PLANT GROWTH PROMOTING RHIZOBACTERIA AND SOYBEAN NODULATION, AND NITROGEN FIXATION UNDER SUBOPTIMAL ROOT ZONE TEMPERATURES

by

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Short title

PLANT GROWTH PROMOTING RHIZOBACTERIA AND SOYBEAN

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DEDICATION

To my mother, Masooma Ahmed and my late father, Haje Ali Dashti

ABSTRACT

Ph. D. Narjes Dashti Plant Science

Soybean [*Glycine max* (L.) Merr.] is a subtropical legume that requires root zone temperatures (RZTs) in the 25 to 30°C range for optimal symbiotic activity. The inability of soybean to adapt to cool soil conditions limits its development and yield in short season areas. In particular, nodulation and N_2 fixation by this subtropical crop species is sensitive to cool (RZT). The objectives of this thesis were to determine whether or not PGPR could be used to help overcome the low RZT inhibition of soybean nodulation, to improve soybean nitrogen fixation and yield under field conditions and to determine the methods by which such increases occurred. The work reported in this thesis has demonstrated that PGPR can increase early season nodulation and total seasonal nitrogen fixation and yield of soybean growing in an area with cool spring soils. The ability of PGPR to stimulate soybean nodulation and growth was shown to be related to their ability to colonize soybean roots, and this was shown to be related to RZT. All steps in early nodulation were stimulated by the presence of PGPR. The beneficial effects of PGPR are exerted through a diffusible molecule excreted into the growth medium. The addition of genistein, a plant-to-bacteria signal molecule already shown to stimulate soybean N_2 fixation at low RZT, plus PGPR causes increases in soybean nodulation, N_2 fixation, and growth that were greater than those caused by the addition of PGPR alone, but only at 25 and 17.5°C, and not at 15°C RZT.

Résumé

Ph. D. Narjes Dashti Phytotechnie

Le soja [*Glycine max* (L.) Merr.] est une légumineuse sous-tropicale qui a besoin d´une température racinaire (TR) de 25 à 30°C pour pouvoir établir une activité symbiotique optimale. L´incapacité d´adaptation du soja aux basses températures édaphiques limite son développement et son rendement dans les régions de courte saison. La nodulation et la fixation azotée de cette plante sont particulièrement sensibles aux basses TR. Les objectifs de cette thèse étaient de déterminer si les PGPR pouvaient aider à surmonter l´inhibition de la nodulation par les basses TR, d´améliorer la fixation azotée et le rendement aux champs et de déterminer les moyens par lesquels de telles améliorations ont lieu. Le travail rapporté dans cette thèse a montré que dans les régions où les TR sont basses au printemps, les PGPR peuvent augmenter la nodulation en début de saison, la fixation azotée totale durant la saison et le rendement du soja. La capacité des PGPR de stimuler la nodulation et la croissance du soja est reliée à leur capacité de coloniser les racines, phénomène lui-même relié aux TR. Tous les stades du début de nodulation étaient stimulés par la présence des PGPR. Les effets bénéfiques des PGPR sont dûs à une molécule qu´ils excrètent et qui diffuse dans le milieu de croissance. L´addition de la genistein (molécule-signal de la plante à la bactérie qui stimule la fixation azotée à basses TR) aux PGPR a causé une amélioration de la nodulation du soja, de la fixation azotée et de la croissance supérieure à celle due à l´addition des PGPR seules, mais seulement à 17,5 et 25°C et non à 15°C.

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CONTRIBUTIONS OF CO-AUTHORS TO MANUSCRIPTS FOR PUBLICATION

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

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

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

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

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

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

Thus, the contents of sections 3 to 9 inclusively are drawn, respectively, from the manuscripts for publication. The manuscripts from which sections 5, 6, 7, 8 and 9 are taken were co-authored by myself, R. K.Hynes and D. L. Smith. Dr. D. L. Smith, my supervisor at the Macdonald Campus of McGill University, provided assistance and supervisory guidance from the outset of the research to the reviewing of the manuscripts before submission for publication. He was also responsible for arranging laboratory, growth bench, and experimental field spaces, and the use of computers, and providing technical assistance.
R. K. Hynes is a researcher at Comino Fertilizer Co. provided expertise in plant growth promoting rhizobacteria, the strains of plant growth promoting rhizobacteria, and reviewed the manuscripts before submission for publication.
The manuscript from which section 3 and 4 were taken were also co-authored by F. Zhang. F. Zhang was a PhD student in Dr. Smith's laboratory. He assisted in establishing the field experiment, data analysis and editing manuscripts.

Section 1

GENERAL INTRODUCTION

1.1. Introduction

Soybean [*Glycine max* (L.) Merr.] is a widely produced N_2-fixing crop, and is a major source of protein and oil for agro-industry world wide. Soybean plants, by forming a symbiosis with the bacterium *Bradyrhizobium japonicum*, can fix up to 200 kg ha^{-1} of atmospheric nitrogen per year. The importance of soybean as a crop is increasing in eastern Canada. In 1993 soybean was tied as the most widely produced crop in Ontario, at 676,000 ha. In Quebec, soybean hectarage has increased from 3,000 in 1989 to approximately 100,000 in 1996. Current Quebec soybean production provides less than 10% of the $200 million local market demand. Soybean production in the maritime provinces has begun over the last five years and the annual hectarage is expanding rapidly.

As a legume, soybean is capable of meeting much of its nitrogen requirement from symbiotic nitrogen fixation and it is of economic importance that they should do so efficiently. Restrictions upon improved soybean yield have been attributed to a decline in N_2 fixation at seed filling due to within plant competition for available photoassimilate (Sinclair and deWit, 1976) or prolonged seedling "N-hunger" prior to the onset of fixation. The production of N fertilizer is economically ($1 billion yr^{-1} in Canada), energetically (equivalent to 30 million barrels of oil) and environmentally (produces 15 million t. of CO_2, ground water polluting-NO_3 and ozone-destroying NO_x) expensive. In eastern Canada the farm community spends $150 million per year for N fertilizer. Nitrogen fixation is the sustainable alternative to N fertilizer.

Recently the cultivation of soybean has been extended into cool temperate areas where soil temperatures, in comparison with those of its natural habitat, are low during the first part of the growing season. Under such conditions root zone temperature (RZT) during the early growth period may be a limiting factor in the growth of the plant. Soybean originated in the subtropics and its nodule development is restricted by the soil temperatures commonly encountered in the early eastern Canadian growing season (Zhang et al., 1995a).

Studies on the effect of sub-optimal RZT on nitrogen fixation by soybean and other subtropical legume crops have indicated that low RZT decease both nodulation and nodule function (Jones and Tisdale 1921; Hardy et al., 1968; Roughley and Date 1986). Matthews and Hayes (1982) showed that an RZT of 10°C range resulted in decreased nodule growth and a four-fold decrease in the total nitrogen fixation per plant when compared to 25°C. This was attributed to the inhibition of infection and nodule initiation by *B. japonicum*. In a recent study by Lynch and Smith (1993) it was observed that a RZT of 15°C severely restricted both infection and nodule development, and delayed the onset of nitrogen fixation until approximately 7 to 8 weeks after inoculation.

As soybean is a subtropical legume RZTs in the 25-30°C range are optimal for symbiotic activity compared to 20-24°C for temperate legumes (Jones and Tisdale, 1921). It is N and not C availability that limits growth at low root zone temperatures (Thomas and Sprent, 1984; Vance and Heich, 1991). In short season areas, the poor adaptability of soybean to cool soils is considered the primary factor limiting yield (Whigham and Minor, 1978).

Over the last decade the understanding of rhizosphere biology has progressed with the discovery of a group of microorganisms, termed plant growth promoting rhizobacteria (PGPR). These PGPR are capable of colonizing the plant root and promote plant growth (Kloepper et al., 1980a). The PGPR *Pseudomonas putida* GR12-2, originally isolated from the rhizosphere of plants growing in the Canadian high arctic (Lifshitz et al., 1986), can both survive the cold Canadian winter and proliferate in the cool spring soil (Hong et al., 1991). The ability to persist and replicate under conditions that are deleterious for most other bacteria could be an important component of the PGPR activity. Therefore, removal of the low RZT restriction on soybean nodulation by PGPR inoculation would allow increased use of this N_2-fixing cash crop, and decreased reliance on potentially polluting N fertilizers.

HYPOTHESES

Based on the information described above, the purpose of this thesis was to improve nitrogen fixation by the soybean-*Bradyrhizobium* symbiosis through the application of PGPR in order to reduce the negative effects of suboptimal RZTs on the soybean N_2 fixation symbiosis under the cool spring conditions prevalent in much of the Canadian soybean production regions. Therefore, it is hypothesized that:

1) PGPR accelerate nodulation, leading to increased nitrogen fixation and improved yield by soybean in areas with cool spring temperatures.

2) The ability of PGPR to survive, grow and multiply in the field under short-season conditions is related to their ability to stimulate soybean nitrogen fixation, growth and yield under field conditions.

3) The ability of PGPR to colonize soybean roots is related to their ability to stimulate soybean N_2 fixation and growth, and this is affected by RZT.

4) Early stages of soybean nodulation (root hair curling, infection thread initiation, infection thread 1/2 way down the root hair, and infection thread reaching the base of the plant root hairs) are affected by PGPR.

5) Plant growth promoting rhizobacteria enhance nodule development of soybean at low RZT through production of diffusible compound(s).

6) Addition of genistein plus PGPR will stimulate soybean nodulation, N_2 fixation and growth more than the addition of PGPR alone.

OBJECTIVES

Following the hypotheses, several objectives were defined. The objectives were:

1. To determine whether PGPR can stimulate soybean nodulation and nitrogen fixation, leading to improved yields, in an area with cool soil temperatures in the spring.

2. To determine the role of PGPR colonization of soybean roots on their ability to stimulate soybean nodulation, N_2 fixation and growth.

3. To determine the effects of RZT on PGPR colonization of soybean roots.

4. To determine the effects of PGPR on the early stages of soybean nodulation over a range of RZT.

5. To determine whether the PGPR effect is due to a diffusible compound.

6. To determine whether the stimulatory effects of PGPR plus genistein are greater than the stimulatory effects of PGPR alone.

Section 2

LITERATURE REVIEW

2.1. THE SOYBEAN CROP

Soybean [*Glycine max* (L.) Merr.] is the world's widely-produced nitrogen (N) fixing crop. By forming a symbiosis with *Bradyrhizobium japonicum*, soybean plants are able to fix 100-200 kg ha^{-1} yr^{-1} of atmospheric N (Smith and Hume, 1987). Numerous studies have demonstrated significant contributions of atmospheric N_2 fixation to soybean nutrition and growth (Weber, 1966; Deibert et al., 1979; Rennie et al., 1982). Most estimates show that soybean derives between 25 and 75% of its N from fixation (Deibert et al., 1979).

Soybean belongs to the family Leguminosae, subfamily Papilionideae, The genus *Glycine* has trifolilated leaves, the flowers are inserted singly at each node of the raceme, a five-toothed calyx with the upper pair of teeth not well united, a glabrous corolla with long clawed petals, a keel which in shorter than the wings, and seed (Hymowitz and Newell, 1981). There are two distinct forms of stem growth habit and floral initiation in soybean. One form is the indeterminate stem, in which the terminal bud continues vegetative activity during most of the growing season. The other form is the determinate stem, in which the vegetative activity of the terminal bud ceases when it becomes an inflorescence.

The main four producers of soybean in the world are USA, Brazil, China, and Argenentina which together make up 90 to 95% of the world's production. The importance of soybean as a crop is increasing in eastern Canada. In 1993, soybean was the most widely produced crop in Ontario, at 676,000 ha. Quebec produces about 35,000 t of soybean protein per year, but imports, from the U.S. and Ontario, some 650,000 t of protein meal, mostly soybean, the bulk of which supplies the dairy industry.

The two major products of soybean are oil and protein. Soybean oil accounts for 20-30% of the total fat and oil production, and 30 to 35% of the total edible vegetable oil production in the U. S. (Smith and Huyser, 1987). Since 1970, soybean oil production has doubled compared to other oilseed crops, such as rapeseed (*Brassica napus* L.) (Johntson, 1987), peanut (*Arachis hypogaea* L.), cottonseed, and sunflower (*Helianthus annuus* L.). The world soybean oil production has increased from 32% in 1965 to over 50% in the 1980s. The other important product of soybean is protein. Soybean meal plays a key role as a protein ingredient in feeds in the USA. Poultry feeds are the largest outlet for meal followed by swine feeds; the two account for 78% of total usage. Soybean meal has been estimated to make up more than 90% of the oilseed meals consumed in poultry feeds. Smaller quantities of soybean meals are fed to beef and dairy cattle (*Bos* spp.). Production of edible protein products for direct human consumption is small as compared to soybean meal for feed uses (Mount et al., 1987). Soybean cultivars grown in the United States generally contain 20-30% oil and 39-45% protein (Hymowitz and Singh, 1987).

Soybean requires at least 2400 Corn Heat Units (Brown, 1981) in one growing season. Canada is at the northern limit of soybean growth range, but in North America, Canadian soybean production has risen sharply in recent years. Temperature is usually the limiting climatic factor in short growing season areas, area such as Canada. Soybean offers a number of advantages over other crops, e.g. its ability to fix N_2, its low phosphorus requirements, its tolerance to low pH and high levels of Al, and its tolerance to high soil moisture contents (Tanaka, 1985). The capacity of soybean to fix N_2 is affected by ecological factors such as temperature, macronutrients, micronutrients, and water regime.

2.2. EFFECT OF LOW RZTs ON SOYBEAN NODULATION AND NITROGEN FIXATION

As a subtropical legume, soybean requires a temperature in a range from 25 to 30°C for optimal symbiotic activity. When RZT drops below this range, legume nodulation and nitrogen fixation are negatively affected. Data from McGill has shown that nitrogen fixing soybean yielded 34% less (910 kg ha^{-1}) than soybean that was heavily fertilized with N (200 kg ha^{-1}) in a year with a cool spring, while there were no differences between the yields of N_2-fixing and N-fertilized soybean when spring weather was warm. At current soybean prices this translates into about $30 million for the Maritimes and Quebec and cooler Ontario soybean production regions. In most of its Canadian production areas soybean is planted into cool soils. This situation is likely to worsen as agronomic data indicates that, with the addition of N fertilizer, earlier seeding increases the yield potential of soybean in these areas. Under eastern Canadian conditions soybean can fix 100-200 kg N ha^{-1} (Smith and Hume, 1987).

Lie (1974) noted that all stages of nodule formation and functioning are affected at low RZT. In a review of the data on environmental effects on the legume-*Rhizobium* symbiosis, Gibson (1971) suggested that low RZTs retard root growth infection more than nodule initiation, nodule development, or N assimilation.

Studies of the effects of sub-optimal RZTs on soybean have shown that these conditions decrease N_2 fixation activity by the nitrogenase enzyme complex (Layzell et al., 1984) and suppress and/or delay root infection and nodulation (Walsh and Layzell, 1986). The effects of low temperature on the function of N_2-fixing nodules may be, in part, due to changes in nodule O_2 permeability (Sinclair and Weisz, 1985; Weisz and Sinclair, 1988). Soybean plants export the N_2 fixed in the nodule mainly in the form of ureide. The solubility of ureide is low and decreases sharply as temperature declines. Therefore low RZTs may also limit the rate of export of fixed N from the nodule (Sprent, 1982). Decreased temperature resulted in progressively less bacteroid tissue (Lie, 1974) and a decrease in its formation rate (Fyson and Sprent, 1982). The effect of low RZT on temperate zone legumes has been investigated. Low RZT decreases both nodulation and N_2 fixation rates (Jones and Tisdale, 1921; Hardy et al., 1968; Roughley and Dart, 1970; Dart and Day, 1971; Waughman, 1977), affecting all stages of nodule formation and function (Lie, 1974). Root hair infection of *Trifolium subterraneum* is more sensitive to low RZT than nodule development or nitrogen assimilation (Gibson, 1971). Lower RZTs decrease nodule growth and total nitrogen fixed per plant by inhibiting infection and nodule initiation (Matthews and Hayes, 1982). These RZTs primarily retard root infection (Lindemann and Ham, 1979).

Work to date in our laboratory has shown that: 1. N_2-fixing soybean plants are more susceptible to low RZT than NO_3-fed plants (Legros and Smith, 1994), 2. the time between soybean inoculation with *B. japonicum* and the beginning of nitrogen fixation increases by 2-2.5 days for every degree between 25 and 17°C (Zhang et al., 1995a), 3. below 17°C the time from inoculation to nitrogen fixation is delayed by a week per degree (Zhang et al., 1994), 4. the greater sensitivity below 17°C is due to an event that occurs within the first 12 h after inoculation, root hair curling, at 25°C RZT (Lynch and Smith, 1994; Zhang and Smith, 1994), 5. the greater inhibition by temperatures below 17°C is due to an inability of the plant to either synthesize or excrete the plant-to-bacterium isoflavone signal molecule (4',5,7-trihydroxyisoflavone, or genistein) at the beginning of symbiosis establishment (Zhang and Smith, 1995b), and most recently, 6. co-inoculation of soybean with *B. japonicum* and some plant growth promoting rhizobacteria (PGPR) strains shortens the time between inoculation and the onset of nitrogen fixation at all temperatures tested, but with a greater degree of shortening (up to 7 days) occurring at lower RZT (Zhang et al., 1996b). Slow nodule development in cool soils prolongs the period of nitrogen deficiency that occurs between the depletion of cotyledonary nitrogen reserves and the beginning of nitrogen fixation. A period of slow growth early on is reflected in growth throughout the remainder of the season. During cool springs the delay in nodulation can be substantial. For instance, during 1992, an unusually cool year, no nodules were observed on field grown soybean plants until July.

Soybean needs qualitatively the same nutrients as other green plants both on macro-scale and micro-scale. Soybean plants need additional Mo and Fe to make nitrogenase and haemoglobin. Combined nitrogen (NO_3^-, NH_4^+, and urea) has been demonstrated to influence symbiotic nitrogen fixation from the initial bidirectional signal exchange between symbionts through to nodule senescence. At nitrate concentrations greater than perhaps 2 mM, both nodule growth and nodule activity are depressed (Streeter, 1981; Eardly et al., 1984).

2.3. OTHER FACTORS AFFECTING SOYBEAN NODULATION AND NITROGEN FIXATION

Water supply also plays an important role in both nodulation and nitrogen fixation. Nodulation increases as the soil water content increases until the soil is water saturated. Water stress negatively affects the number of infection threads formed and inhibits nodulation. As the plants are rewatered, the effects on infection are reversible and immature hairs resume normal growth and become infected. Following successful infection, reduced water supply can prevent nodule development (Sprent and Sprent, 1990). The decrease in the water potential in the nodules may also reduce the nitrogen fixing activity (Pankhurst and Sprent, 1976; Weisz et al., 1985), with a strong reduction in nodule respiration (Pankhurst and Sprent, 1976), accompanied by a reduction in transport of fixed nitrogen out of nodules, and impairment of photosynthate supply from a stressed shoot system (Huang et al., 1975).

Inhibition of nodulation by nitrate has been well documented for over 100 years, but little is known about the mechanisms involved. A study by Darbyshire (1966) indicated that greater delays in nodule formation were caused by nitrate than by either ammonium or urea. Nitrate concentrations higher than 2 mM inhibit both nodule growth and nodule activity (Streeter, 1981; Eardly et al., 1984). A wide range of infection events are affected by nitrate such as, decreased root hair deformation, decreased binding of rhizobia to root hairs, decreased number of infection threads, and increased number of aborted infection events (Streeter, 1988). With long-term exposure of plants to 4 to 8 mM nitrate, effects of nitrate on nodule growth are higher than effects of nitrate on specific nitrogenase activity (Streeter, 1981). Field trials have also shown much greater effects on nodule growth than on specific nodule activity (Miller et al., 1982). A study by Waterer and Vessey (1993) demonstrated that inhibition of symbiotic nitrogen fixation by the presence of mineral nitrogen in the soil is due to a shortage of carbohydrates and or reducing power necessary for the competing metabolic processes of NO_3^- assimilation and symbiotic nitrogen fixation. Recent work by Cho and Harper (1991a,b) and in our laboratory has shown that mineral nitrogen decreases the production of plant-to bacteria signal molecules by soybean roots.

Low pH results in reduction of the growth and multiplication of rhizobia in the soil (Rice et al., 1977), increases in the number of ineffective rhizobia and inhibition of the infection process (Date, 1988). Acid soil may be low in available calcium, magnesium, phosphorus, and molybdenum and contain levels of aluminium and manganese toxic to the host plant (Sprent and Sprent, 1990).

High saline concentrations reduce the water nodule activity as water is withdrawn osmotically from nodules. Bacterial colonization and root hair curling of legume plants maintained at 100 mol m^{-3} NaCl are both reduced when compared to those plants grown at 50 mol m^{-3} conditions. Also, the proportion of root hairs containing infection threads is reduced by about 30% (Zahran and Sprent, 1986).

2.4. PLANT GROWTH PROMOTING RHIZOBACTERIA

Understanding of the rhizosphere biology has progressed with the discovery of a specific group of microorganisms, now called plant growth promoting rhizobacteria (PGPR), that can colonize plant roots and stimulate plant growth and development (Kloepper et al., 1980a). Most of the identified strains of rhizobateria occur within gram-negative genera, of which fluorescent pseudomonads are most characterized, although some strains of *Serratia* have been reported (Kloepper et al., 1986, 1991; Ordentlich et al., 1987). Several gram positive strains of root-colonizing bacteria were reported such as an *Arthrobacter*-like genus (Kloepper et al., 1990) and *Bacillus* (Backman and Turner, 1989; Turner and Backman, 1991). Other documented PGPR, include *Azotobacter* species, *Azospirillum* species, and *Acetobacter* species (Brown, 1974, Elmerich, 1984; Bashan and Levanony, 1990; Tang, 1994).

Several reports related the beneficial effects of PGPR to direct plant growth promotion, disease suppression, associative N_2 fixation and improved access to soil nutrients. Nitrogen fixation promoting rhizobacteria are identified as PGPR that are capable of increasing nodulation as well as plant growth. A different approach was taken recently (Kloepper et al., 1986; Kloepper et al., 1988b) when over 10, 000 bacterial strains were randomly isolated from plant rhizospheres at different locations. After examining their ability to growth at low temperatures, they were tested under greenhouse conditions, using field soils, for their ability to promote the growth and development of soybean and canola plants. Selected strains were then tested in field trials, and some were found to increase growth and yield.

2.5. THE ROLE OF THE PLANT GROWTH-PROMOTING RHIZOBACTERIA

Inoculation of crop seeds with PGPR was of major interest of agricultural production throughout the twentieth century. The commonly applied inoculants have involved nitrogen-fixing strains of *Rhizobium*, which can form a symbiotic association with legumes and fix nitrogen. The capability of soil bacteria that are free-living in the soil (non-symbiotic or "associative") to colonize roots and promote plant growth has been well documented over the past 30 years (Kloepper et al., 1988a). Initial efforts to enhance plant growth with free-living bacteria were centred on either phosphate-solubilizing bacteria such as *Bacillus megaterium* var. *phosphoticum* or on nitrogen-fixing bacteria that are present in the soil as free living bacteria, such as *Azotobacter* (Cooper, 1959; Brown, 1974). The free-living phosphate solubilizing and nitrogen-fixing bacteria were shown to increase the yield up to 25% (Schmidt, 1979). The problem with these bacteria is their inability to compete effectively with other soil microflora, as their population density decline after they were introduced into the rhizosphere. This observation established the "biological balance" (Baker and Cook, 1974) or "microbial equilibrium" (Katznelson, 1985) theory, which stated that the rhizosphere microflora are a distinct collection of organisms that can live and persist in the soil in equilibrium (Kloepper et al., 1988a). The assumption was that after new bacteria are introduced into the rhizosphere their population density will decrease, and the original microbial balance would be reestablished (Kloepper et al., 1988a).

In the late 1970s, researchers at the University of California in Berkeley found that the rhizosphere microbiological equilibrium could be modified by introducing specific strains of rhizosphere pseudomonads (Kloepper et al., 1988a). These pseudomonads were able to colonize roots and alter the balance of both fungal and bacterial microflora throughout the growing season (Kloepper et al., 1980c).

PGPR were reported to increase plant yields 10 to 30% in non-legume crops such as potato, radish, and sugar beet. Numerous reports indicated that PGPR can exert precise effects on diverse hosts, including lentil (Chanway et al., 1989), peanut (Turner and Backman, 1991), bean (Anderson and Guerra, 1985), canola (Kloepper et al., 1988b), cotton (Backman and Turner, 1989; Greenough and Batson, 1989,), pea (Chanway et al., 1989), rice (Sakthivel and Gnanamanikam, 1987), and soybean (Polonenko et al., 1987). The mechanism by which the PGPR promote plant growth is unknown; however, a wide range of mechanisms were postulated such as: mobilization of insoluble nutrients (e.g. phosphate) and resulting enhancement of uptake by the plant (Lifshitz et al., 1987), associative nitrogen fixation (Chanway and Holl, 1991a) production of antibiotics toxic to soil-borne pathogens (De-Ming and Alexander, 1988), production of plant growth regulators that promote plant growth (Kloepper and Schroth., 1981b; Gaskins et al., 1985;), siderophore production [high-affinity iron (III) chelator], exclusively by pseudomonad strains (Neilands and Leong, 1986). Specific Pseudomonad strains have established yield increases, control of soil-borne plant pathogens, promotion of seedling emergence, and promotion of legume nodulation by nitrogen-fixing *(Brady)rhizobium* spp under field conditions..

Some reports suggested beneficial effects of some PGPR on the legume N_2-fixing symbiosis; the bacteria involved are known as nodule promoting rhizobacteria (NPR). Inoculation with NPR, often pseudomonads, and *(Brady)rhizobium* enhances root nodule number or mass (Singh and Subba Rao, 1979; Burns et al., 1981; Grimes and Mount, 1987; Polonenko et al., 1987; Yahalom et al., 1987). The ability of NPR to increase nitrogenase activity was also documented (Iruthayathas et al., 1983; Alagawadi and Gaur, 1988). Burns et al. (1981) reported that co-inoculation of *Azotobacter vinlandii* and *Rhizobium* spp. increased the numbers of nodules on the roots of soybean (*Glycine max*), pea (*Vigna unguiculata*), and clover (*Trifolium repens*). Both field and greenhouse data showed that co-inoculation of *Pseudomonas putida* increased nodulation of beans (*Phaseolus vulgaris*) by *R. phaseoli* (Grimes and Mount, 1984). The mechanism by which NPR increase nodulation and/or N_2 fixation is obscure and the ecology of NPR is poorly understood.

2.6 MECHANISMS BY WHICH RHIZOBACTERIA PROMOTE PLANT GROWTH

2.6.1. PGPR production of plant growth regulators

Plant growth regulators (PGRs) are organic substances that, at very low concentrations, can influence physiological mechanisms of the plants. Several soil microorganisms have the capability to produce active quantities of PGRs, which can effect plant growth and development. Production of PGRs has been illustrated in both culture media and in the soil. Plant growth promoting activity by soil microflora can take different forms such as, the production of phytohormones, or fungicidal or bactericidal indole and phenols. *Azospirillum*, which is a diazotrophic bacterium associated with plant roots, particularly with forage grasses and cereals, in tropical regions, might be of agronomic importance, since it has repeatedly been reported to promote plant growth (Okon, 1994). It is thought to benefit plants by the production by phytohormones, e.g. auxins (Hartmann and Zimmer, 1994).

2.6.2. Enhancement of phosphate uptake by plants

Considerable research efforts have been aimed at evaluating phosphate-solubilizing bacteria. Several ways by which these bacteria, which include fluorescent pseudomonads, may increase the availability of phosphorus to plants were suggested such as, mineralization (solubilization of organic phosphate via the action of phosphatase) or solubilization of unavailable inorganic phosphates by means of organic acids. Lifshitz et al. (1987) reported that a *P. putida* PGPR strain increased the uptake of ^{32}P-labelled phosphate by canola seedlings. Inoculation of seeds with a pseudomonad PGPR resulted in a significant increase of ^{32}P levels in roots and in shoots.

2.6.3. Biological Control of Soil-borne Plant Pathogens by PGPR

phytophathogens can reduce crop yields by 25-57%, which is tremendous loss of crop productivity. Presently, chemical agents (pesticides) are applied to reduce this loss. Other procedures such as fumigation, steam treatment, and solarization of soils were also applied (Gamliel and Katan, 1992). However, many of these chemicals could have hazardous effects on animals, humans, persist and accumulate in natural ecosystems. Currently, biological approaches are being developed to control some plant pathogens. Such biological approaches include the development of plants that are able to resist one or more pathogenic agents (Greenberg and Glick, 1993) and the use of PGPR that can suppress or prevent the phytopathogenic damage (O'Sullivan and O'Gara, 1992; Sivan and Chet, 1992; Cook, 1993; Sutton and Peng, 1993).

The possible use of PGPR as biological control agents is described by the study of the mode of action for fluorescent pseudomonad PGPR reported for potato (Kloepper et al., 1981a). These PGPR strains were not able to promote plant growth under gnotobiotic conditions (Kloepper and Schroth, 1981b). The growth promotion in field soils was accompanied with a 23-64% reduction in the population densities of indigenous rhizoplane fungi and a 25-93% reduction in gram-positive bacterial population densities (Kloepper and Schroth, 1981a). Suslow and Schroth (1982) identified specific strains of root-colonizing bacteria that were pathogenic on sugar beet seedlings and were termed deleterious rhizobacteria (DRB). Several genera of DRB were found to cause growth inhibition and root deformations on crop plants (Suslow and Schroth, 1982; Fredrickson and Elliott, 1985a; Fredrickson and Elliott, 1985b; Gerhardson et al., 1985; Campbell et al., 1986; Schippers et al., 1987;).

Most PGPR strains appear to enhance plant growth indirectly by reductions in populations of DRB. A study by Kloepper (1983) demonstrated that inoculation of potato seed pieces with two strains of fluorescent pseudomonad PGPR, which were responsible for yield increases in the field, caused a reduction in populations of *Erwinia carotovora* on roots, ranging from 95 to 100% fewer than controls without PGPR treatment. Colyer and Mount (1986) and Xu and Gross (1986a,b) confirmed the biological control of *E. carotovora* by selected strains of root-colonizing fluorescent pseudomonads.

2.6.3.1. SOME MECHANISMS BY WHICH RHIZOBACTERIA DEMONSTRATE BIOLOGICAL CONTROL

Biological control of plant pathogens using bacteria as biological control agents has been reviewed by Weller (1988), and examples of rhizobacteria as biological control agents have been reviewed by Schippers (1988). During the last decade, fluorescent pseudomonads were applied successfully as seed inoculants to control soil-borne plant pathogens, as reviewed by Weller (1988). Suppression of *Pythium* root rot by different fluorescent *Pseudomonas* strains was reported on cucumber (Paulitz et al., 1992), wheat (Weller and Cook, 1986), and tulip (Weststeijn, 1990). Generally, bacterial abilities to protect the plants from soil-borne plant pathogens rely on two aspects: the root-colonization capacity of the biocontrol agent and the production of siderophores and antibiotics that control the growth of the plant pathogens.

2.6.3.1.1 Production of siderophores

Iron is one of the soil elements whose availability for direct assimilation by microorganisms frequently limits growth. Since the amount of iron that is available in the soil is much too low to support microbial growth, soil microorganisms secrete iron-binding molecules (siderophores) that bind Fe^{+3}, which is the primary form of iron in nature, carrying it back to the microbial cells, and making it available for microbial growth (Neilands and Leong, 1986; Briat, 1992). Siderophores are high-affinity iron [III] transport agents which are produced when iron is limited (Neilands and Leong, 1986).

PGPR produce and release siderophores molecules that will bind most of the Fe^{+3} that is available in the rhizosphere, and as a result, prevent any pathogens from proliferating because of lack of iron, and thus facilitate plant growth (O'Sullivan and O'Gara, 1992). Pseudomonad PGPR strains are capable of producing siderophore which chelate the ferric iron in the rhizosphere thus inhibiting plant pathogenic or deleterious species, with less affinity for iron (Kloepper et al., 1980b).

Kloepper et al. (1980b) reported that some PGPR strains that induced yield increases for potato were able to produce siderophores which bind to Fe(III). The capacity of *Pseudomonas putida* WCS358 to produce the siderophore: pseudobactin 358 (PSB358), resulted in yield increases in potato tuber (Bakker et al., 1986) and suppression of *Fusarium* wilt in carnation (*Dianthus plumarius*) (Duijff et al., 1993). These findings gave rise to the siderophore theory of biological control by rhizobacteria. The siderophore theory is supported by the following findings: 1. Certain strains that are capable of producing pure siderophores inhibit plant pathogens *in vitro* and also promote plant growth upon addition to soil (Kloepper et al., 1980b); 2. Pseudomonad PGPR (WCS358) promoted plant growth in field trials of continuously cropped potato, while a siderophore-negative mutant, obtain by transposon mutagenesis, did not (Bakker et al., 1987)

One of the main factors that affects iron concentration in the soil solution is the soil pH. As soil pH decreases to below 6, iron concentrations in the soil rise and the siderophores become ineffective (Scher and Baker, 1980). Most aerobic and facultative anaerobic microorganisms produce the siderophores when the iron concentration decreases. Fluorescent pseudomonads are able to produce the fluorescent siderophore, pyoverdin, which allow these bacteria to promote plant growth (Kloepper et al., 1980b; Bakker et al., 1987; Hofte et al., 1991).

Young et al. (1991) showed that ambient temperature strongly affects root colonization by rhizopseudomonad strains ANP15 and 7NSK2, their pyoverdin production and their survival in the soil. Cell yields of both strains were highly reduced at elevated temperatures. Buyer et al. (1993) confirmed that PGPR in the rhizosphere actually synthesize siderophores under iron-limiting conditions. Monoclonal antibodies were used to conduct an enzyme-linked immunosorbent assay (ELISA) to quantify siderophore production by a fluorescent pseudomonad present in a barely rhizosphere.

