS	NS
				July 28, 1997					
0	0	20.3	120.3	7.98	1.443	19.6	153.5	8.29	1.36
0	20	29.6 ***	181.5 ***	9.17 *	1.610 **	22.2	163.0	8.43	1.40
102	0	21.7	128.5	9.75 **	1.588 *	26.4 **	164.5	9.34 *	1.43
102	20	22.8	165.0 **	9.48 **	1.485	33.8 ***	179.5 **	9.16	1.42
268	0	21.8	132.0	8.51	1.480	25.1 *	156.0	8.69	1.3
268	20	20.2	129.8	8.78	1.593 *	23.8	163.8	8.96	1.42
Contrast									
Genistein	20 vs 0	NS	***	NS	NS	*	*	NS	NS
PGPR	102 vs 0	NS	NS	**	NS	***	***	**	NS
PGPR	268 vs 0	**	NS	NS	NS	NS	NS	NS	NS

NS, *, ** and *** = no significant difference and significant at 0.1, 0.05 and 0.001 probability levels, respectively.

Table 11.3 Nitrogen assimilation at the early flowering stage

		August 16, 1996						July 28, 1997	
PGPR	Genistein (μM)	Shoot [N] (%)	Shoot N content (mg)	Root [N] (%)	Root N content (mg)	Nodule [N] (%)	Nodule N content (mg)	Nodule [N] (%)	Nodule N content (mg)
0	0	2.74	544.2	0.834	8.85	4.02	334.4	4.90	6.69
0	20	2.61	627.9 *	0.894	12.96**	4.25	375.3 *	4.96	8.63*
102	0	2.66	518.9	0.816	9.35	3.97	316.3	4.89	7.63
102	20	2.77	584.1	0.783	9.15	4.39 **	346.5	4.66	9.99***
268	0	2.72	564.1	0.771	9.61	4.11	314.8	5.00	7.74
268	20	2.75	527.5	0.856	8.56	4.20	315.9	4.82	8.32
Contrast									
Genistein	20 vs 0	NS	NS	NS	NS	**	*	*	***
PGPR	102 vs 0	NS	NS	NS	**	NS	NS	**	**
PGPR	268 vs 0	NS	NS	NS	**	NS	**	NS	NS

NS, *, ** and *** no significant difference and significant at 0.1, 0.05 and 0.001 probability levels, respectively.

[N] = nitrogen concentration. Shoot, root and nodule N contents are per plant.

Table 11.4 Seed nitrogen concentration and total nitrogen content in 1996 and 1997

		AC Bravor					Maple Glen				
		SHNC (%)	SHNT (mg)	SDNC (%)	SDNT (mg)	NYIELD (kg)	SHNC (%)	SHNT (mg)	SDNC (%)	SDNT (mg)	NYILED (kg)
						1996					
PGPR	0	0.484a	43.6a	5.29a	7.61a	183.0a	0.479a	40.0a	5.51a	7.15a	174.4a
	102	0.488a	37.9a	5.29a	7.86a	180.4a	0.556ab	46.9a	5.56a	7.89a	165.5a
	268	0.448a	36.9a	5.28a	7.94a	180.2a	0.564b	44.7a	5.47a	7.80a	165.8a
Genistein	0	0.464a	35.5a	5.30a	7.80a	182.7a	0.533a	43.5a	5.52a	7.82a	1691a
(μM)	20	0.482a	43.4b	5.28a	7.81a	179.7a	0.532a	44.2a	5.51a	7.40a	168.1a
						1997					
PGPR	0	0.454a	ND	5.42a	10.35b	203.8a	0.401a	ND	5.49a	10.98b	178.9b
	102	0.436a	ND	5.61a	11.02a	216.8a	0.403a	ND	5.60a	11.38a	190.2a
	268	0.431a	ND	5.427a	10.16ab	206.1a	0.401a	ND	5.39a	10.92b	186.1ab
Genistein	0	0.430a	ND	5.39a	10.12b	200.2b	0.410a	ND	5.51a	11.04a	181.9a
(μM)	20	0.451a	ND	5.58a	10.90a	217.6a	0.394a	ND	5.40a	11.15a	188.2a

In each year within columns and PGPR or genistein, means followed by different letters are significantly different by a protected LSD (0.05) test. ND = not determined. NYIELD = nitrogen yield per ha. SHNC = shoot nitrogen concentration. SHNT = shoot nitrogen content per plant. SDNC = seed nitrogen concentration. SDNT = seed nitrogen content per seed.

Preface to section 12

Section 12 is composed of a manuscript by Pan B and Smith DL and will be submitted to *Plant and Soil*.

Legume inoculants have been commercially available for more than 80 years and are effective in increasing yields of legumes in low-N soil which lacks an indigenous *(Brady)rhizobium* population, have small populations or have population comprised of probably ineffective strains. Competition between poorly effective strains already present in agricultural soils and highly effective strains added in commercial inocula has been viewed as one of the most intractable limitations to efficient inoculation with bradyrhizobia under field conditions. The mechanism of strain competitiveness is not well elucidated. In the previous sections, we measured signal production under different conditions and manipulated soybean nodulation and N_2 fixation through the use of signal compounds. This manipulation could change the competitiveness of *B. japomicum* strains and we have tested this possibility here.

Section 12

Genistein Preincubation of *Bradyrhizobium japonicum* Cells Improves Strain Competitiveness under Greenhouse, but not Field Conditions

12.1 Abstract

In the soybean-*B. japonicum* symbiosis, genistein has been identified as one of the major compounds in soybean seed and root extracts responsible for the inducing expression of the *B. japonicum nodYABC* operon. In this study, we have tested the possibility that genistein treatment prior to inoculation can increase the competitiveness of the treated *B. japonicum* strain under both greenhouse and field conditions. Two mutants of the two *B. japonicum* strains each with a different antibiotic resistant marker were selected. They were treated with genistein. The results showed genistein treated mutants had higher level of nodule occupancy than the untreated mutant or parent strain under greenhouse conditions. Mutants from 532C had higher nodule occupancies than mutants from USDA110, especially at 15 °C. In the more complex field environment genistein treated mutants formed fewer nodules than the untreated mutants. The contradictory results of strain competitiveness for greenhouse and field experiments are discussed.

Key words: *Bradyrhizobium japonicum*, soybean, strain competitiveness, genistein

12.2 Introduction

Legume inoculants have been commercially available for more than 80 years and are effective in increasing yields of legumes in low-nitrogen soils which lack an indigenous *(Brady)rhizobium* population or have small populations. However, legume inoculants often do not demonstrably affect yields in many soils where indigenous strains are present at reasonable levels. Inoculant strains are often poor competitors with indigenous strains, which usually occupy the vast majority (80-90%) of soybean nodules (Kuykendall and Weber, 1978). The competitive barrier has been viewed as one of the most intractable limitations to efficient inoculation with bradyrhizobia (Ham, 1980).

Soil temperature is a major factor in the ability of *(Brady)Rhizobium* to persist and compete. Strains differ in their ability to survive and grow over a range of temperatures (Hartel and Alexander, 1984). Weber and Miller (1972) planted soybean plants in pots containing soil with a large population of indigenous rhizobia and found different serogroups predominated in the nodules of plants that were incubated at difference temperatures. Two strains of *R. trifolii* were inoculated onto clover plants; one strain formed the majority of nodules at 12°C, but the other strain was more competitive at 25°C, and the bacterial strain x temperature x variety interaction was highly significant (Hardarson and Jones, 1979).

Several approaches to the competitiveness problem have been attempted, including mass inoculation with *B. japonicum*, isolation of genetic determinants for competitiveness, chemical control of nodulation and soybean breeding for increased competitiveness (reviewed by Triplett and Sadowsky, 1992). But none of these methods seems very promising for enhancement of strain competitiveness. Modern molecular biology studies have shown that the formation of nitrogen fixing nodules is a complex, multi-step process requiring the activity and regulation of both plant and bacterial genes, in which control signals are exchanged between plants and nitrogen fixing bacteria. In the soybean-*B. japonicum* symbiosis, the isoflavonoid genistein, has been identified as one of the major compounds in soybean seed and root extracts responsible for inducing the expression of the *B. japonicum nodYABC* operon (Kosslak et al., 1987). As a result of *nod* gene induction, lipo-chito-oligasaccharide nodulation factors (Nod factors) are produced by the bacterial symbiont (Lerouge et al., 1990), which in turn elicit root hair deformation, cortical cell division and nodulin gene expression in soybean plant roots (for a review see Spaink, 1996).

This research evaluated the competitiveness of *B. japonicum* strains pre-incubated with genistein, under greenhouse and field conditions. The main objective of this research was to determine whether the genistein incubated strains or mutants were more competitive against untreated strains or mutants in forming nodules.

12.3 Materials and Methods

Strains and mutants

The parent strains used in these studies were USDA110 and 532C (Hume and Shelp, 1990). Ten antibiotic resistant marker mutants were selected from each parent strain according to the methods of Kuykendall and Weber (1978). Mutants of 532C were resistant to 100 μg/mL rifampicin, and mutants from USDA110 were resistant to 500 μg/mL streptomycin sulphate. Their nodulation abilities (indicated by nodule number per plant) were tested and compared to the parent strains (data not shown). Mutants selected for antibiotic resistance expressed a variety of reduced nodulation effectiveness conditions and the mutants most similar in nodulation potential to each parent strain were selected for further study of strain competitiveness.

Inoculant preparation

The inoculants were produced by culturing *B. japonicum* stains in yeast extract-mannitol broth (YEM, Vincent, 1970) with appropriate antibiotics in 250-mL flasks shaken at 150 rpm at 25°C for 7 days. For the production of inoculant preincubated with genistein, 100 mL cell suspension from a 4-day-old sub-culture (A_{620}=0.35) was aseptically added to a 500 mL flask of sterile genistein solution in a 2000 mL flask and incubated at 30°C without shaking for 48 h. Following incubation, the cell suspensions were pelleted in sterile centrifuge tubes at 10,000 g for 10 min, washed once with distilled water, and resuspended to an A_{620} of 0.1 (approximately 10^8 cells mL^{-1}). The ratio of the two strains and mutants used as inoculant were 1:1 (v/v) in all experiments.

Plant materials and inoculation

The soybean cultivar AC Bravor was used in all the experiment. For greenhouse experiments, seeds were surface-sterilized in 2% sodium hypochlorite (containing 4 mL L^{-1} Tween 20) for 7 min and then rinsed 10 times with distilled water. Five seeds were sown in each pot. Turface (Applied Industrial Material Corp., Deerfield, IL) and sand (3:1, v/v)

were used as a growing medium. The pots were 20 cm in diameter and 15 cm in deep. When they reached the vegetative-cotyledon stage (VC, unifoliolate leaves unrolled sufficiently that the edges were not touching, Fehr et al., 1971), the seedlings were thinned to 2 plants per pot. One mL of inoculant per plant was applied, using a pipette, onto the rooting medium at the base of the plant for greenhouse experiments. Soybean plants were watered with modified nitrogen-free Hoagland solution (Zhang and Smith, 1995).

For the field experiment, after soybean seeds were sown into the soil by hand, a 20 mL aqueous suspensions of bacteria was applied evenly into open rows of 2 m length with a 60 mL syringe. The inoculation rate was 10^8 bacterial cells per 10 cm row. Immediately after inoculation the seeds were covered with soil.

Nodule sampling

Nodules were sampled 30 days after inoculation for the greenhouse experiment and 50 days after planting for field experiments. In the latter case, four randomly selected plants were harvested in each block for each treatment. All the nodules were removed from each plant and six nodules were selected randomly, making a total of 120 nodule per treatment per experiment.

Identification of nodule isolates

Nodule surface sterilization followed the method of Vincent (1970). The nodules were crushed with a sterilized toothpick. The same toothpick was pressed through the YEM agar in petri dishes with and without appropriate antibiotics. The plates were incubated at 30 °C for 7 days. Doubly infected nodules were given a one-half positive rating for the two strains under consideration.

Counting of indigenous bradyrhizobia

The indigenous bradyrhizobia population was measured using the most probable number (MPN) technique (Vincent, 1970). Soil samples were taken before planting with a

soil depth of 30 cm. MPN testing was repeated three times, giving an average indigenous population of 2,300 cells per gram soil.

Experimental designs

Greenhouse experiment 1: Parent strains were compared with the selected mutants, with or without genistein pre-incubation. The treatments are listed in table 12.1.

Greenhouse experiment 2: USDA110 was used as a reference strain. Mutants with or without genistein preincubation were compared with USDA110. The method of root zone temperature control was the same as in Zhang and Smith (1995). The three controlled root zone temperatures were 15, 20 and 25 °C. The treatments were listed in table 12.1.

Field experiment: The experiment was a random completely block design. Each plot had four rows 2 m long. The soil was a sand-loam. Soybean seeds were sown by hand on May 15, 1997. After seeds were sown into the rows, 20 mL of inoculant was applied evenly into the open rows with a syringe and then the rows were closed. Following emergence, seedlings were thinned to achieve a stand of 45000 plants per ha^{-1}. Samples were taken from the middle two rows. The four treatments used are listed in table 12.1.

12.4 Results

Greenhouse Experiment 1

Genistein preincubation improved *B. japonicum* strain competitive ability (Fig. 12.1). Genistein treated mutants always occurred more frequently in nodules. The results were the same for both mutants of 532C and USDA110.

Greenhouse Experiment 2

Genistein treated mutants had higher nodule occupancy ratios than untreated mutants (Fig. 12.2) for most of the treatments. No difference was found at 25 °C with mutants of USDA110 and at 20 °C with mutant of 532C, although in both cases the genistein treated

mutant still had numerically higher nodule occupancy ratios. The mutant of 532C had higher nodule occupancy ratios than the mutant of USDA110, especially at 15 °C.

Field Experiment

Under field conditions, genistein treated mutants formed fewer nodule than untreated mutants (Fig. 12.3). The 532C mutant formed more nodules than the USDA110 mutant.

12.5 Discussion

Strain competitive ability was improved by genistein preincubation of *B. japonicum* cells in greenhouse experiments. This may have been due to the 'readiness' of the inoculant, therefore the increased speed of nodulation. Improving the 'readiness' of inoculant strains to nodulate has been used in inoculant preparations (Rolfe et al., 1987). When superior mutant *Rhizobium* strains were pretreated with root exudate, inoculated on soybean seeds, and planted in fields where the mutant strain normally were not competitive, the inoculation significantly increased soybean yield (Paau et al., 1990). Paau et al. (1990) reported that the 'readiness' of the rhizobia to nodulate can be altered by adding soybean meal to the fermentation medium and this has an obvious effect on the competitiveness of the inoculant strains. The speed of nodulation is a crucial factor in a strain's competitiveness for nodulation (McDermott and Graham, 1990). Zhang and Smith (1995) show that genistein preincubation of *B. japonicum* cells accelerates nodule development at suboptimal root zone temperatures. The isoflavonoid-inducible resistance to the phytoalexin glyceollin, observed in some strains of *B. japonicum*, may also increase the competitiveness of these strains in the rhizosphere (Parniske et al., 1991).

