# NOTE TO USERS

# Enhanced Soybean Nodulation and Nitrogen Fixation via Modifications of Bradyrhizobial Inoculant and Culture Technologies

By

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Short title:

Enhancement of soybean nodulation and nitrogen fixation

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# ABSTRACT

Soybean (*Glycine max* L. Merr.) and *Bradyrhizobium japonicum* can form a nitrogen fixing symbiosis. This symbiosis is important for most sustainable agriculture systems. This thesis examines two ways to enhance nodulation and nitrogen fixation by this symbiosis: coinoculation of plant growth promoting bacteria (PGPB) with *B. japonicum*, and addition of RNA to a bradyrhizobial culture medium. The optimal coinoculation dose of *Serratia proteamaculans* 1-102 and *S. liquefaciens* 2-68 was determined as $10^8$ cells per plant under both optimal and suboptimal root zone temperatures (RZTs). Nodulation dynamics studies indicated that coinoculation of these two PGPB caused earlier nodule initiation and a higher nodulation rate, contributing to the higher nodule number and nodule weight. The coinoculation also increased nitrogen fixation efficiency under both optimal and suboptimal RZTs. A novel inducible activator only produced by the bacteria after addition of flavonoids to the culture system was prepared and evaluated in greenhouse and field experiments. Fourteen non-bradyrhizobial endophytic bacteria (NEB) were isolated from the surface sterilized root nodules, and three of these, designated NEB4, NEB5 and NEB17, showed soybean plant growth promotion under both greenhouse (with controlled RZTs) and field conditions. Alone, they were neither nodule inducers nor nitrogen fixers. Biolog tests and partial 16S rRNA gene sequence analyses placed the three strains in genus *Bacillus*: NEB4 and NEB5 are *B. subtilis* and NEB17 *B. thuringiensis*. *Bradyrhizobium* species grow slowly, making the culture process long and the cost of inoculant production higher. Addition of commercial yeast RNA to the bacterial culture medium accelerated the bacterial growth rate, shortened the culture time and increased the lipo-chitooligosaccharide (LCO) yield in flask cultures. Inoculation experiments in the greenhouse also showed that bradyrhizobial inoculant produced in the presence of RNA had better symbiotic competency (indicated by greater nodule number, nodule weight and plant weight) than the control. Collectively, these research findings have the potential to improve the overall utility of the nitrogen fixing symbiosis in soybean production and contribute to the use of this symbiosis as a tool in sustainable agriculture systems.

# RESUMÉ

Le soja (*Glycine max* L. Merr) peut former une association symbiotique avec la bactérie *Bradyrhizobium japonicum* pour fixer l'azote. Cette symbiose est une partie importante de n'importe quel système d'agriculture durable. Cette thèse examine deux voies afin d'ameliorer la nodulation et la fixation de l'azote par cette symbiose: la coinoculation avec *le B. japonicum* ainsi que des bactéries favorisant la croissance des plantes (BFSP), et l'ajouts de l'ARN dans le milieu de culture. La dose optimal de coinoculation de $10^8$ cellules par plante a été déterminée pour les organisms *Serratia proteamaculans* 1-102 et *S. liquefaciens* 2-68 sous des températures de zone des racines optimales et suboptimales. Les études de la dynamique de la nodulation ont indiqué que la coinoculation de ces deux BFSP a causé une accélération au niveau du déclenchement et de la vitesse de nodulation, ainsi qu'une augmentation du nombre et du poids des nodules. La coinoculation a également augmenté l'efficacité de la fixation de l'azote sous les températures optimales et suboptimales de la zone des racines. Un activateur produit par les bactéries après l'addition des flavonoïdes dans la culture a été préparé et évalué dans des expériences en serre et sur le terrain. Quatorze souche de bactéries endophytes non-bradyrhizobiales (BEN) ont été isolées à partir de nodules de soya stérilisées en surface. Trois de ceux-ci, indiqué BEN4, BEN5 et BEN17, se sont avérées capable d'augmenter le poids de plants de soya en serre. Ils étaient cependant incapable d'induire la nodulation et la fixation d'azote 'a eux-mêmes. Des essais Biolog et l'analyse des sequences partielles du gène de 16S rRNA ont placé les trois souches dans le genre de *Bacillus*: BEN4 et BEN5 sont des souches appartenant 'a l'espéce *B. subtilis* et BEN17 est une souches appartenant 'a l'espéce *B. thuringiensis*. Les espèces de *Bradyrhizobium* croissent lentement, rendant le processus de culture plus long et le coût de la production d'inoculant plus haut. L'ajout de l'ARN de levure commercialle au milieu de culture (mannitol - extrait de levure) et a accéléré la vitesse de la croissance bactérienne, a raccourci le temps de culture et a augmenté la production de LCO. Les expériences d'inoculation en serre ont également prouvé que l'inoculant bradyrhizobiale produit en présence de l'ARN a démontré une capacité symbiotique plus grande que le control (indiquée par le nombre de nodules, le

poids accrus des nodules et des plants). Collectivement, ces résultats de recherches peuvent améliorer les gains globals de la symbiose avec les bactéries fixant l'azote dans la production du soja et contribuer à l'utilisation de cette symbiose comme un outil dans les systèmes d'agriculture durable.

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I would like to dedicate this thesis to my wife Kuirong and our son Ying, as well as to my parents. They provided constant support, understanding and encouragement that allowed me to pursue my life-long goal with determination and without hesitation. I

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# TABLE OF CONTENTS

# LIST OF FIGURES

Figure

# LIST OF TABLES

Tables

# CONTRIBUTIONS OF CO-AUTHORS TO MANUSCRIPTS FOR PUBLICATION

This thesis has been written in the form of manuscripts to be submitted to scientific journals. The contents of sections 3 to 9, inclusively, are drawn from manuscripts for publication.

The manuscripts from which sections 3 to 9 are taken are all co-authored by D. L. Smith and myself. Dr. D. L. Smith is my supervisor at the Macdonald Campus of McGill University. He 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, bench space in the greenhouse, experimental field space, the use of computers, and providing technical assistance.

The manuscripts on which sections 5, 7 and 9 are based are also co-authored by Dr. X. Zhou, a research associate of Dr. Smith. Dr. Zhou operates the elemental analyzer and helped me to do the nitrogen analyses, and also provided considerable general support in a wide range of areas from organizing summer student helper to doing statistical analyses.

The manuscripts on which sections 4 and 5 are based are also co-authored by Dr. A. Souleimanov, a research associate of Dr. Smith, who takes charge of HPLC operation in the laboratory and helped me to purify the inducible PGPR activator and to assay LCO production. Dr. Souleimanov conducted the HPLC work required to identify the active peak. I cultured *Serratia proteamaculans* 1-102 and added inducers, including genistein, naringenin, apegenin and luteolin. I harvested the cultures and provided the material to Dr. Souleimanov for extraction and HPLC fractionation. I performed the required dilutions and prepared the activator solutions at 8300, 830 and 83 microvolts per ml for the greenhouse and field experiments. I conducted the greenhouse and field experiments. During the summers I supervised an undergraduate student who helped in the collection of samples. I processed the samples and conducted the statistical analyses.

The manuscript on which section 3 is based is also co-authored by T. Charles and B. Pan. Dr. Charles is a professor in Department of Biology, University of Waterloo, who helped me to design the experimentation reported in this section, and offered

guidance during the conduct of the experiments. Dr. Pan, a former Ph. D. student of Dr. Smith, helped me to set up and conduct the greenhouse experiments, to collect the data after the plants were harvested and design some tables and figures in the section.

The manuscript on which section 5 is based was co-authored by B. Driscoll and F. D'Aoust. Dr. Driscoll, a professor in Department of Natural Resource Sciences, Macdonald Campus of McGill University, who offered experimental facilities and technical guidance in my identification of the selected NEB strains. I initiated this line of research. In August 1998, I selected vigorous field grown soybean plants and isolated 14 non-*Bradyrhizobium* endophytic bacteria from the surface disinfected soybean nodules. An undergraduate summer student helped in some of the very routine aspects of the work. Then from December 1998 to February 1999, I conducted greenhouse experiments and selected three plant growth promoting strains from the 14 non-*Bradyrhizobium* endophytic bacterial isolates. I observed the growth of the three selected strains on Ashbey's plates during November 1999, and conducted experiments on their growth responses the three selected strains to the different carbon and nitrogen sources in Ashbey's culture medium in March 2001. Mr. D'Aoust, a Ph.D. candidate supervised by Dr. Driscoll, and I conducted the Gram staining, observations on spore-formation and Biolog tests during the period March to May 2000. Mr. D'Aoust conducted the DNA extraction, PCR amplification and DNA sequencing work from April to July 2001. After the experimental procedure was determined, Mr. D'Aoust and I conducted the DNA extraction, PCR amplification and DNA sequencing work in two parallel procedures. This was done in the period September to October 2000. The phylogenetic analysis based on 16S rRNA gene HV sequences was conducted by Dr. Driscoll.

# Section 1. GENERAL INTRODUCTION

## 1.1 Introduction

Nitrogen is one of the most limiting factors in agricultural production. In the 20th century, application of nitrogen fertilizers improved world grain production substantially, which in turn allowed world population to almost quadruple. However, excessive use of the nitrogen fertilizer has become a serious environmental problem (Smil 1997). In order to deal with the challenge of continued global population growth, food production and supply, and resource and environment protection, modern agriculture must develop more sustainable practices. Fossil fuels and chemicals, used in the past, should be replaced by biological inputs. In this regard, biological nitrogen fixation (BNF) has particular significance (Van Kammen 1997).

BNF uses the capacity of certain nitrogen-fixing bacteria, including the symbiotic, the associative symbiotic and some free-living prokaryotes, mainly bacteria and cyanobacteria, to convert atmosphere $N_2$ into plant-usable ammonia (Pepper 2000). It is estimated that biologically fixed N amounts to 175 Mt per year and that 80% of the biologically fixed N comes through symbiotic systems, among which the rhizobia-legume symbiosis is the best studied and the most critical to agricultural crop production. Legumes provide 20-35% of the protein consumed worldwide. Approximately 250 M ha of legumes are grown worldwide and fix about 90 Mt N per year. To replace this amount of fixed N with synthetic N fertilizer would require 288 Mt of fossil fuel and cost approximately $30 billion USD annually (Vance et al. 1997). Thus, enhancing the use of and improving the efficiency of biological nitrogen fixation by legumes has both environmental and economic significance.

Soybean is one of the world's most important legume crops, and it is able to fix nitrogen symbiotically. Besides its importance as a high protein food for humans and feed supplement for livestock, soybean is also one of the most important oilseed crops. On average, soybean seeds contain 79.2% meal and 17.8% oil. World soybean meal production increased from 30 Mt in 1970 to 70 Mt in 1990 and to 110 Mt by the year 2000. Global soybean oil production was 24.7 Mt in 2000. Besides being used as food,

soybean oil is also an important raw material in pharmaceutical and other organic chemical industrial production. In the 1990's, the soybean oil consumption in the US increased from 10 Mt to 12 Mt, of which at least 10% was consumed in non-food usage, mainly used in synthesis of resins and plastics (Weiss 2000). In Canada, soybean area increased from 1 M ha in 1985/86 to 2 M ha in 1996/97 (Weiss 2000). In Quebec, the area planted to soybean was 3 K ha in 1986, 120 K ha in 1998 and 140 K ha in 2000. Soybean consumption and production is likely to increase during the 21$^{st}$ century.

Soybean is a legume of tropical to subtropical origin and requires warm growing condition (Smith 1995). Thus, the low soil temperatures after seeding, in a short season area such as in Quebec, inhibit the early growth stages, as well as the onset of the nitrogen fixation, of soybean plants. In some areas, environmental pollution has also resulted in some deleterious changes in soil microbial communities. These have become critical factors limiting further increases in soybean production.

In recent decades, plant growth promoting bacteria (PGPB), including rhizobacteria (PGPR) and endophytic bacteria, have been successfully applied in agricultural production. Many PGPB strains, as well as some bio-produced activators, worked well in promoting legume nodulation and nitrogen fixation. They have been added to rhizobial inoculants, thereby promoting legume nodulation and nitrogen fixation. Some strains, such as *Serratia proteamaculans* 1-102 and *S. liquefaciens* 2-68, and active compounds, such as genistein, had also been demonstrated to have efficacy in overcoming the low soil temperature inhibitions of soybean early growth and nitrogen fixation (Zhang et al. 1996b, 1997; Zhang and Smith1995). These two strains of plant growth promoting rhizobacteria (PGPR), *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68, can accelerate soybean seedling early growth, nodulation and nitrogen fixation under both optimal and sub-optimal root zone temperatures (RZT) (Zhang et al. 1996b, 1997). It was speculated that these two *Serratia* strains might promote plant growth by producing a diffusible substance (Dashti 1996). Genistein is one of the soybean produced isoflavone signal molecules triggering the coordinated expression of *nod* genes during the plant-rhizobia recognition and symbiosis establishment processes (Kosslak et al. 1987). Preincubation of bradyrhizobial cells with genistein enhanced the onset of nitrogen

fixation, increased the nodule number, nodule size and plant weight, and this beneficial effect was more obvious with decreasing RZTs (Zhang and Smith 1995, 1997). The effects of genistein treatment and that of the coinoculating PGPR with *Bradyrhizobium japonicum* are additive or antagonistic depending on the RZT conditions (Dashti et al. 2000). Endophytic bacteria are ubiquitous in plant tissues, including legume root nodules. Endophytic bacteria have more intimate relationships with their host plants than general rhizobacteria and are PGPB much more frequently. It seems reasonable that PGPB can be isolated from surface disinfected tissues of at least some of the most vigorous field-growing plants.

Commercial legume crops are often treated with rhizobial inoculants to increase their nitrogen fixation capacity (Pepper 2000). For either the PGPB or active compounds to be effective in promoting legume nodulation and nitrogen fixation they need to be coinoculated with rhizobial preparations. Their application in agriculture could constitute a sustainable input leading to increased legume production. The demand for rhizobial inoculants will be greater as the benefits derived from these inoculants increases. In addition, besides inducing nodule formation in legumes, rhizobia have the potential to be used as PGPB in the production of other crops. They are able to colonize the roots of non-legumes and produce cell-surface glycans, phytohormones, siderophores and HCN. They exhibit antagonistic effects towards many plant pathogenic fungi (Antoun et al. 1998). The production of legume inoculants is always expensive, and this is particularly so for bacteria such as bradyrhizobia, because their slow growth ties up commercial fermentors for a relatively long period of time. Thus, shortening the culture period of the slow growing *Bradyrhizobium* would reduce the cost of inoculant production. Providing the symbiotic competency of the cells could at least be maintained, this will stimulate soybean production and sustainable agriculture practices. There have been many efforts to improve rhizobial culture technology by modification of the culture medium, such as addition of hot water extract of the host seed hulls (Kanuma 1997) and amaranth seed meal (Grassano et al.1999) to rhizobial culture medium. In cultures of *Lactobacillus*, addition of RNA to the culture medium increased the bacterial biomass (Houde 1999). In order to improve the slow growth of bradyrhizobia in culture, in this thesis, the effects of

addition of RNA on bacterial growth, physiology and symbiotic competency were investigate.

## 1.2 Hypotheses

**General hypothesis:**

The overall nitrogen fixing efficiency and efficacy of the soybean-bradyrhizobial symbiosis could be improved via co-inoculation with PGPB strains and the use of RNA additions to *B. japonicum* cultures. The PGPB coinoculations will improve the efficacy to the *B. japonicum* inoculant, resulting in more nodulation, nitrogen fixation, growth and yield. The RNA additions will accelerate growth of the *B. japonicum* cultures leading to more rapid and more efficient production of inoculant.

**Specific hypotheses:**

1. The effect of PGPR varies with coinoculation dose and there is an optimal coinoculation dose for each PGPR; the optimal doses of each strain may vary depending on RZT conditions.

2. PGPR strains enhance soybean nodulation by positively affecting the dynamics of the nodulation process and improve BNF through increasing nitrogen fixation efficiency.

3. The PGPR *Serratia liquefaciens* 2-68 and/or *S. proteamaculans* 1-102 promote soybean nodulation through the production of diffusible activators during the culture process.

4. Effective soybean nodulation and nitrogen fixation promoting bacteria exist within the soybean nodule tissues as bacterial endophytes and may be responsible for particularly vigorous plant growth under field conditions.

5. Addition of commercial yeast RNA to the culture medium will increase the growth rate of *B. japonicum* strains without deleterious effects on the symbiotic competency of the cells produced.

## 1.3 Objectives

**General objectives:**

To improve the utility of the soybean nitrogen fixing symbiosis as a tool of sustainable agriculture.

**Specific objectives:**

1. To further characterize the accelerated soybean nodulation and nitrogen fixation resulting from the coinoculation of *B. japonicum* with PGPB, as part of an effort to better understand the underlying physiological mechanisms.

2. To isolate and identify the PGPR activator(s) produced by *Serratia proteamaculance* 1-102.

3. To evaluate the plant growth promoting effects of the activator on soybean plants under greenhouse and field conditions.

4. To isolate new soybean nodulation and nitrogen fixation promoting bacterial strains from within the nodules of the most vigorous field grown soybean plants.

5. To identify the biological characteristics and taxonomic status of the endophytic isolates.

6. To test on the effect of RNA addition on *Bradyrhizobium japonicum* growth rate and physiological activity.

7. To evaluate the symbiotic competency of the *B. japonicum* cells cultured in medium supplemented with RNA.

## Section 2. GENERAL LITERATURE REVIEW

### 2.1 Soybean and *Bradyrhizobium japonicum*

Soybean [*Glycine max* (L.) Merrill] belongs to the family Leguminosae, subfamily Papilionideae. Soybean was first domesticated in central China, as early as 2800 B.C. (Smith 1995). Because of its tropical to subtropical origins, the optimal temperature range for soybean growth is 25 – 30°C (Smith 1995). Soybean is one of the world's most economically important legume crops. Legume plants and rhizobia form specific symbioses that can fix atmosphere $N_2$ to support plant growth and protein production. Soybean and *Bradyrhizobium japonicum* are two such specific partners, that can form an effective nitrogen fixing symbiosis (Long 1989).

The genus *Bradyrhizobium* consists of two species, *B. japonicum* and *B. elkanii,* as well as some other un-named strains (*B.* spp.*)*. Bradyrhizobia are Gram-negative rods 0.5 – 0.9 × 1.2 – 3.0 μm in size, motile through one or two polar or sub-polar flagella, with a high G + C content (63 – 66%). They are aerobic and have a $T_{opt}$ of 25 – 30 °C and a $pH_{opt}$ of 6.0 – 7.0. Colonies are circular, opaque, rarely transparent, white and convex, and tend to be granular in texture. They are slow growing rhizobia with a generation time more than 8 hours; the diameter of the colony doesn't exceeds 1 mm after 5-7 days of incubation on yeast extract mannitol (YEM) agar plates and broth cultures only develop a moderate turbidity after shaking for 3-5 days. Bradyrhizobia have an alkaline reaction on YEM and are not able to use disaccharides as a carbon source (Krieg 1984). *B. japonicum* can exist free living in soil or infect soybean (*Glycine max* and *G. soja*), cowpeas (*Vigna unguiculata*), mungbeans (*Vigna radiata*) or siratro (*Macroptilium atropurpureum*) root systems as nodule endosymbiotic inhabitants. When living in an endosymbiotic state, as bacteroids in root nodules, *B. japonicum* can fix nitrogen and supply this element to the plant (Stacey et al. 1995).

### 2.2 Soybean Nodulation

The establishment of the symbiotic system, the nodulation process, is a complex process

consisting of a series of interactions between the host and the bacteria. The nodulation process begins with the mutual exchange of diffusible signal molecules followed by attachment of the bacteria to the plant root hairs. The root hair curls and nearly encircles the bacteria. The bacteria invade the plant via an infection thread formed inside the root hairs. When infection threads reach into root cortical cells the bacteria are released into the cytoplasm of the host plant cells. The nodule primordium formed through stimulation by the bacteria-to-plant signal develops into a mature nodule, while the bacteria differentiate into their endosymbiotic forms as bacteroids. Bacteroids produce dinitrogenase, which catalyzes the reduction of nitrogen to produce ammonia that supports plant protein production (reviewed by Mylona et al.1995).

### 2.2.1 Signal Exchange

The signal exchange between host plant roots and the bacteria colonizing the rhizosphere can be characterized as a two-way molecular conversation (Fisher and Long 1992; Spaink 2000). The soybean seedling roots exude isoflavone molecules such as genistein and daidzein (Parke et al. 1985; Lameta and Jay 1987; Rao and Cooper 1994). The isoflavone molecules induce the coordinated expression of bacterial *nod* genes. The *nod* genes encode enzymes involved in the synthesis of lipo-chito-oligosaccharides (LCOs), also known as Nod factors. The backbone of all rhizobial nod factors consists of three to six β -1,4-linked *N*-acetylglucosamines and this has a fatty acid attached to the non-reducing sugar residue. LCOs recognize host receptors at the tip of the root hair and induce root hair deformation and root cortex cell division (Kossalak et al. 1987; Mylona et al. 1995; Prithiviraj et al. 2000). LCO produced by a given rhizobacterial strain has characteristic side groups ($R_1$-$R_5$) on both its non-reducing and reducing terminal glucosamine residues, and these residues play a role in determining the legume host range of a given bacterial strain. A large variety of LCOs is produced by *B. japonicum* strains. The common and most active factor is a pentamer of N-acetylglucosamine acylated at the non-reducing end with vaccenic acid (18:1) and substituted at the reducing terminal sugar with 2-*O*-methylfucose (Sanjuan et al. 1992; Carlson et al. 1993; Stacey et al.1995).

The expression of nod genes is finely timed. Nod factor is required at a nanomolar ($10^{-9}$) level for induction of plant responses that lead to nodule formation. Only the production of Nod factors at the optimal concentration will result in efficient nodule formation. *Bradyrhizobium* species carry all the *nod* genes on the chromsome and have more sophisticated regulation mechanisms for *nod* gene expression than *Rhizobum* (Knight et al. 1986; Schlaman et al.1992; Stacey et al. 1995). The *nod* genes have been characterized as either 'common', such as *nod*DABCIJ, which show significant homology among *Rhizobium* and *Bradyrhizobium* species, or 'host-specific', such as *nodZ* of *B. japonicum, nod*EFLMN of *R. leguminosarum* and *nod*EFGHQ of *R. meliloti* (Horvath et al. 1986; Stacey et al. 1995). Most of the *nod* genes are not expressed until the bacterial inducers of a compatible host plant are present. The *nod*D gene is constitutively expressed and is essential for the expression of the other *nod* genes (Long 1989). In rhizobia, NodD is associated with the cell membrane and can bind to host isoflavone signal molecules with its C-terminal portion. After this binding, NodD binds to the 'nod box', a DNA region upstream of the inducible *nod* genes with its N-terminal region. The *nod* genes are activated and expressed to take part in the synthesis of the Nod factor. In *B. japonicum*, besides NodD, NodV and NodW are alternative isoflavone binding proteins. They too can activate nod gene expression after binding with isoflavone. NodA, NodB and NodC proteins, common *nod* gene products, catalyze the synthesis of the Nod factor backbone (Dudley et al. 1987). The products of the other host specific *nod* genes catalyze side group substitutions (Debelle et al. 1986).

Nod factor exists in two forms: free in the medium following bacterial secretion or as a constituent inserted into the bacterial membrane via its lipid moiety, with its glucosamine backbone extending into the extra-cellular medium (Hirsch 1992). There appears to be a Nod factor receptor on the tip of the root hairs, possibly a plant lectin. In soybean the Nod signal receptor is proposed to be a member of a general class of chitin-binding proteins (Stacey 1999). It is through the putative Nod factor-receptor reaction that this bacterial signal is presumed sensed by the host plant (Diaz et al. 1989). The N-glucosamine residues of the Nod factor react with a sugar-binding site of the receptor. The strength of the interaction between Nod factor and the receptor depends on several

factors, such as the length of the glucosamine backbone, the presence or absence of various side substitutions and the composition of the lipid side chain (Hirsch 1992). It has also been suggested that Nod factors are involved in the inhibition of salicylic acid-mediated defense mechanisms in legumes; this could explain why rhizobia can successfully infect legume plants without triggering a host defense response (Pinton et al. 2001). Besides the flavonoid and the LCO that are exchanged between legume plants and rhizobial cells, it is now believed that a multitude of additional signals from both symbiotic parties are likely involved in the nodulation process. These additional signal molecules include bacterial surface polysaccharides and excreted proteins (Stacey 1999). In the interactions between soybean and *B. japonicum*, the bacterial cyclic β-glucans may play a role as suppressors of host defense responses (Perret et al. 2000).

### 2.2.2 Bacterial Attachment and Root hair Response

Through the Nod factor-receptor reaction, the bacteria attach to the root hair tip, in a two-step process. The first step, a loose binding of the bacteria, as single cells, to the root hair surface, is probably mediated by a bacterial surface located $Ca^{2+}$-binding protein, rhicadhesin. The rhicadhesin activity has been detected in all members of the Rhizobiaceae. The second step is the accumulation and anchoring of the bacteria to the surface of the root hair, leading to a firm attachment, called 'cap formation'. The second step is mediated by plant lectins and bacterial appendages, such as cellulose fimbriae and rhizobial lectins, in the case of *B. japonicum.* Although there are several factors mediating the bacterial attachment, the binding of Nod factor already existing in the cell membrane with the host plant lectin is the most apparent selective mechanism (Vlassak and Vanderleyden 1997). The root hair tip attachment site positions the bacteria at a viable invasion site.

The root hair is an extending tubule of a root surface cell that elongates by tip growth. The root hair tip has a high level of physiological activity and a thin, unpolymerized cell wall. During the attachment stage, the root hair may supply carbon and nitrogen nutrients for the bacteria by means of exudates (Peterson and Farquhar

1996). Following attachment, some root hairs undergo morphological changes: root hair deformation (Had), including swelling, twining, branching and other forms of deformation, and root hair curling (Hac) (Higashi and Abe 1980; Callaham and Torrey 1981). Computer simulation of root hair curling suggests that the attached bacterial cells induce a curling or branching at the region of attachment by affecting root hair growth (van Batenburg et al.1986). Experiments with purified LCO demonstrated that LCO itself is responsible for inducing these root hair responses (Kijne 1992; Prithiviraj et al. 2000). Root hair deformation marks the first visible event in the nodulation process. Root hair deformation and root hair curling may involve a protein termed hadulin. It is one of the root hair-specific proteins induced by the Nod factor (Krause and Broughton 1992). When *B. japonicum* is inoculated onto soybean seedling roots in the laboratory, bacteria became attached to epidermal cells and root hairs within minutes after inoculation. Marked root hair curling occurs within 12 hours (Turgeon and Bauer 1982; Prithiviraj et al. 2000). The root mark method revealed that the infectible cells on soybean roots appear to be located just above the zone of root elongation and just below the position of the smallest emergent root hairs (Bhuvaneswari et al. 1980). So, in general, only short root hairs, which have not yet emerged at the time of inoculation, become tightly curled. Markedly curled root hairs occurred in patches. Longer root hairs do not exhibit pronounced curling although they are often deformed (Turgeon and Bauer 1982).

### 2.2.3 Infection Thread and Symbiosome

In the interaction between *B. japonicum* and soybean, the junction of differentiating trichoblasts are targets of bradyrhizobial infection. The bacteria are entrapped in the curled root hairs. The bacteria produce polysaccharide-degrading enzymes that hydrolyze the plant cell wall in the curled region, and initiate infection thread formation at a hyaline spot. The plasma membrane then invaginates and new plant cell wall material is deposited (Kijne 1992; Mateos et al. 2001). This results in the formation of a tubular structure, the infection thread. Thus, the infection thread is a continuation of the

plant cell wall. The ultrastructure of the infection thread wall is very similar to the normal plant cell wall, but the incorporation of certain nodulins may endow it with unique properties. During the formation of the infection thread, both plant nodulins and hadulin are involved and expressed in the infection threads (Scheres et al. 1990). Infection threads in soybean are relatively narrow, with a diameter of about 1 μm, and contain a single row of linearly aligned bacteria (Turgen and Bauer 1985). The bacteria in the infection thread are surrounded by a matrix consisting of compounds secreted by both the plant and the bacteria (Rae et al. 1992).

The infection thread undergoes an inverted growth form and passes through the root hair cell. This inverted tip growth of an infection thread is similar to normal growth of a root hair in that the nucleus remains a constant distance from the tip. The root hair nucleus precedes the infection thread tip. The bacteria travel via the infection thread following the nucleus. Microtubules might be responsible for anchoring the nucleus to the root hair tip. Concomitant with infection thread formation, cortical cells are mitotically reactivated, forming the nodule primordium. The infection thread grows toward this primordium and, once there, releases bacteria, still surrounded by plant membrane, into the cytoplasm (Kijne 1992; Goodchild and Bergersen 1966). Under greenhouse conditions, soybean infection threads can be observed 24 h after inoculation with *B. japonicum*. By 48 hours, the infection threads have progressed to the bases of the curled root hairs but have not penetrated into the cortex cells (Turgeon and Bauer 1982). In seed-inoculation experiments, electron microscopy photographs show that 10–12 days after inoculation and planting, *B. japoniucum* cells are freed from the infection thread by disintegration of the infection thread wall. The disintegrated wall materials are enclosed in membrane-bound vesicles. As the thread wall is removed in this way, the bacteria are each enclosed in an envelope derived from the thread membrane and then released into the host cytoplasm. The residual thread wall material remaining around a bacterium is removed from the envelope space by vesiculation of the membrane envelope. Both the bacteria and the infection thread wall material enter the host cell by endocytosis (Bassett 1977). Each bacterial cell surrounded by the envelope forms an organelle-like structure termed as symbiosome. In soybean, a symbiosome consists of a

host-derived membrane, the symbiosome membrane, which envelops a microsymbiont and creates an internal space called the symbiosome space. The symbiosome membrane is generally referred to as the peribacteroid membrane (PBM). The symbiosomes continue to divide, with the bacteria and PBM dividing in concert, to increase the bacterial population in the infected host cells (Day 1999).

### 2.2.4 Nodule Development

Root cortical cell division that results in nodule formation is initially anticlinal. Cell division can begin either in the outer or inner cortex of the root and this depends on the type of the nodule that will eventually form. The nodule type will be either indeterminate or determinate, and this depends on the host plant genotype. The indeterminate nodules are elongate and club-shaped. Plants having indeterminate nodules include clover, alfalfa, vetch and pea. The determinate nodules are spherical. Plants having determinate nodules include soybean, mungbean, and common bean. For determinate nodules the initial cortex cell division occurs in the outer cell layers. The determinate nodules lack a meristem; cell division ceases early during nodule development and the final form of the nodule results from cell enlargement rather than cell division (reviewed by Hirsch 1992).

Determinate nodules can be broadly subdivided into central and peripheral tissues. The peripheral tissues consist of nodule parenchyma and nodule cortex separated from each other by the nodule endodermis. Vascular bundles are embedded in the nodule parenchyma. The nodule cortex, at the outside of nodule endodermis, is derived from the root cortical cells that surround the nodule primordium. The first cell division that occurs in response to bacterial infection is anticlinal and in the hypodermis. Later, cell division occurs in the pericycal and inner cortex. Eventually, the two active meristem regions coalesce and give rise to the incipient nodule. The derivatives of the hypodermal cells form the central tissue of the nodule, which is composed of cytoplasm-rich cells. The highly vacuolated derivatives of the inner cortex and pericycle develop into the nodule parenchyma surrounding the central tissue. Vascular strands differentiate from small, dense cytoplasm cells in the nodule parenchyma. The early nodulin gene GmENOD40 is

expressed in the pericycle of the vascular tissue of soybean nodules, while transcripts for the early nodulin genes GmENOD2 and GmENOD13 are detected in nodule parenchyma cells. In the central tissue of the nodule, only some cells are with infected bacteria. The infected cells are larger and denser due to the presence of released bacteria. Interspersed among the infected cells are small, highly vacuolated, uninfected cells. In soybean these interstitial cells, which contain a nodule specific uricase, outnumber the infected cells by a ratio of apporoximately 3:2 (Vandenbosch and Newcomb 1986; reviewed by Hirsch 1992).

As the nodule develops, the bacterial cells inside the symbiosomes differentiate into nitrogen fixing bacteroids. The bacterial cell in each symbiosome stop dividing. After the cell walls become somewhat degraded the vegetative cells differentiate into enlarged pleomorphic bacteroids. Then, in determinant nodules, the PBM of adjacent symbiosomes appear to fuse, forming multiple bacteroid symbiosomes (Day 1999). The rhizobial cells are able to fix nitrogen once they have differentiated into bacteroids. The size and the DNA content per cell in bacteroids are similar to the free-living vegetative cells for *Bradyrhizobium*. *Bradyrhizobium* bacteroids contain three respiratory chains that are different from the free-living cells. In the bacteroid, poly-β-hydroxybutyrate (PHB) is accumulated and dinitrogenase is expressed at up to 10 % of the total soluble protein (reviewed by Werner 1992). Mature infected cells of soybean nodules contain many thousands of multiple bacteroid symbiosomes actively fixing nitrogen (Day 1999).

## 2.3 Soybean Nodule Function

The legume nodules are unique plant organs in which bacteroids fix nitrogen (Hirsch 1992). The nitrogen fixation is catalyzed by the nitrogenase complex of the bacteroids and occurs in the bacteroid cells (Pepper 2000).

### 2.3.1 Nitrogen Fixation

Nitrogen fixation in legume nodules follows the general equation:

$$N_2 + 8e^- + 8H^+ + 16\text{Mg-ATP} \rightarrow 2NH_3 + H_2 + 16\text{Mg-ADP} + 16\ \text{Pi}$$

The nitrogenase complex is made up of two fairly different enzymes, dinitrogenase and dinitrogenase reductase. Dinitrogenase, the larger one, contains Mo, Fe and Co and consists of 4 subunits ($\alpha_2\beta_2$) with a total molecular weight of 200 to 240 KD. It is termed as MoFe protein. Its Mo atom exists in a cofactor with configuration of 7 Fe, 8 S and 1 Mo called FeMoco. This cofactor is the site of $N_2$ binding and reduction. Dinitrogenase reductase, the smaller one, contains 4 Fe atoms and 4 acid labile sulphurs. It is termed the Fe protein and is made up of two identical subunits ($\alpha_2$) with a total molecular weight of 55 – 65KD. For the reduction of the $N_2$ bound in FeMoco, the 8 electrons are transferred from the Fe protein to the MoFe protein in a MgATP-dependent way. Both the Fe protein and the MoFe protein are oxygen sensitive. In the absence of an added reducible substrate nitrogenase reduces protons ($H^+$) to $H_2$ (reviewed by Yates 1992). is made up of two fairly different enzymes, dinitrogenase and dinitrogenase reductase. Dinitrogenase, the larger one, contains Mo, Fe and Co and consists of 4 subunits ($\alpha_2\beta_2$) with a total molecular weight of 200 to 240 KD. It is termed as MoFe protein. Its Mo atom exists in a cofactor with configuration of 7 Fe, 8 S and 1 Mo called FeMoco. This cofactor is the site of $N_2$ binding and reduction. Dinitrogenase reductase, the smaller one, contains 4 Fe atoms and 4 acid labile sulphurs. It is termed the Fe protein and is made up of two identical subunits ($\alpha_2$) with a total molecular weight of 55 – 65KD. For the reduction of the $N_2$ bound in FeMoco, the 8 electrons are transferred from the Fe protein to the MoFe protein in a MgATP-dependent way. Both the Fe protein and the MoFe protein are oxygen sensitive. Besides reducing $N_2$ to $2NH_3$, nitrogenase also reduces pairs of protons ($H^+$) to $H_2$. In the absence of $N_2$ and other reducible substrates, $H_2$ may be the only product of nitrogenase (reviewed by Yates 1992). The produced $H_2$ may be partially or entirely recycled by bacterial strains with uptake hydrogenase ($Hup^+$). Strains that are $Hup^-$ are not able to recycle $H_2$, and in these cases $H_2$ evolution can be measured to monitor nitrogenase activity (Hunt and Layzell 1993).

In eastern Canada field grown soybean can fix 100-200 kg N/ha/yr (Smith and Hume 1987). Soybean gets 25 – 75 % of the total N needed by fixing atmospheric $N_2$ (Deibert et al. 1979). Seed yield in nodulated soybean plants is generally comparable to

plants supplied with N fertilizer (Yates 1992).

### 2.3.2 Ammonia Assimilation

In legume nodules the majority of $N_2$ reduced by the bacteroids is transferred to the plant. It is incorporated into organic compounds through the ammonium-assimilation pathway, which operates in free-living bacteria, but can be repressed in the symbiotic bacteroids (Rosendahl et al. 1999). Most of the fixed nitrogen exists in the form of ammonium ($NH_4^+$) under the physiological conditions inside the bacteroids. Ammonium is initially incorporated into alanine in the bacteriods, then transported into the host cells through the inner and outer membrane of the bacteroid cells and through the peribacteroid membrane of the symbiosome (Waters et al.1998). In soybean nodules, the alanine is synthesized from pyruvate and ammonium by L-alanine dehydrogenase. *B. japonicum* alanine dehydrogenase is sufficiently rapid that the ability of bacteroid to assimilate ammonium into alanine is limited by the availability of ammonium produced by nitrogenase. Alanine is most likely only a transport molecule as glutamate is the central compound of the nitrogen metabolism in most organisms. The use of alanine as a transport molecule would functionally and spatially distinguish bacteroid nitrogen metabolism and transport from plant host cellular nitrogen metabolism. This would facilitate the rapid movement of fixed nitrogen from the bacteroid to plant (Allaway et al. 1999; Emerich et al. 1999). In the host cell, ammonium is further assimilated through catalysis of the enzymes glutamine synthetase, glutamate synthase, asparate aminotransferase and asparagine synthetase. The overall reaction is:

$$2NH_3 + 3ATP + \text{oxaloacetate} + NADH + H^+ \rightarrow$$
$$\text{L-asparagine} + AMP + 2\ ADP + 2\ pi + NAD^+ + PPi$$

Soybean is a ureide-exporting legume. Ammonium in the host cells is used for the amination of specific organic acids to produce amino acids, such as glutamate, aspartate and glycine. The glutamine, glycine and aspartate are then used for purine synthesis in plastids of infected cells. In the neighboring uninfected cells, purine nucleotides are transformed to xanthine and uric acid, which is oxidized to produce allantoin. Allantoin

is further transformed to allantoic acid in the smooth endoplasmic reticulum (ER) of uninfected cells. Uricase II, a nodulin located in the peroxysomes of the uninfected cells, is involved in production of ureides which are exported to the plants via xylem (reviewed by Werner 1992).

### 2.3.3 Carbon Metabolism

During the symbiotic association between rhizobia and their host plants, a series of complex interactions occur. Chief among them is the transfer of reduced carbon sources from the plant to the bacteroid where they are oxidized to produce energy for nitrogen fixation. The major C-compounds available for metabolism of bacteroids are $C_4$-dicarboxylic acids such as malate and succinate (Driscoll et al. 1995). Phytosynthate, namely sucrose, is translocated to nodules via phloem and broken down, by a catabolic form of sucrose synthase, to glucose. Some glucose is oxidized via the Embden-Meyerhof pathway and the tricarboxylic acid (TCA) cycle in host cells to produce malate and succinate. To be utilized by the metabolic machinery of the bacteroid, the carbon sources must traverse both the host-derived peribacteroid membrane and the bacteroid membrane. The peribacteroid membrane is relatively impermeable to sugars but does contain a dicarboxylate transporter capable of rapidly transferring $C_4$-dicarboxylic acids to the bacteroid. Bacteroids are capable of taking up $C_4$-dicarboxylates at rates significantly higher than that of sugars such as glucose (reviewed by Mitsch et al. 1999). $C_4$-dicarboxylates in bacteroids are oxidized dominantly via the TCA cycle producing energy and some intermediates. To maintain the TCA cycle with only $C_4$-dicarboxylates as the sole carbon source, a pathway to generate acetyl-CoA is essential. Acetyl-CoA is readily produced from pyruvate under the catalysis of pyruvate dehydrogenase. Although several enzymatic pathways exist in rhizobial cells, malic enzymes are the dominant means for production of pyruvate from malate in bacteroids (Mitsch et al. 1999). In *B. japonicum* bacteroids, the activity of β-hydroxybutyrate dehydrogenase increases in parallel with the PHB accumulation and nitrogenase activity. Besides supplying C4-dicarboxylic acids to bacteroids, in the infected and uninfected nodule cells, a significant

percentage of the glucose-6-phosphate is used to form starch, stored in the amyloplasts. Considering the amount of the carbohydrate consumed during nodule function, it is estimated that the cost of fixing one gram N in a soybean nodule is 12.1 gram of carbohydrate. Of this 60 % is spent in $N_2$ fixation and $H_2$ evolution (reviewed by Werner 1992).

### 2.3.4 Oxygen Protection and Supply

In legume nodules, the average $O_2$ concentration is very low. The legume nodules exercise physiological control over the $O_2$ concentrations in their infected cells by adjusting their permeability to $O_2$ diffusion. Generally speaking, the nodules have a two-stage mechanism to control the $O_2$ diffusion: coarse control by the nodule inner cortical barrier and fine control through an innate, homeostatic control in the infected cells (Layzell 1999). The nodule parenchyma has very few and small intercellular spaces. Most of the $O_2$ and other gases must enter the nodule by diffusing through the intercellular spaces. Oxygen diffusion is about $10^4$ times faster through air than through water. The plant has the capacity to allow these spaces to fill with water or to keep them partially or maximally emptied of water. The concentration of some organic and inorganic components in the water-filled diffusion barrier is also a part of the diffusion resistance. The potassium concentration in effective and ineffective nodules of soybean is significantly different. In the nodule parenchyma, some nodulin genes such as GmENOD2 and GmENOD13 are expressed, and these protein products might contribute to the development of the oxygen diffusion barrier (Mylona et al. 1995; Hirsch 1992). The leghemoglobin concentration is very high in the infected cells of nodule central tissue. Leghemoglobin, as a late nodulin constituting up to 20% of the total soluble nodule protein, plays a large role in the nodule $O_2$ concentration control. Leghemoglobin is an oxygen carrier protein and can pass $O_2$ very easily from one molecule to another. Leghemoglobin can facilitate $O_2$ diffusion into nodule central tissue. Depending on the capacity of the leghemoglobin, the bacteroids can be provided with sufficient oxygen to generate the required energy, within an overall low oxygen environment (Werner 1992).

Besides the structural oxygen diffusion barrier, physiological substances are responsible for protection of the dinitrogrnase from activated oxygen damage due to both $O_2$ and $H_2O_2$. The leghemoglobins contained in the nodule produce activated oxygen species through spontaneous autoxidation. Adding ascorbic acid, a reducing agent and antioxidant, by irrigation and/or stem infusion, can increase nitrogenase activity, nodule leghemoglobin content, and the activity of ascorbate peroxidase, whereas the concentration of lipid peroxides, an indicator of oxidative damage and onset of senescence, is decreased. The treatment allows nodules to maintain a greater capacity to fix $N_2$ over longer periods (Bashor and Dalton 1999).

Legume plant nodulation and the nodule function are influenced by many ecological factors, both abiotic and biotic. The abiotic factors include combined nitrogen, pH, temperature, water status, salinity and soil texture. The biotic factors include the rhizobial population density in the rhizosphere, the presence of other rhizosphere microbes and the interactions between the plant and the various rhozobacteria, as well as between the plants and the rhizobia themselves (Vlassak and Vanderleyden 1997; Zahran 1999). Of the abiotic factors, low temperature strongly inhibits all stages of soybean nodule formation and function (Lie 1981). In a short season region, such as the Canadian soybean growing area, soil temperature at 10 cm during the first two months of the growing season is often between 10 to 15 $^{o}$C. Thus low root zone temperature (RZT) is one of the prime limiting factors in soybean production in such areas (Zhang and Smith 1994). Of the biotic factors, the interactions between rhizobia and other rhizobacteria affect legume nodulation and nitrogen fixation positively or negatively. Some rhizobacteria produce antibiotics or enzymes that inhibit rhizosphere colonization by other rhizosphere bacterial populations. Antibiotic-synthesizing *Sinorhizobium* has advantages when used as a soil inoculant for soybean (Triplett 1998). Adding nodulation gene-inducing flavones into the bacterial inoculants can enhance the grain yield and protein yield of legumes (Smith and Zhang 1996). When rhizobacterial strains that produce flavonoid-like compounds are co-inoculated with effective *Rhizobium* strains, chickpea growth, nodulation and nitrogen fixation were significantly increased (Parmar and Dadarwal 1999). Whereas when *Serratia marcescens* chiB gene was cloned into

*Sinorhizobium fredii* USDA191 and *Sinorhizobium meliloti* RCR2011, chitinolytic activity was detected and the nodule formation of soybean cultivar McCall was delayed and nodule number was markedly decreased. This negative effect may be due to the hydrolysis of lipo-chitooligosaccharides (Krishnan et al. 1999). The search for strains of rhizobacteria and bacterial endophytes or their products, which promote legume nodulation and nitrogen fixation directly or indirectly, is one of the potentially important ways to improve biological nitrogen fixation in sustainable agricultural systems.

## 2.4 Commercial Legume Inoculants

Legume inoculants provide a means of transporting superior symbiotic nitrogen-fixing rhizobia from fermentor to the legume field. Commercial legume crops in agricultural production are often aided in terms of nitrogen fixation through the application of legume inoculants. Production of legume inoculants on a commercial scale commenced in the UK and the USA at the end of the 19$^{th}$ century and greatly expanded between 1929 and 1940 in the USA. In Canada the use of legume inoculants began in 1905 (Thompson 1980). Although there are several types of inoculant products available, peat based inoculants have been the standard for the inoculant industry (Smith 1992). The USA has probably remained the largest legume inoculant producer in the world, but commercial production is now carried on in all continents in both developed and developing countries (Thompson 1980). Production of commercial inoculants has now become a big business in the United States and internationally (Pepper 2000).

### 2.4.1 Desired Characteristics of Rhizobial Strains for Legume Inoculants

In legume inoculant production, single bacterial strain inoculants are recommended. It is generally felt that the rhizobial strain used in inoculant production should have the following 5 basic characteristics: infective (capable of causing nodule initiation and development), effective (capable of efficient nitrogen fixation), competitive (capable of causing nodule initiation in the presence of other rhizobia), persistent (capable of surviving in soil between crops in successive years) and suitable for inoculant production

(Thompson 1980; Pepper 2000). One attribute of inoculant quality is the number of cells per gram of material. This has varied from $1\times10^7$ up to $4\times10^9$ viable rhizobia per gram. In addition, the inoculant should contain only one strain and should be without contaminating organisms. In the USA, the standard for number of viable bacteria in legume inoculant products is $10^7$- $10^8$ rhizobia per g inoculant (Smith 1987). Canadian standards specify that the inoculant must contain sufficient viable cells for nodule induction when used according to the manufacturer's direction. That is at least the following number of viable cells per seed: $10^3$ per seed for small seeded forage legumes such as alfalfa, clover and birdsfoot trefoil, $10^4$ per seed for sainfoin, and $10^5$ per seed for large-seeded legumes such as bean, pea and soybean (Smith 1992).

### 2.4.2 Legume Inoculant Production

After the desired strain has been selected, the main steps in inoculant production include culture production, carrier preparation, product mixing and curing, and inoculant packaging (Smith 1987).