Generally, plants are not injured by regional depletion of iron caused by PGPR. Most plants are capable of growing at much lower iron concentrations than microorganisms (O'Sullivan and O'Gara, 1992). A number of plants have developed mechanisms by which they can bind to the bacterial iron-siderophore complex, transporting it through the plant, and then unbinding the iron from the siderophore so that the plant can use it (Crowley et al., 1988; Bar-Ness et al., 1991, 1992; Wang et al., 1993). There are several factors that enable the siderophores to act as effective disease-suppressive agents such as, the type of the crop plant, the soil constitution, the bacterium that produces the siderophore, the affinity of the specific siderophore for iron, and the specific phytopathogens being suppressed.

So an awareness of the behaviour of introduced PGPR strains and their siderophore-producing abilities at different temperatures could be of major importance regarding the use of PGPR for promotion of plant growth. However, the influence of PGPR strains on plant growth under different climatological conditions needs additional study before PGPR strains can be introduced into the field.

2.6.3.1.2. Production of antibiotics

One of the most frequently used methods to begin selecting bacteria as potential biological control agents involves screening the bacteria for their ability to produce antibiotics *in vitro* (Fravel, 1988). *Bacillus subtilis* is well known for its ability to produce antibiotics, and for this reason, it has been evaluated during the last 40 years for its potential as a biological control agent (Dunleavy, 1955; Broadbent et al., 1971). Confirmation of the direct involvement of antibiotic production in PGPR for disease suppression came from two distinct types of experiments:

(i) Inability of non-antibiotic-producing mutants of several different disease-suppressive bacterial strains to prevent phytopathogens from causing damage to plants (Gutterson et al., 1986; Thomashow and Weller, 1988; Haas et al., 1991; Howie and Suslow, 1991; Keel et al., 1992). (ii) When a wild type strain of *Pseudomonas fluorescens* was genetically manipulated to overproduce the antibiotics pyoluteorin and 2,4-diacetylphloroglucinol, the resulting strain prevented *Phythium ultimium* from causing disease on cucumber plants more than did the wild-type strain (Maurhofer et al., 1992; Schnider et al., 1994).

Fluorescent pseudomonads produce antibiotics which are toxic to soil-borne plant pathogens (Lesinger and Margraff, 1979). Their capability to produce these metabolites in the rhizosphere was proposed to be the mechanism for biological control of plant diseases. The usage of antibiotic-resistant markers has also allowed direct proof that some specific strains of introduced bacteria could become established in the root zone and remain at stable population densities throughout the growing season (Kloepper and Schroth 1978; Kloepper et al., 1980a).

2.6.3.1.3. Competition

Competition as a mechanism for biological control by rhizobacteria, is through competition among soil microorganisms for infection sites and nutrients. Competition for infection sites was illustrated by Osburn et al. (1989) who reported that *Pseudomonas putida* strain R20, which colonized the pericarp, seeds, and roots of sugar beet, showed no effect on germination of *Pythium sporangia in vitro*. Soil tests showed that strain R20 delayed fungal colonization of the pericarp 4-12 h after planting. By 24 h, 90% of non-treated seeds were infected with *Pythium*, whereas seeds treated with R20 showed 37% infection. Damping-off was 50% less than controls after treatment with R20. It was suggested that biological control originated from protection of the pericarp by possession of pathogen infection sites by rhizobacterial strains.

Pseudomonads are capable of catabolizing a wide range of nutrients and can grow rapidly in the root zone, making them consistent competitors for biological control by competition for nutrients, especially against the slower-growing pathogenic fungi (Weller, 1985). Suslow (1982) reported that PGPR were able to prevent DRB from colonizing sugar beet as they occupied and excluded DRB from the cortical cell junctions, where maximum secretion of nutrients occur. Stephens et al. (1993) concluded that "principal element influencing the ability of a pseudomonads isolate to perform as a biocontrol agent against *Pythium ultimum* on sugarbeets in soil, is their capability to metabolize the components of seed exudate in order to produce compounds inhibitory to *P. ultimum*".

In addition to the widespread antibiosis mechanisms that include the disease control by siderophore, antibiotics and competition, there are a number of other ways in which PGPR can inhibit phytopathogens.

(i) It was demonstrated that different microorganisms, including strains of *Cladosporium werneckii*, *Pseudomomas cepacia* (=*Burkholderia cepacia*), and *Pseudomonas solanacearum* were able to hydrolyze fusaric acid (Toyoda and Utsumi, 1991). Fusaric acid is known as the causative agent of the damage to plants when infected by *Fusarium*. As a result, these bacterial strains prevent plant diseases caused by various species of this fungus.

(ii) When the plants are attacked by pathogens, they respond by synthesizing enzymes that are able to hydrolyze the cell walls of fungal pathogens (Mauch et al., 1988). Some PGPR strains probably produce enzymes that can lyse fungal cells. Lim et al. (1991) isolated a strain of *Pseudomonas stutzeri* that produced extracelluar chitinase and laminarinase enzymes that could digest and lyse *Fusarium solani* mycelia thus preventing the fungus from causing crop loss owing to root rot. In addition, Fridlender et al. (1993) used a ß-1,3-glucanase-producing strain of *Pseudomonads cepacia* that was able to damage fungal mycelia and, thereby, reduce the incidence of plant disease caused by the phytopathogenic fungi *Rhizoctonia solani*, *Sclerotium rolfsii*, and *Phythium ultimum*.

2.6.4. PGPR induction of Systemic Disease Resistance in Plants

Enhancement of plant growth by rhizobacteria through disease control may comprise direct or indirect effects on the pathogen (Davison, 1989). Directly by competing with the pathogen for available nutrients, siderophore production, and production of antibiotics (Weller, 1988), and indirectly by modifying plant defence responses. Induced disease resistance is an active resistance mechanism which depended on the host plant's physical or chemical restrictions, activated by biotic or abiotic agents (Kloepper et al., 1992). Resistance could be induced by pre-inoculation with PGPR strains (van Peer et al., 1991; Wei et al., 1992). Research in past decades illustrated that plants have complex defense mechanisms that can be triggered prior to disease development by environmental factors and microorganisms. One regional defense system activated by pathogen approach, called the hypersensitive response, includes the build up of phytoalexins and intensified synthesis of enzymes involved with phenolic biosynthesis (Dixon et al., 1986). These enzymes involve phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) (Bell et al., 1986; Lawton and Lamb, 1987; Ryder et al., 1987). Other enzymes were documented to be synthesized such as peroxidase (Lagrimini and Rothstein, 1987) and phathogenesis-related proteins (PRP), which include chitinase (Tuzun et al., 1989; Ye et al., 1990). Pathogenesis-related proteins are often present in the intercellular spaces and are acid-soluble (De Tapia et al., 1986).

Several reports have demonstrated that inoculation of plants with rhizobacteria can induce defense responses. Enhanced accumulation of phytoalexinins in stem tissue of Carnation was observed in the presence of the pathogen *Fusarium oxysporum* when the roots were colonized with pseudomonad (Van Peer et al., 1991). Also colonization of plant roots by pseudomonads increased amount of a lignin-like component present in the root (Anderson and Guerra, 1985; Frommel et al., 1991). Systemic impact was also noticed when roots of white bean were colonized by a fluorescent pseudomonad, as the protein profile of intercellular fluids in leaf tissues was modified (Hynes and Lazarovits, 1989).

Scheffer (1983) reported that pre-inoculation of elm trees with four fluorescent pseudomonad strains resulted in reductions in systemic foliar symptoms of *Ophiostoma ulmi*, the Dutch elm disease pathogen. Voisard et al. (1989) studied the mechanisms for biological control of *Thielaviopsis basicola* by PGPR strain CHAO, a strain of *Pseudomonas fluorescens*.

It was suggested that the ability of some pseudomonads to synthesize hydrogen cyanide (to which these pseudomonads are themselves resistant) may be linked to the ability of these strains to inhibit some pathogenic fungi, although the role of the hydrogen cyanide in disease suppression is not considered to be well established (Voisard et al. 1989). Production of HCN was found to be associated with biological control activity and with promotion of root hair growth. Recently, Wei et al. (1992) demonstrated that seed treatment of cucumber with HCN-producing and non-HCN-producing PGPR induced systematic resistance in leaves against *Colletotrichum* *lagenarium*. It was suggested that HCN produced by PGPR strain CHAO might induce plant defence mechanisms (Tuzun and Kloepper, 1994).

Several studies also indicated that PGPR may stimulate production of biochemical responses related to host defence. Such mechanisms involve the buildup of anti-microbial low molecular weight chemicals such as phytoalexins and leaf surface diterpenes (Kuc and Rush, 1985; Tuzun et al., 1989) and/or development of protective layers through the accumulation of biopolymers such as lignin, callose and hydroxyproline-rich glycoproteins (Hammerschmidt and Kuc, 1982; Hammerschmidt et al., 1982; 1984). Plant root colonization by PGPR was also associated with increased peroxidase activity (Albert and Anderson, 1987) and enhanced lignification of stems or leaves in bean (Anderson and Guerra, 1985) and potato (Frommel et al., 1991).

2.7. ECOLOGY OF THE INTRODUCED BACTERIA INTO THE RHIZOSPHERE

2.7.1. Root colonization:

Root colonization is a dynamic process and not a temporary relation between bacteria and roots in soil. It is the process whereby bacteria survive inoculation onto seeds or into soil, divide and proliferate in response to seed exudates rich in carbohydrates and amino acids (Kloepper et al., 1985), adhere to the root surface (Suslow, 1982; Weller, 1983), and colonize the root system in soils containing native microorflora (Kloepper et al., 1980a, Suslow and Schroth, 1982; Weller, 1984). PGPR can multiply and remain in the rhizosphere following inoculation onto crop seeds. The bacteria are allocated in the rhizosphere in a log normal order (Loper et al., 1984) and are sporadically established along the roots (Bahme and Schroth, 1987). Colonization, although difficult to measure, is required for causing an interaction with the plant and other members of the microflora. The role of root colonization by PGPR has been reviewed (Schroth and Hancock, 1982; Suslow, 1982).

Rhizosphere colonization symbolizes a larger ecological niche including root colonization and bacteria that are in close proximity to, although not necessarily attached to roots (Kloepper et al., 1980a). Fluorescent pseudomonads, are highly rhizosphere competent (capable of root colonization), which accounts for their predominance among the PGPR. Pseudomonads hold several characteristics which assist them seed colonization, such as fast growth and motility (Seymour and Doetsch, 1973; Arora et al., 1983; Scher et al., 1985;). However, these traits may not always correlate with root colonization. For example, Howie et al. (1987) found that three nonmotile mutants of *P. fluorescens* colonized wheat roots as well as their motile parents.

Rhizosphere colonization have been reviewed recently by van Elsas and Heijnen (1990), Kloepper and Beauchamp (1992) and Kluepfel (1993). The major problem for successful application of PGPR strains in soil was suggested to be the lack of constant effectiveness of the inoculant (van Elsas and Heijnen, 1990). Several causes were suggested, such as ineffective colonization of the plant, or poor survival of the introduced population. Xu and Gross (1986b) and Bull et al. (1991), demonstrated a positive relationship between root colonization by a PGPR strain and disease suppression, proposing that methodologies which enhance root colonization may also improve the benefits of a PGPR strains in soil. The extent and amount of root colonization required by a PGPR strain in order to enhance plant growth rely on many interrelated considerations. The choice of methods used to try to increase rhizosphere colonization and plant growth should take these factors into account (Stephens, 1994b).

2.8. INTERACTIONS OF INTRODUCED BACTERIA WITH INDIGENOUS RHIZOSPHERE MICROORGANISMS

As the beneficial bacteria are introduced into the rhizosphere, they become involved in a web of complex biological interactions with the host plant and with the surrounding rhizosphere microorganisms. They obtain their nutrients from the root exudates and are accordingly dependent on the host plant, while they affect the host by inducing physiological changes in the plant (Kloepper et al., 1988b). Interactions with indigenous rhizosphere microorganisms could take different forms. It could be neutral, antagonistic (e.g. competition for nutrients, production of antibiotic compounds, parasitism, or predation) or synergistic (i.e. the promotion of *Rhizobium*-induced nodulation of legumes). Several environmental restrictions, including temperature, moisture, and soil type, may affect these microbial interactions.

The genetic marking of bacteria with antibiotic resistance for identification purposes permits the study of population dynamics of soil-inhabiting bacteria. The use of antibiotic-resistant markers allowed the first direct demonstration that some specific strains of introduced bacteria could establish in the root zone and maintain high population densities throughout the growing season (Kloepper and Schroth, 1978; Kloepper et al., 1980a). Certain PGPR that cause obvious increases in plant growth and yield were marked to track their populations during the various stages of plant development (Polonenko et al., 1987).

2.9. SELECTION OF RHIZOSPHERE-COMPETENT STRAINS

There are several factors that play important roles in the selection of the rhizosphere competent strains. These are discussed below:

2.9.1. Crop Specificity

The fitness of a bacterial strain in the rhizosphere may be reliant on the plant species (van Peer and Schippers, 1989, Beauchamp et al., 1991), plant cultivar (Weller, 1986) and the geographical region from which the bacteria were isolated (Mattar and Digat, 1991).

2.9.2. Location on the root

Bacteria isolated from the rhizosphere and from discrete areas in the root, may be phenotypically different from bacteria isolated from other portion of the soil ecosystem. Hozore and Alexander (1991) reported that bacteria taken from the rhizosphere of soybean and re-inoculated onto these plants, were able to colonize roots more efficiently. These studies suggested that bacteria must be selected directly from the rhizosphere and from that area of the plant root in which rhizosphere competence is preferred.

2.9.3. Quantity of inoculum on the seed

One procedure of increasing rhizosphere colonization by certain PGPR strains is by increasing the bacterial inoculum load on the seed. Hebbar et al. (1992) reported that the colonization and spread of *Pseudomonas cepacia* (which is a bio-control agent against *Fusarium moniliforme*) on the roots and rhizosphere of maize is related to the amount of inoculum on the seed. However, the dependence of final colonization status on the initial inoculum level has not been ubiquitous observation. For example, the colonization of introduced pseudomonad strains on maize (Scher et al., 1984) and wheat (Juhnkel et al., 1989) were shown to be irrelevant to the amount of the initial inoculum It is apparent that under certain conditions, increasing the level of inoculum may increase the rhizosphere competence of some, but not all, bacteria.

2.9.4. Co-inoculation of PGPR strains with other microorganisms

Studies have shown that it is feasible to maximize the root and rhizosphere colonization by co-inoculation with other bacteria. Li and Alexander, (1988) reported that co-inoculation of lucerne and soybean roots with antibiotic-producing strains of *Bacillus* sp. and *Streptomyces griseus*, respectively, increased root colonization by *R. meliloti* and *B. japonicum*. Non-antibiotic producing derivatives of *Bacillus* sp. and *S. griseus* did not promote root colonization, suggesting that the benefit of this co-inoculation was a result of antibiotic production. These results suggest that co-inoculation of PGPR strains with other microorganisms may provide a relatively simple and economically viable method of increasing a strain's rhizosphere competence.

2.9.5. Use of Carrier Materials to improve PGPR survival in the rhizosphere

Recent research has focused on the use of chemical polymers as carriers to enhance the survival and rhizosphere colonization of PGPR strains in soil. Alginate has been used to encapsulate *P. cepacia* (Fravel et al., 1985), *Azospirillum* (Fages, 1990) and *Frankia* (Sougoufara et al., 1989). *P. fluorescens* cells entrapped in an alginate matrix and applied to the soil were able to survive better than those added directly to soil (van Elsas et al., 1992). The utilization of chemical polymer beads to distribute bacteria to soil and increase rhizosphere colonization has been found to have significant potential.

2.9.6. Management of the soil

Addition of distinctive clays has been shown to increase bacterial survival in fine textured soils (England et al., 1993). Addition of 5% bentonite clay into a loamy sand soil (prior to inoculation) increased the survival of an introduced *Pseudomonas fluorescens* strain in both bulk soil and the wheat rhizosphere. Mixing of a fresh *P. fluorescens* culture with bentonite clay prior to inoculation also enhanced bacterial survival. Incorporation of organic matter can also influence the survival of a PGPR strain in the soil (Heijnen et al., 1992). Stephens et al. (1994a) reported that when *P. corrugata* 2140R [a biological control agent against take-all (Ryder and Rovira, 1993)] was inoculated into soil, the addition of finely ground barley straw added at 0.6% (w/w) increased the survival of *P. corrugata* 2140R one thousand fold after 29 days. The ecology of pseudomonad PGPR is a relatively new research area. Consequently, there is little understanding of how environmental factors will influence bacterial colonization effects and persistence on roots and the resulting effects on plant growth.

2.10. PGPR STRAIN DISPERSAL THROUGH THE SOIL

Madsen and Alexander (1982) found that neither *B. japonicum* nor *P. putida* moved vertically through soil in the absence of a transporting agent (eg. plant roots, water, earthworms). Similar observations have been reported for *P. fluorescens* (Trevors et al., 1990; van Elsas et al., 1991). This suggests that several transporting agents could be required for the dispersal of PGPR strains through the soil

2.10.1. Water

Percolating water can have a major effect on the distribution of PGPR strains in the rhizosphere (Parke et al., 1986). Kluepfel (1993) reported that roots enhance bacterial movement in the soil by forming channels or pores, through which water carries the bacteria. Increasing the bulk density of soil and the addition of clay to a sandy soil decreased bacterial movement in percolating water (Huysman and Verstraete, 1993a). On the other hand, increasing the rate of irrigation was shown to increase movement of bacteria in soil (Huysman and Verstraete, 1993b). Specific properties of the soil such as pore size and distribution (Dazzo et al., 1972) and the type and amount of the colloidal fraction (Marshall, 1969) can aid bacterial migration. Diverse bacterial characteristics have been reported to influence bacterial migration in percolating water in soil. Huysman and Verstraete (1993a) reported that hydrophobic strains of bacteria migrated through irrigated soil columns 2-3 times more slowly than hydrophilic strains. Issa et al. (1993) reported that heavy textured soils restricted the active movement of rhizobia compared to lighter textured soils.

2.10.2. Earthworms

Earthworms have also been shown to influence the movement of beneficial microorganisms in soil. For example, the earthworm *Lumbricus rubellus*, in the presence of percolating water, enhanced the vertical transport of *Pseudomonas putida* and *Bradyrhizobium japonicum* in soil (Madsen and Alexander, 1982). The earthworm *Lumbricus terrestris* increased the distribution of nodules on the root system of soybean and it was suggested that this reflected the dispersal of *B. japonicum* by *L. terrestris* (Rouelle, 1983). These results suggest that the management of earthworms may be exploited to increase bacterial root colonization by PGPR strains.

2.11. EFFECTS OF PGPR ON PLANTS IN FIELD TRIALS

2.11.1 Yield increase

PGPR ability to increase crop yields under diverse field conditions has been reported (Schroth and Hancock, 1982; Suslow, 1982; Hemming, 1986; Schippers et al., 1987; de-Freitas and Germida, 1992). However, some of these reports demonstrated that seed inoculation with PGPR does not always lead to yield increases. Possible reasons for this disagreement, including iron availability, soil-type, and the nature of the soil microflora and application procedures have already been discussed.

2.11.2. Enhancement of Seedling Emergence

Specific root-colonizing bacteria can increase seedling emergence. This was first reported with strains that caused increases in emergence rates of soybean and canola seedlings under cold field conditions in Canada (Kloepper et al., 1986). The new class of PGPR strains was termed emergence-promoting rhizobacteria (EPR). Inoculation of conifer seeds with *Bacillus* strains caused increased seedling emergence and biomass (Chanway et al., 1991b). Chanway (1995) also reported that seed inoculation with *Bacilus polymyxa* can result in colonization of western hemlock root systems and increase seedling emergence.

2.11.3. Promotion of nodulation of legumes

Specific pseudomonad strains were able to stimulate nodulation of leguminous crops by *Rhizobium* and *Bradyrhizobium*. Grimes and Mount (1987) reported that a *P. putida* strain (M17) increased *Rhizobium* nodulation of bean in field soils. Similarly, Polonenko et al. (1987) tested the effects of fluorescent pseudomonads on nodulation of soybean roots by *B. japonicum*. Certain PGPR strains were capable of enhancing *B. japonicum* nodulation and soybean plant growth in field soil. These strains were termed nodulation-promoting rhizobacteria (NPR). The mode of action of NPR is not known. Work in the laboratory of Dr. D. Layzell has shown that, due to surface to volume relationships, the proportion of bacteroid filled tissue is much higher in large nodules (up to 89%) versus smaller nodules (as little as 30%); the bulk of the remaining tissue is nodule cortex. Hence a soybean plant with large nodules invests less carbon for each nitrogen fixed than a plant with the same nodule dry weight but with smaller nodules. Also, the oxygen concentration inside large nodules is much more precisely regulated than it is in small nodules (unpublished data).

2.12. RECOGNITION BETWEEN *(BRADY)RHIZOBIUM* AND SOYBEAN SYMBIOTIC PARTNERS

The mechanisms of recognition between *(Brady)rhizobium* and soybean could be considered as a form of cell-to-cell interorganismal communication. The exchange of molecular signals between the host plant and (brady)rhizobia is needed for the establishment of effective root nodules. The first step in the exchange of signals involves the production of phenolic compounds, flavonoids and isoflavonoids, by the host plants (Peters and Verma, 1990). These compounds are produced by the part of the root with emerging root hairs, the place which is most susceptible to infection by bradyrhizobia (Verma, 1992). These compounds would induce the expression of *nod* genes in (brady)rhizobia, enhancing the production of the bacterial nod factor

(Kondorosi, 1992) which is a lipo-oligosaccharide (Carlson et al., 1993). The lipo-oligosaccharide induces many of the early steps in nodule development, such as deformation and curling of plant root hairs, the initiation of cortical cell division, and initiation of root nodule meristems (Dénarié and Roche, 1992). There are two major components of soybean root exudate isoflavones, daidzein and genistein, which induce the *nod* genes of *B. japonicum* (Kosslak et al., 1987). Daidzein has less *nod* gene-inducing capability than does genistein (Sutherland et al., 1990).

These isoflavones chiefly occur in the form of 7-*O*-glucoside 6"-*O*-malonate conjugates (Graham et al., 1990). The isoflavone conjugates are stored in vacuoles. There are eleven enzymes, such as phenylalanine ammonia lyase and isoflavone methyltransferase, that are dedicated in the biosynthesis of genistein in soybean roots (Barz and Welle, 1992). The effectiveness of isoflavonoids varies between cultivars (Horvath et al., 1986; Zaat et al., 1988). There is a strong positive relationship between root isoflavone concentrations and soybean nodule numbers (Cho and Harper, 1991b). For example, a hypernodulating soybean mutant, obtained from the cultivar Williams, had higher root concentrations of isoflavone compounds (genistein, daidzein, and coumestrol) than did Williams at 12 days after inoculation. Nitrogen application (urea, NH_4^+, and NO_3^-) decreases the concentrations of isoflavone compounds in soybean plants (Cho and Harper, 1991a), while NO_3^- is the most inhibitory to isoflavone concentrations, and inhibition is concentration-dependent. Exogenous abscisic acid application into the soybean root medium also resulted in a decrease in nodule number and weight in both hypernodulating and wild-type soybean plants, while isoflavonoid concentrations also markedly decreased in response to abscisic acid application (Cho and Harper, 1993).

Zhang et al. (1996c) have shown that the roots of plants germinated and grown at lower RZTs have lower genistein concentrations and contents than plants grown at higher RZTs. The beneficial effect of genistein increased with decreasing RZT (Zhang and Smith, 1995b). At suboptimal RZT (17.5 and 15°C) the most effective concentrations are in the 15 to 20 μM range, whereas at optimal (25°C) RZT 5 μM is most effective. Two studies by Zhang and Smith (1995b), and Zhang et al. (1996d) under controlled environment conditions and under field conditions, have shown that preincubation of *B. japonicum* with genistein hastened the onset of nitrogen fixation, increased the number of nodules produced, increased the size of the nodules, and increased plant growth.

Preface to Section 3

Section 3 is comprised of a manuscript prepared by N. Dashti, R.K. Hynes and D.L. Smith, for submission in 1996. The format has been changed to conform as much as possible to a consistent format within this thesis. All literature cited in this section are listed in the reference section at the end of the thesis. Each table is presented at the end of this section.

Whereas previous work by us has shown that PGPR can help to overcome low RZT inhibition of soybean nodulation and nitrogen fixation under controlled environment conditions, in this section we attempted to determine whether or not PGPR will have the same effects under field conditions, leading to increased seasonal N_2 fixation.

Section 3.

PLANT GROWTH PROMOTING RHIZOBACTERIA ACCELERATE NODULATION AND INCREASE NITROGEN FIXATION ACTIVITY BY FIELD GROWN SOYBEAN [*Glycine max* (L.) Merr.]

3.1. Abstract

Application of plant growth-promoting rhizobacteria (PGPR) has been reported to increase nodulation and nitrogen fixation of soybean over a range of root zone temperatures (RZTs) under controlled environment conditions. Two field experiments were conducted on two adjacent sites in 1994 to evaluate the ability of two PGPR strains (*Serratia liquefaciens* 2-68 or *Serratia proteamaculans* 1-102) to increase nodulation, nitrogen fixation, and total nitrogen yield by two soybean cultivars under field conditions in a short season area. The results of these experiments indicated that co-inoculation of soybean with *B. japonicum* and PGPR increased soybean nodulation and hastened the onset of nitrogen fixation early in the soybean growing season, when the soils were still cool. As a result of the increase in these variables, total fixed nitrogen, fixed nitrogen as a percentage of total plant nitrogen, and the nitrogen yield also increased due to PGPR application. Interactions existed between PGPR application and soybean cultivars, suggesting that application of the PGPR to cultivars with higher yield potentials was more effective. Inoculation with PGPR only also increased soybean nodulation and nitrogen fixation by native *B. japonicum*.

3.2. Introduction

As soybean [*Glycine max* (L.) Merr.] is a subtropical legume, root zone temperatures (RZTs) in the 25-30°C range are optimal for symbiotic activity, compared to 20-24°C for temperate legumes (Jones and Tisdale, 1921). In recent years the cultivation of soybean has been extended into cool temperate areas where soil temperatures, in comparison with those of its natural habitat, are low during the first part of the growing season. Under such conditions, the poor adaptability of soybean to cool soils is considered the primary factor limiting yield (Whigham and Minor, 1978). Studies on the effects of sub-optimal RZT (<25°C) on nitrogen fixation by soybean and other subtropical legume crops have indicated that low RZTs decease both nodulation and nodule function (Jones and Tisdale 1921; Lynch and Smith, 1993; Zhang and Smith, 1994).

Over the last decade understanding of rhizosphere biology has progressed with the discovery of a group of microorganisms, called plant growth promoting rhizobacteria (PGPR), that colonize plant roots and promote plant growth (Kloepper et al, 1980a). The beneficial impacts of PGPR are thought to be direct plant growth promotion by the production of plant growth regulators (Kloepper and Schroth, 1981b; Gaskins et al., 1985,), enhanced access to soil nutrients (Lifshitz et al., 1987), disease control (De-Ming and Alexander, 1988), and associative nitrogen fixation (Chanway and Holl, 1991a). Nitrogen fixation promoting rhizobacteria increase nodulation leading to increased plant growth (Polonenko et al., 1987).

PGPR have been shown to increase plant yields 10 to 30% in a variety of crops with positive effects reported for barley (Iswandi et al., 1987), bean (Anderson and Guerra, 1985), canola (rapeseed) (Kloepper et al., 1988b), cotton (Backman and Turner, 1989; Greenough and Batson, 1989), lentil (Chanway et al., 1989) pea (Chanway et al., 1989), and soybean (Polonenko et al., 1987). Field tests with some Pseudomonad strains have demonstrated yield increases (Kloepper et al., 1986; Kloepper et al., 1988b), control of soil-borne plant pathogens (Kloepper and Scroth, 1981a), promotion of seedling emergence (Kloepper et al., 1986), and promotion of legume nodulation by nitrogen-fixing *(Brady)rhizobium* spp (Grime and Mount, 1987). Grimes and Mount (1987) found that a *Pseudomonas putida* strain (M17), increased *Rhizobium* nodulation of bean in field soils. Similarly, Polonenko et al. (1987) tested the effects fluorescent pseudomonads on nodulation of soybean roots by *Bradyrhizobium japonicum.*

The stimulation of PGPR on legume symbiotic N_2 fixation and plant growth can be affected by environmental factors, such as RZT. Under controlled environment conditions, the effect of the PGPR on soybean nodulation and N_2 fixation (Zhang et al., 1996b), and plant growth and photosynthetic activities (Zhang et al., 1996a) varied with

RZT. However, to date, there have been no investigations of whether co-inoculation of *B. japonicum* with PGPR increases soybean nodulation and N_2 fixation under short season field conditions. Therefore, in this study, we tested the hypotheses that under short season field conditions: 1) co-inoculation of *B. japonicum* with PGPR increases soybean nodulation and N_2 fixation when soybean is planted into cool spring soils, 2) final plant N and protein yield also increase due to improvement of soybean nodulation and N_2 fixation, and 3) inoculation with PGPR only, in the presence of native soil *B. japonicum*, increases soybean nodulation and nitrogen fixation.

3.3. Materials and methods

3.3.1. Field layout

The two experiments were conducted at the Emile A. Lods Research Centre, McGill University, Macdonald Campus, Montreal, Canada. Both of the experiments were performed at each of two adjacent sites. One site was sterilized with methyl bromide (50 g m^{-2}) under a plastic canopy for 72 h (sterilized site). Three days elapsed between removal of the fumigation canopy and planting. The other site was kept unsterilized. The sterilized site was included in this study to prevent possible competition from native *B. japonicum* or interference from other elements of the soil microflora that might obscure PGPR effects. The first experiment was designed as a 3 x 2 x 2 factorial organized in a randomized complete block split-plot with four replications. The main-plot units consisted of PGPR strain applications (no-PGPR application as a control, *Serratia liquefaciens* 2-68 and *Serratia proteamaculans* 1-102). These two strains were chosen based on the reports of Zhang et al. (1996a and 1996b). The two soybean cultivars (Maple Glen and AC Bravor) and the strains of *B. japonicum* [532C (Hume and Shelp, 1990) and USDA110] formed the sub-plot units. In the second experiment, two factors were tested, PGPR application and soybean cultivars with the same design as in experiment 1, except that the two soybean cultivars were the subplot units. At the sterilized site, each sub-plot was 1.6 x 2 m and consisted of three rows of plants with 40 cm between rows. At the unsterilized site, each sub-plot (2 x 3 m) consisted of four rows of plants with 40 cm between rows. The space between plots was 80 cm and between replications 1 m.

For each replication of both experiments one plot of a non-nodulating line of the soybean cultivar "Evans" was included for calculation of soil nitrogen availability and seasonal N_2 fixation. The soil type at both sites was a Chicot light sandy loam. In the previous year, 1993, this experimental field had been planted with oat and barley, while in 1992 it was used to produce a crop of green manure alfalfa. The average nitrogen accumulation in the non-nodulating control plants was 167 kg ha^{-1}. Potassium and phosphate were provided by the spring application of 340 kg ha^{-1} of 5-20-20 according to soil test recommendations.

3.3.2. Inoculum preparation

In experiment 1, the inoculum was produced by culturing *B. japonicum* strains 532C and USDA110 in yeast extract mannitol broth (Vincent, 1970) in 2000 mL flasks shaken at 125 rpm at 25°C. The PGPR strains were cultured in *Pseudomonas* media (Polonenko et al., 1987) in 2000 mL flasks shaken at 250 rpm at room temperature (21-23°C). After reaching the stationary phase (7-days for *B. japonicum* and 1.5-days for PGPR), both *B. japonicum* and PGPR were subcultured. When the subculture reached the log phase (3-days for *B. japonicum* and 1-day for PGPR), each of the *B. japonicum* and PGPR strains was adjusted with distilled water to an A_{620} (*B. japonicum*) and A_{420} (PGPR) value giving a cell density of 10^8 cells mL^{-1}, respectively. Equal volumes of *B. japonicum* and PGPR cultures were mixed and allowed to stand for approximately half an hour at room temperature without shaking.

3.3.3. Planting methods

Seeds of the soybean cultivars 'Maple Glen' and 'AC Bravor' were surface-sterilized in sodium hypochlorite (2% solution containing 4 mL L^{-1} Tween 20), then rinsed several times with distilled water (Bhuvaneswari et al., 1980). These cultivars were selected as they have been developed for production under the short season, cool conditions of eastern Canada and have performed well there. The seeds were planted by hand on May 11 and 18 at the unsterilized and sterilized sites, respectively. The delay in planting the sterilized site was due to the extra time required for the methyl bromide fumigation. Twenty mL of inoculum (for experiment 1), or the same amount of PGPR or distilled water (no PGPR) (for experiment 2) per one meter of row were applied by syringe directly onto the seeds in the furrow. Cross contamination was prevented throughout planting and all subsequent data collection procedures by alcohol sterilization of all implements used. Following emergence seedlings were thinned to achieve a stand of 500,000 plants ha^{-1} (20 plants m^{-1} of row).

3.3.4. ^{15}N application

To be able to measure the seasonal N_2 fixation rates by the isotope dilution method, ^{15}N was applied (1.2 kg ha^{-1}, 99% pure, Isotec Inc., Miamisburg, OH, USA) as double-labelled ammonium nitrate in solution, to a microplot of six plants (30 x 40 cm) within each subplot in the first three replications at both the sterilized and unsterilized sites in both experiments. Each microplot was bordered by plastic sheeting extending 15 cm into the soil to prevent lateral soil losses of the labelled nitrogen. The labelled nitrogen was applied at growth stage V1 (the first unifoliate leaf) (Fehr et al., 1971).