This study confirms previous work showing that, at least for soybean, laboratory and greenhouse comparisons of strain competitiveness for nodule occupancy will probably provide poor predictions of nodule occupancy in the field (Streeter, 1994). In our experiments, the contradictory results of greenhouse and field experiments could be due to several factors. First, the inoculating time for the field experiment was at planting and it

was different from the inoculating time of greenhouse experiments, which was at the VC stage (Fehr et al., 1971). While in the field experiment, there was about half a month before the inoculant had a chance to infect the newly developing soybean roots. In the greenhouse the cells were added to fully formed root systems ready for infection. In the field the bacteria need to adapt to the prevailing environmental conditions and multiply in the soil and host rhizosphere; then they have to compete on the legume roots for infection sites and nodulation. The details of these processes in the soil during this critical time during the period of infection remains largely unknown. Much study needs to be focused on this area as a prime aim of legume inoculation is to maximize survival of inoculated strains during the period between its introduction into the soil and the development of a legume rhizosphere which it can colonize (Brockwell and Bottomley, 1995). Second, *(Brady)Rhizobium* strains vary tremendously in their response to the environment (Schmidt et al., 1985). Under soil conditions, mutants with or without genistein preincubation could respond differently, and therefore affect the competitiveness. This hypothesis is backed up by the data of Ayanaba et al (1986). They found that root exudate treatment increases the nodule occupancy of two out of seven *B. japonicum* strains. Third, environmental factors affect the growth of both plants and bacteria. As plants must develop a rhizosphere to support rhizobial growth as well as form morphologically complete nodules to house the invading rhizobia, it is probable that any factor that adversely affects plant growth will also profoundly affect competitiveness for nodulation. McNeil (1982) found that one strain of *B. japonicum* outcompeted another for nodulation sites in the presence of nitrate, while this was not the case in the absence of nitrate.

The stronger competitiveness of the mutant of 532C in both greenhouse and field experiments, compared with the mutant of USDA110, could be related to its origin of selection. Strain 532C was identified as an efficient N_2 fixer in Ontario soils (Hume and Shelp, 1990) and has been shown to perform well over a range of temperatures (Lynch and Smith, 1994), while USDA110 was selected in a relatively warm area of the United

States of America. The performance of 532C at different temperatures supports the conclusion that the temperature under which experiments are conducted will influence the results of strain competitiveness (Fuhrmann and Wollum, 1989). Different responses of strains to temperature means that effective strains could be selected, whose optimum temperature for nodulation and competitiveness are similar to the soil temperature at the times of inoculation.

Several questions were not answered in this experiment and need to be studied. First, genistein concentration used to incubate *B. japonicum* needs to be studied, as only one genistein concentration was used to incubate the bacteria. Previous experiments showed that a higher genistein concentration was better at promoting nodulation at low at low temperature (Zhang and Smith, 1995). Second, the ecological adaptation, metabolic and genetic changes, once in soil, of genistein preincubated *B. japonicum* cells needs to be elaborated in order to explain the contradictory results of the greenhouse and field experiments and for a better use of this technique under field conditions.

Table 12.1 Treatments tested in greenhouse and field experiments

Greenhouse exp. 1		Greenhouse exp. 2	Field exp.
532Cp + 532Cstr	USDA110p + USDA110rif	USDA110p + USDA110rifG	USDA110rifG
532Cp + 532CstrG	USDA110p + USDA110rifG	USDA110p + USDA110rif	USDA110rif
532CpG + 532Cstr	USDA110pG + USDA110rif	USDA110p + 532CstrG	532CstrG
532CpG + 532CstrG	USDA110pG + USDA110rifG	USDA110p + 532Cstr	532Cstr

P -- parent strain 532C or USDA110, G -- genistein pre-incubation treatment
rif -- rifampcin resistant, str -- streptimycin resistant,

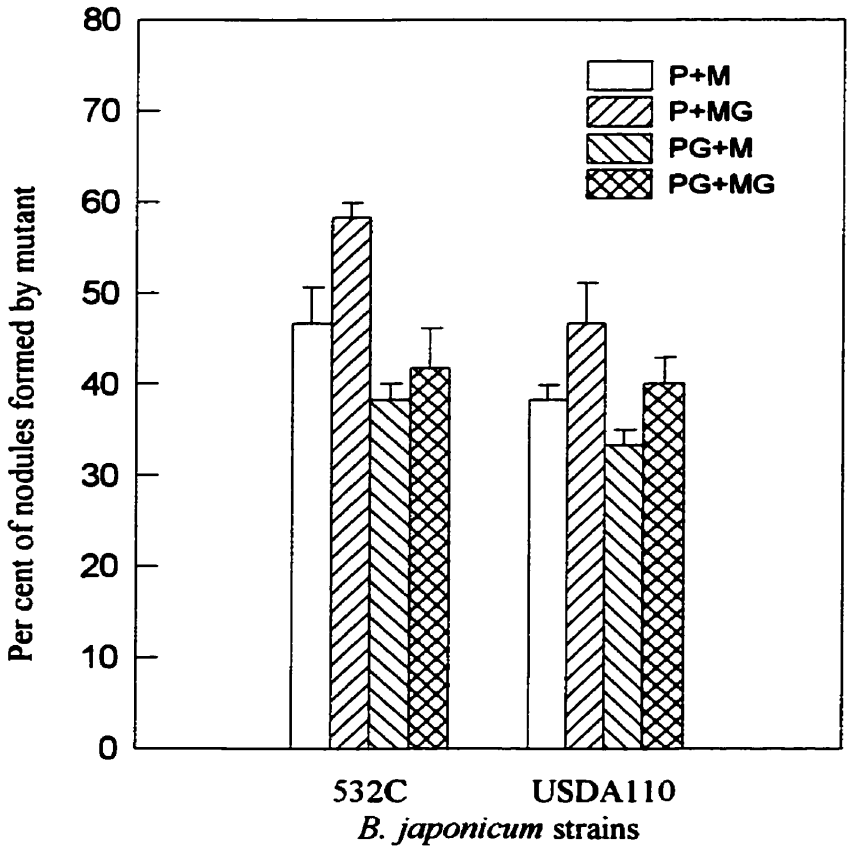

Fig. 12.1 Nodule occupancy ratio of *B. japonicum* strains preincubated with genistein or not compared with the parental strain. p = parental strain. m = mutant. G = genistein treated. + = mutant and parent strain used together.

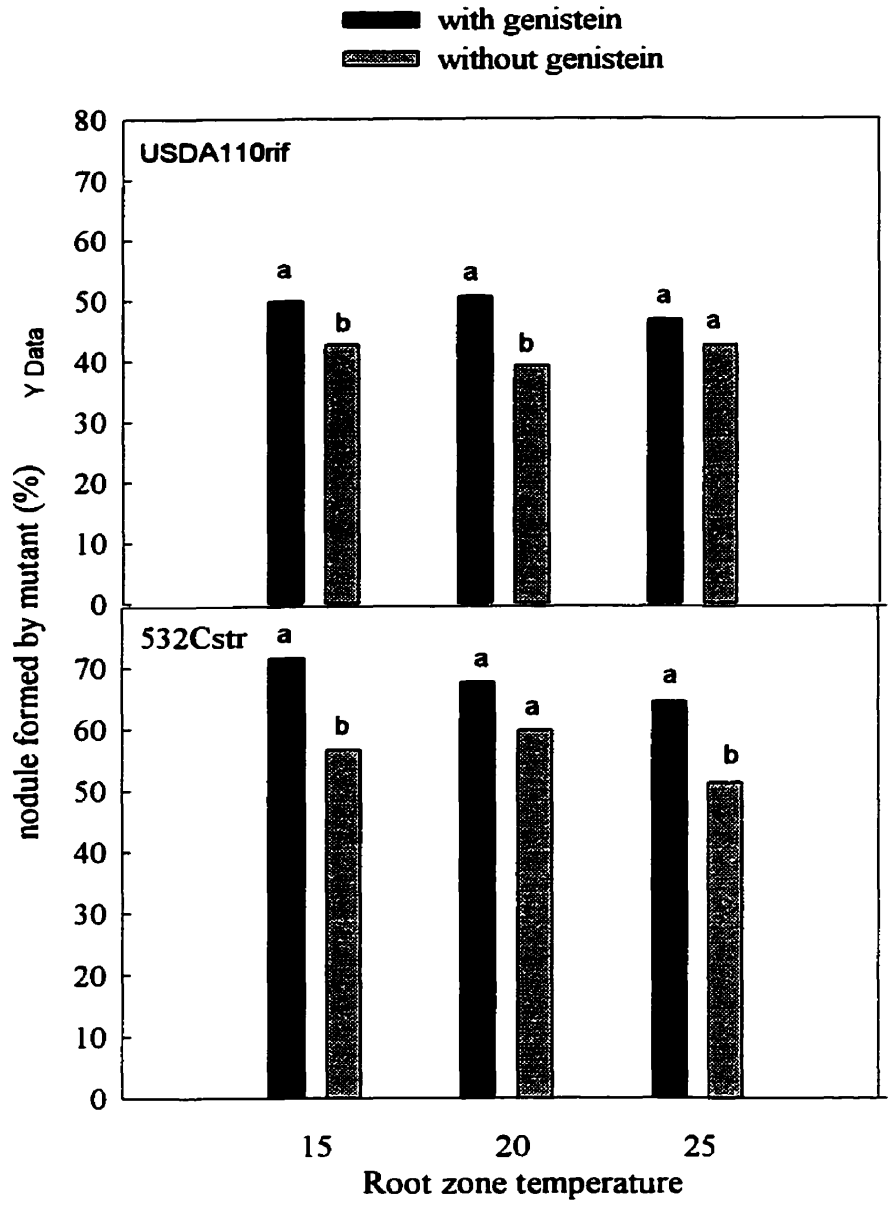

Fig. 12.2 Nodule occupancy ratio of two *B. japonicum* mutant in paired competitiveness assay with USDA110 on soybean cv AC Bravor in greenhouse. Different letters at each temperature with the same mutant indicate significant at 0.05 level.

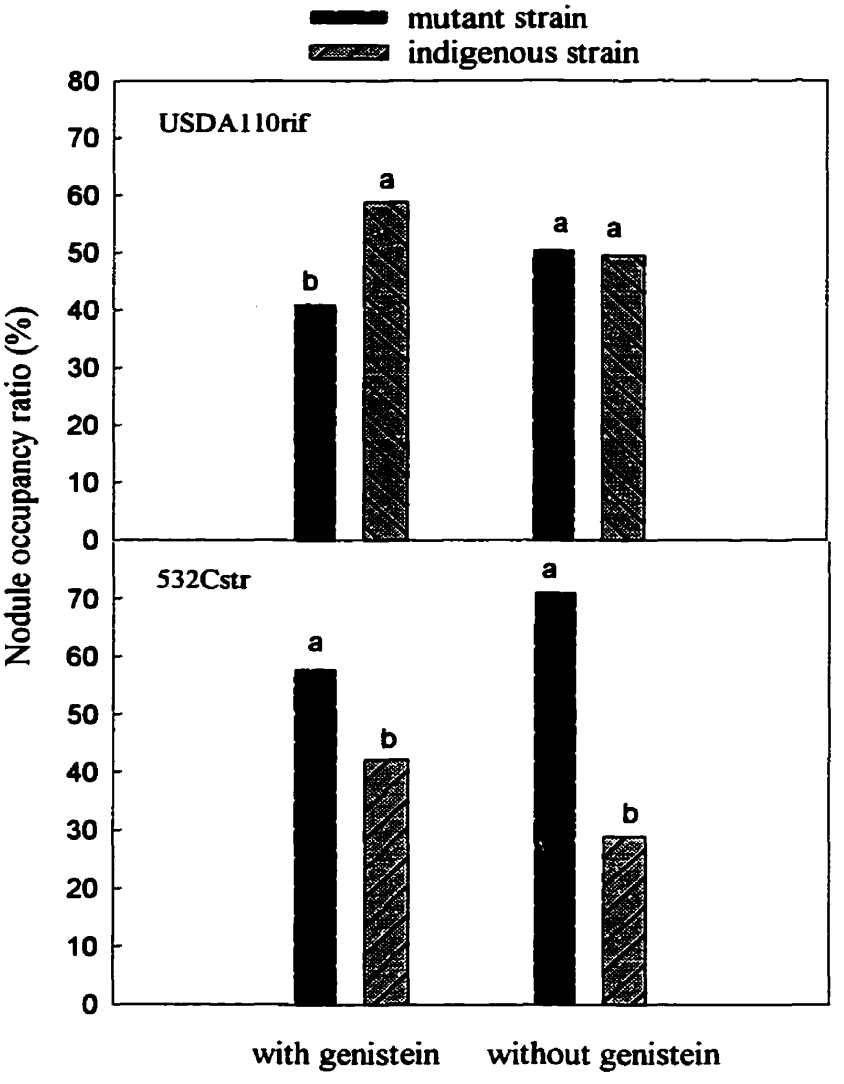

Fig. 12.3 Nodule occupancy ratio in field experiment. Different letters in each mutant with genistein or without genistein indicate significant at 0.05 level.

Section 13. GENERAL DISCUSSION

The interaction between the soil bacterium *(Brady)Rhizobium* and legume plants results in the formation of nitrogen-fixing root nodules. Nodule formation is a complex, multi-step process requiring the activity and regulation of both plant and bacterial genes, which control signal exchange between plants and nitrogen-fixing bacteria. Environmental conditions affect signal production and exchange through their effects on host plants and microsymbionts (Diagram 1).

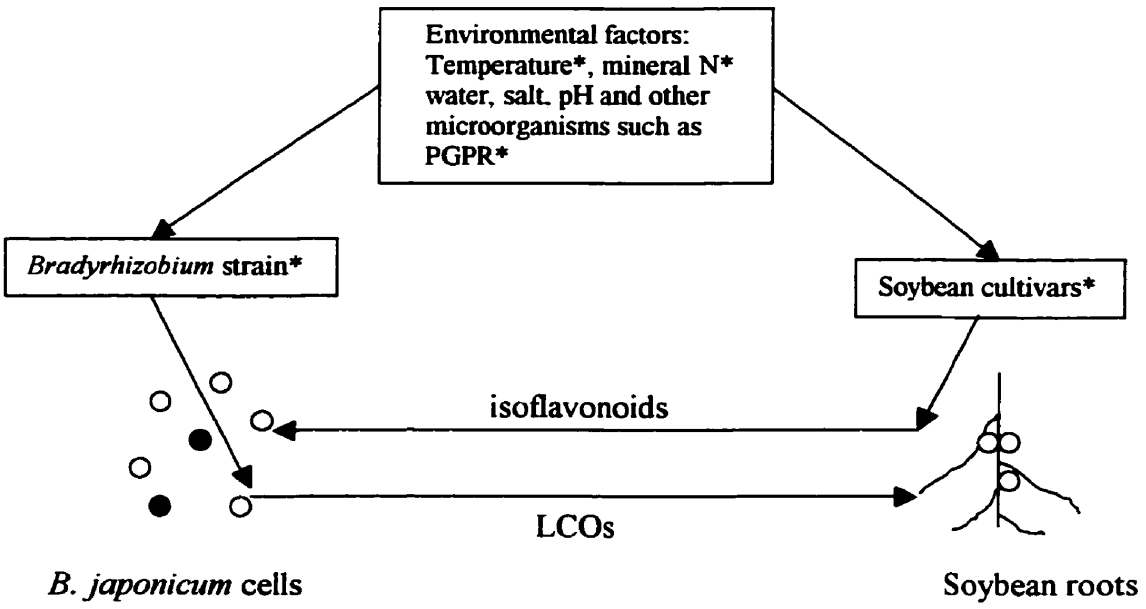

Diagram 1. Signal production and exchange in the soybean-*B. japonicum* symbiosis and factors affecting signal production and exchange (*studied in this thesis and discussed below):

In the soybean-*B. japonicum* symbiosis, genistein and daidzein have been identified as the major isoflavonoid compounds in soybean seed and root extracts responsible for inducing the expression of the *B. japonicum nodYABC* operon (Kosslak et al. 1987). As a result of *nod* gene induction, lipo-chitin oligosaccharides (LCOs) nodulation factors (Nod factors) are produced by the bacterial symbiont (Lerouge et al. 1990), which in turn elicit

root hair deformation, cortical cell division and nodulin gene expression in soybean roots (for a review see Spaink, 1996).