In legume inoculant preparation, large-scale culture is always carried out with fermentation vessels under aerated conditions. Either the strains from the slow growing *Bradyrhizobium* or the fast growing *Rhizobium* are generally cultured in mannitol yeast extract medium (YEM) (Thompson 1980; Somasegaran and Hoben 1994). At 28 °C, production of the fast growing types requires 3 – 5 days and for the slow growing 5 –10 days, to reach a cell population of $1 - 5 \times 10^9$ cell/ml (Burton 1979; Thompson 1980) even in YEM with a higher yeast extract content. It is a long culture period, although not the longest of any bacterial species.

In contrast to other popular bacterial media, YEM has a high carbon to nitrogen ratio; mannitol, the carbon source, is in great surplus (Somasegaran and Hoben 1994). Yeast extract is the only nitrogen source in YEM. The yeast extract concentration in YEM is variable, ranging from 0.4 to as high as 10 g/l (Thompson 1980; Smith 1987; Somasegaran and Hoben 1994). A higher yeast extract content is good for rhizobial growth, however, when the concentration is higher than 3 g/l, the physiologic activities

and symbiotic competency of the bacterial cultures, including nodulation and nitrogen fixation capability, are greatly decreased (Thompson 1980). The situation is the same when amino acids are added to YEM (Strijdom and Allen 1966). Given this situation, there has been continuous publication regarding methods to improve rhizobial culture technology.

Balatti et al. (1991) obtained a high cell concentration in a closed batch culture system using a medium containing yeast extract, glycerol and salts. In order to utilize an inexpensive carbon source during production of *Bradyrhizobium* strains for use in inoculants, Lie et al. (1992) developed a double fermentation technology in which sucrose was prefermented by yeast, and then *Bradyrhizobium* CB756 was inoculated into diluted prefermented medium. This resulted in a high density of bacterial cells. Kanuma (1997) developed a method wherein hot water extract of the host seed hulls or husks was added into the culture medium for better growth of *Rhizobium* strains. Grassano et al. (1999) reported that addition of amaranth seed meal (4 g/l) to YEM, as a replacement for yeast extract, was suitable for culture of both *Bradyrhizobium* and *Rhizobium* strains and the bacterial symbiotic activity was not affected. There has also been work focused on improving the culture methods of rhizobia by adopting continuous culture and solid state fermentation (Smart et al. 1984; Graham-Weiss et al. 1987; Boehnel and Bruns 1992). During the culture of *Lactobacillus*, addition of RNA to the culture medium increased the biomass produced by 30% (Houde 1999). Nucleoside derivatives, including nucleosides, nucleotides and ribonucleic acid, have also been added into the bacterial culture medium as biosynthesis precursors (Nakao 1979; Garcia de Salamone et al. 2001) or nutrient substrates (Schuch et al. 1999). While RNA could be used as a nitrogen source during the culture of rhizobia, this is never done. Addition of RNA, as well as other nucleoside derivatives, to rhizobial culture media should be examined as a way to improve the slow growth of *Bradyrhizobium* strains in culture. In regard to the 5 basic characteristics mentioned above, for research into inoculant production technology, any improvement should be able to maintain or improve the bacterial symbiotic activity while allowing the bacterial growth rate to be increased. A method only proven to increase in the bacterial growth rate is not satisfactory for industrial inoculant production.

## 2.5 Plant Growth Promoting Rhizobacteria (PGPR) and Legume Crops

All bacteria that can actively colonize in the rhizosphere and increase plant growth could be termed as plant growth promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). Experimental field evaluations of PGPR have indicated that they can increase crop yield by over 20% (Kloepper et al. 1991). Some commercially available PGPR based inoculants are being applied to agriculture production systems now (Ryder and Jones 1990; Turner and Backman 1991; Chen et al. 1996; Ryder et al. 1999).

PGPR can affect plant growth either indirectly or directly. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms. The direct promotion of plant growth by PGPR, for the most part, is due to either providing the plant with a compound synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment (Glick 1995).

The research on the effects of rhizobacteria and other microflora on the rhizobia and legume plants begun in 50's of the last century (Robison 1946; Hely et al. 1957; Anderson 1957; Holland and Parker 1966; Barnet 1980). In 1965, Subba-Rao isolated fungi from 9 legume plants belonging to the genera *Trifolium, Melilotus, Trigonella, Cicer, Pisum, Glycine and Phaseolus* and their effect on rhizobia was investigated (Chhonkar and Subba-Rao 1966). The mycoflora of the nodules consisted of *Cephalosporium, Alternaria, Aspergillus, Penicillium, Rhizopus, Acrothecium, Fusarium, Rhizoctonia, Curvularia, Pythium and Trichoderma.* The nodule flora was different from that of the normal roots. Mycocolonization of *Cephalosporium* sp. in soil significantly decreased the fresh weight and nitrogen status of *Trifolium alexandrinum*, while the total number of nodules formed was not significantly decreased. Some soil fungi produce antibiotics and are antagonistic to rhizobia. After their work, few reports on this aspect were published.

Fuhrmann and Wollum (1989) investigated *in vitro* growth responses of *Bradyrhizobium japonicum* strains to soybean rhizosphere bacteria. Of the 115 rhizosphere bacteria tested, 23 strains inhibited one or more *B. japonicum* strain(s). All

of the rhizosphere bacteria that inhibited bradyrhizobia produce fluorescent, yellow-green, diffusible pigments. Nine of these inhibitory strains were pseudomonas. The inhibitory effects of *Pseudomonas* spp. on bradyrhizobial growth might be caused by siderophore-induced ion deprivation. The innate ability of rhizobacteria to antagonize (brady)rhizobia is not manifested in soil systems that contain indigenous microbial populations (Grimes and Mount 1984; Polonenko et al. 1987). Polonenko and coworkers tested the effects of root colonizing bacteria on nodulation of soybean roots by *B. japonicum*. Eighteen strains were isolated, 17 of them belonged to *Pseudomonas* and 1 *Aeromonas*. All the 17 *Pseudomonas* strains antagonized the growth of *B. japonicum* strain USDA110 on agar, 4 of these strains reduced nodulation in a soil-less medium. However none of the 17 strains which exhibited antibiosis to *B. japonicum* interfered with nodulation of soybean in field-soil containing medium. Six of these strains stimulated nodule mass formation by *B. japonicum* strain USDA110. So the conclusion seems to be that root colonizing bacteria do not interfere with nodulation of soybean by *B. japonicum* when the soybean plants are grown in field soil and in certain cases may actually stimulate both nodulation and plant growth.

### 2.5.1 The Effect of PGPR on the Legume Plant Pathogen Resistance

Selected strains of nonpathogenic rhizobacteria can induce a systemic resistance in plants that is effective against pathogens. Inoculation of PGPR *Pseudomonas fluorescens* (S97) onto seeds can induce disease resistance in common bean susceptible to the halo blight bacterial pathogen (Alstrom 1991). Bean plants inoculated with certain pseudomonas strains can increase the levels of some acid-soluble proteins in the leaf extracts of the plants (Zdor and Anderson 1992). *Pseudomonas aeruginousa* 7NSK2 is an effective biocontrol agent of the root pathogen *Pythium splendens* in tomato. Under iron-limiting conditions, this strain produces the siderophores: pyoverdin, pyochelin and salicylic acid (SA), which are responsible for the induced systemic resistance (ISR) (Buysens et al. 1996). It has been demonstrated that SA production by *P. aeruginousa* 7NSK2 is also essential for induction of resistance to *Botrytis cinerea* in bean. Root treatment with

7NSK2 culture in King's B medium can reduce the number of spreading *B. cinerea* lesions on bean leaves by more than 50%, but whether the root treatment interferes with the colonization and nodulation on bean root by *Rhizobium* was not mentioned (Meyer and Hofte 1997).

Alfalfa seedling damping-off is caused by *Phytophthora megasperma* f. sp. medicaginis. *Bacillus cereus* UW85 was isolated from the roots of field-grown alfalfa and selected from 700 isolates. This stain can reduce seedling mortality to zero. Both fully sporulated cultures containing predominantly released spores and sterile filtrates of UW85 cultures are effective in protecting seedlings from damping-off (Handelsman et al. 1990). *Scleotium rolfsii* is a widespread pathogen of several crops. Two hundred and three different bacterial strains were isolated from the rhizosphere of bean, peanut and chickpea plants grown in *Scleotium rolfsii* infected soil. A *Serratia marcescens* strain from the isolates can effectively control the *Scleotium rolfsii* disease. This strain even reduced the damping-off incidence of bean, caused by *Rhizoctonia solani*, by 50% (Ordentlich et al. 1988). *Serratia marcescens* can produce an inducible extracellular chitinase (Monreal and Reese 1969). The biocontrol ability of this *Serratia marcescens* strain to *Scleotium rolfsii* seems to be due to its production of chitinase (Ordentlich et al. 1988).

### 2.5.2 The Effect of PGPR on Legume Plant Growth, Nodulation and Nitrogen Fixation

#### *Azospirillum* and *Azotobacter*

*Azospirillum* sp. is always associated with roots of grasses and other economically important plants. Inoculation of *Azospirillum* in crop plants can increase grain yields (Subba Rao 1979; Vlassak and Reynders 1979; Purushothman et al. 1980). When *Azospirillum brasilense* was inoculated onto soybean seeds, both roots and nodules were colonized. Seed inoculation with *A. brasilense* alone in unsterilized pot soil increased nodulation and yield. However inoculation with *B. japonicum* and *A. brasilense* in combination generally increased yield, but not always significantly (Singh and Subba Rao

1979). The effect of combined inoculation of *Azospirillum* and *Bradyrhizobium* on legume plant nodulation and nitrogen-fixation depends on the genotype competence, especially the genotype of *(Brady)rhizobium* (Iruthayathas et al. 1983; Rai 1983). The cell-ratio of the two bacterial partners is a key factor for manifestation of *Azospirillum* positive effects on nodulation of clovers by *Rhizobium* strains (Plazinski and Rolfe 1985). When the cell concentration of *A. brasilense* was $10^5$ to$10^7$cfu/ml, coinoculation of *R. meliloti* ($10^6$ cfu/ml) with *A. brasilense* to pouch-grown seedlings of *Medicago polymorpha* increased nodulation substantially. Whereas when cell concentrations of *A. brasilense* above or bellow $10^5$ to$10^7$cfu/ml, resulted in much smaller nodulation increases (Yahalom et al. 1987).

*Azotobacter vinelandii* strains are effective in promoting nodulation of leguminous plants, such as *Glycine max, Vigna unguiculata* and *Trifolium repens*. Increased nodulation due to inoculation of *A. vinelandii* also occured in field-grown soybean. The nitrogen fixation ability of *A. vinelandii* is not responsible for its nodulation enhancement. Cell-free extracts and non-viable cell preparations were still active in nodulation enhancement (Burns et al. 1981).

***Bacillus***

*B. cereus* UW85 can enhance soybean nodulation under both field and growth chamber conditions (Halverson and Handelsman 1991). But, in field experiments, increased nodulation of UW85-treated plants appears to be transient: differences only occurred between 25 – 35 days after planting. At 49 days after planting and later, there were no differences in nodulation among treatments observed. Under laboratory conditions, the nodulation increase was only significant at $P = 0.1$. Seed treatment with UW85 can increase acetylene reduction activity by 25 – 73%, as compared with that of the untreated control. In any case, UW85 seed treatment can increase nitrogen availability to the plants and therefore improve plant growth.

Srinivasan et al. (1996) isolated 22 *Bacillus* spp. from the rhizosphere of *Phaseolus vulgaris*. Some of these strains can produce significant amounts of the

phytohormone indoleacetic acid (IAA). The IAA-producing isolates promote root growth and/or nodulation when co-inoculated with *Rhizobium etli* on *Phaseolus vulgaris* under gnotobiotic conditions in a growth chamber. The co-inoculations resulted in increased nodule number, nodule fresh weight, nitrogenase activity, leghemoglobin content and total soluble protein content of the root nodules of the plants.

*B. subtilis* is one of the PGPR strains that promote the growth of several kinds of crop plants. When *B. subtlis* was used to treat peanut seeds, yield increases ranged from -3.5 to 37 %. Only 2 of the 24 tests had negative results. The treatment of peanut seeds with *B. subtilis* was associated with improved germination and emergence, increased nodulation by *Rhizobium* spp., enhanced plant nutrition and increased root growth (Turner and Backman 1991). *B. polymaxa* (H5) is a phosphate-solubilizing bacterium; when co-inoculated with *Rhizobium* on chickpea it can increase the available phosphorus content of soil, plant dry matter content, grain yield, and nitrogen and phosphorus uptake (Alagawadi and Gaur 1988). An antibiotic-producing *Bacillus* sp., when inoculated into soil, can enhance the colonization and the nodulation of soybean roots by *Bradyrhizobium japonicum*. In some tests, the dry weight and seed yield of soybean plants were increased as a result of this treatment (Li and Alexander 1988).

***Pseudomonas***

Seven *Pseudomonas* strains, including *P. fluorescens* G12-22, *P. putida* G2-8, G2-26, G8-4, G8-32, G11-32 and G14-21, were tested on a single cultivar of lentil and pea in the field. None of the strains had any effect on the growth of pea. Inoculation of G2-8 and G11-32 onto lentil resulted in higher root and shoot dry weight and greater acetylene reduction activity than the control. Even for G2-8 and G11-32, the positive effects were not stable and seemed to be dependent on the cultivar and cultivation conditions (Chanway et al. 1989).

*P. syringae* R25 and *P. putida* R105 were tested on pea and field bean in order to assess their influence on growth and acetylene reduction activity. Inoculation of pea with *P. syringae* R25 or *P. putida* R105 alone had no effect on plant growth in pouches. In

soil, however, the isolate R25 inhibited the nitrogenase activity of nodules formed by indigenous rhizobia; strain R105 stimulated pea seedling emergence and nodulation. *P. syringae* R25 inhibited the growth of bean plants in either plant-growth system. *P. putida* R105 had no effect on bean plants in pouches, but reduced plant root biomass and nodulation by indigenous rhizobia in soil. Co-inoculation of pea seeds with *R. leguminosarum* and either of the pseudomonas strains increased shoot, root, and total plant weight in growth pouches ($P < 0.01$), but had no effect in soil. Co-inoculation of field bean with *R. phaseoli* and *P. putida* R105 had no effect on plant biomass in both growth pouches and soil, whereas nodule number and acetylene reduction activity was increased ($P < 0.01$) in the soil. In contrast, co-inoculation of bean with rhizobia and *P. syringae* R25 had severe deleterious effects on seedling emergence, plant biomass, and nodulation in soil and in growth pouches (De Freitas et al. 1993). But it is reported that *P. putida* M17 markedly increased nodulation of common bean, although bean yield and shoot fresh weight were not increased (Grimes and Mount 1984).

*Pseudomonas* sp. CRP55b, when co-inoculated with *Rhizobium* sp. Ca181 and Ca313, increased the chickpea nodule weight, root and shoot biomass and total plant nitrogen. The nodule stimulating pseudomonas enhanced the levels of flavonoid-like compounds in roots. Ethanol extracts of culture supernatant fluids, when applied to seeds, resulted in enhancement of flavonoids in roots and it may be due to the ethanol soluble factor that the bacteria promote nodulation. These rhizobacteria produced fluorescent compounds with an absorption maximum at 252 nm, similar to those of plant flavonoids, but with different Rf values (Parmar and Dadarwal 1999).

*Pseudomonas* strains that produce toxins inhibit winter wheat root growth and the growth of several microorganisms, but do not inhibit pea root growth. When a toxin producing *Pseudomonas* sp. was co-inoculated with *R. leguminosarum*, it colonized roots more rapidly and in greater amounts than rhizobia and the number of nodules formed was greater, but nodule dry weight and pea shoot biomass were not increased (Bolton et al. 1990). *P. strita* used as a phosphate-solubilizing bacterium on chickpea (*Cicer arietinum)* had a positive effect on dry matter content, nitrogen and phosphate uptake and grain yield (Algawadi and Gaur 1988). Inoculation of an antibiotic-producing

*Pseudomonas* sp. into the soil can enhance the colonization of the alfafa rhizosphere by *R. meliloti* during the early period of plant growth (Li and Alexander 1988).

***Serratia***

*S. proteamaculans* 1-102 and *S. fonticola* 2-114 were tested on lentil and pea (Chanway et al. 1989). In the field, 1-102 increased the nodule number for lentil by 50 %, but had no effect on emergence, vigor, acetylene reduction, root weight and shoot weight; 2-114 increased the acetylene reduction ability by about 150%, but had no effect on other variables. Inoculation of 1-102 or 2-114 had no effect on pea either in the field or in the laboratory. In our laboratory, nine PGPR strains were tested for their ability to reduce the negative effect of low root zone temperature (RZT) on soybean under 25, 17.5 and 15 °C controlled RZT conditions in the greenhouse (Zhang et al. 1996b, 1997). Among the 9 strains tested, the 2 *Serratia* types performed best: *S. proteamaculans* 1-102 at 17.5 and 15 °C, *S. liquefaciens* 2-68 at 25 °C. At 15 °C RZT, inoculation of soybean seedlings with 1-102 increased nitrogen fixation approximately 46%; at 25°C RZT, plants inoculated with 2-68 had high levels of fixed nitrogen. The onset of nitrogen fixation by plants inoculated with 1-102 or 2-68 began 2-3 days earlier than that of control plants under 15 or 25 °C RZT conditions. At 25 °C RZT, inoculation of 2-68 increased plant total dry weight, leaf number and area, and the photosynthetic rate by 24 - 25%. At 15 °C RZT, inoculation with 1-102 increased plant leaf area and plant dry weight, as well as plant photosynthetic rate by 30 - 37 % (Zhang et al. 1997). In a field experiment in an area with low spring soil temperatures, co-inoculation of soybean with *B. japonicum* and 2-68 or 1-102 increased soybean grain yield by 23 or 29%, respectively, and protein yield by 60 or 50%, respectively, when the soil was fumigated with methyl bromide prior to seeding. PGPR applied to cultivars with high yield potentials were more effective (Dashti et al. 1997). It was also demonstrated that their culture supernatants had the same beneficial effects on soybean plants as the bacterial broth culture, although not under sub-optimal conditions (Dashti et al. 1997). Combined effects of the PGPR and genistein on

nitrogen fixation in soybean at suboptimal root zone temperatures were more obvious than only application of either the PGPR or genistein (Dashti et al. 2000).

***Streptomyces***

*Streptomyces* is a genus of actinomycete, which always produces chitinase. *S. grieseus* ATCC 10137 can also produce the antibiotic streptomycin. With co-inoculation of this strain and *R. meliloti* or *B. japonicum* resistant to the products of the actinomycete, nodulation of alfafa by *R. meliloti* and of soybean by *B. japonicum* was little or not affected. But, when *S. grieseus* and chitin were combined to treat seeds, both alfafa and soybean colonization and nodulation by *R. meliloti* or *B. japonicum* were enhanced. This treatment also increased dry weight and N content of alfafa and dry weight of soybean (Li and Alexander 1988).

## 2.6 Endophytic Bacteria: Potential Plant Growth Promoting Bacteria

During the growth process, plants are involved in constant interactions with various kinds of microbes. All the microbes interacting with plants are termed as plant-associated microbes. Some of them are epiphytic or plant surface associated, colonizing the rhizosphere (rhizoplane) and/or phyllosphere (phylloplane). The others are the endophytic, colonizing inside plant tissues. Both epiphytic and endophytic microbes, but not all, have been shown to have beneficial interactions with plants, and these are termed beneficial plant-associated microbes (Smith and Goodman, 1999; Andrews and Harris, 2000). Recently, endophytic bacteria have drawn particular attention for their strong potential in acting as plant growth promoting bacteria (PGPB) (Chanway 1996; Hallmann et al.1997; Kozyrovska et al. 1997; Sturz et al. 2000).

### 2.6.1 Definitions of Endophytic Bacteria

The concept of 'endophytes' has evolved. Its precise definition is still being debated by the scientific community (Sturz ,2000; Kobayashi and Palumbo 2000). The term was initially coined by De Barry in 1866. Later it was used specifically to refer to a group of fungi that invade the stems and leaves of plants but cause no symptoms of disease (Wennstrom 1994). Now a proposed clarified definition of endophytes is 'fungi or bacteria which, for all or part of their life cycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues but cause no symptoms of disease.' (Wilson 1995). Gradually, it has been realized that bacterial colonization of internal regions of the plant, including the vascular system, is a common phenomenon. In order to make this concept more logical and more definitive, it has been proposed to eliminate the term 'endorhizosphere', which resulted in conceptual overlap between the rhizobacteria and endophytic bacteria (Kloepper et al. 1992). However, among all the reported endophytic bacteria, only some have been shown to colonize the inside of the host plant tissues (Bailey et al. 1992; Dong et al. 1994; Bacon and Hill 1996; Quadt-Hallmann et al. 1996; Barraquio et al. 1997). Even for them, the precise endophytic stages have not been established in bacterial life cycles (Kobayashi and Palumbo 2000). Given such a confused situation, a much more practical definition for endophytic bacteria has been proposed: 'those bacteria that can be isolated from surface-disinfected plant tissue or extracted from within the plant, and that do not visibly harm the plant.' (Hallmann et al. 1997).

### 2.6.2 Ubiquity of Endophytic Bacteria in Plants

Bacteria have been proposed to exist inside plant without causing disease symptoms for over 50 years (Tervet and Hollis 1948; Hollis 1951). Endophytic bacteria can colonize within host tissue imtercellularly or even intracellularly and therefore are able to form a more intimate relationship with the host plant than most epiphytic strains. Endophytic bacteria have been isolated from flowers, fruits, leaves, stems, roots and seeds of various

plants. Examples of these include alder, aspen, cauliflower, cherry, citrus, corn, cotton, cucumber, elm, grape pine, kallar grass, lemon, maple, oak, peach, pear, potato, pine, rice, *Sinapis*, sorghum, squash, sugar beet, sugar cane, sunflower, tomato, watermelon, and yam (Mundt and Hinkle 1976; Reinhold-Hurek and Hurek 1998; Kobayashi and Palumbo 2000; Sturz et al. 2000). Endophytic bacteria are ubiquitous in most plant species, residing latently or actively colonizing plant tissues locally as well as systemically (Hallmann et al. 1997). Even though endophytic bacteria form nonpathogenic relationships with their hosts, the influence on host growth and development by endophytic bacteria can generally be categorized by effect: (1) plant growth promoting, (2) plant growth inhibiting and (3) plant growth neutral (Sturz et al. 2000).

Diversity associated with endophytic bacteria exists not only in the plant species but also among bacterial taxa. Mundt and Hinkle (1976) identified 46 different bacterial species from the 27 plant species surveyed. From the stem and root of cotton and sweet corn, McInroy and Kloepper (1995) discovered 50 different bacterial species and 46 unidentified bacterial isolates. However, certain trends were apparent with predominant bacterial types isolated as endophytes from a given plant species. Many endophytic bacterial species had previously been identified as plant-associated bacteria (Kobayashi and Palumbo 2000).

### 2.6.3 Endophytic Bacteria in Legumes and Root Nodules

In legumes, endophytic bacteria have been reported in alfalfa (Gagne et al. 1987), clover (Philipson and Blair 1957; Sturz et al. 1997), pea (Elvira-Recuenco and van Vuurde, 2000) and soybean (Manninger and Antal 1970; Oehrle et al. 2000). The bacteria isolated from within legume tissues were various. They belong to the following genera: *Aerobacer* (Philipson and Blair 1957), *Aeromonas* (Oehrle et al. 2000), *Agrobacterium* (Sturz et al. 1997; Oehrle et al. 2000), *Bacillus* (Philipson and Blair 1957; Sturz et al. 1997; Oehrle et al. 2000; Elvira-Recuenco and van Vuurde 2000), *Chryseomonas* (Oehrle et al. 2000), *Curtobacter* (Sturz et al. 1997), *Enterobacter* (Sturz et al. 1997), *Erwinia*

(Gagne et al. 1987), *Flavimonas* (Philipson and Blair 1957; Oehrle et al. 2000), *Pantoea* (Elvira-Recuenco and Vuurde 2000), *Pseudomonas* (Gagne et al. 1987; Sturz et al. 1997) and *Sphingomonas* (Oehrle et al. 2000).

In 1957, Philipson and Blair reported bacteria, including *Aerobacter cloucae, Bacillus megatherium* and *Flavobavterium rhenanus*, existing in clover roots. In alfalfa, 387 isolates were obtained from the roots and xylem of healthy field grown plants. Of these 52% were *Pseudomonas* spp, especially fluorescent species, and 23% were *Erwinia*-like bacteria. Bacterial populations ranged from $6.0 \times 10^3$ to $4.3 \times 10^4$ cfu/g of fresh xylem. They were all normal residents of the root xylem. The main route for them to enter the xylem may be via natural wounds (Gagne et al. 987). In soybean seeds, *Bacillus subtilis* was commonly isolated from the surface disinfected materials (Tenne et al. 1977). In field growing pea, at the flowering stage, five of the 11 tested cultivars showed bacterial colonization within the stems. For most stems, endophytic bacteria existed at a population density of $10^4$ to $10^5$ cfu/g. The populations decreased from the lower to the upper part of the stems (Elvira-Recuenco and Vuurde 2000).

In legume plants, endophytic bacteria other than rhizobia have also been shown to be present in the root nodules. As early as 1902, Beijerinck and Van Delden reported the detection of *Agrobacterium radiobacter* in clover root nodules (reviewed by Sturz et al. 1997). Manninger and Antal (1970) also reported rhizobia and other bacteria in the root nodules of Leguminosae species. Recently it was reported that 29 non-rhizobia bacterial species were isolated from clover tissues and 15 of these resided in the root nodules. Of this 15, eight were nodule specific (Sturz et al. 1997).

### 2.6.4 Endophytic Bacteria Promoting Plant Growth

By residing within the plant tissue, endophytic bacteria may gain an advantage by being sheltered from environmental stresses and microbial competition. They are more apt to be involved in a mutualistic relationship with the host plants. In potato, 10% of endophytic bacteria isolated from potato tubers have beneficial effects on potato growth (Sturz and Christie 1995). *Acetobacter diazotrophicus,* a bacterial endophyte of sugar cane, can use the sucrose contained in sugarcane apoplastic fluids to produce acids and

fix enough nitrogen to supply over 50% of the amount needed for sugarcane growth (Dong et al. 1994). *Azoarcus* sp. in rice and kallar grass, and *Herbaspirillum seropedicae* and *H. rubrisubalbicans* in sugar cane and sorghum are also diazotrophic endophytes (Reinhold-Hurek and Hurek 1998). Endophytic bacteria are a potential group of plant growth promoting bacteria. In red clover, growth promotion or inhibition by endophytic bacteria seems to be dependent upon interactions among internal microflora populations (Sturz and Christie 1995). Of the 29 endophytic species isolated from clover tissues, 15 were tested for their growth promoting effects by being co-inoculated with *Rhizobium leguminosarum*. Three species enhanced nodule number and 3 increased plant total weight (Sturz et al. 1997). In soybean, up to now, plant growth promoting experiments with endophytic bacteria have not been reported.

## 2.7 Conclusions for the Literature Review

Although many references are involved, this literature review offered only necessary, but not all, background information concerned with my research subject. PGPB could promote various aspects of plant growth, including legume nodulation and nitrogen fixation, through a variety of mechanisms. Besides from the rhizosphere, some novel PGPB strains could also be isolated from phylloplane and inside the various plant tissues. Among all the well-demonstrated PGPB strains, in most of the cases, the details of the mechanisms are not understood. The production of bradyrhizobial inoculant is important for improving soybean production. As *Bradyrhizobium* species are slow growing rhizobia, bradyrhizobial culture process is long and needs to be improved. Addition of RNA to the culture medium has been shown to accelerate growth in cultures of other bacterial species, but it is never tested in rhizobial cultures. This literature review gives me confidence in my research program. I believe that (1) the combined use of the selected PGPB and bradyrhizobia in inoculant could promote soybean nodulation and nitrogen fixation; (2) the test on RNA additives in the culture medium may accelerate bradyrhizobial growth and maintain or increase the bacterial symbiotic activity.

## Preface to Section 3

Section 3 is comprised of a manuscript by Bai Y, Pan B, Charles TC and Smith DL and has been submitted to *Soil Biology & Biochemistry*.

Previous work in our laboratory showed that coinoculation of PGPR strains, *Serratia proteamaculans* 1-102 and *S. liquefaciens* 2-68, with *Bradyrhizobium japonicum* increases soybean nodulation, and nitrogen fixation under both optimal and suboptimal root zone temperatures and increases grain and protein yield under field conditions in the short season area where the research was conducted. I conducted two greenhouse experiments, with controlled RZTs to determine the optimal PGPR co-inoculation doses. In one experiment the plants were grown in transparent growth pouches, allowing regular measurements of nodule appearance and development. Data collected from this experiment were used to characterize the effects of the two PGPB strains on the dynamics of soybean nodulation.

The literature cited in this and the following sections are listed together in the reference section at the end of the thesis. Figures and tables for each section are presented at the end of the section.

Of the co-authors, Dr. Pan, a former Ph.D. student of Dr. Smith, helped me to set up and conduct the greenhouse experiments, to collect the data after the plants were harvested and design some tables and figures in the section. Dr. Charles is a professor in Department of Biology, University of Waterloo, who helped design the experimentation reported in this section, and offered guidance during the conduct of the experiments. Dr. Smith is my thesis supervisor. He provided funds, assistance and supervisory guidance from the onset of the research to the reviewing of the manuscripts before submission for publication. He was also responsible for arranging laboratory, bench space in greenhouse, experimental field space, the use of computers, and providing technical assistance. He is the corresponding author for the publication of this section.

# Section 3

## Effects of *Bradyrhizobium japonicum* and plant growth promoting rhizobacteria coinoculation dose at different root zone temperatures on soybean

### 3.1 Abstract

Recent work has shown that two strains of *Serratia* are particularly effective as plant growth promoting rhizobacteria (PGPR) on soybean. However, important details such as the optimum inoculation dose have not been determined. The coinoculation dose effects of *Serratia proteamaculans* 1-102 and *S. liquefaciens* 2-68 with *Bradyrhizobium japonicum* on soybean [*Glycine max* (L.) Merr.] growth, nodulation and nitrogen fixation were investigated under controlled root zone temperatures (RZTs), 25, 20 and 15 °C. The results showed that the growth promoting effects of the two PGPR strains varied with coinoculation dose. The optimal coinoculation dose for both PGPR strains was $10^8$ cells/seedling under all three RZT conditions tested. Under suboptimal RZT conditions, *S. proteamaculans* 1-102 performed better than *S. liquefaciens* 2-68. The coinoculation of PGPR and *Bradyrhizobium japonicum* at their optimal doses increased nodule number, plant dry weight, fixed nitrogen and nitrogen fixation efficiency (NFE).. Analysis of the linear models of the nodulation dynamics made the quantitative description of the nodulation process become possible. The calculated dynamic parameters revealed that optimal PGPR coinoculation shortened the nodule initiation time and increased the average nodulation rate.

**Key words**: plant growth promoting rhizobacteria, coinoculation doses, *Glycine max,* nodulation, nitrogen fixation, nodulation dynamics

## 3.2 Introduction

Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that can actively colonize plant roots and increase plant growth (Kloepper and Schroth 1978). Experimental field evaluations of PGPR have indicated that application of some PGPR strains can increase crop yields by over 20% (Kloepper et al. 1991; Leeman et al. 1995). The application of PGPR can improve crop yield and food quality with little environmental impact. PGPR are a possible alternative to replace the fossil-fuel-based agriculture inputs in the development of sustainable agriculture.

PGPR can increase plant growth by preventing the deleterious effects of phytopathogenic organisms, by providing the plant with a phytohormone or other active compounds, or by facilitating the uptake of certain nutrients from the environment (Glick 1995). Some PGPR strains have been reported to enhance nodulation and symbiotic nitrogen fixation by soybean and other legume plants after being co-inoculated with rhizobia.

Several bacterial genera have been reported to contain PGPR strains that can promote legume growth. These include *Azospirillum* (Singh et al. 1979; Yahalom et al. 1987; Iruthayathas 1983; Groppa et al. 1998), *Azotobacter* (Burns et al. 1981), *Bacillus* (Halverson and Handelsman 1991; Srinivasan 1996), *Pseudomonas* (Grimes and Mount 1984), *Serratia* (Chanway et al. 1989; Zablotowicz et al. 1991; Zhang et al. 1996b) and *Streptomyces* (Li and Alexander 1988). Most experiments with these PGPR were conducted under the optimal growth temperature conditions of the host plants. Only two *Serratia* strains, *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68, were investigated with soybean under both optimal (25 °C) and suboptimal (15 – 17.5 °C) root zone temperatures (RZT) under greenhouse conditions (Zhang et al. 1996b,1997).

Soybean evolved under subtropical conditions and its optimal temperature for growth, nodulation and nitrogen fixation is 25 – 30 °C (Smith 1995). When the temperature is below 25 °C, soybean growth, nodulation and nitrogen fixation are negatively affected. The lower the temperature is, the greater the inhibition. In recent years, soybean production has increased in Canada. The province of Quebec is one of the most northern soybean production areas in North America. During the days following

soybean seeding in Quebec, the soil temperature generally fluctuates around 15 °C. Low soil temperatures during early stages of soybean growth have been shown to be a major factor limiting soybean yield in such short season regions (Whigham and Minor 1978).

It has been demonstrated that when co-inoculated with *B. japonicum*, *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68 have positive effects on soybean growth, nodulation, nitrogen fixation and yield, under either optimal or sub-optimal RZTs in greenhouse (Zhang et al. 1996b, 1997). Even in the field in a short season region, coinoculation of strain1-102 or 2-68 with *Bradyrhizobium* could overcome the low soil temperature inhibition of soybean early growth, and result in increases in both protein production and grain yield (Dashti et al. 1997).

In the agricultural application of bacterial inoculants, the application dose is an important factor that can greatly affect the efficacy of the material. The effects of key environmental factors on the optimum dose are rarely investigated, in spite of its potential importance. In a field experiment with *Bradyrhizobium japonicum* inoculant, it was shown that soybean nodule number and mass, as well as grain yield, were all curvilinearly related to bacterial density from $10^3$ to $10^6$ cells/seed (Hume and Blair 1992). In field experiments, a PGPR strain was applied to several forage species growing in a range of soil conditions. The results showed that the best growth promoting effect of the PGPR could only be obtained with an optimal inoculation dose, and that the optimum dose varied with the soil conditions (Zheng and He 1994). For a tomato growth promoting *Pseudomonas* strain, both the degree of colonization of different tissues and the extent of plant growth promotion depended upon inoculation dose, temperature and plant genotype. The PGPR population on the root surface increased linearly with increasing inoculation density ($10^7$ to $10^8$) and decreased with increasing temperature (10 °C to 30 °C). At 20 °C the growth promotion was best with inoculation densities of 3 to 7 $\times$ $10^8$ cfu/ml for the early maturing cultivars. At 10 °C, the best inoculation density for root colonization and growth promotion was approximately 4 $\times$ $10^8$ cfu/ml (Pillay and Nowak 1997). For *Serratia* strains applied to soybean, several concentrations, from $10^4$ to $10^9$ cfu/ml, were tested under greenhouse and field conditions at optimal root zone temperature (RZT). It was shown that consistent root colonization by the bacteria could be observed at all concentrations of $10^6$ cfu/ml and above, with the maximum root

colonization occurring at $10^8$ and $10^9$ cfu/ml in both greenhouse and field experiments (Zablotowicz et al. 1991). The *Serratia* strains used in this report were previously tested on soybean at an inoculation dose of 108 cells/seedling under both optimal and suboptimal conditions (Zhang et al. 1996b). The objective of this work was to determine whether or not different inoculation doses are needed under different RZT conditions and if higher or lower inoculation doses would be better for overcoming the low RZT inhibitions of soybean nodulation and growth.

### 3.3 Materials and methods

This work was conducted with the soybean cultivar Maple Glen, inoculated with *Bradyrhizobium japonicum* 532C (Hume and Shelp 1990). The two PGPR strains investigated were *Serratia proteamaculans* 1-102 and *S. liquefaciens* 2-68 (Zhang et al. 1996b)

*B. japonicum* 532C was cultured in flasks on a shaker at 200 rev/min, 50 – 75 ml in 250 ml flasks or 100 – 120 ml in 500 ml flasks at room temperature (21 – 28 °C) in YEM culture medium (Vincent 1970). The initial culture time in flasks inoculated from cold slants was approximately 7 d. The subculture time was about 72 h. The PGPR strains were cultured on a shaker at 200 rev/min in flasks, 80 – 100 ml in 250 ml flask or 150 ml – 180 ml in 500 ml flasks at room temperature (21 – 28 °C). The culture medium used was King's Medium B (Atlas 1995). The initial culture time in flasks inoculated with cold slants was approximately 72 h. The subculture time was 24 h. After the bacterial subcultures were harvested, the cell concentration was determined by spectrophotometry at 620 nm for *B. japonicum* (Bhuvaneswari et al. 1980) and at 420 nm for PGPR stains (Dashti et al. 1997).

The experiments were conducted in a greenhouse with an air temperature of 25 ± 2 °C and additional illumination of 300 (mol/$m^2$/s supplied by high pressure sodium lamps (P. L. Light System Canada) for a photoperiod of 16:8 h (day:night). Soybean seeds were surface sterilized in sodium hypochloride (2% solution containing 4 ml Tween 20/l). The seeds were then rinsed with distilled water several times. The seeds were first planted in trays containing Vermiculite and germinated in the greenhouse.

Three or four day old seedlings at the VE stage (Fehr et al.1971) were transplanted into pots filled with Vermiculate, one seedling per pot, or growth pouches (15 ( 16 cm, Mega International, Minneapolis, MN), two seedlings per pouch. In the pouch experiment, the RZT was controlled by water bath systems at 25, 20 and 15 °C respectively. The pot experiment was arranged as a completely randomized design. The pouch experiment was organized following a completely randomized split plot design. The main plots were RZTs. PGPR treatments formed the sub-plots. During the growth process, the plants were watered with modified N-free Hoagland's solution (Hoagland and Arnon 1950), in which $Ca(NO_3)_2$ and $KNO_3$ were replaced with $CaCl_2$, $K_2HPO_4$ and $KH_2PO_4$, to provide a nitrogen-free solution.

The bacterial inoculant was a mixture of *B. japonicum* 532C at $10^8$ cells/ml and PGPR 1-102 or 2-68 at designated cell concentrations. Soybean seedlings were inoculated at the early VC stage by adding 1 ml of mixed inoculant onto the culture medium around the seedling roots in pots, or the root surface in pouches, with a pipette. In the pot experiment the PGPR coinoculation doses were $10^2$, $10^4$, $10^6$, $10^8$ and $10^{10}$ cells/seedling. In the pouch experiment the PGPR coinoculation doses were $10^6$, $10^7$, $10^8$, $10^9$ and $10^{10}$ cells/seedling. The pot experiment and the pouch experiment were each conducted once and each treatment was repeated in 4 pots or 3 pouches. In the controls, PGPR cell suspensions were replaced by distilled water or PGPR culture medium diluted in the same way as that for the highest PGPR dose ($10^{10}$).

In order to investigate changes in the dynamics of the nodulation process due to the imposed treatments, nodule number was counted every 3 – 5 d through the transparent plastic growth pouches. Nodulation dynamic curves were determined by plotting the nodule numbers against days after inoculation (DAI) (Chanway et al. 1989; Srinivasan et al. 1997). For calculation of some dynamic variables that characterize the soybean nodulation process, linear models were selected through trial and error (Mead et al. 1993).

The plants were harvested at 63 DAI. After harvesting, data on nodule number, nodule weight, root weight and shoot weight were collected. All the samples were weighed after not less than 48 h drying at 70 – 80 °C. Dried material was ground with a Moulinex coffee mill (Moulinex Appliances Inc., Virginia Beach, VA, USA). Total

nitrogen was determined by the Kjeldahl method (Kjeltec system, which included digestion system 20 and a 1002 distilling unit, Tecator AB, Hoganas, Sweden). Fixed N was calculated as total N minus average seed N (11.45 mg/seed). The nitrogen fixation efficiency (NFE) was defined as the nitrogen fixed by unit nodule mass or unit nodule nitrogen, and expressed as the ratio of fixed N to nodule weight or to nodule N.

The data were analyzed statistically with the GLM procedure of SAS system (Littell et al. 1991). When analysis of variance indicated differences among means, comparisons among the treatment means were conducted with an ANOVA protected least significance difference (LSD) tests (Steel and Torrie 1980).

### 3. 4 Results

*Coinoculation dose - pot experiment results*

There were differences (P = 0.05) among inoculation doses for soybean nodule number, nodule weight, root weight and shoot weight (Table 3.1). None of the dose levels of the two PGPR strains had negative effects on soybean growth and nodulation. The $10^8$ dose coinoculation treatments of 1-102 and 2-68 increased nodule number by 46.9 and 55.1%, nodule weight by 27.9 and 37.0%, root weight by 29.2 and 35.0%, and shoot weight by 28.6 and 29.1%, respectively. Adding King's Medium B to 532C inoculant (data not shown) was not different from the control of 532C in distilled water. . The results showed that the optimal coinoculation dose in the pot experiment for both PGPR 1-102 and 2-68 is $10^8$ cells/seedling.

*Coinoculation dose - pouch experiment results*

Based on the pot experiment results, the coinoculation doses of both 1-102 and 2-68 in the pouch experiment were $10^6 - 10^{10}$ cells/seedling at all three RZTs. When the results were analyzed over the three RZTs, both RZT and PGPR treatments had significant effects on the measured variables ($0.05 \geq P$); there was no difference between 25 and 20 RZTs, and both these RZTs were superior to 15 RZT (P = 0.01). Potential interactions between RZT and PGPR were not significant, except for the nitrogen fixation efficiency (NFE) expressed in a ratio of fixed N to nodule N (P = 0.01). Given the

objective of this work (see in the Introduction section), the data for each RZT were presented seperately. Like the pot experiment results, adding King's Medium B to 532C inoculant in the pouch experiment had no effect on the measured variables under all three RZT (data not shown).

The effects of PGPR coinoculation doses on nodule number, root weight and shoot weight are shown in Fig. 3.2. Compared with the 532C alone control, none of the coinoculation doses of the two PGPR strains had negative effects on either growth or nodulation under any of the three RZTs. Among the tested coinoculation doses for each PGPR strain, $10^8$ cells per seedling caused the largest increases in plant growth and promoted plant growth most consistently.

At 25 °C RZT (Table 3.2), coinoculation with 2-68 at $10^8$ cells per seedling increased nodule number by 38.6%, root weight by 31.6% and shoot weight by 26.3%. Coinoculation with 1-102 at $10^8$ dose increased nodule number by 37.5%, root weight by 26.3% and shoot weight by 1.3%. . At 15 °C RZT (Table 3.2), coinoculation with 2-68 at at $10^8$ cells per seedling increased nodule number by 31.1%, root weight by 17.6% and shoot weight by 34.5%. At 15 °C RZT coinoculation with 1-102 at $10^8$ dose increased nodule number by 53.8%, root weight by 23.5% and shoot weight by 37.9%. Strain 1-102 performed better than 2-68 under suboptimal RZT. At 20 °C RZT, coinoculation at $10^8$ cells per seedling was effective, and the situation was similar to that at 25 °C RZT. At 25 °C RZT, coinoculation of 2-68 and 1-102 at $10^8$ cells per seedling increased the amount of fixed nitrogen in the pouch experimentby 46.1 and 49.3 % respectively (Fig. 3.3). At 20 and 15 °C RZT, coinoculation of 1-102 at $10^8$ cells per seedling increased fixed N by 63.1 and 180% respectively. However, at 20 and 15 °C RZT, coinoculation of 2-68 at $10^8$ cells per seedling had no effect on the amount of nitrogen fixed, although this coinoculation treatment increased the amount of nitrogen fixed by 46.5 and 94.2% respectively and $10^8$ cells per seedling was more effective than the other tested doses of strain 2-68. So, based on the data for nodulation, growth and nitrogen fixation, $10^8$ cells per seedling was the optimal coinoculation dose for both 1-102 and 2-68 at all the three tested RZT. Other coinoculation doses, especially $10^7$ and $10^9$ cells per seedling, sometimes showed effects but these were not consistent. Compared with the increase in nodule number and nodule weight (data not shown) due to the tested coinoculation

treatments, the amount of fixed nitrogen was increased the most. It is reported in the published literature that some PGPR increase nitrogen fixation by improvement of the nodule nirtrogen fixation efficiency more than nodule number and nodule weight (Groppa et al. 1998). It is obviously reasonable to deduce that the increase in the fixed nitrogen was not only due to a nodule number or nodule mass increase, but also due to an improvement of the nitrogen fixation efficiency (NFE) of the treated plants. NFE was expressed here as fixed N/nodule N and fixed N/nodule weight (Table 3.3). As with other variables $10^8$ cells per seedling provided the most consistent improvement in NFE under all three RZTs. At 25 °C RZT and $10^8$ cells per seedling, coinoculation of 1-102 increased NFE by 26.2 and 44.1 % and coinoculation of 2-68 increased NFE by 23.3 and 30.9% when NFE was expressed as Fixed N/nodule weight and Fixed N/nodule N. At 15 °C RZT and $10^8$ cells per seedling, 1-102 performed better than 2-68. Coinoculation of 1-102 increased NFE by 154.2and 149.7% and coinoculation with 2-68 increased NFE by 75.7and 84.1% when NFE was expressed as Fixed N/nodule weight and Fixed N/nodule N. Adding King's Medium B to the 532C inoculant (data not shown) had no effect on nitrogen fixation.

The interaction between RZT and PGPR treatment was significant for NFE expressed in Fixed N/nodule N ($P = 0.01$). However, this was due to specific treatments, such as 1-102 and 2-68 at $10^8$ to $10^{10}$ cells per seedling at 20 °C RZT; this resulted in higher NFE than the same treatment at 25 °C RZT. At 15 °C RZT, all treatments had lower NFEs than at 25 and 20 °C RZT. However, this did not alter the overall conclusion that $10^8$ cells per ml was the optimum co-inoculation dose for the two PGPRs tested (Table 3.3).

*Soybean nodulation dynamics in the pouch experiment*

Nodulation is a result of interactions between infecting rhizobia and their host plants. In the pouch experiment, the nodulation process was monitored different RZTs for the control and at the optimal coinoculation dose for the two PGPR strains. When nodule numbers were plotted against DAI, the curve describing the nodulation process was S-shaped (Fig. 3.1). Based on these S-shaped dynamic curves, the nodulation process could only be described qualitatively. The whole nodulation process could be divided into

three stages. In the first stage, the nodule number was increased slowly. In the second stage, the nodulation rate, the average increase in the number of nodules per day, increased most rapidly. Most of the nodules were formed at the second stage. In the third stage, the nodulation rate gradually approached to zero, and the maximum nodule number was reached. At the suboptimal (15 °C) RZT, the maximum nodule number was reduced and the nodulation initiation, the appearance of the first nodule, was delayed. The nodulation period, the time span from the first nodule appearance to attainment of maximum nodule number, was not greatly changed by the suboptimal RZT. Coinoculation of the two PGPR strains enhanced the nodulation process by increasing the nodule number. However, except for maximum nodule number, it was very difficult to quantitatively estimate the other dynamic parameters shown in Table 3.4 based on the S-shaped dynamic curves.