3.3.5. Data collection

One month after planting, the onset of N_2 fixation was tested for. Acetylene reduction activity assays were used as a +/- measure of nitrogenase activity. From each sub-plot four plants were randomly selected, uprooted and detopped; the roots then were exposed to 10% acetylene in a sealed 1L Mason jar for 10 min. A 0.5 mL gas aliquot was then extracted and analyzed by gas chromatography (Hardy et al., 1968). When acetylene reduction activity was detected in all the PGPR application plots, the number, weight, and nitrogen concentration of nodules were measured.

The final nodulation data were taken from plants harvested on August 13, when the plants reached reproductive stage 6 [pod(s) containing a green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf (Fehr et al., 1971)]. The plants were uprooted, the roots were washed with distilled water and the nodules were removed, counted and weighed. Microplot materials were harvested by hand at harvest maturity, oven-dried at 70°C for at least 48 h, and weighed. The seeds were threshed by hand and ground using a Moulinex coffee mill

(Moulinex Appliances Inc., Virginia Beach, VA, USA). The above-ground plant tissue from each microplot was ground to pass a 1 mm screen of a Wiley mill (A. H. Thomas Co., Philadephia, PA, USA). The nitrogen concentration of grain and other plant tissues was then determined by Kjeldahl analysis (Kjeltec system, Tecator AB, Hoganas, Sweden). Following Kjeldahl analysis, a sample of the distillate obtained from the shoot and grain material of each microplot was dried and the ammonium present converted to nitrogen gas by the application of the Dumas method (Preston et al., 1981) before measuring the $^{15}N:^{14}N$ ratio of each sample by emission spectrometry (Jasco N-150 ^{15}N analyzer, Japan Spectroscopic Co., Tokoyo, Japan). The proportion of the total plant nitrogen derived from N_2 fixation was then determined following the formula described by Lynch and Smith (1993):

$$\text{N\% from fixation} = \{1 - [(^{15}\text{N}:^{14}\text{N of fixing plant})/(^{15}\text{N}:^{14}\text{N of control plant})]\} \times 100.$$

3.3.6. Statistical analysis

The data were analyzed statistically by analysis of variance using the Statistical Analysis System (SAS) computer package (SAS Institute Inc., 1988), except for the onset of nitrogen fixation data (presented in Table 3.1), which were compared by the Cochran Q test (Hollander, 1973). When analysis of variance showed treatment effects ($p \leq 0.05$), the least significant difference (LSD) test was applied to make comparisons among the means ($p \leq 0.05$) (Steel and Torrie, 1980).

3.4. Results

3.4.1. Native Soil B. japonicum Levels

At the sterilized site, the fumigation with methyl bromide was not completely effective and the control plants formed nodules. At the unsterilized site, in experiment 2, a small number (less than 4 per plant) of large nodules were formed. Because at least some contamination occurred, the non-nodulating plants "Evans" were used as the reference for estimating seasonal N_2 fixation. Because of the contamination at the sterilized site, comparisons between strains could not be made with confidence, therefore, only the effects of PGPR application, soybean cultivar and the two way interaction between

PGPR application and soybean cultivar were tested.

3.4.2. PGPR effects on nodulation and onset of N_2 fixation

Acetylene reduction activity was used as a +/- indicator of the onset of N_2 fixation in experiment 1 and showed that inoculation with PGPR resulted in a two to four days earlier onset of N_2 fixation on both sites (Table 3.1).

B. japonicum USDA110 or 532C co-inoculated with PGPR 2-68 increased both nodule weight per plant and weight per nodule for both AC Bravor and Maple Glen at early soybean growth stages relative to inoculation of the *B. japonicum* strains alone at the unsterilized site (Table 3.2). This increase in mass was due to the formation of larger nodules, not to increased nodule number per plant (Table 3.2). At the second sampling, August 13, Maple Glen plants co-inoculated with *B. japonicum* USDA110 and PGPR 2-68 had increased nodule numbers compared to the plants receiving *B. japonicum* USDA110 alone at the unsterilized site (Table 3.2). At the sterilized site, Maple Glen plants receiving PGPR 1-102 caused a 100% increase in the individual nodule weight (Table 3.3).

3.4.3. Effects of PGPR on seasonal N_2 fixation

Co-inoculation of AC Bravor plants with *B. japonicum* USDA110 and PGPR 1-102 increased seasonal N_2 fixation by 184% as compared to plants inoculated with *B. japonicum* USDA110 alone by the N difference method and 92% by the ^{15}N dilution method at the unsterilized site (Table 3.4), however, there was no increase in the seasonal N_2 fixation for the same treatment combination at the sterilized site (Table 3.5). Co-inoculation with *B. japonicum* USDA110 and PGPR 2-68 increased seasonal N_2 fixation by 145 and 100% relative to *B. japonicum* USDA110 alone for AC Bravor plants by the N difference method at both the sterilized and unsterilized sites, respectively (Table 3.4 and 3.5). The same combination increased the seasonal N_2 fixation 94 and 88% by the ^{15}N dilution method at both the unsterilized and sterilized sites, respectively (Tables 3.4 and 3.5). Co-inoculation with *B. japonicum* USDA110 and PGPR 1-102 increased the total plant nitrogen yield of AC Bravor plants by 63% relative to *B. japonicum* USDA110 alone at the unsterilized site (Table 3.4) but caused no increase at the sterilized site (Table 3.5). Co-inoculation with *B. japonicum* USDA110 and PGPR 2-68 increased the total plant nitrogen yield of AC Bravor plants by 50% relative to *B. japonicum* USDA110 alone at the unsterilized site (Table 3.4) and by 31% increase at the sterilized site (Table 3.5).

Co-inoculation with *B. japonicum* USDA110 and PGPR 1-102 increased seasonal N_2 fixation by Maple Glen plants 30% as estimated with the ^{15}N dilution method, when compared to the *B. japonicum* USDA110 alone at the unsterilized site (Table 3.4), however, there was no increase in the seasonal N_2 fixation for the same treatment combination at the sterilized site (Table 3.5). Maple Glen plants co-inoculated with *B. japonicum* USDA110 and PGPR 2-68 had seasonal N_2 fixation levels, estimated by the ^{15}N dilution method, 50% higher than those of plants inoculated with USDA110 alone at the unsterilized site (Table 3.4). The same combination did not increase the seasonal N_2 fixation at the sterilized site (Table 3.5). Interactions between PGPR application, *B. japonicum* strains, and soybean cultivars existed for total fixed N and fixed N as a percentage of total plant N indicating that 532C and USDA110 had different sensitivities to PGPR preincubation at the unsterilized site (Table 3.4). Interactions between PGPR application and soybean cultivars indicated that AC Bravor tended to be more responsive to both PGPR treatments for total fixed N, fixed N as a percentage of total plant N, and N yield at the unsterilized site (Table 3.5).

3.4.4. PGPR effects on soybean nodulation and N_2 fixation through native B. japonicum

In experiment 2, where plots were not inoculated with *B. japonicum*, application of PGPR onto seeds in the furrow increased the number of nodules at the sterilized site at the first sampling date (Table 3.6). At crop maturity, nodule number was again increased by PGPR at the sterilized site. PGPR application, in the absence of *B. japonicum* inoculation, also increased seasonal N_2 fixation at both the unsterilized and sterilized sites (Table 3.7). The final total nitrogen yield of plants receiving PGPR 2-68 was 57% greater than that of plants receiving only distilled water at the unsterilized site.

3.5. Discussion

The two PGPR strains *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 used in this study were selected based on work reported by Zhang et al. (1996a,b), in which nine PGPR strains were tested for effects on soybean nodulation and nitrogen fixation over a range of RZTs under controlled environment conditions. In our field study, the onset of nitrogen fixation by plants receiving bradyrhizobia preincubated with PGPR was two to three days earlier than those receiving no PGPR treatment at both the unsterilized and sterilized sites (Table 3.1). Application of PGPR directly onto the seeds in the furrow at the time of planting also improved plant nodulation and N_2 fixation. The findings of this field study agreed with results from controlled environment work, in which co-inoculation of some PGPR with *B. japonicum* reduced the negative effects of low RZT on soybean nodulation and nitrogen fixation (Zhang et al., 1996b). In addition, recent work has shown that inoculation of soybean with PGPRs in the presence of *B. japonicum* increased soybean grain yield, grain protein yield, and total plant protein production under short season areas (Dashti et al., 1996).

Since nodule dry weight per plant was increased and the onset of nitrogen fixation was hastened by *B. japonicum* co-inoculation with PGPR, total fixed nitrogen and nitrogen yield per plant were increased (Table 3.5). Sprent (1979) postulated that an increase of 10% in the period of nodule activity by a grain legumes, particularly between the onset of N_2 fixation and the attainment of maximum fixation, could double the seasonal level of nitrogen fixed. In our experiment, the period of nodule function was about 70 days (late-June to early-September). PGPR application resulted in a two to four day increase in the duration of N_2 fixation (Table 3.1). PGPR application increased the total amount of nitrogen fixed. It seems likely that some of this increase in total fixed nitrogen was due to earlier nitrogen fixation, hastened by PGPR application under short season conditions where soils were stressfully cool in the early growing season, with the remainder being due to increased nodules mass.

The mechanisms of growth and nitrogen fixation promotion by PGPR are not well understood; however, a wide range of possibilities have been suggested, including both direct and indirect effects. Direct effects include an increase in mobilization of insoluble nutrients and consequent enhancement of uptake by the plants (Lifshitz et al., 1987), production of antibiotics (Li and Alexander, 1988), production of plant growth regulators that stimulate plant growth (Gaskins et al., 1985), and associative nitrogen fixation (Chanway and Holl, 1991a). Indirect effects include enhancement of symbiotic nitrogen fixation through increase of root nodule number or mass (Grimes and Mount, 1984; Yahalom et al., 1987; Zhang et al., 1996b) and increased nitrogenase activity (Iruthayathas et al., 1983; Alagawadi and Guar, 1988). There has been little investigation of species in the genus *Serratia* as potential PGPR. A study by Sneh et al.(1985) showed that *Serratia liquefaciens* can exert biological control effect on *Fusarium*-wilt in Carnation.

The results of experiment 2, which investigated the effects of PGPR application without *B. japonicum* addition on soybean growth and development, were different from those observed in experiment 1. At the unsterilized site, although both PGPRs did not increase plant nodule number, they increased total fixed nitrogen and N yield (Tables 3.6 and 3.7). At the sterilized site, both *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 increased nodule number, total nitrogen fixed, and N yield.

The average proportional increases in nodule number per plant, nodule weight per plant, total fixed nitrogen, and N yield were generally larger in experiment 1 than in experiment 2. There are two possible explanations for this observation. First, in experiment 1, PGPR were preincubated with *B. japonicum* for a period of at least 30 minutes before inoculation, during which PGPR-*B. japonicum* interactions might have occurred. Plant growth promoting rhizobacteria produce many phytohormones and signal molecules (Burr and Caesar, 1984; Davison, 1988; Kapulnik, 1991), such as genistein, the plant-to-bacteria signal involved in the soybean nodule infection and formation processes. Therefore, inoculation of soybean plants with *B. japonicum* and PGPR could have resulted in higher relative increases in nitrogen fixation and subsequent soybean growth and yield than *B. japonicum* or PGPR alone. Second, the

PGPR may have stimulated overall plant growth, leading to greater nitrogen demand by the developing soybean plants, leading in turn to greater nodulation and nitrogen fixation. The data of Zhang et al. (1996a), showing improved soybean photosynthesis and growth due to PGPR prior to the onset of nitrogen fixation, argue against the former of these two possibilities.

The cultivar AC Bravor tended to be more responsive to inoculation with PGPR plus *B. japonicum* than Maple Glen at the sterilized site (Tables 3.3 and 3.5). AC Bravor is a later-maturing cultivar and has a higher potential yield than Maple Glen (Conseil Des Productions Végétales du Québec recommendations); however, at crop physiological maturity it had lower nodule numbers, nodule weight per plant and total nitrogen yield than Maple Glen at the sterilized site (Tables 3.3 and 3.5). Therefore, there was less likely to be a nitrogen limitation to the growth and development of Maple Glen than AC Bravor. The increased nodule number and dry matter per plant of AC Bravor due to PGPR application would have reduced any nitrogen limitation; therefore, the increases in total fixed nitrogen and nitrogen yield were greater than those of Maple Glen. At the unsterilized site, the same pattern was found for nodule number and nodule weight per plant for PGPR 1-102 (Table 3.2). PGPR 2-68 showed a different pattern as it increased nodule number and nodule weight per plant for Maple Glen plants. An interaction also existed between PGPR strain and *B. japonicum* strain (Tables 3.2 and 3.4). The combination of USDA110 with PGPR 2-68 or 1-102 resulted in greater increases in total fixed nitrogen, fixed nitrogen as a percentage of total plant nitrogen, and nitrogen yield than was the case for strain 532C (Table 3.4).

In the second experiment, plots were not inoculated with *B. japonicum*, and inoculation with PGPR did not increase nodule number at the unsterilized site. However, nodule number increased in the early growing season and at the final harvest at the sterilized site (Table 3.6). This resulted in an increase in the total fixed nitrogen, tissue N concentration, and N yield (Table 3.7). It seems that the PGPRs were able to stimulate the native soil *B. japonicum*, resulting in increased soybean nodulation and N_2 fixation in the absence of other soil microflora which might have interfered with this effect.

In summary, this is the first field experiment showing that co-inoculation of *B. japonicum* with PGPR increased soybean nodulation and nitrogen fixation. PGPR application increased nodule dry matter per plant and hastened the onset of nitrogen fixation, especially for early-planted soybean (unsterilized site). Total fixed nitrogen, fixed nitrogen as a percentage of total plant nitrogen, and total nitrogen yield were all increased in at least some cases due to PGPR application. Interactions existed between PGPR application and soybean cultivar indicating that PGPR applied to potentially more nitrogen-stressed plants was more effective. Overall, from this study it was clear that preincubation of *B. japonicum* with PGPR can increase soybean nodulation and nitrogen fixation in the early part of the growing season when soil temperatures are low.

Table 3.1. The time of onset of nitrogen fixation by soybean plants inoculated with *B. japonicum* alone or co-inoculated either with PGPR 2-68 or 1-102 at both the unsterilized and sterilized sites. The onset of nitrogen fixation was indicated by detection of acetylene reduction activity.

Site	PGPR	June 13	June 15	June 17	June 21	June 23	June 28
unsterilized	2-68	0/16	2/16	13/16	—	—	—
	1-102	0/16	6/16	16/16	—	—	—
	Control	0/16	1/16	9/16	—	—	—
	Probability	NS	0.05	0.05			
sterilized	2-68	—	—	—	0/12	11/12	12/12
	1-102	—	—	—	0/12	8/12	12/12
	Control	—	—	—	0/12	1/12	9/12
	Probability				NS	0.05	5.05

Values in the table indicate the number of plots in which nitrogen fixation was detected over the total number of plots. Within each site, the data were compared by the Cochran Q test.

Table 3.2. Effects of PGPR strain, *B. japonicun* strain, and soybean cultivar on soybean nodule number, nodule weight, and nodule nitrogen concentration at two harvest stages at the unsterilized site (experiment 1).

			Sampling on June 17					Sampling on August 13		
PGPR	*B. japonicum*	cultivar	Nodule number	Nodule weight		Nodule nitrogen		Nodule number	Nodule weight (mg)	
				($plant^{-1}$)	($Nodule^{-1}$)	($mg\ g^{-1}$)	($mg\ plant^{-1}$)		($plant^{-1}$)	($nodule^{-1}$)
1-102	USDA110	AC Bravor	13.92	24.60	2.78	42.81	2.75	84.17	519.40	6.18
		Maple Glen	14.00	97.13	7.94	34.14	3.33	68.67	646.55	9.53
	532C	AC Bravor	10.58	42.20	4.40	40.99	1.71	71.00	632.89	9.36
		Maple Glen	15.33	56.82	3.61	39.44	2.29	90.42	804.54	9.61
2-68	USDA110	AC Bravor	13.33	78.86	6.00	40.26	3.17	52.92	502.96	10.49
		Maple Glen	13.30	76.27	6.35	40.27	3.08	103.00	923.00	9.01
	532C	AC Bravor	11.33	52.18	4.90	44.57	2.32	43.33	537.64	12.60
		Maple Glen	14.92	86.43	6.52	39.15	3.35	90.75	742.23	8.49
Control	USDA110	AC Bravor	18.33	11.57	0.62	47.63	0.57	37.83	380.99	10.07
		Maple Glen	19.33	15.73	0.82	32.46	0.51	34.08	410.64	13.03
	532C	AC Bravor	10.50	7.27	0.70	46.38	0.34	46.25	427.32	11.67
		Maple Glen	12.33	6.67	0.53	41.09	0.29	60.38	440.39	10.54
$LSD_{0.05a}$			8.29	19.59	2.78	10.86	1.79	33.32	274.11	4.83
$LSD_{0.05b}$			9.29	20.33	3.33	10.25	1.64	35.86	321.77	6.80
PGPR			NS	***	**	NS	***	**	*	NS
B.japonicum			*	*	NS	NS	NS	NS	NS	NS
cultivar			NS	***	*	***	NS	**	***	NS
PGPR **B.japonicum*			NS	NS	NS	NS	NS	NS	NS	NS
PGPR*cultivar			NS	**	NS	NS	NS	**	NS	NS
PGPR**B.japonicum**cultivar			NS	***	**	NS	NS	NS	NS	NS

Values represent the five plants in the ^{15}N microplot (area equal to 0.12 m^2) from each subplot unit. Means within the same column were compared by an ANOVA protected LSD test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, **, and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

Table 3.3. Simple effect means for PGPR strain and soybean cultivar effects on soybean nodule number, nodule weight, and nodule nitrogen concentration at two harvest stages at the sterilized site (experiment 1).

		Sampling on June 17					Sampling on August 13		
PGPR	cultivar	Nodule number	Nodule weight		Nodule nitrogen		Nodule number	Nodule weight (mg)	
			(plant^{-1})	(Nodule^{-1})	(mg g^{-1})	(mg plant^{-1})		(plant^{-1})	(nodule^{-1})
1-102	AC Bravor	11.90	21.69	1.78	34.56	0.79	57.58	180.13	3.35
	Maple Glen	14.17	29.17	2.26	33.20	0.95	78.17	211.59	2.66
2-68	AC Bravor	16.08	22.27	1.21	28.82	0.67	55.08	113.99	2.17
	MapleGlen	21.25	33.78	1.66	35.78	1.19	61.00	108.04	1.90
Control	AC Bravor	14.10	15.23	1.18	28.48	0.44	43.00	97.11	2.19
	Maple Glen	20.75	21.18	1.03	32.53	0.69	59.50	125.93	2.14
$LSD_{0.05a}$		14.33	18.62	0.94	8.93	0.61	21.33	73.77	1.05
$LSD_{0.05b}$		12.09	20.79	0.98	11.34	0.58	25.83	77.14	1.47
PGPR		NS	NS	*	NS	NS	*	**	NS
cultivar		NS	**	NS	NS	***	***	NS	*
PGPR*cultivar		NS	NS	NS	NS	NS	NS	NS	NS

Values represent the five plants in the ^{15}N microplot (area equal to 0.12 m^2) of each subplot unit. Means within the same column were compared by an ANOVA protected LSD test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, **, and *** indicate no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

Table 3.4. Simple effects means for PGPR strain, *B. japonicum* strain, and soybean cultivar for fixed nitrogen, nitrogen concentration and yield at final harvest stages at the unsterilized site (experiment 1).

PGPR	*B. japonicum*	cultivar	N-difference Total fixed N (kg ha^{-1})	N-difference Fixed N as % of total plant N	^{15}N dilution Total fixed N (kg ha^{-1})	^{15}N dilution Fixed N as % of total plant N	N Concentration (mg g^{-1})	N yield (kg ha^{-1})
1-102	USDA110	AC Bravor	249.46	56.88	113.96	26.44	25.20	417.39
		Maple Glen	166.04	49.50	105.66	31.48	30.93	333.98
	532C	AC Bravor	87.30	34.09	80.98	31.25	28.84	255.23
		Maple Glen	79.95	33.26	76.66	31.29	31.79	247.88
2-68	USDA110	AC Bravor	214.99	53.54	115.08	29.16	26.57	382.92
		Maple Glen	162.37	47.81	121.74	37.34	24.41	330.31
	532C	AC Bravor	152.24	48.11	92.24	28.76	25.28	323.08
		Maple Glen	114.85	39.09	86.75	30.36	29.26	282.79
Control	USDA110	AC Bravor	87.76	34.40	59.32	23.69	27.93	255.69
		Maple Glen	127.58	42.58	81.38	27.75	24.82	295.52
	532C	AC Bravor	123.64	40.51	69.87	23.87	28.81	288.26
		Maple Glen	41.54	19.2	57.66	27.60	28.01	209.48
$LSD_{0.05a}$			53.90	8.89	23.38	5.53	4.25	51.22
$LSD_{0.05b}$			49.73	8.99	22.32	5.10	4.30	49.65
PGPR			***	***	***	***	NS	***
B.japonicum			***	***	***	NS	***	***
cultivar			***	***	NS	***	*	***
PGPR *B.japonicum			***	**	*	*	NS	***
PGPR*cultivar			NS	NS	NS	NS	***	NS
PGPR*B.japonicum*cultivar			***	***	NS	NS	*	***

Values represent the five plants in the ^{15}N microplot (area equal to 0.12 m^2) of each subplot unit. Means within the same column and site were analyzed by an ANOVA protected LSD test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, **, and *** indicate no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

Table 3.5. Simple effects means of PGPR strain and soybean cultivar on fixed nitrogen, nitrogen concentration and yield at final harvest stages at the sterilized site (experiment 1).

		N-difference		^{15}N dilution			
PGPR	Cultivar	Total fixed N (Kg ha^{-1})	Fixed N as % of total plant N	Total fixed N (Kg ha^{-1})	Fixed N as % of total plant N	N Concentration (mg g^{-1})	N yield (Kg ha^{-1})
1-102	ACBravor	100.30	38.14	59.21	21.90	24.23	268.33
	MapleGlen	36.60	19.03	38.87	18.89	28.05	198.65
2-68	AC Bravor	143.15	44.63	88.14	28.06	27.23	311.08
	Maple Glen	86.38	33.66	47.35	19.89	25.09	250.89
Control	ACBravor	68.98	26.68	46.80	20.33	26.18	236.91
	Maple Glen	66.25	26.58	48.88	22.25	26.13	236.69
$LSDa_{0.05}$		53.94	14.59	18.26	8.57	6.19	59.27
$LSDb_{0.05}$		47.35	12.54	18.06	8.00	4.97	52.53
PGPR		**	**	**	NS	NS	**
Cultivar		**	**	***	*	NS	***
PGPR*Cultivar		NS	NS	***	*	NS	NS

Values represent the five plants in th ^{15}N microplot (area equal to 0.12 m^2) of each subplot unit. Means within the same column and *B. japonicum* strain or or soybean cultivar were analyzed by an ANOVA protected LSD test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the main plot factor. NS, *, **, and *** indicate no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

Table 3.6. Main effects of PGPR applied alone onto seed in furrow at the time of planting on soybean nodule number, nodule weight at both sterilized and unsterilized sites (experiment 2).

		First sampling*			Sampling on August 13		
Site	PGPR	Nodule number	Nodule weight (mg)		Nodule number	Nodule weight (mg)	
			($plant^{-1}$)	($nodule^{-1}$)		($plant^{-1}$)	($nodule^{-1}$)
unsterilized site							
	1-102	3.38	13.00	3.78	20.83	262.50	13.21
	2-68	3.30	14.38	4.56	19.92	250.54	13.97
	Control	3.80	18.00	4.63	22.70	286.42	12.22
	Probability	NS	NS	NS	NS	NS	NS
sterilized site							
	1-102	9.33	16.86	1.79	18.46	52.44	2.89
	2-68	10.50	12.00	1.35	15.13	48.40	3.10
	control	4.30	9.28	1.94	14.50	36.44	2.68
	Probability	0.05	NS	NS	0.05	NS	NS

* The date of first sampling was the same as experiment 1 for the unsterilized site, June 17, for the sterilized was June 28. Means within the same column and site were analyzed by an ANOVA protected t test.

Table 3.7. Effects of PGPR applied alone onto soybean seeds in the furrow at the time of planting on fixed nitrogen tissue, nitrogen concentration and yield at final harvest at both the unsterilized and sterilized sites (experiment 2).

Treatment	N-difference		^{15}N dilution			
	Total fixed N (kg ha^{-1})	Fixed N as % of total plant N	Total fixed N (kg ha^{-1})	Fixed N as % of total plant N	N Concentration (mg g^{-1})	N yield (kg ha^{-1})
Unsterilized site						
1-102	49.76	22.62	64.90	29.82	22.03	217.69
2-68	114.79	40.00	83.45	31.03	21.41	278.54
Control	9.17	4.92	36.64	21.34	23.16	177.10
Probability	0.05	0.05	0.05	0.05	NS	0.05
Sterilized site						
1-102	77.86	27.06	44.38	19.19	25.47	241.85
2-68	49.00	20.88	41.40	19.37	25.22	214.40
Control	32.83	19.00	36.06	18.47	17.67	205.27
Probability	0.05	0.05	NS	NS	0.05	0.05

Values represent the five plants in the ^{15}N microplot (area equal to 0.12 m^2) from each subplot unit. Means within the same column and site were analyzed by an ANOVA protected t test.

Preface to Section 4

Section 4 is comprised of a manuscript by N. Dashti, R.K. Hynes, and D.L. Smith, accepted by the journal of *plant and soil* for publication in 1996. The format has been changed to conform as much as possible to a consistent format within this thesis. All literature cited in this section is listed in the reference section at the end of the thesis. Each table for section 4 is presented at the end of this section.

Following the demonstration that some PGPR increase soybean nodulation and nitrogen fixation under short season conditions, this section addresses the possibility that this technology not only accelerates soybean nitrogen-fixing ability, but also increases soybean final grain and protein yield.

Section 4

APPLICATION OF PLANT GROWTH-PROMOTING RHIZOBACTERIA TO SOYBEAN [*Glycine max* (L.) Merr.] INCREASES PROTEIN AND DRY MATTER YIELD UNDER SHORT-SEASON CONDITIONS

4.1. Abstract

We previously reported that application of plant growth-promoting rhizobacteria (PGPR) increased soybean growth and development and, specifically, increased nodulation and nitrogen fixation over a range of root zone temperatures (RZTs) in controlled environment studies. In order to expand on the previous studies, field experiments were conducted on two adjacent sites, one fumigated with methyl bromide and one nonfumigated, in 1994. Two experiments were conducted at each site, one involving combinations of two soybean cultivars and two PGPR strains, the other involving the same factors, but also in combination with two strains of *Bradyrhizobium japonicum*. Soybean grain yield and protein yield were measured. The results of these experiments indicated that co-inoculation of soybean with *B. japonicum* and *Serratia liquefaciens* 2-68 or *Serratia proteamaculans* 1-102 increased soybean grain yield, protein yield, and total plant protein production, compared to the nontreated controls, in an area with low spring soil temperatures. Interactions existed between PGPR application and soybean cultivar, suggesting that PGPRs applied to cultivars with higher yield potentials were more effective. PGPRs applied into the rhizosphere without addition of *B. japonicum* only increased plant leaf area and seed number at the fumigated site. Overall, inoculation of soybean plants with PGPRs in the presence of *B. japonicum* increased soybean grain yield, grain protein yield, and total plant protein production under short season conditions.

4.2. Introduction

Soybean [*Glycine max* (L.) Merr.] is a subtropical legume and, as such temperatures of 25 to 30°C are optimal for its growth, nodulation and N_2 fixation. In areas with relatively short growing seasons, temperature is the major limiting factor for soybean growth and development. When soybean is grown under conditions of suboptimal root zone temperature (RZT) (below 25°C), plant growth and development are inhibited, and final total dry matter production and grain yield decrease (Summerfield and Wien, 1982). In eastern Canada soybean production is at its northernmost North American limit.

Over the last two decades the understanding of rhizosphere biology has progressed with the discovery of a specific group of microorganisms, known as plant growth promoting rhizobacteria (PGPR), that colonize the plant root and promote plant growth (Kloepper et al., 1980a,b). Plant growth-promoting rhizobacteria have been shown to increase plant yields 10 to 30% in non-legume crops such as potato, radish, and sugar beet (Kloepper et al., 1980a,b).

The beneficial effects of the PGPR are direct plant growth promotion, mobilization of insoluble nutrients (e.g. phosphate) resulting enhancement of uptake by the plant (Lifshitz et al., 1987), and production of antibiotics toxic to soil-borne pathogens (De-Ming and Alexander, 1988). Co-inoculation studies with PGPR and *B. japonicum* have also demonstrated that increased soybean plant root and shoot weight, grain yield, plant vigour, nodulation and nitrogen fixation can result from the presence of the PGPR (Verma et al., 1986; Yahalom et al., 1987; Li and Alexander, 1988). Compared to inoculation with *B. japonicum* alone, soybean yield increased following inoculation with a mixed culture of *B. japoniucm* and certain PGPR strains (Yahalom et al., 1987). Similarly, increases in grain yield, nodule dry matter, and nitrogenase activity were also obtained in chick pea inoculated with a mixture of *Azospirillum brasilense* and *Rhizobium* strains (Rai, 1983). Recently, tripartite associations of soybean plants, nitrogen-fixing bacteria, and PGPR, or vesicular arbuscular mycorrhizae were investigated at different root zone temperatures (RZTs) (Zhang et al., 1995c; Zhang et al., 1996a,b). Zhang et al. (1996a,b) found that some PGPR increased legume plant growth, development, nodulation and nitrogen fixation over a range of temperatures, and especially under low RZT conditions.

In an attempt to overcome the decreases in soybean grain and protein yield commonly seen under low soil temperature conditions, mixtures of *B. japonicum* and

PGPR were used to inoculate soybean plants grown over a range of RZTs under controlled environment conditions (Zhang et al., 1996a,b) to improve either nodulation, nitrogen fixation, or plant growth and development. Application of PGPR increased soybean plant growth, development, nodulation, and nitrogen fixation, especially under low RZTs. However, to date, there have been no investigations of whether these positive effects exist under field conditions in short season areas. Therefore, the objectives of this study was to test the hypotheses that: 1) co-inoculation of *B. japonicum* with PGPR in cool spring soybean production areas increases soybean grain and grain yield, and 2) PGPRs applied directly into the soil rhizosphere, without *B. japonicum*, increase soybean protein and grain yield under short-season field conditions.

4.3. Materials and methods

4.3.1. Field layout and site preparation

Two experiments were conducted at the Emile A. Lods Research Centre, McGill University, Macdonald Campus, Montreal, Canada. They were performed at two adjacent sites. One site was fumigated with methyl bromide (50 g m^{-2}) applied under a plastic canopy for 72 hours, to prevent possible interference from soil microfloral or faunal elements that might obscure PGPR treatment effects. Three days elapsed between removal of the fumigation canopy and planting. The soil of the other site was left nonfumigated.

The experimental design was a 3 x 2 x 2 factorial organized in a randomized complete block split-plot with four replications. The first experiment included three factors, PGPR application, *B. japonicum* strain, and soybean cultivar. The main-plot units consisted of PGPR strain applications (no-PGPR application as a control, *Serratia liquefaciens* 2-68 and *Serratia proteamaculans* 1-102). The two strains tested were chosen based on the results of a previous controlled environment experiment (Zhang et al., 1996a,b). The subplot units were formed by the combination of soybean cultivars and *B. japonicum* strains. The soybean cultivars, Maple Glen and AC Bravor were selected as they have been developed for production under the short season, cool conditions of eastern Canada. Both are widely grown and yield well in this area. The

B. japonicum strains tested were 532C (Hume and Shelp, 1990) and USDA110, both of which are or have been included in commercial inoculants used in eastern Canada. In the second experiment two factors were tested, PGPR strains, and soybean cultivars. The design of the experiment was the same as experiment 1. Three levels of PGPR applications (no-PGPR control, *S. liquefaciens* 2-68, and *S. proteamaculans* 1-102) formed the main plot units, and two soybean cultivars (Maple Glen and AC Bravor) were the subplot units. At the nonfumigated site, each sub-plot (2 x 3 m) consisted of four rows of plants with 40 cm between rows. The space between plots was 80 cm and between replications 1 m. At the fumigated site, each sub-plot was 1.6 x 2 m and consisted of three rows of plants, also with 40 cm between rows. The space between plots was 80 cm and between replications, 2 m. The soil type at both sites was a Chicot light sandy loam. In the previous year, 1993, this experimental field was planted with oat and barley, while in 1992 it was used to produce a crop of green manure alfalfa. The soil nitrogen available for soybean uptake proved to be reasonably high. The average nitrogen accumulation in non-nodulating Evans seeded at the same site was 167 kg ha^{-1}. Potassium and phosphate were provided by spring application of 340 kg ha^{-1} of 5-20-20, N, P_2O_5, K_2O (recommended procedures following a soil test).

4.3.2. Inoculum preparation

For experiment 1, the inoculum was produced by culturing *B. japonicum* strains 532C and USDA110 in yeast extract mannitol broth (Vincent, 1970) in 2000 mL flasks shaken at 125 rpm at room temperature (23-25°C). The PGPR strains were cultured in *Pseudomonas* media (Polonenko et al. 1987) in 2000 mL flasks shaken at 250 rpm at room temperature (21-23°C). After both *B. japonicum* and PGPR reached the stationary phase (7-days for *B. japonicum* and 1.5-days for PGPR), they were subcultured under the same conditions as described above. When the subculture reached the log phase (3-days for *B. japonicum* and 1-day for PGPR), *B. japonicum* and PGPR strains were each adjusted with distilled water to an A_{620} and A_{420} value, respectively, giving a cell density of 2 x 10^8 cells mL^{-1}. Before inoculation equal volumes of *B. japonicum* and PGPR cultures were mixed and allowed to stand for a minimum of 30 min at room temperature.