13.1 **Temperature, flavonoid and nodualtion**

Low temperature is the major factor limiting soybean nodulation (Zhang and Smith, 1995) and yield (Whigham and Minor, 1978) in short growing season areas. Measurements taken at the Macdonald Campus of McGill university meteorology facility indicate a mean soil temperature at a depth of 10 cm of 10 °C in mid-May (the usual planting time for soybean here), 15 °C in June, 18 °C in July, 19 °C in August, 13 °C in September (Lynch and Smith, 1993b). Our data (Section 3, Figs. 3.1-3.4) indicate that changes in daidzein and genistein concentrations and contents of soybean roots above 15 °C could not be the cause of inhibition of nodulation. On the other hand, the effect of temperature on isoflavonoid release is not known. Isoflavonoid biosynthesis and excretion are different processes, and RZT could have different effects on them. Graham (1991) pointed out several lines of evidence which suggest that the release of isoflavones may be a specific and regulated phenomenon, and that soybean seeds may possess a feedback mechanism to regulate the exudation of their very large stores of isoflavone signal molecules. Our data (section 4) showed that daidzein and genistein content and concentration in root exudates do not correspond to the content and concentration inside the roots, suggesting that their excretion is subject to regulation. In view of the role of these compounds in symbiotic interactions with micro-organisms their secretion and accumulation in the rhizosphere are of interest.

Daidzein to genistein ratios were higher at 15 and 17.5 °C than at 25 °C. Daidzein is a less efficient *nod* gene inducer that genistein (Kosslak et al. 1987). The implications of this change due to temperature needs to be studied.

13.2 **Mineral N effects on soybean flavonoid synthesis and exudation**

Our data showed that mineral N inhibits soybean shoot and root daidzein and genistein contents and concentrations. Our results confirm other results observed in soybean roots (Cho and Harper, 1990). The biochemical mechanism underlying this

reduction is still not clear. But this inhibition did not necessarily mean a decrease in nodulation, at least under our experimental conditions. Mathews et al. (1989) reported similar results for alfalfa. Isoflavonoids perform other functions in plants, including serving as natural auxin transport inhibitors (Jacobs and Rubery, 1988) and producing cytokinin-like activities (Binns et al., 1987). Both auxin and cytokinin were suggested as playing roles in nodule formation (Hirsch et al., 1989; Long and Cooper, 1988). High concentrations of isoflavonoids can also inhibit bacterial growth (Section 7). Several flavonoids have also been found to antagonize the induction process (Firmin et al., 1986). Nitrate effects on the synthesis and release of those antagonizing compounds is not known. It is important to understand the synthesis and exudation of both inducing and inhibiting compounds in the developing root (Peters and Long, 1988).

Less or no mineral N inhibition was found on daidzein and genistein exudation, depending on N level in the medium. This result suggested that exudation and synthesis were affected differently by mineral N. Our studies also showed that temperature, soybean cultivar, soil mineral N level, and cultivation methods all influence flavonoid synthesis and exudation. Variation among soybean cultivars for daidzein and genistein contents and concentrations suggests that appropriate soybean cultivars could be selected for different environmental conditions. But more questions need to be answered to make this selection meaningful.

It is interesting to find that daidzein and genistein had different patterns of change, over time, among roots, shoots and exudates, and over the three levels of ammonium nitrate. Daidzein and genistein have different abilities to induce *nod* gene expression (Kosslak et al., 1987). Soybean plants can alter the effectiveness of a stronger inducer in the rhizosphere by releasing weaker inducers which would compete for binding sites (Phillips, 1992). Graham (1991) reported that soybean roots could selectively exude the conjugated forms of daidzein and genistein. From my data on genistein and daidzein ratios we can see that genistein content deceased as soybean plants grew. This means more daidzein and less genistein was synthesized inside the root and shoot. This could affect root nodule establishment, as genistein is more effective than daidzein in inducing *nod* gene expression. Daidzein content and concentration were

much higher than genistein in soybean roots. The implication of these higher daidzein contents and the change in ratio of genistein to daidzein at different growth stages and mineral N conditions for nodulation and N fixation need to be further investigated.

Conjugates of the isoflavones daidzein and genistein are major metabolites within the dry seed and in seedling roots after germination (Graham, 1991). It has been reported that following inoculation with *Rhizobium meliloti* alfalfa seedlings exude isoflavonoid conjugates (Dakora et al., 1993). D'Arcy-Lameta (1986) reported that a hydrolysis of polyphenolic conjugates takes place at the time of exudation. This observation suggests that isoflavonoid conjugates are also playing an important role in signalling between roots and bradyrhizobia. Both the formation and hydrolysis of these isoflavone conjugates are subject to an intricate regulation program, and they may play a direct role in the responses of soybean tissues to elicitor treatments (Graham, 1991). In our study, we only measured daidzein and genistein concentrations and contents. The quantity, regulation and function of the conjugate forms of isoflavones among soybean cultivars may be important and needs to be studied.

Nodulation and N fixation is a complex process involving both host plant and bradyrhizobia. Flavonoid changes could only explain part of the story. Mineral N condition (Section 7) and low temperature (Zhang and Smith, 1997) also affect bradyrhizobial signal production.

The growth environment of soybean used for HPLC measurement in our experiment, in the sterilized growth pouches or pots, was totally different from field conditions. The microorganisms associated with the rhizosphere are very active in degrading isoflavonoids to CO_2 (Rao, 1990). It may be possible to find a different correlation between isoflavonoids and nodulation under field conditions.

13.3 Genistein on mineral N inhibition of nodulation

Genistein addition to the rhizosphere or preincubation of *B. japonicum* with genistein improved nodulation, N fixation and in some cases plant growth and yield. The most obvious explanation is that genistein addition may promote *nod* gene expression (Kosslak et al. 1987) of the *B. japonicum* leading to higher levels of Nod factor

production by *B. japonicum* cells. The expression of bradyrhizobial *nod* genes led to the production of the bacterial Nod factors, which are able to induce many of the early events in nodule development and regulate plant morphogenesis (Spaink 1996). At low root zone temperatures, the positive effects of genistein on nodulation were mainly due to the alteration of the time-course of each nodulation stage (Zhang and Smith 1995). It is not clear at this time if similar alterations could be caused by genistein in the presence of mineral N. Barz (1970) reported that microorganisms in the rhizosphere are very active at degrading the isoflavonoids. Thus the addition of genistein to the rooting medium may compensate for some of the degradation.

On the bradyrhizobial side, our data showed that mineral N inhibited *nod* gene expression and addition of higher levels of genistein could partially overcome this inhibition. The induced production of Nod factors could, in turn, increase isoflavonoid exudation by the host root (van Brussel et al., 1990) and compensate for the inhibition of isoflavonoid caused by mineral N. The results also indicated that nitrate, as well as ammonium sulphate and urea, exert their effects early in the nodulation process and, at least partially, through the bacterial cells. Calvert et al. (1984) reported that, for soybean nodule formation, the initial stages in the infection process appear to be the most sensitive steps. Inhibition is substantially alleviated by delaying exposure to nitrate until 18 h after inoculation (Malik et al., 1987). It means that most of the nitrate-sensitive events of infection were functionally complete within less than 18 h. Those sensitive events may include isoflavonoid concentration in the rhizosphere, bacterial reception of those isoflavonoids and bacteria-to-plant signal production by the common and specific *nod* genes. Stacey et al. (1997) reported that *nod* gene expression in *B. japonicum* is a complex system and involves more regulatory proteins than just NodD. NodV and NodW comprise a second isoflavone recognition system (the first being NodD) in *B. japonicum* (Sanjuan et al., 1994). The effects of nitrate on the second isoflavone recognition system are not clear at this time. Yuen and Stacey (1996) reported that organic acids could also inhibit *nod* gene expression in *B. japonicum*. Changes in root extract pH were found to be associated with ammonium nitrate levels (section 5). It is possible that the pH changes in root extract could affect the content or concentration of

different organic acids in nodule, and hence affect gene expression. Genistein could also affect nodulation through alteration of the composition and molecular mass distribution of exocellular polysaccharides produced by *B. japonicum* (Dunn et al. 1992).

The most consistent results of genistein manipulation in several experiments were increases in nodule size and weight. Since flavonoids can function as modulators of polar auxin transport (Jacobs and Rubery 1988), we speculate that they could locally disturb the auxin-cytokinin balance, leading to the induction of nodule meristems (Schmidt et al. 1994). D'Arcy-Lameta and Jay (1987) reported that low micromolar concentrations of flavonoids stimulate the growth rate of rhizobia. But the function of flavonoids inside soybean roots in nodulation is not clear. Flavonoid injection might be used to answer this question. Increased nodule size may also be due to the effects of genistein on Nod factors, which trigger the formation of the root nodules by initiating cell divisions at distinct sites, and possibly by affecting regulation of the plant cell cycle (Verma 1992).

The improvement of *nod* gene expression, nodulation and N fixation by genistein was concentration dependent and varied with soil mineral N levels. As symbiotic N fixation is a complex process and isoflavonoids have many functions in both host plants and in bradyrhizobia. Thus, much work remains to be done before the impact of genistein on soybean nodulation is fully understood.

13.4 Bradyrhizobium strains, their competitiveness and signal production

Signal manipulation provides a new way of improving strain competitiveness, which has limited the efficiency of inoculants since they were introduced. Our data showed that strain competitiveness was improved in greenhouse experiments by genistein preincubation of *B. japonicum* cells. This may have been due to the 'readiness' of the inoculant and therefore increased speed of nodulation (Rolfe et al., 1987; Paau et al, 1990). The speed of nodulation is a crucial factor in a strain's competitiveness for nodulation (McDermott and Graham, 1990). Zhang and Smith (1995) showed that genistein preincubation of *B. japonicum* cells accelerates nodule development at suboptimal root zone temperatures. The isoflavonoid-inducible resistance to the

phytoalexin glyceollin, observed in some strains of *B. japonicum*, may also increase the competitiveness of these strains in the rhizosphere (Parniske et al., 1991).

Our data also suggested that signal treatment had to be combined with other factors such as strain selection. *(Brady)Rhizobium* strains vary tremendously in their response to the environment (Schmidt et al., 1985). Strain 532C, used in our study, showed stronger competitiveness than USDA110. Soybean cultivar (Section 6), soil N condition (Section 5), and temperature (Section 3) could also affect strain competitiveness, through their effects on signal production and exchange between host plant and *Bradyrhizobium* cells.

Our experiment raised more questions than it answered. To explain the contradictory results of greenhouse and fieldwork, further study is needed to investigate the survival of inoculants treated with signal during the period between introduction into the soil and the development of a legume rhizosphere which it can colonize. The details of processes in the soil during this critical infection period of time are largely unknown.

13.5 PGPR effect on soybean nodulation and mineral N inhibition

The effects of PGPR on soybean nodulation, nitrogen fixation, plant growth and yield were inconsistent and varied according to experiment condition, soybean cultivar and PGPR strain. Similar effects were found in other studies (Suslow, 1982; Grimes and Mount, 1984; Lynch, 1990; Zhang et al., 1996). PGPR affects nodulation and N fixation through its promotion of plant growth or the reverse. PGPR could interrupt early signal production or balance, at a critical stage of nodule formation (Bhuvaneswari et al, 1980; McDermott and Graham, 1990).

Nitrate inhibition of nodulation and nitrogen fixation by soybean plants is related, at least in part, to alterations in root isoflavonoid synthesis and exudation (Cho and Harper, 1991) and resulting *B. japonicum nod* gene expression. Stimulation of isoflavonoid biosynthesis in plants is a common feature of many infecting microorganisms irrespective of whether they are bacteria, fungi, viruses or nematodes (for a review see Dakora and Phillips, 1996), and hence these infections can be expected to promote *nod* gene expression through indirect increases in those compounds. It would be interesting to

measure the isoflavonoid change in soybean root systems when plants were inoculated with PGPR strains.

The isoflavonoid genistein is the most active compound in promotion of *nod* gene expression in *B. japonicum* (Kosslak et al., 1987). One form of commercial genistein is from the fermentation of *Pseudomonas* sp. (INC, 1995). If strains of PGPR producing genistein, and at the same time benefiting plant growth, could be found they could be co-inoculated with *B. japonicum* to overcome negative environmental conditions, which act through interruption of isoflavonoid synthesis and exudation and subsequent inhibition of *nod* gene expression by *B. japonicum*. Further work needs to be done to answer questions such as the effects of nitrate level on the growth of PGPR themselves and their exudation processes, especially the exudation of active compounds.

PGPR can increase the growth and yield of non-legume crops (Gaskin et al., 1985) through the production of plant growth hormones (De Freitas and Germida, 1990; Glick, 1995). Soybean nodulation and nitrogen fixation processes involve several phytohormones (for a review see Arshad and Frankenberger, 1998). Changes in the phytohormone balance are a necessary requirement for elicitation of nodule formation (Hirsch and Fang, 1994). Prinsen et al. (1991) have demonstrated that *nod*-inducers (isoflavonoids) also stimulate the production of IAA, suggesting that nodule morphogenesis may be controlled by the highly specific *nod* signal in combination with phytohormones, such as auxins, released by rhizobia. So the effects PGPR on soybean nodulation could be related to changes in the production, exudation and/or balance interruption of those plant phytohormones and/or isoflavonoids in the rooting medium and inside the nodules.

Section 14

SUMMARY AND CONCLUSIONS

Based on the research findings of this thesis, the following conclusions can be drawn.