The linear regression formulas made quantitative description of nodulation dynamics possible. Based on the linear models, several dynamic parameters of soybean nodulation were calculated (Table 3.4). The selected nodulation dynamic linear model at 25 and 20 °C RZT was different from that at 15 °C RZT. At 25 and 20 °C RZT, the linear model was $P = a + b$ log DAI, in which probit of the nodule number ($P$) was regressed against log of days after inoculation. At 15 °C RZT, the linear model was $Y = a + b$ log DAI. In the equation, $Y$ stands for the accumulated nodule number percentage (by taking the maximum nodule number as 100%) as the nodulation is proceeding.

By comparison of the dynamic parameters of the controls under diffeent RZT conditions, it could be seen that the suboptimal RZT (15 °C) inhibited soybean nodulation initiation ($DAI_{ini}$) and maximum nodule number ($NN_{max}$) greatly, but had less effect on nodulation period (NP) length. At 25 °C RZT, $DAI_{ini}$ was 5.9 (DAI), $NN_{max}$ was 37.3 (nodule/plant) and NP was 46.1 (d). Whereas at 15 °C RZT, they were 19.7, 19.3 and 44.5 respectively. The average nodulation rate, $NN_{max}/NP$, was 0.81 (nodule/day) at 25 °C and 0.43 at 15 °C.

Compared with the controls, PGPR coinoculation resulted in the nodulation initiation about 1, and 1.5 - 2 d earlier under 25 and 15 °C RZT conditions respectively, and the nodulation period longer under both optimal and suboptimal RZTs. PGPR coinoculation increased the $NN_{max}$ ($P = 0.05$), as well as the average nodulation rates.

That could be the apparent reason for the nodule number increase due to the PGPR coinoculation. The average nodulation rates for the PGPR coinoculation were about 1.10 at 25°C and 0.56 (nodules/day) at15 °C, which were 30% greater than the control.

### 3.4 Discussion

The optimal coinoculation dose for two PGPR strains is $10^8$ cells/seedling in both the pot and pouch experiments. This is very similar to the optimal root-colonizing dose reported by Zablotowicz et al. (1991). Our work has also shown that this optimal coinoculation dose is not altered by RZT conditions. Fortunately, previous research on these two PGPR stains used a dose of $10^8$ cells/soybean seedling (Zhang et al. 1996b; Zhang et al. 1997; Dashti et al. 1997).

Coinoculation of the PGPR strains with *B. japonicum* 532C can increase nodule number, nodule weight, fixed nitrogen, plant weight and seed yield, as well as other physiological activities (Zhang et al. 1996b; Zhang et al. 1997; Dashti et al. 1997). In most cases, the positive effect of the PGPR coinoculation with rhizobia in legume plants resulted in an increase of total nitrogen and/or total biomass per plant. This increase was often related to an increase in nodule number and nodule weight. But sometimes, although nodule number and nodule weight were not increased, the increase in total N and/or total biomass still occurred, due to an increase in NFE, that is, the increase of dinitrogenase activity, glutamine synthetase activity and leghaemoglobin concentration in the nodules (Groppa et al. 1998). Our experimental results indicated that, coinoculation of the PGPR stains, especially at the optimal coinoculation dose, increased NFE, as well as the nodule number and weight. Both increases in nodule number and weight, and the increase in NFE contributed to the increase in fixed N. However, the mechanism for increases in nodule number and weight or in NFE remain to be investigated. Besides the increased enzymes and leghaemoglobin, the NFE should also be improved by a longer working duration of the enzymes due to an improved physiological state of the plants.

Plotting nodule number against time always produces an S-shaped curve (Chanway et al. 1989; Srinivasan et al. 1997). Using the model $P = a + b$ log DAI at 25 and 20 °C RZT and $Y = a + b$ log DAI at 15 °C RZT, a linear relationship between nodule

number and time was formulated. The model changes under different RZT conditions showed that nodulation dynamics was greatly affected by the low RZT. Although the biological meanings of the coefficient *a* and *b* are poorly understood, the dynamic parameters calculated by using the linear formulas provided quantitative descriptions and allowed better understanding of the nodulation process. It had already been demonstrated that infection thread development, the time for infection thread to reach the base of the root hair, needs 2.5 days under 25 °C RZT and about 8 days under 15 °C RZT (Zhang and Smith 1994). Compared with the optimal RZT condition, 15 °C RZT also tripled the time needed for nodulation initiation (Table 3-4). PGPR coinoculation promotes infection thread development (Dashti et al. 1997), and this may partly explain why the PGPR coinoculation results in an acceleration of nodulation initiation.

The linear model of the soybean nodulation dynamics was initially used to describe the nodulation process. Whether it is generally applicable needs to be verified. It has become clear that the host plant dominates the regulation of the biological nitrogen fixation process (Shantharam and Mattoo 1997). The host plant regulates the nitrogen fixation partly through the regulation of the nodulation process. Plant culture conditions also have strong effects on the nodulation process. In our pot and pouch (at 25 °C RZT) experiments, plant cultivar, bacterial strain, inoculation dose and greenhouse conditions were all same. However the nodule number per plant in pot culture was about twice as much as that in the pouch culture. The linear nodulation model formulated based on the pouch experiment results may be not available to describe the nodulation dynamics of the pot-cultured plants. Our models have offered a better way for describing the nodulation process dynamically and quantitatively, although the methodology still needs to be perfected.

In conclusion, the optimal coinoculation doses for *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68, with regard to promotion of soybean growth, nodulation and nitrogen fixation was $10^8$ cells/seedling. Coinoculation of the PGPR strains in their optimal doses increased nodule number, nodule weight, plant weight, and fixed nitrogen. The changes due to RZT conditions did not alter the optimal coinoculation doses, although it strongly affected the nodulation dynamics. Interestingly, coinoculation of these two PGPR strains

increases nitrogen fixation not just through increases in nodule number and weight, but also through an improvement of the physiological activity of the nodules, i.e., the nitrogen fixation efficiency (NFE).

**Fig. 3.1. Effects of coinoculation of PGPR strains in $10^8$ cells per seedling with *Bradyrhizobium japonicum* 532C on soybean nodulation dynamics under various root zone temperatures (RZTs)**

Each data point in the graphs represents a mean and is given ± 1 unit of standard error. n = 3.

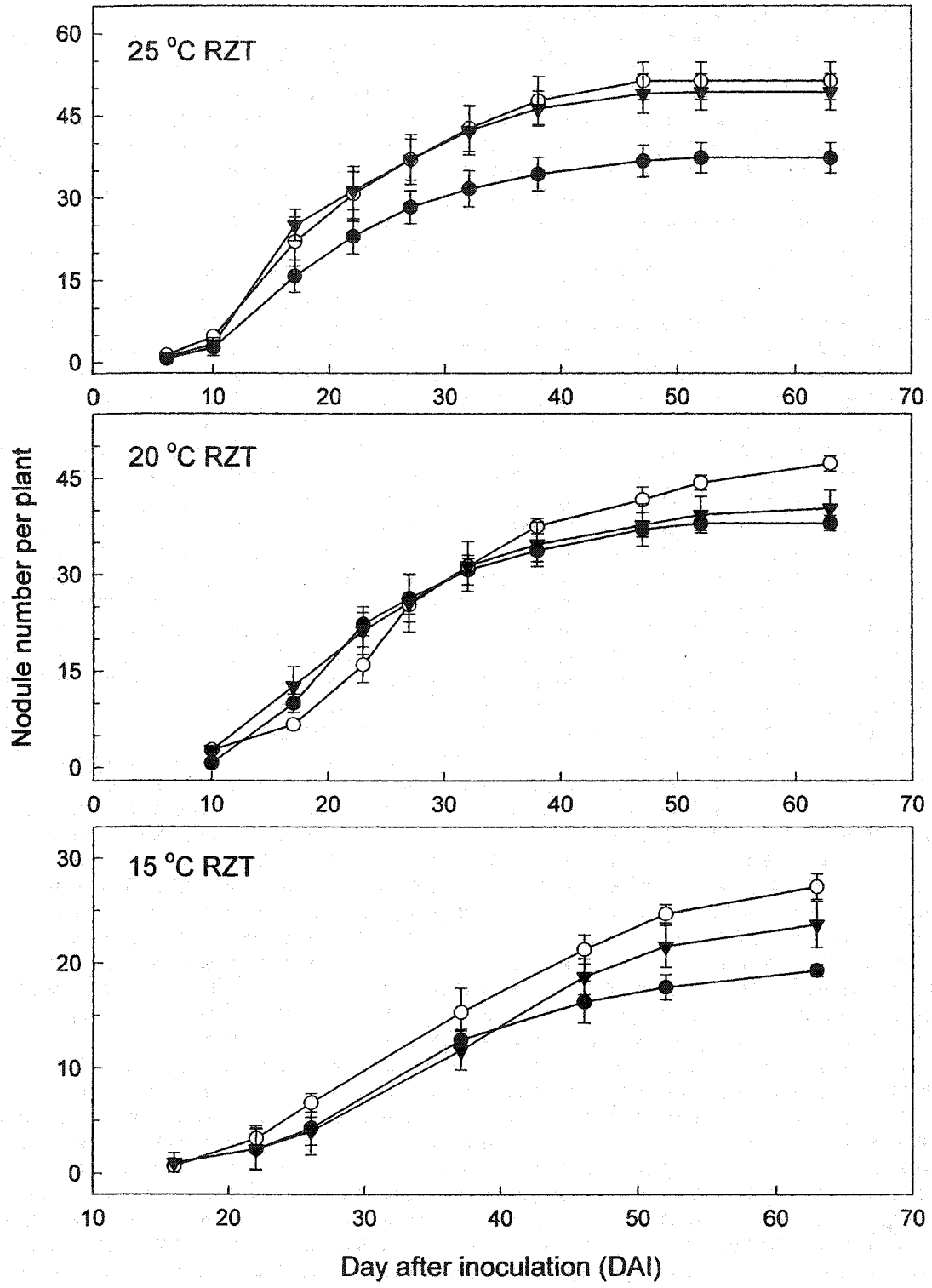

25 °C RZT
20 °C RZT
15 °C RZT
Nodule number per plant
Day after inoculation (DAI)
Bradyrhizobium japonicum control
Coinoculation of Serratia proteamaculans1-102 at 10^8 cells per seedling
Coinoculation of Serratia liquefaciens 2-68 at 10^8 cells per seedling

**Table 3.1. The effect of PGPR coinoculation dose on soybean nodulation and growth in the pot experiment**

| Treatment | Nodule number (per plant) | Nodule dry weight (mg/plant) | Root dry weight (g/plant) | Shoo dry weight (g/plant) |
|---|---|---|---|---|
| Control | 73.5 d | 154.1 cde | 1.20 cd | 3.64 b |
| 1-102 $10^2$ | 80.8 cd | 149.3 de | 1.13 cd | 3.80 b |
| 1-102 $10^4$ | 90.0 bcd | 160.9 cde | 1.36 abc | 4.07 ab |
| 1-102 $10^6$ | 95.3 abc | 188.2 abc | 1.38 abc | 4.55 a |
| 1-102 $10^8$ | 108.0 ab | 199.1 ab | 1.55 ab | 4.65 a |
| 1-102 $10^{10}$ | 95.0 abcd | 162.8 cde | 1.30 abc | 4.16 ab |
| 2-68 $10^2$ | 81.0 cd | 138.3 e | 1.22 bcd | 3.60 b |
| 2-68 $10^4$ | 86.0 cd | 155.9 cde | 1.31 abcd | 4.08 ab |
| 2-68 $10^6$ | 95.5 abc | 179.5 abc | 1.34 abc | 4.24 ab |
| 2-68 $10^8$ | 114.3 a | 211.4 a | 1.62 a | 4.70 a |
| 2-68 $10^{10}$ | 98.5 bcd | 174.0 bcd | 1.39 abc | 4.18 ab |

Treatments: control, inoculation of *Bradyrhizobium japonicum* 532C in distilled water; 1-102, coinoculation of *Serratia proteamaculans* 1-102 with *B. japonicum* 532C; 2-68, coinoculation of *S. liquefaciens* 2-68 with *B. japonicum* 532C. $10^n$, the PGPR coinoculation dose at $1 \times 10^n$ cells per seedling. Means, within the same column, followed by the same letter are not different (P = 0.05) by an ANOVA protected LSD test. n = 4.

**Table 3.2. The effect of PGPR coinoculation dose on soybean nodule number, root weight and shoot weight under different root zone temperatures in the growth pouch experiment.**

| Treatments | | Root zone temperature (RZT) 25 °C | 20 °C | 15 °C | 25 °C | 20 °C | 15 °C | 25 °C | 20 °C | 15 °C |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Nodule number per plant | | | Root dry weight (g/plant) | | | Shoot dry weight (g/plant) | | |
| Control | | 37.3 cd | 38.0 cd | 19.3 cd | 0.19 c | 0.20 d | 0.17 | 0.76 e | 0.67 e | 0.58 e |
| 1-102 | $10^6$ | 35.3 d | 35.0 d | 18.0 d | 0.21 bc | 0.21 cd | 0.19 | 0.85 cde | 0.85 cd | 0.62 de |
| 1-102 | $10^7$ | 39.7 bcd | 37.3 cd | 21.7 bcd | 0.24 ab | 0.23 bcd | 0.19 | 0.89 bcde | 0.90 abcd | 0.64 cde |
| 1-102 | $10^8$ | 51.3 a | 47.3 a | 29.7 a | 0.24 ab | 0.27 bc | 0.21 | 0.95 abcd | 1.00 ab | 0.80 ab |
| 1-102 | $10^9$ | 50.3 a | 45.0 ab | 25.0 abc | 0.25 a | 0.24 bc | 0.19 | 0.93 abcd | 1.02 ab | 0.82 a |
| 1-102 | $10^{10}$ | 47.7 ab | 40.7 bcd | 20.7 bcd | 0.26 a | 0.27 a | 0.25 | 0.80 de | 0.98 abc | 0.69 bcde |
| 2-68 | $10^6$ | 36.7 cd | 35.7 d | 19.0 d | 0.22 abc | 0.20 d | 0.19 | 0.87 cde | 0.90 abcd | 0.67 bcde |
| 2-68 | $10^7$ | 40.0 bcd | 40.0 cde | 20.7 bcd | 0.23 abc | 0.21 cd | 0.19 | 0.97 abc | 1.04 a | 0.72 abcd |
| 2-68 | $10^8$ | 51.7 a | 43.0 abc | 25.3 ab | 0.25 a | 0.24 bc | 0.20 | 0.96 abcd | 0.96 abc | 0.78 ab |
| 2-68 | $10^9$ | 45.0 abc | 37.7 cd | 21.0 bcd | 0.25 a | 0.21 cd | 0.20 | 1.06 a | 0.94 abcd | 0.76 abc |
| 2-68 | $10^{10}$ | 38.0 cd | 27.7 e | 19.3 cd | 0.24 ab | 0.21 bcd | 0.21 | 1.04 ab | 0.87 bcd | 0.69 bcde |

Treatments: control, inoculation of *Bradyrhizobium japonicum* 532C in distilled water; 1-102, coinoculation of *Serratia proteamaculans* 1-102 with *B. japonicum* 532C; 2-68, coinoculation of *S. liquefaciens* 2-68 with *B. japonicum* 532C. $10^n$, the PGPR coinoculation dose at $1 \times 10^n$ cells per seedling. Means, within the same column, followed by the same letter are not different (P = 0.05) by an ANOVA protected LSD test. n = 3.

**Table 3.3. The effect of PGPR coinoculation dose on soybean nitrogen fixation under different root zone temperatures in the growth pouch experiment.**

| Treatments | Root zone temperature (RZT) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 25 °C | 20 °C | 15 °C | 25 °C | 20 °C | 15 °C | 25 °C | 20 °C | 15 °C |
| | Fixed nitrogen (mg/plant) | | | Fixed N/nodule N | | | Fixed N/nodule weight | | |
| Control | 21.7 d | 15.7 cde | 3.5 cde | 11.2 e | 10.2 d | 4.5 d | 0.56 c | 0.46 b | 0.22 e |
| 1-102 $10^6$ | 27.0 abcd | 15.5 de | 3.5 de | 17.6 a | 11.7 bcd | 6.5 cd | 0.73 ab | 0.45 b | 0.34 cde |
| 1-102 $10^7$ | 30.4 abc | 21.4 abcd | 7.1 abc | 17.4 ab | 12.8 abc | 8.5 bc | 0.69 ab | 0.56 ab | 0.45 abc |
| 1-102 $10^8$ | 32.4 a | 25.6 ab | 9.8 a | 16.2 abcd | 14.0 a | 11.3 a | 0.70 ab | 0.61 a | 0.56 a |
| 1-102 $10^9$ | 30.8 abc | 25.9 a | 7.5 ab | 13.0 e | 14.4 a | 6.8 cd | 0.61 bc | 0.65 a | 0.31 de |
| 1-102 $10^{10}$ | 27.0 abcd | 24.6 ab | 2.9 e | 11.2 e | 13.2 ab | 6.5 cd | 0.55 c | 0.56 ab | 0.40 bcde |
| 2-68 $10^6$ | 26.0 cd | 18.2 bcde | 5.9 bcde | 13.3 de | 11.2 bcd | 10.5 ab | 0.69 abc | 0.53 ab | 0.52 ab |
| 2-68 $10^7$ | 30.4 abc | 22.7 abcd | 5.4 bcde | 16.6 abc | 12.5 abc | 8.6 abc | 0.77 a | 0.57 ab | 0.43 abcd |
| 2-68 $10^8$ | 31.7 ab | 23.0 abc | 6.8 abcd | 14.6 bcd | 14.5 a | 8.3 bc | 0.69 ab | 0.66 a | 0.39 bcd |
| 2-68 $10^9$ | 31.9 ab | 19.1 abcde | 6.2 bcde | 14.0 bcde | 12.8 abc | 9.1abc | 0.70 ab | 0.62 a | 0.45 abcd |
| 2-68 $10^{10}$ | 25.1 cd | 13.9 e | 4.9 bcde | 12.5 e | 12.1 bc | 9.6 ab | 0.64 abc | 0.54 ab | 0.44 abcd |

Treatments: control, inoculation of *Bradyrhizobium japonicum* 532C in distilled water; 1-102, coinoculation of *Serratia proteamaculans* 1-102 with *B. japonicum* 532C; 2-68, coinoculation of *S. liquefaciens* 2-68 with *B. japonicum* 532C. $10^n$, the PGPR coinoculation dose at $1 \times 10^n$ cells per seedling. Means, except for Fixed N/nodule weight at 25 °C RZT ($P = 0.10$), within the same column, followed by the same letter are not different ($P = 0.05$) by an ANOVA protected LSD test. $n = 3$.

**Table 3.4. Soybean nodulation dynamic regression equations and some calculated parameters under various root zone temperatures (RZTs) and the optimal PGPR coinoculation dose, in the growth pouch experiment.**

| RZT (°C) | Treatment | Regression equation | NNmax (Nodule number per plant) | $DAI_{1n1}$ (DAI) | $DAI_{15}$ (DAI) | $DAI_{50}$ (DAI) | $DAI_{85}$ (DAI) | NP (Days) |
|---|---|---|---|---|---|---|---|---|
| 25 | Control | $P = 4.7210$ Log DAI – 0.9874 | 37.3 | 5.9 | 11.4 | 18.5 | 30.2 | 46.1 |
| 25 | 1-102 | $P = 4.2579$ Log DAI – 0.4223 | 51.3 | 4.8 | 10.9 | 18.8 | 32.2 | 47.2 |
| 25 | 2-68 | $P = 4.4151$ Log DAI – 0.6102 | 51.7 | 5.0 | 11.1 | 18.7 | 31.4 | 47.0 |
| 20 | Control | $P = 5.8180$ Log DAI – 2.8430 | 38.0 | 8.7 | 15.0 | 22.3 | 33.1 | 43.3 |
| 20 | 1-102 | $P = 4.0935$ log DAI – 0.7242 | 47.3 | 6.3 | 14.3 | 25.0 | 43.9 | 56.7 |
| 20 | 2-68 | $P = 4.3592$ Log DAI – 0.7309 | 43.0 | 5.8 | 12.2 | 20.6 | 35.0 | 57.2 |
| 15 | Control | $Y$ (%) = 211.40 Log DAI – 272.20 | 19.3 | 19.7 | 23.1 | 33.4 | 48.5 | 44.5 |
| 15 | 1-102 | $Y$ (%) = 180.20 Log DAI – 226.57 | 29.7 | 17.8 | 21.4 | 33.0 | 50.8 | 47.7 |
| 15 | 2-68 | $Y$ (%) = 180.29 Log DAI – 226.43 | 25.3 | 18.3 | 22.1 | 34.1 | 52.8 | 49.5 |

Treatments: control, incolation of *Bradyrhizobium japonicum* 532C alone; 1-102, coinoculation of *Serratia proteamaculans* 1-102 with *B. japonicum* 532C; 2-68, coinoculation of *S. liquefaciens* 2-68 with *B. japonicum* 532C. $P$, probit of nodule number. $Y$ (%): percentage of nodule number. $NN_{max}$: the average maximum nodule number per plant; $DAI_{ini}$: days after inoculation for the first nodule appearance; $DAI_{15}$: time needed to reach to 15% of the $NN_{max}$; $DAI_{50}$: time needed for reach to 50% of the $NN_{max}$; $DAI_{85}$: time needed to reach to 85% of the $NN_{max}$; NP: time span from the first nodule appearance to attainment of $NN_{max}$.

## Preface to Section 4

Section 4 is comprised of a manuscript by Bai Y, Souleimanov A and Smith DL and has been accepted by *Journal of Experimental Botany*.

In Section 3, I determined the optimal coinoculation dose of the two *Serratia* strains and determined some nodulation dynamic parameters under different RZT conditions. The results also repeated the previous work in our laboratory that *S. proteamaculans* 1-102 performed better than *S. liquefaciens* 2-68 in promoting soybean growth and nodulation under suboptimal RZT condition. I hypothesized (Specific hypotheses 3) that the *Serratia* PGPR strains promote soybean nodulation through the production of diffusible activator during the culture process. In this section by *using S. proteamaculans* 1-102 strain, the diffusible activator was prepared and its effectiveness in promoting soybean nodulation and growth was well demonstrated in greenhouse experiments.

Of the co-authors, Dr. Souleimanov, a research associate of Dr. Smith, is in charge of HPLC operation in the laboratory. In this experiment, Dr. Souleimanov conducted the HPLC fractionation and isolated the active peak using a bioassay based on the stimulation of soybean seed germination. Dr. Smith is my thesis supervisor. He 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, bench space in greenhouse, experimental field space, the use of computers, and providing technical assistance. Dr. Smith is the corresponding author for publication of this section.

## Section 4

# An inducible activator produced by a *Serratia proteamaculans* strain and its soybean growth promoting activity under greenhouse conditions

### 4.1 Abstract

*Serratia proteamaculans* 1-102 (1-102) promotes soybean-bradyrhizobia nodulation and growth but the mechanism is unknown. After adding isoflavonoid inducers to 1-102 culture, an active peak with a retention time of about105 min in our HPLC fractionation was isolated using a bioassay based on the stimulation of soybean seed germination. The plant growth promoting activity of this material was compared with 1-102 culture (cells) and supernatant under greenhouse conditions. The activator was applied to roots in 83, 830 and 8300 HPLC microvolt units (μVs) per seedling when plants were inoculated with bradyrhizobia or sprayed onto the leaves in same concentrations at 20 days after inoculation. The root-applied activator, especially at 830 μVs per seedling, enhanced soybean nodulation and growth at the same level as 1-102 culture under both optimal and sub-optimal root zone temperatures. Thus, this activator stimulating soybean seed germination is also responsible for the plant growth promoting activity of 1-102 culture. However, when sprayed onto the leaves, the activator did not increase growth and in higher concentrations decreased average single leaf area. The results suggest that this inducible activator might be a lipo-chitooligosaccharide (LCO) analog. LCOs act as rhizobia-to-legume signals stimulating root nodule formation. The activator could provide additional "signal" increasing in the signal quality (the signal to noise ratio, SNR) of the plant-rhizobia signal exchange process.

**Keywords:** Soybean, plant growth promoting rhizobacteria,
*Serratia proteamaculans*, inducible activator

## 4.2 Introduction

Legume nodulation is a complex process involving interactions between the host plants and rhizobia. This process is also affected by many biotic and abiotic environmental factors (Hungria and Stacey 1997; Vlassak and Vanderleyden 1997). The first stage in the establishment of the symbiotic system is signal exchange between legume plants and rhizobia. The plant-to-bacteria signals are isoflavonoids which induce bradyrhizobial *nod* gene expression, in the case of soybean, mainly genistein and daidzein (Rao and Cooper 1994). The rhizobia-to-plant return signals are lipo-chitooligosaccharides (LCOs), so called Nod factors, which play pivotal roles in root nodule formation. LCOs are oligosaccharides of β-1,4-linked N-acetyl-D-glucosamine and of some specifically modified side groups. LCOs are synthesized via sophisticated biochemical processes catalyzed by a series of *nod* gene encoded enzymes (Perret 2000). All individual rhizobial strains produce specific structurally diverse LCO mixtures (Spaink 1996) and the major LCO molecule produced by *Bradyrhizobium japonicum* 532C is Nod *Bj* V ($C_{18:1}$; MeFuc) (Prithiviraj et al. 2000).

During the signal exchange process, environmental factors affecting either signal production or signal perception can affect nodulation and subsequent nitrogen fixation. Sub-optimal (15 – 17.5°C) root zone temperatures (RZTs), pH stress and mineral nitrogen inhibit production of isoflavonoids by soybean roots as well as subsequent nodulation and nitrogen fixation (Streeter 1988; Cho and Harper 1990; Zhang and Smith 1994; Zhang and Smith 1996a; Pan and Smith 1998). High temperature (39 °C) increases the release of the isoflavonoid signals from soybean seeds during the first 24 h, but the compounds released have decreased nod gene inducing activities (Hungria and Stacey 1997). Addition of genistein to the inoculant or the rhizosphere could at least partially alleviate the deleterious effects of these environmental factors (Zhang and Smith 1995; Smith and Zhang 1996; Zhang and Smith 1996b; Hungria and Stacey 1997; Zhang and Smith 1997; Pan et al. 1998). Besides inhibiting the synthesis and excretion of isoflavonoids by soybean roots, low RZTs also suppress bacterial *nod* gene expression, and this also could be partially overcome by genistein application (Zhang et al. 1996a). In addition, LCOs are sensitive to chitinase and related hydrolases which cleave and inactivate Nod factors

in the host rhizosphere (Perret et al. 2000). When *Sinorhizobium fredii* and *S. meliloti* were transconjugated with a chitinase gene from a *Serratia marcescens* strain, the enzyme was expressed and nodulation of soybean and alfalfa were impeded (Krishnan et al. 1999).

Many plant growth promoting rhizobacteria (PGPR) have beneficial effects on legume growth and at least some PGPR strains enhance legume nodulation and nitrogen fixation by affecting signal exchange between the plants and rhizobia. Co-inoculation of some *Pseudomonas* and *Bacillus* strains, along with effective *Rhizobium* spp., stimulates chickpea growth, nodulation and nitrogen fixation (Parmar and Dadarwal 1999). Seed colonization by these PGPR or application of the ethyl acetate extract of the culture supernatant increase the concentration of flavonoid-like compounds in roots, and the rhizobacteria themselves are capable of producing fluorescent flavonoids similar to those produced by the plant (Parmar and Dadarwal 1999). These lines of evidence indicate that PGPR may produce signal molecule analogs and/or stimulate the plant to produce more signal molecules. It may also be reasonable to postulate that some rhizobacteria produce LCO analogs or improve conditions for signal exchange.

Some *Serratia* strains, such as *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68, have beneficial effects on legume plant growth (Chanway et al. 1989; Zhang et al. 1996b). They are both able to partially overcome the effects of sub-optimal RZT on soybean nodulation and $N_2$ fixation. Strain 1-102 generally performed better than 2-68 at sub-optimal RZTs (Zhang et al. 1996b). Their culture supernatants had the same beneficial effects on soybean plants as the bacterial cultures, although not under sub-optimal conditions (Dashti 1997). Combined application of these PGPR and genistein improved $N_2$ fixation in soybean at suboptimal root zone temperatures (Dashti et al. 2000). Given their effects on soybean plants, we hypothesize that the PGPR strains exert their influence via the production of specific compounds after they have been inoculated into plant rhizospheres. In testing this hypothesis, a series of experiments were designed and carried out with *S. proteamaculans* 1-102. A plant growth stimulating substance (activator) in the HPLC fractions from 1-102 culture treated with isoflavonoid inducers was first isolated through a bioassay for its ability to stimulate soybean seed germination and then its activity in promoting soybean growth and nodulation was further evaluated

under greenhouse conditions. The objective of the results presented here was to demonstrate that the inducible activator produced by 1-102 was the compound responsible for the bacterial promotion of soybean plant growth and nodulation under both optimal and suboptimal RZT conditions.

## 4.3 Materials and methods

*Activator preparation*

PGPR strain *Serratia proteamaculans* 1-102 (1-102) was cultured in King's Medium B (Atlas 1995). The initial broth inoculum was inoculated with slant material and cultured in 250 ml flasks containing 100 ml medium for 72 h on a shaker (Model 4530 Table Top Orbital Shaker, Forma Scientific Inc., Mariotla, Ohio, USA) at 150 rev per min and 28 °C. Subcultures were then inoculated with the initiation broth inoculum at a 1% inoculation ratio and cultured in 4 l flasks containing 1 l of medium for 96 h under the same conditions as the initial culture. During the culture period, isoflavonoid inducers (Sigma-Aldrich Canada Ltd. Oakville, Canada), including genistein (G6766), naringenin (N5893), apigenin (A9914) and luteolin (L9283) were added at final concentrations of 1 μM each (Kosslak *et al.*, 1987). At the end of the 96 h culture period, the culture broth was extracted with butanol at a 40% (v/v) final concentration. The organic phase was collected and evaporated in a low-pressure rotary evaporation system (Yamota RE500, Yamato, USA) at 50 °C. The residue was re-suspended in 18% acetonitrile (AcN/$H_2O$, v/v) as a crude preparation. This crude preparation was further purified through HPLC fractionation, using a Waters system equipped with two model 510 pumps, a WISP 712 auto-sampler, a model 441 absorbence detector and a fraction collector (Waters, MA, USA). The crude sample was loaded onto a C18 reverse-phase column (Vydac 218TP54, 300 Å, 5 μm, 4.6 × 250 mm). The elution was performed as follows: 0 – 45 min with isocratic 18% acetonitrile; 45 – 110 min with a gradient from 18 to 60.7 % acetonitrile; 110 – 115 min with a gradient from 60.7 to 100% acetonitrile; 115 – 120 min, with a reversed gradient from 100 to 18% acetonitrile. The absorbence of the eluted fractions was monitored at 214 nm. The HPLC elutes were collected as 120 fractions, one minute of elution time per fraction, and maintained at 4 °C until use.

Besides 1-102 culture treated with the inducers, the culture without added the inducers, and the culture medium alone (culture medium that had never grown cells) were also subjected to the same extraction procedures described above. All the 120 HPLC fractions from these three samples were tested with a bioassay method based on soybean seed germination as a test for the presence of activator.

*Bioassay for the active HPLC peak*

The bioassay of the active peak was conducted by following a 3-step focusing strategy. In the first step, all the 120 fractions were divided to three parts: I-i, 0 – 40 min; I-ii, 41 – 80 min and I-iii, 81 – 120 min. and tested for ability to stimulate soybean seed germination. In the second step, the selected active part I-iii (81 – 120 min) was further divided to 4 parts, II-i, 80 –90 min; II-ii, 91 -100 min and II-iii, 101 – 110 min and II-iv, 111- 120 min. and bioassayed, and part II-iii (101-110 min) was identified. In the third bioassay step, part II-iii was subdivided to 3 parts: III-i 101-103 min, III-ii 104-106 and III-iii 107-110. In the bioassay, soybean seeds (cultivar OAC Bayfield) were surface sterilized in sodium hypochloride (2% solution containing 4 ml/l of Tween 20) (Bhuvaneswari et al. 1980). A filter paper disc was put in the bottom of each sterilized Petri dish (100 × 15 mm, Fisher Scientific, Ontario, Canada) in order to have an even distribution of the added solution. Ten soybean seeds were placed on the filter paper of each dish. Each treatment was replicated 5 times in 5 separate dishes, and the entire experiment was conducted twice. At each step the treatment solution was prepared as follows: all of the one minute samples corresponding to a section of the HPLC chromatogram that was to be assayed were combined and the resulting material was serially diluted with distilled water to 1:500, 1:5000 and 1:50000. Ten ml of each solution was added to each of a set of 5 dishes. The time of solution addition was taken as the beginning of the germination period. All the dishes were kept in an incubator (Conviron E15 Growth Chamber, Controlled Environments Ltd., Winnipeg, Canada) at 26 ± 1 °C, with 70 - 80% humidity, good ventilation and without lightening. A seed was judged to be germinated when the root tip had clearly penetrated the seed coat. The number of germinated seed in each dish was recorded periodically during the 66 h germination process. The germination rate was expressed as a percentage of the total

number of seeds in the dish. The bioassay eventually identified part III-ii, 104 – 106 min. as the potential activator. This peak was only present in cultures where the 1-102 cells had been treated with flavonoid inducers. The relative concentration of the activator was given as the area under the HPLC peak, measured in micro volts (μVs). The applied active solutions were equal to 8300 μVs/ml (1: 500), 830 μVs/ml (1: 5000) and 83 μVs/ml (1:50000). In the following greenhouse experiments, only this selected activator from culture treated with inducers was tested in these 3 relative concentrations.

*Greenhouse experiments*

The effect of the activator on soybean plant growth was evaluated under greenhouse conditions by comparison with 1-102 cultures, 1-102 culture supernatant. The experiment was conducted in both pot (20 cm diameter) and pouch (15 × 16 cm, Mega International, Minneapolis, MN) culture systems. When pouch culture was adopted, the RZT was controlled by water bath systems at 25 and 15 °C. The greenhouse air temperature was 25 ± 2 °C with additional illumination of 300 $\mu M/m^2/s$ supplied by high pressure sodium lamps (P. L. Light System, Montreal, Canada) for a photoperiod of 16:8 h (day : night). The inocula used in the experiment were *Bradyrhizobium japonicum* 532C (532C), 532C plus 1-102 culture, 532C plus 1-102 culture supernatant, 532C plus activator at three relative concentrations (83, 830 and 8300 μVs per plant). In the pot experiment, as described below, all three concentrations of the activator were also sprayed onto seedling leaves.

The soybean seeds (OAC Bayfield) were surface sterilized in sodium hypochloride (Bhuvaneswari et al. 1980) and planted in trays containing Vermiculite and germinated in the greenhouse. Three to four day old healthy seedlings, at the VE stage (Fehr and Caviness 1971), were transplanted into pots containing Vermiculite (VIL Vermiculite Inc. Montreal & Toronto, Canada) or pouches suspended in a water bath. Inoculation was conducted when the seedlings were 10 days old, at the early VC stage.

*B. japonicum* 532C was cultured in yeast extract mannitol culture medium (YEM) (Vincent 1970) on a shaker (Model 4580 Refrigerated Console Incubater Orbital Shaker. Forma Scientific Inc., Marietla, Ohio, USA) at 150 rev per min and 28 °C. The initial culture was inoculated with slant material and cultured for 7 d. The subculture time was

72 – 96 h. The cell concentration of the *B. japonicum* 532C culture was estimated by spectrophotometry at 620 nm (Bhuvaneswari et al. 1980). The *B. japonicum* culture was diluted with distilled water to $A_{620nm}$ = 0.08 (approximately $10^8$ cells/ml), and the inoculation dose was $10^8$ cells per seedling (Zhang and Smith 1994).

*S. proteamaculans* 1-102 was produced in the same way as for activator preparation but for only 24 h and no isoflavonoid inducer was applied. The cell concentration of the 1-102 culture was estimated by spectrophotometry at 420 nm (Pan et al. 1999). The culture was diluted with distilled water to $A_{420nm}$ = 0.10 (approximately $10^8$ cells/ml) and the inoculation dose for 1-102 was also $10^8$ cells per seedling. The culture supernatant was prepared from a 24 h culture by centrifugation at 4000 rev per min for 15 min. When it was co-inoculated with 532C, it was diluted with distilled water at the same rate as the culture inoculant.

When the purified activator was applied to roots by co-inoculation with the bradyrhizobia or the leaves by spraying, it was diluted with distilled water to final concentrations of 83 μVs/ml (Act1), 830 μVs/ml (Act2) or 8300 μVs/ml (Act3). The activator solutions were applied at 1 ml per plant, either to roots or the leaves. The sprayings of the activator in the pot experiment were conducted at 20 days after inoculation (DAI). When the spraying was conducted, the pots were far enough apart that the seedlings did not touch each other.

The plants were cultured without application of any mineral nitrogen and harvested at 50 DAI. During the growth period, the plants were watered with N-free Hoagland's solution (Hoagland and Arnon 1950), in which $Ca(NO_3)_2$ and $KNO_3$ were replaced with 1 mM $K_2HPO_4$ and 1mM $KH_2PO_4$. After harvesting, data were collected on leaf number and leaf area (not applicable to 15°C RZT samples as most of leaves had senesced by then), nodule number, nodule weight, root weight and shoot weight. The weights of nodules, shoots and roots were collected after they had been dried at 70 – 80 °C for not less than 48 h.

*Data analysis*

All the data were analyzed statistically with the SAS system (Littell et al. 1991). When analysis of variance indicated differences, comparisons among treatment means

were conducted with an ANOVA protected least significance difference (LSD) test (Steel and Torrie 1980). In general, differences were considered significant at $P \leq 0.05$. However, in some cases differences significant at probabilities between 0.05 and 0.1 are described. When this happens the P values are given in the text.

### 4.4 Results

The HPLC profiles of the three extracted materials, 1-102 culture with the inducers, 1-102 culture without the inducers and the culture medium alone, are shown in Fig. 4.1. The 1-102 culture with the inducers had a novel peak with retention time 104.83 min. (Fig. 4.1-A). At the same position, as well as in the neighboring regions, the medium control had no peak (Fig. 4.1-C). The 1-102 culture without inducers had a peak with retention time 103.15 min. (Fig. 4.1-B), and this peak disappeared in the culture with the inducers (Fig. 4.1-A). After the 3-step bioassay, based on stimulation of soybean seed germination, we focused on fraction III-ii, with a retention time 104 – 106 min, in which the novel peak in Fig. 4.1–A was included. The final bioassay results with 104-106 min fractions are shown in Fig. 4.2. Compared with the medium alone control, the fraction from both cultures with and without the inducers resulted in higher germination rate. Although all three tested concentrations were active, the lowest, 1:50000 (83 μVs/ml) caused the greatest stimulation of germination (Fig. 4.2-III).

In the pouch experiment, co-inoculation of 1-102 culture increased nodule number by 30%, nodule weight by 45.5% and plant weight by 31.3% under 25 °C RZT, and nodule number by 81.1% and plant weight by 14.6% under 15 °C RZT, compared with the 532C-alone control (Table 4.1). Addition of the PGPR supernatant to the *B. japonicum* inoculant increased nodule weight by 53.7% and plant weight by 31.2% under 25 °C RZT, that is at essentially the same level with coinoculation of *B. japonicum* with the 1-102 culture. However, at 15 °C RZT, the application of 1-102 supernatant failed to cause an increase in the plant weight, although nodule number was increased.

In the pouch experiment, addition of the activator, Act2 at 830 μVs per plant, to the *B. japonicum* inoculant caused the largest increases of all the treatments. It increased all the measured variables at both RZTs. Thus, the effects of the activator were similar to

co-inoculation of 1-102 culture with *B. japonicum* cells. Act2 increased nodule number by 31.9 and 80.2%, nodule weight by 81.2 and 46.6%, and plant weight by 39.2 and 27.2% under 25 and 15 °C RZT, respectively (Table 4.1). Act 1 at 83 μVs plant$^{-1}$ and Act3 at 8300 μVs plant$^{-1}$ had little positive effect at 25 °C RZT in the pouch experiment. However, at 15 °C RZT it increased nodule number and plant weight at levels similar to co-inoculation of 1-102 culture. Act1 also increased nodule weight at 15 °C RZT (Table 4.1). Thus, soybean plants are more sensitive to this activator higher RZT, in the pouch experiment.

In the pot experiment, Act2 resulted in the largest increases in nodule number (50%), nodule weight (47.5%) and plant weight (35%)(Fig. 4.3), relative to the 532C-alone control. In spite of these large numerical increases, the levels of these variables for this treatment were not different from those resulting from treatment with the 1-102 culture, 1-102 supernatant, Act1 and the Act3. Spray treatment with the activator, Act1LS at 83 μVs per plant increased nodule number ($P = 0.06$), but the other two spray treatments, Act2LS and ActT3LS, did not increase any of the measured variables (Fig. 4.3). Act2LS and Act3LS decreased single leaf area (Table 4.2), although the leaf number per plant and the total leaf area per plant were not different between leaf-applied activator treatments and the control (data not shown). The higher the concentration of the activator applied on leaves, the more the average single leaf area was decreased.

## 4.5 Discussion

Numerous publications have reported that co-inoculation of PGPR with rhizobia improves legume nodulation and nitrogen fixation, however, in only a few cases has the mechanism of PGPR stimulation been investigated (Derylo and Skrupska 1993; Srinivasan et al. 1996; Parmar and Dadarwal 1999). Finding the mechanisms by which PGPR promote legume growth and nodulation remains a major challenge. Recent progress in the understanding of legume-rhizobia interactions (Perret et al. 2000) has encouraged us to approach the PGPR mechanisms with new and more specific questions. The work included in this paper showed that root application of the activator had essentially the same efficacy as live 1-102 cells in promoting soybean plant growth and

nodulation. This strongly suggested that the activator is the compound responsible for the PGPR efficacy and that our hypothesis that the live cells in the rhizosphere produce the activator after being activated by the isoflavonoid inducers secreted by plant roots is correct.

In the greenhouse experiments, promotion of soybean plant growth and nodulation by the 1-102 culture supernatant only at an RZT in the optimum range confirms the results of Dashti (1997). Regardless of culture system or RZT, Act1, the 1-102 supernatant and the 1-102 culture were not different from each other (Table 4.1 and Fig. 4.3). This suggested that even without the application of inducers, the PGPR cells produce low concentrations of the activator during the culture process. This was supported by the variable effectiveness of applications of the activator at the different concentrations. Among the three applied concentrations, Act2 performed the best, both the higher and the lower doses, Act3 and Act1, showed diminished effectiveness in promoting plant growth and nodulation. Thus, Act 2 was the optimal concentration and it may well be that the concentration of the activator in the supernatant was lower than this, being sufficient to promote plant growth and nodulation at an optimal RZT, but insufficient for this growth promotion under the lower RZT condition. The activity of 1-102 cells at the lower RZT indicates that they were able to produce sufficient activator for soybean plant growth stimulation; presumably isoflavonoid inducers produced by soybean roots played a role in activating bacterial biosynthesis of the activator. The purified activator caused stimulation of soybean plant growth at all concentrations and at both optimal and suboptimal RZTs, suggesting that even the lowest concentration of isolated activator was greater than the concentration in the culture supernatant.

Bioassay methods are generally more sensitive in detecting physiologically active substances than the physico-chemical analyses. In our bioassay experiments, besides fraction III-ii, which contained the active peak (104.85 min) from 1-102 culture with the inducers, the same fraction from cultures without inducers also showed activity in stimulating seed germination, although it displayed no obvious peak in the profile (Fig. 4.1). This could be explained by a very low concentration of the activator, below detection by HPLC. This is possible. For instance, LCOs show high activity in the stimulation of nodulation in soybean systems at $10^{-8}$ M, but this is well below the level of

HPLC detection. For stimulation of root hair deformation, LCO activity has been seen at concentrations as low as $10^{-11}$ M (Relic et al. 1993).

The efficacy of root applications of the activator was constant in both pouch and pot experiments. However, the leaf applications were not as effective as the root applications. When the activator was sprayed on soybean leaves, decreased leaf area per leaf and the lack of increased plant growth and nodulation were observed. That the activator, which would normally be produced in the rhizosphere, has effects on plant development when applied on the leaves is interesting. Our experimental results showed that decreases in average single leaf area occurred only at higher concentrations of activator, Act2LS and Act3LS. Besides activator concentration, activator effectiveness may be also related to the plant development stage when the spray was conducted. For soybean seedlings grown in the greenhouse under N-free culture conditions, 20 DAI is the beginning of the period of rapid leaf area expansion (personal observations). When leaf area is increasing, it may become a competitive sink for both nitrogen and carbohydrate. If the activator stimulates translocation from the leaves to other sinks, leaf area expansion will be limited by the spray application. This could, in turn, diminish total plant photosynthesis and subsequently, nitrogen fixation through plant auto-regulation mechanisms for maintaining nitrogen-carbon balance (Shantharam and Mattoo 1997).

Rhizobia produce a series of LCOs with different substitutions and/or modifications of the basic structure of three to five 1,4-β-linked *N*-acetylglucosaminosyl fragments (Hungria and Stacey 1997). Besides playing a key role in legume root nodule formation, LCOs are also involved in other plant morphogenesis activities, such as induction of cell cycle genes in suspension cell cultures and induction of mitosis in the protoplast cultures, and even in some aspects of animal morphogenesis (Spaink 1996). There is increasing evidence that some plants and animals are also capable of producing and recognizing signal molecules that structurally resemble the rhizobial LCOs, i.e. LCO analogs (Spaink 1996). Our results imply that the activator in question could be a LCO analog. As with LCOs from rhizobia, the activator was produced in measurable amounts following exposure of the cells to the isoflavonoid inducers. The activator and LCO were prepared by following a similar extraction procedure. Their HPLC purification programs

were also very similar (following the same procedure LCO *Bj* V [$C_{18:1}$ MeFuc] has an HPLC retention time of 83-86 min), and both of them were monitored with UV light of 214 nm wavelength. Both the activator and LCOs stimulate soybean seed germination (Prithiviraj, LCOs stimulate soybean and other crop seed germination, personal communication). If this is the case, it not only supports our initial hypothesis, but also allows us to further describe the mechanism of the plant growth promoting activity by *Serratia proteamaculans* 1-102.

During signal communication, signal quality is described in terms of signal to noise ratio (SNR). Only when the SNR becomes greater than a critical value does the communication succeed. The plant-bacteria signal exchange process is a two-way molecular conversation occurring in the rhizosphere (Fisher and Long 1992). The SNR concept may also be applicable to the legume-rhizobia signal exchange. A LCO analog might increase the SNR of the molecular conversation between legume and rhizobia. LCOs have multiple functions in inducing root nodule formation and other responses related to the infection process in host plants (Prithiviraj et al. 2000). An LCO analog might perform any one or a number of these multiple functions. Furthermore, besides the host plant (Perret et al. 2000; Prithiviraj et al. 2000), other rhizobacteria may also produce LCO hydrolases in the rhizosphere. LCO analogs maybe act as competitive substrates of hydrolysis enzymes.