4.3.3. Planting methods

Seed of the soybean cultivars 'Maple Glen' and 'AC Bravor' were surface-sterilized in sodium hypochlorite (2% solution containing 4 mL L^{-1} Tween 20), then rinsed several times with distilled water (Bhuvaneswari et al., 1980). The seeds were planted by hand on May 11 and 18 at the nonfumigated and fumigated sites, respectively. The delay in planting the fumigated site was due to the extra time required for the methyl bromide application. Twenty mL of combined *B. japonicum*-PGPR inoculum (for experiment 1), or the same volume and cell density of PGPR inoculum or the same volume of distilled water (no PGPR application) (for experiment 2) per one meter row were applied by syringe directly onto the seed in the furrow. Cross contamination was prevented throughout planting and all subsequent data collection procedures by alcohol sterilization of all implements used. Following emergence seedlings were thinned to achieve a stand of 500,000 plants ha^{-1} (20 plants m^{-1} of row).

4.3.4. Data collection

Plant samples were taken on August 13, at which time plants were at reproductive stage 6 (pod containing a green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf [Fehr et al., 1971]), to investigate growth variables, such as leaf number, leaf area, pod number and seed number. Leaf number and area per plant were determined using a Delta-T area meter (Delta-T Devices Ltd., Cambridge, UK). Pod number and seed number per plant were counted by hand. Grain yield was determined from a one meter row of plants taken from the middle row of each plot. Plants were harvested by hand at harvest maturity, then shelled with a plot combine (Wintersteiger, Salt Lake City, UT), oven-dried at 70°C for at least 48 hours, and weighed. Six additional plants, also from the middle row, were hand-harvested, and oven-dried at 70°C, after which seeds were manually separated from shoots. Total shoot weight and harvest index were determined from these plants, which had been enclosed within wire mesh from flowering to maturity to allow the collection of senescent leaves. The dried seeds from each plot were ground using a Moulinex coffee mill (Moulinex Appliances Inc., Virginia Beach, VA, USA). The nitrogen concentration of seeds was then determined by Kjeldahl analysis (Kjeltec system, Tecator AB, Hoganas, Sweden). The protein concentration was calculated by multiplying the nitrogen concentration by 6.25.

4.3.5. Statistical analysis

Results were analyzed statistically by analysis of variance using the Statistical Analysis System (SAS) computer package (SAS Institute Inc., 1988). When analysis of variance showed significant treatment effects, the least significant difference (LSD) test was applied to make comparisons among the means at the 0.05 level of significance (Steel and Torrie, 1980).

4.4. Results

4.4.1. Temperature and seed emergence

The average daily temperature for both air and soil (at a depth of 5 cm) was below 15°C through early June, and remained below 20°C until mid-July. These conditions slowed the rate of seedling emergence, particularly for the plants at the nonfumigated site (early planted). For the May 11 planting, at the nonfumigated site, seedlings emerged on May 25, 14 days after planting (DAP). At the fumigated site, the seeds were planted on May 18 and the seeding emerged at 9 DAP, only 2 days after the nonfumigated site. Consequently, there was no effect of the delayed planting due to fumigation on subsequent plant growth and yield.

4.4.1.1. Experiment 1

The nodule number of uninoculated plants in experiment 2 indicated that the native soil population of *B. japonicum* in the nonfumigated soil was low, with uninoculated plants forming few nodules. However, at the fumigated site, fumigation with methyl bromide was not completely effective and the un-inoculated plants in experiment 2 formed also few nodules. Therefore neither of the two interactions relating to *B. japonicum* strain nor the three way interaction were tested at the fumigated site.

Many growth variables, such as plant height, time of crop maturity, harvest index, and seed moisture content at harvest maturity, were not affected by the inoculation of PGPR at either site (data not shown). Overall, leaf number was increased by PGPR application. The leaf area of AC Bravor receiving USDA110 was increased by inoculation with a mixture of *B. japonicum* and PGPR at the nonfumigated site (Table 4.1), while at the fumigated site leaf area was increased by PGPR application across all levels of *B. japonicum* strain and soybean cultivar, except for PGPR 2-68 with Maple Glen (Table 4.2). Differences between the two PGPR strains were not observed for any plant growth variables at either site.

Averaged over the two PGPR strains, the number of seeds formed on AC Bravor inoculated with a mixture of *B. japonicum* USDA110 and PGPR increased by 52% at the nonfumigated site (Table 4.1), while at the fumigated site the number of seeds of AC Bravor plants receiving *B. japonicum* and PGPR increased by 58% (Table 4.2). This increase in seed number was due to increases in their pod numbers (Tables 4.1 and 4.2). Since seed number per plant increased and seed protein concentration did not decrease, total grain yield and protein yield also increased. At the nonfumigated site, averaged over the two PGPR strains, the final grain yield of AC Bravor and Maple Glen inoculated with a mixture of *B. japonicum* USDA110 and PGPR increased by 23 and 21%, respectively (Table 4.1). At the fumigated site the grain yields of AC Bravor receiving *B. japonicum* and either *S. proteamaculans* 1-102 or *S. liquefaciens* 2-68 were 23 and 29% higher than plants receiving only *B. japonicum* (non-PGPR control plants), respectively (Table 4.2). Again, there was no difference between the two PGPR strains for increase in soybean grain yield.

The main effect of PGPR on protein concentration was significant at both sites. Averaged over all the treatments, the protein concentrations of plants receiving PGPR application were 7.5 and 5.1% higher than those of the non-PGPR control plants at the nonfumigated and fumigated sites, respectively (Tables 4.1 and 4.2). Because both final grain yield and protein concentration were increased by inoculating with a mixture of *B. japonicum* and PGPR, the final grain protein and total plant protein yields also increased at both sites. At the nonfumigated site, the grain protein yield of AC Bravor receiving the mixture of USDA110 and *S. proteamaculans* 1-102 was 22% higher than its corresponding control plants (Table 4.1). At the fumigated site, the protein yield of AC Bravor plants receiving *S. liquefaciens* 2-68, and *S. proteamaculans* 1-102 application increased by 60, and 50% over AC Bravor receiving only *B. japonicum* (Table 4.2). The total plant protein yield increase due to PGPR application generally followed the same pattern as the grain protein yield (Tables 4.1 and 4.2).

Two way interactions between either PGPR strain and soybean cultivar, or PGPR strain and *B. japonicum* strain existed for most of the yield related variables investigated in these studies at both sites (Tables 4.1 and 4.2). The combination of AC Bravor and *B. japonicum* USDA110 was more sensitive to PGPR application than Maple Glen and 532C.

4.4.1.2. Experiment 2

Plant growth-promoting rhizobacteria, directly applied onto seeds in the furrow at the time of planting, did not have any effect on growth variables and yield compared to control plants at the nonfumigated site (Table 4.3) but increased leaf area and seed number at the fumigated site (Table 4.4). Generally speaking, at the fumigated site, the effects of PGPR application directly into rhizosphere soil, on soybean growth variables, yield components, and final grain and protein yield were different from those observed in experiment 1.

4.5. Discussion

Plant growth-promoting rhizobacteria strains *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 were selected following previous studies (Zhang et al., 1996a,b) in which nine PGPR strains were tested for effects on soybean plant growth, development, nodulation and nitrogen fixation over a range of RZT under controlled environment conditions. Specifically, *S. liquefaciens* 2-68 performed well at optimal RZT (25°C), while *S. proteamaculans* 1-102 performed best at suboptimal RZTs ranging from 18 to 15°C. In our current studies, co-inoculation with PGPR and *B. japonicum* improved plant growth, development, yield components, and final grain and protein yield in the presence and absence of methyl bromide fumigation. Application of PGPR, without *B. japonicum,* directly onto the seeds in the furrow at the time of planting increased only leaf area and seed number at the fumigated site. These results agreed with those previously found under controlled environment conditions (Zhang et al., 1996a). However, the effects of PGPR application on plant growth, development, and final grain and protein yield were not different between *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102. This could be due to variations in field soil temperature during the entire soybean growing season.

Inoculation of soybean plants with a mixture of *B. japonicum* and PGPR not only increased plant dry matter accumulation, but also increased grain protein and total protein production at both sites in experiment 1 (Tables 4.1 and 4.2). Zhang et al. (1996b) reported that co-inoculation of some PGPR with *B. japonicum* could reduce the negative effects of low RZT on soybean nodulation and nitrogen fixation. In addition, a recent study at McGill University found that co-inoculation of PGPR and *B. japonicum* accelerated the processes of soybean nodulation and the onset of nitrogen fixation under short-season field conditions (Dashti et al., unpublished). Sprent (1979) postulated that an increase of 10% in the period of nodule activity of a grain legume, particularly between the onset of nitrogen fixation and the attainment of maximum fixation, could double the seasonal level of nitrogen fixation. In a controlled environment experiment, the onset of nitrogen fixation by plants co-inoculated with *B. japonicum* and the most effective PGPR strains began 2 to 3 days earlier than those receiving only *B. japonicum* (Zhang et al., 1996b). Therefore, it is possible that application of PGPR increased grain and total protein yield under field conditions.

The mechanisms of growth and nitrogen fixation promotion by PGPR are not well understood; however, a wide range of possibilities have been suggested, including both direct and indirect effects. The direct effects include an increase in mobilization of insoluble nutrients followed by enhancement of uptake by the plants (Lifshitz et al., 1987), production of antibiotics toxic to soil-born pathogens (Li and Alexander, 1988), and production of plant growth regulators that stimulate plant growth (Gaskins et al., 1985). Indirect effects include positive effects on symbiotic nitrogen fixation by enhancement of root nodule number or mass (Grimes and Mount, 1984; Yahalom et al., 1987; Zhang et al., 1996b) and increased nitrogenase activity (Iruthayathas et al., 1983; Alagawadi and Gaur, 1988). There has been little investigation of species in the genus *Serratia* as potential PGPR. A study by Sneh et al.(1985) has shown that *Serratia liquefaciens* can exert biological control effect on *Fusarium*-wilt in Carnation.

The results of experiment 2, which investigated the effects of PGPR application without *B. japonicum* addition on soybean growth and development, were different from those observed in experiment 1. At the nonfumigated site, although both PGPR *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 numerically increased plant growth variables such as leaf area and seed numbers, there were no statistically significant differences among treatments (Table 3). At the fumigated site, both *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 increased leaf area and seed number.

The average proportional increases in plant growth variables, yield components and final grain and protein yield were generally larger in experiment 1 than in experiment 2. There are two possible explanations for this observation. First, as described above, PGPR application may not have acted effectively in the absence of *B. japonicum* under short season field conditions. Second, in experiment 1, PGPR were preincubated *with B. japonicum* for a period of at least 30 minutes before inoculation, during which a number of PGPR-*B. japonicum* interactions might have occurred. Plant growth promoting rhizobacteria produce many phytohormones and signal molecules (Burr and Caesar, 1984; Davison, 1988; Kapulnik, 1991), such as genistein, a plant-to-bacteria signal involved in the soybean nodule infection and formation processes. Preincubation of *B. japonicum* inocula with genistein increased nodule number and hastened the onset of nitrogen fixation at suboptimal RZT under both controlled environment (Zhang and Smith, 1995a) and short-season field (Zhang and Smith, 1996c) conditions. Therefore, co-inoculation of soybean plants with *B. japonicum* and PGPR could have resulted in higher relative increases in nitrogen fixation and subsequent soybean growth and yield than with *B. japonicum* or PGPR alone.

Table 4.1. Effects of PGPR application, *B. japonicum* strains, and soybean cultivars on soybean growth variables, grain yield, final protein and grain yield in a nonfumigated field trial (experiment 1)

			Leaf(plant^{-1})		Number (plant^{-1})		1000 seeds	Yield (t ha^{-1})			Protein Concentration
PGPR	*B. japonicum*	Cultivar	Number	Area(cm^2)	Pod	Seed	Weight(g)	Grain	Grain Protein	Total Protein	(mg\g)
1-102	USDA110	AC Bravor	31.3	976.2	29.0	69.0	190.33	5.4	2.2	2.7	401.3
		Maple Glen	19.2	518.3	20.2	50.0	190.33	4.3	1.7	2.0	386.5
	532C	AC Bravor	27.8	977.1	28.7	68.3	161.00	5.0	1.9	2.1	395.4
		Maple Glen	15.2	425.6	19.6	48.3	190.33	3.1	1.3	1.5	365.0
2-68	USDA110	AC Bravor	31.3	830.0	28.3	70.3	194.67	5.4	1.9	2.7	391.5
		Maple Glen	26.3	805.4	31.3	75.0	189.00	4.9	1.9	2.3	416.6
	532C	AC Bravor	29.0	1044.2	27.0	45.0	195.00	4.6	1.7	2.1	386.5
		Maple Glen	16.4	628.0	18.3	46.0	169.33	3.3	1.3	1.8	380.5
Control	USDA110	AC Bravor	16.0	516.0	16.0	45.7	180.33	4.4	1.8	2.1	373.8
		Maple Glen	21.4	543.0	24.4	57.3	195.67	3.8	1.4	1.8	365.0
	532C	AC Bravor	21.1	910.0	26.3	59.0	201.67	4.9	1.8	2.0	380.5
		Maple Glen	14.8	533.0	24.1	35.0	196.00	3.1	1.2	2.1	333.5
LSD_a			7.6	240.4	7.0	15.4	36.20	0.5	0.3	0.3	32.4
LSD_b			6.9	239.5	6.7	16.3	35.14	0.6	0.4	0.3	30.0
PGPR			***	**	**	***	NS	***	***	***	***
B.japonicum			**	NS	NS	***	NS	***	***	***	**
cultivar			***	***	NS	NS	NS	***	***	***	**
*PGPR *B.japonicum*			NS	NS	***	**	NS	***	***	***	NS
PGPR*cultivar			**	**	*	NS	NS	**	***	***	*
*PGPR*B.japonicum**cultivar			NS	NS	NS	***	NS	NS	***	***	NS

Means of leaf number and leaf area, and seed number represent four plants from each subplot unit, at crop maturity. Means of 1000 seed weight calculated from the one meter middle row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference at the 0.1, 0.05, and 0.01 levels, respectively.

Table 4.2. Effects of PGPR application and soybean cultivars on soybean growth variables, grain yield, final protein and grain yield in a fumigated field trial (experiment 1).

		leaf(plant^{-1})		number(plant^{-1})		1000 seed	yield (t ha^{-1})			protein concentration
PGPR	cultivar	number	area(cm^2)	pod	seed	weight(g)	grain	grain protein	total protein	(mg/g)
1-102	AC Bravor	52.9	893.3	46.2	80.5	160.5	3.8	1.5	1.8	396.7
	Maple Glen	41.8	473.7	36.0	57.7	152.1	2.1	0.8	1.0	382.6
2-68	AC Bravor	56.2	995.8	38.2	73.1	164.7	4.0	1.6	2.0	396.5
	Maple Glen	23.8	239.0	19.8	47.8	155.8	2.3	1.1	1.3	373.8
Control	AC Bravor	28.2	472.8	24.7	48.7	154.0	3.1	1.0	1.4	371.2
	Maple Glen	16.0	189.7	18.7	47.8	155.2	3.1	1.0	1.4	365.7
LSD_a		7.1	165.0	8.2	12.6	11.6	0.6	0.3	0.3	15.4
LSD_b		6.0	134.9	9.8	15.4	8.7	0.7	0.3	0.4	12.4
PGPR		***	***	***	**	**	***	***	***	***
cultivar		***	***	***	***	*	***	***	***	***
PGPR*cultivar		***	***	**	***	*	***	***	***	*

Means of leaf number and leaf area, and seed number represent four plants from each subplot unit, at crop maturity. Means of 1000 seed weight calculated from the one meter middle row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference or significant differences at the 0.1,0.05, and 0.01 levels, respectively.

Table 4.3. Effects of PGPR application and soybean cultivars on soybean growth variables, grain yield, final protein and grain yield in a nonfumigated field trial (experiment 2)

		leaf(plant^{-1})		number(plant^{-1})		1000 seeds	yield (t ha^{-1})		
PGPR	cultivar	number	area(cm^2)	pod	seed	weight(g)	grain	grain protein	total protein
1-102	AC Bravor	32.0	754.8	28.3	52.7	177.1	3.2	1.2	1.7
	Maple Glen	23.6	500.8	26.3	73.3	170.9	3.3	1.0	1.4
2-68	AC Bravor	30.3	767.8	20.7	49.7	179.0	3.6	1.2	1.4
	Maple Glen	28.7	866.2	21.7	50.7	177.9	3.2	1.0	1.4
Control	AC Bravor	23.0	740.4	16.0	35.3	151.3	3.4	1.5	1.7
	Maple Glen	22.7	720.3	15.3	35.0	163.1	3.5	1.0	1.6
LSD_a		15.6	510.2	14.8	29.7	20.0	0.4	0.4	0.4
LSD_b		14.6	445.9	13.9	29.6	18.3	0.4	0.3	0.3
PGPR		NS	NS	NS	NS	NS	NS	NS	NS
cultivar		NS	NS	NS	NS	NS	NS	NS	NS
PGPR*cultivar		NS	NS	NS	NS	NS	NS	NS	NS

Means of leaf number and leaf area, and seed number represent four plants from each subplot unit, at crop maturity. Means of 1000 seed weight calculated the one meter middle row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

Table 4.4. Effects of PGPR application and soybean cultivars on soybean growth variables, grain yield, final protein and grain yield in a fumigated field trial (experiment 2).

		leaf ($plant^{-1}$)		number ($plant^{-1}$)		1000 seeds	yeild ($t\ ha^{-1}$)		
PGPR	cultivar	number	area(cm^2)	seed	pod	weight(g)	grain	grain protein	total protein
1-102	Maple Glen	38.0	627.3	82.7	34.0	162.5	2.7	1.1	1.1
	AC Bravor	62.0	987.5	86.0	29.5	147.7	3.3	1.3	1.6
2-68	Maple Glen	27.0	338.5	45.7	20.2	151.0	2.6	0.9	1.3
	AC Bravor	53.2	756.2	77.7	33.5	151.3	2.7	1.2	1.2
Control	Maple Glen	40.5	849.3	62.2	26.2	155.3	3.0	1.3	1.6
	AC Bravor	41.3	683.8	39.6	21.5	148.0	2.5	0.9	1.3
LSD_a		19.3	143.0	13.2	6.1	10.0	0.9	0.3	0.4
LSD_b		17.0	161.6	16.1	8.9	11.0	0.7	0.3	0.3
PGPR		NS	**	***	NS	NS	NS	NS	NS
cultivar		NS	***	NS	NS	NS	NS	NS	NS
PGPR*cultivar		NS	***	***	***	NS	NS	NS	NS

Means of leaf number and leaf area, and seed number represent four plants from each subplot unit, at crop maturity. Means of 1000 seed weight calculated from the one meter middle row of each subplot unit at harvest maturity. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference or significant differences at the 0.1, 0.05, and 0.01 levels, respectively.

Preface to Section 5

Section 5 is comprised of a manuscript by N. Dashti, R.K. Hynes, and D.L. Smith, prepared for publication in 1996. The format has been changed as much as possible to a consistent format within this thesis. All literature cited in this thesis are listed in the reference section at the end of the thesis. Each table is presented at the end of this section.

After demonstrating that PGPR were able to increase soybean growth, development, nodulation and nitrogen fixation under short season conditions in sections 3 and 4, this section tested wether the ability of PGPR to survive, grow and multiply in the field under short-season conditions was related to their ability to stimulate soybean nitrogen fixation, growth and yield under field conditions.

Section 5

GROWTH, SURVIVAL, AND ROOT COLONIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA UNDER SHORT-SEASON CONDITIONS

5.1. Abstract

Co-inoculation of *B. japonicum* with plant growth promoting rhizobacteria (PGPR) has been shown to increase soybean [(*Glycine max*. (L.) Merr.] nodulation, nitrogen fixation, growth, and development compared to controls not inoculated with PGPR, in an area with low spring soil temperatures. We studied the growth and survival of two plant growth promoting rhizobacteria (PGPR) *Serratia liquefaciens* 2-68 and *Serratia proteamaculans* 1-102 inoculated on soybean plants under cool spring conditions. Two field experiments were conducted on two adjacent sites, one fumigated with methyl bromide and one not fumigated, in 1994. Two experiments were conducted at each site, one involving combinations of two soybean cultivars, two strains of *Bradyrhizobium japonicum* and two PGPR strains, the other involving the same factors, but without *B. japonicum*. The population density of PGPR applied into the rhizosphere without addition of *B. japonicum* increased over time indicating that the PGPR were able to survive and proliferate. Overall, PGPR inoculated onto soybean roots was able to grow, survive, and colonize the roots better at the fumigated site. PGPR 2-68 achieved higher population densities on both the root and in the soil (rhizosphere) which demonstrates their ability to colonize the root more rapidly.

5.2. Introduction

Soybean [*Glycine max* (L.) Merr.] is a subtropical legume which requires temperatures of 25 to 30°C for its growth, nodulation and N_2 fixation. Temperature is the major limiting factor for soybean growth and development in areas with relatively short growing seasons. When soybean is grown under conditions of suboptimal root zone temperature (RZT) (below 25°C), plant growth and development are inhibited, and final total dry matter production and grain yield decrease (Summerfield and Wien, 1982). In eastern Canada soybean production is at its northernmost North American limit.

recently, understanding of rhizosphere biology has advanced with the discovery of a specific group of microorganisms, called plant growth promoting rhizobacteria (PGPR), that colonize the plant root and promote plant growth (Kloepper et al, 1980a). Following reports have evidenced that PGPR can exert positive effects on different hosts, including barley (Iswandi et al., 1987), bean (Anderson and Guerra, 1985), canola (Kloepper et al., 1988b), cotton (Backman and Turner, 1989; Greenough and Batson, 1989), peanut (Turner and Backman, 1991), lentil (Chanway et al., 1989) pea (Chanway et al., 1989), rice (Sakthivel and Gnanamanikam, 1987), and soybean (Polonenko et al., 1987). Several genera, chiefly *Pseudomonas*, *Azospirillum*, *Azotobacter*, and *Bacillus*, have been demonstrated to promote plant growth (Rovira, 1963; Gaskins et al., 1985).

A wide range of mechanisms has been suggested by which PGPR increase plant growth, such as direct plant growth promotion, mobilization of insoluble nutrients (e.g. phosphate) resulting enhancement of uptake by the plant (Lifshitz et al., 1987), production of antibiotics (De-Ming and Alexander, 1988) and associative N_2 fixation (Chanway and Holl, 1991a). Co-inoculation of PGPR and *B. japonicum* can increase soybean plant root and shoot weight, grain yield, plant vigour, nodulation and nitrogen fixation (Verma et al., 1986; Yahalom et al., 1987; Li and Alexander, 1988). Compared to inoculation with *B. japonicum* alone, soybean yield increased following inoculation with a mixed culture of *B. japoniucm* and certain PGPR strains (Yahalom et al., 1987). Recently, tripartite associations of soybean plants, nitrogen-fixing bacteria, and PGPR, or vesicular arbuscular mycorrhizae were investigated at different root zone temperatures (RZTs) (Zhang et al., 1995c; Zhang et al., 1996a,b). Zhang et al. (1996a,b) found that some PGPR increased legume plant growth, development, nodulation and nitrogen fixation over a range of temperatures, and especially under low RZT conditions.

Root colonization is the mechanism by which bacteria survive inoculation onto seeds or into soil, proliferate in response to seed or root exudates rich in carbohydrates and amino acids (Kloepper et al., 1985), bind to the root surface (Suslow, 1982, Weller, 1983), and disperse over the developing root system (Kloepper et al., 1980a, Suslow and Schroth, 1982; Weller, 1984). In the root zone, rhizobacteria are efficient microbial competitors that can displace native root-colonizing microorganisms (Kloepper and Schroth, 1981a). The bacteria are distributed in the rhizosphere in a log normal pattern with a sharp increase as the root surface is approached (Loper et al., 1984) and are sporadically located along roots (Bahme and Schroth, 1987).

The detection and enumeration through time of populations of rhizobacteria following inoculation under field conditions is important for understanding root colonization. In addition, the ability to distinguish between the introduced bacteria and the indigenous microflora is limited by the available marking systems (Kloepper and Beauchamp, 1992). The most commonly used marker for root colonization studies has been antibiotic resistance (Kluepfel et al., 1991).

In this study, PGPR were marked to follow their populations during plant development. This study presents quantitative data concerning PGPR colonization of soybean plant roots and their persistence during the life of the plant. The objectives of this study were to : 1) evaluate the growth and survival of two PGPR strains inoculated on soybean plants under short season conditions, and 2) evaluate the relationship between the ability of PGPR to colonize the roots of the soybean plants and their ability to stimulate soybean nodulation, nitrogen fixation, plant growth and yield.

5.3. Materials and methods

5.3.1. Field layout and site preparation

Two experiments at two adjacent sites were conducted at the Emile A. Lods Research Centre, McGill University, Macdonald Campus, Montreal, Canada. One site was fumigated with methyl bromide (50 g m^{-2}) applied under a plastic canopy for 72 hours, to prevent possible interference from soil microfloral or faunal elements. Three days elapsed between removal of the fumigation canopy and planting. The soil of the other site was left unfumigated.

The experiment was arranged in a 3 x 2 x 2 factorial organized in a randomized complete block split-plot with four replications. Experiment 1 included three factors, PGPR application, *B. japonicum* strain, and soybean cultivar. The main-plot units consisted of PGPR strain applications (*Serratia liquefaciens* 2-68 and *Serratia proteamaculans* 1-102). The two strains tested were chosen based on the results of a previous controlled environment experiment (Zhang et al., 1996a,b). The subplot units were formed by the combination of soybean cultivars and *B. japonicum* strains. The soybean cultivars, Maple Glen and AC Bravor were selected as they have been developed for production under the short season, cool conditions of eastern Canada. Both are widely grown and yield well in this area. The *B. japonicum* strains tested were 532C (Hume and Shelp, 1990) and USDA110, both of which are or have been included in commercial inoculants used in eastern Canada. In experiment 2, only two factors were tested, PGPR strains, and soybean cultivars. The design of experiment 2 was the same as experiment 1. Two levels of PGPR (*S. liquefaciens* 2-68, and *S. proteamaculans* 1-102) formed the main plot units, and two soybean cultivars (Maple Glen and AC Bravor) were the subplot units.

5.3.2. Inoculum preparation

For experiment 1, the inoculum was produced by culturing *B. japonicum* strains 532C and USDA110 in yeast extract mannitol broth (Vincent, 1970) in 2000 mL flasks shaken at 125 rpm at room temperature (23-25°C). Two rifampacin resistant PGPR strains (1-102 *Serratia proteamaculans* and 2-68 *Serratia liquefaciens)* were tested in this experiment. The PGPR strains were cultured in *Pseudomonas* media (Polonenko et al. 1987) in 2000 mL flasks shaken at 250 rpm at room temperature (21-23°C). After both *B. japonicum* and PGPR reached the stationary phase (7-days for *B. japonicum* and 1.5-days for PGPR), they were subcultured under the same conditions as described above. When the subculture reached the log phase (3-days for *B. japonicum* and 1-day for PGPR), *B. japonicum* and PGPR strains were each adjusted with distilled water to an A_{620} and A_{420} value, respectively, giving a cell density of 2 x 10^8 cells mL^{-1}. Before inoculating, equal volumes of *B. japonicum* and PGPR cultures were mixed and allowed to stand for a minimum of 30 min at room temperature.

5.3.3. Planting methods

Seed of the soybean cultivars 'Maple Glen' and 'AC Bravor' were surface-sterilized in sodium hypochlorite (2% solution containing 4 mL L^{-1} Tween 20), then rinsed several times with distilled water (Bhuvaneswari et al., 1980). The seeds were planted by hand on May 11 and 18 at the unfumigated and fumigated sites, respectively. The delay in planting the fumigated site was due to the extra time required for the methyl bromide application. Twenty mL of combined *B. japonicum*-PGPR inoculum (for experiment 1), or the same volume and cell density of PGPR inoculum (for the treatment) or the same volume of distilled water (for the control) (for experiment 2) were applied by syringe directly onto the seed in the furrow. Cross contamination was prevented throughout planting and all subsequent data collection procedures by alcohol sterilization of all implements used.

5.3.4. Enumeration of bacteria

Root and soil samples were collected twice during the experiment. The first sample was collected June 20 when the plants were beginning to bloom (one open flower at any node on the main stem(R1)). The second sample was collected on August 13 when the plants had reached reproductive stage 6 [pod(s) containing a green seed that fills the pod cavity at one of the four uppermost nodes on the main stem with a fully developed leaf] (Fehr et al., 1971). Number of culturable PGPR cells in the bulk soil (rhizosphere) was determined by transferring 10 g of the soil into a 250-mL Erlenmeyer flask, containing 90 mL of sterile distilled water. The flasks were shaken for 30 min at room temperature (200 rpm). Serial 10-fold dilutions were made and plated on King's B agar (proteose peptone, 20 g; K_2HPO_4, 1.5 g; $MgSO_4$ $7H_2O$, 1.5 g; glycerol, 10 g; agar, 15 g; demineralized water, 1L; pH 7.2) supplemented with 100 mg L^{-1} cycloheximide (to inhibit fungal growth) and 100 mg L^{-1} rifampicin. Plant roots and adhering soil were separated from the bulk soil (rhizosphere) by careful, manual shaking. Roots and adhering soil were then transferred into a 250-mL Erlenmeyer flask, containing 90 mL of sterile distilled water. Shaking, dilution and plating procedures were similar to those described for the bulk soil (rhizosphere). The plates were incubated for 24 hr and the number of cfu g^{-1} of dry soil and root were calculated. For the purpose of this paper "root associated PGPR" are defined as the microbial population on both the root and the soil attached to the root, while "rhizosphere" is the 5-10 cm region around the plant root where materials released from the root increase the microbial population and its activities (Prescott et al., 1993).

5.3.5. Statistical analysis

Results were analyzed statistically by analysis of variance using the Statistical Analysis System (SAS) computer package (SAS Institute Inc., 1988). When analysis of variance showed significant treatment effects, the least significant difference (LSD) test was applied to make comparisons among the means at the 0.05 level of significance (Steel and Torrie, 1980).

5.4. Results

5.4.1. Temperature and seed emergence

The average daily temperature for both air and soil (at a depth of 5-10 cm) was below 15°C through early June, and remained around 20°C until mid-July. By August, the temperature start to drop until it reached 10°C by the end of September (Zhang and Smith, 1995a).

5.4.1.1. Experiment 1

The microbial densities associated with the root and in the soil (rhizosphere) of the soybean plants are presented in table 5.1. The data indicates differences among the PGPR strains. PGPR 2-68 showed more cfu g^{-1} root in the first sampling than PGPR 1-102, while there was no significant difference between the two PGPR at the second sampling at the unfumigated site. PGPR 2-68, which had higher population densities on the root, also showed higher microbial activities in the soil (rhizosphere) than PGPR 1-102. There was no difference between the two PGPRs in the cfu g^{-1} root or cfu g^{-1} soil at the second sampling of the unfumigated site (Table 5.1).

At the fumigated site, the same general pattern was observed as PGPR 2-68 showed higher cfu g^{-1} root in both the first and second sampling than PGPR 1-102 which indicate that this strain was able to multiply and colonize the plant roots more effectiently than PGPR 1-102. There was no difference between the two PGPR in the number of cfu g^{-1} root or cfu g^{-1} soil at the second sampling (Table 5.2). There was no difference observed between the two soybean cultivars, AC Bravor and Maple Glen, at both fumigated and unfumigated sites (Tables 5.1 and 5.2)

At the unfumigated site, the population density of both PGPR 2-68 and 1-102 on the root and in the soil had decreased by the second sampling, except in the case of PGPR 2-68 co-inoculated with *B. japonicum* onto soybean cultivar AC Bravor, where the population density remained very high both on the root and in the soil at the unfumigated site (Table 5.1). Conversly, the population density of both PGPR 1-102 and 2-68 increased by the second sampling both on the roots and in the soil at the fumigated site.

5.4.1.2. Experiment 2

There was no difference in the number of cfu g^{-1} root at the first sampling between PGPR 2-68 and 1-102, however, at the second sampling PGPR 2-68 inoculated directly into soybean cultivar AC Bravor had the highest population density (Table 5.3). There was no difference in the number of cfu g^{-1} soil between the first and second sampling for either PGPR (Table 5.3). There was not a great reduction in the population density of either PGPR associated with the root between the first and second sampling. At the fumigated site, there were more cfu g^{-1} root for PGPR 2-68 than PGPR 1-102 at the first sampling, while there was no difference between the two PGPR at the second sampling. PGPR 2-68 inoculated onto AC Bravor plants had higher population densities in the soil (rhizosphere) than PGPR 2-68 inoculated onto Maple Glen plants. There was no difference between the two PGPR in terms of the number of cfu g^{-1} root or cfu g^{-1} soil at the second sampling at the fumigated site (Table 5.4). There was an increase in the population densities for both PGPR associated with the root and in the soil (rhizosphere) by the second sampling (Table 5.4).

5.5. Discussion

Plant growth-promoting rhizobacteria strains *S. liquefaciens* 2-68 and *S.*

proteamaculans 1-102 were selected following previous controlled environment studies (Zhang et al., 1996a,b) in which nine PGPR strains were tested for effects on soybean plant growth, development, nodulation and nitrogen fixation over a range of RZTs under controlled environment conditions. *S. liquefaciens* 2-68 was shown to perform well at an optimal RZT (25°C), while *S. proteamaculans* 1-102 performed best at suboptimal RZTs ranging from 18 to 15°C.