1. Temperature affected genistein and daidzein contents and concentrations in soybean roots. The effect of temperature varied among soybean cultivars.
2. Addition of genistein at the onset of nitrogen fixation improved soybean nodulation and nitrogen fixation. Soybean cultivars had different sensitivities to genistein addition.
3. Daidzein and genistein distribution patterns varied with plant organs, ammonium nitrate levels, and plant development stages. Ammonium nitrate inhibited daidzein and genistein contents and concentrations in soybean root and shoot extracts, but not in root daidzein and genistein exudate. In both synthesis and excretion, daidzein and genistein were not affected equally by ammonium nitrate treatments.
4. Changes in root daidzein and genistein contents of soybean cultivars varied in response to mineral N levels. The amount of daidzein and genistein excreted by soybean roots did not correspond to the inside root daidzein and genistein contents and concentrations.
5. *Nod* gene expression of *B. japonicum* cells was inhibited by mineral nitrogen. Addition of genistein partially overcame this inhibitory effect.
6. Genistein additions increased nodule weight and nodule nitrogen fixation under greenhouse conditions, but interactions existed among soybean cultivars, genistein treatment and nitrate levels.
7. Similar results were found under field conditions. Soybean yield was increased on sandy-loam soil by preincubation of *B. japonicum* cells with genistein.
8. Some PGPR strains can mitigate the negative effects of nitrate on soybean nodulation and nitrogen fixation, however, this is influenced by soybean genotype.
9. Applying PGPR together with genistein preincubation of *B. japonicum* cells improved soybean nodulation and increased yield. The improvement varied among soybean cultivars and PGPR strains.

10. Preincubation of *B. japonicum* cells with genistein improved strain competitiveness under greenhouse, but not field, conditions.

Section 15.

CONTRIBUTIONS TO KNOWLEDGE

The research presented in this thesis has answered several questions relevant to the basic biology of soybean N_2 fixation and to the mineral N inhibition of nodulation. This research contributed to the basic knowledge needed by agronomists to exploit the potential offered by the symbiosis in their quest for sustainable agricultural production.

Original contributions to knowledge include the following:

1. *Temperature affects soybean isoflavonoid production*: In this work we demonstrated, for the first time, that temperature affected genistein and daidzein contents and concentrations in soybean roots.
2. *Mineral N affect soybean isoflavonoid distribution, production and excretion.* In this work we found that daidzein and genistein distribution patterns varied with plant organs, ammonium nitrate levels, and plant development stages. We extended previous work on isoflavonoid synthesis to their exudation and found that mineral N inhibited daidzein and genistein contents and concentrations in soybean root and shoot extracts, but not in root daidzein and genistein exudates. In both synthesis and excretion, daidzein and genistein were not affected equally by mineral N treatments.
3. *Soybean cultivar variation exists for isoflavonoid synthesis and excretion under different mineral N levels.* We found that variation existed among soybean cultivars for genistein production. Soybean cultivars had different sensitivities to genistein addition and this interacted with mineral N level. These results provided a new possible criterion for selection of soybean cultivars based on signal production and excretion.
4. *Genistein partially overcomes inhibition of nod gene expression of B. japonicum.* *Nod* gene expression by *B. japonicum* cells was inhibited by mineral nitrogen. Addition of higher concentrations of genistein partially overcame this inhibitory effect. We studied mineral N inhibition from the bacterial side and provided a way

of improving inoculant efficiency with the use of signal molecules.

5 *PGPR, genistein and mineral N interact in terms of effects on soybean nodulation, N_2 fixation and yield.* Applying PGPR together with genistein preincubation of *B. japonicum* cells improved soybean nodulation and increased yield. The improvement varied among soybean cultivars and PGPR strains. We also found that PGPR strain could, to some extent, mitigate the negative effects of nitrate on soybean nodulation and nitrogen fixation, however, this is, again, influenced by soybean genotype.

6 *Genistein addition affects B. japonicum strain competitiveness.* Preincubation of *B. japonicum* cells with genistein improved strain competitiveness under greenhouse, but not under field conditions. This study potentially opened a new way to manipulate inoculants and increase their efficiency.

Setion 16

REFERENCES

Alexander M. 1985. Enhancing nitrogen fixation by use of pesticides: A review. Adv. Agron. 38:267-282

Almendras AS and PJ Bottomley. 1987. Influence of lime and phosphate on nodulation of soil-grown *Trifolium subterraneum* L. by indigenous *Rhizobium rifolii.* Appl. Environ. Microbiol. 53:2090-2097

Appelbaum E. 1990. The *Rhizobium/Bradyrhizobium*-legume symbiosis. In: *Molecular Biology of Symbiotic Nitrogen Fixation*. Ed. Gresshoff PM, CRC Press, Boca Raton, FL, p 131-158

Arshad M and Frankenberger Jr WT. 1998. Plant growth-regulating substances in the rhizosphere: microbial production and functions. Adv. Agron. 62:45-151

Atkins CA. 1994. Symbiotic nitrogen fixation. In *Mechanisms of plant growth and improved production: modern approaches*. Ed. Basra AS, Marcel Dekker Inc., New York, p 73-106

Athar M and Jhonson DA. 1996. Influcence of drought on competition between selected *Rhizobium meliloti* and naturalized soil rhizobia in alfalfa. Plant Soil 184:231-241

Athar M and Jhonson DA. 1997. Effect of drought on the growth and survival of *Rhizobium meliloti* strains from Pakistan and Nepal. J. Arid Environ. 35:335-340

Ayanaba A, Haugland RA, Sadowsky MJ, Upchurch RG, Weiland KD and Zablotowicz RM. 1986. Rapid colored-nodule assay for assessing root exudate-enhanced competitiveness of *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. 52:847-851

Baev N, Amar M, Defez R and Iaccarino M. 1992. The expression of the nodD and nodABC genes of *Rhizobium leguminosarum* is not a response to combined nitrogen. FEMS Microbiol. Lett. 97:205-208

Banfalvi Z, Nieuwkoop AJ, Schell MG, Besl L and Stacey G. 1988. Regulation of *nod* gene expression in *Bradyrhizobium japonicum*. Mol. Gen. Genet. 214:420-424

Barbour WM, Hatterman DR and Stacey G. 1991. Chemotaxis of *Bradyrhizobium japonicum* to soybean exudates. Appl. Environ. Microbiol. 57:2635-2639

Barea J M and Azcon-Aguilar C. 1983. Mycorrhiza and their significance in nodulating nitrogen-fixing plants. Adv. Agron. 36:1-54

Barnet YM. 1991. Ecology of legume root-nodule bacteria. In: *Biology and Biochemistry of Nitrogen Fixation*. Eds. Dilworth MJ and Glenn AR, Elsevier, Amsterdam. p 199-228

Barz W. 1970. Bacterial degradation of plant products. I. Isolation of rhizosphere bacterium capable of degrading flavonoids. Phytochem. 9:1745-1749

Barz W and Welle R. 1992. Biosynthesis and metabolism of isoflavones and pterocarpan phytoalexins in chickpea, soybean and phytopathogenic fungi. In: *Recent Advances in Phytochemistry 26: Phenolic Metabolism in Plants*. Eds. Stafford HA and Ibrahim RK, Plenum Press, New York, p 139-146

Bassam BJ, Djordjevic MA, Redmond JW, Batly M and Rolfe BG. 1988. Identification of a nodD-dependent locus in the *Rhizobium* strain NGR234 activated by phenolic factors secreted by soybeans and other legumes. Mol. Plant-Microbe Interact. 1:161-168

Beck DP and Munns DN. 1984. Phosphate nutrition of *Rhizobium* spp. App. Environ. Microbiol. 47:278-282

Bhagwat AA, Tully RE and Keister DL. 1991. Isolation and characterization of a competition-defective *Bradyrhizobium japonicum* mutant. Appl. Environ. Microbiol. 57:3496-3501

Bhuvaneswari TV, Goodman RN and Bauer WD. 1980. Early events in the infection of soybean [*Glycine max* (L.) Merr.] by *Rhizobium japonicum* 1. Location of infectible root cells. Plant Physiol. 66:1027-1031

Binns AN, Labriola J and Black RC. 1987. Initiation of auxin autonomy in *Nicotiana glutinosa* cells by the cytokinin-biosynthesis gene from *Agrobacterium tumefaciens*. Planta. 171:534-548

Bohlool BB and Schmidt EL. 1974. Lectins: A possible basis for specificity in the *Rhizobium*-legume root nodule symbiosis. Science 185:269-271

Bohlool BB, Ladha JK, Garrity DP and George T. 1992. Biological nitrogen fixation for sustainable agriculture: A perspective. Plant Soil 141:1-11

Brignoli E and Lauteri M. 1991. Effects of salinity on stomatal conductance, photosynthetic capacity, and carbon isotope discrimination of salt-tolerant (*Gossypium hirsuium* L.) and salt-sensitive (*Phaseolus valgaris* L.) C_3 non-halophytes. Plant Physiol. 95:638-645

Brockwell J and Bottomley PJ. 1995. Recent advances in inoculant technology and prospects for the future. Soil Biol. Biochem. 27:683-697

Brockwell J, Bergersen FJ, Gault RR, Herride DF, Mathews A, Morthorpe LJ and Roughley RJ. 1988. Population dynamics of soybean rhizobia and the implications for the N economy of rotational cropping systems. In *Proceedings of the Fifth Australian Soybean Research Workshop*, Tamworth, N.S.W. p 86-92

Broughton WJ, Samrey U, Pankhurst CE, Schneider GM and Vance CP. 1984. Ecology and microblology of symbiotic diazotrophs. In: Advances in Nitrogen Fixation Research. Eds. Veeger C and Newton WE, The Hague: Nijhoff, p 279-85

Broughton WJ, Heycke N, Priefer U, Schneider GM and Stanley J. 1987. Ecological genetics of *Rhizobium meliloti*: diversity and competitive dominance. FEMS Microbial. Lett. 40:245-249

Brunel B, Cleyet-Maral JC, Normand P and Bardin R. 1988. Stability of *Bradyrhizobium japonicum* inoculants after introduction into soil. Appl. Environ. Microbiol. 54:2636-2642

Burn J, Rossen L and Johnston AWB. 1987. Four classes of mutations in the nodD gene of *Rhizobium leguminosarum* biovar. *viciae* that affect its ability to autoregulate and/or activate other nod genes in the presence of flavonoid inducers. Genes Dev. 1:456-464

Caetano-Anollés G and Gresshoff PG. 1991. Plant-genetic control of nodulation. Annu. Rev. Microbiol 45:345-82

Calevert HE, Pence MK, Pierce M, Malik NS and Bauer WD. 1984. Anatomical analysis of the development and distribution of *Rhizobium* infections in soybean roots. Can. J. Bot. 62:2375-2384

Canada Department of Agriculture. 1991. The methods of testing legume inoculant and pre-inoculated seed products. Feed and Fertilizer Division. Food, Production and Inspection Branch, Ottawa, Ontario.

Cardenas L, Dominguez J, Quinto C, Lopez-Lara I, Lugtenberg B, Spaink H, Rademaker G, Harverkamp J and Thomaas-Oates J. 1995. Isolation, chemical structures and biological activity of the lipo-chitin oligosaccharide nodulation signals from *Rhizobium etli*. Plant Mol. Biol. 29:453-464

Carlson RW, Sanjuan J, Bhat R, Glushka J, Spaink HP, Wijes AHW, van Brussel ANA, Stokkermans TJW, Peters NK and Stacey G. 1993. The structures and biological activities of the lipo-oligosaccharide nodulation signals produce by type I and II strains of *Bradyrhizobium japonicum*. J. Biol. Chem. 268:18372-18381

Carrol BJ, Gresshoff PM and Delves AC. 1988. Inhereitance of supernodulation in soybean and estimation of the genetically effective cell number. Theor. Appl. Genet. 74:54-58

Carroll BJ and Mathews A. 1990. Nitrate inhibition of nodulation in legumes. In: *Molecular Biology of Symbiotic Nitrogen Fixation*, Ed. Gresshoff PM, CRC Press, Boca Raton, FL. p 158-180

Challdra R and Pareek RP. 1985. Role of host-genotype in effectiveness and competitiveness of chick pea (Clcer arientinum) *Rhizobium*. Trop. Agric. 62:90-94

Chanway CP, Hynes RK and Nelson LM. 1989. Plant growth-promoting rhizobacteia: effects on growth and nitrogen fixation of lentil (*Lens esculenta* Moench) and Pea (*Pisum sativum* L.). Soil Biol. Biochem. 21:511-517

Chanway CP, Radley RA and Holl FB. 1991. Inoculation of confier seed with plant growth promoting *Bacillus* strains cause increased seedling emergence and biomass. Soil Biol. Biochem. 23:575-580

Chen PC and Phillips DA. 1977. Induction of root nodule senescence by combined nitrogen in *Pisum sativum* L. Plant Physiol. 59:440-442

Cho, MJ and Harper JE. 1990. Effect of various N sources on isoflavonoid concentration in wild type and nodulation-mutant soybean roots. In *Nitrogen Fixation: Achievement and Objectives*. Eds. Gresshoff PM, Roth LE, Stacey G and Newton WE, New York: Chapman and Hall. p 255

Cho MJ and Harper JE. 1991A. Effect of inoculation and nitrogen on isoflavonoid concentration in wild-type and nodulation-mutant soybean roots. Plant Physiol. 95:435-442

Cho MJ and Harper JE. 1991b. Effect of localized nitrate application on isoflavonoids-concentration and nodulation in spilt-root systems of wild type and nodulation mutant soybean plants. Plant Physiol. 95:1106-1112

Cho MJ and JE Harper. 1993. Effect of abscisic acid application on root isoflavonoid concentration and nodulation of wild-type and nodulation-mutant soybean plants. Plant Soil. 152:145-149

Cooper JB and Long SR. 1994. Morphogenetic rescue of *Rhizobium meliloti* nodulation mutants by trans-zeatin secretion. Plant Cell 6:215-225

Cregan PB and Keyser HH. 1988. Influence of *Glycine* spp. on competitiveness of *Bradyrhizobium japonicum* and *Rhizobium fredii*. Appl. Environ. Microboil. 54:803-808

Cren M, Kondorosi A and Kondorosi E. 1995. NolR controls expression of the *Rhizobium melilloti* nodulation genes involved in the core Nod factor synthesis. Mol. Microbiol. 15:733-747

Cunningham S, Kollmeyer WD and Stacey G. 1991. Chemical control of interstrain competition for soybean nodulation by *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. 57:1886-1892

Curley RC and Burton JC. 1975. Compatibility of *Rhizobium japonicum* with chemical seed protectants. Agron J. 67:807-808

Dakora FD, Joseph CM and Phillips DA. 1993. Alfalfa (*Medicago sativa L.*) root exudates contain isoflavonoids in the presence of *Rhizobium meliloti*. Plant Physiol. 101:819-824

Dakora FD and Phillips DA. 1996. Diverse function of isoflavonoids in legumes transcend anti-microbial definitions of phytoalexins. Physiol. Mol. Plant Pathol. 49: 1-20

Dakora FD. 1995. Plant flavonoids: biological molecules for useful exploitation. Aust. J. Plant Physiol. 22:87-99

Darbyshire JF. 1966. Studies on the physiology of nodule formation. IX. The influence of combined nitrogen, glucose, light intensity and day length on root hair infection in clover. Ann. Bot. 30:623-638

D'Arcy Lameta A. 1986. Study of soybean and lentil root ecudates II. Identification of some polyphenolic compounds, relation with plantlet physiology. Plant Soil 92:113-123

D'Arcy Lameta A and Jay M. 1987. Study of soybean and lentil root exudates III. Influence of soybean isoflavonoids on growth of rhizobia and some rhizospheric microorganisms. Plant Soil 101:267-272

Dart PJ and Wildon DC. 1970. Nodulation and nitrogen fixation by *Vigna sinensis* and *Vicia atropurpurea*: the influence of concentration, form, and site of application of combined nitrogen. Aust. J. Agri. Res. 21:45-56