**Fig. 4.1. HPLC profiles (part) of the three samples: A, *Serratia proteamaculans* 1-102 culture with inducers; B, *S. proteamaculans* 1-102 culture without inducers; C, King's Medium B.**

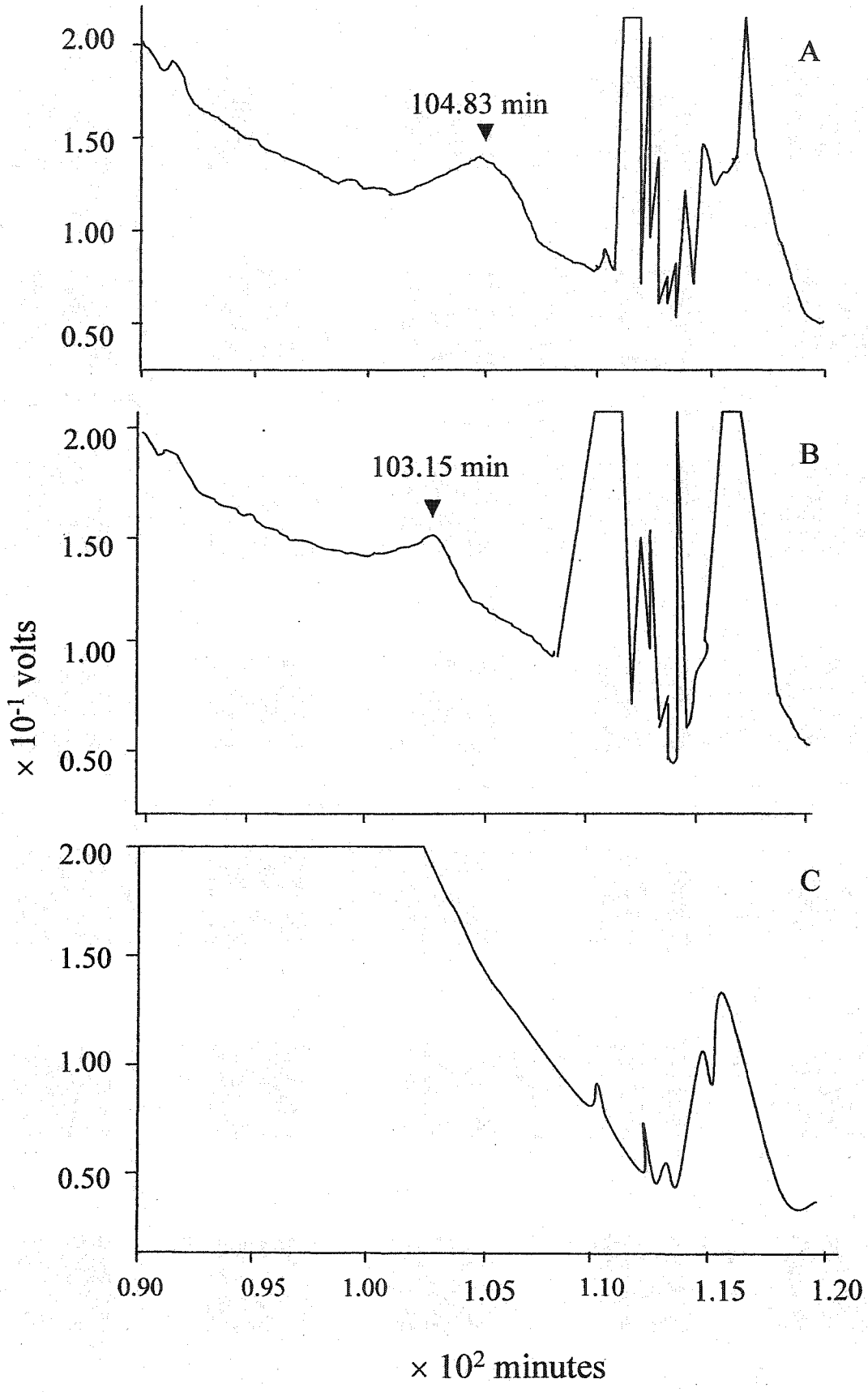

2.00
1.50
1.00
0.50
A
104.83 min
2.00
1.50
1.00
0.50
B
103.15 min
× 10-1 volts
2.00
1.50
1.00
0.50
C
0.90
0.95
1.00
1.05
1.10
1.15
1.20
× 10² minutes

**Fig. 4.2. Stimulation of soybean seed germination by a HPLC fraction (104 –106 min) from the three samples: *Serratia proteamaculans* 1-102 culture with inducers; *S. proteamaculans* 1-102 culture without inducers and King's Medium B.**

The relative concentration of the tested fraction: I, 1: 500; II, 1: 5000; III, 1: 50000

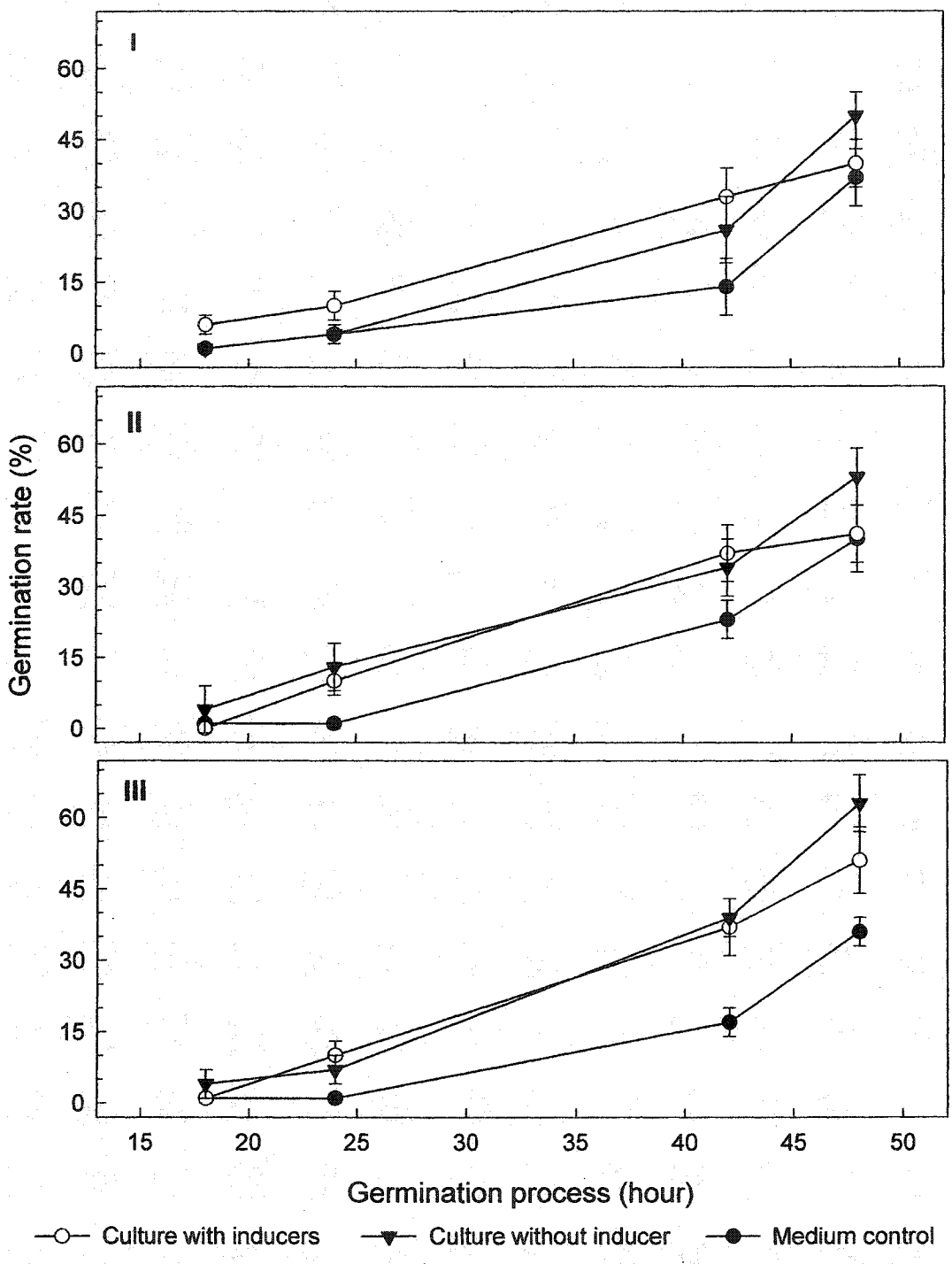

I
II
III
Germination rate (%)
0
15
30
45
60
15
20
25
30
35
40
45
50
Germination process (hour)
Culture with inducers
Culture without inducer
Medium control

**Fig. 4.3. The effects of activator applications on soybean nodule number (I), nodule weight (II) and plant weight (III) in the pot experiment.**

Treatments: Control, *Bradyrhizobium japonicum* 532C inoculant; 102C, *Serratia proteamaculans* 1-102 culture coinoculation; 102S, *Serratia proteamaculans* 1-102 culture supernatant applied in inoculant; Act1, 2 and 3, the activator applied in inoculant at 83, 830 and 8300 microvolts per plant, respectively; Act1LS, Act2LS and Act3LS, leaf spraying of Act1, Act2 and Act3. Bars associated with the same letters are not different (P = 0.05) by an ANOVA protected LSD test. n = 5.

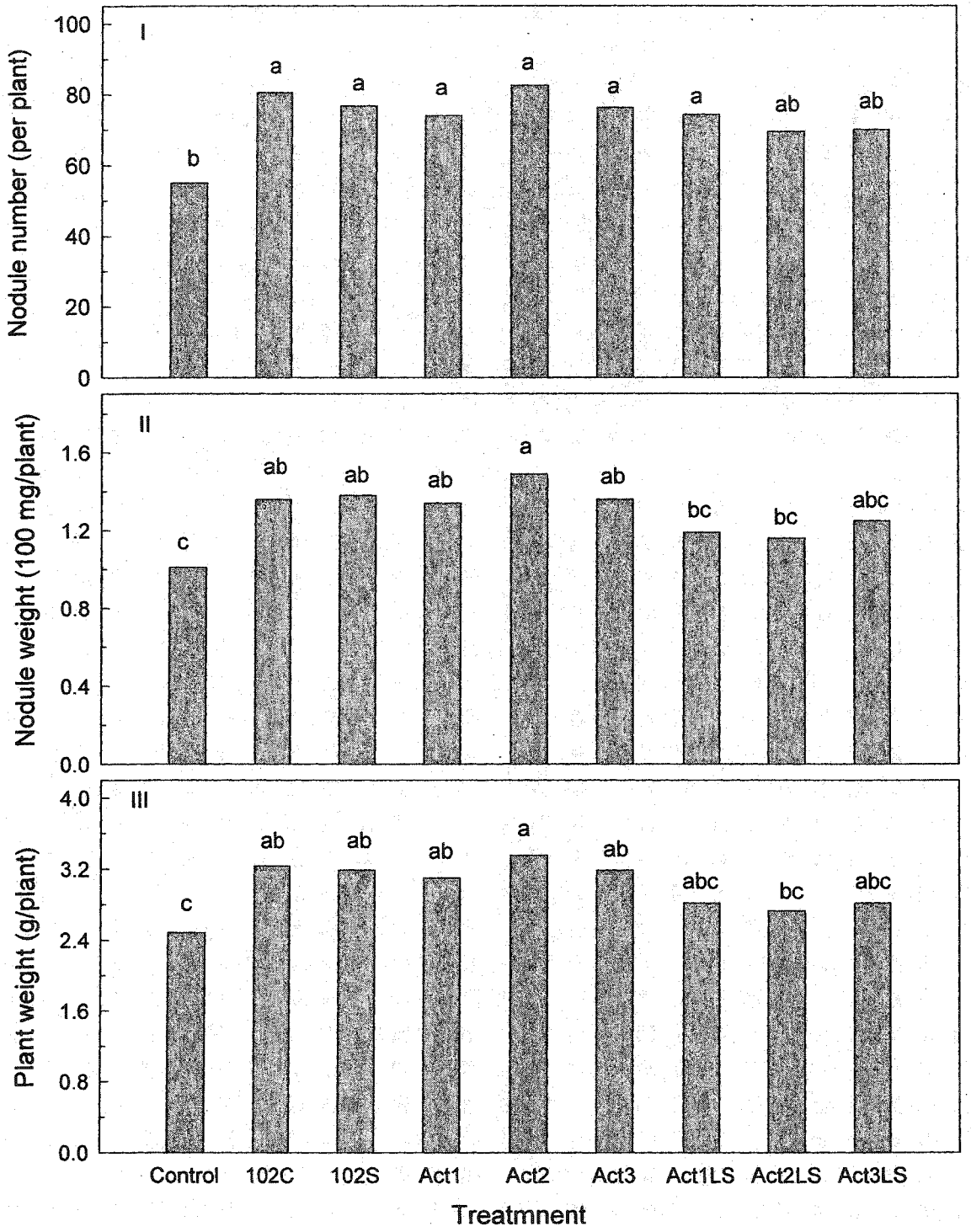

I
Nodule number (per plant)
100
80
60
40
20
0
b
a
a
a
a
a
a
ab
ab
II
Nodule weight (100 mg/plant)
1.6
1.2
0.8
0.4
0.0
c
ab
ab
ab
a
ab
bc
bc
abc
III
Plant weight (g/plant)
4.0
3.2
2.4
1.6
0.8
0.0
c
ab
ab
ab
a
ab
abc
bc
abc
Control
102C
102S
Act1
Act2
Act3
Act1LS
Act2LS
Act3LS
Treatmnent

**Table 4.1. The effects of root application of the activator at the different root zone temperatures (RZTs) on soybean nodule number, nodule weight and plant weight in the pouch experiment.***

| Treatment | Nodule number (per plant) | Nodule weight (g/plant) | Plant weight (g/plant) |
|---|---|---|---|
| 15°C RZT | | | |
| 532C control | 27.75 c | 0.0631 c | 1.0235 c |
| 532C + 1-102 Culture | 50.25 a | 0.0750 abc | 1.1726 ab |
| 532C + 1-102 Supernatant | 46.50 ab | 0.0737 bc | 1.1605 bc |
| 532C + Act1 (83 μVs/plant) | 51.00 a | 0.0890 ab | 1.2384 ab |
| 532C + Act2 (830 μVs/plant) | 50.00 a | 0.0925 a | 1.3018 a |
| 532C + Act3 (8300 μVs/plant) | 39.00 b | 0.0678 c | 1.2085 ab |
| 25°C RZT | | | |
| 532C control | 46.25 c | 0.0750 c | 1.5777 c |
| 532C + 1-102 Culture | 60.25 a | 0.1091 ab | 2.0718 ab |
| 532C + 1-102 Supernatant | 54.50 abc | 0.1148 ab | 2.0706 ab |
| 532C + Act1 (83 μVs/plant) | 56.25 ab | 0.0998 bc | 1.8902 abc |
| 532C + Act2 (830 μVs/plant) | 61.00 a | 0.1359 a | 2.1965 a |
| 532C + Act3 (8300 μVs/plant) | 49.75 bc | 0.0947 bc | 1.6756 bc |

* Treatment: 532C, *Bradyrhizobium japonicum* 532C; 1-102, *Serratia proteamaculans* 1-102; Act, activator. Means, within the same column and RZT level, followed by the same letter are not different (P = 0.05) by an ANOVA protected LSD test. n = 4.

**Table 4.2. Soybean average single leaf area variation due to PGPR coinoculation or application of the isolated activator to roots or leaves in the pot experiment.***

| Treatment | Single leaf area ($cm^2$/leaf) | |
|---|---|---|
| 532C control | 40.6 | a |
| 532C + 1-102 Culture | 36.4 | ab |
| 532C + 1-102 Supernatant | 36.0 | ab |
| 532C + Act1 (83 μVs/plant) | 36.3 | ab |
| 532C + Act2 (830 μVs/plant) | 35.3 | ab |
| 532C + Act3 (8300 μVs/plant) | 35.2 | ab |
| 532C + Act1 leaf spraying | 36.1 | ab |
| 532C + Act2 leaf spraying | 31.4 | bc |
| 532C + Act3 leaf spraying | 29.2 | c |

* Treatment: 532C, *Bradyrhizobium japonicum* 532C; 1-102, *Serratia proteamaculans* 1-102; Act, activator. Means followed by the same letters are not different (P = 0.05) by an ANVOA protected LSD test. n = 5.

## Preface to Section 5

Section 5 is comprised of a manuscript by Bai Y, Souleimanov A, Zhou X and Smith DL and will be submitted to *Crop Science*.

Based on the previous findings from our laboratory, I hypothesized that after the PGPR strain *Serratia proteamaculans* 1-102 was inoculated into the rhizosphere, the cells interact with the roots of soybean plants, leading to the production of a compound or compounds that are responsible for its plant growth promoting effects. In Section 4 of this thesis, we isolated an active HPLC peak from flavonoid-induced cultures of *S. proteamaculans* 1-102 and demonstrated its activity in a greenhouse experiment. In this section, I evaluated the growth promotion ability of the inducible activator under field conditions, both by adding it to *B. japonicum* inocula at the time of seeding, and by spraying it on the leaves at V3 and R# development stages.

Of the co-authors, Dr. Souleimanov, a research associate of Dr. Smith, is in charge of HPLC operation in the laboratory and helped me to purify the inducible PGPR activator. Dr. Zhou, another research associate of Dr. Smith, operates the elemental analyzer and helped me to do the nitrogen analysis, and also provided considerable general support in a wide range of areas from organizing summer student helper to statistics. Dr. Smith is my thesis supervisor. He 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, bench space in greenhouse, experimental field space, the use of computers, and providing technical assistance. Dr. Smith will be the corresponding author for publication of this section.

# Section 5

## An inducible activator is responsible for the soybean plant growth promoting activity of *Serratia proteamaculans* 1-102 under short season field conditions

### 5.1 Abstract

The inducible activator produced by *Serratia proteamaculans* 1-102 (1-102) has been shown to promote soybean [*Glycine max* (L.) Merr.] growth and nodulation under both optimal and suboptimal root zone temperatures in greenhouse experiments. In order to test the effectiveness of this activator under filed conditions in a short season area, two field experiments were conducted in Quebec. The activator was applied into the seed furrow at planting at a relative concentration of 830 HPLC detector microvolts (measure of peak area) per plant by adding it to *Bradyrhizobium japonicum* 532C (532C) inoculant. It was also applied to the leaves at V3 or R3 growth stages. During the growing season, plants were harvested three times (V3, R3 and harvest maturity). Compared with 532C alone control, the furrow application of the activator increased nodule number, nodule weight, plant weight, grain yield and total nitrogen at the same level as coinoculation of 1-102 culture (1-102C). However, the effectiveness of leaf application of the activator was dependent on the application times: spraying at R3 worked well, but spraying at V3 failed to have effect on the measured variables. These results indicated that the inducible activator was responsible for the soybean plant growth promoting activity of *S. proteamaculans* 1-102. The activator performed as well as 1-102C not only in the greenhouse, but also in field in a short season area.

**Key words:** Serratia proteamaculans, plant growth promoting rhizobacteria, plant growth promoting activator, soybean

## 5.2 Introduction

Legume plant nodulation is a specialized and complex interaction between the host plants and appropriate symbiotic rhizobia. Soybean [*Glycine max.* (L.) Merr] and *Bradyrhizobium*, as well as some *Sinorhizobium,* species are specialized symbiotic partners (Pepper 2000). Besides the two parties involved in this symbiotic interaction, many environmental factors, both biotic and abiotic, can affect nodulation and subsequent nitrogen fixation and grain yield (Lie 1974; Nishijima et al. 1988; Zhang and Smith 1994; Dubey 1996; Zhang et al. 1996b; Dashti et al. 1997; Shantharam and Mattoo 1997; Vlassak and Vanderleyden 1997). Enhancing biological nitrogen fixation by exploitation of beneficial factors that promote legume growth and nodulation in agricultural systems has both environmental and economic advantages over applying nitrogen fertilizers and other agricultural chemicals (Zahran 1999). Plant growth promoting rhizobacteria (PGPR) or plant growth promoting bacteria (PGPB) are among the beneficial biotic factors known to benefit legume crop production.

In recent decades, many PGPR strains have been reported to have beneficial effects on legume growth, nodulation, nitrogen fixation and grain yield. Co-inoculation of these PGPR with rhizobia is a potential alternative way to improve legume production. In general, PGPR could have beneficial effects on host plant growth via a direct or indirect mechanism (Glick 1995). Whether they exert their beneficial effect directly or indirectly, some kind of metabolite is likely to be the responsible compound (Garcia de Salamone et al. 2001). Some metabolites, including antibiotics, siderophores, and HCN, work in an indirect manner, increasing plant growth by decreasing the activities of pathogens or other deleterious microbes. And some other metabolites, such as phytohormones and other plant growth regulators, work in a direct way, acting on targets inside plant cells, tissues or organs (Garcia de Salamone et al. 2001). Some of the PGPR strains that benefit the growth of legume plants are able to act through enhancement of nodulation and nitrogen fixation. It is well known that both legumes and rhizobia produce signal molecules that are involved in the early events of the nodulation process (Spaink et al. 1991; Prithiviraj et al. 2000). It has been demonstrated that these signal molecules, as well as some of their analogs, might be a new category of metabolites

through which the PGPR exert their beneficial effects on the host legumes. *Pseudomonas* and *Bacillus* are common residents of both the rhizosphere and the rhizoplane of at least some legume plants (Gorden et al. 1973; Hassanjadeh et al. 1991; Parmar and Dadarwal 1999). When some *Pseudomonas* and *Bacillus* strains are co-inoculated with effective *Rhizobium* spp., they stimulate chickpea growth, nodulation and nitrogen fixation (Parmar and Dadarwal 1999). Besides seed inoculation with the PGPR, the application of the ethyl acetate extracts of the culture supernatant was also effective. Both of the treatments increased flavonoid concentrations in roots (Parmar and Dadarwal 1999). The rhizobacteria-produced metabolite was isolated from the extract and determined to be a flavonoid-like compound (Parmar and Dadarwal 1999). These results indicate that PGPR may produce signal molecule analogs and/or stimulate the plant to produce more signal molecules. In legume-rhizobia interactions, plant signals are flavonoids and work as *nod* gene activators. After the rhizobial *nod* genes are activated by the flavonoid inducers, lipo-chitooligosaccharides (LCOs), the bacterial Nod factors, are synthesized (Werner 2001). Some LCO molecules are bound on the bacterial cell surface and some are excreted into the rhizosphere. LCOs are representatives of a general class of signal involved in plant and animal morphogenesis and some plants and animals also produce LCO analog signal molecules (Spaink 1996). LCOs elicit various effects on the process of plant morphogenesis, and especially on elements of the legume nodulation process, such as root hair deformation, cortical cell division and the expression of nodulins and defence-related enzymes. LCOs modulate rhizobial host specificity in the legume-rhizobial interaction. LCOs may also be involved in the inhibition of salicylic acid-mediated defense mechanisms in legumes, and this could explain why rhizobia prevent the triggering of host defense responses (Hirsch 1992; Stacey et al. 1995; Spaink 1996; Pinton et al. 2001). It is reasonable that some PGPR strains might interact with both the legume plant and the rhizobia in the rhizosphere. They might exert their effects on these interactions by producing LCO analogs or by stimulating the rhizobia to produce more LCO.

Some *Serratia* strains have been shown to have beneficial effects on legume plant growth under both greenhouse and field conditions and their mechanisms have not been well investigated (Chanway et al. 1989; Zablotowicz et al. 1991). We have studied the

effects of *Serratia proteamaculans* 1-102 and *S. liquefaciens* 2-68 on soybean plant growth and nodulation. Both strains enhance soybean growth, nodulation and nitrogen fixation either under controlled root zone temperatures (RZTs) in a greenhouse or under field conditions in a short season area. They were able to help the host plants overcome the negative effects of sub-optimal RZT (≤ 17.5 °C) on plant nodulation and growth; 1-102 generally performed better than 2-68 when applied at sub-optimal RZTs (Zhang et al. 1996b). It was also demonstrated that their culture supernatants had the same beneficial effects on soybean plants as the bacterial culture only under optimal, but not under sub-optimal, RZT conditions (Dashti 1997). Combined application of the PGPR culture and genistein had greater positive effects on soybean nodulation and nitrogen fixation than the addition of either PGPR or genistein (Dashti et al. 2000). Given these experimental results, we hypothesized that the PGPR exert their influence via production of some specific compound(s) in the plant rhizospheres. These compounds would be produced as a result of interactions between the PGPR and host plants, or between the PGPR and the nodule inducing rhizobia. By adding flavonoid inducers into a 1-102 culture system, an unidentified activator was produced by the bacterial cells (Section 4). The HPLC purified activator stimulated soybean seed germination and was responsible for the soybean plant growth promoting efficacy of 1-102 culture under both optimal and sub-optimal RZT conditions in a greenhouse (Section 4). In order to determine whether the activator has the same efficacy as 1-102 culture under field conditions, two field experiments were conducted in Quebec, a short season area in eastern Canada.

## 5.3 Materials and methods

*Experimental conditions and plant material*

The experiment was conducted at the Emile A. Lods research station of McGill University, Quebec, Canada (45° 25' 45'' N latitude and 73° 56' 00'' W longitude). Two experiments were performed at two sites, in the same year (2000). Experiment 1 (Exp-1) was on sandy-loan soil and the previous crop was barley. Experiment 2 (Exp-2) was on clay-loam soil and the previous crop was corn. Nitrogen fertilizer was not applied to either experiment. The experiments were structured following a completely randomized

block design with 3 replications. Each plot area was 5 m × 1.6 m. The space between the blocks was 1.5 m and the space between the plots was 0.2 m. The designed plant density was 400 plants per plot with a 10 cm seedling space and 20 cm row space, or 500,000 plants per ha. The soybean cultivar 'OAC Bayfield' was used in both experiments. The seeding date was May 31, 2000. Planting was performed with a small plot seeder configured so that the furrow was left open. The inoculants were added by hand and the seeds were covered immediately afterward. The inoculants were sprayed into the opened furrows with 60 mL sterilized plastic syringes. The inoculation dose for each inoculant was 10 mL per meter of furrow.

*Activator preparation*

Activator preparation followed the procedures previously described (Section 4). *S. proteamaculans* 1-102 (1-102) was cultured in King's Medium B (Atlas, 1995). The initial broth medium was inoculated with slant material and cultured at 28 °C in 250 mL flasks containing 100 mL medium for 72 h on a shaker (Table Top Orbital Shaker, Model 4530, Forma Scientific Inc., Mariotla, Ohio, USA) at 150 rev per min. The subculture was inoculated with the initiation broth inoculum at a 1% inoculation ratio and cultured in 4 L flasks containing 1 L of medium for 96 h under the same conditions as for the initial culture. During the culture period, isoflavonoid inducers (Sigma-Aldrich Canada Ltd., Oakville, Canada), including genistein (G6766), naringenin (N5893), apigenin (A9914) and luteolin (L9283) were added at final concentrations of 1 μM each (Kosslak et al., 1987). At the end of the 96 h culture period, the culture broth was extracted with butanol at a 40% (v/v) final concentration. The organic phase was collected separately and evaporated in a low-pressure rotary evaporation system (Yamota RE500, Yamato, USA) at 50 °C. The residue was re-suspended in 18% acetonitrile ($AcN/H_2O$, v/v) as a crude preparation. This crude preparation was further purified through HPLC fractionation, using a Waters system equipped with two model 510 pumps, a WISP 712 auto-sampler, a model 441 absorbence detector and a fraction collector (Waters, MA, USA). The crude sample was loaded onto a C18 reverse-phase column (Vydac 218TP54, 300 Å, 5 μm, 4.6 × 250 mm). The elution procedure was 0 – 45 min with isocratic 18% acetonitrile; 45 – 110 min with a gradient from 18 to 60.7 % acetonitrile; 110 – 115 min

with a gradient from 60.7 to 100% acetonitrile; 115 – 120 min, with a reversed gradient from 100 to 18% acetonitrile. The absorbance of the eluted fractions was monitored at 214 nm.

The activator was isolated from the chromatographic fractions through a 3-step focusing bioassay method based on soybean seed germination (Section 4). The isolated fraction stimulating soybean seed germination had a retention time of 104 – 106 min in preparation chromatography. The activator was further purified following the same HPLC program as used in the initial isolation. The relative concentration of the activator was expressed as μVs (HPLC detector microvolts) $mL^{-1}$. The final purified activator was maintained in 18% acetonitrile at 4 °C until use.

*Inoculant preparations*

In this experiment, *Bradyrhizobium japonicum* 532C (532C)(Hume and Shelp 1990), *Serratia proteamaculans* 1-102 (1-102) and the activator produced by 1-102 were used. 532C was cultured in yeast extract mannitol medium (YEM)(Vincent 1970) with an initial culture period of 7 days and a subculture period of 4 days at 28 °C, in both cases on a 200 rev $min^{-1}$ shaker. The cell density in inoculants was adjusted to $10^8$ cells $mL^{-1}$. 1-102 was cultured in King's Medium B (Atlas 1995) with an initial culture period of 3 days and a subculture period of 1 day, at 28 °C and on a 200 rev $min^{-1}$ shaker. The cell density in the broth culture was determined at 620 nm for 532C (Bhuvaneswari et al. 1980) and at 420 nm for 1-102 (Dashti et al. 1997) using spectrophotometry. The cell density of the 1-102 culture used in the inoculants, mixtures with 532C (1-102C), was adjusted to $10^8$ cells $mL^{-1}$. Based on the previous green house experiment results (Section 4), the activator was applied in a relative concentration of 830 μVs $mL^{-1}$. Besides being applied by co-inoculation with 532C in the furrows (F-act), the activator was also sprayed on the soybean seedling leaves at early vegetative (V3; L-act1) and early reproductive (R3; L-act2) stages (Fehr et al. 1971). A solution of 18% acetonitrile was diluted in the same way as the activator and used as the control for both in furrow and leaf applications.

*Data collection*

The plants were harvested three times. The first harvest was conducted on July 17 (V3 growth stage, the 3rd node developed) and the second on August 16 (R3 growth stage, beginning pod), 2001. When harvesting, five randomly selected plants were removed from each plot by uprooting with a spade. Data on leaf number, leaf area ($cm^2$), nodule number, nodule weight, shoot weight and root weight were collected. All weight data were recorded after the samples were oven dried at 70 – 80 °C for at least for 48 h. Plant weight was defined as shoot weight plus root weight. The final harvest was conducted on October 10 (R8, harvest maturity), 2001. Plants in the central 1 m of the two center rows (area of 0.4 $m^2$) in each plot were collected by hand. Plant number, branch number, and pod number were counted for each plot. After the roots were removed, the shoots were oven dried at 70 – 80 °C for not less than 48 h. In this case, the shoot weight was taken as the total weight, i.e, the biological yield. The shoots were mechanically threshed to remove the seeds. The seed weight and the 100-seed weight were also determined. The seed weight was taken as the economic yield. Seed yield is given at 0% moisture. The difference between the shoot weight and seed weight was defined as stem weight. The harvest index was expressed as the ratio of the economic yield (the seed weight) to the biological yield (the total weight). The total number of seeds and the seed number per pod were calculated by using the variables seed weight, 100-seed weight and pod number. The nitrogen content of the stem and the seeds were determined separately using an element analyzer (NC250b Elementory Analyzer, ThermoQuest Italic S.P.A., Italy) and used to calculate stem and seed nitrogen yield. The total nitrogen was defined as the sum of stem nitrogen and seed nitrogen.

*Statistical analysis*

All data were analyzed using the GLM procedure of SAS (Littell et al. 1991). Before conducting the ANOVA, a test of homogeneity of variances between the two experiments was conducted using the Bartlett's test (Steel and Torrie 1980). When analysis of variance indicated differences due to treatments, comparisons among the treatment means of each experiment were conducted with an ANOVA protected least

significance difference (LSD) test (Steel and Torrie 1980). In the text, P value was always offered and differences were considered significant at either $P < 0.5$ or $P < 0.1$.

## 5.4 Results

*Effects of the activator on the soybean growth and nodulation at the V3 stage*

Leaf number, leaf area, and plant weight in Exp-1 were all lower than in Exp-2 ($P = 0.01$; Table 5.1) for material harvested at the V3 stage (July 17). However, nodule number and nodule weight in Exp-1 were higher than in Exp-2 ($P = 0.01$). Although there were differences between the two experiments, the comparison of some variables from the different treatments within each experiment were valuable. Compared with the 532C alone control, treatment with 1-102C and F-act increased nodule number ($P = 0.10$) in both Exp-1 and Exp-2, and nodule weight ($P = 0.10$) in Exp-1. F-act performed as well as 1-102C. F-act increased nodule number by 59.2% and 65.9% while 1-102C increased by 60.5% and 60.4% in Exp-1 and Exp-2 respectively. F-act increased nodule weight by 38.7% while 1-102C increased this variable by 42.3% in Exp-1. The L-act1 treatment caused no detectable differences. The acetonitrile controls had no effect on any of the plant growth and nodulation variables measured at the V3 stage (data not shown).

*The effect of the activator on the soybean growth and nodulation at the R3 stage*

There were still large differences ($P = 0.01$) between the two experiments at the R3 (Aug. 17) harvest (Table 5.2), and the pattern of these differences was the same as for the first harvest. Nodule number and nodule weight were higher in Exp-1 than in Exp-2. Leaf number, leaf area and plant weight were lower in Exp-1 than in Exp-2. However, in both Exp-1 and Exp-2, the positive effects of F-act were at a level similar to 1-102C (Table 5.2). Compared with the 532C alone control, F-act increased nodule number by 34.6% and nodule weight by 26.6% in Exp-1 ($P = 0.05$) and nodule number by 63.9% and nodule weight by 65.2% in Exp-2 ($P = 0.10$). While 1-102C increased nodule number by 34.6% and nodule weight by 21.0% in Exp-1 ($P = 0.05$) and nodule number by 88.8 % and nodule weight by 71.4 % in Exp-2 ($P = 0.10$). L-act1 was not different from the 532C control in both experiments. L-act2 resulted in numeral increase in nodule number and nodule weight but this was only significant in Exp-2 at $P = 0.10$.

Considering of the fact that L-act2 was applied 10 days before the harvest, it seemed that leaf applying could influence plant growth more rapidly than the furrow application.

In each experiment there were no differences among treatments for leaf number and leaf weight. F-act resulted in an increase in plant weight (P = 0.01) in both experiments, while 1-102Ccaused a positive effect only in Exp-1. As with the first harvest, the acetonitrile controls had no effect on plant growth and nodulation (data not shown).

*Application of the activator increased grain yield and nitrogen yield*

The final harvest results of both experiments showed positive effect of application of the activator either by co-inoculation with 532C in the seed furrow or by spray application onto the leaves at the R3 stage. Compared with the 532C alone control, F-act increased total weight by 20.9 and 16.4%, seed weight by 22.9 and 21.7 % in Exp-1 (P = 0.10) and Exp-2 (0.05), respectively. L-act2 increased total weight by 24.9 and 20.4%, and seed weight by 28.1 and 22.4% in Exp-1 (P = 0.10) and Exp-2 (P = 0.05), respectively. 1-102Cincreased nodule number and nodule weight at the same level as F-act and L-act2. L-act1 and the acetonitrile controls (data not shown) were not different from the 532C alone control treatment. The increased seed weight was mainly due to increased pod number (P = 0.1) and total seed number (P= 0.1 in Exp-1 and P = 0.05 in Exp-2). The 100-seed weight and the harvest index (data not shown) were not different among the applied treatments in both Exp-1 and Exp-2. Differences in total weight, seed weight, total seed number and 100-seed weight existed between the two experiments (Table 5.3). However, at final harvest, all of these four variables in Exp-1 were greater than in Exp-2. This was consistent with nodule number and nodule weight in previous harvests, but contrary to leaf area and plant weight data.

As with the total weight and seed weight, total N and seed N in Exp-1 were much higher than in Exp-2 (data not shown, P = 0.01). The harvest index was 0.520 in Exp-1 and 0.423 in Exp-2. The nitrogen yields were increased in both experiments by activator treatments F-act and L-act2, and at similar levels to that of 1-102C. The bulk of the total N was in the seeds (Fig. 5.1).

## 5.5 Discussion

In previous greenhouse experiments, we demonstrated the ability of root applications of the activator to stimulate soybean nodulation and plant growth, at a suitable application dose, under both optimal and sub-optimal RZT conditions (Section 4). In these field experiments the efficacy of the activator was clearly demonstrated: under short season field conditions, at 830 μVs per plant, either applied in furrow or sprayed on leaves at the R3 stage, the activator showed same efficacy as coinoculation of the PGPR culture. These results support the greenhouse finding that the activator is responsible for the plant growth promoting activity of *S. proteamaculans* 1-102.

In the previous greenhouse experiments, we had found that under N-free culture conditions leaf spraying of the activator, at an early soybean plant developmental stages (20 days after inoculation) did not have positive effects (Section 4). In these field experiments, under N limited conditions, the effect of the activator sprayed on the leaves was dependent on spraying time. The L-act2, sprayed at early R3 stage, performed well and L-act1, spayed at early V3 stage, failed to have any positive effect on soybean plants. In the previous greenhouse experiment, we found that spraying the activator on leaves at early development stages resulted in reduced single leaf area and higher activator concentrations resulted in even smaller single leaf area. We postulated that this phenomenon was due to the influence of activator spraying on the nutrient translocation (Section 4). In these field experiments, L-act1 application did not result in reduced average single leaf areas, so the greenhouse observation and explanation for the observed phenomenon needs to be further investigated. The positive results of L-act2 showed that the activator was effective when applied leaves at the R3 stage and suitable leaf application caused positive effects more rapidly than root applications. In practice, application of the activator in the furrow is much more convenient and economic than spraying on leaves. However, comparison the effects of these two applications of the activator on plant physiological activity would be informative for further understanding of its mechanisms.

At all the three harvests, there were large differences between the two experiments. However the situations were changed after R3 stage; at the first and second

harvests, nodule number and weight were greater for Exp-1 than Exp-2, but all other measured variables of Exp-1 were lower than Exp-2. At the final harvest, most of the variables (Table 5.3) were greater for Exp-1 than Exp-2. Given that the two experiments were conducted on the same farm and during the same year, the differences between the experiments might be explained by different soil nutrient levles. The soil at the Exp-1 site was a sandy-loam with a previous barley crop. The soil P and K were 180 and 219 kg/ha, respectively (soil test). The soil in Exp-2 was clay-loam with a previous corn crop. The soil P and K were 347 and 520 kg/ha, respectively (soil test). In general, more fertilizer N was applied to the corn than the barley and more soil residual fertilizer would be expected after the corn crop (Pan and Smith 2000b), so the soil at the Exp-2 site may have had a higher background nutrient level than at the Exp-1 site. Higher nutrition level would have supported better vegetative plant growth but inhibited the nodulation process (Abaidoo et al. 1990; Rai 1992). At the first two harvests, the plants in Exp-2 had better plant growth but fewer nodules and less nodule weight. This was consistent with higher soil nutrient levels at the Exp-2 site. After the R3 growth stage, soil nitrogen was likely to have been largely exhausted even at the Exp-2 site. N nutrition was likely to have become a more serious limiting factor for continued plant reproductive growth and fixed N became the major N nutrient source. The nitrogen accumulation rate in soybean plants was greatly increased at about 8 weeks after seeding, and about 70% of the plant nitrogen was accumulated after the R3 stage (Weiss 2000). At the final harvest, biological yield, economic yield, and N yield were all higher in Exp-1 than Exp-2. Exp-1 had a greater seed number, higher 100-seed weight (Table 5.3) and larger harvest index. No matter what caused the differences between the two experiments, the relative performances of the different treatments in each experiment supported the same conclusion: the activator is responsible for the plant growth promoting effects of the PGPR in question, as it showed the same plant growth stimulating ability as the PGPR coinoculation.

Many PGPR strains have been reported to have beneficial effect on the symbiotic interactions between the legumes and the rhizobia (Lie 1974; Nishijima et al. 1988; Zhang and Smith 1994; Dubey 1996; Zhang et al. 1996b; Dashti et al. 1997; Shantharam and Mattoo 1997; Vlassak and Vanderleyden 1997). When a PGPR strain is coinoculated with rhizobia, the symbiotic interaction between plants and rhizobia will be unavoidably

affected by the coinoculated PGPR. This results in a tripartite system. How the three parties interact with each other in the rhizosphere needs to be further clarified. It is known that some PGPR exert their beneficial effects on legume-rhizobial interactions by producing flavonoid-like compounds (Parmar and Dadarwal 1999). Such PGPR should be involved in signal exchange between the two symbiotic partners. On the other hand, addition of flavonoids (Pan and Smith 2000a and 2000b; Dashti et al. 2000) and LCO preparations (Prithiviraj et al. 2000) also have beneficial effects on the symbiotic interactions. Our field experiments and previous greenhouse experiments on the unidentified activator produced by *S. proteamaculans* 1-102 was based on a hypothesis that *S. proteamaculans* 1-102 exerts its positive effect on soybean growth by producing some specific metabolite(s) which stimulates the soybean-bradyrhizobial symbiotic interaction. Based on available experimental results, this metabolite was conjectured to be an LCO analog, proposed to increase the signal to noise ratio (SNR) of plant-rhizobia communication in the rhizosphere (Section 4). Although these field experimental results do not offer further information regarding the chemical structure of the activator or its mechanisms, they do demonstrate its efficacy in promoting soybean plant nodulation and growth. No matter what kind of compound the activator eventually proves to be, the fact that it is produced more abundantly after addition of flavonoid inducers into the PGPR culture system, and its effectiveness in promoting soybean growth and nodulation under both greenhouse and field conditions offer novel clues toward understanding the subtle three-party interactions between legume hosts, rhzobia and PGPR in rhizosphere.

**Fig. 5.1. Total and seed nitrogen yield in the two field experiments on *Serratia proteamaculans* and its inducible activator.**

Treatment: 532C, inoculation of *Bradyrhizobium japonicum* 532C alone; 1-102C, coinoculation of *Serratia proteamaculans* 1-102 with *B. japonicum* 532C; F-act, applied activator in furrow with *B. japonicum* 532C inoculant; L-act1, sprayed activator on leaves at V3 growth stage; L-act2, sprayed activator on leaves at R3 growth stage. Different letters on each bar indicate differences at P = 0.10 level for total nitrogen in both experiments and seen nitrogen in the experiment-1 and at P = 0.05 level for seed nitrogen in the experiment 2. The capital letters are for comparison of total nitrogen and the small letters are for comparison of seed nitrogen among the treatments in each experiment. n = 3.

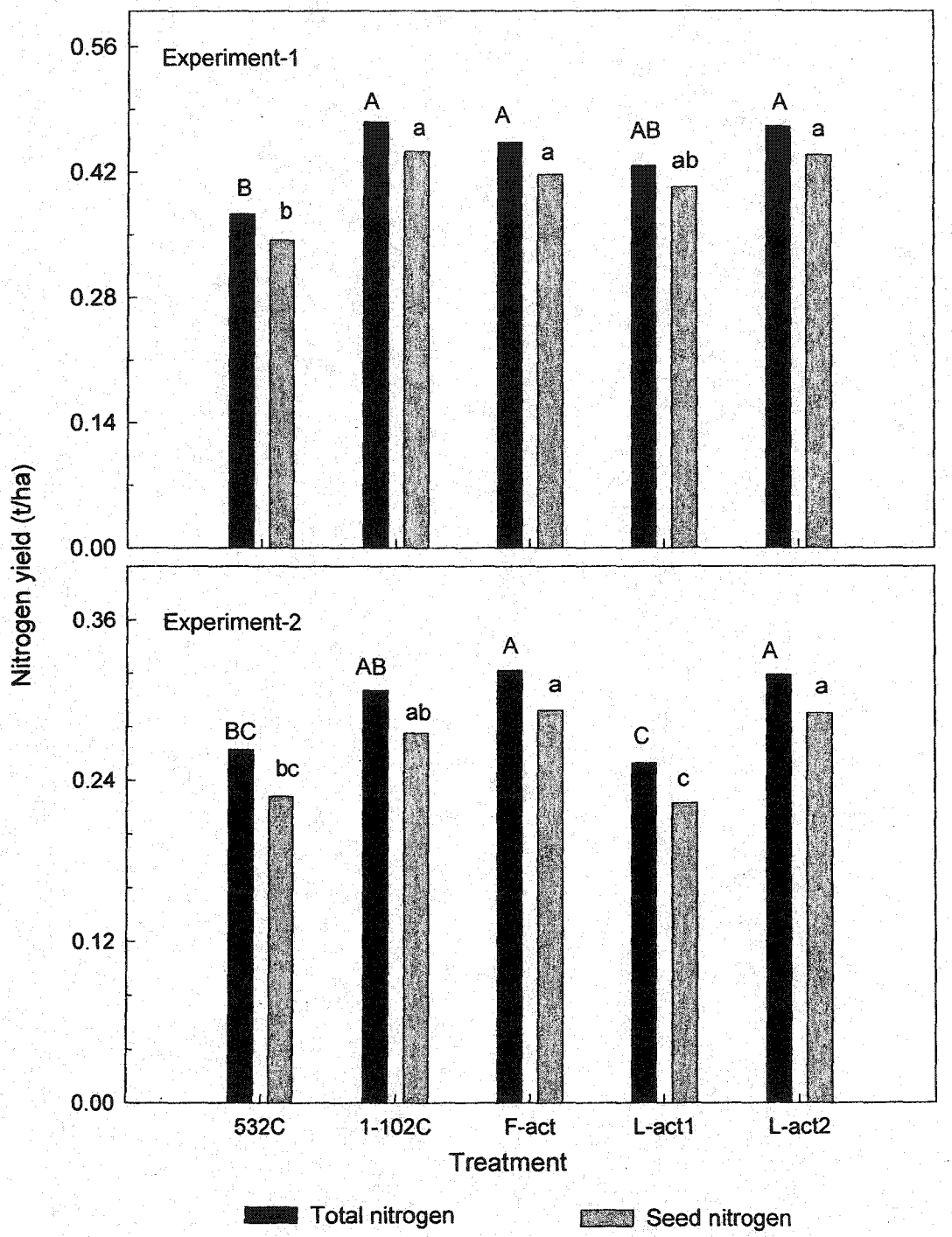

Experiment-1
Experiment-2
Nitrogen yield (t/ha)
0.56
0.42
0.28
0.14
0.00
0.36
0.24
0.12
0.00
B
b
A
a
A
a
AB
ab
A
a
BC
bc
AB
ab
A
a
C
c
A
a
532C
1-102C
F-act
L-act1
L-act2
Treatment
Total nitrogen
Seed nitrogen

**Table 5.1. The effect of the applied PGPR and the activator treatments on soybean growth and nodulation at the V3 stage in the two field experiments, and overall comparisons between experiments for each variable.**

| | Leaf number (per plant) | Leaf area ($cm^2$/plant) | Nodule number (per plant) | Nodule weight (10 mg/plant) | Plant weight (g/plant) |
|---|---|---|---|---|---|
| Experiment-1 | 11.6 b | 504.2 b | 31.3 a | 12.4 a | 4.83 b |
| Experiment-2 | 19.6 a | 1154.0 a | 17.6 b | 9.0 b | 10.5 a |
| In experiment-1 (n=3) | | | | | |
| 532C | 11.1 | 502.0 | 25.5 b | 11.1 b | 4.97 |
| 1-102C | 11.4 | 561.0 | 40.9 a | 15.8 a | 5.69 |
| F-act. | 11.5 | 572.2 | 42.3 a | 15.4 a | 5.63 |
| L-act1 | 12.6 | 508.0 | 26.6 b | 11.1 b | 4.90 |
| In experiment-2 (n=3) | | | | | |
| 532C | 18.5 | 1054.7 | 14.7 b | 7.4 | 9.61 |
| 1-102C | 18.8 | 1296.0 | 23.6 a | 10.5 | 11.69 |
| F-act. | 19.9 | 1268.0 | 23.4 a | 11.9 | 11.57 |
| L-act1 | 18.4 | 1010.3 | 16.1 b | 8.3 | 10.05 |

Treatment: 532C, inoculation of *Bradyrhizobium japonicum* 532C alone; 1-102C, coinoculation of *Serratia proteamaculans* 1-102 with *B. japonicum* 532C. F-act, applied activator in furrow with *B. japonicum* 532C inoculant; L-act1, sprayed activator on leaves at V3 growth stage. Means within the same column and experiment, or for comparisons between the experiments, followed by the same letter are not different by an ANOVA protected LSD test. When no letters are given there was no significant effect indicated in the initial ANOVA analysis. The comparison of variables within experiments (nodule number and nodule weight) $P = 0.05$. The overall comparison between the two experiments was conducted at $P = 0.01$.