Colonization of soybean plants varied among PGPR strains and soil conditions. In experiment 1, at the unfumigated site, PGPR 2-68 colonized soybean plant roots more efficiently than PGPR 1-102 at the first sampling, while there was no difference by the second sampling which indicates that PGPR 2-68 was able to grow and colonize the root more effectively initially but over time, PGPR 1-102 was able to grow and colonize the roots as effectively as PGPR 2-68. PGPR 2-68 was able to proliferate successfully in the soil (rhizosphere) as indicated by high cfu values at both samplings. Another interesting observation is that the population densities of both PGPR 2-68 and 1-102 with the different combinations of *B. japonicum* and soybean cultivars had decreased in the second sampling as compared to the first, except for the combonation of PGPR 2-68, *B. japonicum* strain USDA110 and soybean cultivar AC Bravor where the population density had increased in the second sampling compared to the first at the unfumigated site. These observations indicate that PGPR 2-68 cells can survive and colonize the roots of the soybean plants effectively in the presence of other soil microflora elements, and that they can tolerate the change in field conditions, including temperature, over time without reductions in population density.

At the fumigated site, where presumably, no other microflora competes with the PGPR, PGPR 2-68 showed the same pattern as in unfumigated conditions. In addition, the population density of both PGPR had increased by the second sampling as compared to the first. These observations suggest that both PGPR were able to survive and increase in number in the absence of other microflora that may compete with them and reduce their population density.

The results of experiment 2, in the absence of *B. japonicum*, were different from those observed in experiment 1. There was no difference between the two PGPR in the number of cfu g^{-1} root at the first sampling, while at the second sampling the combination of PGPR 2-68, *B. japonicum* USDA110 and AC Bravor had the highest number of cfu g^{-1} root. There was no difference in the cfu g^{-1} soil between the two PGPR strains at both samplings of the unfumigated site.

At the fumigated site, in experiment 2, the same pattern was seen as in experiment 1. PGPR 1-102 also had more cfu g^{-1} of both roots and rhizosphere soil. There was no difference in the number of cfu g^{-1} root or cfu g^{-1} soil between the two PGPR at the second sampling for both experiments.

The colonies which grew on the plates used to establish PGPR numbers in the rhizosphere and associated with the roots were all uniform in the appearance, indicating that the plates did not contain other rifampicin resistant soil bacteria

Root colonization by introduced bacteria is considered as an important step in the interaction of beneficial bacteria with the host plant. A rapid growth rate was suggested to be an important characteristic for successful rhizosphere colonization (Rovira et al., 1983; Schorth and Weinhold, 1986). De Weger et al. (1987) suggested that non-motile mutants colonize the roots less effeciently than the corresponding wild types, while others found that non-motile mutants and the corresponding wild types do not differ in their colonizing ability (Parke et al., 1986; Scher et al., 1988). Chemotaxis of bacteria to exudates was reported (Reinhold et al., 1985; Scher et al., 1985), but the direct relationship between chemotaxis and successful colonization remains unclear. Movement along the root was also reported to be very important for a successful root colonization (Chao et al., 1986; Schippers et al., 1987). Adherence has also been suggested as an important feature for rhizospheere competence and survival (Schippers et al., 1987; Vesper, 1987). Cells of bacteria in the genus *Serratia* are motile (Prescott, 1993).

Fluorescent pseudomonads, isolated from the crop rhizosphere, are characterized as a highly rhizosphere competent as they are capable of root colonization. This accounts for their predominance among the PGPR. Several traits of the Pseudomonads aid them in seed colonization, such as higher cell division and motility (Arora et al., 1983; Scher et al., 1985). However, these traits may not be directly relative to subsequent root colonization. For example, Howie et al. (1987) found that three nonmotile mutants of *P. fluorescence* colonized wheat roots as effectively as their motile parents. Flourescent pseudomonads are able to establish high population densities in the rhizosphere (Suslow, 1982; Bahme and Schorth, 1987), an important characteristic for the production of consistent plant growth responses (Kloepper et al., 1980a; Suslow, 1982; Kloepper et al., 1985; Bahme and Schroth, 1987; Klein et al., 1990; ; Kloepper et al., 1991; Parke, 1991;). In general, there has been little investigation of species in the genus *Serratia* as potential PGPR. A study by Sneh et al.(1985) has shown that *Serratia liquefaciens* can exert biological control effect on *Fusarium*-wilt in Carnation.

Studies on rhizosphere colonization have been reviewed recently by van Elsas and Heijnen (1990), Kloepper and Beauchamp (1992) and Kluepfel (1993). Van Elsas and Heijnen, (1990) reported that lack of consistent effectiveness of the inoculant prevent successful application of PGPR strains in to the soil. This always was related to ineffective colonization of the plant, as well as poor survival and/or low activity of the introduced population. Xu and Gross (1986b) and Bull et al. (1991), demonstrated a positive relationship between root colonization by a PGPR strain and disease suppression, suggesting that methodologies which improve root colonization may also improve the performance of a PGPR strain in soil. The extent and amount of root colonization needed by a PGPR strain to increase plant growth rely on numerous interrelated factors. The choice of methods used to try to increase rhizosphere colonization and plant growth has to take these factors into consideration (Stephens, 1994a).

Hebbar et al. (1992) reported that the colonization and spread of *Pseudomonas cepacia* (which acts as a bio-control agent against *Fusarium moniliforme*) on the roots and in the rhizosphere of maize depends on the amount of inoculum on the seed. However, this was not a universal observation. For example, the colonization of introduced pseudomonad strains on maize (Scher et al., 1984) and wheat (Juhnkle et al., 1989) was shown to be independent from the initial inoculum level. It is obvious that under certain conditions, increasing the level of inoculum could increase the rhizosphere competence of some, but not all, bacteria.

In a previous study (in preparation), we found that co-inoculation with PGPR and *B. japonicum* improved plant growth, development, yield components, and final grain and protein yield under field conditions at both fumigated and unfumigated sites. Also application of PGPR with the *B. japonicum* directly onto the seeds in the furrow at the time of planting also improved plant growth at the fumigated site. The effects of PGPR *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 on plant growth, development, and final protein were shown to be not different, this was attributed to variations in field soil temperature during the entire soybean growing season. In addition, a recent study at McGill university found that co-inoculation of PGPR and *B. japonicum* accelerated the processes of soybean nodulation and the onset of nitrogen fixation under short season condition (Dashti et al., unpublished).

Zhang et al., (1996a,b), in a controlled environment experiment, showed that both *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 stimulate plant growth, development, and plant photosynthesis. At an optimal RZT (25°C) *S. liquefaciens* 2-68 was reported to increase plant leaf development and dry matter accumulation, while at 15°C RZT *S. proteamaculans* 1-102 increased plant dry matter accumulation.

In summary, the results of this study indicated that PGPR 2-68 was able to grow and survive better than PGPR 1-102 under short season conditions. A previous work (unpublished data), has shown that the combination of PGPR 2-68 with AC Bravor plants had increased leaf area, seed number, and grain protein yield suggesting that there is a relationship between the ability of these PGPR to colonize the roots of the soybean plants and their ability to stimulate soybean nodulation, nitrogen fixation, plant growth and physiological activities under short season conditions.

Table 5.1. Root associated and rhizosphere PGPR colony forming units (log cfu gm dry root^{-1}) and (log cfu gm dry soil^{-1}) for PGPR strains, *B. japonicum* strains, and soybean cultivars in a nonfumigated field trial (experiment 1)

			(log cfu gm dry root^{-1})		(log cfu gm dry soil^{-1})	
PGPR	*B. japonicum*	cultivar	1st sampling	2nd sampling	1st sampling	2nd sampling
1-102	USDA110	AC Bravor	6.08	5.83	4.02	3.34
		Maple Glen	5.77	5.50	3.94	3.08
	532C	AC Bravor	5.66	5.25	4.18	2.98
		Maple Glen	5.59	5.24	3.71	3.82
2-68	USDA110	AC Bravor	6.70	7.16	4.52	4.76
		Maple Glen	6.58	6.15	4.66	2.96
	532C	AC Bravor	6.20	6.30	4.76	2.97
		Maple Glen	6.44	6.80	4.68	3.31
LSD_a			0.49	1.0	0.2	0.67
LSD_b			0.69	1.8	0.3	0.62
PGPR			*	NS	**	NS
B. japonicum			**	NS	**	*
cultivar			NS	NS	NS	NS
PGPR **B. japonicum*			NS	NS	***	**
PGPR*cultivar			NS	NS	*	***
PGPR**B. japonicum**cultivar			NS	NS	**	***

Means within the same column were analyzed by an ANOVA protected t test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference at the 0.1, 0.05, and 0.01 levels, respectively.

Table 5.2. Root associated and rhizosphere PGPR colony forming units (log cfu gm dry root^{-1}) and (log cfu gm dry soil^{-1}) for PGPR strains, *B. japonicum* strains, and soybean cultivars in a fumigated field trial (experiment 1)

			(log cfu gm dry root^{-1})		(log cfu gm dry soil^{-1})	
PGPR	*B. japonicum*	cultivar	1st sampling	2nd sampling	1st sampling	2nd sampling
1-102	USDA110	AC Bravor	5.78	6.94	3.24	4.91
		Maple Glen	5.61	6.57	3.34	5.09
	532C	AC Bravor	5.03	6.28	3.56	3.88
		Maple Glen	5.50	7.30	3.37	4.55
2-68	USDA110	AC Bravor	7.58	7.81	4.54	4.74
		Maple Glen	7.75	7.60	4.89	4.87
	532C	AC Bravor	7.56	7.89	4.44	4.50
		Maple Glen	7.13	7.30	4.53	5.21
LSD_a			0.5	1.2	0.86	0.95
LSD_b			0.8	1.3	0.72	0.78
PGPR			***	NS	***	NS
B. japonicum			***	NS	*	NS
cultivar			NS	NS	*	NS
PGPR **B. japonicum*			NS	NS	**	NS
PGPR*cultivar			NS	NS	**	NS
PGPR **B. japonicum**cultivar			**	NS	NS	NS

Means within the same column were analyzed by an ANOVA protected t test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference at the 0.1, 0.05, and 0.01 levels, respectively.

Table 5.3. Root associated and rhizosphere PGPR colony forming units (log cfu gm dry root^{-1}) and (log cfu gm dry soil^{-1}) for PGPR strains and soybean cultivars in a nonfumigated field trial (experiment 2)

		(log cfu gm dry root^{-1})		(log cfu gm dry soil^{-1})	
PGPR	cultivar	1st sampling	2nd sampling	1st sampling	2nd sampling
1-102	AC Bravor	5.66	5.32	3.99	2.98
	Maple Glen	5.81	5.54	3.68	3.78
2-68	AC Bravor	6.67	6.68	4.12	3.08
	Maple Glen	6.23	5.66	4.24	3.37
LSD_a		0.97	0.83	0.29	0.99
LSD_b		1.00	0.91	0.28	0.98
PGPR		NS	*	*	NS
cultivar		NS	NS	NS	NS
PGPR*cultivar		NS	**	*	NS

Means within the same column were analyzed by an ANOVA protected t test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference at the 0.1, 0.05, and 0.01 levels, respectively.

Table 5.4. Root associated and rhizosphere PGPR colony forming units (log cfu gm dry root^{-1}) and (log cfu gm dry soil^{-1}) for PGPR strains, *B. japonicum* strains, and soybean cultivars in a fumigated field trial (experiment 2)

		(log cfu gm dry root^{-1})		(log cfu gm dry soil^{-1})	
PGPR	cultivar	1st sampling	2nd sampling	1st sampling	2nd sampling
1-102	AC Bravor	5.74	6.3	3.69	3.67
	Maple Glen	5.30	6.7	2.97	4.44
2-68	AC Bravor	7.26	7.8	4.31	4.57
	Maple Glen	6.91	7.3	2.98	4.38
LSD_a		0.89	0.7	0.74	2.1
LSD_b		0.81	1.9	0.93	1.5
PGPR		***	NS	*	NS
cultivar		NS	NS	***	NS
PGPR*cultivar		NS	*	NS	NS

Means within the same column were analyzed by an ANOVA protected t test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparison of means across levels of the same main plot factor. NS, *, **, and *** indicated no significant difference at the 0.1, 0.05, and 0.01 levels, respectively.

Preface to Section 6

Section 6 is comprised of a manuscript by N. Dashti, R.K. Hynes and D.L. Smith, prepared for submition in 1996. The format has been changed to conform as much as possible to a consistent format within this thesis. All literature cited in this section are listed in the reference section at the end of the thesis. Each table is presented at the end of this section.

After showing that the ability of PGPR to stimulate soybean N_2 fixation and yield under field conditions, we attempted in this section, to show that the ability of PGPR, which peformed well in the field, to colonize the roots of soybean plants was directly affected by RZT and to relate these RZT effects to previously published performances of these strains under controlled environment conditions.

Section 6.

ROOT AND RHIZOSPHERE COLONIZATIONOF SOYBEAN [*Glycine max* (L.) MERR.] BY PLANT GROWTH PROMOTING RHIZOBACTERIA AT LOW ROOT ZONE TEMPERATURES

6.1. Abstract

Co-inoculation of plant growth promoting rhizobacteria (PGPR) with *B. japonicum* has been shown to increase soybean [(*Glycine max* (L.) Merr.] nodulation, nitrogen fixation, growth, and physiological activity at suboptimal root zone temperatures (RZTs). We studied the survival and growth of seven plant growth promoting rhizobacteria (PGPR) inoculated on soybean in a sterile rooting medium, under low RZTs. Three RZTs were tested: 25, 17.5 and 15°C. In general, population densities varied with temperature. At each temperature, populations of some PGPR strains increased either on the root or in the rooting medium (rhizosphere). Root zone temperature affected the distribution of PGPR populations between the root surface and in the rooting medium (rhizosphere). The strains with higher population densities on the root, which reflects their ability to colonize the root more rapidly, were as follows: 15°C- PGPR 1-102 *Serratia proteamaculans*, 17.5°C, G11-32 *Pseudomonas putida*, and 25°C 2-68 *Serratia liquefaciens*. These PGPR strains had lower population densities in the rooting medium (rhizosphere) at these temperatures. Other PGPR strains were not able to effectively colonize the roots of the soybean plants, and their population densities remained very high in the rooting medium (rhizosphere). The strains which colonized soybean roots best at 25 and 15°C were previously shown to be effective at promoting soybean growth at 25 and 15°C.

6.2. Introduction

Recently, there has been interest in the use of soil bacteria which, when applied to seeds, tubers or roots, are able to stimulate plant growth and crop yield. These organisms have been termed plant growth promoting rhizobateria (PGPR) (Kloepper et al., 1980a). PGPRs have been shown to increase plant yields by 10 to 30% in nonlegume crops such as potato, radish, sugar beet, wheat and canola.

A wide range of mechanisms has been postulated by which PGPR can increase plant growth such as: production of plant growth regulators that stimulate plant growth (Kloepper and Schroth, 1981a,b; Gaskins et al., 1985), supply of N by symbiotic nitrogen fixation, mobilization of insoluble nutrients (e.g phosphate) and subsequent enhancement of uptake by the plant (Lifshitz et al., 1987), production of antibiotics (De-Ming and Alexander, 1988), production of siderophores [high-affinity iron (III) chelators], and associative nitrogen fixation (Chanway and Holl, 1991a).

Root colonization is an active process and not a transitory relationship between bacteria and roots of the plant. It is defined as the process by which bacteria survive inoculation onto seeds or into soil, multiply relying on seed or root exudates rich in carbohydrates and amino acids (Kloepper et al., 1985), adhere to the root surface (Suslow, 1982, Weller, 1983), and colonize the root system (Kloepper et al., 1980a; Suslow and Schroth, 1982; Weller, 1984). PGPR can multiply and survive in the rhizosphere after inoculation. The bacteria are distributed in the rhizosphere in a log normal pattern with a sharp increase in population density as the root surface is approached (Loper et al., 1984) and are sporadically located along roots (Bahme and Schroth, 1987). Although colonization is difficult to measure, it is a prerequisite for effects on plant growth. The role of root colonization by PGPR was reviewed (Schroth and Hancock, 1982; Suslow, 1982).

Inoculation with root colonizing bacteria and *Rhizobium* has been demonstrated to affect symbiotic nitrogen fixation by enhancing root nodule number or mass (Singh and Subba Rao, 1979; Burns et al., 1981; Polonenko et al., 1987; Yahalom et al., 1987) and by increasing nitrogenase activity (Iruthayathas et al., 1983; Alagawadi and Guar, 1988). The mechanism by which nitrogen fixation is stimulated is unknown.

Soybean is a legume of tropical origin and, as such, has difficulty nodulating and fixing nitrogen below 25°C (Jones and Tisdale, 1921; Hardy et al., 1968; Roughley and Date, 1986; Legros and Smith, 1994; Lynch and Smith, 1994). Recently, the tripartite association of nitrogen-fixing bacteria, PGPR and soybean plants was investigated at different RZTs. Zhang et al. (1996a,b) has shown that co-inoculation of *B. japonicum* with some PGPR strains increased soybean nodulation and nitrogen fixation and increased soybean growth and development, but the stimulatory strains varied with RZT. Therefore, our objectives in the present study were: 1) to evaluate the growth and survival of nine PGPR strains inoculated on soybean plants at three RZTs, 2) to determine the ability of PGPR to colonize the roots of soybean plants under low RZTs, and 3) evaluate the relationship between the ability of these PGPR to colonize the roots of the soybean plants and their ability to stimulate soybean nodulation, nitrogen fixation, plant growth and physiological activities.

6.3 Materials and Methods

6.1.1. Plant materials

The soybean cultivar "Maple Glen" was used in these experiments. It was selected because it was developed for production in eastern Canada, where seasons are short and the springs are cool. It is widely grown and yields well in this area. Soybean seed was surface-sterilized in sodium hypochlorite (2% solution containing 4 ml L^{-1} Tween 20), then rinsed with distilled water several times (Bhuvaneswari et al., 1980). The seeds were then planted in trays containing a sterilized Turface (Applied Industrial Materials Corp., Illinois, USA):sand (1:1 vol.) mixture (rooting medium). Seven-day-old seedlings at the VC stage [unifoliolate leaves were unrolled sufficiently that the edges were not touching (Fehr et al., 1971)] were transplanted into sterilized 13 cm plastic pots containing the same medium, on a Conviron growth bench (Model GB48, Controlled Environments Ltd., Winnipeg, Manitoba). The growth bench light (300 μmol m^{-2} s^{-1}) was provided by cool white fluorescent tubes. The photoperiod was 16:8 h (day:night). As this work was attempting to isolate RZT effects, air temperature was held constant at 25°C. RZTs were controlled by circulating cooled water around the pots, with eight pots sealed to the bottom of each tank. A hole drilled in the tank bottom below each pot allowed the pots to drain when watered. Plants were then acclimatized for 24 hours prior to inoculation. During the period of plant growth, plants were watered with a modified 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. Prior to each watering the added Hoagland's solution was temperature adjusted to the treatment RZTs.

6.3.2. Inoculum

The *B. japonicum* inoculum was produced by culturing strain 532C (Hume and Shelp 1990) in yeast extract mannitol broth (Vincent 1970) in 250 mL flasks shaken at 125 rpm at room temperature. Strain 532C has been shown to perform well over a range of temperatures (Lynch and Smith 1993). Seven rifampicin resistant PGPR strains (Table 6.1) were tested in this experiment. The genetic marking of bacteria with antibiotic resistance for identification purposes allows the study of population dynamics of soil-inhabiting bacteria. All the PGPR strains were cultured in *Pseudomonas* media (Polonenko et al. 1987) in 250 mL flasks shaken at 250 rpm at room temperature. After reaching the stationnary phase (7-days for *B. japonicum* and 1.5-days for PGPR), both *B. japonicum* and PGPR were subcultured using the procedure described above. When they reached the log phase (3-days for *B. japonicum* and 1-day for PGPR), the *B. japonicum* culture was adjusted with distilled water to an $O.D._{620}=0.08$ (approximately 10^8 cells mL^{-1}) (Bhuvaneswari et al. 1980) and each of the PGPR strains was adjusted with distilled water to an $O.D._{420}$ value giving a cell density of 10^8 cells mL^{-1} (Table 6.2). Before inoculation *B. japonicum* 532C and an equal volume of the appropriate PGPR strain were incubated together for at least half an hour at room temperature without shaking. The inoculum was then cooled to the corresponding RZT and applied by pipette to the rooting medium at the base of the plant.

6.3.3. Experimental design

The experiment was arranged in a completely randomized split-plot design with 3 replications. The main-plot units consisted of three RZTs: 25, 17.5, and 15°C. The combinations of *B. japonicum* strain 532C and PGPR (Table 6.1) formed the sub-units and there were a total of 8 units within each main-plot. A *B. japonicum* only control was included in each replicate.

6.3.4 Enumeration of bacteria

The roots and rooting medium (rhizosphere) samples were collected 50 days after inoculation (DAI). Number of culturable PGPR cells in the rooting medium was determined by transferring 10 g of the rooting medium (containing no roots) into a 250-mL Erlenmeyer flask, containing 90 mL of sterile distilled water. The flasks were shaken for 30 min at room temperature (200 rpm). Serial 10-fold dilutions were made and plated on King's B agar (proteose peptone, 20 g; K_2HPO_4, 1.5 g; $MgSO_4$ $7H_2O$, 1.5 g; glycerol, 10 g; agar, 15 g; demineralized water, 1L; pH 7.2) supplemented with 100 mg L^{-1} cycloheximide (to inhibit fungal growth) and 100 mg L^{-1} rifampicin. Plant roots and adhering rhizosphere rooting medium were separated from the bulk rooting medium by careful, manual shaking. Roots and adhering rhizosphere rooting medium were then transferred into a 250-mL Erlenmeyer flask, containing 90 mL sterile distilled water. Shaking, dilution and plating procedures were similar to those described for the bulk rooting medium. The plates were incubated for 24 hr and the cfu g^{-1} of dry rooting medium and root were calculated. For the purpose of this paper " rhizosphere" is defined as a region around the plant root where materials released from the root increase the microbial population and its activities (Prescott et al., 1993).

6.3.5 Statistical analysis

Results were analyzed statistically by analysis of variance (ANOVA) using the SAS system (SAS Institute Inc. 1988). When analysis of variance showed significant treatment effects Duncan's multiple range test was applied to make comparisons among the means at the 0.05 or 0.01 levels of significance (Steel and Torrie 1980).

6.4. Results

The microbial densities associated with the roots of the soybean plants are presented in Table 6.2. The data indicate differences among the PGPR strains that vary with temperatures. The density of total bacteria was higher for PGPR 31-34, G11-32, 36-43, 63-49, and 2-68 than 1-102 and 1-104 at 25°C.

At 17.5°C PGPR 2-68, 31-34, G11-34 and 63-49 showed more cfu g^{-1} root than 36-43. PGPR 36-43 was less able to colonize soybean roots at 17.5 than at 25°C.

PGPR strain 31-34, and 2-68 had high population densities on the root and less microbial activities in the rooting medium (Table 6.3). G11-32 had high population densities both associated with the root and in the rooting medium.

At 15°C RZT strains 1-102, 31-34, G11-32 and 36-43 had high cfu g^{-1} root values, which indicate that these isolates were able to multiply and colonize the plant roots at this low RZT (Table 6.1). Strains 63-49, 2-68, and 1-104 had lower microbial densities associated with the root, which indicated their relative inability to colonize roots under low RZT conditions. At 25°C most of the PGPR produced more cfu g^{-1} root than at 17.5 or 15°C RZT, however, PGPR 1-102 and 1-104 produced fewer cfu g^{-1} root at 25° than at 15°C.

PGPR 1-102 showed the highest rate of root associated colonization at 15°C, and the lowest at 25°C. Clearly, the effect of RZT on root colonization by PGPR is very strain specific. When the cfu g^{-1} dry root was compared to the cfu g^{-1} dry rooting medium across PGPR strains and RZTs, it was clear that isolate 1-102 colonized soybean roots more extensively at 15°C RZT but had lower rhizosphere cell densities. The same was true for isolate 2-68 at 25°C RZT where it colonized the roots more extensively at 25°C RZT and its cell densities were lower in the rooting medium.

At 25°C 1-102 had the lowest population density on the root and in the rooting medium indicating that this RZT was probably outside of its optimum range for survival and growth. PGPR strains 31-34 and G11-32 were able to colonize soybean roots effectively at all three RZT tested (15, 17.5 and 25°C). PGPR 63-49 was able to colonize the roots very effectively at 17.5 and 25 but not 15°C. PGPR G11-32 had the highest cfu g^{-1} dry root and in the rooting medium, at all three RZT.

In general, PGPR cell densities were higher associated with the roots than in the rooting medium at all three RZTs. The single exception was PGPR G11-32 which had high microbial densities both in the rooting medium and associated with the roots at all RZTs tested. PGPR 1-104 had the lowest cfu values both on the roots and in the rooting medium at all three RZTs.

The total microbial populations both associated with the roots of the soybean plants and in the rooting medium are presented in Table 6.4. The total bacterial density was higher for PGPR 31-34, G11-32, and 2-68 than 36-43, 63-49, 1-102 and 1-104 at 25°C.

At 17.5°C PGPR 2-68 and G11-34 showed higher total bacterial densities than 31-34, 36-43, 63-49, 1-102 and 1-104. PGPR 1-102 has the lowest total microbial population at both 17.5 and 25°C, while PGPR strain G11-32 and 2-68 had the highest total population densities at 17.5 and 25°C.

At 15°C RZT strains 1-102, 31-34, and G11-32 had the highest total bacterial densities which indicate that these isolates were able to multiply and colonize the plant roots effectively at this low RZT. Strains 63-49 and 2-68 had the lowest total microbial densities which indicated their relative inability to proliferate effectively at low RZTs.

6.5.Discussion

Colonization of soybean plants varied among PGPR strains and over temperatures. Some PGPR strains colonized soybean plant roots more efficiently than others, others proliferated more successfully in the rooting medium. At 15°C RZT, PGPR 1-102 had a high number of cfu g^{-1} dry root (Table 6.2) while it had the lowest cfu g^{-1} dry rooting medium (Table 6.3). At 17.5°C RZT PGPR 31-34 and 63-49 had high numbers of cfu g^{-1} dry root (Table 6.2) while they had the lowest number of cfu g^{-1} dry rooting medium (Table 6.3). The same pattern was seen for PGPR 2-68 which at 25°C had a high number of cfu g^{-1} dry root (Table 6.2) but had the lowest cfu g^{-1} dry rooting medium value (Table 6.3). These results indicated that the colonization of soybean roots by PGPR is altered by temperature.

These observations suggest that a PGPR inoculated onto soybean roots at its optimum temperature will tend to colonize the root extensively but will proliferate relatively less in the rooting medium, while outside the optimum temperature range the reverse may be true.

The total microbial populations varied among the PGPR strains depending on the RZTs. Some PGPR strains had higher overall population densities at higher temperatures (25°C) while others had higher total population densities at lower temperatures (15°C). This indicated that PGPR strains can survive at their optimum temperature range, grow and colonize the soybean roots effectively and still have high population densities in the rooting media.

In general, rhizosphere populations varied with temperatures. However, there are few studies regarding environmental effects such as soil temperature (especially low RZT), pH, salinity etc. on PGPR effectiveness (Garibaldi, 1971; Broeze et al., 1978).

The genus *Serratia* has received little attention as a PGPR, most of the research on PGPR has been focused on pseudomonads. Fluorescent pseudomonads, isolated from the crop rhizosphere, are highly rhizosphere competent as they are capable of root colonization, which accounts for their predominance among the PGPR. Pseudomonads have several characters which appear to aid them in seed colonization, such as rapid cell division and motility (Arora et al., 1983; Scher et al., 1985). However, these traits may not relate to their ability to colonize the root or stimulate plant growth. For example, Howie et al. (1987) showed that three nonmotile mutants of *P. fluorescence* colonized wheat roots as effectively as their respective motile parents. Several other genera, chiefly *Azospirillum*, *Azotobacter*, and *Bacillus*, have been demonstrated to promote plant growth (Rovira, 1963; Gaskins at al., 1985).

Many of the PGPR strains which stimulate the growth of potatoes were identified as fluorescent pseudomonads (Baker, 1968; Brown, 1974; Burr and Caeser, 1984; Xu and Gross, 1986a; Bahme and Schroth, 1987). They have the ability to establish high population densities in the rhizosphere (Suslow, 1982; Bahme and Schorth, 1987), a characteristic essential for the production of stable plant growth responses (Kloepper et al, 1980a; Suslow, 1982; Kloepper et al., 1985; Bahme and Schroth, 1987; Klein et al, 1990; Kloepper et al., 1991; Parke, 1991).

Several factors were suggested that may influence colonization such as the ability of certain microorganisms to attach to the root. The presence of polysaccharides on the cell surface seems to play a role in plant-microbe associations such as crown gall by *Agrobacterium tumefaciens* (Matthysse et al., 1981; Douglas et al., 1982, 1985; Thomashow et al., 1987) and the nodulation of legumes by *(Brady)Rhizobium* species (Dazzo et al., 1984; Leigh et al., 1985; Cangelosi et al., 1987; Smit et al., 1987). Studies on rhizosphere colonization have been reviewed recently by van Elsas and Heijnen (1990), Kloepper and Beauchamp (1992) and Kluepfel (1993). The major problem for a successful application of PGPR strains in soil was the lack of stable effectiveness of the inoculant (van Elsas and Heijnen, 1990). This were always due to ineffective colonization of the plant, as well as low activity of the introduced population. Xu and Gross (1986b) and Bull et al. (1991), demonstrated a positive relationship between root colonization by a PGPR strain and disease suppression, suggesting that methodologies which improve root colonization may also improve the performance of a PGPR strain in soil.

Several studies suggest that the ability of a bacterial strain to survive and grow in the rhizosphere may dependent on the plant species (van Peer and Schippers, 1989, Beauchamp et al., 1991) and even plant cultivar (Weller, 1986). One method of increasing rhizosphere colonization by certain PGPR strains may be through maximizing the bacterial inoculum load on the seed. Hebbar et al. (1992) reported that the colonization and spread of *Pseudomonas cepacia* (which acts as a bio-control agent against *Fusarium moniliforme*) on the roots and in the rhizosphere of maize correlated with the amount of inoculum on the seed. However, this was not always true. For example, root colonization by introduced pseudomonad strains on maize (Scher et al., 1984) and wheat (Juhnkle et al., 1989) has been shown to be independent of the initial inoculum level. It is obvious that under certain conditions, increasing the level of inoculum may increase the rhizosphere competence of some, but not all, bacteria.

Beneficial bacteria which are introduced into the rhizosphere are involved in a complex of biological interactions with the host plant. They are nourished by root exudates and as a result dependent on the host plant. At the same time, they may affect the host plant by inducing physiological changes (Kloepper et al., 1988b).

In a recent study, Zhang et al. (1996a,b) have shown that co-inoculation of *B. japonicum* with some PGPR strains increased soybean nodulation and nitrogen fixation and increased soybean growth and development, but the stimulatory strains vary with RZTs. PGPR 1-102 *S. proteamaculans* was shown to increase soybean root nodulation. nitrogen fixation, plant growth and development at suboptimal RZTs (17 and 15°C) while PGPR 2-68 *S. liquefaciens* was shown to have the same effect on soybean plants at 25°C. Our results showed that PGPR 1-102 *Serratia proteamaculans* which was most stimulatory at 15°C (Zhang et al., 1996a,b), showed best root colonization at that temperature, while PGPR 2-68 (*Serratia liquefaciens*) which was shown to stimulate plant growth and development at 25°C (Zhang et al., 1996a), showed best root colonization at that temperature.

Our data indicate that some PGPR are able to grow better and can colonize soybean roots effectively at lower RZTs while, at the same time, their numbers in the rooting medium decline. Those PGPR which are not able to colonize the root will be present in the rooting medium in relatively high numbers. Other PGPR are able to colonize roots at higher temperatures, and their numbers were higher in the root and lower in the rooting medium at such temperatures. Also, in as much as the PGPR strains which colonized the roots well have been shown to be best at promoting soybean growth at each RZT, some strains that colonized the roots well were shown not to be effective at plant growth promotion (Zhang at al., 1996a,b). It seems likely that an ability to effectively colonize plant roots, as affected by PGPR strain, plant type and environmental conditions, it is necessary, but not sufficient condition for the stimulation of plant growth.

In summary, the results of this study indicated that the ability of PGPR strains to grow, multiply, and survive is strain specific and temperature dependent. Some PGPR strains are able to grow and multiply effectively at low RZTs and colonize the roots effectively. Others are able to grow and multiply effectively at higher RZTs and colonize the roots effectively. It was shown that in the optimum RZT range an effective PGPR will be heavily present on the root, but be relatively less present in the surrounding rooting medium, while outside the optimum RZT range the reverse was true. Also, the ability of the PGPR to colonize the root effectively could be a prerequisite to the stimulation of growth, nodulation and nitrogen fixation of soybean plants.

Table 6.1. Species identification and the geographical origins of the PGPR strains tested in this study.

Strain number	Strain identification	Source
31-34	*Pseudomoas putida*	Mould Bay Soil
G11-32	*Pseudomonas putida*	Grise Fiord, NWT
36-43	*Pseudomonas fluorescens*	Canola rhizosphere Clyde, AB
63-49	*Pseudomonas fluorescens*	Canola rhizosphere Winnipeg, MB
2-68	*Serratia liquefaciens*	James Bay Soil, NWT
1-102	*Serratia proteamaculans*	Yellowknife, NWT
1-104	*Pseudomonas putida*	Yellowknife, NWT

Table 6.2. Root associated PGPR colony forming units (log cfu gm dry root^{-1}) for different PGPR strains at different temperatures.