Dashti N, Zhang F, Hynes RK and Smith DL. 1997. Application of plant growth promoting rhizobacteria to soybean (*Glycine max* [L.] Merr.) increases protein and dry matter yield under short-season conditions. Plant Soil 188: 33-41

Dashti N, Zhang F, Hynes RK and Smith DL. 1998. Nodulation and seasonal nitrogen fixation of field grown soybean co-inoculated with *Bradyrhizobium japonicum* and plant growth promoting bacteria. Plant Soil 200:205-213

Dashti N. 1996. Plant growth promoting rhizobacteria and soybean nodulation, and nitrogen fixation under suboptimal root zone temperatures. Ph.D. thesis, McGill University, Montreal, Canada, p 120-178

Date RA. 1988. Nodulation difficulties related to low pH. In: *Current Perspectives in Nitrogen Fixation* Eds Gibson AH and Newton WE, Australian Academy of Sciences, Canberra, p 216-262

Date RA. 1991. Nodulation success and persistence of recommended inoculum strains for subtropical and tropical forage legumes in northern Australia. Soil Boil. Biochem. 23:533-541

De Freitas JR and Germida JJ. 1990. Plant growth promoting rhizobacteria for winter wheat. Can. J. Microbiol. 36:265-272

De Jong AJ, Heidstra R, Spaink HP, Hartog MV, MeijerEA, Hendriks T, Lo Schiavo F, Terzi M, Bisseling T, Van Kammen A and Devries SC. 1993. *Rhizobium* lipoolisaccharides rescue a carrot somatic embryo mutant. Plant Cell 5:615-620

Dehio C and de Bruijn FJ. 1993. The early nodulin gene *SrEnod2* from *Sesbania rostrata* is inducible by cytokinin. Plant J 2:117-128

Delgado MJ, Ligero F and Lluch C. 1994. Effects of salt stress on growth and nitrogen fixation by pea, faba-bean, common bean and soybean plants. Soil Biol. Biochem. 26:371-376

Demont-caulet N, Maillet F, Tailler D, Jacquinet JC, Promé JC, Nicolaou KC, Truchet G, Beau JM and Dénarié, J. 1999. Nodule-inducing activity of synthetic *Sinorhizobiun meliloti* nodulation factors and related lipo-chitoligosaccharides on alfalfa, Importance of the acyl chain structure. Plant Physiol. 120:83-92

Dénarié J and Cullimore J. 1993. Lipo-oligosaccharide nodulation factors: a minireview on new class of signalling molecules mediating recognition and morphogenesis. Cell 74:951-954

Dénarié, J, Debellé F and Rosenberg C. 1992. Signaling and host range variation in nodulation. Annu. Rev. Microbiol. 46:496-531

Dénarié, J, Debellé F and Promé JC. 1996. Rhizobium lipo-chitooligasaccharidic nodulation factors: signaling molecules mediating recognation and morphogenesis. Annu. Rev. Biochem. 65:503-535

Diatloff A and Brockwell J. 1976. Symbiotlc properties of *Rhizobium japonicum* and competitive success in nodulatlon of two *Glycine max* cultivars by effective and ineffective strains. Aust. J. Exp. Agric. Anim. Husb. 16:514-521

Diaz del C, Hunt S and Layzell DB. 1992. O_2 regulation and O_2-limitation of nitrogenase activity in root nodules of pea and lupin. Physiol. Plant. 86:269-278

Dixon RA and Lamb CJ. 1990. Molecular communication in interactions between plants and microbial pathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41: 339-367

Dixon ROD. 1969. Rhizobia. Annu. Rev. Microbiol. 23:137

DjordJevic M A, Sargen C L, Innes R W, Keumpel P L and Rolfe BG. 1985. Host range genes also affect strain competitiveness in *Rhizobium trifolii. In Nitrogen Fixation Research Progress.* Eds. Evans HJ, Bottomley PJ and Newton WE, Dordrecht, Holland: Nijhoff, p 117

Djordjevic MA, Redmond JW, Batley M and Rolfe BG. 1987. Clovers secrete specific phenolic compounds which either stimulate or repress nod gene expression in *Rhizobium trifolii*. EMBO J. 6:1173-1179

Dockendorff TC, Sanjuan J, Grob P and Stacey G. 1994. NolA represses *nod* gene expression in *Bradyrhizobium japonicum*. Mol. Plant-Microbe Interact. 7:596-602

Dooner HK. 1983. Coordinate genetic regulation of flavonoid biosynthetic enzymes in maize. Mol. Gen. Genet. 189:136-141

Dostanova RK, Llyshev LK and Toibaeva KA. 1979. Phenol compounds of pea roots with salting of the medium. Fiziol. Biokhim. Kult. Rast. 11:40-47 (Chm Abstr. 1979, 90:135118y)

Dowling DN, and Broughton WJ. 1985. Predominance of fast-growing *Rhizobium japonicum* in a soybean field in the People's Republic of China. Appl. Environ. Microbiol. 54:1171-1176

Duelli DM and Noel KD. 1997. Compounds exudated by *Phaseolus vulagaris* that induce a modification of *Rhizobium etli* lipopolysaccharide. Mol. Plant-Microbe Interact.

10:903-910

Dughri MH and Bottomley PJ. 1983. Effect of acidity and the composition of an indigenous soil population of *Rhizobium trifolii* found in nodules of *Tritolrum subterraneum*. Appl. Environ. Microbiol. 46:1207-1213

Duke SH, Schrader LE and Miller MG. 1977. Low temperature effects on soybean [*Glycine max* (L.) Merr. cv Wells.] mitochondrial respiration and several dehydrogenase during imbibition and germination. Plant Physiol. 60:716-722

Duke SH, Schrader LE, Henson CA, Servaites JC, Vogelzang RD and Pendleton JW. 1979. Low root temperature effects on soybean nitrogen metabolism and photosynthesis. Plant Physiol. 63:956-962

Duke SH, Schrader LE, Miller MG and Hiece RL. 1978. Low temperature effects on soybean [*Glycine max* (L.) Merr. cv Wells.] free amino acid pools during germination. Plant Physiol. 62:642-647

Dunn MF, Pueppke SG, and Krishnman HB. 1992. The *nod* gene inducer genistein alters the composition and molecular mass distribution of extracellular polysaccharides production by *Rhizobium fredii* USDA193. FEMS Microbiol. Lett. 97:107-112

Dusha I and Konodorosi A. 1993. Genes at different regulatory levels are required for the ammonia control of nodulation in *Rhizobium meliloti*. Mol. Gen. Genet. 240:435-444

Dusha I, Bakos A, Kondorosi A, De Bruijn FJ and Schell J. 1989. The *Rhizobium meliloti* early nodulation genes (*nodABC*) are nitrogen-regulated: Isolation of a mutant strain with efficient nodulation capacity on alfalfa in the presence of ammonium. Mol Gen Gene 219:89-96

Eardly BD, Hannaway DB and Bottomley PJ. 1984. Nitrogen nutrition and yield of seedling alfalfa as affected by ammonium nitrate fertilization. Agron. J. 77:57-62

Earl CD, Ronson CW and Ausubel FM. 1987. Genetic and structural analysis of the *Rhizobium meliloti fixA, fixB, fixc*, and *fixX* genes. J. Bacteriol. 169:1127-1136

Earl HJ and Tollenaar M. 1997. Maize leaf absorptance of photosynthetically active

radiation and its estimation using a chlorophyll meter. Crop Sci. 37:436-440

Earnshaw MJ. 1981. Arrhenius plots of root respiration in some arctic plants. Arct. Alp. Res. 13:425-430.

Eggleston G, Huber MC, Liang RT, Karr AL and Emerich DW. 1996. *Bradyrhizobium japonicum* mutants deficient in exo- and capsular polysaccharides cause delayed infection and nodule initiation. Mol. Plant-Microbe Interact. 9:419-423

Ehrhardt DW, Atkinson EM and Long SR. 1992. Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. Science 256:998-1000

Estabrook EM and Sengupta-Gopalan C. 1991. Differential expression of phenylalanine ammonia-lyase and chalcone synthase during soybean nodule development. Plant Cell 3:299-308

Evans IJ and Downie JA. 1986. The nodI gene product of *Rhizobium leguminosarum* is closely related to ATP-binding bacterial transport proteins; nucleotide sequence analysis of the *nodI* and *nodJ* genes. Gene 43:95-101

Evans LS, Lewin K F and Vella FA. 1980. Effect of nutrient medium pH on symbiotic nitrogen hxation by *Rhizobium leguminosarum* and *Pisum sativum*. Plant Soil 56:71-80

Faizah AW, Broughton WJ and John CK. 1980 Rhizobia in tropical legumes. Xl. Survival in the seed environment. Soil Biol. Biochem. 12:219-227

Fehr WR, Caviness CE, Burmood DT and Penning JS. 1971. Stages of development descriptions for soybean, *Glycine max* (L.) Merrill. Crop Sci. 11: 929-930

Firmin J L, Wilson KE, Rossen L and Johnston AWB. 1986. Flavonoid activation of nodulation genes in *Rhizobium* reversed by other compounds present in plants. Nature 324 90-92

Fisher HM. 1993. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. 58:352-386

Franco AA and Munns DN. 1982. Acidity and aluminum restraints on nodulation, nitrogen fixation and growth of *Phaseolus vulgaris* in solution culture. Soil Sci. Soc. Am.

J. 46:296-301

Fuhrmann JJ and Wollum AG. 1989. Symbiotic interaction between soybean and competing strains of *Bradyrhizobium japonicum*. Plant Soil 119: 139-145

Fuhrmann JJ, Davey CB and Wollum AG. 1986. Desiccation tolerance of clover rhizobia in sterile soils. Soil Biol. Biochem. 21:131-135

Fuhrmann JJ. 1993. Population diversity groupings of soybean bradyrhizobia. Adv. Agron. 50:67-103

Fujita K, T Masuda and Ogata S. 1991. Analysis of factors controlling dinitrogen fixation in wild and cultivated soybean (*Glycine max*) plants by reciprocal grafting. Soil Sci. Plant Nutr. 37:233-240

Fyson A and Sprent JI. 1982 The development of primary root nodule on *Vicia faba* L. grown at two root temperatures. Ann. Bot. 50:681-692

Gaskins MH, Albrecht SL and Hubbel DH. 1985. Rhizosphere bacteria and their use to increase productivity: A review. Agric. Ecol. Environ. 12:99-116

Geremia RA, Mergaert P, Geelen D, Van Montagu M and Holstars M. 1994. The NodC protein of *Azorhizobium caulinodaus* is an N-acetyly lucosaminyltransferase. Proc. Natl. Acad. Sci. USA 91:2669-2673

Gianinazzi-pearson V, Branzanti B and Gianinazzi S. 1989. *In vitro* enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungi by host root exudates and plant flavonoids. Symbiosis 7:243-255

Gibson AH and Harper JE. 1985. Nitrate effect on nodulation of soybean by *Bradyrhizobium japonicum*. Crop Sci. 25:497-501

Gibson AH. 1971. Factors in the physical and biological environment affecting nodulation and nitrogen fixation by legume. Plant Soil, Special Vol. 139-152

Glick BR. 1995. The enhancement of plant growth by free-living bacteria. Can. J. Microbiol. 41:109-117

Gottfert M, Holzhduser D and Hennecke H. 1992 Structural and functional analysis of two different *nodD* genes in *Bradyrhizobium japonicum* USDA110. Mol. Plant-Microbe Interact. 5:257-265

Gottfert M, Lamb JW, Gasser R, Semenza J and Hennecke H. 1989. Mutation analysis of the *Bradyrhizobium japonicum* common *nod* genes and further *nod* box linked genomic DNA regions. Mol Gen. Genet. 215:407-415

Govers F, Gloudemans T, Moerman M, Van Kammen A and Bisseling T. 1985. Expression of plant genes during the development of pea root nodules. EMBO J. 4:861-867

Govers F, Nap JP, van Kammen A and Bisseling T. 1987. Nodules in the developing root nodule. Plant Physiol. Biochem. 25:309-322

Graham D and Patterson BD. 1982. Responses of plants to low, nonfreezing temperatures: protein, metabolism, and acclimation. Annu. Rev. Plant Physiol. 33:347-372

Graham PH, Ocampo G, Ruiz LD and Duque A. 1980. Survival of *Rhizoblum phaseoli* in contact with chemical seed protectants. Agron. J. 72:625-27

Graham TL. 1991. Flavonoid and isoflavonoid distribution in developing soybean seedling tissues ans in seed and root exudates. Plant Physiol. 95:594-603

Graham TL and Graham MY. 1991. Glyceollin elicitors induce major but distinctly different shifts in isoflavonoid metabolism in proximal and distal soybean cell populations. Mol. Plant-Microbe Interact. 4:60-68

Graham TL, Kim JE and Grahan MY. 1990. Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with *Phytophthora megasperma*. Mol. Plant-Microbe Interact. 3:157-166

Graham-Weiss L, Bennett ML and Paau AS. 1987. Production of bacterial inoculants by direct fermentation on nutrient-supplemented vermiculite Appl. Environ. Microbiol. 53:2138-2140

Gresshoff PM. 1993. Molecular genetic analysis of nodulation genes in soybean. Plant

Breeding Rev. 11:275-318

Gresshoff PM, Krotzky A, Mathews A, Day D A, Schuller KA, Olssen J, Delves AC and Carroll BJ. 1988. Suppression of the symbiotic supernodulation symptoms of soybean. J. Plant Physiol. 132:417-423

Griffith GW and Roughley RJ. 1992. The effect of moisture potential on growth and survival of root nodule bacteria in peat culture and on seed. J Appl. Bacteriol. 73:1-13

Grimes HD and Mount MS. 1984. Influence of *Pseudomonas putida* on nodulation of *Phaseolus vulgaris*. Soil Biol. Biochem. 6:27-30

Gross DC and Vidaver AK. 1978. Bacteriocin like substances produced by *Rhizobium japonicum* and other slowgrowing rhizobia. Appl. Environ. Microbiol. 36:936-43

Ham GE. 1980. Inoculation of legumes with *Rhizobium* in competition with naturalized strains. In: *Nitrogen Fixation. Vol. 2: Symbiotic Associations and Cyanobacteria*. Eds. Newton WE and Orme-Johnson WH, University Park Press, Baltimore, Maryland, p 131-138

Hamel C, Neeser C, Barrantes-Cartin U and Smith DL. 1991. Endomycorrhizal fungal species mediate ^{15}N-transfer from soybean to corn in nonfumigated soil. Plant Soil 138:41-47

Hardarson G and Jones DG. 1979. Effect of temperature on competition amongst strains of *Rhizobium trifolii* for nodulation of two white clover varieties. Annu. Appl. Biol. 92:229-36

Hardarson G and Zapata F. 1984. Effect of plant genotype and nitrogen fertilizer on symbiotic nitrogen fixation by soybean cultivars. Plant Soil 82:397-405

Hardarson G, Golbs M and Danso SKA. 1989. Nitrogen fixation in soybean (*Glycine max* L. Merrill) as affected by nodulation patterns. Soil Biol. Biochem. 21:783-787

Harper JE. 1974. Soil and symbiotic nitrogen requirements for optimum soybean production. Crop Sci. 14:255-260