**Table 5.2. The effect of the applied PGPR and the activator treatments on soybean growth and nodulation at the R3 stage in the two field experiments, and overall comparisons between experiments for each variable.**

| | Leaf number (per plant) | Leaf area ($100cm^2$/plant) | Nodule number (per plant) | Nodule weight (10mg/plan) | Plant weight (g/plant) |
|---|---|---|---|---|---|
| Experiment-1 | 27.6 b | 18.4 a | 73.4 a | 75.5 a | 27.07 b |
| Experiment-2 | 38.4 a | 27.9 b | 27.3 b | 28.4 b | 47.67 a |
| In the experiment-1 (n=3) | | | | | |
| 532C | 27.1 | 16.1 | 64.1 b | 66.9 b | 23.12 c |
| 1-102C | 27.9 | 21.3 | 86.3 a | 86.5 a | 32.55 a |
| F-act. | 28.5 | 23.1 | 86.3 a | 84.7 a | 32.43 a |
| L-act1 | 30.2 | 15.8 | 71.9 ab | 71.5 ab | 25.73 bc |
| L-act2 | 28.2 | 20.6 | 77.7 ab | 79.7 ab | 29.31 abc |
| In the experiment-2 (n=3) | | | | | |
| 532C | 37.3 | 24.8 | 20.5 c | 22.4 c | 44.23 b |
| 1-102C | 42.5 | 30.8 | 38.7 a | 38.4 a | 54.33 ab |
| F-act. | 45.3 | 33.9 | 33.6 a | 37.0 a | 57.67 a |
| L-act1 | 37.4 | 27.3 | 23.1 bc | 23.7 bc | 47.03 ab |
| L-act2 | 39.6 | 32.7 | 32.4 ab | 34.3 ab | 52.38 ab |

Treatment: 532C, inoculation of *Bradyrhizobium japonicum* 532C alone; 1-102C, coinoculation of *Serratia proteamaculans* 1-102 with *B. japonicum* 532C. F-act, applied activator in furrow with *B. japonicum* 532C inoculant; L-act1, sprayed activator on leaves at V3 growth stage; L-act2, sprayed activator on leaves at R3 growth stage. Means within the same column and experiment, or for comparisons between the experiments, followed by the same letter are not different by an ANOVA protected LSD test. When no letters are given there was no significant effect indicated in the initial ANOVA analysis. For comparison of nodule number and nodule weight, the P value was 0.05 in experiment 1 and 0.10 in Exp-2. For comparison of plant weight in each experiment the P value was 0.1. The overall comparison between the two experiments was conducted at P = 0.01.

**Table 5.3. The effect of the applied PGPR and the activator treatments on yield related variables in the two field experiments, and overall comparisons between experiments for each variable.**

| Treatment | Pod number ($10^6$ pods/ha) | Total weight (t/ha) | Seed weight (t/ha) | Total seed number ($10^6$ pods/ha) | 100 seed weight (g) |
|---|---|---|---|---|---|
| Experiment-1 | 13.65 a | 12.73 a | 6.62 a | 32.62 a | 20.31 a |
| Experiment-2 | 13.08 a | 11.99 b | 5.07 b | 29.53 b | 17.21 b |
| In the experiment-1 (n=3) | | | | | |
| 532C | 11.15 b | 11.08 b | 5.63 b | 27.52 b | 20.37 |
| 1-102C | 15.25 a | 13.69 a | 7.23 a | 36.82 a | 19.70 |
| F-act. | 14.28 a | 13.47 a | 6.93 a | 33.92 a | 20.41 |
| L-act1 | 13.18 ab | 11.50 ab | 6.46 ab | 31.65 ab | 20.43 |
| L-act2 | 14.87 a | 13.84 a | 7.21 a | 34.78 a | 20.77 |
| In the experiment-2 (n=3) | | | | | |
| 532C | 12.51 b | 10.84 c | 4.57 c | 26.36 c | 17.30 b |
| 1-102C | 14.57 a | 12.41 ab | 5.45 ab | 31.49 ab | 17.33 ab |
| F-act. | 13.37 ab | 12.61 ab | 5.58 a | 32.80 a | 17.13 b |
| L-act1 | 12.54 b | 11.37 bc | 4.50 c | 27.79 bc | 16.10 b |
| L-act2 | 13.73 a | 13.05 a | 5.59 a | 30.05 abc | 18.63 a |

Treatment: 532C, inoculation of *Bradyrhizobium japonicum* 532C alone; 1-102C, coinoculation of *Serratia proteamaculans* 1-102 with *B. japonicum* 532C. F-act, applied activator in furrow with *B. japonicum* 532C inoculant; L-act1, sprayed activator on leaves at V3 growth stage; L-act2, sprayed activator on leaves at R3 growth stage. Within each column and experiment, or for the comparison between experiments, means followed by the same letter are not different by an ANOVA protected LSD test. The P value for difference was 0.10 in experiment-1 and 0.05 in experiment-2. The overall comparison between the two experiments was conducted at P = 0.01 level.

## Preface to Section 6

Section 6 is comprised of a manuscript by Bai Y, D'Aoust F, Smith DL and Driscoll BT published *Canadian Journal of Microbiology* in 2002, Vol. 48, 230-238.

In Section 3, 4, and 5, I investigated the mechanisms of the soybean plant growth promoting efficacy of the two *Serratia* strains, in which the optimal coinoculation dose, nodulation dynamics and the responsible active compounds were determined. In this section of the thesis I report on the isolation of non-bradyrhizobial endophytic bacteria from surface disinfected root nodules detached from field-grown soybean plants that were particularly vigorous. Of the primary isolates, three enhanced soybean nodulation and plant growth. These three strains were identified to the level of species.

Of the co-authors, Mr. D'Aoust is a Ph.D. candidate supervised by Dr. Driscoll. Mr. D'Aoust and I conducted the experimental procedures on bacterial identification. Dr. Smith is my thesis supervisor. He 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, bench space in greenhouse, experimental field space, the use of computers, and providing technical assistance. Dr. Driscoll is a professor in Department of Natural Resource Sciences, Macdonald Campus of McGill University. He provided experimental facilities and technical guidance in my identification of the selected NEB strains. Dr. Driscoll is the corresponding author for publication of this section.

# Section 6

# Isolation of plant-growth promoting *Bacillus* strains from soybean root nodules

## 6.1 Abstract

Endophytic bacteria reside within plant tissues, and have often been found to promote plant growth. Fourteen strains of putative endophytic bacteria, not including endosymbiotic *Bradyrhizobium* strains, were isolated from surface-sterilized soybean [*Glycine max.* L. Merr] root nodules. These isolates were designated as non-*Bradyrhizobium* endophytic bacteria (NEB). Three isolates (NEB4, NEB5 and NEB17) were found to increase soybean plant weight when co-inoculated with *B. japonicum* under nitrogen-free conditions, compared to plants inoculated with *B. japonicum* alone. In the absence of *B. japonicum*, these isolates neither nodulated soybean, nor did they affect soybean growth. All three isolates were Gram positive spore-forming rods. While Biolog tests indicated that the three isolates belonged to the genus *Bacillus*, it was not possible to determine the species. Phylogenetic analysis of 16S rRNA gene hypervariant region sequences demonstrated that both NEB4 and NEB5 are *B. subtilis* strains, and that NEB17 is a *B. thuringiensis* strain.

**Key words:** root nodule, endophytic bacteria, plant-growth promoting bacteria, *Bacillus subtilis, Bacillus thuringiensis.*

## 6.2 Introduction

Plants are constantly involved in interactions with a wide range of bacteria. These plant-associated bacteria colonize the rhizosphere (rhizobacteria), the phyllosphere (epiphytes), and the inside of plant tissues (endophytes). Endophytes are sheltered from environmental stresses and microbial competition by the host plant, and they seem to be

ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots, and seeds of various plant species (Kobayashi and Palumbo 2000). Depending on their effect on the host plant, endophytic bacteria can be categorized into three groups: plant-growth promoting, plant-growth inhibiting and plant-growth neutral (Sturz et al. 2000).

Bacterial strains that have beneficial effects on plant health are referred to as beneficial plant-associated bacteria, plant-growth-promoting bacteria (PGPB), or plant-growth promoting rhizobacteria (PGPR; Andrews and Harris 2000). PGPB can promote plant growth directly or indirectly, via biocontrol of host plant diseases, production of phytohormones or improvement of plant nutritional status (Glick 1995).

Rhizobia are perhaps the best known beneficial plant-associated bacteria because of the importance of the nitrogen fixation that occurs during the *Rhizobium*-legume symbiosis. The co-inoculation of other PGPB with rhizobia is becoming a practical method in the development of sustainable agriculture, because of yield increases seen compared to inoculation with rhizobia alone. PGPB that have been tested as co-inoculants with rhizobia include strains of the following well-known rhizobacteria: *Azospirillum* (Yahalom et al. 1987), *Azotobacter* (Burns et al. 1981), *Bacillus* (Srinivasan et al. 1996), *Pseudomonas* (Chanway et al. 1989), *Serratia* (Chanway et al. 1989; Zhang et al. 1997) and *Streptomyces* (Li and Alexander 1988).

Endophytic bacteria have been isolated from legume plants such as alfalfa (Gagne et al. 1987), clover (Sturz et al. 1997), pea (Elvira-Recuenco and van Vuurde 2000) and soybean (Oehrle et al. 2000). Bacteria of several genera have been isolated from legume tissues, including: *Aerobacter*, *Aeromonas*, *Agrobacterium*, *Bacillus*, *Chryseomonas*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Flavimonas*, *Pseudomonas* and *Sphingomonas* (Gagne et al. 1987; Sturz et al. 1997; Elvira-Recuenco and van Vuurde 2000; Oehrle et al. 2000). Sturz et al. (1997) reported the isolation of fifteen non-rhizobial species from clover root nodules, eight of which were found only in root nodule tissues.

Endophytic PGPB have the potential to be used as agricultural inoculants (Hallmann et al. 1997; Sturz et al. 2000). PGPB have been isolated by screening the rhizosphere, phyllosphere, and the tissues of plants showing particularly vigorous growth in the field. This approach was used to isolate the effective plant-growth-promoting

*Bacillus megaterium* strain B153-2-2 from a field-grown soybean plant (Liu and Sinclair 1993). Our goal was to isolate endophytic PGPB from the root nodules of particularly vigorous field-grown soybean plants.

## 6.3 Materials and Methods

*Bacterial strains and growth conditions*

*Bradyrhizobium japonicum* 532C is a Hup$^-$ strain adapted to Canadian soils (Hume and Shelp 1990). Wild-type strains of *Staphylococcus aureus* and *Bacillus cereus* were from the Microbiology Unit collection, Department of Natural Resource Sciences, McGill University. *Bradyrhizobium japonicum* was cultured at 28°C using yeast extract mannitol (YEM; Vincent 1970). NEB strains were cultured at 28°C using King's Medium B (Atlas 1995), or at 30°C using Luria Bertani (LB) broth (Sambrook et al. 1989), or Ashbey's nitrogen-free medium (Atlas 1995) with different combinations of the following; mannitol (15 g/l), dextrose (15 g/l), $NH_4NO_3$ (0.5 g/l), proteose peptone (1 g/l; Anachemica Canada, Inc., Montreal QC), and yeast extract (1 g/l; Anachemica). Liquid cultures were grown in flasks or test tubes, with rotary agitation (200 rpm), and plates were prepared by solidifying the media with 1.5% [w/v] agar (Anachemica). Culture densities were estimated by optical density by $A_{620}$ for *B. japonicum*, or $A_{420}$ for the NEB strains (Dashti et al. 1997).

*Isolation of endophytic bacteria from surface-sterilized nodules*

On August 14, 1998, twenty vigorous soybean [*Glycine max.* L. Merr] seedlings at the R3 stage (Fehr et al. 1971) were selected from five fields at the A. E. Lods Agronomy Research Center, Macdonald Campus, McGill University. The fields had been sown with soybean cultivars OAC Bayfield and OAC Maple Glen, and inoculated with *B. japonicum* 532C, as described (Dashti et al. 1997). The roots were washed thoroughly with tap water, and 80 healthy nodules were detached along with a portion of the root. The nodules were placed into sterilized flasks and were surface-sterilized by rinsing with 95% ethanol for 15 sec, and then with acidified 0.1% $HgCl_2$ solution for 3–5 min, depending

on the size of the nodule. The nodules were then rinsed with three cycles of 4-5 changes of sterile $H_2O$, followed by soaking in sterile $H_2O$ for 15 min.

Twenty nodules, four from each of the five fields, were placed into separate sterile Eppendorf tubes with 1 ml of sterile $H_2O$. To confirm nodule surface sterility, the tubes were vortexed (2 min), 0.1 ml of the surface-wash water was spread on YEM plates, and the plates were incubated at 28°C for 4 days. Immediately following surface-sterilization, the nodules were crushed aseptically, nodule contents were streaked onto YEM plates, and the plates were incubated at 28°C. Non-*Bradyrhizobium* colonies were chosen on the basis of colony morphology and growth rate. After four days, non-*Bradyrhizobium* colonies were picked, and then were purified by single-colony streaking on three successive King's Medium B plates. A total of 14 strains with distinct colony morphologies (three strains from one nodule, two each from three nodules, and one each from five nodules) were kept for further study. Isolates were only retained from nodules that were confirmed to have been surface-sterilized. The putative nodule endophyte strains were designated as NEB (non-*Bradyrhizobium* endophytic bacteria).

*Soybean cultivation in growth pouches*

Growth pouch experiments were arranged following a completely randomized split plot design with three replicates per inoculation treatment (Mead et al. 1993). Soybean (cultivar OAC Bayfield) seeds were surface-sterilized (2% NaOCl, 4 ml/l Tween 20, 3 minutes), rinsed with several changes of sterile $H_2O$, and then germinated in trays of vermiculite in a greenhouse. The greenhouse conditions were: air temperature 25±1°C, with supplemental illumination of 300 $\mu mol/m^2/s$ via high pressure sodium lights (P.L. Light System Canada) for a photoperiod of 16:8 h (day:night). Single four-day-old healthy seedlings at the VE stage (Fehr et al. 1971) were transplanted into each growth pouch (15×16 cm, Mega International, Minneapolis, MN) and suspended in a 25°C water bath in the greenhouse. The plants were watered as needed with N-free Hoagland's solution, in which $Ca(NO_3)_2$ and $KNO_3$ were replaced with $CaCl_2$, $K_2HPO_4$ and $KH_2PO_4$, as recommended (Hoagland and Arnon 1950). Six days following transplantation, the seedlings were inoculated with $10^8$ cells from late-log phase cultures of the NEB strains

(King's Medium B), 72h sub-cultures of *B. japonicum* (YEM), or co-inoculated with combinations of both. Control plants were inoculated with 1 ml of sterile distilled $H_2O$ or suitably-diluted sterile King's Medium B. Inoculation with the medium had no effect on plant growth or nodulation relative to inoculation with sterile distilled $H_2O$.

Plants were harvested 55 days following inoculation, and nodule number, nodule dry weight, root dry weight, and shoot dry weight data were collected, each on a per plant basis. Dry weight data were determined from samples dried at 70°C for a minimum of 48 h. Plant dry weight values were the sum of shoot plus root dry weight values for each plant. When analysis of variance indicated differences among means, comparisons among the treatment means were conducted with an ANOVA protected least significance difference (LSD) test (Steel and Torrie 1980).

The three strains (NEB4, NEB5, NEB17) that had positive effects on soybean growth and/or nodulation when co-inoculated with *B. japonicum* 532C, were tested with soybean plants, as above, in the absence of *B. japonicum*. Control plants were inoculated with 1 ml of sterile distilled $H_2O$. Nodule number and plant dry weight were determined as above, and the nitrogen content of dried plants (shoot plus root) was determined using the Kjeldahl method (Kjeltec system, with Digestion System 20, and a 1002 Distilling Unit, Tecator AB, Hoganas, Sweden), as previously described (Bremner 1965). Control values for plant dry weight and nitrogen content were 864 ± 68 mg/plant and 11.1 ± 1.4 mg/plant, respectively (mean ± SD, n=6).

*Phenotypic characterization of NEB strains*

NEB4, NEB5 and NEB17 cells were harvested from 24 h King's Medium B plates for cytological staining and microscopy. The cultures were tested for the presence of spores using the Schaeffer-Fulton staining method, and for Gram reaction. As all three strains were found to be Gram positive, they were assayed for carbon utilization using Biolog GP Microplates (Biolog Inc., Hayward, CA), following the manufacturer's instructions. *Staphylococcus aureus* and *Bacillus cereus* were used as controls. All strains were cultured on plates of Biolog Universal Growth Medium (BUGM; Biolog Inc.) plus 1% [w/v] glucose, at 30°C for 9 h. Glucose was added to the BUGM in an

attempt to limit the degree of sporulation, as directed by the manufacturer for dealing with putative *Bacillus* species. Aliquots (150 µl) of the cell suspensions were distributed into each of the 96 wells, and then the Microplates were incubated at 30°C. Colourimetric changes were measured by determining the $A_{595}$, after 4 h and 24 h, using a 3550-UV Microplate Reader (BioRad Laboratories, Mississauga, ON). Readings were standardized against the control well containing no carbon source. Standardized absorbance values greater than 0.1 were scored as positive. Putative identifications were made using MicroLog1 v. 3.50 software plus database (Biolog), and only similarity index (SIM) values above 0.5 were considered significant for identification purposes (Biolog).

*Extraction of plasmid and bacterial genomic DNA*

Genomic DNA was extracted from cultures of NEB4, NEB5, and NEB17 grown to stationary phase in LB broth at 30°C, using the standard lysozyme/SDS/Pronase protocol (Sambrook et al. 1989). The DNA was purified using DNeasy Tissue kits (Qiagen, Mississauga, ON). Plasmid DNA was isolated from cultures grown in LB plus ampicillin (50 µg/ml) at 37°C, using QIAprep spin miniprep kits (Qiagen) according to the manufacturer's instructions. Agarose gel electrophoresis (0.8% agarose, TAE buffer pH 8.0) and staining with 0.5 mg/l ethidium bromide was done as previously described (Sambrook et al. 1989). DNA concentrations were estimated relative to the *Hin*dIII-digested lambda DNA standard (Gibco-BRL, Life Technologies, Burlington, ON) using an AlphaImager (Alpha Innotech, Mississauga, ON). PCR products, and plasmid DNA to be used as template in DNA sequencing reactions, were excised from agarose gels and purified using QIAEX II gel extraction kits (Qiagen).

*PCR amplification and DNA sequencing*

The complete 1.6 kb 16S rDNA region was amplified using the universal bacterial 16S rDNA primers 27f [5' - AGA GTT TGA TCM TGG CTC AG], and 1492r [5' - TAC GGY TAC CTT GTT ACG ACT T] (Ritchie et al. 1997). Primers BhvF1 [5' - TGT AAA ACG ACG GCC AGT GCC TAA TAC ATG CAA GTC GAG CG], and BhvR1 [5' - CAG GAA ACA GCT ATG ACC ACT GCT GCC TCC CGT AGG AGT], were used

to amplify approximately 350 bp containing the hypervariant (HV) region of *Bacillus* 16S rDNA (Goto et al. 2000). PCR reactions (50 µl) contained: 25 to 50 ng of purified genomic DNA; 10 pmol of each primer; PCR buffer (Gibco-BRL); 1.5 mM $Mg^{2+}$ (Gibco-BRL); and 200 µM dNTPs (Roche, Laval, QC, Canada). Template DNA was denatured at 94°C for 3 min, then 2.5 U *Taq* DNA polymerase (Gibco-BRL) was added, and the reaction was cycled 30 times as follows: denaturation for 1 min at 92°C; annealing for 1 min 60°C; extension for 1 min at 72°C. This was followed by a final extension for 5 min at 72°C. A PTC-100 thermocycler (MJ Research, Waltham, MA) was used.

PCR products were ligated into the vector pGEM-T Easy, and ligation products were transformed into $CaCl_2$-competent *E. coli* DH5α cells, using the materials and protocols supplied with the vector (Promega Inc., Madison, WI, USA). Plasmid DNA was isolated from positive clones, and purified prior to sequencing, as described above. DNA sequencing was done using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Mississauga, ON), and standard T7 and SP6 promoter sequencing primers (Gibco-BRL). Sequencing reactions were run on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Nucleotide sequences were compiled using Sequencher v. 3.0 (Gene Codes Corporation, Inc., Ann Arbor, MI). The NEB4 (275 nucleotides), NEB5 (275 nucleotides), and NEB17 (277 nucleotides) 16S rRNA gene HV sequences were deposited in GenBank under accession numbers AF406704, AF406705, and AF406706, respectively.

*Phylogenetic analysis*

DNA sequences were compared to the nr nucleotide databases using the standard nucleotide-nucleotide BLAST (blastn) search algorithm (Altschul et al. 1997). Phylogenetic analysis was done using MacVector v. 7.0 (Oxford Molecular Ltd., Genetics Computer Group, Madison WI). Nucleotide sequences were aligned using the CLUSTAL W algorithm (Thompson et al. 1994). Phylogenetic trees were reconstructed by the neighbor-joining method (Saitou and Nei 1987), using the distance matrix from the alignment. Distances were calculated using both the Kimura (Kimura 1980) and Tamura-Nei (Tamura and Nei 1993) methods. Gaps were ignored, no gamma correction shape

was specified, and for the Kimura method, the transition:transversion ratio was estimated by the algorithm (average = 1.81). Phylogenetic trees were subjected to bootstrap analysis with 1000 replications (Felsenstein 1985). 16S rDNA sequence of the following strains (type strains, unless otherwise indicated) were obtained from GenBank (accession numbers in brackets): *B. thermoglucosidasius* (AB021197); *B. stearothermophilus* (AB021196); *B. weihenstephanensis* (AB021199); *B. mycoides* (AB021192); *B. thuringiensis* WS2625 (Z84587); *B. mojavensis* (AB021191); *B. vallismortis* (AB021198); *B. atrophaeus* (AB021181); *B. subtilis* (X60646); *B. carboniphilus* (AB021182); *B. psychrosaccharolyticus* (AB021195); *B. marinus* (AB021190); *B. flexus* (AB021185); *B. niacini* (AB021194); *B. megaterium* (D16273); and the out-group, the Gram positive bacterium *Alicyclobacillus acidoterrestris* DSM 3922T (X60742).

## 6.4 Results

*Isolation of bacterial strains from soybean root nodules*

We wished to isolate non-*Bradyrhizobium* bacteria from within soybean root nodules. To reduce the possibility of isolating rhizobacteria from the surface of the nodules, only nodules that were confirmed to have been surface-sterilized were used. As *Bradyrhizobia* require nearly a week to form colonies on YEM plates, colonies that arose from crushed nodule contents were picked after an incubation of only four days, and no colonies that had a similar morphology to the soybean endosymbiont, *B. japonicum*, were chosen. Colonies of putative non-*Bradyrhizobium* endophytes (NEB) were observed on plates from nine out of 17 crushed nodules. Of the 17 NEB strains isolated, 14 had distinct colony morphologies, and so were selected for further study.

*Effects of the NEB strains on the growth of soybean plants*

Soybean seedlings were co-inoculated with *B. japonicum* 532C and each of the 14 distinct NEB isolates. Plant weight, nodule number and nodule weight were determined 55 days after inoculation (Fig 6.1.). While the majority of the isolates had no significant effects on soybean growth and development, three (NEB4, NEB5 and NEB17) appeared

to have positive effects. Plants co-inoculated with these strains had significantly higher nodule and plant weights, and NEB5 and NEB17 seemed to increase nodule number per plant. These strains also had positive effects on soybean growth when the root zone temperature was lowered (results not shown). Isolates NEB10, NEB11 and NEB12, seemed to be the poorest performers overall, with some significant decreases in plant weight and nodule number compared to the control. The remaining isolates had no significant effects on soybean growth or nodulation. All further experiments were limited to the soybean-growth promoting strains NEB4, NEB5, and NEB17. Once it had been determined that the eleven other isolates had no positive effects on soybeans, they were discarded.

There was no evidence that the positive soybean-growth effects of NEB4, NEB5, and NEB17 were as a result of supplying the plants with fixed nitrogen. The strains were each inoculated onto soybean seedlings, as above, but in the absence of *B. japonicum* 532C. None of these strains were able to form root nodules with soybean, and the plants appeared chlorotic and stunted, similar to uninoculated control plants. Neither the plant weights nor their nitrogen contents were significantly different from uninoculated control plants (results not shown).

*Phenotypic characterization the NEB strains*

Distinct colony morphologies were observed for NEB4, NEB5 and NEB17 on King's Medium B plates. NEB4 and NEB5 colonies both had slimy capsules, and produced red, water-soluble, pigments. NEB17 colonies had a waxy appearance, with no pigment. All three strains were determined to be Gram positive spore-forming rods.

NEB4, NEB5, and NEB17 cultures showed no significant growth after 7 days in Ashbey's nitrogen-free broth (Fig. 6.2), or after 30 days on plates of the same medium (results not shown). We therefore concluded that these strains were unable to fix nitrogen aerobically. All three strains responded best when nitrogen was provided in complex form, with identical growth with either peptone (Fig. 6.2) or yeast extract (results not shown). With $NH_4NO_3$ as sole nitrogen source in Ashbey's broth, with either carbon source, NEB4 and NEB5 grew poorly, and NEB17 grew very poorly. With respect to

carbon sources, NEB4 and NEB5 showed similar growth when supplied with either mannitol or dextrose, whereas NEB17 showed much better growth with dextrose. The results for growth of these strains on Ashbey's plates with the same additions mirrored those for liquid cultures (results not shown).

The NEB strains could not be identified at the species level using the Biolog system, due to a very high percentage of false-positive results. This result was anticipated, however, as spore-forming bacteria, such as *Bacillus* species, frequently yield false-positives in Biolog tests. This phenomenon is discussed in the Biolog technical literature, and has been observed by others (Baillie et al. 1995). Despite numerous attempts, the SIM values for the NEB strains, and the *B. cereus* control (0.315), were below the threshold of 0.5 acceptable for species identification. The SIM value for the (non spore-forming) *S. aureus* control was, however, 0.563. The Biolog database matches with the highest SIM values were to *B. subtilis* for both NEB4 (0.242) and NEB5 (0.426). For NEB17, the best matches were to *B. mycoides* (0.483), *B. cereus* (0.417), and *B. thuringiensis* (0.417). Therefore, while these tests indicated that the NEB strains were *Bacillus* species, they did not provide identifications at the species level.

*Analysis of 16S rDNA sequences*

Single PCR products of the expected size (1.6 kb) were amplified from NEB4, NEB5 and NEB17 using bacterial 16S rDNA primers. The PCR products were cloned, and single strand sequences of 400-450 nucleotides from both ends of each clone were determined. The NEB4 and NEB5 sequences were identical to each other. BLAST comparisons, done to verify that the clones contained 16S rDNA, revealed that the NEB17 sequences had very high homology to the 5' and 3' ends of the *B. thuringiensis* WS2625 16S rRNA gene, and that the NEB4 and NEB5 sequences had very high homology to the 5' and 3' ends of *B. subtilis* 16S rRNA genes.

As all indications were that the three NEB strains were *Bacillus* species, we utilized PCR primers designed to amplify the hypervariant (HV) region of *Bacillus* 16S rDNA (Goto et al. 2000). The PCR amplifications yielded single PCR products of the expected size, approximately 350 bp, for each strain. The PCR products were cloned, and

nucleotide sequences were generated for both strands. The NEB4 and NEB5 HV sequences (275 nucleotides) were identical, and were identical to those of thirteen *B. subtilis* strains. The NEB17 HV sequence (277 nucleotides) was identical to *B. thuringiensis* strain WS2625.

A neighbor-joining dendrogram was generated using the HV sequences from the NEB strains and representative *Bacillus* sequences from GenBank (Fig. 6.3). As expected, NEB4 and NEB5 clustered with *B. subtilis*, and NEB17 clustered with *B. thuringiensis* WS2625. The separation of the NEB4/NEB5/*B. subtilis* cluster from the *B. vallismortis*/*B. mojavensis*/*B. atrophaeus* cluster was supported by a bootstrap value of 100%. The separation of the NEB17/*B. thuringiensis* WS2625 cluster from the *B. weihenstephanensis*/*B. mycoides* cluster also had 100% bootstrap support. The same tree topology and high bootstrap values were achieved using Tamura-Nei distances (results not shown). The phylogenetic relationships between species related to the NEB strains, and those between HV sequences of other *Bacillus* species, particularly those from the *B. megaterium* and *B. stearothermophilus* clusters, were reconstructed as previously reported (Goto et al. 2000).

## 6.5 Discussion

Fifty-three percent of the surface-sterilized soybean nodules tested carried non-*B. japonicum* bacteria, and several carried more than one morphologically-distinct strain. Endophytic bacteria have been isolated from legume root nodules previously. For example, Sturz et al. (1997) characterized 15 bacterial species from red clover nodules, and estimated endophyte population densities to be in the range of $10^4$ viable bacteria per g fresh nodule. In plant tissue in general, endophytic bacterial populations have been reported between $10^2$ and $10^4$ viable bacteria per g (Kobayashi and Palumbo 2000).

There is much debate as to how to define an endophyte (reviewed in Kobayashi and Palumbo 2000). For example, Hallman et al. (1997) suggested that bacteria that are isolated from surface-sterilized plant tissues, and that do no apparent harm to the plant, could be considered endophytes. Other definitions suggest that it is necessary to

demonstrate that the bacterial colonization is of internal plant tissues (Kobayashi and Palumbo 2000). While our results indicate that NEB4, NEB5, and NEB17 are PGPB, we have not pursued definitive tests that could prove whether these strains are endophytes or epiphytes. As the strains were isolated from surface-sterilized root nodules, we labeled them as putative endophytes, however, we recognize that they may not prove to be colonizing internal plant tissues. In future studies it would be interesting to attempt to re-isolate the NEB strains from surface-sterilized root nodules of plants co-inoculated with *B. japonicum*. This type of study, and perhaps microscopic examination of nodule tissue, would aid in determining if these strains should be defined as endophytes. Until that work is done, the strains should simply be considered as PGPB. One reason for this uncertainty is that, although the root nodules used in our experiments appeared to be surface-sterilized, the three strains examined in detail (NEB4, NEB5, and NEB17) were all endospore formers. Bacterial endospores bound to the surface of the nodules may have survived the surface sterilization.

Of the fourteen isolates, only NEB4, NEB5, and NEB17 improved soybean nodulation and plant weight when co-inoculated with *B. japonicum* 532C under N-free culture conditions, compared to plants inoculated with *B. japonicum* alone. These three strains were incapable of either nodule formation or nitrogen fixation with soybean, and did not enhance plant growth when inoculated without *B. japonicum*. None of the strains appeared to be able to fix nitrogen for growth in N-free media. Whether these three NEB strains could promote soybean growth under conditions with full or limited N supply needs to be determined. Further experiments to determine if these strains can colonize other plant tissues or plant species would be of interest, and it may be useful to try to determine if the increased plant weight was due to increased nodule weight, or vice-versa.

Endophytic bacteria isolated from red clover have also been reported to promote host plant growth and nodulation more often when co-inoculated with *Rhizobium leguminosarum* than when applied alone (Sturz et al. 1997). Our group has previously described PGPR strains, *Serratia proteamaculans* 1-102 and *S. liquefaciens* 2-68, which enhance soybean nodulation and improve both plant growth and grain yield (Zhang et al. 1996b; Dashti et al. 1997).

*Bacillus* species have been shown to have positive soybean-growth effects. *Bacillus megaterium* B153-2-2 appears to enhance plant growth, and nodulation by *B. japonicum*, by inhibiting the growth of the phytopathogen *Rhizoctonia* on the host plant (Liu and Sinclair 1993). In addition to legumes, *Bacillus* endophytes have been isolated from many different plant tissues, such as cotton (Misaghi and Donndelinger, 1990), potato (Sturz and Christie 1995), citrus (Araujo et al. 2001), oak, maple, cauliflower, grape, corn and sunflower (Kobayashi and Palumbo 2000).

Colony morphology, carbon and nitrogen use in Ashbey's media, and Biolog tests indicated that three NEB isolates of interest were *Bacillus* strains. The Biolog tests did not yield conclusive species identifications, and the difficulty of identifying *Bacillus* species (*B. thuringiensis* in particular) has been documented previously (Baillie et al. 1995). The oxidation of reserves by germinating spores is thought to be at least one reason why these species frequently yield false-positive results in Biolog tests.

As preliminary analysis of 16S rDNA sequences also indicated that the NEB strains were *Bacillus* species, it was possible to perform phylogenetic analysis using the approximately 275 nucleotides from the HV region of the 16S rDNA. Goto et al. (2000) demonstrated that many *Bacillus* strains may be reliably classified to the species level on the basis of the sequences of a minimum of 219 nucleotides from the HV region, and validated this for numerous strains in the *B. subtilis* cluster (*B. subtilis*/*B. mojavensis*/*B. atrophaeus*/*B. vallismortis*).

The situation with respect to the identification of *B. thuringiensis* strains is more complex. *Bacillus thuringiensis* falls into the "*B. cereus* group" of species, including *B. cereus*, *B. mycoides*, *B. anthracis*, and *B. weihenstephanensis.* Numerous phenotypic and genotypic characterization methods have been applied to this group, and strains of one species are often found to be closer taxonomically to another species, than to their own type strain (Bourque et al. 1995; Lechner et al. 1998; Daffonchio et al. 2000). Indeed, it has been suggested that *B. anthracis*, *B. cereus*, and *B. thuringiensis* may belong to one species (Helgason et al. 2000). Although no system for classifying these species has been agreed upon, we found the results of sequencing the HV region strongly suggested that NEB17 is a *B. thuringiensis* strain, as this sequence was identical to *B. thuringiensis*

WS2625 and to nothing else. Sequences with lower homology to NEB17 were from various species within the "*B. cereus*" group.

Phylogenetic analysis of the HV sequences of the NEB strains and key *Bacillus* species (Fig. 6.3), demonstrated that both NEB4 and NEB5 are strains of *B. subtilis*, and NEB17 is a *B. thuringiensis* strain related to *B. thuringiensis* WS2625. The clusters into which the NEB HV sequences fit were the same as those reported by Goto et al. (2000), and had high bootstrap support. In addition, the sequences obtained for all three NEB strains were 100% identical to previously characterized *Bacillus* strains. Therefore, we have designated the nodule isolates as *B. subtilis* NEB4, *B. subtilis* NEB5, and *B. thuringiensis* NEB17, and propose that these strains may be useful in agriculture, as co-inoculants with *B. japonicum*. As all three strains form endospores, they should be readily adaptable to commercial formulation and application to field crops (Liu and Sinclair 1993).

**Fig. 6. 1. Effects of NEB strains on soybean plants coinoculated with *B. japonicum*.** Plants were cultured in growth pouches with N-free Hoagland's solution, harvested 55 days after inoculation, and then nodule number (A), nodule weight (B), and plant weight (C) were determined. Control plants (532C) were inoculated with *B. japonicum* 532C alone, all other plants were inoculated with *B. japonicum* 532C plus one of the NEB strains, as indicated. The bars represent the mean values (n = 6), and the letters above each bar indicate significance at the P = 0.05 level.

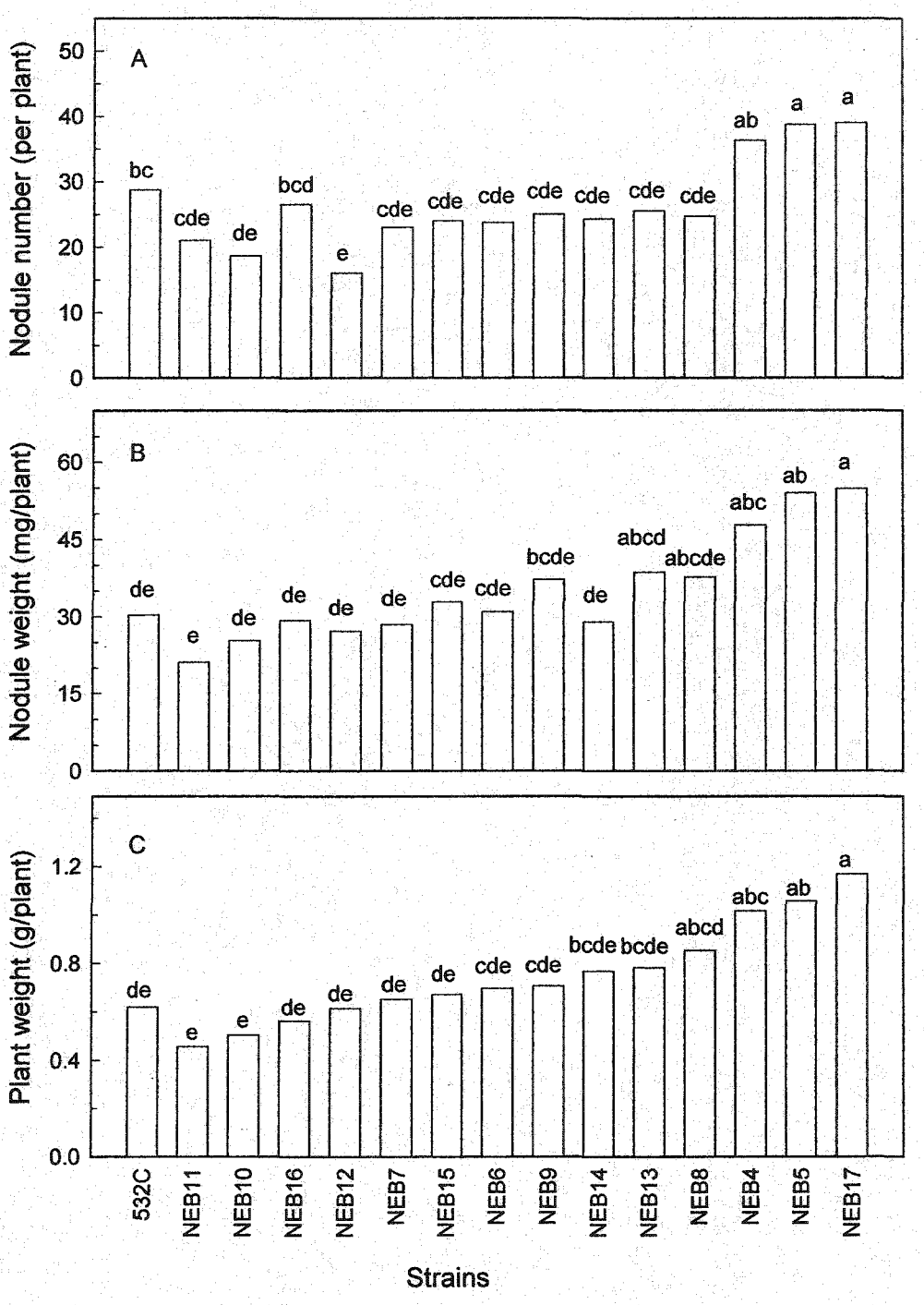

A
Nodule number (per plant)
0
10
20
30
40
50
bc
cde
de
bcd
e
cde
cde
cde
cde
cde
cde
cde
ab
a
a
B
Nodule weight (mg/plant)
0
15
30
45
60
de
e
de
de
de
de
cde
cde
bcde
de
abcd
abcde
abc
ab
a
C
Plant weight (g/plant)
0.0
0.4
0.8
1.2
de
e
e
de
de
de
de
cde
cde
bcde
bcde
abcd
abc
ab
a
532C
NEB11
NEB10
NEB16
NEB12
NEB7
NEB15
NEB6
NEB9
NEB14
NEB13
NEB8
NEB4
NEB5
NEB17
Strains

**Fig. 6. 2. Growth of NEB17 (A, D), NEB5 (B, E), and NEB4 (C, F) in Ashbey's broth with different carbon and nitrogen sources.**

Media contained either mannitol (A, B, C), or glucose (D, E, F) as carbon source. The Ashbey's broth was nitrogen-free (closed circles), or supplemented with either 0.5 g/l $NH_4NO_3$ (open circles), or 1 g/l peptone (closed triangles). Note that peptone is a complex nutrient containing both carbon and nitrogen compounds. Data are the mean (n = 3) optical density, and error bars are the standard deviation.

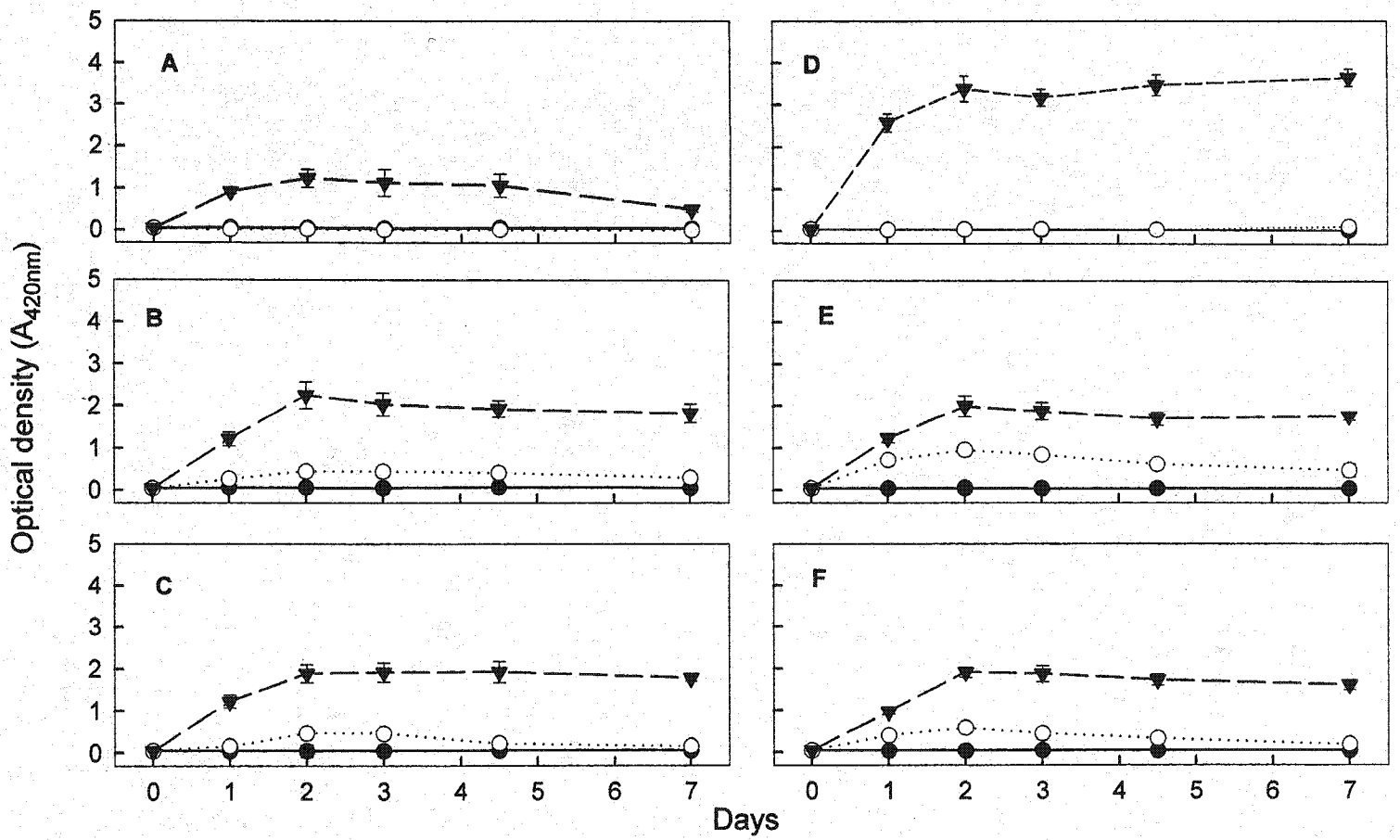
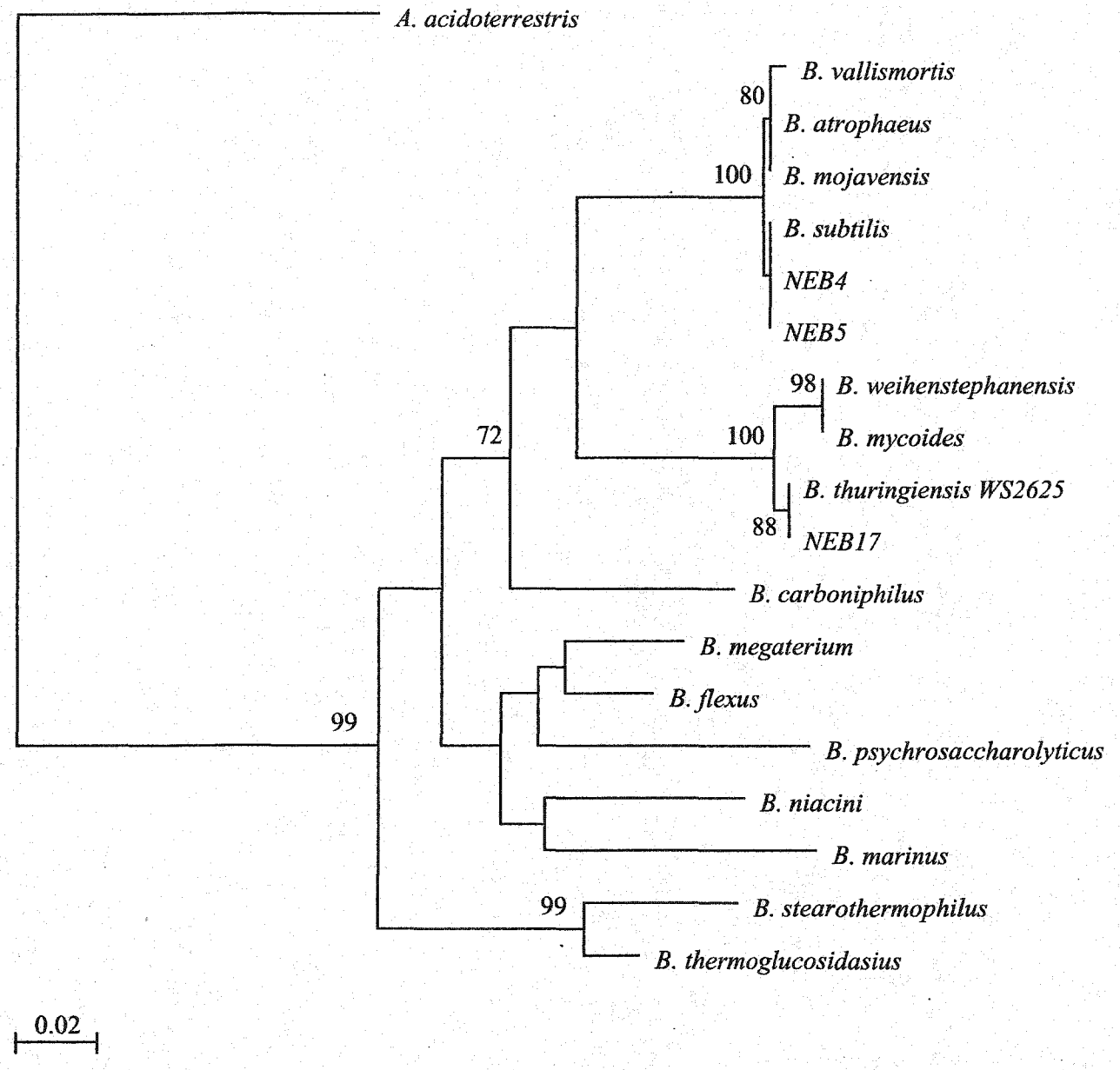

A
B
C
D
E
F
Optical density ($A_{420nm}$)
Days
0
1
2
3
4
5
6
7

**Fig. 6.3. Phylogenetic relationships between NEB4, NEB5, NEB17, and representative *Bacillus* species based on 16S rDNA HV sequences.**

The dendrogram was generated by the neighbor-joining method, with Kimura distances, and is rooted to the out-group *A. acidoterrestris*. Nodes with greater than 70% bootstrap support (1000 replications) are indicated. The bar represents 0.02 nucleotide substitutions per site. Accession numbers are reported in the Methods.