	(log cfu gm dry root^{-1})		
PGPR STRAINS	15°C	17.5°C	25°C
31-34	8.73	8.86	8.49
G11-32	8.81	8.92	8.71
36-43	8.55	7.93	8.29
63-49	8.11	8.55	8.59
2-68	8.21	8.80	8.61
1-102	8.90	8.29	7.61
1-104	8.42	8.38	7.85
$LSD_{0.05a}$	0.45		
$LSD_{0.05b}$	0.48		

Means within the same column were analyzed by an ANOVA protected LSD test. $LSD_{0.05a}$ is for comparisons of means within the same main-plot unit and $LSD_{0.05b}$ is for comparisons of means across levels of the main plot factor.

Table 6.3. Rhizosphere PGPR colony forming units (log cfu gm dry rooting media^{-1}) for different PGPR strains at three different temperatures.

	log cfu gm dry rooting media^{-1}		
PGPR STRAINS	15°C	17.5°C	25°C
31-34	5.08	4.48	5.45
G11-32	5.30	5.40	5.56
36-43	4.99	4.99	5.35
63-49	4.92	4.86	4.67
2-68	5.10	5.29	5.38
1-102	4.82	3.96	4.58
1-104	5.05	5.10	5.21
$LSD_{0.05a}$	0.17		
$LSD_{0.05b}$	0.16		

Means within the same column were analyzed by an ANOVA protected LSD test. $LSD_{0.05a}$ is for comparisons of means within the same main- plot unit and $LSD_{0.05b}$ is for comparisons of means across levels of the main plot factor

Table 6.4. Root associated and rhizosphere PGPR colony forming units (log cfu gm dry root^{-1} and rooting media^{-1}) for different PGPR strains at three different temperatures.

	log cfu gm dry root^{-1} and rooting media^{-1}		
PGPR STRAINS	15°C	17.5°C	25°C
31-34	8.7	8.8	8.5
G11-32	8.8	8.9	8.7
36-43	8.6	7.9	8.3
63-49	8.1	8.6	8.6
2-68	8.5	8.7	8.6
1-102	8.9	8.4	7.6
1-104	8.4	8.4	7.9
$LSD_{0.05a}$	0.4		
$LSD_{0.05b}$	0.5		

Means within the same column were analyzed by an ANOVA protected LSD test. $LSD_{0.05a}$ is for comparisons of means within the same main- plot unit and $LSD_{0.05b}$ is for comparisons of means across levels of the main plot factor.

Preface to Section 7

Section 7 is comprised of a manuscript by N. Dashti, R.K. Hynes, and D.L. Smith, prepared for submission in 1996. The format has been changed to conform as much as possible to a consistent format within this thesis. All literature cited in this section is listed in the reference section at the end of the thesis. Each table or figure is presented at the end of this section.

After having demonstrated that PGPR promote soybean nodulation, N_2 fixation and growth under cool spring soil conditions and that RZT play a definite role in the effectiveness of the PGPR in previous sections. In this section we attempted to determine the effects of PGPR on the early stages in the establishment of soybean nodules and how the stimulatory effect is transmitted from the PGPR to the soybean roots.

Section 7

PLANT GROWTH PROMOTING RHIZOBACTERIA AND SYMBIOSIS ESTABLISHMENT BETWEEN SOYBEAN [*Glycine max* (L.) Merr.] AND *Bradyrhizobium japonicum* AT LOW ROOT ZONE TEMPERATURES

7.1. Abstract

Low root zone temperatures (RZTs) have more effect on infection and early nodule development than on subsequent development of the nitrogen fixation symbiosis by soybean [*Glycine max* (L.) Merr.]. We have recently shown that some plant growth-promoting rhizobacteria (PGPR) stimulate nodule formation, and/or nitrogen fixation at low RZTs. However, there have been no studies regarding the effects of PGPR application on the infection of soybean at low RZTs. Two controlled environment experiments were conducted. In experiment 1, the effects of PGPR on the early stages of symbiosis establishment between soybean and *Bradyrhizobium japonicum* at low root zone temperatures were tested. Soybean plants were maintained at 25, 17.5, or 15°C RZT and inoculated with *B. japonicum* strain 532C alone (control), or *B. japonicum* with either *Serratia proteamaculans* (1-102), or *Serratia liquefaciens* (2-68). In experiment 2, PGPR cells were centrifuged from log phase cultures and the resulting supernatant was filter sterilized and tested for PGPR associated stimulation of the early stages of symbiosis development at 15°C and 25°C RZT. At 15°C, soybean plants were inoculated with *B. japonicum* strain 532C alone, or inoculated with *B. japonicum* treated with PGPR growth media alone, with *S. proteamaculans* (1-102), with *S. proteamaculans* (1-102) supernatant applied only once (at the same time as plant inoculation), or with *S. proteamaculans* (1-102) supernatant every day. At 25°C, soybean plants were subjected to the same treatments as at 15°C, except that *S. proteamaculans* (1-102) was replaced with *Serratia liquefaciens* (2-68). Previous work has shown *S. proteamaculans* (1-102) to be most effective at 15°C and *Serratia liquefaciens* (2-68) to be most effective at 25°C. Early symbiotic establishment between soybean and *B. japonicum* was examined microscopically. The results showed that: (1)

at 25°C PGPR 2-68 reduced the time required for root hair curling, infection thread initiation, and the infection thread to reach the base of the root hair, (2) at 15°C PGPR 1-102 shortened the duration of all the measured steps of the infection process, (3) at both 15 and 25°C, daily watering of soybean plants with the PGPR supernatant reduced the time required for root hair curling, infection thread initiation, and the infection threads to reach the base of the root hairs, more than inoculation with the PGPR, (4) the frequency of occurance of every measured infection stage was the highest at both 15 and 25°C for soybean plants watered daily with the PGPR supernatants. Taken together these results indicate that the PGPR tested accelerate the early stages of the soybean-*B. japonicum* symbiosis and that they do so through a substance released into their growth medium.

7.2 Introduction

Soybean [*Glycine max* (L.) Merr.] is a subtropical legume which requires root zone temperatures (RZTs) in the 25 to 30°C range for optimal symbiotic activity (Jones and Tisdale, 1921). Studies of sub-optimal RZT effects on N_2 fixation by soybean and other subtropical legumes has indicated that low RZTs decrease both nodulation and nodule function (Jones and Tisdale, 1921; Hardy et al., 1968; Layzell et al., 1984; Roughley and Date, 1986; Lynch and Smith, 1994). When soil temperature drops below the optimal range (25 to 30°C for soybean), legume nodulation and N_2 fixation are negatively affected.

Matthews and Hayes (1982) have shown that decreasing RZT below 25°C results in decreased nodule growth and total N_2 fixation per plant, while at a RZT of 15°C, the plant net nitrogenase activity is reduced by 25% (Walsh and Layzell, 1986), and nodulation ceases in plants at 10°C RZT (Matthews and Hayes, 1982). The infection and early nodule development processes are the most sensitive to suboptimal RZTs (Lindemann and Ham, 1979; Matthews and Hayes, 1982; Lynch and Smith, 1993).

All stages of nodule formation and functioning are affected by low RZT (Lie, 1974). Lynch and Smith (1993) observed that a RZT of 15°C severely restricted both infection and nodule development, and delayed the onset of nitrogen fixation until approximately 7 to 8 weeks after inoculation. In Canadian soybean growing areas soil temperatures at a depth of 10 cm during the early growing season often range from 10 to 15°C. Soybean production in eastern Canada is at the northern most North American limit of the crop.

Zhang et al. (1995a) demonstrated that: 1) RZTs less than 17°C strongly inhibited both infection and nodule development, 2) the early nodule development stages (within 14 days after inoculation) were very sensitive to RZT, 3) an early infection step (within 12 hours after inoculation) is most sensitive to low RZTs, and 4) before flowering, inoculated plants at RZTs between 17 and 25°C fixed some nitrogen, but plants at 15°C RZT had not began to fix nitrogen.

Recently, the knowledge of rhizosphere biology has been advanced by the discovery of a specific group of microorganisms, known as plant growth promoting rhizobacteria (PGPR), that colonize plant roots and enhance plant growth (Kloepper et al., 1980a,b). There were several mechanisms suggested for the beneficial effects of the PGPR such as: direct plant growth promotion, mobilization of insoluble nutrients (e.g. phosphate) and subsequent enhancement of uptake by the plant (Lifshitz et al., 1987), production of antibiotics (De-Ming and Alexander, 1988), and associative nitrogen fixation (Chanway and Holl, 1991a). Some PGPR were shown to increase legume nodulation and nitrogen fixation (Grimes and Mount, 1987; Chanway et al., 1989), often resulting in increased plant growth.

The exact mechanism by which PGPR increase nodulation and/or N_2 fixation is unknown and the ecology of PGPR is poorly understood. Consequently, there is little understanding as to how environmental factors affect bacterial colonization and persistence on roots and the resulting effects on plant growth. Direct growth promotion by the PGPR occurs when a rhizobacterium produces metabolites that promote plant growth without any direct interactions with elements of the native soil microflora (Kloepper et al., 1991). We have recently shown, in a controlled environment study (Zhang et al., 1996b), that some plant growth-promoting rhizobacteria (PGPR) stimulate nodule formation, and/or nitrogen fixation at low RZTs. Those results also demonstrated that the effects of PGPR were altered by RZT. Plant physiological events and growth in general were increased by PGPR, and soybean nodulation and N_2 fixation was improved. The two PGPR strains, 1-102 (*Serratia proteamaculans*) and 2-68 (*Serratia liquefaciens*), tested here were shown to be the best of seven PGPR strains tested, including pseudomonads, for stimulation of soybean nitrogen fixation (Zhang et al., 1996a,b). They exerted their effects first on overall plant physiology and secondarily on nodulation (Zhang et al., 1996a,b).

Until now, there have been no investigations of the effects of PGPR application on the various infection stages of soybean at low temperatures. The objectives of this experiment were to: 1) determine the effects of PGPR application at three RZTs (25, 17.5°C and 15°C) on the early infection stages of soybean-*Bradyrhizobium japonicum* symbiotic establishment, and 2) test the hypothesis that PGPR exert their beneficial effects through a diffusible substance or substances whose production does not require the presence of plant roots.

7.3 Materials and methods

7.3.1. Plant materials

Seeds of the soybean cultivar Maple Glen were surface sterilized in sodium hypochlorite (2% solution containing 4 mL L^{-1} Tween 20), then rinsed with distilled water several times (Bhuvaneswari et al., 1980). The seeds were then planted in trays containing a sterilized Turface (Applied Industrial Materials Corp., Illinois, USA):sand (1:1 vol.) mixture. Seven-day-old seedlings at the VC stage [unifoliolate leaves were unrolled sufficiently that the edges were not touching (Fehr et al., 1977), were transplanted into sterilized 13 cm plastic pots containing the same medium, on a Conviron growth bench (Model GB48, Controlled Environments Ltd., Winnipeg, Manitoba). The growth bench light (300 $\mu E\ m^{-2}\ s^{-1}$) was provided by Coolwhite fluorescent tubes and the photoperiod was 16:8 h (day:night). As this work is attempting to isolate RZT effects, air temperature was held constant at 25°C. RZTs were controlled by circulating cooled water around the pots, with eight pots sealed to the bottom of each tank. A hole drilled in the tank bottom below each pot allowed the pots to drain when watered. After transplanting into the pots, the plants were acclimatized for 24 hours before the inocula were applied.

7.3.2 Inoculum preparation

7.3.2.1. Experiment 1

The *Bradyrhizobium* portion of the inocula was produced by culturing *B. japonicum* strain 532C in yeast extract mannitol broth (Vincent, 1970) in 2 L flasks shaken at 125 rpm at room temperature (23-25°C). The PGPR strains tested in this experiment were *Serratia liquefaciens* (2-68) and *Serratia proteamaculans* (1-102). These two strains were chosen based on the results of a previous controlled environment experiment in which seven PGPR strains were tested for their ability to enhance soybean nodulation and nitrogen fixation at both optimal and suboptimal RZT. *S. proteamaculans* 1-102 increased nodule size and nitrogen fixation at 15°C RZT, while *S. liquefaciens* 2-68 caused similar effects at 25°C RZT (Zhang et al., 1996a,b). The PGPR strains were cultured in *Pseudomonas* media (Polonenko et al. 1987) in 250 ml flasks shaken at 250 rpm at room temperature (21-23°C). After reaching the stationnary phase (7-days for *B. japonicum* and 1.5-days for PGPR), both *B. joponicum* and PGPR were subcultured using the procedure described above. When they reached the log phase (3-days for *B. japonicum* and 1-day for PGPR), each of the PGPR strains was adjusted with distilled water to an $O.D._{420}$ value giving a cell density of 10^8 cells mL^{-1}. Before inoculation equal volumes of *B. japonicum* and PGPR cultures were mixed and allowed to stand for more than 30 min at room temperature without shaking. The inoculum was cooled to the corresponding RZT temperature and applied by pipette to the rooting medium at the base of the plant. Each inoculated plant received 1 mL of the inoculum. Control plants were inoculated only with *B. japonicum*. Plants were watered with a modified 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. Prior to each watering the added Hoagland's solution was temperature adjusted to the treatment RZT.

7.3.2.2. Experiment 2

The same procedure was followed for the production of the *B. japonicum* as in experiment 1. The PGPR strains tested in this experiment were, again, *S. liquefaciens* (2-68) and *S. proteamaculans* (1-102). The PGPR strains were cultured as described above. After reaching the stationary phase (1.5-day), the two PGPR were subcultured using the procedure described above. When they reached the log phase each of the PGPR strains was adjusted with distilled water to an $O.D._{420}$ value giving a cell density of 10^8 cells mL^{-1}. The two PGPR cell suspensions were pelleted in sterile centrifuge tubes at 7000g for 15 minutes, after which, the supernatant was collected and filter sterilized. Before inoculation equal volumes of *B. japonicum* and PGPR supernatant were mixed and allowed to stand for more than 30 min at room temperature without shaking. Each inoculated plant received 1 mL of the inoculum. The inoculum was cooled to the corresponding RZT temperature and applied by pipette to the rooting medium at the base of the plant. Plants were watered with a modified 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. Prior to each watering the added Hoagland's solution was temperature adjusted to the treatment RZT. Plants receiving daily applications of PGPR supernatant were given 50 mL of sterile supernatant, adjusted to the appropriate RZT, every day for 10 days for PGPR1-102 at 15°C, or 6 days, for PGPR2-68 at 25°C. This material was applied instead of the Hoagland's solution.

7.3.3. Experimental design

7.3.3.1. Experiment 1

The experiment was arranged in a completely randomized design with 4 replications. Three different RZTs used: 25 [optimal temperature for soybean nodulation and nitrogen fixation (Jones and Tisdale, 1921; Dart and Day, 1971)], 17.5 [sub-optimal temperature, but still above the critical point of 17°C, below which soybean nodulation and nitrogen fixation were strongly inhibited (Lynch and Smith, 1993)], or 15°C [at this RZT soybean nodulation was strongly inhibited (Lynch and Smith, 1993)]. Recent work has shown that between 25 and 17°C RZT the onset of N_2 fixation was delayed by 2 days for each degree decrease in RZT and the relationship between RZT and the onset of N_2 fixation is linear from 25 to 17°C. However, the onset of N_2 fixation was delayed by 15 days, or 7.5 days per °C, when the RZT decreased from 17 to 15°C (Zhang et al, 1995a).

7.3.3.2 Experiment 2

The experiment was arranged in a completely randomized design with 3 replications. Two root zone temperatures, 25 and 15°C, were used. At 25°C, the treatments were combinations of *B. japonicum* 532C and PGPR 2-68, *B. japonicum* and supernatant of PGPR 2-68 applied only at the time of *B. japonicum* inoculation, and supernatant of PGPR 2-68 applied daily beginning at the time of *B. japonicum* inoculation. Two controls were included, *B. japonicum* alone and *B. japonicum* inoculated with the *Pseudomonas* media used to grow the PGPR. At 15°C, soybean plants were inoculated with *B. japonicum* strain 532C alone, or treated with the *Pseudomonas* media alone, with *S. proteamaculans* (1-102), with *S. proteamaculans* (1-102) supernatant applied only at the time of *B. japonicum* inoculation, or with daily watering of *S. proteamaculans* (1-102) supernatant beginning at the time of *B. japonicum* inoculation. The two controls included at 25°C were also included at 15°C RZT.

7.3.4. Harvest and data collection

Plants were harvested each day, from 0.5 day after inoculation (DAI) until 6 DAI for plants grown at 25°C, and one DAI until 10 DAI for plants grown at 15°C. Plant roots were washed in distilled water and the six uppermost plant roots were taken for microscopic observation. Plant roots were stained with 1% aniline blue for 10 minutes and observed under a light microscope (Jenalumar, Jena Instruments Ltd., Jena, Germany).

7.3.5. Statistical analysis

Results were analyzed statistically by analysis of variance using the SAS system (SAS Institute Inc., 1988). When analysis of variance showed significant treatment effects, the least significant difference (LSD) test was applied to make comparisons among the means at the 0.05 levels of significance (Steel and Torrie, 1980).

7.4. Results

7.4.1 Experiment 1

Root hair curling and infection thread initiation

The effects of RZT on morphological changes during the early nodule infection stages of soybean, as observed by light microscope, was reported by Zhang and Smith (1994). In the current study, for the control plants (no PGPR treatment) at 25°C RZT, root hair curling commenced at 0.5 DAI while the infection thread initiation occurred at 1 DAI (Table 7.1). For the plants grown at suboptimal RZTs, all the infection steps were delayed and the duration of these stages were increased. Root hair curling commenced at 1 and 2 DAI for plants grown under suboptimal RZTs of 17.5 and 15°C, respectively.

Application of PGPR 2-68 shortened the duration of root hair curling and infection thread initiation at 25 and 17.5°C RZTs. Root hair curling was completed at 0.5 DAI at an optimal RZT (25°C) and infection thread initiation commenced immediately after. For plants maintained at suboptimal RZTs of 17.5 and 15°C, root hair curling commenced at 1 and 2 DAI, respectively, while the infection thread initiation was completed at 2 and 4 DAI. There was little difference between plants receiving PGPR 2-68 and those receiving no PGPR treatment at 15°C RZT for root hair curling and infection thread initiation. In both cases, root hair curling ended at 2 DAI while infection thread initiation ended by 4.5 DAI (Table 7.1).

For the plants receiving PGPR 1-102, root hair curling commenced at 0.5 DAI, while infection thread initiation occurred at 1 DAI for plants grown under optimal RZT (25°C). For plants grown under suboptimal RZTs of 17.5 and 15°C, root hair curling commenced at 1 and 1.5 DAI (Fig 7.1), respectively. Application of PGPR 1-102 did not affect the duration of the stages at an optimal RZT (25°C) or at 17.5°C, but at 15°C it shortened all measured stages relative to the control.

Elongation of infection threads was affected by RZT. At 25°C RZT Infection threads of the control plants (no PGPR) reached the base of root hairs at 3.5 DAI, for an elapsed time of 3 days after infection thread initiation (Table 7.1). Infection thread growth of the plants maintained at the sub-optimal RZTs, 17.5 and 15°C, was decreased, with the infection thread reaching the base of the plant root hairs at 6 and 8 DAI, respectively. Application of PGPR 2-68 shortened the time required for root hair curling, infection thread initiation, and the infection thread to reach the base of the root hair at both 25 and 17.5 but not at 15°C, while application of PGPR 1-102 shortened the duration of all the stages at 15 but not at 25 and 17.5°C.

7.4.2. Experiment 2

The same pattern was seen in terms of temperature and PGPR effects on the time required for root hair curling, infection thread initiation, infection threads to reach 1/2 way down the root hair, and infection threads to reach the base of the root hairs. There was no difference observed between *B. japonicum* alone and *B. japonicum* plus growth medium at either 15 or 25°C, removing the possibility that any observed effects were due to constituents of the pseudomonad growth medium. At 15°C, there was no difference in the duration of the different stages for plants receiving PGPR 1-102 or PGPR 1-102 supernatant at inoculation. For plants receiving PGPR 1-102 supernatant daily, the durations of all the stages were shortened relative to the PGPR1-102 treatment (Table 7.2). The frequency of the occurance of root hair curling, infection thread initiation, infection thread 1/2 way down the root hair, and infection thread reaching the base of the root hair were all increased for plants receiving daily watering with PGPR 1-102 supernatant (Fig 7.2). At 25°C, there was no difference in the duration of the different stages between plants receiving PGPR 2-68 or PGPR 2-68 supernatant only at the time of plant inoculation. For plants watered daily with PGPR 2-68 supernatant, the durations of all the stages were shortened relative to other PGPR 2-68 treatments (Table 7.2). The frequency of occurance of root hair curling, infection thread initiation, infection thread 1/2 way down the root hair, and infection thread reaching the base of the root hair were greater for plants watered daily with PGPR 2-68 supernatant than those inoculated with PGPR 2-68 or watered with its supernatant only at the time of plant inoculation (Fig 7.1).

7.5 Discussion

Plant growth-promoting rhizobacteria strains *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 were selected following previous controlled environment studies (Zhang et al., 1996a,b) in which nine PGPR strains were tested for effects on soybean plant growth, development, nodulation and nitrogen fixation over a range of RZT under controlled environment conditions. Inoculation of soybean plants with PGPR strains produced a wide range of effects which varied among strains of PGPR and over RZTs.

In experiment 1, application of PGPR 2-68 accelerated root hair curling, infection thread initiation, infection thread 1/2 way down the root hair, and infection thread reaching the base of the root hair when compared to the control (no PGPR) at both 17.5 and 25°C (Table 7.1). On the other hand, PGPR 1-102 accelerated all measured variables compared to the control (no PGPR) at 15°C (Table 7.1). Both *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68 were reported to stimulate plant growth, development, and plant physiological activities (Zhang et al., 1996a). At an optimal RZT (25°C), *S. liquefaciens* 2-68 was reported to increase plant leaf development and dry matter accumulation, while at 15°C RZT *S. proteamaculans* 1-102 increased plant dry matter accumulation (Zhang et al., 1996a).

In experiment 2, no differences were observed between application of PGPR 2-68 and application of PGPR 2-68 supernatant at inoculation for the four measured infection stages at 25°C (Table 7.2). The same result was observed for PGPR 1-102 and PGPR 1-102 supernatant addition at 15°C. These results indicate that the effect of the PGPR is due to a substance or substances present in the PGPR supernatant. Plant growth promoting rhizobacteria have been shown to produce many phytohormones and signal molecules (Burr and Caesar, 1984; Davison, 1989; Kapulnik, 1991), such as genistein, a plant-to-bacteria signal involved in the soybean nodule infection and formation processes. Molecules of this type could have been present in the supernatant and, as a result, cause morphological changes of the root hair.

If signal molecules were being produced, they are unlikely to have been analogues of genistein, or to stimulate the development of the N_2 fixing symbiosis the same way genistein does (Zhang and Smith, 1995b). There are two pieces of evidence that argue this point. First, Zhang and Smith (1995b) found that genistein addition to *B. japonicum* cause a stimulation of photosynthesis only at the time when nodulation was completed and N_2 fixation began. Zhang et al., (1996a) found that PGPR stimulated photosynthesis prior to the onset of N_2 fixation. Second, while Zhang and Smith (1995b) found that genistein addition to *B. japonicum* inocula accelerated root hair curling, it did not shortened the duration of time between root hair curling and infection thread initiation, or the time required for developing infection threads to reach the half way or all the way down the root hairs. We found that PGPRs or their supernatants shortened all measured stages of infection (Tables 7.1 and 7.2).

For plants watered daily with PGPR 2-68 supernatant, the durations of all the stages were shortened relative to plants inoculated with the PGPR itself (Table 7.2). The frequency of the occurance of root hair curling, infection thread initiation, infection thread 1/2 way down the root hair, and infection thread reaching the base of the plant root was also increased (Fig 7.1). The same pattern was observed for plants watered daily with PGPR 1-102 supernatant (Table 7.2 and Fig 7.2). The most probable explanation for this is that, as we made the PGPR inocula by spinning down the cells, almost all the growth stimulating molecules were discarded with the supernatant. It would then have taken some time for the PGPR to synthesize more of the plant growth stimulating substance(s). When we added the supernatant only at inoculation, the roots were exposed to the growth stimulating substance(s) sooner than when plants are inoculated with PGPR cells, but experience it/them only transiently. However, when we watered every day with the supernatant the roots were exposed to the growth stimulating substance(s) from the time of inoculation and constantly thereafter. Thus, the roots were affected more by the growth stimulating molecule(s) when they were added constantly than would be the case with once only supernatant application or inoculation with PGPR cells.

The observation that the supernatant of PGPR cells not previously exposed to plant tissues produce growth stimulating substances indicates that unlike the signal exchange process between legumes and their N_2 fixing symbionts, the production of the bacteria-to-plant effector molecule(s) in the PGPR system does not require a signal molecule from the plant.

Both *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68 were reported to stimulate plant growth, development, and photosynthesis (Zhang et al., 1996b). At an optimal RZT (25°C), *S. liquefaciens* 2-68 was reported to increase plant leaf development and dry matter accumulation, while at 15°C RZT *S. proteamaculans* 1-102 increased plant dry matter accumulation.

Some reports have shown positive effects of PGPR on the legume N_2-fixing symbiosis. The bacteria involved have been termed nodule promoting rhizobacteria (NPR). Inoculation with NPR, often pseudomonads, and *(Brady)Rhizobium* has been demonstrated to positively affect symbiotic nitrogen fixation by enhancing root nodule number or mass (Singh and Subba Rao, 1979; Burns et al., 1981; Grimes and Mount, 1987; Polonenko et al., 1987; Yahalom et al., 1987) and by increasing nitrogenase activity (Iruthayathas et al., 1983; Alagawadi and Gaur, 1988).

The mechanisms of growth and nitrogen fixation promotion by PGPR are not well understood; however, a wide range of possibilities have been postulated, as discussed above, including both direct and indirect actions. Although it seems likely that some diffusible molecule(s) mediates these effects on the plants, this has not been proven.

This study suggests that both PGPR 1-102 and 2-68 produce diffusible growth promoting substance(s), and that 1-102 does this well at high temperatures (25°C) and 2-68 at lower temperatures, or that 1-102 makes the substance(s) available to the plant at higher temperatures due to effective rhizosphere colonization at high temperatures, as we have shown in a previous study (Dashti et al., unpublished data), and 2-68 makes the substance (s) available to the plant only at low RZT because it is able to colonize the rhizosphere effectively only at low RZT (Dashti et al., unpublished data). Since addition of the PGPR supernatant resulted in stimulation which was strain specific and temperature dependent, our data suggests that each PGPR releases a different growth stimulating substance. Although, given that they have similar effects on plant growth, they may be similar molecules.

In summary, this is the first time it has been shown that: 1) at 25 and 17.5°C, application of PGPR 2-68 shortened the time elapsed from the beginning of root hair curling until the infection thread reached the base of the root hair, 2) at 15°C PGPR 1-102 shortened the duration of the root hair curling, infection thread initiation and infection thread growth to the base of the root hair, 3) at both 15 and 25°C treatment of the soybean plants with PGPR supernatant, at inoculation only or through daily applications, reduced the time required for root hair curling, infection thread initiation, and the infection threads to reach the base of the root hair, 4) the frequency of the occurance of the different stages was the highest at both 15 and 25°C for soybean plants treated daily with PGPR supernatants.

Table 7.1. Effect of the plant growth promoting rhizobacteria on root hair curling, infection thread initiation,infection thread 1/2 way down the root hair, and infection thread reaching the base of the plant root hairs at 15, 17.5 and 25°C RZT.

	D0.5*	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
15°C											
PGPR1-102	0.0	1.0	1.6	2.4	2.6	2.7	3.2	3.6	4.0	4.0	4.0
PGPR2-68	0.0	1.0	1.4	2.2	2.3	2.5	2.9	3.2	3.5	3.7	4.0
Control	0.0	0.5	1.2	2.0	2.1	2.4	3.0	3.2	3.5	3.8	4.0
$LSD_{0.05}$	0.0	0.08	0.3	0.32	0.18	0.26	0.26	0.23	0.23	0.13	0.0
17.5°C											
PGPR1-102	0.0	1.0	1.7	2.0	2.5	2.7	3.3	3.8	4.0		
PGPR2-68	0.0	1.4	1.9	2.4	3.3	3.8	4.0	4.0	4.0		
Control	0.0	1.0	1.5	2.1	2.5	2.8	3.5	3.9	4.0		
$LSD_{0.05}$	0.0	0.13	0.13	0.13	0.13	0.13	0.16	0.13	0.0		
25°C											
PGPR1-102	0.6	1.5	2.5	3.1	3.6	3.8	4.0				
PGPR2-68	0.8	2.1	2.9	3.6	4.0	4.0	4.0				
Control	0.5	1.6	2.3	3.2	3.7	4.0	4.0				
$LSD_{0.05}$	0.21	0.23	0.13	0.28	0.12	0.13	0.0				

Means within the same column were analyzed by an ANOVA protected LSD test. $LSD_{0.05}$ is the comparisons of the means within the same temperature and sampling time. D = days after inoculation. *values indicate infection stages: 1 -root hair curling, 2 - infection thread initiation, 3 - infection thread 1/2 way down the root hair, and 4 - infection thread reaching the base of the plant root hairs; non-integer values indicate the average stage observed on each day.

Table 7.2. Effect of the plant growth promoting rhizobacteria, supernatant (S), or daily watering with supernatant (SW) on root hair curling, infection thread initiation, infection thread 1/2 way down the root hair, and infection thread reaching the base of the plant root hairs at 15, 17.5 and 25°C RZT.

	0.5D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10
15°C											
PGPR1-102	0.0	1.10	1.30	2.30	2.65	3.00	3.30	3.50	3.70	3.90	4.00
PGPR1-102 (S)	0.0	1.10	1.30	2.30	2.70	2.95	3.25	3.45	3.65	3.90	4.00
PGPR1-102 (SW)	0.0	1.20	1.40	2.40	3.00	3.40	3.50	3.70	4.00	4.00	4.00
Control$_a$ (*B. japomicum*)	0.0	0.90	1.20	2.00	2.20	2.40	2.70	2.90	3.20	3.70	4.00
Control$_b$ (Madium)	0.0	0.85	1.15	2.00	2.20	2.40	2.70	2.90	3.20	3.70	4.00
$LSD_{0.05}$	0.0	0.18	0.10	0.10	0.17	0.19	0.25	0.15	0.14	0.13	0.00
25°C.											
PGPR2-68	0.88	2.23	3.12	3.80	4.00	4.00					
PGPR2-68 (S)	0.90	2.30	3.20	3.80	4.00	4.00					
PGPR2-68 (SW)	1.20	2.40	3.44	4.00	4.00	4.00					
Control$_a$ (*B. japomicum*)	0.70	1.70	2.30	3.40	3.80	4.00					
Control$_b$ (Madium)	0.66	1.68	2.30	3.33	3.72	4.00					
$LSD_{0.05}$	0.19	0.20	0.19	0.28	0.07	0.00					

Means within the same column were analyzed by an ANOVA protected LSD test. $LSD_{0.05}$ is the comparisons of the means within the same temperature and sampling time. D = days after inoculation. [a] values indicate infection stages: 1 -root hair curling, 2 - infection thread initiation, 3 - infection thread 1/2 way down the root hair, and 4 - infection thread reaching the base of the plant root hairs; non-integer values indicate the average stage observed on each day.

Fig. 7.1. The frequency of root hair curling, infection thread initiation, infection thread 1/2 way down the root hair, and infection thread reaching the base of the plant root hairs for different treatments over time at 25°C. Each point is the mean (±s.e.) value for ten observations.