Hartel PG and Alexander M. 1984. Temperature and desiccation tolerance of cowpea

Vigna unguiculara rhizobia. Can. J. Microbiol. 30:820-823

Hartel, PG and Alexander M. 1983 Growth and survival of cowpea rhizobia in acid, aluminum-rich soils. Soil Sci. Soc. Am. J 47:502-506

Hartiwig UA, Joseph CM and Phillips DA. 1991. Flavonoids released naturally from alfalfa seeds enhance growth rate of *Rhizobium meliloti*. Plant Phisyiol. 95:797-803

Hartwig UA, Boller B and Nosberger J. 1987. Oxygen supply limits nitrogenase activity of clover nodules after defoliation. Ann. Bot. 59:285-291

Hegde SV and Brahmaprakash GP. 1992. A dry granuar inoculant of *Rhizobium* for soil application. Plant Soil 144:309-311

Heidstra R and Bisseling T. 1996. Nod factor-induced host responses and mechanisms of Nod factor perception. New Phytol. 133:25-43

Hely FW, Hutchings RJ and Zorin M. 1980. Methods of rhizobial inoculation and sowing techniques for *Trifolium subteraneum* L. establishment in a harsh winter environment. Aust. J. Agri. Res. 31:703-712

Herridge DF. 1984. Effects of nitrate and plant development on the abundance of nitrogenous solutes in root-bleeding and vacuum-extracted exudates in soybean. Crop Sci. 25:173-179

Hirsch AM, Bhuvaneswari TV, Torrey JG and Bisseling T. 1989. Early nodulin genes are included in alfalfa root outgrowths elicited by auxin transport inhibitors. Proc. Natl. Acad. Sci. USA 86:1244-1248

Hirsch AM and Fang Y. 1994. Plant hormones and nodulation: what's the connection? Plant Mol. Biol. 26:5-9

Hirsch AM. 1992. Developmental biology of legume nodulation. New Phytol. 122:211-237

Hoagland DR and Arnon DI. 1950. A water culture method for growing plants without soil. California Agricultural Experimental Station Circle 347:23-32

Hodgson ALM and Stacey G. 1986. Potential for *Rhizobium* improvement. CRC Crit. Rev. Biotechnol. 9:125-145

Horvath B, Kondorosi E, John M, Schmidt J, Torok I, Gyorgypal Z, Barabas I, Wieneke U, Schell J and Kondorosi A. 1986. Organization, structure and symbiotic function of *Rhizobium meliloti* nodulation genes determining host specificity for alfalfa. Cell. 46:335-343

Huang CY, Boyer JS and Vanderhoef LN. 1975. Limitation of acetylene reduction (nitrogen fixation) by photosynthesis in soybean having low water potentials. Plant Physiol. 56:228-232

Hume DJ and Shelp BJ. 1990. Superior performance of the Hup$^-$ *Bradyrhizobium japonicum* strain 532C in Ontario soybean soil field trials. Can. J Plant Sci. 70:661-666

Hungria M, Joseph CM and Phillips DA. 1991. *Rhizobium nod*-gene inducers exuded naturally from roots of common bean (*Phaseolus vulgaris* L.). Plant Physiol. 93:795-764

Hungria M and Phillips DA. 1993. Effects of a seed colour mutation on rhizobial nod-gene inducing flavonoids and nodulation in common bean. Mol. Plant-Microbe Interact. 6:418-422

Hungria M and Stacey G. 1997. Molecular signals exchanged between host plants and rhizobia: basic aspects and potential application in agriculture. Soil Biol. Biochem. 29:819-830

Hunt S, King BJ, Canvin DT and Layzell DB. 1987. Steady and nonsteady state gas exchange characteristics of soybean nodule in relation to the oxygen diffusion barrier. Plant Physiol. 84:164-172

Hunt S and Layzell DB. 1993. Gas exchange of legume nodules and the regulation of nitrogenase activity. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44:483-511

Hunt S, King BJ and Layzell DB. 1989. Effects of gradual increase in O_2 concentration on nodule activity in soybean. Plant Physiol. 91:315-321

Hunt PG, Matheny TA and Wollum AG. 1988. Yield and N accumulation responses of late-season determinate soybean to irrigation and inoculation with various strains of

Bradyrhizobiun japonicum. Communica. Soil Sci. Plant Analysis 19:1601-1612

Hylnowitz T and Newell CA. 1981. Taxonomy of the genus *Glycine* domestication and uses of soybean. Econ. Bot. 35:272-288

ICN. 1995. ICN Pharmaceuticals Inc., Costa Mesa, CA., USA. 308

Imsande J. 1989. Rapid dinitrogen fixation during soybean pod fill enhances net photosynthetic output and seed yield: A new perspective. Agron. J. 81:549-556

Imsande J. 1998. Nitrogen deficit during soybean pod fill and increased plant biomass by vigorous N_2 fixation. Euro. J. Agron. 8:1-11

Iruthayathas EE, Gunasekaran, S and Vlassak K. 1983. Effect of combined inoculation of *Azospirillum* and *Rhizobium* on nodulation and nitrogen fixation of winged bean and soybean. Sci. Hort. 20:231-240

IS-MPMI meeting report. 1997. Mol. Microbe-Plant Interact 10:6-12

Jacobs M and Rubery PH. 1988. Naturally occurring auxin transport regulators. Science 241:346-349

Jain V, Garg N and Nainawatee HS. 1990. Naringenin enhanced efficiency of *Rhizobium meliloti*-alfalfa symbiosis. World J. Microbiol. 6:434-436

John M, Röhrig H, Schmidt J, Wieneke U and Schell J. 1993. *Rhizobium* NodB protein involved in nodulation signal synthesis is a chitooligosaccharide deacetylase. Proc. Natl. Acad. Sci. USA 90:625-629

Johntson JW, Welch LF and Kurtz LT. 1975. Environmental implications of N fixation by soybean. J. Environ. Qual. 4:303-306

Johntson RR. 1987 Crop management. In *Soybeans Improvement, Production, and Uses*, Ed. Wilcox JR, ASA-CSSA-SSSA, Madison, Wisconsin. p 355-390

Jones DG and Hardarson G. 1979. Variation within and between white clovers in their preference for strains of *Rhizobium trifolii*. Soil Biol. Biochem. 1:57-61

Jones FR and Tisdale WB. 1921. Effect of soil temperature upon the development nodules on the root certain legumes. J. Agric. Res. 22:17-37

Kape R, Pamiske M, Braaandt S and Wemer D. 1992. Isoliquiritigenin, a strong *nod* gene and gyceollin resistance-inducing flavonoid from soybean root exudate. Appl. Environ. Microbiol. 58:1705-1710

Kaplan, DT, Keen NT and Thoason IJ. 1980. Association of glyceollin with the incompatible response of soybean roots to *Meloidogyne incognita*. Physiol. Plant Pathol. 16:319-327

Kapulnik Y, Joseph CM and Phillips DA. 1987. Flavone limitations to root nodulation and symbiotic nitrogen fixation in alfalfa. Plant Physiol. 84:1193-1196

Keyser HH and Li F. 1992. Potential for increasing biological nitrogen fixation in soybean. Plant Soil 141:119-135

Kloepper JW, Schroth MN and Miller TD. 1980. Effects of rhizosphere colonization by plant growth promoting rhizobacteria on potato plant growth and yield. Phytopathol. 70:1078-1082

Kloepper, JW, Lifshitz, R and Zablotowicz RM. 1989. Free-living bacterial inocula for enhancing crop productivity. Trends in Biotechnol. 7:39-43

Kloepper, JW, Scher, FM, Laliberté, M and Tipping B. 1986. Emergence-promoting rhizobacteria: description and implications for agriculture. *In Iron, Siderophores, and Plant Diseases.* Ed. Swinburne TR. Plenum Press, New York, p 155-164

Knight CD, Rossen L, Robertson JG, Wells B and Downie JA. 1986. Nodulation inhibition by *Rhizobium leguminosarum* multicopy *nodABC* genes and analysis of early stages of plant infection. J Bacteriol. 166:552-558

Kondorosi E, Pierre M and Michel C. 1992. Identification of No1R, a negative transacting factor controlling the *nod* regulation in *Rhizobium meliloti*. J. Mol. Biol. 222:885-896

Kosslak RM and Bohlool BB. 1984. Suppression of nodule development of one side of a

root system of soybeans caused by prior inoculation of the other side. Plant Physiol. 75:125-130

Kosslak RM, Bohlool BB, Dowdle S and Sadowski MJ. 1983. Competition of *Rhizobium japonicum* strains in early stages of soybean nodulation. Appl. Environ. Microbiol. 46:870-873

Kosslak RM, Bookland R, Barkei J, Paaren H and Appelbaum ER. 1987. Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*. Proc. Natl. Acad. Sci. USA 84:7428-7432

Kosslak RM, Joshi RS, Bowen BA, Paaren HE and Appelbaum ER. 1990. Strain-specific inhibition of *nod* gene induction in *Bradyrhizobium japonicum* by flavonoid compounds. Appl. Environ. Microbiol. 56:1333-1341

Kouchi H and Hala S. 1993. Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. Mol. Gen. Genet. 238:106-119

Krishnan HB and Puppke SG. 1993. Flavonoid inducers of nodulation genes stimulate *Rhizobium fredii* USDA257 to export proteins into the environment. Mol. Plant-Microbe Interact. 6:107-113

Kucey RMN and Paul EA. 1982. Carbon flow, photosynthesis, and N_2 fixation in mycorrhizal and nodulated faba beans (*Vicia faba* L.) Soil Biol. Biochem. 14:407-412

Kumarasinghe RMK and Nutman PS. 1979. The influence of temperature on root hair infection of *Trifolium parviflorum* and *T. glomeratum* by root nodule bacteria. I. The effects of constant root temperature on infection and related aspects of nodule development. J. Exp. Bot. 30:503-515

Kuykendall LD, Weber DF. 1978. Genetically marked *Rhizobium* identifiable as inoculum strain in nodules of soybean plants grown in fields populated with *Rhizobium japonicum*. Appl. Environ. Microbiol. 36:915-919

Latimore M Jr, Giddens J and Ashley DA. 1977. Effect of ammonium and nitrate upon photosynthate supply and nitrogen fixation by soybeans. Crop Sci. 11:399-404

Lawson C, Rolfe B, and Djordjevic M. 1995. *Rhizobium* inoculation induces condition-dependent changes in the flavonoid composition of root exudates in *Trifolium subterraneum*. Aust. J. Plant Physiol. 23:93-101

Lawson CGR, Carroll BJ and Gresshoff PM. 1988. Alleviation of nitrate inhibition of soybean nodulaton by high inoculum does not involve bacterial nitrate metabolism. Plant Soil 110:123-127

Layzell DB, Rochmann P and Canvin DT. 1984. Low temperatures and nitrogenase activity in soybean. Can. J. Bot. 62:965-971

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Prome JC and Denarie J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamineoligosaccharide signal. Nature 344:781-784

Lersten NR and Carlson JB. 1987. Vegetative morphology. In: *Soybean. Improvement, Production, and Uses*. Ed. Wilcox JR, 2nd Edition, ASA-CSSA-SSSA, Madison, Wisconsin. p 49-94

Li DM and Alexander A. 1988. Co-inoculation with antibiotic-producing bacteria to increase colonization and nodulation by rhizobia. Plant Soil 108:211-219

Li, DM and Alexander GE. 1979. Co-inoculation with antibiotic-producing bacteria to increase colonization and nodulation by rhizobia. Plant Soil 108:211-219

Lie TA. 1974. Environmental effects on nodulation and symbiotic nitrogen fixation, In *The Biology of Nitrogen Fixation*. Ed. Quispel A, North-Holland, Amsterdam, p 555-582

Lie TA. 1981. Environmental physiology of the legume-*Rhizobium* symbiosis. In: *Nitrogen Fixation Volume 1. Ecology.* Ed. Broughton WJ, Clarendon Press, Oxford. p 104-134

Ligero F, Caba JM, Lluch C and Olivares J. 1991. Nitrate inhibition of nodulation can be overcome by the ethylene inhibitor amonoethoxyvinylglycine. Plant Physol. 97:1221-1225

Lindemann WC and GE Ham. 1979. Soybean plant growth, nodulation, and nitrogen

fixation as affected by root temperature. Soil Sci. Soc. Am. J. 43:1134-1137

Long SR. 1989. *Rhizobium*-legume nodulation: Life together in the underground. Cell 56:203-214

Long SR. 1996. *Rhizobium* symbiosis: Nod factors in perspective. Plant Cell 8:1885-1898

Long SR and Cooper J. 1988. Overview of symbiosis. In *Molecular Genetics of Plant-Microbe Interactions*. Eds. Palacios R and Verma DPS, St. Paul, Minnesota: APS Press. p 163-187

Lynch DH and Smith DL. 1993. Soybean [*Glycine max* (L.) Merr.] nodulation and N_2 fixation as affected by period of exposure to a low root zone temperature. Physiol. Plant. 88:212-220

Lynch DH and Smith DL. 1993b. Early seedling and seasonal N_2-fixing symbiotic activity of two soybean [*Glycine max* (L.) Merr.] cultivars inoculated with *Bradyrhizobium* strains of diverse origin. Plant Soil 157:289-303

Lynch DH and Smith DL. 1994. The effects of low temperature stress on two soybean (*Glycine max*) genotypes when combined with *Bradyrhizobium* strains of various genotypic origin. Physiol. Plantarum. 90:105-113

Lynch, JM. 1982. The Rhizosphere. In: *Experimental Microbial Ecology*. Eds. Burns RG and Slater JH, Blackwell Scitific Publicatios, Oxford, UK p 395-411

Lynn DG and Chang M. 1986. Phenolic signals in cohabitation: implications for plant development. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41:497-

Malik NSA, Calvert HE and Bauer WD. 1987. Nitrate induced regulation of nodule formation in soybean. Plant Physiol. 84:266-271

Materon LA and Vincent JW. 1980. Host specificity and interstrain competition with soybean rhizobia. Field Crops Res. 3:215-24

Mathews A, Kosslak RM, Sengupta-Gopala C, Appelbaum ER, Carroll BJ and Gresshoff PM. 1989. Biological characterization of root exudates and extracts from

nonodulating and supernodulating soybean mutants. Mol. Plant-Microbe Interact. 2:283-290

Matthews DJ and Hayes P. 1982. Effect of root zone temperature on early growth, nodulation and nitrogen fixation in soybeans. J. Agric. Sci. Camb. 98:371-376

Maury P, Sue S, Berger M. and Planchon C. 1993. Response of photochemical process of photosynthesis to dinitrogen fixation in soybean. Plant Physiol. 101:493-497

Maxwell CA and Phillips DA. 1990. Concurrent synthesis and release of nod-gene-inducing flavonoids from alfalfa roots. Plant Physiol. 93:1552-1558

McDermott TR and Graham PH. 1990. Competitive ability and efficiency in nodule formation of strains of *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. 56:3035-3039

McDermott TR and Graham PH. 1989. *Bradyrhizobium japonicum* inoculant mobility, nodule occupancy, and acetylene reduction in the soybean root system. Appl. Environ. Microbiol. 55:2493-2498