A. acidoterrestris
B. vallismortis
80
B. atrophaeus
100
B. mojavensis
B. subtilis
NEB4
NEB5
98
B. weihenstephanensis
72
100
B. mycoides
B. thuringiensis WS2625
88
NEB17
B. carboniphilus
B. megaterium
B. flexus
99
B. psychrosaccharolyticus
B. niacini
B. marinus
99
B. stearothermophilus
B. thermoglucosidasius
0.02

## Preface to Section 7

Section 7 is comprised of a manuscript by Bai Y, Zhou X and Smith DL and will be submitted to *Annals of Botany.*

Suboptimal root zone temperatures strongly inhibit soybean nodulation and nitrogen fixation. In short growing season areas, such as Quebec, the average soil temperature at 10 cm is 10 – 15°C from mid-May to June, a time when the nitrogen fixing nodules of field grown soybean plants are usually developing. One potential method for overcoming this low RZT limitation to soybean production is coinoculation with of *B. japonicum* with specific PGPB. I evaluated whether or not the three non-bradyrhizobial endophytic *Bacillus* strains isolated and identified through the work reported in the previous section (Section 6) were effective in this capacity. Field experiments were conducted under short season conditions and soybean development and final grain yield were determined.

Of the co-authors, Dr. Zhou, a research associate of Dr. Smith operates the elemental analyzer, conducted the nitrogen analysis, and also provided considerable general support in a wide range of areas from organizing summer student helper to statistics. Dr. Smith is my thesis supervisor. He 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, bench space in greenhouse, experimental field space, the use of computers, and providing technical assistance. Dr. Smith is the corresponding author for the publication of this section.

# Section 7

## Enhanced soybean plant growth due to co-inoculation of *Bacillus* strains with *Bradyrhizobium japonicum*

### 7.1 Abstract

Nodulation and subsequent nitrogen fixation of soybean [*Glycine max* (L.) Merr.] plants are inhibited by low root zone temperatures (RZTs). Plant growth promoting bacteria can help overcome these deleterious effects. Three *Bacillus* strains, *B. subtilis* NEB4 and NEB5, and *B. thuringensis* NEB17, were isolated from inside the nodules of vigorous field-grown soybean plants in 1998, and were shown to have plant growth promoting activity on pouch-grown soybean plants under greenhouse conditions. To test their ability to improve soybean nodulation and growth under low RZTs, these strains were co-inoculated onto soybean plants, with *Bradyrhizobium japonicum*, under greenhouse conditions at RZTs of 25, 17 and 15 °C, and under field conditions in a short growing season area. In all cases the experiments were conducted by using soybean cultivar OAC Bayfield. All the three *Bacillus* strains enhanced soybean nodulation and growth in greenhouse and field experiments. Coinoculation with NEB17 provided the largest and most consistent increases in nodule number, nodule weight, shoot weight, root weight, total biomass, total nitrogen fixation and grain yield. The other two strains provided positive responses in only one of the two years of field-testing. Thus, *B. thuringensis* NEB17 would be suitable for use as a plant growth promoting bacterium in soybean production systems in short growing season regions.

**Keywords:** Soybean, *Bradyrhizobium japonicum*, nodulation, nitrogen fixation, plant growth promoting bacteria, *Bacillus subtilis*, *Bacillus thuringiensis*

## 7.2 Introduction

Legume-rhizobia symbioses actively fix nitrogen and are critical to agricultural crop production (Smith and Hume 1987; Vance et al. 1997; Pepper 2000). Enhancement of legume nitrogen fixation by co-inoculation of rhizobia with some plant growth promoting bacteria (PGPB) is a practical way to improve nitrogen availability in sustainable agriculture production systems. Most of the PGPB strains tested by co-inoculation with *Rhizobium* or *Bradyrhizobium* species are general rhizobacteria (Subba Rao 1979; Burns et al. 1981; Li and Alexander 1988; Chanway et al. 1989; Halverson and Handelsman 1991; Srinivasan et al. 1996; Zhang et al. 1996b). However, in recent years, endophytic bacteria have drawn particular attention as a group of potential PGPB (Hallmann et al. 1997; Sturz et al. 2000).

Most of the well-known PGPB are rhizobacteria, i.e., they are isolated from the plant rhizosphere or rhizoplane. These rhizobacteria can promote plant growth directly or indirectly. Indirect effects are related to production of metabolites, such as antibiotics, siderophores or HCN that decrease the growth of phytopathogens and other deleterious microorganisms. Direct effects are dependent on production of plant growth regulators, or improvements in plant nutrient uptake (Kloepper 1993; Glick 1995). Some rhizobacterial strains promote legume nodulation and nitrogen fixation by producing flavonoid-like compounds and/or stimulating the host legume to produce more flavonoid signal molecules (Parmar and Dadarwal 1999). Endophytic bacteria probably promote host plant growth through similar mechanisms although, the intimate nature of their endophytic habitats may allow other mechanisms (Hallmann et al. 1997).

Endophytic bacteria are ubiquitous in plant tissues including those of legumes, and have been isolated from flowers, fruits, leaves, stems, roots and seeds (Kobayashi and Palumbo 2000), as well as root nodules of legume crops (Sturz et al. 1997). Endophytic bacteria reside intercellularly or even intracellularly within host tissues and therefore are able to form more intimate relationships with the host plant than most other plant-associated bacteria. By residing within plant tissues, endophytic bacteria may also gain advantages for themselves, by being sheltered from environmental stresses and microbial competition. Although some endophytic isolates may inhibit host plant growth

(Sturz 2000), it has been shown that a higher proportion of bacterial endophytes is PGPB than is the case for bacteria found on the rhizoplane or in the rhizosphere (Hallman et al. 1997).

*Bacillus* are spore-forming Gram positive rod-shaped bacteria. They are highly tolerant of adverse ecological conditions. *Bacillus* species comprise one of the most common soil bacteria groups and they are frequently isolated from the rhizospheres of plants. *Bacillus* species are also common plant endophytes (Liu and Sinclair 1989; Misaghi and Donndelinger 1990; Sturz and Christie 1995; Kobayashi and Palumbo 2000; Araujo et al. 2001). Because of their spore-forming ability, plant growth promoting *Bacillus* strains are readily adaptable to commercial formulation and field application (Liu and Sinclair 1993).

Soybean is one of the most important agricultural legumes for oil and protein production. Soybean needs relatively warm temperatures for development, for example, the optimum temperature for its symbiotic nitrogen fixation is 25-30 °C. However, in some short growing season regions, such as eastern Quebec, the mean soil temperature at a depth of 10 cm is 10 °C in mid-May and 15 °C in June (Lynch and Smith 1993). Suboptimal root zone temperatures (RZTs), such as these, strongly inhibit soybean early growth and, especially, establishment of the soybean-*B. japonicum* nitrogen fixing symbiosis. As such they are a major limiting factor for soybean production in short season areas (Whigham and Minor 1978; Zhang and Smith 1995). Co-inoculation of some PGPB, such as *Serratia proteamaculans* 1-102 and *S. liquefaciens* 2-68, enhances soybean nodulation and nitrogen fixation under suboptimal RZTs, under both greenhouse and field conditions (Zhang et al. 1996b, 1997; Dashti et al. 1997, 1998).

In 1998, we isolated 14 strains of non-bradyrhizobial endophytic bacteria (NEB) from inside soybean root nodules of particularly vigorous field-grown plants. Based on greenhouse run pouch experiments at 25 °C RZT, three of the 14 isolates were selected as potential PGPB and identified as *Bacillus* strains (Section 6). The objective of this work was to determine whether or not these PGPB strains would be able to improve soybean nodulation, nitrogen fixation and growth under low RZT in the greenhouse, and under field conditions in a short season area, where spring soil temperatures are low.

## 7.3 Materials and methods

*Preparation of the bacterial inoculants*

This work was conducted with the soybean [*Glycine max*. (L.) Merr.] cultivar OAC Bayfield, coinoculated with *Bradyrhizobium japonicum* strains 532C or USDA110, and with one of the three endophytic bacterial strains: *Bacillus subtilis* NEB4 (NEB4), *B. subtilis* NEB5 (NEB5) *and B. thuringensis* NEB17 (NEB17) (isolated and maintained in our laboratory, Section 6).

*B. japonicum* was cultured in flasks on a shaker at 200 rev per min, 50 – 75 ml in 250 ml flasks or 100 – 120 ml in 500 ml flasks, at 28 °C in yeast extract mannitol (YEM) culture medium (Vincent 1970). The initial culture time in flasks inoculated from cold slants was approximately 7 days. The subculture time was not less than 72 h. The cell density in the culture was determined by spectrophotometry at 620 nm, taking $A_{620nm}$ reading 0.08 as approximately $10^8$ cells/ml (Bhuvaneswari et al. 1980). The *Bacillus* strains were cultured on a shaker at 200 rev $min^{-1}$ in flasks, 80 – 100 ml per 250 ml flask or 150 – 180 ml per 500 ml flask, at 28 °C. The culture medium for *Bacillus* culture was King's Medium B (Atlas 1995). The initial culture time in flasks inoculated with cold slants was approximately 72 h. The subculture time was 30 h. After the bacterial subcultures were harvested and the cell concentration was determined at 420 nm (Dashti et al. 1997).

The bacterial cultures were diluted with distilled water. The inoculants were prepared by mixing *B. japonicum* and one of the three tested *Bacillus* strains. The cell density in the inoculants was $10^8$ cells/ml for both *B. japonicum* and the co-inoculated *Bacillus* strain. Under greenhouse conditions the inoculants were applied immediately after preparation, while for the fieldwork there was a delay of not more than 24 h.

*Green house experiment*

In the greenhouse experiments the only *B. japonicum* strain used was 532C (Hume and Shelp 1990). The greenhouse conditions were: air temperature of 25 ± 2 °C, additional illumination of 300 $\mu mol/m^2/s^1$ supplied by high pressure sodium lamps (P. L. Light System Canada) for a photoperiod of 16:8 h (day : night). Soybean seeds were

surface sterilized in sodium hypochloride (2% solution containing 4 ml Tween20/l). The seeds were then rinsed several times with distilled water. The seeds were first planted in trays containing Vermiculite and germinated in the greenhouse. Three or four day old seedlings at the VE stage (Fehr et al. 1971) were transplanted into pots filled with Vermiculite (one seedling per pot) or growth pouches (15 × 16 cm, Mega International, Minneapolis, MN, one seedling per pouch). In the pouch experiment, the RZT was controlled by water bath systems at 25, 20 and 15 °C respectively (Zhang et al. 1996b). Six days after transplanting the seedlings, they were inoculated with the 532C-NEB mixtures at the rate of 1 ml per plant. Control plants were inoculated with 532C alone or a mixture of 532C and King's Medium B (without bacteria).

The pot experiment was arranged as a completely randomized design with 5 replicates. The pouch experiment was organized following a randomized complete block design with 6 replicates. The levels of RZT and inoculations were combined factorially and were allocated to the blocks in a split-plot fashion. The main plots were RZTs. NEB coinoculation treatments formed the sub-plots. During the growth process, the plants were watered with modified N-free Hoagland's solution (Hoagland and Arnon 1950), in which $Ca(NO_3)_2$ and $KNO_3$ were replaced with 1 mM $CaCL_2$, 1 mM $K_2HPO_4$ and 1 mM $KH_2PO_4$, to provide a nitrogen-free solution. The plants were harvested at 55 days after inoculation (DAI). After harvesting, data on nodule number, nodule weight, shoot weight and root weight were collected. All the samples were weighed after not less than 48 h of drying at 70 – 80 °C. The plant weight in greenhouse experiment was calculated as shoot weight plus root weight.

*Field experiment*

The field experiment was structured following a randomized complete block design with three blocks. The *B. japonicum* and NEB strains, along with the appropriate controls, were combined factorially, resulting in 3 blocks of 12 plots each. The tested factors were three bradyrhizobial strains (no inoculant control in which the indigenous *B. japonicum* community was relied upon for nodulation, *B. japonicum* 532C and *B. japonicum* USDA110), and four NEB treatments (no NEB as a control, NEB4, NEB5 and NEB17). The experiment was conducted at the Emile A. Lods Research Centre (45° 25'

45'' N latitude and 73° 56' 00'' W longitude) of McGill University in 1999, on a clay-loam type soil where the previous crop was corn, and in 2000 on a sandy-loam type soil where the previous crop was barley. The soybean cultivar was OAC Bayfield. Meanwhile, non-nodulating Evans was planted in the same way one plot in each block. Each plot was 5 × 1.6 m with 0.2 m between adjacent plots. The plant population was 400 plants/plot (500,000 plants/ha) with 10 cm between plants within the row and 20 cm between rows. The sowing date was May 20 in 1999 and May 17 in 2000. The soybean seed was sown mechanically. The seeds in the furrows were not covered until the inoculants were added. The inoculants were sprayed into the open furrows by hand, using 60 ml sterilized plastic syringes. The inoculation dose for all inoculants was 1 ml per seed.

The plants were harvested three times during whole growing season, at V3, R3 and harvest maturity (R8) stages (Fehr et al. 1971). At the first and second harvest, 5 plants were randomly taken from each plot. After washing the roots with tap water, data on nodule number, nodule weight, shoot weight and root weight were collected in the same way as for greenhouse samples. At the final harvest, plants in the central 1 m of each of the two center rows (an area of 0.4 $m^2$) of each plot were collected by hand. Plant number was determined, and branch number and pod number were counted for each plant. After the roots were detached, the shoots were oven dried at 70 – 80 °C for not less than 48 h. The shoot weight, including the seeds, was taken as the total weight, i.e. the biological yield or total aboveground biomass. The shoots were mechanically threshed to remove the seeds. The seed weight and the 100-seed weight were also determined. The seed weight was taken as the economic yield. Seed yield is given at 0% moisture. Stem weight was calculated as the difference between the shoot weight and seed weight. The harvest index was expressed as the ratio of the economic yield (the seed weight) to the biological yield (the total weight or total aboveground biomass). The total number of seeds and the seed number per pod were calculated using the variables seed weight, 100-seed weight and pod number. The nitrogen concentrations (%) of the stem and the seed were determined separately using an Element Analyzer (NC2500 Elementary Analyzer, ThermoQuest Italic S.P.A., Italy). The nitrogen yield in stem or seed was calculated by

stem or seed weight times their respective nitrogen concentration. The total nitrogen yield was defined as a sum of stem and seed nitrogen yields.

*Data analysis*

For the two field experiments, the homogeneity of variance was assessed with a Battlett's test. The data were not homogenous and they were not pooled across experiments in subsequent ANOVA analyses. All the data collected in greenhouse or field experiments were analyzed with ANOVA procedure of the SAS system (Littell et al. 1991). When analysis of variance indicated differences among means, comparisons among the treatment means were conducted with an ANOVA protected least significance difference (LSD) test (Steel and Torrie 1980). In general, differences were considered significant when detected at P □ 0.05. However, in some cases differences at $0.05 \leq P$ □ 0.1 are discussed in the text. When this happens the P value is provided.

## 7.4 Results

*Greenhouse experiment*

The general patterns of responses to the treatments were the same in growth pouch and pot culture systems. In the pouch experiment, there was no interaction between NEB and RZT. Compared with 25 °C, the optimal RZT for soybean growth and nodulation, 15 °C RZT greatly inhibited the plant nodulation and growth, while 20 °C RZT had little inhibitory effect (Table 7.1). Coinoculation of the three *Bacillus* NEB strains generally promoted soybean plant growth and nodulation under either optimal or suboptimal RZT conditions (Table 7.1). Coinoculation of NEB 17 resulted in constant plant growth promotion, regardless of RZT (Table 7.1), whereas responses to coinoculation with NEB4 and NEB5 were less consistent. Inclusion of NEB17 in the inoculant resulted in increases in nodule number, nodule weight, shoot weight and root weight. NEB5 performed almost as well as NEB17. NEB4 stimulated nodule number and shoot weight at 15°C RZT, and root weight and shoot weight at 20 °C RZT, but had no effect on the four measured variables at 25 °C RZT (Table 7.1). Compared with the 532C alone control, in the pot experiment (data not shown), coinoculation with NEB17 increased nodule number, nodule weight, shoot weight and root weight ($P = 0.05$).

Coinoculation with NEB5 increased nodule number, nodule weight and shoot weight (P = 0.05). Coinoculation with NEB4 increased only nodule number (P = 0.05). In both pouch and pot experiments, the King's medium B controls (data not shown) were not different from the 532C alone control.

*Field experiments (Bacillus strains)*

The Bartlett's test showed that the seed weight data was not homogenous between the two filed experiments ($\chi^2$ = 19.23, $\alpha$ = 0.00001). There were also no interactions among the applied levels of *B. japonicum* inoculants and *Bacillus* NEB strains for any of the measured variables in both 1999 and 2000. This occurred in spite of the different growth conditions, due to different soil conditions (soil types and nutrient levels) and weather conditions (Fig. 7.1) between the experiments in 1999 and 2000, which contributed to different levels of overall plant growth responses. The overall growth conditions of 2000 would be considered to be better than in 1999. In 1999 the average total biomass production was 10.08 t/ha and seed production was 5.33 t/ha compared, whereas these were13.95 and 7.83 t/ha in 2000. Similar differences also existed when the respective within growth season harvests (at V3 and R3 stages) were compared across years. However, the included plots of non-nodulating Evans accumulated more total nitrogen in 1999 (219.0 kg/ha) than in 2000 (153.3 kg/ha). This indicated higher soil nitrogen at the 1999 site than the 200l site. The higher soil nitrogen in 1999 resulted in fewer nodules and less nodule weight than in 2000. The average nodule number and nodule weight were 18.4 (per plant) and 0.083 (g/plant) at the V3 stage and 48.5 and 0.46 at the R3 stage in 1999. The average nodule number and nodule weight were 47.6 and 0.29 at the V3 stage and 73.7 and 0.74 at the R3 stage in 2000.

At both V3 and R3 stages, none of the three selected NEB strains had any negative effects on soybean plant growth and nodulation (Table 7.2). At the V3 stage, the nodule number was increased by 34.7% (NEB17, 2000) to 185% (NEB4, 1999); the nodule weight was increased by 21.5% (NEB4, 2000) to 36.8% (NEB17, 1999); and the root weight was increased by 13.7% (NEB4, 2000) to 38.0% (NEB4, 1999). Only in 2000 experiment was the shoot weight was increased by coinoculation of NEB4 and NEB17. At the R3 stage, the nodule number was increased in 46.1% (NEB17, 2000) to

66.3% (NEB17, 1999); the nodule weight was increased by 27.1% (NEB4, 1999) to 69.6% (NEB5, 2000); the shoot weight was increased by 41.4% (NEB17, 2000) to 68.4% (NEB17, 1999); and the root weight was increased by 22.8% (NEB4, 2000, not significant) to 46.8% (NEB5, 2000). These data show that the three co-inoculated NEB strains were all reasonably effective in promoting plant growth up to the R3 stage.

As at the V3 and R3 stages, all the measured variables at the final harvest were larger in 2000 than in 1999 (Table 7.3). However, compared with no-NEB coinoculation control, in 1999, coinoculation of each NEB strain increased total weight ($P = 0.08$) by 13.2 to 16.6%, and seed weight by 14.9 to 16.5 %. In 2000, only the coinoculation of NEB17 increased total weight (27.3%) and seed weight ($P = 0.07$, 22.9%). In 2000, the total seed number was increased in parallel with seed weight due to coinoculation of NEB17.

In both years the nitrogen concentration (%) of either stems or seeds were not different among the treatments. In 1999, the stem nitrogen concentration was between 0.58 – 0.62 % and seed nitrogen concentration between 5.77 – 6.19%. In 2000, the stem nitrogen concentration was between 0.52 – 0.62% and seed nitrogen concentration between 6.36 – 6.62%. The total nitrogen yield and the seed nitrogen yield paralleled the biological and the economic yields (Table 7.3). In 1999, coinoculation of the three NEB strains resulted in increases in total nitrogen and seed nitrogen yield, relative to the control. Among the PGPB treatments, NEB17 caused the greatest responses, increasing the total nitrogen yield by 24.8% and the seed nitrogen yield by 22.3%. In 2000, only coinoculation of NEB17 increased the total nitrogen yield by 25.8% and the seed nitrogen content by 23.4% ($P = 0.07$) over the control.

*Field experiments (B. japonicum strains)*

At the final harvests, there were few differences among the three *B. japonicum* levels (no-inoculant, *B. japonicum* 532C and *B. japonicum* USDA110) in 2000 (Table 7.4). In 1999, both the inoculated bradyrhizobial strains increased biological and economic yields relative to the control (no-inoculant) ($P = 0.10$).

## 7.5 Discussion

In the selection of plant growth promoting bacterial strains, the final evaluations must be made under field conditions. The field environment is generally much more stressful, and the conditions there are more complex, than controlled environment conditions. Thus, results obtained in greenhouse experiments do not necessarily reflect the potential for plant growth promotion in the field. Because of this, after the 3 strains of a set of 14 NEB isolates were evaluated and selected primarily through greenhouse experiments (Section 6), their performances were further evaluated in two plant culture systems in a greenhouse and finally in the field. These results showed that *B. thuringensis* NEB17 is a soybean growth promoting bacterial strain whose effects are stable across the various tested experimental conditions. Whereas the other two *B. subtilis* strains, NEB4 and NEB5, sometimes showed plant growth promoting effects, and were not consistent. Thus, among these three strains, NEB17 is most suitable for application to soybean production in short season regions. In the greenhouse experiment the performance of NEB17 was not altered by the changes in RZT, indicating that it may also be effective under longer season conditions, where spring soil temperature is warmer.

There were obvious differences, due to weather and soil conditions, between the experiments conducted in 1999 and 2000. Different soil types and different previous crops result in different soil nitrogen levels (Dashti et al. 1997; Pan and Smith 2000). When compared with the 30-year average, monthly average temperatures from May to September in 1999 were high and were higher than in 2000. This was especially so from May to July when it was 2.5-3.5 °C higher in 1999 than in 2000. In 1999, the precipitation in May was low, but in September it was much higher than in 2000 and than in the past 30 years (Fig. 7.1). In spite of the very different field conditions in 1999 and 2000, NEB17 caused similar effects on plant nodulation and growth. This corroborates the greater stability for NEB17 observed in the greenhouse experiments.

There were differences between the inoculant and no-inoculant *B. japonicum* in 1999, but not in 2000. The lack of differences among the three levels of *B. japonicum* in 2000 indicated that an indigenous *B. japonicum* population was sufficient to allow nodulation and nitrogen fixation levels not be improved by the application of *B. japonicum* inoculants. In these two year's experiments, the two applied *B. japonicum*

strains, 532C and USDA110, performed at the same level, as previously observed (Dashti et al. 1997).

*Bacillus* NEB 17 had beneficial effects on soybean growth similar to those previously reported for the two *Serratia* strains, *S. proteamaculance* 1-102 and *S. liquefaciens* 2-68 (Zhang et al. 1996b, 1997; Dashti et al. 1997, 1998). We have determined that *S. proteamaculans* 1-102 produces flavonoid inducible activator(s) that cause the positive effects on signal exchange between bradyrhizobia and soybean plants (Section 4). Whether NEB17 and the *S. proteamaculans* 1-102 promote plant growth through similar or different mechanisms remains to be investigated. Based on the experimental results reported here, we know that NEB17 did not exert its plant growth promoting effects through biocontrol of any insect or disease organisms. There was no apparent insect or disease pressure either in the greenhouse or in the field. However, the current data do not allow us to conclude that the NEB strains have no biocontrol potential in all situations. In fact, some *Bacillus* PGPB strains probably promote plant growth through biocontrol of disease and insect pests (Liu and Sinclair 1989, 1990; Handelsman et al. 1990).

In the both greenhouse and field experiments, N was the limiting nutrient for plant growth. Thus, we think that the increased nodulation and subsequent nitrogen fixation resulted in the measured increases in plant growth and grain yield. Although no direct nitrogen fixation data were collected, the higher total nitrogen in NEB coinoculation treatments than in the no-NEB coinoculation control in field is a direct demonstration of higher nitrogen fixation. Coinoculation with the NEB increased nodule number and nodule weight, and root weight was increased more often than shoot weight. Better root development means better nutrient uptake capability and some PGPB are known to exert their plant growth promoting effects via stimulating better root growth through production of indole-3-acetic acid (Barbieri etal. 1986; Barbieri and Galli 1993; Dubeikovesky et al. 1993; Srinivasan et al. 1996). This may be also contribute to the positive growth promoting effects of the coinoculated NEB strains on soybean plants.

The NEB strains tested here might also produce plant growth regulators or other activators that cause promotion of root growth and enhancement of nodulation. This possibility needs to be investigated through additional research.

*Bacillus* species are among the bacteria most often isolated from plant tissues (Kobayashi and Palumbo 2000). Philipson and Blair (1957) isolated *B. megaterium* from clover root tissues. Sturt et al. (1997) isolated 32 endophytic bacteria from red clover, six *Bacillus* strains were included (*B. azotoformans*, *B. brevis*, *B. circulans*, *B. insolitus*, *B. megaterium* and *B. subtilis*) and four of these were resident within root nodules. In the inoculation experiments, *B. brevis* and *B. insolius* promoted clover nodulation when co-inoculated with *Rhizobium leguminosarium* (Sturz et al. 1997). *Bacillus* species exist in the soybean seed tissues and could survive common surface sterilization procedures (Tenne et al. 1977). *Bacillus subtilis* (Ehrenberg) Cohn, the most constantly soybean seed borne bacterial species, always resulted in soybean seed decay (Schiller 1977; Sinclair 1993). Oehrle et al. (2000) reported that some soybean seedborne *Bacillus* spp. inhibited the attachment of *B. japonicum* to the soybean seedling root surface. Thus, soybean seedborne *Bacillus* strains often show negative effects on soybean growth and symbiosis establishment. The three *Bacillus* strains used in this work were spore-forming rods isolated from surface disinfected soybean root nodules (Section 6), not seeds, and showed growth promoting effects on soybean plants. Their spore-forming characteristics could easily be adapted to commercial formulations in the future inoculant production (Liu and Sinclair 1993).

In conclusion, all three NEB strains tested showed plant growth promoting effects. The growth promotion provided by these strains was apparently related to improved root development and enhanced nodulation, which resulted in better nutrient uptake capability and increased N supply. One of them, NEB17, was superior in this regard because it provided best and most consistent effects. NEB17 seems to be suitable for use as a plant growth promoting bacteria in soybean production in short season regions.

**Fig. 7.1. Monthly average temperature (I) and precipitation (II) during the growing season.**

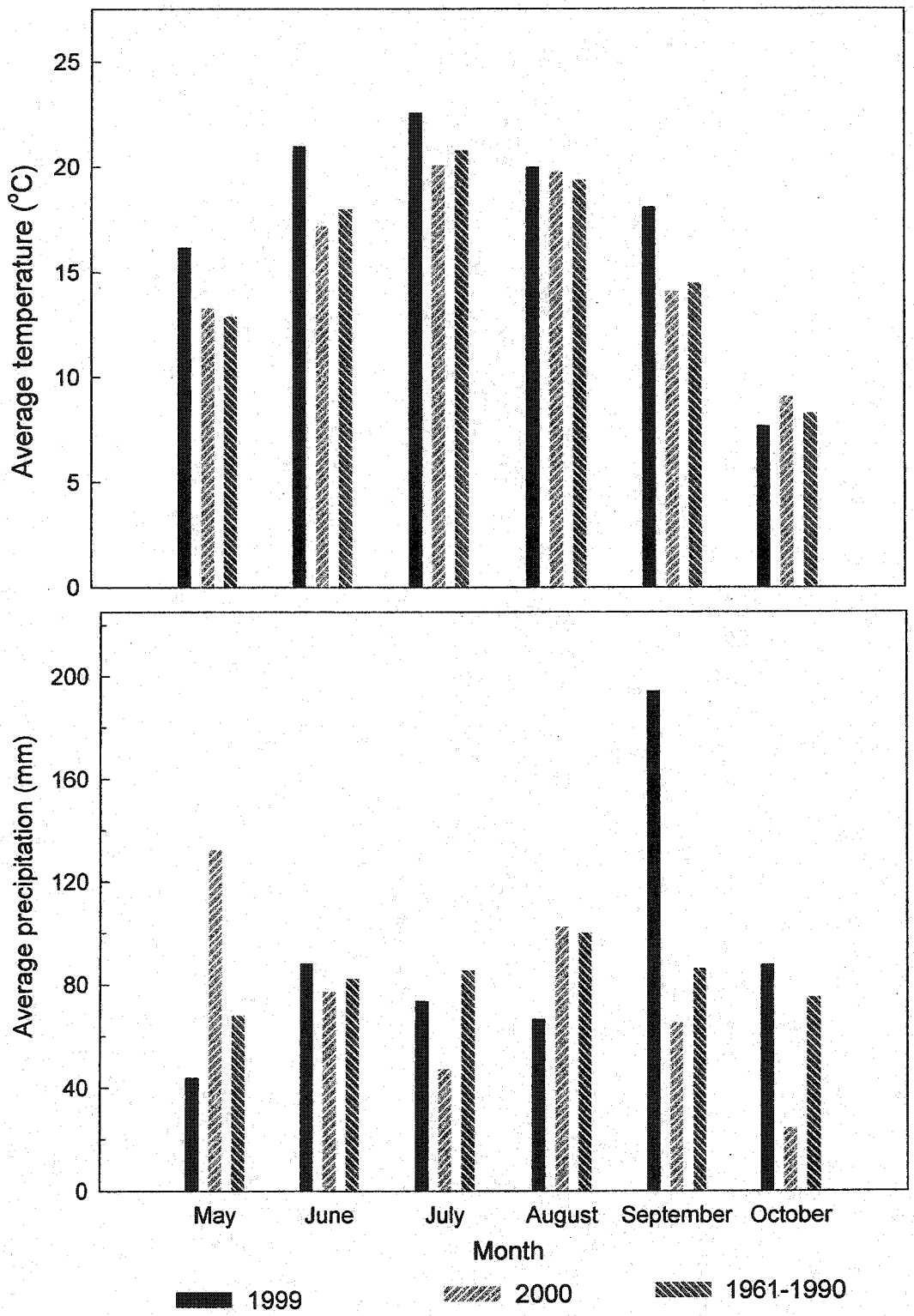

Average temperature (°C)
25
20
15
10
5
0
Average precipitation (mm)
200
160
120
80
40
0
May
June
July
August
September
October
Month
1999
2000
1961-1990

**Table 7.1. Pouch experiment results for the three *Bacillus* NEB strains at three root zone temperatures (RZTs) under greenhouse conditions**

| Treatment | | Nodule number (per plant) | Nodule weight (g/plant) | Root weight (g/plant) | Shoot weight (g/plant) |
|---|---|---|---|---|---|
| 15 °C RZT | | | | | |
| | 532C Control | 14.5 c | 0.019 b | 0.224 b | 0.601 b |
| | 532C + NEB4 | 24.3 b | 0.024 ab | 0.274 ab | 0.741 a |
| | 532C + NEB5 | 25.0 b | 0.027 a | 0.296 a | 0.752 a |
| | 532C + NEB17 | 30.9 a | 0.029 a | 0.300 a | 0.746 a |
| 20 °C RZT | | | | | |
| | 532C Control | 31.8 b | 0.032 c | 0.244 b | 0.820 b |
| | 532C + NEB4 | 34.0 b | 0.040 b | 0.337 a | 1.088 a |
| | 532C + NEB5 | 45.2 a | 0.046 a | 0.353 a | 1.131 a |
| | 532C + NEB17 | 45.2 a | 0.048 a | 0.345 a | 1.115 a |
| 25 °C RZT | | | | | |
| | 532C Control | 32.6 b | 0.034 c | 0.250 b | 0.828 c |
| | 532C + NEB4 | 37.3 ab | 0.041 bc | 0.319 ab | 0.944 bc |
| | 532C + NEB5 | 43.0 a | 0.048 b | 0.331 a | 1.091 ab |
| | 532C + NEB17 | 44.0 a | 0.051 a | 0.327 a | 1.186 a |

Treatments: 532C, *Bradyrhizobium japonicum* 532C; NEB4 and NEB5, *Bacillus subtilis* strains; NEB17, *Bacillus thuringiensis* strain. The plants were harvested at 55 days after inoculation. Within the same column and root zone temperature, means followed by the same letter are not different ($p = 0.05$) by an ANOVA protected LSD test. $n = 6$.

**Table 7.2. Effects of coinoculation of NEB strains with *Bradyrhizobium japonicum* strains on nodulation variables at V3 and R3 stages of field grown soybean plants in 1999 and 2000**

| Inoculant | 1999 experiment | | | | 2000 experiment | | | |
|---|---|---|---|---|---|---|---|---|
| | Nodule number (per plan) | Nodule weight (g/plant) | Shoot weight (g/plant) | Root weight (g/plant) | Nodule number (per plant) | Nodule weight (g/plant) | Shoot weight (g/plant) | Root weight (g/plant) |
| V3 stage harvest | | | | | | | | |
| Control | 8.5 b | 0.068 b | 3.26 | 0.50 b | 35.7 b | 0.214 b | 3.20 b | 0.64 b |
| NEB4 | 24.3 a | 0.086 a | 3.61 | 0.69 a | 51.1 ab | 0.293 a | 4.94 a | 0.83 a |
| NEB5 | 21.9 a | 0.091 a | 3.37 | 0.62 a | 55.3 a | 0.300 a | 5.28 a | 0.89 a |
| NEB17 | 23.9 a | 0.093 a | 3.55 | 0.62 a | 48.1 ab | 0.339 a | 5.39 a | 0.91 a |
| R3 stage harvest | | | | | | | | |
| Control | 37.2 c | 0.380 c | 19.43 b | 1.53 b | 49.9 b | 0.519 b | 20.88 b | 1.58 b |
| NEB4 | 44.9 b | 0.483 b | 21.92 a | 1.78 a | 82.4 a | 0.814 a | 30.87 a | 1.94 ab |
| NEB5 | 49.7 b | 0.453 ab | 20.59 ab | 1.74 a | 89.6 a | 0.880 a | 31.96 a | 2.32 a |
| NEB17 | 62.2 a | 0.510 a | 22.37 a | 1.86 a | 72.9 ab | 0.748 a | 29.54 a | 2.17 a |

Inoculant: Control, *Bradyrhiozobium japonicum* inoculation without NEB coinoculated; NEB4, NEB5 and NEB17, coinoculation of *Bacillus subtilis* NEB4, *B. subtilis* NEB5 *and B. thuringiensis* NEB17 with *B. japonicum*. Within the same column and harvest stage, means followed by the same letter are not different (P = 0.05) by an ANOVA protected LSD test. n = 9.

**Table 7.3. Effects of coinoculation of NEB strains with *Bradyrhizobium japonicum* strains on yield and yield components of field grown soybean plants at harvest maturity in 1999 and 2000**

| Inoculant | Total weight ( t/ha) | Total seed ($10^6$ seeds/ha) | Seed weight (t/ha) | 100-seed weight (g) | Total nitrogen (kg/ha) | Seed nitrogen (kg/ha) |
|---|---|---|---|---|---|---|
| 1999 experiment | | | | | | |
| Control | 9.12 b | 26.17 | 4.82 b | 18.46 | 301.7 b | 277.0 b |
| NEB4 | 10.63 a | 29.21 | 5.54 a | 19.05 | 357.3 a | 325.8 a |
| NEB5 | 10.76 a | 28.75 | 5.61 a | 19.55 | 364.3 a | 335.5 a |
| NEB17 | 10.34 a | 29.76 | 5.61 a | 18.93 | 376.5 a | 347.0 a |
| 2000 experiment | | | | | | |
| Control | 12.37 b | 34.24 b | 7.22 b | 21.07 | 496.1 b | 471.4 b |
| NEB4 | 13.37 ab | 36.30 b | 7.77 b | 21.40 | 527.8 b | 492.5 ab |
| NEB5 | 13.28 b | 33.57 b | 7.44 b | 21.34 | 528.6 b | 493.3 ab |
| NEB17 | 15.74 a | 41.26 a | 8.87 a | 21.53 | 624.1 a | 581.5 a |

Inoculant: Control, NEB4, NEB5 and NEB17, same as in Table 7.2. Within the same column and experiment, means followed by the same letter are not different ( at P = 0.05 level in 1999 experiment and at P = 0.10 level in 2000 experiment by an ANOVA protected LSD test. Where columns are not followed by letters the means were not different (P = 0.10). n = 9.

**Table 7.4. Effects of *Bradyrhizobium japonicum* inoculants on variables related to dry matter and nitrogen yield of soybean plants grown under field conditions in 1999 and 2000.**

| | Total weight (t/ha) | Seed weight (t/ha) | Total Nitrogen (t/ha) | Seed Nitrogen (t/ha) |
|---|---|---|---|---|
| 1999 experiment | | | | |
| No-inoculant control | 8.36 b | 4.44 b | 0.278 | 0.252 |
| *B. japonicum* USDA110 | 10.06 a | 5.37 a | 0.353 | 0.324 |
| *B. japonicum* 532C | 10.42 a | 5.51 a | 0.353 | 0.325 |
| 2000 experiment | | | | |
| No-inoculant control | 13.30 | 7.54 | 0.512 | 0.477 |
| *B. japonicum* USDA110 | 13.97 | 7.79 | 0.544 | 0.509 |
| *B. japonicum* 532C | 14.18 | 8.15 | 0.576 | 0.541 |

Within the same column and year, means followed by the same letter are not different at p = 0.10 level by an ANOVA protected LSD test. When there were no differences among the means there are no letters following the means of that year. n=12.

## Preface to Section 8

Section 8 is comprised of a manuscript by Bai Y, Souleimanov A and Smith DL and will be submitted to *FEMS Microbiology Letters*.

*Bradyrhizobium japonicum* is a typical slow-growing rhizobial species. It has a generation time > 6 hours and requires a long culture time during inoculant preparation. This increases the cost of inoculant production. During the past several decades there has been considerable research effort focused on methods to accelerate the growth of *B. japonicum* in culture via technological improvements, including the culture medium formulation. During the work reported in Sections 3 to 7 I grew *B. japonicum* cultures and was often frustrated by their slowness to grow. Although there are no previous reports of RNA use in rhizobial culture media, its use has reported in the culture of other bacteria. Addition of RNA the culture medium has been shown to increase the biomass of a *Lactobacillus* strain. The experiments reported in this section were designed to test the possibility that RNA additions to the culture medium accelerate the growth rate of cultures of *B. japonicum*.

Of the co-authors, Dr. Souleimanov, a research associate of Dr. Smith, is in charge of HPLC operation in the laboratory and assayed LCO production. Dr. Smith is my thesis supervisor. He 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, bench space in greenhouse, experimental field space, the use of computers, and providing technical assistance. Dr. Smith will be the corresponding author for publication of this section.

# Section 8

## RNA addition to the culture medium accelerates growth and increases lipo-chitooligosaccharide yield of *Bradyrhizobium japonicum*

### 8.1 Abstract

The time required to produce *Bradyrhizobium japonicum* cultures suitable for use in inoculants is long as the bacterium grows slowly. Methods to shorten the production time would be of economic benefit to the inoculant industry. The effects of commercial yeast RNA addition to yeast extract mannitol medium (YEM) on growth and lipo-chitooligosaccharide (LCO) production by *B. japonicum* 532C was investigated. The experiments were conducted in shaker agitation cultures. The positive effect of RNA addition on growth was additive with yeast extract additions, and was not replaceable by the latter. Growth dynamic analysis revealed that RNA addition shortened the generation lag, and increased the generation rate. These resulted in increased total growth (*TG*), in spite of the shortened log phase. The time to maximum *TG* was 12-days for the control with 0.5 g/l yeast extract, but was shortened by 2.5, 3.5 and 5 days when RNA was added at 0.05, 0.1 and 0.2 g/l, respectively. The positive effect of RNA on *TG* was verified for other two *B. japonicum* strains, USDA3 and 110. LCO production is critical for rhizobial physiologic and symbiotic activities. RNA addition also increased the LCO yield, although no effect on LCO production per $10^{10}$ cells. RNA addition stimulated bacterial growth and had no side effects on other physiological activities. This technique should have potential for industrial application in production of the bacterial inoculants.

**Keywords:** *Bradyrhizobium japonicum*, culture medium, RNA additions, growth dynamics, lipo-chitooligosaccharide production

## 8.2 Introduction

The rhizobia are a group of bacteria capable of entering into a nitrogen fixing symbiosis with legumes (Madigan et al. 2000; De Lajudie et al. 1998). Traditionally rhizobia are divided into the slow and the fast growers, based on their growth dynamic characteristics. The genus *Rhizobium* is a typical fast grower and *Bradyrhizobium* a typical slow grower (Elkan and Bunn 1991). Despite their genetic and physiological differences, they share many characteristics related to their capacity to establish a successful $N_2$-fixing symbiosis with their specific legume hosts. They can all recognize plant signal molecules, such as isoflavonoids, and produce reply signal molecules, Nod factors, in response. All the Nod factors produced by rhizobia, with the exception of one minor Nod factor produced by *Sinorhizobium fredii* USDA191 (Bec-Ferte et al. 1996), consist of an oligo-saccharide backbone of β-1,4- linked *N*-acetyl-*D*-glucosamine (Spaink 2000). A fatty acyl group is always attached to the nitrogen of the non-reducing sugar. This backbone structure is a chitin fragment, so the rhizobial nod factors are often referred to as lipo-chitooligosaccharides (LCOs). LCOs are synthesized via coordinated processing of the glucosamine precursors by the products of *nod* genes (Perret et al. 2000). LCO production is an essential prerequisite for establishment of a successful symbiosis between rhizobia and legumes.

With the increasing emphasis on the development of sustainable agriculture, biological nitrogen fixation will play an increasingly important role in grain production (van Kammen 1997). A key aspect in this is the development of more economic technologies for the production of rhizobial inoculants, especially for production of slow-growing bradyrhizobial inoculants. Rhizobia are generally cultured in mannitol yeast extract medium (YEM) (Thompson 1980; Somasegaran and Hoben 1994). Production of slow growing rhizobia requires 5 –10 days (Burton 1979; Thompson 1980) even in YEM with a higher yeast extract content. A reduction in this period would have economic benefit. In contrast to other popular bacterial media, YEM has a high carbon to nitrogen ratio; mannitol, the carbon source, is in great surplus (Somasegaran and Hoben 1994). Yeast extract is the only nitrogen source in YEM. It also supplies some growth factors (Burton 1979). The yeast extract concentration in YEM is variable, ranging from 0.4 to

as high as 10 g/l (Thompson 1980; Smith 1987; Somasegaran and Hoben 1994). Increasing the yeast extract content is good for rhizobial growth, however, when the concentration is higher than 3 g/l, the physiologic activity of the bacterial cultures, especially the infectious capability, is decreased (Thompson 1980). The situation is the same when amino acids are added to YEM (Strijdom and Allen 1966). Given this situation, there have been numerous publications regarding methods to improve rhizobial culture technology.

Culture medium is always one of the first considerations in the studies of microbial production technology (Banoub et al. 1987; Balatti et al. 1991; Wagner et al. 1995; Choi et al. 1999). Balatti et al. (1991) obtained a high cell concentration in a closed batch culture system by using a medium containing yeast extract, glycerol and salts. In order to be able to utilize an inexpensive carbon source during production of *Bradyrhizobium* strains for use in inoculants, Lie et al. (1992) reported a double fermentation technology in which sucrose was prefermented by yeast, and then *Bradyrhizobium* CB756 was inoculated into diluted prefermented medium. This resulted in a high density of bacterial cells. Kanuma (1997) developed a method wherein hot water extract of the host seed hulls or husks was added into the culture medium for better growth of *Rhizobium* strains. Grassano et al. (1999) reported that addition of amaranth seed meal (4 g/l) to YEM, as a replacement for yeast extract, was suitable for culture of both *Bradyrhizobium* and *Rhizobium* strains. There has also been work focused on improving the culture methods of rhizobia by adopting continuous culture and solid state fermentation (Smart et al. 1984; Graham-Weiss et al. 1987; Boehnel and Bruns 1992).

As our understanding of the interactions between rhizobia and their host plants has improved, more and more bacterial metabolite products have been produced. The products produced via *Bradyrhizobium* culture include siderophores (van Rossum et al. 1994; Abd-Alla, 1999), rhizobiotoxin (Yuhashi et al. 2000), lipopolysaccharides (Banoub et al. 1987) and some other products (Choi et al. 1999; Jimenez-Zurdo et al. 1996). To produce these metabolite products, selection of a suitable culture medium was always conducted. LCO products have also been included. A technology for synthesis of Nod Bj-IV (C18:1, Fuc, Gro) produced by *B. japonicum* USDA61 was developed by coupling reactions of oligosaccharides with glycerols (Ikeshita et al. 1995).

Nucleoside derivatives, including nucleotides, nucleosides and nucleic acids, are another important group of biosynthesized nitrogenous compounds. Nucleotide derivatives are seldom used as ingredients of microbial culture media, except when a special medium is needed for isolation or selection of mutants with specific nutritional deficiencies. However, in the production of inosine by bacterial fermentation, yeast crude RNA was added into the culture medium as an adenine source (Nakao 1979). Recently it was reported that nucleosides could be readily taken up from the growth medium, as a carbon source, by some *Bacillus subtilis* strains (Schuch et al. 1999). In research focused on producing probiotic food materials via culture of *Lactobacillus* strains, adding commercial yeast RNA into the culture medium increased bacterial biomass by as much as 30% (Houde 1999). Based on these reports, we tested the addition of commercial yeast RNA to YEM as a way to improve the otherwise slow growth of *B. japonicum* strains. The effect of RNA additions on bacterial growth dynamics, LCO yield, and LCO production ($\mu g/10^{10}$ cells) were investigated.