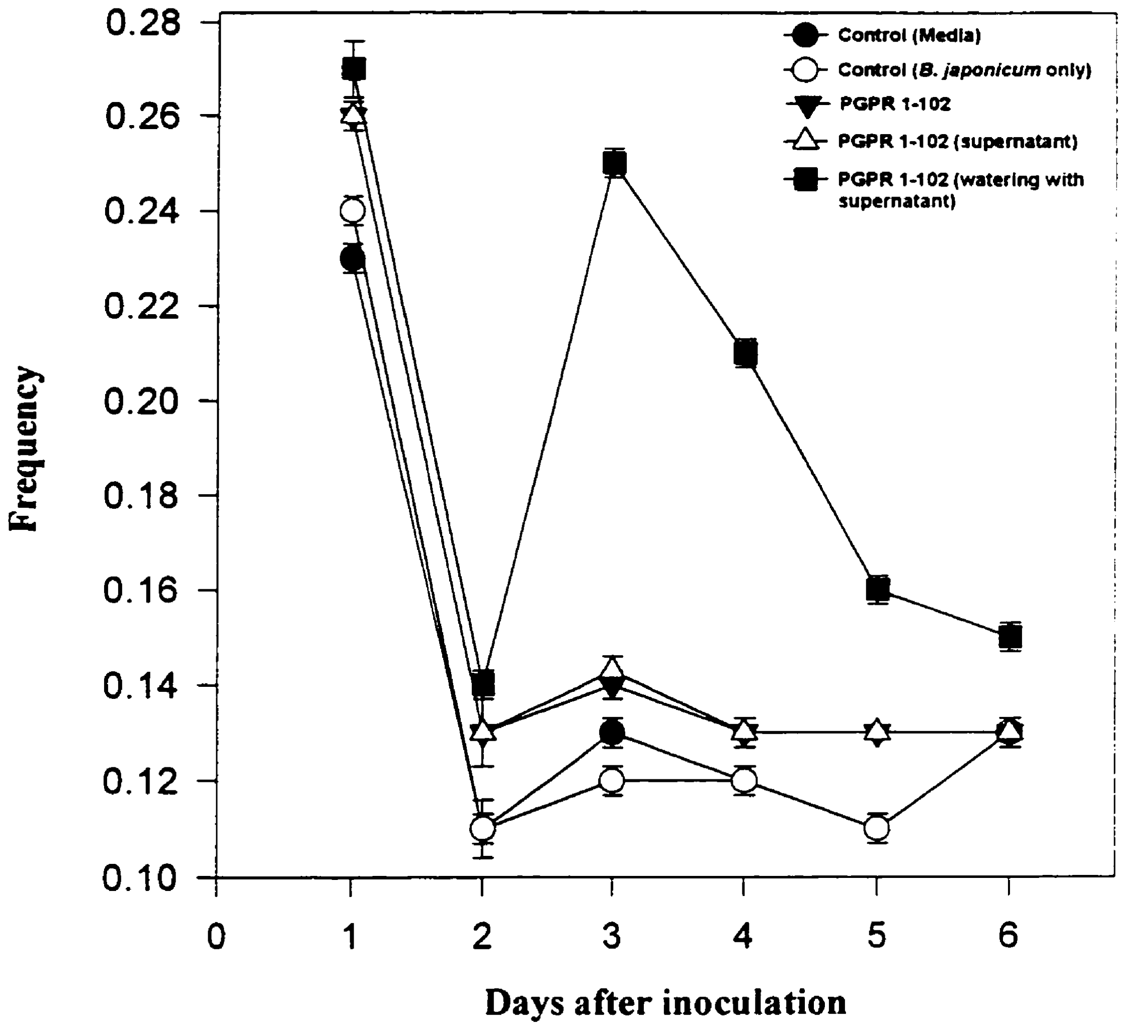
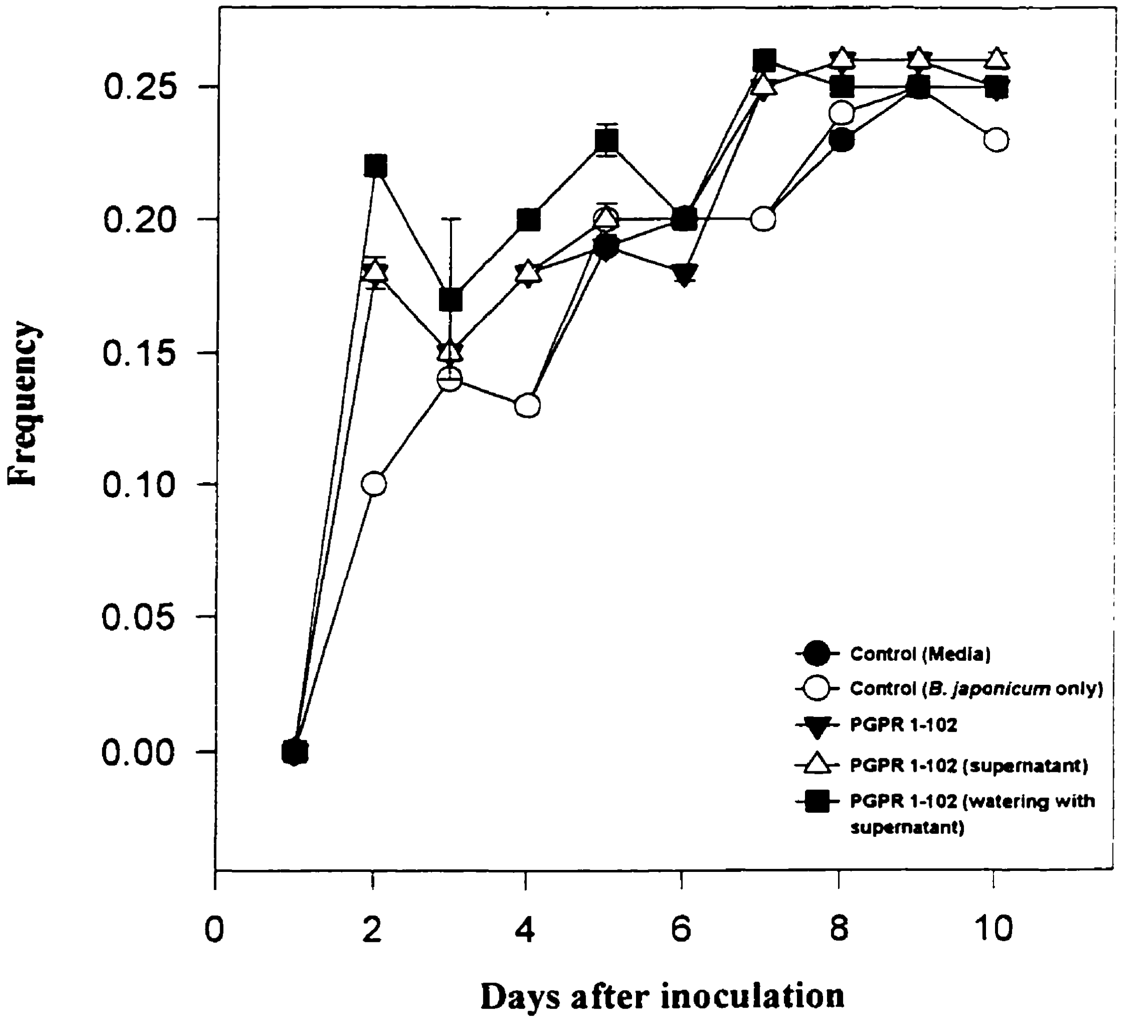

Control (Media)
Control (B. japonicum only)
PGPR 1-102
PGPR 1-102 (supernatant)
PGPR 1-102 (watering with supernatant)
Frequency
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0
1
2
3
4
5
6
Days after inoculation

Fig. 7.2. The frequency of root hair curling, infection thread initiation, infection thread 1/2 way down the root hair, and infection thread reaching the base of the plant root hairs for different treatments over time at 15°C. Each point is the mean (±s.e.) value for ten observations.



Frequency
0.25
0.20
0.15
0.10
0.05
0.00
0
2
4
6
8
10
Days after inoculation
Control (Media)
Control (B. japonicum only)
PGPR 1-102
PGPR 1-102 (supernatant)
PGPR 1-102 (watering with supernatant)

Preface to Section 8

Section 8 is comprised of a manuscript by N. Dashti, R.K. Hynes, and D.L. Smith, prepared for publication in 1996. The format has been changed to conform as much as possible to a consistent format within this thesis. All literature cited in this section are listed in the reference section at the end of the thesis. Each table for section 8 is presented at the end of this section.

As we had demonstrated the ability of PGPR to stimulate soybean nodulation, N_2 fixation and growth under field conditions and shown that this is related to RZT, and as it has already been shown that the same is true for genistein, a plant-to-bacteria signal molecule, in this section we have tested the hypothesis that the addition of PGPR and genistein together will result in greater stimulation of nodulation and N_2 fixation than either alone.

Section 8

CO-INOCULATION OF *Bradyrhizobium japonicum* PREINCUBATED WITH GENISTEIN AND PLANT GROWTH PROMOTING RHIZOBACTERIA ACCELERATES SOYBEAN [*Glycine max* (L.) Merr.] NODULATION AND NITROGEN FIXATION AT SUBOPTIMAL ROOT ZONE TEMPERATURES

8.1. Abstract

Application of plant growth-promoting rhizobacteria (PGPR) has been shown to increase nodulation and nitrogen fixation of soybean [*Glycine max* (L.) Merr.] over a range of root zone temperatures (RZTs), as has preincubation of *Bradyrhizobium japonicum* cultures with the plant-to-bacteria signal molecule, genistein. A controlled-environment experiment was conducted to examine the combined ability of both PGPR and genistein to reduce the negative effects of low root zone temperature (RZT) on soybean nodulation and nitrogen fixation. Each of two PGPR strains, *Serratia proteamaculans* 1-102 and *Serratia liquefaciens* 2-68 were co-inoculated with *B. japonicum* USDA110 or 532C preincubated with different concentrations of genistein (0, 15, or 20μM). The resulting inocula were added to a soybean rooting medium to test their ability to reduce the negative effects of low RZT on soybean growth and development by improving the physiological status of the plants. Three RZTs were tested: 25 (optimal), 17.5 (somewhat inhibitory), and 15°C (very inhibitory). At each temperature PGPR strains and genistein increased the number of nodules formed and the amount of fixed nitrogen, but the most stimulatory combination of PGPR, genistein concentration and *B. japonicum* strain varied with temperature. The combinations that were most stimulatory at each temperature were as follows: at 15°C - *S. proteamaculans* 1-102, genistein concentration 0 μM and *B. japonicum* USDA110, at 17.5°C - *S. proteamaculans* 1-102, genistein concentration 15 μM and *B. japonicum* USDA110, and at 25°C - *S. proteamaculans* 1-102, genistein concentration 5 μM and *B. japonicum* USDA110. In at least some cases, these stimulatory effects can be attributed to additive effects of both PGPR and genistein in enhancing the number of

nodules formed and the amount of nitrogen fixed by soybean plants. The combinations of PGPR and genistein showed additive effects, when compared to PGPR or genistein alone at higher RZT (25°C), while they show antagonistic effects at lower RZT (15°C).

8.2. Introduction

As a subtropical legume, soybean [*Glycine max* (L.) Merr.] requires temperatures in the 25 to 30°C range for optimal symbiotic activity (Jones and Tisdale, 1921). When root zone temperatures (RZTs) drop below this, soybean nodulation and nodule function are negatively affected (Jones and Tisdale, 1921; Hardy et a., 1968; Roughley and Date, 1986; Legros and Smith, 1994; Lynch and Smith, 1994). In areas with short growing seasons, such as the Canadian soybean production regions, the poor adaptability of soybean to cool soils is considered the primary factor limiting yield (Whigham and Minor, 1978).

Infection and early nodule development processes are most sensitive to low RZT (Lindemann and Ham, 1979; Lynch and Smith, 1993; Zhang and Smith, 1994). When the RZT drops below 25°C, but remains above 17°C, the time between inoculation and the onset of N_2 fixation is delayed by 2 to 3 days for each °C decrease in the temperature, whereas RZTs between 17.5 and 15°C are more strongly inhibitory, and each °C delays the onset of N_2 fixation by about a week (Zhang et al., 1995a).

Certain rhizosphere microorganisms, collectively referred to as plant growth promoting rhizobacteria (PGPR), can colonize plant roots and promote plant growth (Kloepper et al., 1980a). PGPRs can increase plant growth, development, and yield in such nonlegume crops as potato, radish, sugar beet, wheat and canola (Gaskins et al., 1985; Polonenko et al., 1987). Co-inoculation of *(Brady)rhizobium* with PGPR has been shown to increase soybean plant nodulation and nitrogen fixation under normal growth conditions (Verma et al., 1986; Li and Alexander, 1988). Similarly, an increase in grain yield, nodule dry matter, and nitrogenase activity was also obtained in chick pea inoculated with a mixture of *Azospirillum brasilense* and *Rhizobium* strains (Rai, 1983). Grimes and Mount (1984) found that a *Pseudomonas putida* strain (M17), which had been selected as a potential biological control agent, markedly increased

Rhizobium nodulation of bean in field soils. Polonenko et al. (1987) found similar effects of fluorescent pseudomonads on nodulation of soybean roots by *B. japonicum*.

Numerous studies indicated that co-inoculation of *Bradyrhizobium* and certain PGPR, can positively affect symbiotic nitrogen fixation by enhancing both root nodule number and mass (Polonenko et al., 1987; Yahalom et al., 1987), and by increasing nitrogenase activity (Iruthayathas et al., 1983; Alagawadi and Gaur, 1988).

Plant growth promotion by the PGPR has been attributed to the production of plant growth regulators (Kloepper and Schroth, 1981; Gaskins et al., 1985), increased access to soil nutrients (Lifshitz et al., 1987), disease suppression (De-Ming and Alexander, 1988), and associative nitrogen fixation (Chanway and Holl, 1991a).

Zhang et al. (1996b) investigated the tripartite association of nitrogen-fixing bacteria, PGPR and soybean plants at different RZTs. They have shown that co-inoculation of *B. japonicum* with some PGPR strains increased soybean nodulation and nitrogen fixation at both optimal and suboptimal RZTs, although, the PGPR strains exerting stimulatory effects varied with RZT. The effects of PGPR on legume plant growth and development were apparent before the onset of nitrogen fixation and were therefore through improved overall plant physiological activities, whereas after the onset of nitrogen fixation they may also have been due to improvement of plant nodulation and nitrogen fixation.

Genistein, the most important plant-to-bacterial signal in the soybean-*B. japonicum* symbiosis, is a part of the earliest phase of the nodulation process, the release of signal molecules that trigger the coordinated expression of a series of bacterial nodulation (nod) genes in the bacterial symbiont (Long, 1989, Peters and Verma, 1990). The isoflavones daidzein and genistein are the major components of the soybean root extracts responsible for inducing the *nod* genes of *B. japonicum* (Kosslak et al., 1987). Zhang et al. (1996c) have shown that the roots of plants germinated and grown at lower RZTs have lower genistein concentrations and contents than plants grown at higher RZTs. The beneficial effects of genistein increased with decreasing RZT (Zhang and Smith, 1995b). At suboptimal RZTs (17.5 and 15°C) the most effective concentrations are in the 15 to 20 μM range, whereas at an optimal (25°C) RZT 5 μM is most effective.

Two studies by Zhang and Smith (1995b), and Zhang et al. (1996d) under controlled environment conditions and under field conditions, have shown that preincubation of *B. japonicum* with genistein hastened the onset of nitrogen fixation, increased the number of nodules produced, increased the size of the nodules, and increased plant growth.

Zhang and Smith (1995b) showed that genistein stimulation of photosynthesis was only seen after the onset of N_2 fixation, while PGPR stimulation of photosynthesis was apparent prior to the onset of N_2 fixation. Thus, while the effects of genistein are through nitrogen fixation, the effects of PGPR are on overall plant vigour (Zhang et al.,1996a). Since PGPR and genistein seem to work by different mechanisms, their beneficial effects might reasonably be additive. As both PGPR and genistein have been shown to stimulate plant nodulation and nitrogen fixation, but by different mechanisms, a controlled environment experiment was conducted to test the hypothesis that the inclusion of both PGPR and genistein in *B. japonicum* inocula would increase soybean nodulation and nitrogen fixation at suboptimal and optimal RZTs more than the addition of either PGPR or genistein alone.

8.3. Materials and methods

8.3.1. Experimental design

Two controlled environment experiments were conducted. Both were arranged in completely randomized designs with three replications. Three different RZTs 15, 17.5, and 25°C were tested. Combinations of *B. japonicum* strains, genistein levels and PGPR strains were used as the treatments. At each RZT three PGPR levels (no PGPR, *Serratia proteamaculans* 1-102 and *Serratia liquefaciens* 2-68), two *B. japonicum* strains [USDA110 and 532C (Hume and Shelp, 1990)], and two genistein concentrations were factorially combined. The two genistein concentrations tested were 0 and 5 μM, 0 and 15μM, and 0 and 20μM at 25, 17.5 and 15°C RZT, respectively (Zhang and Smith, 1995b). The combination of PGPR (*S. liquefaciens* 2-68), *B. japonicum* (532C), and genistein concentrations (5, 15 and 20 μM at 25, 17.5 and 15°C, respectively), were excluded due to insufficient space. The different genistein concentrations were chosen based on a report by Zhang and Smith (1995b). They found that at suboptimal RZTs (17.5 and 15°C) the most effective genistein concentrations were in the 15 to 20 μM range, whereas at an optimal (25°C) RZT 5 μM was most effective.

8.3.2. Plant materials

Seeds of the soybean cultivar Maple Glen were surface sterilized in sodium hypochlorite (2% solution containing 4 mL L^{-1} Tween 20) for 5 minutes, then rinsed with distilled water several times (Bhuvaneswari et al., 1980). The seeds were then planted in trays containing a sterilized Turface (an inert calcined clay; Applied Industrial Materials Corp., Deerfield, IL, USA): sand (1:1 vol.) mixture. Two ten-day-old seedlings at the vegetative cotyledonary (VC) stage [unifoliolate leaves unrolled sufficiently that the edges were not touching (Fehr et al., 1971)] were transplanted into each sterilized, 13-cm plastic pot containing the same rooting medium. In experiment 1, the seedlings were grown on a Conviron growth bench (Model GB48, Controlled Environments Ltd., Winnipeg, Canada) at an irradiance of 300 μmol m^{-2} s^{-1} and a 16:8 h (day:night) photoperiod with a constant air temperature of 25°C. Experiment 2, was established in a greenhouse facility where the level of environmental control was comparable to the growth bench used in experiment 1 but with a higher light intensity (800-1200 μmol m^{-2} s^{-1}). In both experiments, the pots were sealed to the bottoms of plastic tanks (68 x 42 cm) and RZTs ($\pm$ 0.5°C) were controlled by circulating cooled water around the pots, with eleven pots in each tank. A hole drilled through the bottom of the tank beneath each pot allowed the pots to drain when watered. Plants were then acclimatized for 24 h prior to inoculation. During the period of plant growth, plants were watered with a modified Hoagland's solution (Hoagland and Arnon, 1950), in which $Ca(NO_3)_2$ and KNO_3 were replaced with $CaCl_2$ (1 mM), K_2HPO_4 (1 mM) and KH_2PO_4 (1 mM), to provide a nitrogen-free solution. Prior to each watering, the added Hoagland's solution was adjusted to the temperature of the treatment RZT.

8.3.3. *Inoculum*

Two PGPR strains, *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68, were included in this study, based on the reports of Zhang et al. (1996a and 1996b). All PGPR strains were cultured in *Pseudomonas* medium (Polonenko et al., 1987) in 250-mL flasks, shaken at 250 rpm, at room temperature (20 to 23°C). After reaching the stationary phase (36 h), the PGPR were subcultured. When the subculture reached the log phase (24 h), each of the PGPR strains was adjusted spectrophotometrically with distilled water to an A_{420} value giving a cell density of 10^8 cells mL^{-1}. *B. japonicum* strains 532C and USDA110 (Hume and Shelp, 1990) were cultured in yeast extract mannitol broth (Vincent, 1970) in 250 mL flasks shaken at 125 rpm at room temperature. For production of *B. japonicum* with genistein, genistein (4', 5, 7-trihydroxyisoflavone, purity of 98%, Sigma, Mississauga, Ontario, Canada), as a 10 mM stock solution in 100% methanol, was added to the cultures. The final genistein concentrations of the cultures were equal to the treatment requirements. Cultures were incubated at 23°C without shaking for 48h (Halverson and Stacey, 1984). Following incubation, the cell suspensions were pelleted in sterile centrifuge tubes at 7000g for 10 minutes, washed once with distilled water, and resuspended to an A_{620} of 0.08 (approximately 10^8 cells mL^{-1}). The suspension was then mixed with each PGPR (1:1 cell population) strain before inoculation. The inoculum was cooled to the corresponding root temperature and 1 mL was applied by pipette to the rooting medium at the base of the plant.

8.3.4. *Harvest and data collection*

Plants from both experiments were harvested at 50 days after inoculation (DAI) and the following data were collected: number of nodules; nodule dry weight; plant dry weight and plant nitrogen concentration (Kjeltec system, with digestion system 20 and 1002 distilling unit, Tecator AB, Hoganas, Sweden). The amount of nitrogen fixed per plant was calculated from the total plant nitrogen content minus the amount of nitrogen in the original seed (11.5 mg per seed; average of 25 seeds). The nitrogen concentration of different plant tissues (nodules, shoots, and roots) was also measured.

8.3.5. Statistical analysis

Results were analyzed statistically by analysis of variance (ANOVA) with SAS (SAS Institute Inc., 1988). When analysis of variance showed significant treatment effects, the least significant difference (LSD) test was applied to make comparisons among the means at the 0.05 levels of significance (Steel and Torrie, 1980).

8.4. Results

8.4.1. Nodulation

The effects of different combinations of *B. japonicum*, genistein concentration and PGPR strains on nodule formation varied with RZT.

8.4.1.1. At 25°C

Co-inoculation of soybean plants with a mixture of *B. japonicum* 532C and PGPR 1-102 increased nodule dry weight per plant (56%), nodule size (53%) and the ratio of nodule dry weight to plant dry weight (47%) over *B. japonicum* 532C alone in experiment 1 (Table 8.1). *B. japonicum* 532C preincubated with 5 μM genistein increased nodule number (23 and 30%), nodule dry weight per plant (84 and 23%) and the ratio of nodule dry weight to plant dry weight (50 and 13%) over *B. japonicum* 532C alone in experiments 1 and 2, respectively (Table 8.1). The combination of *S. proteamaculans* 1-102, 5 μM genistein and *B. japonicum* 532C increased only nodule size (9%) in experiments 1 and 2 when compared with *B. japonicum* 532C preincubated with 5 μM genistein. The same combination increased nodule number (13 and 16%), nodule dry weight per plant (20 and 24%), and the ratio of nodule dry weight to plant dry weight (7 and 12%) in experiments 1 and 2, respectively, when compared with *B. japonicum* 532C co-inoculated with PGPR 1-102.

Co-inoculation of soybean plants with *B. japonicum* 532C and PGPR 2-68 increased nodule dry weight per plant (26%), nodule size (40%) and the ratio of nodule dry weight to plant dry weight (13%) over *B. japonicum* alone in experiment 1 (Table 8.1).

PGPR 1-102 co-inoculated with *B. japonicum* USDA110 increased nodule number (27 and 32%), nodule dry weight per plant (40 and 66%), nodule size (14 and

29%) and the ratio of nodule dry weight to plant dry weight (28 and 55%) over *B. japonicum* USDA110 alone in experiments 1 and 2, respectively. *B. japonicum* USDA110 preincubated with 5 μM genistein increased nodule number (28 and 36%), nodule dry weight per plant (63 and 81%), nodule size (29 and 36%) and the ratio of nodule dry weight to plant dry weight (38 and 60%) in experiments 1 and 2, respectively, over the *B. japonicum* USDA110 alone (Table 8.1). The combination of *S. proteamaculans* 1-102, 5 μM genistein and *B. japonicum* USDA110 increased nodule dry weight per plant (27 and 22%), nodule size (44 and 39%) and the ratio of nodule dry weight to plant dry weight (22 and 16%) in experiments 1 and 2, respectively, when compared with *B. japonicum* USDA110 co-inoculated with PGPR 1-102. The same combination increased nodule dry weight per plant (10 and 12%), nodule size (28 and 32%) and the ratio of nodule dry weight to plant dry weight (13 and 12%) in experiments 1 and 2, respectively, when compared with *B. japonicum* USDA110 preincubated with 5 μM genistein.

Co-inoculation of soybean plants with a mixture of *B. japonicum* USDA110 and PGPR 2-68 increased nodule dry weight per plant (100 and 132%), nodule size (129 and 164%) and the ratio of nodule dry weight to plant dry weight (55 and 80%) over *B. japonicum* USDA110 alone in experiments 1 and 2, respectively (Table 8.1). The combination of *S. liquefaciens* 2-68, 5 μM genistein and *B. japonicum* USDA110 decreased nodule dry weight per plant (23 and 26%), nodule size (34 and 38%), and the ratio of nodule dry weight to plant dry weight (2 and 6%) when compared to *B. japonicum* USDA110 co-inoculated with PGPR 2-68 in experiments 1 and 2, respectively. The same combination increased nodule size (17%) and the ratio of nodule dry weight to plant dry weight (10%) in experiment 1 and increased nodule size (21%) in experiment 2 when compared to *B. japonicum* USDA110 preincubated with 5 μM genistein.

8.4.1.2. At 17.5°C

Plants receiving a mixture of *B. japonicum* 532C and PGPR 1-102 had increased nodule number (12%), nodule dry weight per plant (128%), nodule size (114%) and the ratio of nodule dry weight to plant dry weight (140%) in experiment 2 over *B. japonicum* 532C alone (Table 8.2). *B. japonicum* 532C preincubated with 15 μM genistein increased nodule number (27%) and the ratio of nodule dry weight to plant dry weight (13%) over *B. japonicum* 532C alone in experiment 1 and increased nodule number (24%), nodule dry weight per plant (100%), nodule size (71%) and the ratio of nodule dry weight to plant dry weight (80%) in experiment 2 (Table 8.2). The combination of *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* 532C increased nodule dry weight per plant (125 and 80%) and nodule size (116 and 67%) in experiments 1 and 2, respectively, when compared with *B. japonicum* 532C preincubated with 15 μM genistein. The same combination increased nodule number (13%), nodule dry weight per plant (58%), nodule size (33%), and the ratio of nodule dry weight to plant dry weight (39%) in experiment 2 when compared with *B. japonicum* 532C co-inoculated with PGPR 1-102 alone.

Co-inoculation of soybean plants with of *B. japonicum* 532C and PGPR 2-68 increased nodule number (33%), nodule dry weight per plant (12%) and the ratio of nodule dry weight to plant dry weight (27 %) over *B. japonicum* 532C alone in experiment 1 (Table 8.2).

Soybean plants co-inoculated with *B. japonicum* USDA110 and PGPR 1-102 had increased nodule dry weight per plant (48%), nodule size (56%) and the ratio of nodule dry weight to plant dry weight (23%) in experiment 1 and increased nodule number (51%), nodule dry weight per plant (181%), nodule size (88%) and the ratio of nodule dry weight to plant dry weight (166%) in experiment 2 over *B. japonicum* USDA110 alone (Table 8.2). *B. japonicum* USDA110 preincubated with 15 μM genistein increased nodule number (37%), nodule dry weight per plant (118%), nodule size (56%) and the ratio of nodule dry weight to plant dry weight (54%) in experiment 1, while this treatment increased nodule number (51%), and nodule size (25%) in experiment 2 over the *B. japonicum* USDA110 alone (Table 8.2). This treatment increased nodule dry weight increased almost 4 times, while ratio of nodule weight to plant weight increased 3 times in experiment 2 over the *B. japonicum* USDA110 alone.

The combination of *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* USDA110 increased nodule number (72%), nodule dry weight per plant (50%), and the ratio of nodule dry weight to plant dry weight (25%) in experiment 1 while it increased nodule dry weight per plant (35%), nodule size (67%) and the ratio of nodule dry weight to plant dry weight (20%) in experiment 2, when compared with *B. japonicum* USDA110 co-inoculated with PGPR 1-102 (Table 8.2). *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* USDA110 increased nodule number (14%) in experiment 1 while it increased nodule size 2.5 times in experiment 2 more than *B. japonicum* USDA110 preincubated with 15 μM genistein.

Co-inoculation of soybean plants with *B. japonicum* USDA110 and PGPR 2-68 increased nodule number (34%), nodule size (125%), while nodule dry weight per plant and the ratio of nodule dry weight to plant dry weight increase 3 times over *B. japonicum* USDA110 alone in experiment 2 (Table 8.2). On the other hand, the combination of *S. liquefaciens* 2-68, 15 μM genistein and *B. japonicum* USDA110 decreased nodule number (13 and 25%), nodule dry weight per plant (8 and 41%) and the ratio of nodule dry weight to plant dry weight (12 and 44%) when compared to *B. japonicum* USDA110 and PGPR 2-68 in experiments 1 and 2, respectively. Also, the same combination decreased nodule number (19 and 34%), nodule dry weight per plant (38 and 56%) and the ratio of nodule dry weight to plant dry weight (25 and 45%) when compared to *B. japonicum* USDA110 preincubated with 15 μM genistein.

8.4.1.3. At 15°C

Co-inoculation of soybean plants with a mixture of *B. japonicum* 532C and PGPR 1-102 increased nodule number (143 and 52%), and nodule dry weight per plant (32 and 88%) in experiments 1 and 2 over *B. japonicum* 532C alone (Table 8.3). *B. japonicum* 532C preincubated with 20 μM genistein increased nodule number (108%) in experiment 1 and increased nodule dry weight per plant (122%), nodule size (109%) and the ratio of nodule dry weight to plant dry weight (114%) in experiment 2 over *B. japonicum* 532C alone (Table 8.3). The combination of *S. proteamaculans* 1-102, 20 μM genistein and *B. japonicum* 532C decreased nodule number (48 and 40%), and nodule dry weight per plant (69 and 44%) in experiments 1 and 2, respectively, when compared with *B. japonicum* 532C preincubated with PGPR 1-102. The same combination also decreased nodule dry weight per plant (71 and 53%), and nodule size (50 and 46%) in experiments 1 and 2, respectively, when compared with *B. japonicum* 532C preincubated with 20 μM genistein.

Co-inoculation of soybean plants with a mixture of *B. japonicum* 532C and PGPR 2-68 decreased nodule size (33%) and ratio of nodule weight to plant weight (40%) compared to *B. japonicum* 532C alone in experiment 1 (Table 8.3).

PGPR 1-102 co-inoculated with *B. japonicum* USDA110 increased nodule number (2.5 times), nodule dry weight per plant (3 times) and ratio of nodule weight to plant weight (2.5) times over *B. japonicum* USDA110 alone in experiment 2. *B. japonicum* USDA110 preincubated with 20 μM genistein increased nodule number (51 and 79%), nodule dry weight per plant (65 and 115%) and ratio of nodule weight to plant weight (29 and 100%) in both experiments 1 and 2, respectively, over the *B. japonicum* USDA110 alone (Table 8.3).

The combination of *S. proteamaculans* 1-102, 20 μM genistein, and *B. japonicum* USDA110 decreased nodule number (18 and 55%), nodule dry weight per plant (44 and 60%), and ratio of nodule weight to plant weight (88 and 55%) in experiments 1 and 2, respectively, compared with *B. japonicum* USDA110 co-inoculated with PGPR 1-102. The same combination decreased nodule number (20 and 31%), nodule dry weight per plant (39 and 41%), and the ratio of nodule dry weight to plant dry weight (94 and 44%) per plant in experiments 1 and 2, respectively, compared with *B. japonicum* USDA110 with 20 μM genistein (Table 8.3).

Co-inoculation of soybean plants with a mixture of *B. japonicum* USDA110 and PGPR 2-68 decreased nodule dry weight per plant (33%), nodule size (33%) and the ratio of nodule dry weight to plant dry weight (57%) compared to *B. japonicum* USDA110 alone in experiment 1 (Table 8.3). There was no difference between the combination of *S. liquefaciens* 2-68, 20 μM genistein and *B. japonicum* USDA110 when compared to either *B. japonicum* USDA110 co-inoculated with PGPR 2-68 or *B.*

japonicum USDA110 preincubated with 20 μM genistein.

8.4.2. Nitrogen fixation

8.4.2.1. At 25 °C

There was no difference in the plant nitrogen fixation (as indicated by total plant nitrogen content minus seed nitrogen) between soybean plants receiving a mixture of *B. japonicum* 532C and PGPR 1-102 and *B. japonicum* 532C alone in experiments 1 and 2 (Table 8.1). *B. japonicum* 532C preincubated with 5 μM genistein fixed 28 and 69% more nitrogen than *B. japonicum* 532C alone in experiments 1 and 2, respectively (Table 8.1). The combination of *S. proteamaculans* 1-102, 5 μM genistein and *B. japonicum* 532C was not different in the amount of plant nitrogen fixed from the *B. japonicum* 532C preincubated with 5 μM genistein treatment in experiment 2. The same combination resulted in 12 and 24% more plant nitrogen fixed in experiments 1 and 2, respectively, than *B. japonicum* 532C co-inoculated with PGPR 1-102. The same combination resulted in 11 and 43% more plant nitrogen fixed in experiments 1 and 2, respectively, than *B. japonicum* 532C alone.

Co-inoculation of soybean plants with a mixture of *B. japonicum* 532C and PGPR 2-68 resulted in 10 and 52% more plant nitrogen fixed, in experiments 1 and 2, respectively, than *B. japonicum* 532C alone (Table 8.1). Plant nitrogen fixation resulting from PGPR 1-102 co-inoculated with *B. japonicum* USDA110 was increased 10 and 41% over *B. japonicum* USDA110 alone in experiments 1 and 2, respectively. *B. japonicum* USDA110 preincubated with 5 μM genistein increased plant nitrogen fixation by 21 and 41% over the *B. japonicum* USDA110 alone in experiments 1 and 2, respectively (Table 8.1). Nitrogen fixation by plants receiving *S. proteamaculans* 1-102, 5 μM genistein and *B. japonicum* USDA110 was 7% greater than *B. japonicum* USDA110 co-inoculated with *S. proteamaculans* 1-102 in experiment 1. There was no difference in the plant nitrogen fixation for plants receiving *S. proteamaculans* 1-102, 5 μM genistein and *B. japonicum* USDA110 and those receiving *B. japonicum* USDA110 preincubated with 5 μM genistein.

Co-inoculation of soybean plants with a mixture of *B. japonicum* USDA110 and

PGPR 2-68 resulted in 33 and 57% more fixed nitrogen than *B. japonicum* USDA110 alone in experiments 1 and 2, respectively (Table 8.1). The combination of *S. liquefaciens* 2-68, 5 μM genistein and *B. japonicum* USDA110 resulted in (28 and 20%) less fixed nitrogen than *B. japonicum* USDA110 and PGPR 2-68 in experiments 1 and 2, respectively. The same combination resulted less fixed nitrogen (20 and 11%) compared *B. japonicum* USDA110 preincubated with 5 μM genistein in experiments 1 and 2, respectively.

8.4.2.2. At 17.5 °C

Nitrogen fixation by soybean plants receiving *B. japonicum* 532C co-inoculated with PGPR 1-102 was (19%) greater in experiment 1 than plants receiving *B. japonicum* 532C alone (Table 8.2). *B. japonicum* 532C preincubated with 15 μM genistein resulted in (23 and 65%) more plant nitrogen fixed than *B. japonicum* 532C alone in experiments 1 and 2, respectively (Table 8.2). The combination of *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* 532C resulted in an average plant nitrogen fixation level not different from the *B. japonicum* 532C preincubated with 15 μM genistein treatment. The same combination resulted in 54% more plant nitrogen fixed than *B. japonicum* 532C co-inoculated with PGPR 1-102 in experiment 2.