Mckay IA and Djordjevic MA. 1993. Production and excretion of Nod metabolites by *Rhizobium leguminosarum* bv. *trifolii are* disrupted by the same environmental factors that reduce nodulation in the field. Appl. Environ. Microbiol. 59:3385-3392

McLoughlin TJ, Bordeleau LM and Dunican LK. 1984. Competition studies with *Rhizobium trifolii in* a field experiment. J. Appl. Bacleriol. 56:131-35

McNeil DL. 1982. Variations in ability of *Rhizobium japonicum* strains to nodulate soybeans and maintain fixation in the presence of nitrate. Appl. Environ. Microbiol. 44:647-652

Mellor RB and Collinge DB. 1995. A simple model based on known plant defence reactions is sufficient to explain most aspects of nodulation. J. Exp. Bot. 46:1-18

Miller JC, Scott JS, Zary KW and O'Hair SK. 1982. The influence of available nitrate levels on nitrogen fixation in three cultivars of cowpea. Agron. J. 74:14-18

Miller, JH. 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, NY

Millor RB and Werner D. 1990. Legume nodule biochemistry and function. In: *Molecular Biology of Symbiotic Nitrogen Fixation*, Ed. Gressorff PM, CRC Press, Boca Raton, FL. p 111-130

Minami E, Kouchi H, Carlson RW, Cohn JR, Kolli VK, Day RB, Ogawa T and Stacey G. 1996. Cooperative action of lipo-chitin nodulation signals in the induction of the early nodulin, *ENOD2*, in soybean roots. Mol Plant-Microbe Interact. 9:574-583

Moll W. 1968. The diffusion coefficient of myoglobin in muscle homogenates. Arch. Gesamte Physiol. 299:247-251

Mullen MD, Israel DW and Wollum AG. 1988. Effects of *Bradyrhizobium japonicum* and soybean [*Glycine max* (L.) Merr.] phosphorus nutrition on nodulation and dinitrogen fixation . Appl. Environ. Microbiol. 54:2387-2392

Musser RL, Thomas SA and Kramer PJ. 1983. Short and long term effects of root and shoot chilling of Ransom soybean. Plant physiol. 73:778-783

Newcomb W, Sippell D and Peterson RL. 1979. The early morphogenesis of *Glycine max* and *Pisum sativum* root nodule. Can. J. Bot. 57:2603-2616

Noel D. 1992. Rhizobial polysaccharides required in synbiosis with legumes. In *Molecular Signals in Plant-Microbe Communication*. Ed. Verma DPS. CRC Press, Inc., Boca Raton, Fla. p 341-358

Nutman PS. 1956. The influence of the legume in root-nodule symbiosis. Biol. Rev. 31:109-151

Osa-Afiana LO and Alexander M. 1982. Differences among cowpea rhizobia in tolerance to high temperature-and desiccation in soil. Appl. Environ. Microbiol. 43:435-439

Ozawa T and Yamuguchi M. 1986. Fractionation and estimation of particle-attached and unattached *Bradyrhizobium japonicum* strains in soils. Appl. Environ. Microbiol.

52:911-914

Paau AS, Bennett ML, Kurtenbach CJ, Graham LL. 1990. Improvement of inoculant efficiency by strain improvement and formulation manipulation. In: *Nitrogen Fixation: Achievements and Objectives.* Eds. Gresshoff PM, Roth LE, Stacey G and Newton WE , Chapman and Hall, New York London, p 617-624

Pan B, Zhang F, Smith DL. 1998. Genistein addition to the soybean rooting medium increases nodulation. J. Plant Nutri. 21:1631-1639

Pan B. and Smith DL. 1998. Genistein and daidzein concentrations and contents in seedling roots of three soybean cultivars grown under three root zone temperatures. J. Agron. Crop Sci. 200:77-82

Pankhurst CE and Sprent JI. 1976. Effects of temperature and oxygen tension on the nitrogenase and respiratory activities of turgid and water-stressed soybean and French bean root nodules. J. Exp. Bot. 27:1-9

Pankhurst CE and Biggs DR. 1980. Sensitivity of *Rhizobium* to selected isoflavonoids. Can. J. Bot. 26:542-545

Parker AK and Dangerfield JA. 1975. Influence of bacterial inoculations on growth of containerized Douglas-fir seedlings. Can. Forest Service Bi-Monthly Res. Notes. 31:14-15

Parniske M, Ahlborn B and Werner D. 1991. Isoflavonoid-inducible resistance to the phytoalexin glyceollin in soybean rhizobia J. Bacteriol. 173:3432-3439

Pate JS, Atkins CA. 1983. Nitrogen uptake, transport and utilization. In: *Nitrogen Fixation Vol. 3 Legumes*, Ed. Broughton WJ, Oxford University Press, Oxford, P 245-298

Peoples MB and Craswell ET. 1992 Biological nitrogen fixation: Investments, expectations and actual contributions to agriculture. Plant Soil 141:13-39

Peters NK, Frost JW and Long SR. 1986. A plant flavone, luteolin, induces expression of *Rhizobium meliloti* nodulation genes. Science 233:977-980

Perters NK and Long SR. 1988. Alfalfa root exudates and compounds which promote or

inhibit induction of *Rhizobium meliloti* nodulation genes. Plant Physiology. 88:396-400

Perters NK and Verma DPS. 1990. Phenolic compounds as regulators of gene expression in plant-microbe interactions. Mol. Plant-Microbe Interact. 3:4-8

Phillips DA. 1992. Flavonoids: plant signals to soil microbes. In Phenolic Metabolism in Plants. Eds. Stafford HA and Ibrahim RK, Plenum Press, New York. Recent Adv. Photochem. 26:201-231

Polonenko DR, Scher FM, Kloepper JW, Singleton CA, Laliberte M and Zaleska I. 1987. Effects of root colonizing bacteria on nodulation of soybean roots by *Bradyrhizobium japonicum.* Can. J. Microbiol. 33:498-503

Prinsen E, Chauvaux N, Schmidt J, John M, Wieneke N, De Greef J, Schell J and Van Onckelen. 1991. Stimulation of indole-3-aceatic acid production in *Rhizobium* by flavonoids. FEBS Lett. 282:53-55

Promé JC and Demont N. 1996 Nodulation factors. In *Plant-Microbe Interactions,* Eds Stacey G and Keen NT, Vol. 1, Chapman and Hall, New York, p 272-307

Pueppke SG, Bolanos-Vasquez MC, Werner D, Bec-Ferte MP, Prome JC and Krishnan HB. 1998. Release of isoflavonoids by soybean cultivars McCall and Peking and their perception as signals by the nitrogen-fixing symbiont *Sinorhizobium fredii.* Plant Physiol. 117:599-608

Purcell LC, Vadez V, Sinclair TR, Serraj R and Nelson R. 1998. Screen of soybean germplasm for N2 fixation drought tolerance. 16th North American Symbiotic nitrogen Fixation Conference. Cancun, Mexico

Quispel A. 1974. *The Biology of Nitrogen Fixation.* North-Holland Pub. Co. Amersterdam.

Ralston EJ and Imsande J. 1983. Nodulation of hydroponically grown soybean plants and inhibition of nodule development by ammonium nitrate. J. Exp. Bot. 34:1371-1378

Rao AS. 1990. Root flavonoids. The Botanical Review 56:1-84

Rao RV and Keister DL. 1978. Infection threads in root-hairs of soybean (*Glycine max*) plants inoculated with *Rhizobium japonicum*. Protoplasma 97:311-316

Raper CD and Kramer PJ. 1987. Stress physiology. In: *Soybean: Improvement, Production, and Uses*, Ed.Wilcox JR, 2nd Edition, ASA-CSSASSSA, Madison, Wisconsin. p 589-642

Recourt, K Schripsema J, Kijne JW van Brussel AAN and Lugtenberg BJJ. 1991. Inoculation of *Vicia sativa* subsp. *nigra* roots with *Rhizobium leguminosarum* biovar *viciae* results in release of *nod* gene activating flavonones and chalcones. Plant Mol. Biol. 16:841-852

Rice WA, Penney DC and Nyborg M. 1977. Effects of soil acidity on rhizobia numbers, nodulation and fixation by alfalfa and red clover. Can. J. Soil Sci. 57:197-203

Richardson AE, Djordjevic MA, Simpson and Rolfe BG. 1988a The effects of pH, Ca and Al on the exudate from clover seedlings of compounds that induce the expression of nodulation genes in *Rhizobium trifolii*. Plant Soil 109:37-47

Richardson AE, Djordjevic MA, Simpson and Rolfe BG. 1988b. Expression and induction of nodulation genes in *Rhizobium trifolii* at low pH and in the presence of Ca and Al. Appl. Environ. Microbiol. 54:2541-2548

Rigaud J and Puppo A. 1977. Effect of nitrate upon leghemoglobin and interaction with nitrogen fixation. Biochem. Biophys. Acta 497:702-706

Robert JM and Triplett EW. 1996. Toward more productive, efficient, and competitive nitrogen-fixing symbiotic bacteria. CRC Cri. Rev. Plant Sci. 15:191-234

Roberts GP and Brill WJ. 1981. Genetics and regulation of nitrogen fixation. Annu. Rev. Microbiol. 35:207-235

Robson AD and Bottomley PJ. 1991. Limitations in the use of legumes in agriculture and forestry. In: *Biology and Biochemistry of Nitrogen Fixation*. Eds. Dilworth MJ and Glenn AR. Elsevier Publisher, Amsterdam p 320-349

Rohrig HT, Schmidt U, Wieneke E, Kondorosi I, Barlier J and Schel MJ. 1994.

Biosynthesis of lipooligosaccharides nodulating factors:*Rhizobium* NodA protein is involved in N-acylation of the chitooligosaccharide backbone. Proc. Natl. Acad. Sci. USA 91:3122-3126

Rolfe B and Gresshoff PM. 1988. Genetic analysis of legume nodule initiation. Annu. Rev. Plant Physiol. 39:297-319

Rolfe BG, Redmond JW, Bateley M, Djordjevic MAL. 1987. European Patent Application No. 245931

Rolfe BG. 1988. Flavones and isoflavones as inducing substances of legume nodulation. Biofactors 1:3-10

Rossen L, Shearman CA, Jphnston, AWB and Dowinie JA. 1985. The *nod*D gene of *Rhizobium leguminosarum* is autoregulatory and in the presence of plant exudate induces the *nodA,B,C* genes. EMBO J. 4:3369-3373

Rossiter RC. 1969. Physical and ecological study on the oestrogenic isoflavonoids in subterranean clover (*T. subterraneaneum L.)* VII Effects of nitrogen supply. Aust. J. Agric. Res. 20:11043-11051

Roughley RJ, Nutman PS and Chandler MR. 1981. Effects of host plant selection and temperature on the structure of root nodule of red clover *(Trifolum pratense L.)*. Plant Soil. 6:112-124

Roughley RJ. 1970. The influence of root temperature, *Rhizobium* strain and host selection on the structure and nitrogen-fixing efficency of the root nodules of *Trifolium subterraneum*. Annu. Bot. 34:631-646

Roughley RJ and Date RA. 1986. The effect of strain of *Rhizobium* and of temperature on nodulation and early growth of *Trifolium semipilosum*. Exp. Agric. 22:123-131

Ryan CA and Faemer EE. 1991. Oligosaccharide signals in plants: a current assessment. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:651-674

Ryo GJ and Phung T. 1984. Effect of nitrogen form and counterion on establishment of the *Rhizobium trifolii-Trifolium repens* symbiosis. J. Experi. Bot. 35:2011-2019

Sadowsky MJ, Cregan PB, Golrt M, Sharma A, Gerhold D, Rodriguez-Quinones F, Keyser HH, Hennecke H and Stacey G. 1991. The *Bradyrhizobium japonicum nodM* gene and its involvement in the genotype-specific nodulation of soybeans. Proc. Natl. Acad. Sci., USA 88:637-641

Salomonsson AC, Theander O and Aman, P. 1978. Quantitive determination by GLC of phenolic acids as ethyl derivatives in cereal straws. J Agric. Food Chem. 26:830-835

Sanjuan J, Carlson RV, Spaink HP, Bhat UR, Barbiour WM, Glushka J and Stacey G. 1992. A 2-methylfucose moiety is present in the lipo-oligosaccharide nodulation signal of *Bradyrhizobium japonicum.* Proc. Natl. Acad. Sci. USA, 89:8789-8793

Sanjuan J, Grob P, Gottfert M, Hennecke H and Stacey G. 1994. NodW is essential for full expression of the common nodulation genes in *Bradyrhizobium japonicum.* Mol. Plant-Microbe Interact. 7:364-369

SAS Institute Inc. 1988. *SAS User's Guide.* Statistical analysis Institute Inc., Cary, NC

Savoure A, Magyar Z, Pierre MB, Schultze M, Dudits D, Kondorosi A, Kondorosi E. 1994. Activation of the cell cycle machinery and the isoflavonoid biosynthesis pathway by active *Rhizobium meliloti* Nod signal molecules in *Medicago microcallus* suspensions. EMBO J. 13:1093-1102

Schippers B, Scheffer RJ, Lugtenberg BJJ and Weisbeek PJ. 1995. Biocoating of seed with plant growth promoting rhizobacteria to improve plant establishment. Outlook on Agric. 24:179-18

Schmidt EL and Robert FM. 1985. Recent advances in the ecology of *Rhizobium.* In: *Nitrogen Fixation Research Progress.* Eds. Evans HJ, Bottomely PJ and Newton WE, Dordrecht, Holland:Nijhoff, p 379-385

Schmidt PE, Broughton WJ and Werner D. 1994. Nod factors of *Bradyrhizobium japonicum* and *Rhizobium* sp. NGR34 induce flavonoid accumulation in soybean root exudate. Mol. Plant-Microbe Interact. 7:384-390

Schmidt PE, Parniske M and Wemer D. 1992. Production of the phytoalexin glyceollin I by soybean roots in response to symbiotic and pathogenic infection. Bot. Acta. 105:18-25

Schuller KA, Day DA, Gibson AH and Gresshoff PM. 1986. Enzymes of ammonia assimilation and ureide biosynthesis in soybean nodules: effect of nitrate. Plant Physiol. 80:646-650

Schultze M and Kondorosi A. 1995. What makes nodulation signals host-plant specific? Trends in Microbiol. 3:370-372

Serraj R, Sinclair TR and Purcell LC. 1999. Symbiotic N2 fixation response to drought. J. Exp. Bot. 50:143-155

Sharma SB and Signer ER. 1990.Temporal and spatial regulation of the symboitic genes of *Rhizobium meliloti* in planta revealed by transposon Tn5-gusA. Genes and Development 4:344-356

Sinclaire TR and Serraj R. 1995. Dinitrogen fixation sensitivity to drought among grain legume species. Nature 378:344

Siqueira JO, Nair MG, Hammerschmidt R and Safir GR. 1991. Significance of phenolic compounds in plant-soil-microbial systems. CRC Cri. Rev. Plant Sci. 10:63-121

Smil V. 1997. Global population and the nitrogen cycle. Scientific American, July, 76-81

Smit G, Puvanesarajah V, Carlson RW, Barbour WM and Stacey G. 1992. *Bradyrhizobium japonicum nodD*$_1$ can be specifically induced by soybean flavonoids that do not induce the *nodYABCSUIJ* operon. J. Biol. Chem. 267:310-318