**8.3 Methods and materials**

The bacterial strains used in this work were *Bradyrhizobium japonicum* 532C, USDA3 and USDA110. The basic culture medium was YEM and, except when specifically indicated, the added amount of yeast extract was 0.5 g/l (Somasegaran and Hoben 1994). Commercial yeast RNA and genistein, used to induce the *B. japonicum nod* genes, were purchased from Sigma products. The bacterial culture conditions were 28 °C while being shaken at 200 RPM with 250 ml flasks containing 60 ml of medium. The initial culture time, following inoculation with a slant or plate, was 7 days. The inoculation ratio in the subculture was about 1% (v/v). The cell concentration of the bacterial culture was determined spectrophotometrically at 620 nm with an LKB Biochrom 4050 ultrospec® (Fisher Scientific, Montreal, Canada), assuming an $A_{620\ nm}$ of 0.08 indicates $10^8$ cells/ml (Bhuvaneswari et al. 1980).

In an experiment to study the effect of yeast extract concentration on *B. japonicum* growth, yeast extract was added to YEM in concentrations ranging from 0 to 8.0 g/l. Based on the results of this work, an experiment on the combined application of

RNA (0.05 to 0.8 g/l) and yeast extract (0.5 to 4.0 g/l) concentrations was conducted. In subsequent experiments, yeast extract was always added at 0.5 g/l, whereas RNA was added at four levels: 0 (RNA0), 0.05 (RNA1), 0.1 (RNA2) and 0.2 g/l (RNA3). Bacterial growth dynamics were analyzed for each RNA level. Growth dynamic variables were determined by plotting $\log_2$ bacterial cell concentrations against culture times (Monod 1949; Lockhart 1960; Bergersen 1961). The dynamic parameters were defined as follows. The generation lag ($L_g$) was the time needed for the first doubling of the initial cell population. The log phase was the duration of the exponential growth (*LP*). The generation time ($G_t$) was the time needed for finishing cell division once or doubling the cell population during the log phase. The growth rate (*R*) was the number of generations formed per day during the log phase. The total growth (*TG*) was the calculated cell concentration. Net growth (*NG*) was calculated by subtracting initial cell concentrations from *TG*. The generations (*G*) was the number of cell division cycles completed during a given culture process.

In research on LCO production, the bacterial culture was grown in 4 l flasks containing 2 l of medium, or 500 ml flasks containing 250 ml of medium, under the conditions described above. After the bacteria were subcultured for 5 days, 50 μM genistein, in a methanol solution, was added into the culture broth to a final concentration of 5 μM. Growth of the subculture continued for 4 days after the addition of the genistein. Following this, 40% volume of HPLC-grade 1-butanol was added into the broth with sufficient shaking to allow extraction of LCO into the organic phase. The upper LCO containing layer was collected using a glass separating funnel and then concentrated in a low-pressure rotary evaporator system (Yamato RE500, Yamato, USA) at 50 °C, until only a few ml of liquid remained. The residue was resuspended in 18% (v/v) acetonitrile-distilled water solution and kept in the dark at 4 °C in a sealed glass vial. The LCO concentration was determined by HPLC (Waters system, equipped with two Water Model 510 pumps, WISP 712 autosampler, model 441 absorption detector, Waters, MA, USA). The analysis column was a C18 reversed-phase type (Vydac, CA, USA; catalog no. 218TP54) with a flow rate of 1.0 ml/min and a Vydac guard column (catalog no. 218GK54). As a baseline 18% acetonitrile was run through the system for at least 10 min prior to injection, the sample was then loaded and an isocratic elution was

conducted with 18% acetonitrile for 45 min, in order to remove all non-polar light fractions. Thereafter, gradient elution (18 to 82% acetonitrile) was run for 90 min. The LCO was eluted at 94 – 96 min of HPLC run time. By comparison with a known standard (gift of G. Stacey, University of Tennessee), the LCO concentration was calculated from the elution peak area (Prithiviraj et al. 2000a). The LCO yield was expressed as μg/l of initial culture broth. LCO production by the cells was calculated against the cell concentration and expressed as μg of LCO/$10^{10}$ cells.

The data were analyzed statistically with GLM procedure of SAS (Littell et al. 1991). When the analysis of variance indicated differences among means, comparisons among the treatment means were conducted with a protected least significance difference (LSD) tests (Steel and Torrie 1980).

## 8.4 Results

*RNA stimulation of the growth of B. japonicum 532C in spite of yeast extract level*

Increasing the concentration of yeast extract in YEM caused a proportional increase bradyrhizobial growth (data not shown). When the RNA and yeast extract were added to YEM over a selected concentration range and at the same time, all five RNA addition levels increased the growth of *B. japonicum* 532C at all yeast extract levels (Fig. 8.1). As the added RNA concentration increased, the bacterial growth rate increased. At lower addition levels, the relative RNA stimulation was greater. As added RNA levels increased from 0.2 to 0.8 g/ l, the stimulating effect was almost linear, regardless of the yeast extract concentration. This indicated that RNA and yeast extract stimulate bacterial growth through different mechanisms. The effect of RNA additives on the cell growth could not be duplicated by increasing yeast extract concentration.

*Growth dynamics and RNA addition levels*

Compared with the control (RNA0), all the three RNA addition levels increased total growth (*TG*) at every point in the culture process (Fig. 8.2 and Table 8.1). This was due to two positive changes in growth dynamic parameters. In cultures with added RNA, the generation lag ($L_g$) and the generation time ($G_t$) were both shortened. This resulted in

a higher growth rate (R) and, subsequently, an increased *TG* or net growth (*NG*). These beneficial effects were linearly related to the tested RNA addition concentrations: $L_g$ (day) = 1.77 – 0.432 RNA (g/l) ($R^2$ = 0.9481), and *R* (generation/day) = 0.306 RNA (g/l) - 0.714 ($R^2$ = 0.9475). Because of the shortened $L_g$ and increased *R*, the culture time needed to attain a *TG* level similar to the control was correspondingly shortened. For instance, the time for the control to reach the maximum *TG* of $28.5 \times 10^8$ cells/ml was 12 days. For the RNA addition treatments, RNA1, RNA2 and RNA3, only 9.5, 8.5 and 7 days, respectively, were required, in spite of the fact that they had shortened log phases (*LP*) (Table 8.1).

The *TG* values for 4 and 8-day cultures of *B. japonicum* strains USDA3 and USDA110 were affected by RNA additions in the same way as demonstrated above for strain 532C (Fig. 8. 3).

*LCO production and added RNA concentrations*

LCO yield per liter of culture broth was increased by the RNA addition treatments (Table 8.2). In the culture broth, each cell should work as an independent LCO producing unit if they were all in a normal physiological state. The increased LCO yield could have been due to the greater numbers of cells in the cultures; LCO production, at $\mu g/10^{10}$ cells, was not different among the tested RNA addition levels. Given these findings, the only conclusion that could be drawn is that RNA additions had no effect on LCO production ($\mu g/10^{10}$ cells), so that the increased cell concentration in RNA addition treatments was the cause of increased LCO yield.

## 8.5 Discussion

Rhizobia can utilize a variety of sugars and organic acids as carbon sources and a number of nitrogenous compounds as nitrogen sources. Besides this, several growth factors, such as biotin and thiamin, are also needed for growth in culture (Bergersen 1961; Thompson 1980; Chemardin et al. 1989; Wagner et al. 1995). Yeast extract contains about 9.5% (w/w) N, of which 73% is amino N from a variety of the standard 20 amino acids. Yeast extract also contains vitamins such as biotin, niacin, pyridoxine and thiamin (Burton

1979). Among the reported growth substrates in rhizobial media, yeast extract has a very complex and potentially subtle role. Yeast extract was always considered as a nitrogen source and growth factor supplier (Bergersen 1961), whereas some authors thought it also could be a source of reduced carbon, iron, potassium and magnesium (Hunt and Stieber 1986; Wagner et al. 1995). However, when the yeast extract concentration was higher than 3.0 g/l, there were some deleterious effects on cell morphology, viable cell concentration and rhizobial symbiotic activity (Thompson 1980). The high amino acid content of yeast extract may be the real reason for the deleterious effects. In spite of increasing yeast extract contents, RNA additions, at the tested concentrations, accelerated bradyrhizobial growth and showed a potential as a new tool for improving the culture technology of the slow growing rhizobia.

Generally, the generation time (*Gt*) for the fast growing rhizobia is < 6 hours, whereas for the slow growing rhizobia it is > 6 hours (Elkan and Bunn 1991) or 2-4 hours and 6-12 hours respectively (Thompson 1980). In our experiment the determined *Gt* in the control was as long as 38.5 h, typical of these growth conditions (Prithiviraj et al. 2000). And even after it was shortened by RNA addition, e.g. RNA3, it was still 19.0 h. This difference might have been due to the culture conditions. Thompson used a YEM recipe with 100 ml/l yeast water which containing 10% yeast extract. Our experimental results showed that *TG* increased as yeast extract concentration increased and higher yeast extract concentrations could undoubtedly further shorten the *Gt* of the culture. In addition, we determined that the *LP*, log phase, of the control culture was 6 days, which was in accord with that reported by Bhuvaneswari et al. (1980). RNA additions shortened the bacterial culture period, but it seemed that $5 \times 10^9$ cells/ml was the maximum *TG* in the given culture conditions. This is in the general quality range necessary for inoculant preparation (Thompson 1980). Better *TG* results could be expected by adopting a controlled fermentor system, a higher concentration of yeast extract and a higher concentration of RNA addition. Even at the present yield level, only the shortened fermentation period due to RNA addition could result in a higher instrument utility, easier mechanical maintenance and, subsequently, a lower production cost.

RNA addition increased LCO yield and had no effect on LCO production of the cultures. LCO production depends on multiple bacterial physiological characteristics, such as substrate utilization, energy synthesis, the expression and function of the *nod* genes, precursor supply and processing, as well as the cell membrane structural and functional status. LCO production might be taken as an indication of overall cell physiological activities. The effect of RNA addition on LCO production implied that RNA additives had no deleterious effects on bacterial physiological activity. The LCO production program in our laboratory was established based on bacterial culture in YEM without RNA additives (Prithiviraj et al. 2000). Through the optimization of a program specifically matching RNA additions to the culture, it should be possible to further improve LCO production and, therefore, to further increase the LCO yield.

The mechanisms by which RNA stimulates bradyrhizobial growth are unknown. The added RNA may be used as nutrient substrate, as a source of material for production of RNA and DNA in the bradyrhizobial cells, or as a precursor of other physiologically active compounds. In these cases, RNA concentration should decrease during cell growth. The added RNA may work as physiologically active compounds directly, then the added RNA might not be consumed during cell growth. It will be very interesting to investigate the mechanisms as recent experiments have also demonstrated the positive effect of RNA additives on *Rhizobium* growth.

**Fig. 8.1. The effect of RNA additions to culture media with different yeast extract concentrations on the growth of *Bradyrhizobium japonicum* 532C.**

Data were from 96-h cultures. The experiment was conducted twice with three replicates of each treatment each time.

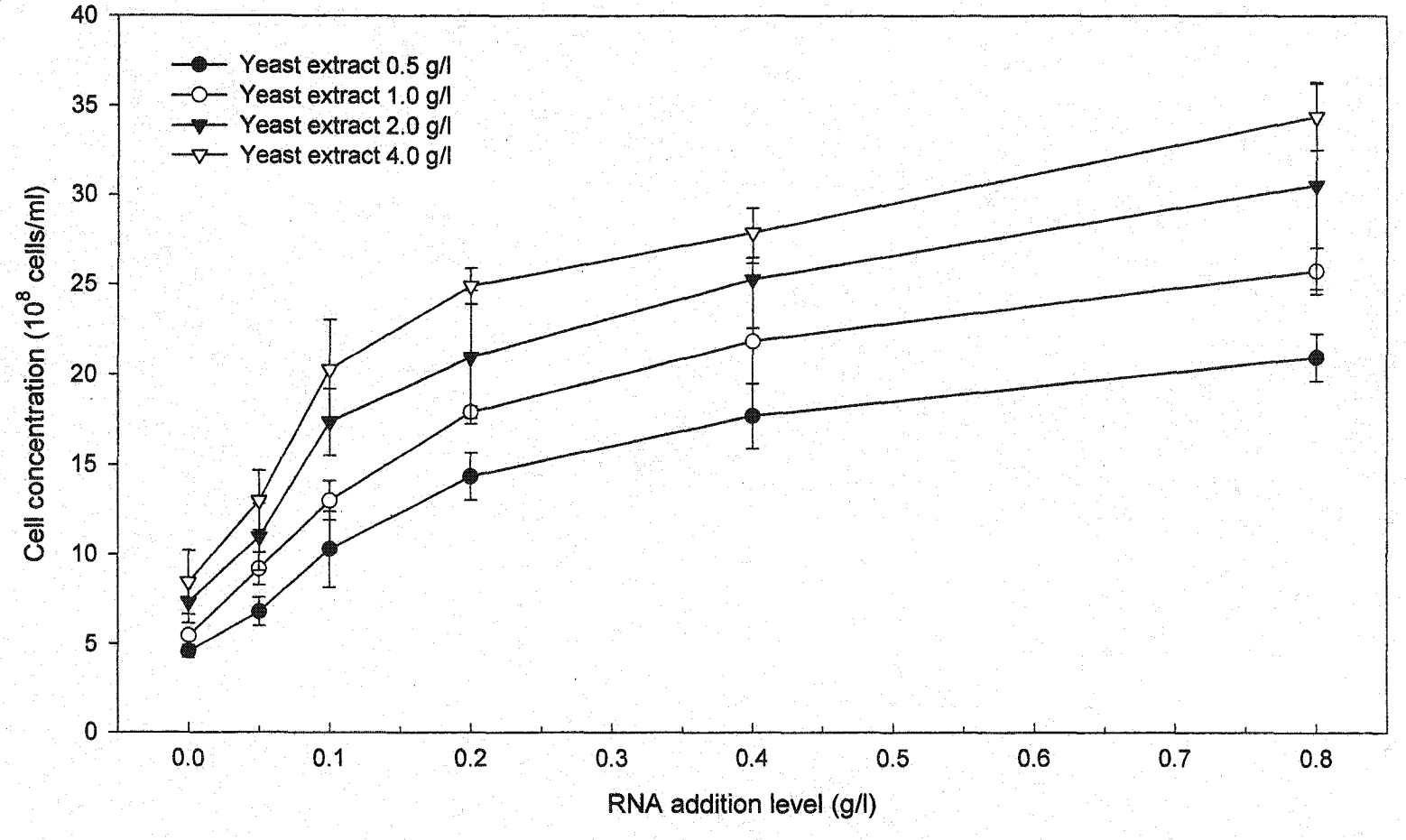

Yeast extract 0.5 g/l
Yeast extract 1.0 g/l
Yeast extract 2.0 g/l
Yeast extract 4.0 g/l
Cell concentration ($10^8$ cells/ml)
RNA addition level (g/l)
0
5
10
15
20
25
30
35
40
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

**Fig. 8.2. The growth dynamics of *Bradyrhizobium japonicum* 532C with different levels of RNA added to the culture medium.**

The experiment was conducted twice with two replicates each time. Points represent M ± SD.

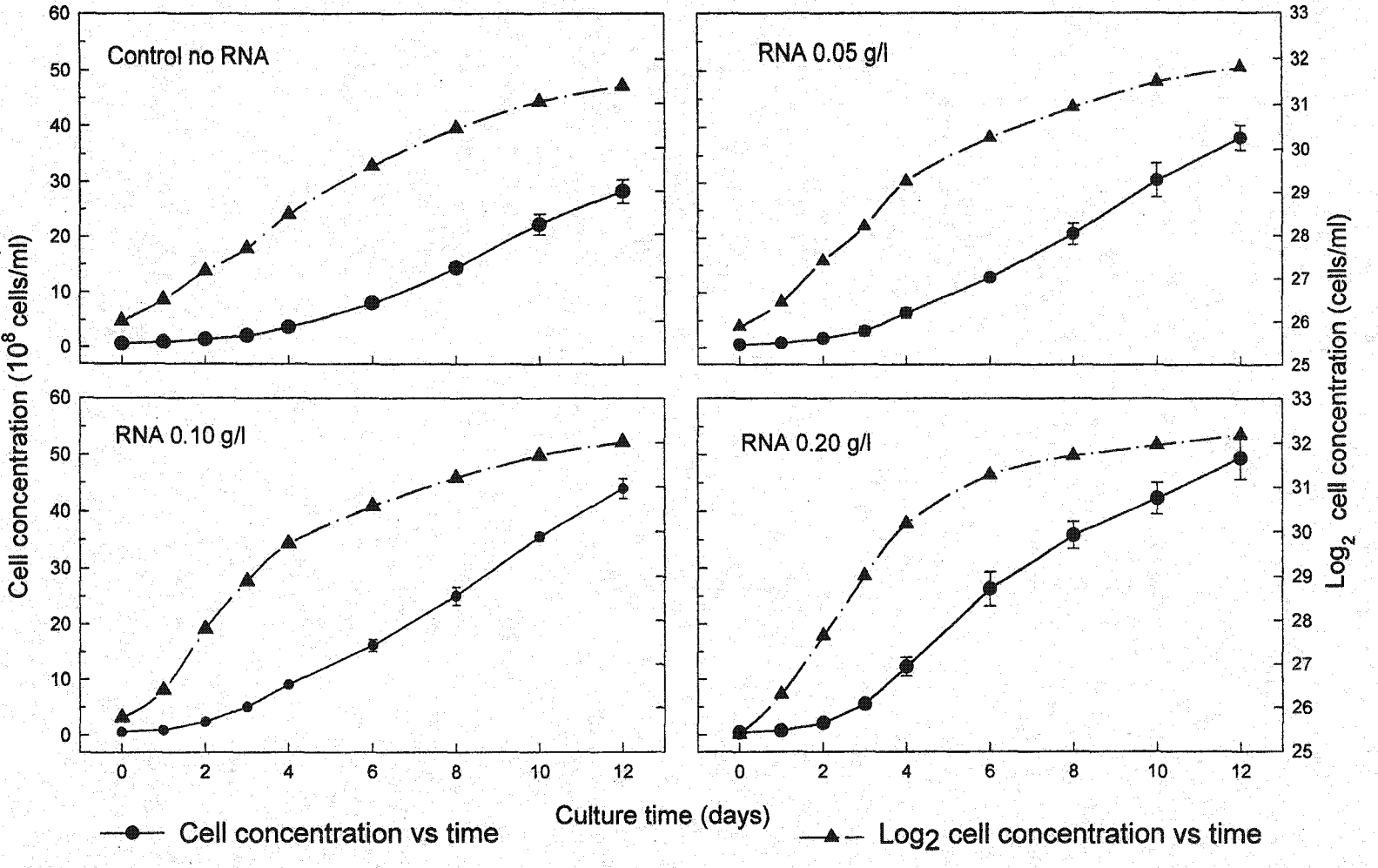

Control no RNA
RNA 0.05 g/l
RNA 0.10 g/l
RNA 0.20 g/l
Cell concentration ($10^8$ cells/ml)
$Log_2$ cell concentration (cells/ml)
Culture time (days)
Cell concentration vs time
$Log_2$ cell concentration vs time

**Fig. 8.3. Growth response of *Bradyrhizobium japonicum* USDA3 (I) and USDA110 (II) to RNA addition levels in the culture medium.**

The experiment was conducted twice with two replicates each treatment each time. Histogram bars indicated the values of the means. Bars associated with the same letters are not different at P = 0.10 (I) or 0.05 (II). The upper case letters are for comparisons among 8-day cultures and lower case letters for comparisons among 4-day cultures.

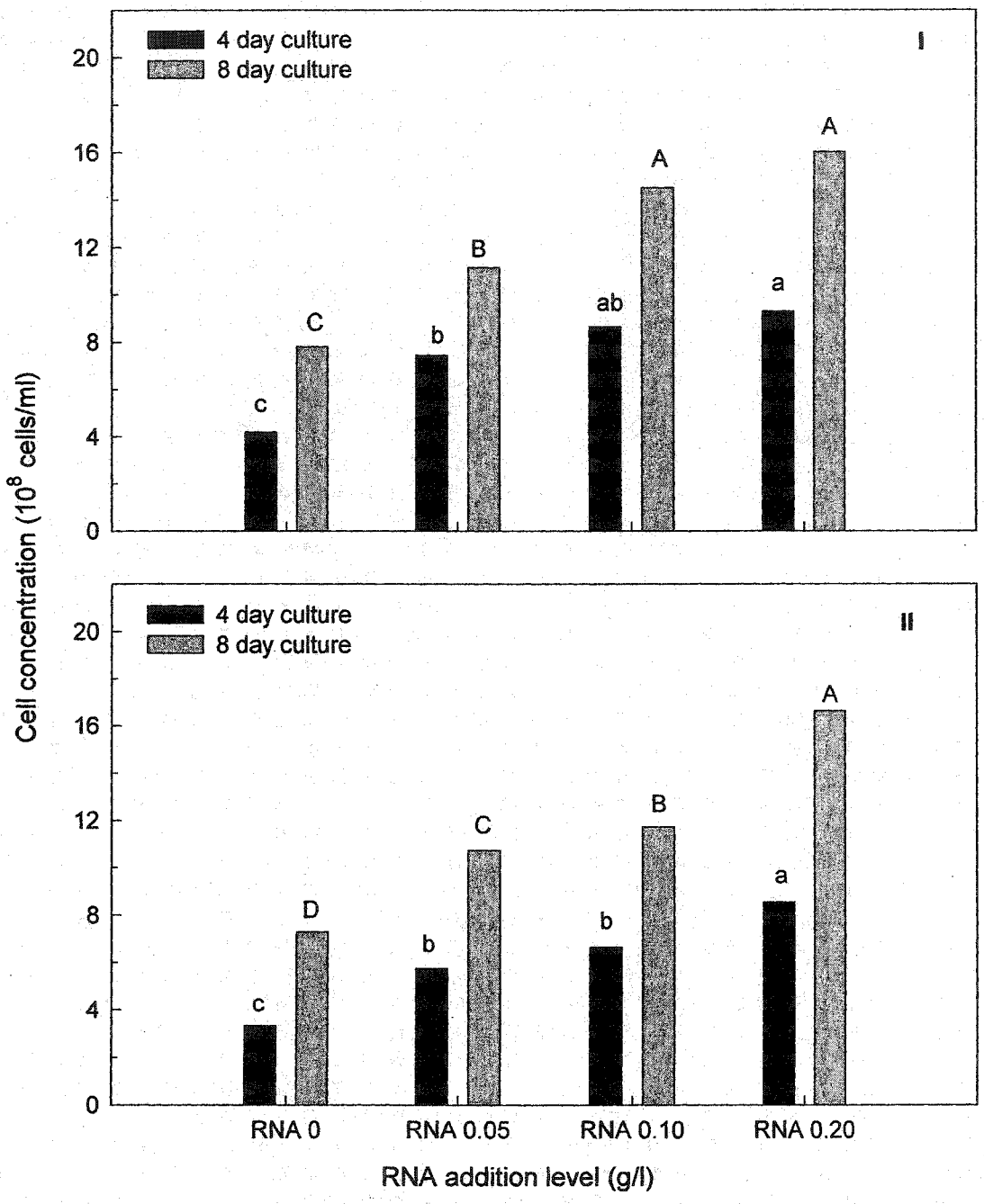

4 day culture
8 day culture
I
20
16
12
8
4
0
c
C
b
B
ab
A
a
A
4 day culture
8 day culture
II
20
16
12
8
4
0
c
D
b
C
b
B
a
A
RNA 0
RNA 0.05
RNA 0.10
RNA 0.20
RNA addition level (g/l)
Cell concentration ($10^8$ cells/ml)

**Table 8.1. Effects of RNA addition to yeast extract mannitol (YEM) culture medium on growth dynamic variables of *Bradyrhizobium japonicum* 532C**

| Medium (RNA addition g/l) | Generation Lag (days) | Generation Time (hours) | Log Phase (days) | Growth Rate (Generation per day) | Net growth ($10^8$ cells/ml) [Generations] In 4-day culture | In 8-day culture | In 12-day culture |
|---|---|---|---|---|---|---|---|
| YEM (RNA 0) Control | 1.89 | 38.5 | 6 | 0.625 | 3.05 d [2.46] | 13.83 c [4.43] | 27.83 d [5.40] |
| YEM (RNA 0.05) | 1.48 | 25.9 | 5 | 0.926 | 5.83 c [3.36] | 20.27 b [5.05] | 37.77 c [5.93] |
| YEM (RNA 0.10) | 1.20 | 21.7 | 4 | 1.111 | 8.47 b [3.96] | 24.52 b [5.43] | 43.42 b [6.24] |
| YEM (RNA 0.20) | 0.99 | 19.0 | 4 | 1.266 | 11.85 a [4.78] | 35.35 a [6.32] | 49.05 a [6.79] |

Only the data in the 'Net growth' column were determined experimentally, all others were calculated based on linear regression formulas developed for log phases; means in same column followed by the same letter are not different (P = 0.05) from each other.

**Table 8.2. Effects of RNA addition to yeast extract mannitol (YEM) culture medium on lipo-chitooligosaccharide (LCO) yield and production by *Bradyrhizobium japonicum* 532C**

| RNA Addition Level in Culture Medium (g/l) | LCO Yield in Culture Broth (μg/l) | LCO Production (μg/$10^{10}$cells, at culture termination) |
|---|---|---|
| RNA 0 | 1225.0 b | 9.15 a |
| RNA 0.05 | 2152.5 a | 10.90 a |
| RNA 0.10 | 2028.2 a | 8.75 a |
| RNA 0.20 | 2058.1 a | 8.54 a |

The experiment was repeated three times. Means in same column followed by the same letter are not different (P = 0.05).

## Preface to Section 9

Section 9 is comprised of a manuscript by Bai Y, Zhou X and Smith DL and will be submitted to *Journal of Applied Microbiology*.

In Section 8, I showed that the addition of RNA to the culture medium accelerated bradyrhizobial growth and increased bacterial lipo-chitooligosaccharide production. However, previous efforts at increasing the growth rate of cultures of *B. japonicum* have sometimes resulted in reduced symbiotic competency for the resulting cells. Increasing the growth rate while decreasing the ability of the bradyrhizobia to nodulate soybean plants is not acceptable for inoculant production. This section of the thesis reports greenhouse experimentation conducted to determine the symbiotic competency of cells from *B. japonicum* cultures that have developed more quickly because of RNA additions to their growth medium.

Of the co-authors, Dr. Zhou, a research associate of Dr. Smith, helped me to do the statistical analysis in this section. Dr. Smith is my thesis supervisor. He 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, bench space in greenhouse, experimental field space, the use of computers, and providing technical assistance. Dr. Smith will be the corresponding author for publication of this section.

# Section 9

## Addition of RNA to the culture medium improves the symbiotic activity of *Bradyrhizobium japonicum*

### 9.1 Abstract

In attempt to improve inoculant production for slow-growing *Bradyrhizobium* species, a technological key is to have the bacterial culture retain a high level of symbiotic activity while its growth rate is increased. For this purpose, addition of commercial yeast RNA to yeast extract mannitol (YEM) medium at levels of 0 – 0.2 g/l was tested for effects on nodulation competency, along with different bacterial culture times and inoculation doses, in greenhouse experiments. The experimental results showed that addition of RNA to YEM had no deleterious effects on the symbiotic activity of *B. japonicum* 532C while the bacterial growth was accelerated indicating that addition of RNA to YEM could be used as a novel technological means in the preparation of *B. japonicum* inoculant. The highest RNA addition level RNA3 (0.2 g/l) actually increased soybean nodule number (20.5%), nodule weight (29.2%) and plant weight (16.1%) at 55 days after inoculation (DAI) ($0.05 \leq P \leq 0.01$). However, it had no detectable effects on soybean nodulation and growth during the first 20 DAI. Application of RNA to conventionally grown cultures immediately prior to use as inoculant revealed that the RNA itself inhibited soybean nodulation and growth. This demonstrated that it was improvement of the bacterial physiological and symbiotic activity due to RNA addition to the culture medium during bacterial growth, rather than the RNA additives themselves, that was responsible for the positive effect of the bacterial culture on plant nodulation and growth. The relative positive effects of the different RNA addition levels in the culture medium were present in both the pot-Vermiculite and the growth-pouch cultured plants and at both $10^8$ and $10^5$ cells/seedling inoculation doses. The bacterial culture time had effect on the symbiotic activity of *B. japonicum* 532C cultures. At the RNA3 addition level, the 12 day culture time resulted in lower bacterial symbiotic activity than 8 day culture. So the best culture condition for preparation of *B. japonicum* inoculant was addition of 0.2 g/l RNA to YEM cultured for 8 days.

**Keywords:** *Bradyrhizobium japonicum*, culture medium, inoculant preparation, soybean, symbiotic activity, nodulation and growth.

## 9.2 Introduction

*Bradyrhizobium* is a typical slow growing genus of rhizobia (Elkan and Bunn 1991). Generally, their generation time is > 6 hours or 6-12 hours, whereas it is < 6 hours or 2-4 hours for the fast growing rhizobia, such as the genus *Rhizobium* (Thompson 1980; Elkan and Bunn 1991). Rhizobia are generally cultured in yeast extract mannitol medium (YEM) (Thompson 1980; Somasegaran and Hoben 1994). Production of agricultural inoculants of slow growing rhizobia always require 5 –10 days (Burton 1979; Thompson 1980), even in YEM with a higher yeast extract content and under industrial settings. Although not the longest of all bacterial culture processes, it is a very long culture period. Shortening the bradyrhizobial culture period would benefit both the inoculant production industry and the application of such inoculants in the development of sustainable agriculture.

Many papers concerning the improvement of the bradyrhizobial, as well as rhizobial, growth dynamics and culture technology have been published. It has been shown that increasing the yeast extract content in YEM enhances bacterial growth. Yeast extract contains about 9.5% (w/w) N, of which 73% is amino N from a variety of the standard 20 amino acids. Yeast extract also contains vitamins such as biotin, niacin, pyridoxine and thiamin (Burton 1979). Yeast extract was always considered as a nitrogen source and a supplier of growth factors (Bergersen 1961), and some authors have postulated that it could be a source of reduced carbon, iron, potassium and magnesium (Hunt and Stieber 1986; Wagner et al. 1995). It is the only potential source of nitrogen and growth factors in the YEM. The yeast extract content of YEM recipes varies from as low as 0.4 g/l to as high as 10 g/l (Thompson 1980; Smith 1987; Somasegaran and Hoben 1994). Although a higher content of yeast extract in YEM promotes more rapid bacterial growth, it had been demonstrated that yeast extract concentrations higher than 3 g/l diminish specific aspects of bacterial physiological and, especially, symbiotic activity (Thompson 1980). The same situation occurs when amino

acids are added to YEM as a supplementary nitrogen source (Strijdom and Allen 1966). This implies that the amino acid component of yeast extract might be responsible for its negative effect on the bacterial cultures. Thus increases in the yeast extract content or addition of amino acids to YEM may improve bradyrhizobial growth rate but this does not result in material that is desirable for the production of agricultural inoculants.

Grassano et al. (1999) reported that addition of amaranth seed meal (4 g/l) to YEM, as a replacement for yeast extract, was suitable for culture of both *Bradyrhizobium* and *Rhizobium* strains in the production of the inoculants. Other attempts at improvements to *Bradyrhizobium* and *Rhizobium* culture techniques have been reported, but sometimes were limited to the effects on the bacterial growth, and not concerned with evaluation of the bacterial symbiotic activity (Balatti et al. 1991; Lie et al. 1992; Boehnel and Bruns 1992). However, for bacterial inoculant production the challenge is to keep the bacterial symbiotic activity at a high level while increasing bacterial growth.

Following earlier reports of beneficial effects of RNA, as well as some other nucleotide derivatives, applied to other bacterial cultures (Nakao 1979; Schuch et al. 1999; Houde 1999), we added commercial yeast RNA to YEM in an attempt to develop a novel means to improve the *Bradyrhizobium* culture technology. We have already shown that adding RNA to YEM at levels from 0.05 to 0.2 g/l accelerates growth rate and increases lipo-chitooligosaccharide yield of *B. japonicum* cultures. The culture period for reaching the maximal biomass in YEM was shortened by 20.8 – 41.7% by adding RNA at 0.05 to 0.2 g/l (Section 8). In this paper, the symbiotic activity of *B. japonicum* 532C cultured with RNA amended medium was evaluated under greenhouse conditions.

## 9.3 Materials and methods

*Bacterial inoculant preparation*

The bacterial strain used in this work was *Bradyrhizobium japonicum* 532C (Hume and Shelp 1990). The basic culture medium was YEM with a yeast extract content of 0.5 gl (Somasegaran and Hoben 1994). The bacterial culture conditions were 28°C while being shaken at 200 rev/min in 250 ml flasks containing 60 ml of medium broth. The initial culture time, following inoculation with a slant or plate, was 7 days.

The inoculation ratio in the subculture was 1% (v/v). Except for the culture time, culture conditions for the subculture were the same as that of the initial culture. Based on knowledge of the bacterial growth dynamics (Section 8), in this experiment three different subculture times were tested: 4, 8 and 12 days for both the control culture and all the three RNA addition treatments. Commercial yeast RNA was purchased from Sigma products. In the basic YEM for the subcultures, RNA was added at four levels: 0 (RNA0, medium control), 0.05 (RNA1), 0.1 (RNA2) and 0. 2 g/l (RNA3). After the culture was harvested, the cell concentration was determined by spectrophotometry at 620nm with an LKB Biochrom 4050 Ultrospec® (Fisher Scientific, Montreal, Canada), assuming an $A_{620nm}$ of 0.08 indicates $10^8$ cells/ml (Bhuvaneswari et al. 1980). All the inoculants were prepared by diluting the cultures with distilled water to $10^5$ or $10^8$ cells/ml and used immediately after they were prepared.

*Plant culture and inoculation*

The experiments were conducted in a greenhouse with an air temperature of 25 ± 2 °C and additional illumination of 300 $\mu mol/m^2/s^1$ supplied by high pressure sodium lamps (P. L. Light System, Montreal, Canada) for a photoperiod of 16:8 h (day:night). The soybean cultivar used in the experiments was OAC Bayfield. Soybean seeds were surface sterilized in sodium hypochloride (2% solution containing Tween 20 at 4 ml/l for 3 min.; Bhuvaneswari et al. 1980). The seeds were then rinsed several times with distilled water and planted in trays containing Vermiculite and germinated in the greenhouse. Seedlings at the VE stage (Fehr and Coviness 1971)(3 to 4 days old under our conditions), were transplanted into pots filled with Vermiculite, one seedling/pot, or growth pouches (15 × 16 cm, Mega International, Minneapolis, MN), one seedling/pouch. In this experiment, both the pot and pouch culture techniques were adopted and RNA addition level, along with culture time and inoculation dose, was investigated. In the pouch experiment, the root zone temperature was controlled at 25 °C by a water bath system. The experiment was organized following a split-split plot design. The main plot was bacterial culture times. Inoculation dose formed the sub-plots. The different RNA addition levels were arranged within each sub-plot (Mead et al. 1993). Soybean seedlings were inoculated at the VC stage (Fehr and Coviness 1971), i.e., 6 days

after transplanting or 10 days after germination. Each seedling was inoculated by adding 1 ml of one of the inoculants (24 inoculants: 3 culture times × 4 RNA levels × 2 inoculation doses, Table 9.1) onto the culture medium around the seedling roots in pots, or the root surface in pouches, with a pipette. In order to investigate possible direct effects of RNA on plant nodulation and growth, RNA-control (RC) inoculants were prepared by adding RNA into 4 day-old control cultures immediately prior to their use as inocula. The RNA was added at concentrations of 0 (RC0), 0.0068 (RC1), 0.0118 (RC2) and 0.0156 (RC3) g/l. The concentrations were same as those in inoculants 1, 2, 3 and 4, respectively (Table 9.1). RC inoculants were also inoculated onto soybean plants at 1 ml/seedling. During the growth process, all the plants were watered with modified N-free Hoagland's solution (Hoagland and Arnon 1950), in which $Ca(NO_3)_2$ and $KNO_3$ were replaced with 1 mM $CaCl_2$, 1 mM $K_2HPO_4$ and 1 mM $KH_2PO_4$, to provide a nitrogen-free culture condition for the plant growth.

*Data collection and analysis*

The plants were harvested twice at 20 and 55 DAI respectively, but the plants used to test the effect of RC inoculants were harvested only once, at 55 DAI. After harvesting, data on nodule number, nodule weight, root weight and shoot weight were collected. All samples were weighed after not less than 48 h drying in a 70 – 80 °C dryer. In this case, the plant weight is the sum of shoot weight and root weight. All the data were analyzed statistically with the GLM procedure of SAS (Littell et al. 1991; Cody and Smith 1997). When analysis of variance indicated differences among means, comparisons among the treatment means were conducted with least significance difference (LSD) tests (Steel and Torrie 1980).

## 9.4 Results

Among the three tested factors, RNA addition level (R), bacterial culture time (T) and inoculation dose (D), the effect of R and D on the three variables, nodule number, nodule weight and plant weight, were significant in both pouch and pot cultures (Table 9.2). But there was no effect of T on nodule number in pouch or pot cultures, and it did not affect

plant weight in pot cultures. The R × T and R × D interactions occurred only in pouch culture for nodule weight and plant weight (Table 9.2). Given that pot culture supported better plant growth, the interactions that occurred in the pouch culture system are not presented.

*Bacterial symbiotic activity and RNA addition to YEM*

In order to evaluate the effect of the bacteria cultured at different RNA addition levels on early stages of soybean nodulation and growth the first harvest was conducted at 20 DAI. There were no differences among the tested RNA addition levels (RNA0, RNA1, RNA2 and RNA3) either in pouch or pot cultures at the 20 DAI harvest (data not shown). This demonstrated that *B. japonicum* 532C cultured in RNA added medium retained its normal symbiotic activity during the early stages of soybean nodulation and growth. All of the inoculated bacteria cells, either cultured in basic YEM alone or in RNA amended YEM medium, had similar performances with regard to nodulation and growth of host plants before 20 DAI.

At the final harvest (55 DAI), either in pouch or pot culture, none of the RNA addition treatments showed deleterious effects on the bacterial symbiotic activity. RNA3, the highest tested RNA addition level, increased nodule number by 19.8%, nodule weight by 39.1% and plant weight by 17.9% in pouch culture and nodule number by 21.1%, nodule weight by 21.6% and plant weight by 14.6% in pot culture (Fig. 9.1). In the pot experiment, RNA2 also increased nodule number, nodule weight and plant weight, to the same degree as RNA3. Collectively, these experimental results demonstrated that all the tested RNA addition treatments had no negative effect on bacterial symbiotic activity. While greatly increasing the bacterial growth rate, the highest RNA addition treatment, RNA3 (0.2 g/l), as well as RNA2 (0.1 g/l) in some cases, improved the symbiotic activity of *B. japonicum* 532C.

Given the lack of RNA addition effects at 20 DAI, it can be concluded that all of the positive effects developed between 20 and 55 DAI. Taking the data of each treatment at 20 DAI as 100%, during the subsequent 35 day growth period nodule number increased by 24.0 and 64.1%, nodule weight by 567.1 and 799.1% and plant weigh by 374.8 and 440.0% for RNA3 in pouch and pot cultures, respectively. Whereas during

this period, for the control RNA0, nodule number increased by 13.3 and 38.7%, nodule weigh by 446.1 and 653.0% and plant weight by 298.4 and 331.6% in pouch and pot cultures respectively. For RNA1 and RNA2 nodule number increased at a level similar to RNA 3, but the increase in nodule weight was much nearer to RNA0, resulting in less increase in plant weight than RNA3. This implied that the application of the bacteria cultured in YEM with RNA additives stimulated plant growth mainly via increase in nodule mass. This was also supported by the correlation analysis of the three measured variables. In either pouch or pot culture, the correlation coefficient between the nodule weight and plant weight was much higher than that between the nodule number and plant weight. The former is 0.90 and 0.92, the latter is 0.53 and 0.63, in pouch and pot cultures, respectively.

*RNA-control (RC) inoculants and nodulation and plant growth*

In both pot and pouch versions of the experiment, RC inoculants (RNA added to the cultures immediately prior to inoculation, instead of being present throughout the bacterial growth) caused inhibition of both nodulation and plant growth, and this inhibition increased as the RNA concentration in the inoculants increased. In pot culture, RC2 and RC3 inhibited nodule weight by 19.4 to 29.1% and plant weight by 16.6 to 19.1%, respectively. The inhibition was even greater in pouch culture than in pot culture, although the effects on absolute nodule number were different between the pot and the pouch experiments (Table 9.3). This result showed that the RNA additives in the culture medium were not directly responsible for the positive effect of the RNA additions on the soybean seedling nodulation and growth during the whole growth period. It was the improvement of the bacterial physiological properties due to the addition of RNA to YEM that resulted in the improved bacterial symbiotic activity and subsequently the better soybean nodulation and growth.

*The effect of bacterial culture time and inoculation dose on the bacterial symbiotic activity*

In pouch culture, 8-day is the best culture time in regard to the plant nodulation and growth (Fig. 9.2). There were interactions between RNA addition level and bacterial

culture time (R × T, Table 9.2) in the pouch experiment. For the RNA0 control culture, when culture time was increased from 4 to 8 to 12 days, the symbiotic activity of the bacterial inoculant increased. However, for all the three RNA addition treatments, the 8 day cultures had the highest symbiotic activities (data not shown). In pot culture, only the differences in nodule weight between the culture times were significant (P = 0.05), for which 8 and 12 day cultures were superior to the 4 day culture. At the RNA3 addition level treatment in pot cultures, 8-days of culture resulted in the highest symbiotic activity (data not shown). In view of the acceleration of bacterial growth caused by RNA addition treatments (Table 9.1) and the resulting higher symbiotic activity (Fig. 9.1), the optimal culture time should be 8 day when RNA addition has been adopted.

In both pouch and pot culture, the higher inoculation dose, $10^8$ cells/plant, was always better than $10^5$ cells/plant density (Fig. 9.3). The interaction between RNA addition level and inoculation dose (R × D, Table 9.2) indicated only that inoculant from the 0.2 g/l RNA addition culture at the $10^5$ inoculation dose resulted in higher nodule weight and plant growth than the inoculant from the RNA0 control culture at the $10^8$ inoculation dose (data not shown). This does not alter the general conclusion that the higher inoculation dose was better than the lower inoculation dose in this experiment.

## 9.5 Discussion

In the experiment, two different plant culture methods were utilized. Essentially the same set of inoculants was applied in both the pot-Vermiculite and the growth-pouch cultures in the experiment. The pot-Vermiculite culture was always superior to the growth-pouch for soybean seedling nodulation and growth under the given nitrogen–free experimental conditions. This was much more obvious at 55 DAI than at 20 DAI, probably because plant growth before 20 DAI was mainly supported by nitrogenous nutrients contained in the seed that gave rise to the plant, rather than the N from nitrogen fixation (Zhang et al. 1996b, 1997). At 55 DAI harvest, the measured variables in the growth-pouch system could generally reach to about 50 – 75 % of those values in the pot-Vermiculite culture. In the pouch culture, the RNA1 and RNA2 treatments didn't result in higher plant growth than RNA0 control. There were differences in the occurrence of

interactions between pot and pouch culture experiments. However, the superiority of the RNA3 treatment to RNA0, at 8 days of culture, as opposed to at 4 and 12 days of culture, and of the $10^8$ inoculation dose to the $10^5$ inoculation dose, were all demonstrated in the two culture systems. Based on analysis results of RNA addition levels and bacterial culture times, it could be concluded that the best bacterial culture condition is addition of 0.2 g/l RNA in YEM and culturing for 8 days. Under these conditions, the bacterial total growth was at the same level as the total growth in the basic YEM cultured at 12 days (Table 9.1). This meant that addition of 0.2 g/l RNA into YEM had two advantages over the original culture technology. While the bacterial growth rate was increased, the symbiotic activity of the bacterial culture was also improved. This is an important objective in research on the improvement of inoculant production for slow growing *Bradyrhizobium* species.

In Section 8, I demonstrated that RNA addition levels from 0.05 to 0.8 g/l all accelerated the growth rate of *B. japonicum*, in spite of a YEM yeast extract content that was low (0.5 g/l) or high (4 g/l). In order to avoid the possibility that higher RNA additions, greater than 0.2 g/l, might have deleterious effects on rhizobial physiological and symbiotic activities, in this experiment, only RNA additions equal to and lower than 0.2 g/l were tested. We expected that these tested RNA addition levels would have no negative effects on bacterial symbiotic activity, and could provide data supporting RNA addition as a novel technology for preparation of inoculant from *B. japonicum* cultures. In this case, the results showed that RNA addition to the YEM medium had no deleterious effect on the symbiotic abilities of *B.* japonicum 532C. It was beyond our expectation that the RNA3 treatment, and sometimes the RNA2 treatment, actually improved the bacterial symbiotic activity, resulting in increased plant nodulation and growth. These results indicate that RNA additions higher than 0.2 g/l in YEM might further improve the bacterial growth and symbiotic activity. This remains to be tested. Thus, the best culture conditions concluded from the present experiment, 0.2 g/l RNA addition and an 8 day culture period, are relative to the tested conditions. If RNA addition is further increased, the optimal culture time for the bacteria to well develop their symbiotic activity may be further shortened.

When the RNA was not present during the growth of the culture, but was added immediately prior to the use of the cultures as inoculants (RNA controls), RNA addition inhibited soybean nodulation and growth, and this inhibition increased as the added RNA concentration increased. This implies that the improved bacterial symbiotic activity may be due to improved physiological status of the bacteria during the culture process, but not due the involvement of the RNA additives in interactions between the plant and the inoculated bacteria. This latter point may be responsible for the negative effects of the RNA additives in the control inoculants on the plant nodulation and growth. However, the present experimental results do not provide any further information regarding the mechanisms underlying this observation. This and the mechanisms leading to RNA stimulation of bacterial growth in culture both merit additional research effort. Only in the same inoculation dose level was the comparisons of the symbiotic activity of the different inoculants reasonable. Soybean nodulation and seed yield were improved when the inoculation dose increased from $10^5$ to $10^6$ *B. japonicum*/seed and it was recommended to increase the Agriculture Canada standard of $10^5$ *B. japonicum*/seed (Hume and Blair 1992). Although $10^8$ dose is often adopted in our greenhouse and field experiments (Zhang et al. 1996b; Dashti et al.1997; Pan and Smith 2000; Prithiviraj et al. 2000), it is possible that the $10^8$ cells/plant was higher than a necessary cell density for nodulation of a soybean plant. If that is true, the excessive inoculation dose may interfere in the comparisons of the symbiotic activity of the different inoculants. In case this situation occurs, in our experiment both $10^8$ and $10^5$ inoculation doses were adopted. The experimental results showed that the higher inoculation dose was better than the lower. However, both of them were available in the comparisons of the different RNA addition levels and the different bacterial culture times. This made the conclusions more reliable than using only one inoculation dose.

In conclusion, the work presented in this paper demonstrated that all the tested RNA additions to YEM had no deleterious effects on the symbiotic activity of the bacterial cultures. The higher RNA addition levels actually improve the bacterial symbiotic activity, in some cases, while the bacterial growth rate was increased. This and our previous work have provided a good foundation for further research on this subject,

and indicated the high possibility for use of RNA addition as a novel method to improve bradyrhizobial inoculant production technology.

**Fig. 9.1. The effect of RNA addition levels in the culture medium on the symbiotic activity of *Bradyrhizobium japonicum* 532C at 55 days after inoculation in pouch and pot cultures of soybean plants.**

Graphs: I, nodule number; II, nodule weight; III, plant weight. Histogram bars associated with the same letters, within each graph, are not different (P = 0.05) by an ANOVA protected LSD test. n =30.

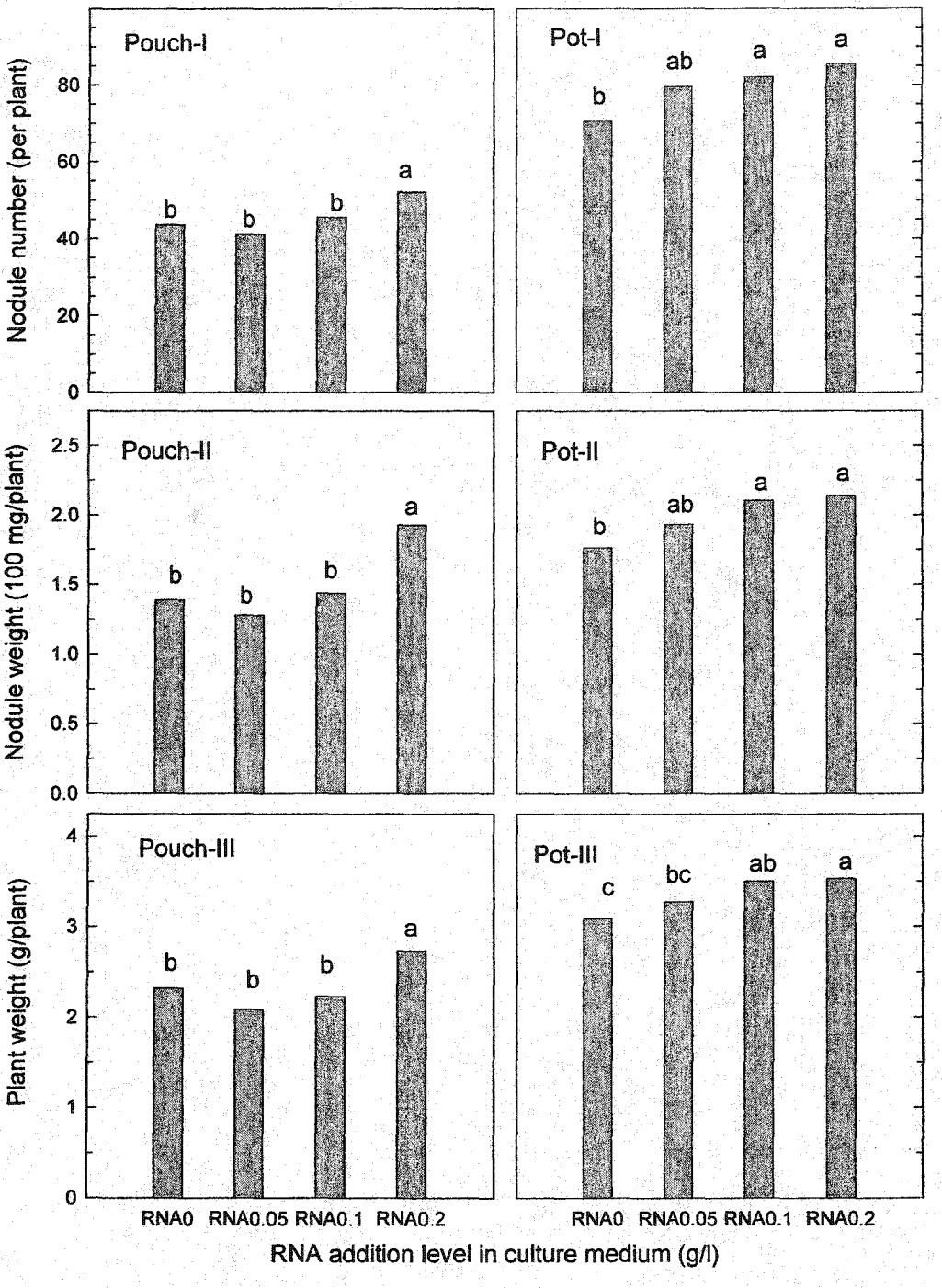

Pouch-I
Pot-I
Pouch-II
Pot-II
Pouch-III
Pot-III
Nodule number (per plant)
Nodule weight (100 mg/plant)
Plant weight (g/plant)
RNA0
RNA0.05
RNA0.1
RNA0.2
RNA addition level in culture medium (g/l)

**Fig. 9.2. The effect of bacterial culture time on the symbiotic activity of *Bradyrhizobium japonicum* 532C at 55 days after inoculation in pouch and pot cultures of soybean plants.**

Graphs: I, nodule number; II, nodule weight; III, plant weight. Histogram bars associated with the same letters, within each graph, are not different (P = 0.05) by an ANOVA protected LSD test. n = 40.

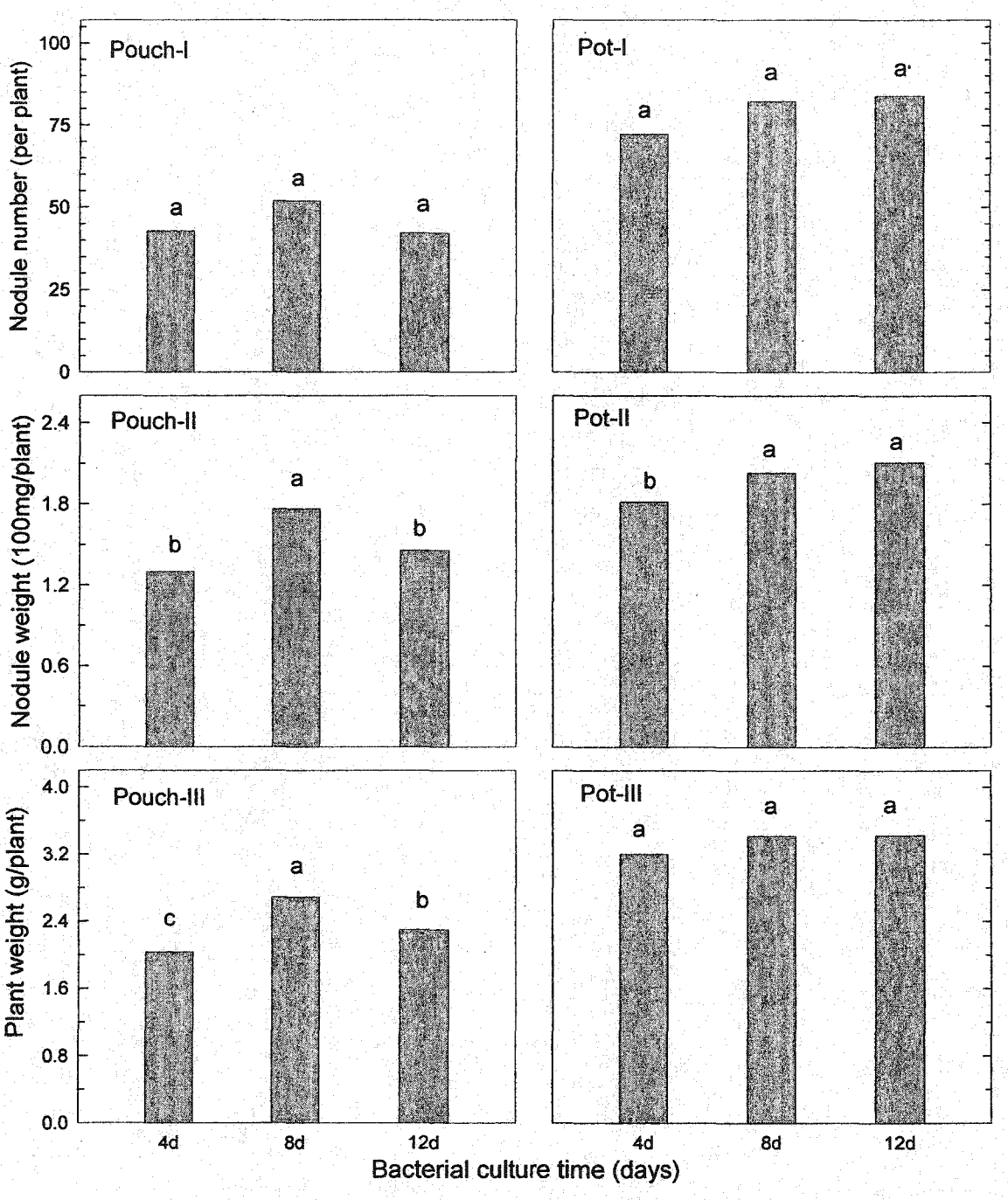

Pouch-I
Pot-I
Pouch-II
Pot-II
Pouch-III
Pot-III
Nodule number (per plant)
Nodule weight (100mg/plant)
Plant weight (g/plant)
Bacterial culture time (days)
4d
8d
12d
0
25
50
75
100
0.0
0.6
1.2
1.8
2.4
0.8
1.6
2.4
3.2
4.0
a
b
c

**Fig. 9.3. The effect of inoculation dose on the symbiotic activity of *Bradyrhizobium japonicum* 532C at 55 days after inoculation in pouch and pot cultures of soybean plants.**

Graphs: I, nodule number; II, nodule weight; III, plant weight. Histogram bars associated with the same letters, within each graph, are not different (P = 0.05) by an ANOVA protected LSD test. n = 60.

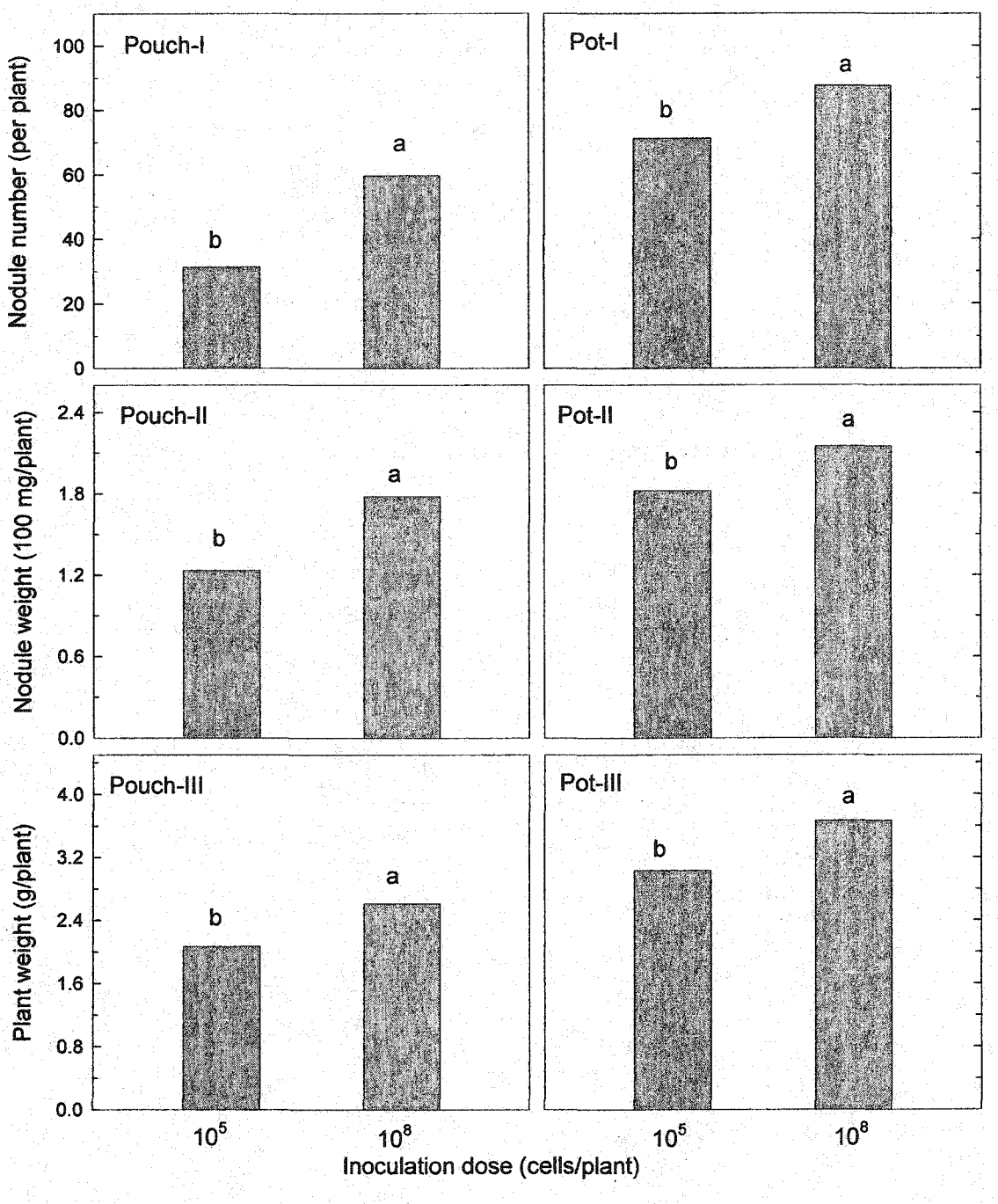

Pouch-I
Pot-I
Pouch-II
Pot-II
Pouch-III
Pot-III
Nodule number (per plant)
Nodule weight (100 mg/plant)
Plant weight (g/plant)
Inoculation dose (cells/plant)
10^5
10^8
a
b
0
20
40
60
80
100
0.0
0.6
1.2
1.8
2.4
0.8
1.6
3.2
4.0

**Table 9.1. The preparation parameters (culture time, RNA addition level and culture dilution) for the 12 *Bradyrhizobium japonicum* 532C inoculants ($10^8$ cells/ml) used in the experiment.**

| Inoculant | Culture time (days) | RNA addition Levels (g/l) | $A_{620nm}$ in 1/10 diluted culture | Cell concentration ($10^8$cells/ml) | Culture volume (ml) added into 50ml inoculant | $A_{620nm}$ in inoculant |
|---|---|---|---|---|---|---|
| 1 | 4 | 0 | 0.026 | 3.5 | 14.3 | 0.064 |
| 2 | 4 | 0.05 | 0.054 | 7.3 | 6.9 | 0.067 |
| 3 | 4 | 0.1 | 0.063 | 8.5 | 5.9 | 0.073 |
| 4 | 4 | 0.2 | 0.096 | 13.0 | 3.9 | 0.073 |
| 5 | 8 | 0 | 0.150 | 20.4 | 2.5 | 0.074 |
| 6 | 8 | 0.05 | 0.241 | 32.8 | 1.5 | 0.069 |
| 7 | 8 | 0.1 | 0.248 | 33.7 | 1.5 | 0.070 |
| 8 | 8 | 0.2 | 0.286 | 39.0 | 1.3 | 0.074 |
| 9 | 12 | 0 | 0.283 | 38.5 | 1.3 | 0.068 |
| 10 | 12 | 0.05 | 0.309 | 42.0 | 1.2 | 0.074 |
| 11 | 12 | 0.1 | 0.327 | 45.8 | 1.1 | 0.075 |
| 12 | 12 | 0.2 | 0.343 | 47.5 | 1.1 | 0.082 |

The $10^5$ cells/ml inoculants were obtained by dilution of the $10^8$ cells/ml cultures with distilled water.

**Table 9.2. Analysis of variance for the data in the experiment**

| | Nodule number (per plant) | Nodule weight (g/plant) | Plant weight (g/plant) |
|---|---|---|---|
| POUCH CULTURE | | | |
| RNA addition level (R) | ** | ** | ** |
| Bacterial culture time (T) | NS | ** | ** |
| Inoculation dose (D) | ** | ** | ** |
| R × T | NS | ** | ** |
| R × D | NS | * | * |
| T × D | NS | NS | NS |
| R × T × D | NS | NS | NS |
| POT CULTURE | | | |
| RNA addition level (R) | * | ** | * |
| Bacterial culture time (T) | NS | * | NS |
| Inoculation dose (D) | ** | ** | ** |
| R × T | NS | NS | NS |
| R × D | NS | NS | NS |
| T × D | NS | NS | NS |
| R × T × D | NS | NS | NS |

* Significant at P = 0.05; ** Significant at P = 0.01; NS, not significant.

**Table 9.3. The effect of RNA additions to *Bradyrhizobium japonicum* 532C culture immediately prior to use as an inoculant (RNA-control, RC) on soybean nodulation and growth in the pot and the pouch cultures.**

| | Nodule number (per plant) | | Nodule weight (g/plant) | | Plant weight (g/plant) | |
|---|---|---|---|---|---|---|
| In the pouch experiment (n=4) | | | | | | |
| RC0 | 51.3 | a | 0.1404 | a | 2.1416 | a |
| RC1 | 41.6 | ab | 0.1287 | ab | 1.9359 | ab |
| RC2 | 38.8 | bc | 0.1038 | bc | 1.6998 | b |
| RC3 | 29.3 | c | 0.0845 | c | 1.6103 | b |
| In the pot experiment (n=5) | | | | | | |
| RC0 | 73.8 | a | 0.2211 | a | 3.5928 | a |
| RC1 | 72.8 | a | 0.2203 | a | 3.5608 | a |
| RC2 | 81.0 | a | 0.1782 | ab | 2.9961 | b |
| RC3 | 79.2 | a | 0.1567 | b | 2.9081 | b |

*RNA concentrations in RC inoculants were 0 (RC0), 0.0068 (RC1), 0.0115 (RC2) and 0.0158 (RC3) g/l. Within the same column and experiment, means followed by the same letter are not different (P = 0.05) by an ANOVA protected LSD test.

# Section 10. General Discussion

The formation of nitrogen fixing root nodules on soybean (*Glycine max* L. Merr) is the result of subtle interactions between the plants and their endosymbiont, *Bradyrhizobium japonicum*. In effective root nodules, the bradyrhizobial bacteroids produce dinitrogenase, which catalyzes the reduction of $N_2$ to $NH_3$. Thus, the root nodule is a specific organ for nitrogen fixation, like the leaf is a specific organ for photosynthesis. Nodulation and nitrogen fixation are controlled by both symbiotic partners. Nodulation and nitrogen fixation are also affected by many biotic and abiotic factors. The theme of this thesis is the improvement of soybean nodulation and nitrogen fixation, to expand their use in sustainable agriculture systems.

## 1. Soybean RZT and plant growth promoting bacteria (PGPB)

Soybean is a legume of tropical to subtropical origin and requires warm growing conditions, eg. 25–30°C soil temperature for optimal symbiotic activity (Jones and Tisdale 1921; Smith 1995). In Quebec, at a depth of 10 cm, the mean soil temperature is 10°C in mid-May and 15°C in June (Lynch and Smith 1993). Low soil temperature during the early development stages of soybean plants inhibits plant growth, and this can be a major limiting factor for soybean production in short season areas. Soybean nodulation and nitrogen fixation are sensitive to low RZT and the response is more obvious at early stages of soybean nodulation than on nitrogen fixation (Layzell et al. 1984; Roughley and Date 1986; Lynch and Smith 1994; Zhang and Smith 1994). Suboptimal RZT (15 – 17.5°C) delay infection thread elongation (Zhang and Smith 1994), and inhibits flavonoid synthesis and excretion by soybean roots (Zhang and Smith 1996b; Pan and Smith 1998). My data (Section 3) indicated that 15°C RZT delayed the onset of nodulation by about 12 days and lowered the nodulation rate by 50%. However, coinoculation of *B. japonicum* with some specifically selected PGPB, such as *Serratia proteamaculans* 1-102 and *S. liquefaciens* 2-68 could relieve the low RZT inhibition of soybean nodulation, nitrogen fixation and growth. This thesis also describes the isolation of new PGPB strains from soybean root nodules. Three of these, *Bacillus thuringiensis*

strain NEB17, and *B. subtilis* strains NEB4 and NEB5, worked well in overcoming low RZT inhibition of soybean nodulation and nitrogen fixation under both greenhouse and field conditions (Sections 6 and 7). In a greenhouse experiment at 15°C RZT, coinoculation of the *S. proteamaculans* 1-102 caused nodule initiation to occur about 2 days earlier and increased the average nodulation rate by 30%, resulting in a 51% increase in nodule number, a 280% increase in fixed nitrogen and a 35% increase in plant dry matter (Section3). Under the same conditions, coinoculation of *B. japonicum* with *B. thuringiensis* NEB17 increased nodule number by 113%, nodule weight by 53% and plant dry matter 27% (Section 7). Under short season field conditions coinoculation of *S. proteamaculans* 1-102 increased grain yield by 19 - 29% and total nitrogen yield by 23 – 27% (Section 5), whereas coinoculation of *B. thuringiensis* NEB17 increased grain yield by 17 – 23% and total nitrogen yield by 25 – 26% (Section 7). All these PGPB strains could be used in soybean production to relive the low soil temperature inhibition of soybean nodulation under short season conditions.

The two genera of PGPB investigated here (*Serratia* and *Bacillus*) do not have identical effects on soybean nodulation, nitrogen fixation, growth and yield (Sections 3, 6 and 7). However, they do have generally similar effects. Suggesting that they might act through a similar mechanism, eg. production of similar active compounds which alleviate the same bottle neck in nodulation and processes that follow this. At this time we have no clear evidence as to the exact mechanism, however, isolation of a NEB growth activator with an overall structure or conformation similar to that of the activator produced by *S. proteamaculans* 1-102 could provide compelling evidence, should this happen. Work of this nature is included in the suggestions for future research.

## 2. PGPB and the nodulation process

Soybean nodulation is a multi-stage process. It begins with signal exchange between the two symbiotic partners. Soybean produces flavonoid molecules, mainly genistein and daidzein, which activate the bacterial nod genes (Kosslak et al. 1987). In response, the bacteria produce nod factor, LCOs, through the coordinated activities of many metabolic pathways. LCOs constitute the return signal and play a role in root hair deformation,

guiding bacterial attachment to root hair tips and stimulating the formation of nodule primodia (Perret et al. 2000). Applying the signal compounds, either flavonoids or LCOs, to the soybean rhizosphere is effective in enhancing the soybean nodulation process (Zhang and Smith 1995; Dashti et al. 2000; Pan and Smith 2000a, 2000b; Prithiviraj et al. 2000). Accumulation of knowledge regarding this signal exchange and the role it plays in soybean nodulation is potentially helpful in understanding how PGPB enhance the nodulation process.

The general mechanisms by which PGPB exert their effects have been thoroughly reviewed by several authors (Glick 1995; Hallmann et al. 1997; Sturz et al. 2000). There is a growing body of evidence suggesting that specific metabolites are responsible for the effects of many PGPB (Garcia de Salamone et al. 2001). Investigations of nodulation enhancement by PGPBs have shown that signal-like metabolites play a role in at least some of these relationships (Parmar and Dadarwal 1999). In this thesis I have conducted experiments in which *S. proteamaculans* 1-102 is induced to produce a plant growth activator by adding flavonoids to the bacterial culture system. A novel active HPLC peak was identified and isolated, and its activity in enhancing soybean nodulation was demonstrated under both greenhouse and field conditions (Sections 4 and 5). I have proposed that this activator as a LCO analog that functions by increasing the signal to noise ratio during the signal exchange process. The nodulation dynamics studies showed that coinoculation of PGPB, *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68, with bradyrhizobia, enhanced the soybean nodulation process by causing earlier nodule initiation and higher average nodulation rates than the control (Section 3). Whether the inducible activator actually works in such a way remains to be proven. Besides producing signal-like compounds, PGPB could exert their positive effect on legume nodulation by producing other active metabolites such as antibiotics, chelators and vitamins, etc. In this thesis we have also demonstrated the efficacy of coinoculation of the three NEB strains in promoting soybean plant growth and enhancing soybean nodulation (Section 6 and 7). And their ability to do this may, like *S. proteamaculans* 1-102, be also related to activator compounds they produce.

## 3. The effect of PGPR on the nitrogen fixation

In the legume-rhizobia symbiosis nitrogen fixation happens in bacteroids and is catalyzed by dinitrogenase. Dinitrogenase is sensitive to oxygen and requires a low $pO_2$ environment inside the nodules. Nitrogen fixation is a very energy intensive process and the bacteroid energy substrates are mainly malate and succinate produced by the oxidation of sucrose, the product of plant photosynthesis (Driscoll et al. 1995; Mitsch et al. 1999). Thus, the nitrogen fixation conducted in the nodule depends on the bacteroids and is also affected by the physiological status of the whole plant. PGPB enhance nitrogen fixation by increasing nodule number and nodule weight. The two *Serratia* and the three *Bacillus* NEB strains all increased nodule number and nodule weight under either nitrogen free plant culture conditions in the greenhouse, or under field conditions. This is a general phenomenon in coinoculation of PGPB with rhizobia in legume growth experiments (Iruthayathas et al. 1983; Halverson and Handelsman 1991; Srinivasan et al. 1997). The nitrogen fixation activity, indicated by accumulated nitrogen, is often proportional to both nodule number and nodule weight. However, both total nitrogen content and plant dry matter are more closely related to nodule weight than nodule number (Section 9). Under some circumstances, even though the legume nodule number and nodule weight were not increased, the amount of fixed nitrogen was increased (Groppa et al. 1998). This was due to the improved nitrogen fixation efficiency of the nodules. In this thesis, it was demonstrated that coinoculation of PGPB with *B. japonicum* caused increases in nitrogen fixation that was only partially explained by increases in nodule number and nodule weight. The ratio of fixed nitrogen/nodule weight or fixed nitrogen/nodule nitrogen was greater in the PGPR treatment than in the control. Thus, we concluded that coinoculation of the *Serratia* strains also increased the nitrogen fixation efficiency (Section 3). This may be due to the more dinitrogenase expression or the higher dinitrogenase activity. It had been demonstrated that coinoculation of the tested *Serratia* strains with *B. japonicum* also increased soybean stomatal conductance, transpiration rate and photosynthetic rate (Zhang et al. 1997). These physiological changes, due to coinoculation of PGPB, will allow the plants to increase production of reduced carbon, which could supply root nodules with more energy substrate, allowing

higher levels of dinitrogenase activity. In this thesis, all the conducted experiments were either N-free, as with the greenhouse experimentation, or N-limited, as in the field (Sections 3, 4, 5, 6, 7 and 9). Under such experimental conditions, higher nitrogen fixation activity always resulted in better plant growth. The improved plant growth will lead to a greater N requirement, further stimulating nitrogen fixation efficiency and total nitrogen fixation (Zhang et al. 1997). In the end, no matter how PGPB exert their beneficial effects on the host plants or on the symbiotic bacteria, their effects increase overall legume-rhizobia nitrogen fixation.

## 4. Non-bradyrhizobial endophytic bacteria (NEB) and their effects on soybean growth

Endophytic bacteria have two basic characteristics: 1) they reside inside plant tissues and 2) they have no pathogenic effects on the host plants (Hallmann et al. 1997; Kobayashi and Palumbo 2000). According to this definition symbiotic bradyrhizobia in soybean root nodules are authentic endophytic bacteria, and so they are termed endosymbionts (Perret et al. 2000). Although some NEB isolates were obtained from within surface disinfected soybean nodules, it is not clear that they are specific nodule endophytes. They could also present in other plant tissues. It is also possible that some bacteria could survive the surface sterilization methods used, and the isolated strains could be surviving surface resident contaminants, rather than true NEB. Thus, these NEB can only be considered as putative endophytes (Section 6). It should also be noted that the relationship of these NEB with bradyrhizobia, both in the rhizosphere and within the nodule, is unknown. The work conducted as a part of this thesis resulted in the isolation of 14 bacteria from surface sterilized soybean nodules, three of which showed interesting levels of plant growth promotion. These three were spore-forming Gram positive rods, eventually identified as stains of genus *Bacillus*: *B. subtilis* NEB4 and NEB5 and *B. thuringiensis* NEB17 (Section 6).

*Bacillus* strains are one group of the common plant associated bacteria. They can reside in the phyllosphere and the rhizosphere or within various tissues of many kinds of plants. Some of them, including red clover endophytic strains, have shown plant growth

promoting effects on legume crops (Alagawadi and Gaur1988; Liu and Sinclair 1989,1990; Halverson and Handelsman 1991; Srinivasan et al. 1996). When the three selected NEB strains were inoculated into the soybean plant rhizosphere alone, no nodules were formed on the roots. Although some *Bacillus* species are capable of fixing nitrogen (Dalton 1980), our experimental results showed that none of these three NEB strains were nitrogen fixers, either when they were applied as an inoculant in the soybean rhizosphere or when grown on plates or in flask culture. They can utilize ammonium nitrate ($NH_4NO_3$) as a sole nitrogen source, but grow only weakly when this is the case (Section 6). Thus, at most these NEB strains could be cohabitants with the bradyrhizobial cells inside root nodules. The bacteroids are in the nitrogen-fixing zone (nodule parenchyma), the most inner part of a nodule (Hirsch 1992) and, if the NEB strains are really endophytes, they may occupy these tissues as well, or other tissues such as the nodule cortex, nodule endodermis, or vascular bundles. Sturz et al. (1997) reported that in red clover some bacterial endophytes were isolated only from nodules, i.e., they were nodule specific, while some were isolated from both nodule and root tissues.

In the soybean inoculation experiments, the ability of the NEB to promoting plant growth and enhance soybean nodulation and nitrogen fixation was evaluated under controlled RZT in greenhouse and short season field conditions. All three strains were effective under all the tested conditions. However, the *B. thuringiensis* strain, NEB17, caused larger growth enhancement and was more consistent in causing these positive responses than the two *B. subtilis* strains (Sections 6 and 7). Greenhouse experiments were conducted under nitrogen-free and controlled environment conditions. Field experiments were nitrogen limited. Even in the control plots, in which no NEB was applied, neither disease nor insect damage was observed. Thus, the plant growth promoting efficacy of these NEB strains was not related biocontrol of any disease or insect. These NEB might also produce some kind of active metabolites that promote root growth and benefit nodulation and/or nitrogen fixation like other well know PGPB examples (*Serratia proteamaculans* in Sections 4 and 5; Derylo and Skrupska 1993; Srinivasan et al. 1996; Parmar and Dadarwal 1999). Some strains of *B. thuringiensis* produce paracrytsalline protein bodies that are toxic to specific groups of insects (Pepper 2000). However, in our experiments, the beneficial effect of *B. thuringiensis* NEB17 was

not related to the production of such a toxic protein, as there was not insect pressure on the plants under either field or greenhouse conditions (Sections 6 and 7). Whether the selected NEB strains have potential in biocontrol of diseases or pest insects needs to be investigated. Although these NEB strains were not superior to the two *Serratia* strains (Section 3) in promoting soybean plant nodulation and nitrogen fixation, these NEB strains had the advantage of easier commercial formulation and agricultural application than the *Serratia* strains, because all the three NEB strains are spore-forming bacteria (Liu and Sinclair 1993).

## 5. RNA additives and bradyrhizobial growth and symbiotic activity

Compared with most bacterial groups, rhizobial growth rates are low and their culture times are long. *B. japonicum* is a typical slow growing rhizobial species with a generation time > 6 hours. Whereas fast growing species, such as *Rhizobium spp.*, have generation times < 6 hours. The slow growing trait of *B. japonicum* makes its culture time longer than that of *Rhizobium* spp., not to mention those of many other bacteria. The longer culture time makes the cost of inoculant production higher. Research attempting to improve rhizobial culture practices and lower inoculant production costs has been reported for decades (Strijdom and Allen 1966; Skinner et al. 1977; Balatti et al. 1991; Lie et al. 1992; Kanuma 1997; Grassano et al. 1999). Based on some reported positive effects of nucleosides and related compounds, including RNA, on the growth and metabolite production of other bacteria (Nakao 1979; Schuch et al. 1999; Houde 1999; Garcia et al. 2001), commercial yeast RNA was added to the culture medium to test the possibility that it might accelerate bradyrhizobial growth. Addition of RNA from 0.05 – 0.20 g/l to the culture medium increased the growth rate of *B. japonicum* 532C by 48 – 103% and shortened the culture time by 21 - 41%. This positive effect of RNA additives on cell growth was also verified in other two *B. japonicum* strains, USDA3 and USDA110 (Section 8).

LCOs are oligosaccharide compounds modified by attached side groups. LCO biosynthesis requires the coordinated operation of many nodulation genes (*nod*, *nol* and *noe* genes)(Perret et al. 2000). After synthesis, LCOs are secreted from the cells. The

*nodI* and *nodJ* genes play a role in the secretion of LCOs (Spaink 2000). We suppose that bradyrhizobial cells are able to synthesize LCOs at consistent levels only when their overall metabolism is functioning normally. Thus, as a part of the evaluation of RNA effects on bacterial physiology, we compared LCO production between the control and the treatments. RNA additives had no negative effect on LCO production by the cells and the LCO yield per volume of culture medium was increased by 65 – 76% (Section 8).

The mechanism(s) by which added RNA increases bradyrhizobial growth remain unknown. The RNA additives may have added to the positive effects of yeast extract on bacterial growth, as yeast extract contains nucleoside derivatives, but adding pure RNA removed the potential negative effects of high amino acid levels that would have occurred if RNA levels were increased by adding additional yeast extract (Date 1972; Skinner et al. 1977). Commercial RNA may be a mixture of different types of nucleoside relatives. Whether only some specific polymer molecules or the monomer are necessary for RNA stimulation of rhizobial growth, or the whole mixture is required can only be determined through additional experimentation.

## 6. Possible application of RNA additives in the inoculant production

*B. japonicum* is the rhizobial species used in all widely produced soybean inoculants. Any improvement in inoculant culture technology should at least maintain bacterial symbiotic competency while the growth rate is increased. Bradyrhizobial cells begin to produce LCO after they receive plant flavonoid signals in the host plant rhizosphere. Only after sufficient LCO is produced is the signal exchange loop closed. My results indicate that RNA additives have no negative effect on LCO production by bradyrhizobial cells (Section 8), which suggests that the bacterial symbiotic competency is not reduced by RNA additives in the culture medium. This was demonstrated through inoculation experiments in different plant culture systems under greenhouse conditions (Section 9). The inoculants cultured with addition of RNA showed the same nodulation capability in the early nodulation stages (within 20 days after inoculation). At the latter stages (22 – 55 days after inoculation), they showed an ability to increase nodule number and nodule weight. Addition of RNA at 0.2 g/l and a culture time of 8 days was the best

condition for inoculant production. Compared with the 8-day control culture, such an inoculant affected nodule number by - 4.7 to 18.8%, nodule weight by 15.2 and 76.2% and plant weight 14.3 and 44.7% in pot-Vermiculite and growth-pouch culture systems, respectively. Compared with the 12-day control culture, the inoculant cultured under the best conditions increased nodule number by 15.2 and 76.2%, nodule weight by 15.8 and 57.6% and plant weight by 20.6 and 13.2%, respectively, in pot and pouch culture systems (Section 9). All these results support the applicability of RNA additives in the bradyrhizobial inoculant production.

In order to reveal whether the RNA additives themselves have direct positive effects on legume nodulation, RNA was also added into the control cultures just prior to their inoculation onto soybean plant roots. The results showed that addition of RNA in this way had negative effects on nodulation. They mainly reduced nodule development, resulting in decreased nodule weight and, subsequently, less plant weight (Section 9). Based on these results I concluded that the improved symbiotic activity of inoculants cultured in YEM medium modified by addition of RNA was due to improvement of bacterial physiological activities during the culture process, with no positive direct effect of the RNA additives on the plants, or the interactions between the host and the bradyrhizobial cells. We expected that the application of this method to industrial inoculant production would lower the inoculant cost, by shortening the time required, and lead to the production of a better quality product with normal LCO production potential and improved symbiotic competency.

While the PGPB data suggest that the growth enhancement provided by these organisms (Section 4) is from LCO like compounds, the improved symbiotic competency of *B. japonicum* cells cultured in the presence of RNA additions does not appear to be related to LCO production, or at least not during the culture phase, as LCO production/cells was not altered by RNA additions (Section 8).

## 7. Prospects for enhancement of the legume-rhizobia nitrogen fixation

Biological nitrogen fixation provides 65% of the total annual fixed nitrogen in the world and it is considered to be a major sustainer of life on earth (Newton 1999). Optimization

of the nitrogen fixing legume-rhizobia symbiosis will be one of the major goals of research on biological nitrogen fixation. In addition to genetic improvement of crop cultivars and bacterial strains, the work conducted in this thesis shows two potential technologies able to promote the use of the legume-rhizobia symbiosis: the use of PGPB in the inoculants and RNA additives in the culture medium.

Coinoculation of PGPB with rhizobia, or so-called double inoculants, is still considered to be a new technology in inoculation of diazotrophs (Martinez-Drets and Castro 1999). For example, in tropical areas most legume species are highly dependent on mycorrhizal associations. Double inoculation of rhizobia and arbuscular mycorrhizal fungi increased the shoot dry weight of *Anadenanthera peregrina* by 4 – 5 times, compared to rhizobial mono-inoculation (Franco et al. 1999). It is reported that coinoculation of some PGPB strains with *B. japonicum* strain C145 could increase soybean dry matter production by 53 to 108% at 50 days after planting (Olmedo and Thuar 1999). The results presented in this thesis are not as good as these two examples, indicating that there may be considerable additional potential in using dual inoculant technology in legume agricultural production.

The higher inoculant cost due to the slow growth of bradyrhizobia in culture has been a difficult problem for the inoculant industry. Besides improving the culture technology, in the past few decades a great deal of effort has been directed at finding fast growing species that can nodulate soybean and eventually replace the slow growing *B. japonicum* in soybean production. In Hohghu County, China, a total of thirty-three fast growing isolates were isolated. These fast growing species are all belong to genus *Sinorhizobium*, for example *S. fredii* and *S. xinjiangensis* (Camacho et al. 1999). There have been no reports as to their efficacy in legume production systems.

However, follow up work recently conducted in our laboratory has shown that addition of RNA to YEM also increases the growth rate of the fast growing rhizobial species, including *Rhizobium* NGR234, *R. leguminosarum* 127K105 and *S. meliloti* 1021. Thus, addition of RNA to culture medium as an improvement in culture technology is possible for fast growing rhizobia as well.

It had been reported that genistein pre-incubation of bradyrhizobia and the two *Serratia* PGPB strains showed additive effects on soybean nodulation and nitrogen

fixation under 25 and 17.5°C RZT (Dashti et al. 2000). As to the application of the two techniques presented in this thesis, they could have an additive effect on soybean nitrogen fixation and growth when PGPB strains are coinoculated with bradyrhizobial cells produced in culture medium with RNA additives. This possibility would seem to be especially high for the NEB strains as they appear to be the natural coinhabitants, along with *B. japonicum,* of soybean nodule tissues. In addition, the NEB may well work through production of LCO type compounds, while the increased symbiotic competency of rhizobial cells cultured in the presence of RNA appears not to be due to increased LCO production. Thus, these two technologies seem to operate through different mechanisms, increasing the likely hood that their effects could be additive. If their additive effect is proven through greenhouse and field experiments, the dual inoculant will have much higher symbiotic competency and without any substantial increase in production cost. The shortened bradyrhizobial culture time will off set at least some of the extra cost associated with the culture of NEB strains.

# Section 11. CONCLUSIONS

Based on the research results in this thesis, the following may be concluded:

1. The effect of the two *Serratia* strains on soybean nodulation and growth varies with coinoculation dose. Their optimal inoculation dose was $10^8$ cells/plant under both optimal and suboptimal RZT conditions.
2. Soybean nodulation dynamics, under greenhouse conditions, could be described by a linear regression model. At 25 and 20 °C RZTs, soybean nodulation could be described by the same model, but a different model was required at 15 °C. At 15 °C RZT, nodule initiation was delayed and the nodulation rate was reduced.
3. Besides increasing nodule number and weight, via earlier nodule initiation and more rapid nodulation than the control, coinoculation of *B. japonicum* with *Serratia* also improves nodule nitrogen fixation efficiency.
4. *S. proteamaculans* 1-102 produces an inducible activator, following addition of flavonoids to its culture system. The purified activator promotes soybean nodulation, nitrogen fixation and plant growth to the same degree as the bacterial cultures, under both greenhouse and field conditions. This implies that this *Serratia* strain exerted its positive effect on soybean plant growth via production of the activator, as a response to a chemical stimulus (flavonoid) from the host plants.
5. The evidence presented here supports the possibility that the inducible activator produced by *S. proteamaculance* 1-102 is a LCO analog. Both the activator and LCO were produced only after flavonoid inducers were applied. They were isolated following a similar HPLC program and were detected at the same UV wavelength during HPLC purification. They have similar biological activities in that they both stimulate soybean seed germination and promote soybean growth and nodulation. This activator may function by increasing the signal-noise ratio during signal exchange between the host plant and bradyrhizobial cells.
6. The effect of the *S. proteamaculance* 1-102 produced inducible activator on soybean growth is dose dependent. Its optimal dose was the same for both root and leaf applications. When applied by leaf spaying, its effect was altered by the application

time, i.e. the plants showed different responses to the activator at different developmental stages.

7. Bacterial species other than bradyrhizobia are also present within the tissues of soybean root nodules, i.e. as endophytes. Most of these NEB have no deleterious effect on soybean nodulation, nitrogen fixation or growth, and some of them have positive effects.
8. The three most strongly growth promoting NEB strains, NEB4, NEB5 and NEB17, isolated by me were all Gram positive, spore-forming rods. They were neither root nodule inducers nor symbiotic or free-living nitrogen fixers. They can use ammonium nitrate as a nitrogen source, but only poorly. Biolog tests and partial 16S rRNA gene sequence analyses identified them all as *Bacillus* species: NEB4 and NEB5 are *B. subtilis* strains and NEB17 a *B. thuringiensis* strain.
9. When coinoculated with *B. japonicum*, all the three selected NEB strains enhance soybean nodulation, nitrogen fixation and growth under both optimal and suboptimal RZT conditions in the greenhouse, as well as under short season field conditions. Among them, *B. thuringiensis* NEB17 provided the largest and most consistent growth promotion. In the experiments reported here, there was no evidence that this growth promotion was due to biocontrol of any disease or insect pest.
10. *Bradyrhizobium japonicum* strains are slow growing rhizobia. Addition of RNA to YEM culture medium accelerates the bacterial growth rate and greatly shortens the bacterial culture time. The positive effect of RNA addition on bacterial growth could not be duplicated by increasing yeast extract concentration in the culture medium. The RNA additives also increase the bacterial LCO yield and improved the bacterial symbiotic competency. These primary results indicate the potential for adopting RNA additives in the industrial preparation of bradyrhizobial inoculant.

## Section 12. CONTRIBUTIONS TO KNOWLEDGE

The work reported in this thesis has focused on the interactions between *B. japonicum,* soybean plants and plant growth promoting bacteria (PGPB), with the overall objective of enhancing soybean nodulation, nitrogen fixation and, ultimately, growth. The following are considered to be the original contributions to the basic knowledge resulting from this research effort.

1. The efficacy of either the plant growth promoting bacteria or the active metabolite of *S. proteamaculance* 1-102 are dose dependent, and I have shown the optimum doses in the work presented in this thesis.
2. Coinoculation of plant growth promoting bacteria with *B. japonicum* enhances the soybean nodulation process by causing earlier nodule initiation and more rapid subsequent nodule formation. I have demonstrated this to be the case with two bacterial strains from the genus *Serratia.*
3. Higher nodule number and higher nodule weight generally lead to higher levels of nitrogen fixation and plant growth. Nodule weight is more closely related to nitrogen fixation activity than nodule number. However, besides increasing nodule number and nodule weight, coinoculation of PGPB can enhance nitrogen fixation by improving the nitrogen fixation efficiency of the root nodules.
4. One of the mechanisms by which the PGPB enhance soybean nodulation and nitrogen fixation is production of metabolites that positively affect the signal exchange between the host plants and bradyrhizobial cells. Some times this activator is produced based on interactions between the PGPB cells and the soybean plants, i.e. the compounds produced by the plant stimulate the PGPB to produce the activator. The production of a specific activator, and its effects on plant growth were demonstrated in this thesis.
5. In soybean root nodules, there are some NEBs that are effective in enhancing soybean nodulation and nitrogen fixation. However, they are neither nodule inducers nor nitrogen fixers. They exert their effects when coinoculated with *B. japonicum.*

During the course of the work conducted for this thesis a novel set of these NEBs was isolated and three were shown to be PGPBs.

6. Addition of RNA to the bradyrhizobial culture medium increases the bacterial growth rate and LCO yield, and shortens the culture time. The positive effect of the RNA additives is not replaceable by increasing yeast extract concentration in the culture medium.
7. Addition of RNA to the bradyrhizobial culture medium improves bacterial symbiotic competency, leading to faster nodulation and the formation of more nodules and more nodule mass. This is through an effect of the RNA on the bradyrhizobia and not a direct effect of the RNA on the plant.

## Section 13. SUGGESTIONS FOR FUTURE RESEARCH

The work presented in this thesis is in three parts: 1) investigations into the mechanisms of plant growth promotion by two *Serratia* strains, 2) isolation, selection and identification of NEB isolates as plant growth promoting bacteria, and 3) improvement in the culture technology of bradyrhizobial inoculants via modifying the bacterial culture medium. For each of these three parts, there are some very interesting lines of research that could be further pursued.

1. **Prepare enough of the inducible activator produced by *S. proteamaculans* 1-102 for its structure to be identified.** This will offer direct evidence as to whether or not it is an LCO analog, as we have postulated. Knowledge of the activator's structure, whether it is an LCO analog or not, will provide some information regarding the PGPB's mechanism of action.
2. **Determine the activator responsible for the promotion of soybean nodulation, nitrogen fixation and growth by *B. thuringiensis* NEB17.** It has been demonstrated that this strain had the greatest and most stable plant growth promoting effect of the NEB strains evaluated, under both greenhouse and field conditions. NEB17 may produce some kind of active compound that has positive effects on the host plants, the bradyrhizobial cells or both.
3. **Test the effects of higher RNA addition levels on bradyrhizobial growth rate and symbiotic activity.** In this thesis only 0.05, 0.10 and 0.20 g/l were tested. However the experimental results, especially in the inoculation experiment, indicated that the highest concentration tested resulted in the greatest promotion of bacterial and plant growth. Thus, higher concentrations may well result in further stimulation of growth by both bradyrhizobia and plants inoculated with them.
4. **Verify the positive effect of RNA addition to the culture medium on a wider range of *Bradyrhizobium, Sinorhizobium* and *Rhizobium* strains.** In this work only 3 bradyrhizobial strains were used in bacterial growth investigations and only one strain was used to study effects on the physiological and symbiotic activities. It is

possible that the conclusions for this small group of strains are also applicable to most or all rhizobia species and strains.

5. **Determine the mechanisms by which the RNA addition exerts its positive effect on bradyrhizobial growth rate, physiological activity and symbiotic competency.** RNA additives may be used as a nutrient substrate or some kind of physiological active compound. Commercial yeast RNA is a mixture of poly- or oligo-ribonucleotides and even some monomers of nucleosides and nucleotides. It is possible only some specific, and not all, components are responsible for the detected positive effects. Work should be done to test all of the possible forms of the RNA, when added separately or in combination, to rhizobial cultures.
6. **Test the combined effect of coinoculation with PGPB and RNA addition to a bradyrhizobial culture medium on soybean nodulation, nitrogen fixation, and growth.** The effectiveness of both ways of enhancing nodulation and nitrogen fixation were proven separately in this thesis. If these two factors were combined, their effects may be additive, resulting in a larger overall enhancement of nodulation, nitrogen fixation and growth than either of these methods alone, leading to a further increase in the utility of the nitrogen fixing symbiosis in agricultural systems.

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