Smith DL and Hume DJ. 1987. Comparison of assay methods for nitrogen fixation utilizing white bean and soybean. Can. J. Plant Sci. 67:11-19

Smith RS, Ellis MA and Smith RE. 1981. Effect of *Bradyrhizobium japonicum* inoculant on soybean nodulation in tropical soil. Agron. J. 73:505-508

Spaink HP. 1996. Regulation of plant morphogenesis by lipo-chitin oligosaccharides. CRC Cri. Rev. Plant Sci. 15:559-582

Spaink HP, Aarts A, Stacey G, Blomberg GV, Lugtenberg, and Kennedy EP. 1992. Detection and separation of *Rhizobium* and *Bradyrhizobium* Nod metablites using thin

layer chromatograpgy. Mol. Plant-Microbe Interact. 5:72-80

Spaink HP, Okker RJH, Wijffelman CA, Tak T, Goosen-De Roo L, Pees E, Van Brussel AAN and Lugtenberg BJJ. 1989. Symbiotic properties of rhizobia containing a flavonoid-independent hybrid *nodD* product. J. Bacteriol. 171:4045-4053

Spaink HP, Wijffelman CA, Pees E, Okker RJH and Lugtenberg BJJ. 1987. *Rhizobium phaseoli* symbiotic plasmid. J Bacteriol. 167:487-491

Spaink HP. 1996. Regulation of plant morphogenesis by lipo-chitin oligosaccharides. Critical Rev. Plant Sci. 15:559-582

Sprent JI and Sprent P. 1990. *Nitrogen Fixing Organisms.* Chapman and Hall, New York. P 249

Sprent JT. 1982. Nitrogen fixation by grain legume in U.K. Phil. Trans. Royal Soc. London B. 296:387-395

Stacey G, Gresshoff PM and Keen NY. 1992. Friends and foes: new insights into plant microbe. In: *Nitrogen Fixation: Achievements and Objectives*. Eds. Stacey G and Newton WE, Chapman and Hall, New York London, p 617-624

Stacey G. 1995. *Bradyrhizobium japonicum* nodulation genetics. FEMS Microbiol. Lett. 127:1-9

Stacey G, Sanjuan J, Luka S, Dockendorff T and Carlson RW. 1995. Signal exchange in the *Bradyrhizobium*-soybean symbiosis. Soil Biol. Biochem. **27**:473-483

Stacey G, Cohn J, Day B, Loh J, Garcia M, Paik J, Yuen J. 1997. The complex interaction between *Bradyrhizobium japonicum* and its symbiotic host plants. In *Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture.* Eds. Legocki A, Bothe H and Pühler A, Springer-Verlag, Berlin, Germany. p 29-32

Staehelin C, Granado J, Muller J, Wiemken A, Mellor RB, Felix G, Regenass M, Broughton WJ and Boller T. 1994. Perception of *Rhizobium* nodulation factors by tomato cells and inactivation by root chitinases. Proc. Natl. Acad. Sci. USA 91:2196-2200

Staehelin C, Schultze M, Kondorosi E and Kondorosi A. 1995. Lipo-chitooligasaccharide nodulation signals from *Rhizobium meliloti* induce their rapid degradation by the host plant alfalfa. Plant Physiol. 108:1607-1614

Stafford HA. 1992. *Flavonoid Metabolism*. Boca Raton, CRC Press, 202-207

Steel RGD and Torrie JH. 1980. *Principles and Procedures of Statistics: A Biometrical Approach*. Second edition, McGraw-Hill Book Company.

Stevens DE. 1982. The effect of temperature on facilitated oxygen diffusion and its relation to warm tuna muscle. Can. J. Zool. 60:1148-1152

Stokkermans TJW and Peters NK. 1994. *Bradyrhizobium elkanii* lipo-oligosaccharide signals induce complete nodule structures on *Glycine soja* Siebold et Zucc. Planta 193: 413-420

Stokkermans TJW, Ikeskita S, Cohm J, Carlson RW, Stacey G, Ogawa T and Peters NK. 1995. Structural requirements of synthetic and natural product lipo-chitin oligosaccharides for induction of nodule primordia on *Glycine soja*. Plant Physiol. 108:1587-1595

Streeter GJ. 1988. Inhibition of legume nodule formation and N_2 fixation by nitrate. CRC Cri. Rev. Plant Sci. 7:1-23

Streeter GJ. 1994. Failure of inoculant rhizobia to overcome the dominance of indigenous strain for nodule formation. Can. J. Microbiol. 40:513-522

Subba Rao NS. 1984. Interaction of nitrogen-fixing microorganisms with other soil microorganisms. In: *Current Developments in Biological Nitrogen Fixation*, Ed. Subba Rao NS, New Delhi: Oxford Univ. p 3764

Suslow TV. 1982. Role of root-colonizing bacteria in plant growth. In *Phytopathogenic Procaryotes*. Eds. Mount MS and Lacy GH, Academic Press, New York, p 187-233

Sutherland TD, Bassam BJ, Schuller LJ and Gresshoff PM. 1990. Early nodulation signals of the wild type and symbiotic mutants of soybean [*Glycine max)*. M]. Mol. Plant-Microbe Interact. 3:122-128

Sutton JM, Lea EJA and Dowine JA. 1994. The nodulation signaling protein NodD from *Rhizobium leguminosarum* Biovar *viciae* forms ion channels in membrances. Proc. Natl. Acad. Sci. USA. 91:9990-9994

Sutton JC and Peng G. 1993. Manipulation and vectoring of biocontrol organisms to manage foliage and fruit diseases in cropping systems. Annu. Rev. Phytopathol. 31: 473-493

Taller BJ and Stutevant DB. 1991. Advances in molecular genetics of plant-microbe interactions. In: *Cytokinin Production by Rhizobia.* ed. By Henneck H and Verma DPS, Academic Publishers. Dordrecht, Newtherlands p 215-221

Thomas RJ and Sprent JI. 1984. The effects of temperature on vegetative and early reproductive growth on a cold-tolerant and a cold-sensitive line of *Phaseolus vulgaris L. I.* Nodulation, growth and partitioning of dry matter, carbon and nitrogen. Ann. Bot. 53:579-588

Tiller SA, Parry AD and Edwards R. 1994.Changes in the accumulation of flavonoid and isoflavonoid conjugates associated with plant age and nodulation in alfalfa (*Medicago sativa*). Physiol. Plant. 91:27-36

Torrey JG. 1986. Endogenous and exogenous influcences on the regulation of lateral root formatiom. In: *New Root Formation in Plant and Cuttings.* Ed. Jackson MB, Martinus Nijhoff Publishers, Dordrecht p 31-66

Trappe JM. 1982. Synoptic keys to the genera and species of zygomycetous mycorrhizal fungi. Phytopathol. 72:1102-1109

Trinick MJ. 1985. *Rhizobium* strain competition for host nodulation. In *Proc. World Soybean Research Conference 3,* Ed. Shibles R. Boulder Colo: Westview.

Triplett EW and Sadowsky MJ. 1992. Genetics of competition for nodulation of legumes. Annu. Rew. Microbiol. 46:399-428

Truchet G, Roche P, Lerouge P, Vasse T, Camut S, Billy F de, Prome JC, Denarie J. 1991. Sulphated lipo-oligosaccharider signals of *Rhizobium meliloti* elicit root nodule organogenisis in alfafa. Nature 351:670-673

Turgeon BG and Bauer WD. 1982. Early events in the infection of soybean by *Rhizobium japonicum.* Time course and cytology of the initial infection process. Can. J. Bot. 60:152-161

van Brussel AAN, Bakhuizen R, van Spronsen PC, Spaink HP, Tak T, Lugtenberg BJJ and Kijne JW. 1992. Induction of preinfection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of *Rhizobium.* Science 257:70-72

van Brussel AAN, Recourt K, Pees E, Spaink HP, Tak T, Wijffelman CA, Kijne JW and Lugutenberg BJJ. 1990. A biovar-specific signal of *Rhizobium legumonosarum* bv. *viciae* induces increased nodulation gene-inducing activity in root exudate of *Vicia sativa* subsp. *nigra*. J. Bacteriol. 172:5394-5401

van Rhijn P and J Vanderleyden. 1995. The *Rhizobium*-plant symbiosis. Microbiol. Rev. 59:124-142

Vance CP. 1997. Enhanced agricultural sustainability through biological nitrogen fixation. In *Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture.* Eds. Legocki A, Bothe H and Pühler A. Springer, Springer-Verleg Berlin, German, p 197-186

Vance CP and Heichel GH. 1991. Carbon in N_2 fixation: Limitation or exquisite adaptation? Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:373-392

Velagaleti RR, Marsh S, Kramer D, Fleischman D and Corbin J. 1990. Genotypic differences in growth and nitrogen fixation among soybean (*Glycine max* (L.) Merr.) cultivars grown under salt stress. Trop. Agric. 67:169-177

Verma DPS. 1992. Signals in root nodule organogenesis and endocytosis of *Rhizobium*. Plant Cell 4:373-382

Verma DPS, Ball S, Guerin C and Wanamker L. 1979. Leghemoglobin biosynthesis in soybean root nodule. Characterization of the synthesis of the major leghemoglobin. Biochem. 18:476-483

Verma DPS. 1992. Signals in root nodule organogenisis and endocytosis of *Rhizobium*. Plant Cell 4:373-382

Vessey JK, Walsh KB and Layzell DB. 1988. Oxygen limitation of nitrogen fixation in stem girdled and nitrate-treated soybean. Physiol. Plant. 73:113-121

Vessy JK and Waterer J. 1992. In search of the mechanism of nitrate inhibition of nitrogenase activity in legume nodules: Recent development. Physiol. Plant. 84:171-176

Vigue JT, Harper JE, Hageman RH and Peters DB. 1977. Nodulation of soybean grown hydroponically on urea. Crop Sci. 17:169-172

Vincent, J.M. 1970. A manual for the practical study of root-nodule bacteria. IBP Handbook No. 15. Blackwell, Oxford

Vincent IM and Waters LM. 1953. The influence of the host on competition amongst clover root-nodule bacteria. J. Gen. Microbiol. 9:357-70

Walsh KB and Layzell DB. 1986. Carbon and nitrogen assimilation and partitioning in soybeans exposed to low remperatures. Plant Physiol. 80:249-255

Walsh KB, Canny MJ and Layzell DB. 1987. Carbohydrate supply and N_2 fixation in soybean: the effect of varied day length and stem girdling. Plant Physiol. 85:137-144

Walsh KB. 1995. Physiology of the legume nodule and its response to stress. Soil Biol. Biochem. 27:637-655

Wang SP, Stacey G. 1990. Ammonia regulation of *nod* genes in *Bradyrhizobium japonicum*. Mol. Gen. Gene 223:329-331

Weaver RW and Denck LR. 1974a. Effect of inoculum rate on competitive nodulation of *Glycine max* L. Merrill. 1. Greenhouse Studies. Agron. J. 66:229

Weaver RW and Derick LR. 1974b. Effect of inocuium rate on competitive nodulation of *Glycine max* L. Memll.II. Field studies. Agron. J. 66:233-36

Weber CF, Miller VL. 1972. Effect of soil temperature on *Rhizobium japonicum* serogroup distribution in soybean nodules. Agron. J. 64:72-98

Weisz PR and Clair TR. 1988. Soybean nodule gas permeability, nitrogen fixation and

diurnal cycles in soil temperature. Plant Soil. 109:227-234

Werner D and Rschel E. 1978. Differentiation of nodules of *Glycine max.* Planta. 141:169-177

Whigham DK and Minor HC. 1978. Agronomic characteristics and environmental stress. In: *Soybean Physiology, Agronomy, and Utilization.* Ed. Norman GA. Academic Press, New York: p 77-118

Wojtaszek P, M Stobiecki and Gulewicz K. 1993. Role of nitrogen and plant growth regulators in the exudation and accumlation of isoflavonoids by roots of intact white (Lupin (*Lupinus albus* L.) plants. J. Plant Physiol. 142:689-694

Williams LE, DeJong TM and Phillips DA. 1982. Effect of changes in carbon-exchange rate on soybean nodule activity. Plant Physiol. 69:432-436

Wolfson JL. 1987. Impact of *Rhizobium* nodules on *Sitona hispidulus,* the clover root curculio. Entomol. Exp. Appl. 43:237-243

Yahalom E, Okon Y and Dovrat A. 1987. *Azospirillum* effects on susceptibility to *Rhizobium* nodulation and on nitrogen fixation of several forage legumes. Can. J. Microbiol. 33:510-514

Yoshida S and Yatazawa M. 1967. The effects of combined nitrogen on nodule formation in soybean seedlings. J. Sci. Soil Manure 38:21-24

Young JPW. 1996. Phylogeny and taxonomy of rhizobia. Plant Soil. 186:45-52

Yuen JP and Stacey G. 1996. Inhibition of nod gene expression in *Bradyrhizobium japonicum* by organic acids. Mol. Microbe-Plant Interact. 9:424-428

Zaat SA, Wuffelman CA, Mulder, IHM, van Brussel ANN and Lugtenberg BJJ. 1988. Root exudates of various host plants of *Rhizobium leguminosarum* contain different sets of inducers of *Rhizobium* nodulation genes. Plant Physiol. 86:1298-1303

Zahran HH and Sprent JI. 1984. Effects of sodium chloride and polyethylene glycol on root hair infection and nodulation and NH_4NO_3 fertilized *Vicia faba L.* plants by *Rhizobium leguminosarum.* Planta 167:303-309

Zahran HH. 1991. Conditions for successful *Rhizobium*-legume symbiosis in saline environments. Biol. Fertil. Soils 12:73-80

Zapata F, Danso SKA, Hardarson G and Fried M. 1987. Time course of nitrogen fixation in field-grown soybean using nitrogen-15 methodology. Agron. J. 79:172-176

Zhang F and Smith DL. 1994. Effects of low root zone temperatures on the early stages of symbiosis establishment between soybean [*Glycine max* (L.) Merr.] and *Bradyrhizobium japonicum*. J. Experi. Bot. 45:1467-1473

Zhang F and Smith DL. 1995. Preincubation of *Bradyrhizobium japonicum* with genistein accelerates nodule development of soybean at suboptimal root zone temperatures. Plant Physiol. 108:961-968

Zhang F, Dashti N, Hynes RK, Smith DL. 1996. Plant growth promoting rhizobacteria and soybean [*Glycine max* (L) Merr.] growth and physiology at suboptimal root zone temperatures. Ann. Bot. 79:243-249

Zhang F and Smith DL. 1996a. Genistein accumulation in soybean [*Glycine max* (L) Merr.] root systems under suboptimal root zone temperature. J. Experi. Bot. 47:785-792

Zhang F and Smith DL. 1996b. Inoculation of soybean [*Glycine max*. (L.)] with genistein-preincubatited *Bradyrhizobium japonicum* or genistein directly applied into soil increases soybean protein and dry matter yield under short season conditions. Plant Soil 179:233-241

Zhang F and Smith DL. 1997. Application of genistein to inocula and soil to overcome low spring soil temperature inhibition of soybean nodulation and nitrogen fixation. Plant Soil 192:141-151