Co-inoculation of soybean plants with a mixture of *B. japonicum* 532C and PGPR 2-68 resulted in 39% more plant nitrogen fixed over *B. japonicum* 532C alone in experiment 2 (Table 8.2).

The nitrogen fixation by plants receiving PGPR 1-102 co-inoculated with *B. japonicum* USDA110 increased 48 and 51% over *B. japonicum* USDA110 alone treatment in experiments 1 and 2, respectively. *B. japonicum* USDA110 preincubated with 15 μM genistein resulted in 75 and 76% more fixed nitrogen than *B. japonicum* USDA110 alone in experiments 1 and 2, respectively (Table 8.2). The nitrogen fixation by plants receiving *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* USDA110 was 16 and 34% greater than *B. japonicum* USDA110 receiving *S. proteamaculans* 1-102 in experiments 1 and 2, respectively. Nitrogen fixation by plants receiving *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* USDA110 was 15% higher than those receiving *B. japonicum* USDA110 preincubated with 15 μM genistein in experiment 2.

Co-inoculation of soybean plants with a mixture of *B. japonicum* USDA110 and PGPR 2-68 resulted in (89 and 19%) more fixed nitrogen when compared to *B. japonicum* USDA110 alone in experiments 1 and 2, respectively (Table 8.2). The combination of *S. liquefaciens* 2-68, 15 μM genistein and *B. japonicum* USDA110 resulted in 24% more fixed nitrogen, in experiment 2, when compared to the *B. japonicum* USDA110 and PGPR 2-68 treatment. The same combination resulted in less fixed nitrogen (21 and 16%) compared to *B. japonicum* USDA110 preincubated with 15 μM genistein in experiments 1 and 2, respectively.

8.4.2.3. At 15 °C

After 50 days, the duration of this experiment, plants inoculated with *B. japonicum* USDA110 or 532C alone had not fixed any nitrogen in either experiment at 15°C RZT (Table 8.3). This was also true for some other combinations, such as *B. japonicum* USDA110 coinoculated with PGPR 2-68 and 20 μM genistein in experiment 1. Plant nitrogen fixation was increased by co-inoculating with *S. proteamaculans* 1-102 for either *B. japonicum* USDA110 or 532C when compared to *B. japonicum* alone in experiments 1 and 2, respectively (Table 8.3). Nitrogen fixation by plants receiving either *B. japonicum* USDA110 or 532C preincubated with 20 μM genistein was greater than by plants inoculated with *B. japonicum* alone in both experiments.

The combination of *B. japonicum* USDA110, 20 μM genistein and *S. proteamaculans* 1-102 decreased the amount of fixed nitrogen by 33 and 71% when compared to *B. japonicum* USDA110 co-inoculated with *S. proteamaculans* 1-102 in experiments 1 and 2, respectively. Nitrogen fixation by soybean plants receiving *B. japonicum* 532C, 20 μM genistein and *S. proteamaculans* 1-102 was decreased by 26 and 45% compared to *B. japonicum* 532C and *S. proteamaculans* 1-102 in experiments 1 and 2, respectively.

No other relative comparisons were possible as no nitrogen was fixed in plants inoculated with *B. japonicum* in the absence of genistein or PGPR.

8.4.3. Nitrogen distribution

Nodule, shoot, and root nitrogen concentrations were assayed separately. The differences of nitrogen concentration ratio of nodule to shoot tissues between treatments indicated that the applied treatments variably affected nitrogen transfer from root-nodules to shoot tissues. For example, at 15°C plants inoculated with *B. japonicum*USDA110 and *S. proteamaculans* 1-102 had a lower nitrogen concentration ratio for nodule to shoot tissues (2.3 and 1.3) when compared to plants receiving *B. japonicum* USDA110alone (2.8 and 2.7) in experiments 1 and 2, respectively.

The same combination was not different from those receiving *B. japonicum* and 20 μM genistein in experiment 1 and 2. At 17.5 and 25°C RZT, both the plant nodule:shoot and shoot:root nitrogen concentration ratios were not different among PGPR treatments or when compared to control plants receiving *B. japonicum* or genistein or PGPR alone (Tables 8.4 and 8.5).

8.5. Discussion

Co-inoculation of soybean plants with PGPR strains produced a wide range of effects which varied among PGPR and over RZTs. *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68 were reported to enhance nodulation and nitrogen fixation at suboptimal RZTs (Zhang et al., 1996b). At an optimal RZT (25°C), *S. liquefaciens* 2-68 was reported to increase nodule size and nitrogen fixation, while *S. proteamaculans* 1-102 had the same effect but at a lower RZT (15°C). *S. proteamaculans* 1-102 was reported to perform very poorly at 25°C (Zhang et al., 1996a,b). Those results indicated that the effects of PGPR on soybean plants are affected by RZT. The same patterns of results were observed in both experiments 1 and 2 of the current work. At an optimal RZT (25°C), *S. liquefaciens* 2-68 increased nodule dry weight per plant, nodule size and ratio of nodule weight to plant weight. The increase in the ratio of nodule weight to plant weight was due to increased nodule sized (as indicated by the higher average weight per nodule). At 15°C RZT *S. proteamaculans* 1-102 increased nodule number, nodule dry weight per plant, nodule size and ratio of nodule weight to plant weight, again, confirming the results of Zhang et al. (1996b).

A wide range of possibilities have been postulated to explain the mechanisms of PGPR, including an increase in mobilization of insoluble nutrients and subsequent enhancement of uptake by the plants (Lifshitz et al., 1987), production of antibiotics toxic to soil-borne pathogens (Li and Alexander, 1988), associative N_2 fixation (Chanway and Holl, 1991a), and production of plant growth regulators that stimulate plant growth (Gaskins et al., 1985). Given that our plants were growing in nutrient solution, without pathogen pressure, and that members of the genus *Serratia* are not known to fix N_2, our results suggest the fourth possibility. PGPR applied to legume crops have also been demonstrated to positively affect symbiotic nitrogen fixation by enhancing root nodule number or mass (Grimes and Mount, 1984; Polonenko et al., 1987; Yahalom et al., 1987; Zhang et al., 1996b) and increasing nitrogenase activity (Iruthayathas et al., 1983; Alagawadi and Gaur, 1988). Previous studies have shown that co-inoculation of *Pseudomonas* sp. (including *P. fluorescens* and *P. putida*) with *B. japonicum* increased nodule formation and weight of soybean plants in different rooting media (soil and perlite mix) (Polonenko et al., 1987); in the present study, both *Serratia* species increased these two variables. The enhancement of nodule weight per plant by PGPR or genistein additions always led to increased nitrogen fixation (Tables 8.1, 8.2, and 8.3). Increases in nodule weight per plant can be due to increases in nodule number, increases in the weight per nodule or both. In this work both were increased by PGPR and genistein additions and, although there were few notable exceptions, the weight per nodule generally increased more. Both the number of successful infections and the growth of nodules resulting from successful infections were stimulated by genistein and PGPR additions.

Genistein (the most effective plant-to-bacterium signal in the soybean-*B. japonicum* N_2 fixing symbiosis) has been reported to increase soybean nodulation at suboptimal RZTs. Genistein effects increased with decreasing RZT (Zhang and Smith, 1995b). At suboptimal RZTs (17.5 and 15°C) the most effective concentrations are in the 15 to 20 μM range, whereas at an optimal (25°C) RZT 5 μM was most effective.

At 25°C, addition of PGPR plus genistein generally resulted in greater, and approximately additive, increases than the addition of either alone. However, while co-inoculation of soybean plants with *B. japonicum* USDA110 and PGPR 2-68 increased nodule dry weight per plant (26%), nodule size (40%) and the ratio of nodule dry weight to plant dry weight (13%) over *B. japonicum* alone in experiment 1 (Table 8.1) or preincubation of *B. japonicum* USDA110 with 5 μM genistein, which was reported to be most effective at 25°C (Zhang et al., 1995b), the addition of PGPR 2-68 and genistein resulted in an antagonistic interaction between the PGPR and the genistein. Nodule dry weight per plant, nodule size and the ratio of nodule dry weight to plant dry weight were decreased when compared to *B. japonicum* USDA110 co-inoculated with PGPR 2-68 (Table 8.1). The combination of *B. japonicum* USDA110 with PGPR 2-68 or 5 μM genistein showed the largest proportional increases of any of the possible combinations of *B. japonicum* strains, PGPR strains and genistein, when compared to *B. japonicum* USDA110 alone, but when genistein and PGPR were added together an antagonistic effect was observed.

In the same way, the largest proportional increases in nodulation variables and nitrogen fixation (ranging up to 2-3 times), due to the addition of either PGPR or genistein occured at 15°C RZT and it was at this RZT that antagonistic effects were observed between PGPR and genistein additions. Although the underlying cause for this is unclear, it does appear that when the increases are largest, due to the addition of PGPR or genistein alone the probability of antagonistic interactions between the two is greatest.

Zhang et al. (1995a) demonstrated the existence of a linear relationship between RZT and the time from inoculation with *B. japonicum* to the onset of nitrogen fixation between 25 and 17.5°C RZTs. In this RZT range the time between inoculation and the onset of nitrogen fixation was delayed by 2 days for each degree decrease in RZT. However, the onset of nitrogen fixation was delayed by one week per °C as the RZT decreased from 17.5 to 15°C. The results from the second experiment showed that the nitrogen concentrations of plant shoots and whole plants grown at 15°C were lower than those at 17.5 and 25°C. These results agreed with those of Zhang et al. (1996b). Our data showed stimulation of nodulation and nitrogen fixation due to genistein addition. At optimal (25°C) RZT *B. japonicum* USDA110 co-inoculated with 5 μM genistein increased nodule number, nodule dry weight per plant, nodule size and ratio of nodule weight to plant weight in experiments 1 and 2. At suboptimal RZT (17.5°C) *B. japonicum* USDA110 pre-inoculated with 15 μM genistein increased nodule number, nodule dry weight per plant, nodule size and ratio of nodule weight to plant weight relative to the controls in both experiments 1 and 2 (Table 8.2). At 15°C *B. japonicum* USDA110 co-inoculated with 20 μM genistein resulted in increased nodule number, nodule dry weight per plant, nodule size and ratio of nodule weight to plant weight relative to the controls in both experiments 1 and 2 (Table 8.3).

Some PGPR and genistein combinations showed additive effects. The combination of PGPR 1-102, *B. japonicum* USDA110 and 5μM genistein had an additive effect on the nodule dry weight per plant in both experiments 1 and 2 at 25°C. At 25°C RZT many of the additive effects were approximately complete, with the increases due to genistein addition being nearly the same in the presence or absence of PGPR. On the other hand, the combination of PGPR 2-68, *B. japonicum* USDA110 and 5μM genistein showed an antagonistic effect on almost all plant variables at 25°C in contrast with PGPR 2-68, *B. japonicum* USDA110 alone which was previously reported to increase plant nodule number, nodule weight, nodule size and nitrogen fixation capacity (Zhang et al., 1996b) and which data in our current work support.

. At 17.5°C, there was still evidence of additivity, for instance, the combination of PGPR 1-102, *B. japonicum* USDA110 and 15μM genistein had an additive effect on the nodule number in experiment 1 and on nodule size in experiment 2. However, the level of additivity was not complete, with the increases due to the addition of genistein being smaller, or non existent, in the presence of genistein than in its absence. As at 25°C RZT, the combination of *B. japonicum* USDA110, PGPR 2-68 and 5 μM genistein resulted in antagonism between the genistein and the PGPR.

At 15°C, the combination of PGPR 1-102, *B. japonicum* USDA110, and 20μM genistein had an antagonistic effect on all measured variables. PGPR 1-102 performed better by itself than with genistein at RZT 15°C.

The additive effects observed at 25 and 17.5°C RZT could be explained in that genistein and PGPR may work by different mechanisms, stimulating different aspects of soybean plant physiology at optimum RZTs. At a suboptimal RZT (15°C) or for the combination of *B. japonicum* USDA110, PGPR 2-68 and 5μM genistein at 25°C, the effects are antagonistic. The cause of the antagonistic effects is unclear and requires further study.

The frequent increases in the ratio of nodule weight to plant dry weight demonstrate that plants treated with genistein or PGPR, or both required more nodule mass to achieve each gram of accumulated dry weight. Thus, while the additions of PGPR, genistein or both, increase nodule dry weight per plant, and plant nitrogen fixation, it appears that the efficiency with which the additional nodule mass is able to support plant growth is less than for the nodule mass formed without the addition of these materials. These decreases in efficiency could be due to decreased relative efficiency for nitrogenase or greater restrictions of O_2 entry in to the nodules (Hunt and Layzell, 1993).

The nitrogen distribution data indicated that the applied treatments variably affected nitrogen translocation from root-nodules to shoot tissues. The nitrogen concentrations of plant shoots and whole plants grown at 15°C were lower than those at 17.5 and 25°C RZT. These results agree with those of Zhang et al. (1996b). *B. japonicum* and *S. proteamaculans* 1-102 showed a lower nitrogen concentration ratio for nodule to shoot tissues (2.3) and (1.3) when compared to plants receiving *B. japonicum* alone (2.8) and (2.7) in experiments 1 and 2, respectively. Co-inoculation of *B. japonicum* with *S. proteamaculans* 1-102 was shown to increase nitrogen concentration of plant shoots at 15°C RZT.

In summary, the results of this study indicated that 1) some PGPR strains combined with genistein can enhance soybean nodulation and nitrogen fixation, 2)

PGPR *S. proteamaculans* 1-102 which was previously shown to perform poorly at 25°C was shown in this study to be effective at 25°C when combined with 5 μM genistein, 3) the stimulations due to combinations of PGPR and genistein were probably due to PGPR stimulation prior to the onset of N_2 fixation on PGPR plus genistein effects after the onset of nitrogen fixation, 4) the combinations of PGPR and genistein had an additive effect compared to PGPR or genistein alone at the highest RZT tested (25°C), and partial additivity at 17.5°C, while they showed antagonistic effects at a lower RZT (15°C). Overall these findings confirm that adding PGPR or genistein, along with *B. japonicum*, to soybean roots each accelerates nitrogen fixation and shows, in addition, that adding the two together caused stimulations of N_2 fixation that, in some cases, were greater than those caused by either PGPR or genistein alone.

Table 8.1. Effects of *B. japonicum* strains, PGPR strains and genistein addition on soybean nodulation and nitrogen fixation at 25° C.

PGPR strain	*B. japonicum* strain	Genistein (μ M)	nodule number ($plant^{-1}$)	nodule dry weight (mg) ($plant^{-1}$)	$nodule^{-1}$	$plant^{-1}$ (mg)	plant N concentration ($mg\ g^{-1}$)	fixed N ($mg\ plant^{-1}$)
Experiment 1								
1-102	USDA110	0	117.4	184.5	1.6	0.037	31.8	162.6
		5	103.0	235.0	2.3	0.045	32.5	173.3
	532C	0	86.8	202.1	2.3	0.044	30.7	141.9
		5	97.7	242.8	2.5	0.047	30.2	159.3
2-68	USDA110	0	84.0	265.8	3.2	0.045	32.4	197.6
		5	95.3	203.9	2.1	0.044	29.9	143.1
	532C	0	78.7	162.7	2.1	0.034	31.6	157.9
Control	USDA110	0	92.7	131.5	1.4	0.029	32.0	148.2
		5	119.0	213.7	1.8	0.040	32.8	179.4
	532C	0	83.7	129.4	1.5	0.030	29.6	143.1
		5	102.7	237.6	2.3	0.045	33.7	182.5
$LSD_{0.05}$			**6.6**	**15.7**	**0.2**	**0.0028**	**1.5**	**8.4**
Experiment 2								
1-102	USDA110	0	96.0	168.0	1.8	0.031	34.93	163.7
		5	83.0	205.0	2.5	0.036	34.82	168.7
	532C	0	67.0	172.0	2.6	0.034	27.26	111.0
		5	78.0	212.7	2.7	0.038	32.87	158.2
2-68	USDA110	0	64.0	235.7	3.7	0.036	32.6a	183.0
		5	75.7	174.0	2.3	0.034	33.79	146.4
	532C	0	58.7	132.7	2.3	0.024	33.59	151.7
Control	USDA110	0	72.7	101.5	1.4	0.020	28.4b	116.3
		5	99.0	183.7	1.9	0.032	32.93	163.7
	532C	0	63.7	169.3	2.7	0.032	25.21	100.0
		5	82.7	207.7	2.5	0.036	34.27	168.8
$LSD_{0.05}$			**5.9**	**14.4**	**0.2**	**0.003**	**4.27**	**20.7**

Means the same column an experiment were analyzed by an ANOVA protected LSD test.

Table 8.2. Effects of *B. japonicum* strains, PGPR strains and genistein addition on soybean nodulation and nitrogen fixation at 17.5°C.

PGPR strain	*B. japonicum* strain	Genistein (μ M)	nodule number (plant^{-1})	nodule dry weight (mg) (plant^{-1})	nodule dry weight (mg) nodule^{-1}	plant^{-1} (mg)	plant N concentration (mg g^{-1})	fixed N (mg plant^{-1})
Experiment 1								
1-102	USDA110	0	15.7	22.3	1.4	0.016	23.7	23.2
		15	27.0	33.4	1.4	0.020	30.0	26.9
	532C	0	6.3	13.6	1.2	0.011	23.9	26.0
		15	26.7	27.2	1.3	0.018	29.0	28.6
2-68	USDA110	0	22.3	22.3	1.0	0.017	29.0	29.6
		15	19.3	20.6	1.1	0.015	25.9	21.8
	532C	0	21.3	25.3	1.2	0.019	22.6	19.5
Control	USDA110	0	17.3	15.1	0.9	0.013	22.3	15.7
		15	23.7	33.0	1.4	0.020	28.0	27.5
	532C	0	16.0	22.5	1.2	0.015	25.9	21.9
		15	20.3	12.8	0.6	0.017	29.1	26.9
$LSD_{0.05}$			**2.3**	**2.2**	**0.3**	**0.002**	**2.5**	**3.5**
Experiment 2								
1-102	USDA110	0	92.2	137.7	1.5	0.080	31.5	35.2
		15	84.8	185.7	2.5	0.096	32.7	47.3
	532C	0	75.3	108.8	1.5	0.072	29.7	27.6
		15	85.3	172.3	2.0	0.10	31.8	42.5
2-68	USDA110	0	81.8	144.3	1.8	0.097	27.1	27.7
		15	61.0	84.8	1.4	0.054	30.3	34.4
	532C	0	91.4	153.5	1.7	0.096	28.4	32.7
Control	USDA110	0	61.0	49.0	0.8	0.030	27.7	23.3
		15	92.2	193.8	1.0	0.099	32.7	41.0
	532C	0	67.2	47.7	0.7	0.030	28.4	23.5
		15	83.0	95.6	1.2	0.054	32.5	38.8
$LSD_{0.05}$			**6.9**	**7.9**	**0.2**	**0.005**	**2.6**	**4.4**

Means the same column an experiment were analyzed by an ANOVA protected LSD test.

Table 8.3. *B. japonicum* strains, PGPR strains and genistein addition on soybean nodulation and nitrogen fixation at 15°C.

PGPR strain	*B. japonicum* strain	Genistein (µ M)	nodule number (plant^{-1})	nodule dry weight (mg)		plant^{-1} (mg)	plant N concentration (mg g^{-1})	fixed N (mg plant^{-1})
				(plant^{-1})	nodule^{-1}			
Experiment 1								
1-102	USDA110	0	11.0	7.7	0.7	0.008	29.0	15.2
		20	3.0	1.3	0.5	0.002	26.2	10.2
	532C	0	9.0	2.9	0.3	0.007	30.6	8.7
		20	4.7	0.9	0.2	0.001	27.2	11.7
2-68	USDA110	0	8.7	2.9	0.4	0.006	28.0	2.9
		20	9.3	2.2	0.2	0.006	26.7	0.0*
	532C	0	3.0	1.3	0.4	0.003	23.7	0.0*
Control	USDA110	0	7.5	4.3	0.6	0.014	24.2	0.0*
		20	11.3	7.1	0.6	0.018	30.3	9.7
	532C	0	3.7	2.2	0.6	0.005	23.5	0.0*
		20	7.7	3.1	0.4	0.005	32.2	9.7
$LSD_{0.05}$			**1.4**	**1.1**	**0.1**	**0.002**	**1.8**	**1.4**
Experiment 2								
1-102	USDA110	0	33.3	18.3	0.55	0.020	29.0	15.7
		20	15.0	7.3d	0.49	0.009	22.7	4.6
	532C	0	22.0	9.0c	0.41	0.011	25.3	8.9
		20	13.3	5.0e	0.37	0.006	22.7	4.9
2-68	USDA110	0	18.3	7.2d	0.39	0.010	20.3	3.4
		20	21.2	9.3c	0.45	0.013	23.0	4.8
	532C	0	12.8	7.1d	0.57	0.010	19.3	2.6
Control	USDA110	0	12.2	5.7d	0.47	0.008	18.0	0.0*
		20	21.8	12.3	0.56	0.016	24.0	7.1
	532C	0	14.5	4.8f	0.33	0.007	16.7	0.0*
		20	15.3	10.7	0.69	0.015	23.4	3.6
$LSD_{0.05}$			**3.8**	**2.2**	**0.16**	**0.005**	**4.0**	**2.3**

Means the same column an experiment were analyzed by an ANOVA protected LSD test. *Zero presented that the total fixed nitrogen per plant (plant nitrogen content-seed nitrogen content) were zero or minors (because of depletion of seed nitrogen reserves as plants growing in nitrogen free media).

Table 8.4. Effects of co-inoculation of *B. japonicum* with PGPR on nitrogen concentrations of different soybean tissues and nitrogen concentration ratios for nodule:shoot, nodule:root, and shoot:root at three temperatures. (experiment 1)

PGPR	*B. japonicum*	Genistein (μ M)	nitrogen concentrations (mg g^{-1}) nodule	shoot	root	nitrogen concentrations ratios nodule:shoot	nodule:root	shoot:root
15°C								
1-102	USDA110	0	56.7	24.7	12.6	2.3	4.5	1.4
		20	47.3	19.7	11.7	2.4	4.0	1.7
	532C	0	61.0	17.9	12.9	3.4	4.7	1.4
		20	46.0	21.3	11.3	2.2	4.1	1.9
2-68	USDA110	0	53.1	18.0	13.0	2.9	4.1	1.4
		20	49.0	18.9	12.3	2.6	4.0	1.5
	532C	0	37.7	18.1	12.4	2.1	3.0	1.5
Control	USDA110	0	46.0	16.6	11.8c	2.8	3.9	1.4
		20	55.3	22.3	13.2	2.5	4.2	1.7
	532C	0	46.0	17.6	11.4	2.6	4.0	1.5
		20	65.7	17.9	13.0	3.7	5.1	1.4
$LSD_{0.05}$			**4.5**	**3.4**	**1.8**	**0.5**	**0.6**	**0.4**
17°C								
1-102	USDA110	0	32.6	21.1	14.1	1.5	2.7	1.5
		15	35.4	25.0	14.8	1.4	2.4	1.7
	532C	0	32.3	17.2	14.1	1.9	2.3	1.2
		15	41.0	24.3	13.4	1.7	3.1	1.8
2-68	USDA110	0	52.4	21.2	9.6	2.1	5.5	2.5
		15	52.2	20.5	13.7	2.5	3.8	1.5
	532C	0	42.7	23.3	14.8	2.0	2.9	1.4
Control	USDA110	0	35.3	19.3	12.2	1.8	2.9	1.6
		15	48.0	23.7	15.7	2.0	3.1	1.5
	532C	0	37.7	17.0	10.4	2.2	3.6	1.6
		15	50.7	20.4	13.5	2.5	3.8	1.5
$LSD_{0.05}$			**8.2**	**2.8**	**2.6**	**0.5**	**1.1**	**0.4**
25°C								
1-102	USDA110	0	52.8	35.9	16.1	1.5	3.3	2.2
		5	50.2	38.2	16.1	1.3	3.1	2.4
	532C	0	39.5	28.0	14.3	1.5	2.8	2.0
		5	50.4	34.6	13.5	1.5	3.7	2.6
2-68	USDA110	0	49.0	32.3	16.5	1.5	3.0	2.0
		5	50.7	33.8	16.2	1.5	3.1	2.1
	532C	0	51.5	34.8	14.5	1.5	3.6	2.4
Control	USDA110	0	40.9	30.3	13.8	1.3	3.0	2.2
		5	49.7	32.5	16.5	1.5	3.0	2.0
	532C	0	37.7	24.0	13.9	1.6	2.7	1.7
		5	52.8	36.0	14.3	1.5	3.7	2.5
$LSD_{0.05}$			**10.0**	**4.4**	**2.5**	**0.37**	**0.8**	**0.4**

Means the same column an experiment were analyzed by an ANOVA protected LSD test.

Table 8.5. Effects of co-inoculation of *B. japonicum* with PGPR on nitrogen concentrations of different soybean tissues and nitrogen concentration ratios for nodule:shoot, nodule:root, and shoot:root at three temperatures. (experiment 2)

			nitrogen concentrations (mg g^{-1})			nitrogen concentrations ratios		
PGPR	*B. japonicum*	Genistein (μM)	nodule	shoot	root	nodule:shoot	nodule:root	shoot:root
15°C								
1-102	USDA110	0	46.2	35.4	13.8	1.3	3.3	2.6
		20	47.1	31.7	12.4	1.5	3.8	2.6
	532C	0	47.0	19.5	12.4	2.4	3.8	1.6
		20	46.8	24.1	12.8	1.9	3.7	1.9
2-68	USDA110	0	44.4	21.3	13.9	2.1	3.2	1.5
		20	63.6	20.4	13.1	3.1	4.9	1.6
	532C	0	39.2	21.8	12.0	1.8	3.3	1.8
Control	USDA110	0	43.0	15.7	10.3	2.7	4.2	1.5
		20	54.7	22.3	13.2	2.5	4.1	1.7
	532C	0	39.1	18.9	9.7	2.1	4.0	1.9
		20	52.4	19.9	10.6	2.6	4.9	1.9
$LSD_{0.05}$			**15.2**	**1.4**	**2.1**	**0.6**	**1.4**	**0.3**
17°C								
1-102	USDA110	0	48.4	25.4	15.4	1.9	3.1	1.6
		20	55.0	25.8	16.8	2.1	3.3	1.5
	532C	0	43.0	30.3	15.8	1.4	2.7	1.9
		20	49.4	28.4	17.7	1.7	2.8	1.6
2-68	USDA110	0	44.7	23.2	13.5	1.9	3.3	1.7
		20	46.2	28.8	15.8	1.6	2.9	1.8
	532C	0	41.7	27.3	16.1	1.5	2.6	1.7
Control	USDA110	0	41.7	26.3	15.2	1.6	2.7	1.7
		20	53.7	27.1	17.4	2.0	3.1	1.6
	532C	0	41.0	27.5	16.6	1.5	2.5	1.7
		20	52.0	28.5	17.1	1.8	3.0	1.7
$LSD_{0.05}$			**5.6**	**6.9**	**1.9**	**0.6**	**0.5**	**0.4**
25°C								
1-102	USDA110	0	49.1	43.6	16.6	1.4	3.0	2.1
		20	46.9	35.0	19.5	1.3	2.4	1.8
	532C	0	42.7	28.5	17.6	1.5	2.4	1.6
		20	45.9	35.2	18.3	1.3	2.5	1.9
2-68	USDA110	0	48.1	32.1	17.5	1.5	2.7	1.8
		20	48.0	24.4	17.4	2.0	2.8	1.4
	532C	0	45.3	34.1	16.4	1.3	2.8	2.1
Control	USDA110	0	47.3	31.4	17.0	1.5	2.8	1.8
		20	47.6	35.7	17.7	1.3	2.7	2.0
	532C	0	41.4	29.7	15.4	1.4	2.7	1.9
		20	48.0	34.4	18.6	1.4	2.6	1.8
$LSD_{0.05}$			**3.9**	**1.9**	**2.5**	**0.17**	**0.5**	**0.3**

Means the same column an experiment were analyzed by an ANOVA protected LSD test.

Preface to Section 9

Section 9 is comprised of a manuscript by N. Dashti, R.K. Hynes, and D.L. Smith, prepared for publication in 1996. The format has been changed to conform as much as possible to a consistent format within this thesis. All literature cited in this section are listed in the reference section at the end of the thesis. Each table for section 9 is presented at the end of this section.

As the previous section showed that the stimulation of soybean nodulation and nitrogen fixation by PGPR plus genistein was greater than either of these factors alone at 25°C and 17°C, but not at 15°C RZT, in this section we examined these effects on the growth and development of soybean plants.

Section 9

CO-INOCULATION OF *Bradyrhizobium japonicum* PREINCUBATED WITH GENISTEIN AND PLANT GROWTH PROMOTING RHIZOBACTERIA ACCELERATES SOYBEAN [*Glycine max* (L.) Merr.] PHYSIOLOGY AND DEVELOPMENT AT SUBOPTIMAL ROOT ZONE TEMPERATURES

9.1. Abstract

Application of plant growth-promoting rhizobacteria (PGPR) has been reported to enhance soybean growth and development at suboptimal root zone temperatures (RZTs) as has preincubation of *Bradyrhizobium japonicum* cultures with the plant-to-bacteria signal molecule, genistein. A controlled-environment experiment was conducted to examine the combined effect of both PGPR and genistein on soybean growth and photosynthesis at suboptimal RZTs. Each of two PGPR strains, *Serratia proteamaculans* 1-102 and *Serratia liquefaciens* 2-68 were applied to *B. japonicum* USDA110 or 532C preincubated with different concentrations of genistein (0, 15, or 20μM). The resulting inocula were added to a soybean rooting medium to test their ability to reduce the negative effects of low RZT on soybean growth and development by improving the physiological status of the plants. Three RZTs were tested: 25, 17.5, and 15°C. At each temperature some combination of PGPR strains and genistein concentration increased plant growth and development, but the most stimulatory combinations of PGPR strains, genistein concentration and *B. japonicum* strains varied with temperature. The combinations that were most stimulatory at each temperature were as follows: at 15°C - *S. proteamaculans* 1-102, genistein concentration 0 μM and *B. japonicum* USDA110, at 17.5°C - *S. proteamaculans* 1-102, genistein concentration 15 μM and *B. japonicum* USDA110, and at 25°C - *S. proteamaculans* 1-102, genistein concentration 5 μM and *B. japonicum* USDA110. In at least some cases, these stimulatory effects can be attributed to additive effects of both PGPR and genistein in enhancing soybean growth and early development. At 25°C, some combinations of PGPR strains, genistein concentration and *B. japonicum* strains has shown an additive

effects, while at 15°C others has antagonistic effects.

9.2. Introduction

Soybean [*Glycine max* (L.) Merr.] is a plant of tropical to subtropical origin, which requires temperatures in the 25 to 30°C range for optimal growth and development (Jones and Tisdale, 1921). When soybean is grown under suboptimal temperatures (below 25°C), plant growth and development are inhibited, and final total dry matter production and grain yield are decreased (Summerfield and Wien, 1982). Soybean production in eastern Canada is at the northernmost North American limit of the crop. In most of the Canadian soybean production area, early vegetative growth of soybean occurs under elevated shoot temperatures but low root zone temperatures (RZTs).

Studies of low RZT effects on nitrogen fixation by soybean have indicated that low RZTs decreased both nodulation and nodule function (Jones and Tisdale, 1921; Hardy et al., 1968, Roughley and Date, 1986; Legros and Smith, 1994; Lynch and Smith, 1994). Between 25°C (an optimum RZT) and 17°C, the time between soybean inoculation and the onset of nitrogen fixation was linearly delayed by 2 days for each degree decrease in temperature, whereas at RZT between 17 and 15°C nodulation and onset of nitrogen fixation were delayed by one week for each degree of decrease in temperature (Zhang et al., 1995a).

Plant growth promoting rhizobacteria (PGPR), are rhizosphere microorganisms which can colonize plant roots and promote plant growth (Kloepper et al., 1980a). PGPRs can dramatically increase plant growth, development, and yield in such nonlegume crops as potato, radish, sugar beet, wheat and canola (Gaskins et al., 1985; Polonenko et al., 1987). Co-inoculation of *(Brady)Rhizobium* with PGPR has been shown to increase soybean plant root and shoot weight, seed yield, plant vigour, nodulation and nitrogen fixation under normal growth conditions (Verma et al., 1986; Li and Alexander, 1988). Soybean yield has been shown to increase following inoculation with a mixed culture of *B. japonicum* and certain PGPR strains, when compared to inoculation with *B. japonicum* alone (Yahalom et al., 1987). Similarly, an increase in grain yield, nodule dry matter, and nitrogenase activity was obtained in chick pea inoculated with a mixture of *Azospirillum brasilense* and *Rhizobium* strains (Rai, 1983). The beneficial effects of PGPR are thought to be direct plant growth promotion by the production of plant growth regulators (Gaskins et al., 1985, Kloepper and Schroth, 1981), increased access to soil nutrients (Lifshitz et al., 1987), disease control (De-Ming and Alexander, 1988) and associative nitrogen fixation (Chanway and Holl, 1991a).

Recently, the tripartite association of nitrogen-fixing bacteria, PGPR and soybean plants was investigated at different RZTs. Zhang et al. (1996a,b) have shown that co-inoculation of *B. japonicum* with some PGPR strains increased soybean nodulation, nitrogen fixation, growth and development at both optimal and suboptimal RZTs, although, the PGPR strains exerting stimulatory effects varied with RZT. The effects of PGPR on legume plant growth and development were apparent before the onset of nitrogen fixation and were therefore through improved overall plant physiological activities, whereas afterward the onset of nitrogen fixation they may also have been due to improvement of plant nodulation and nitrogen fixation.

Symbiotic N_2 fixation is a complex process involving physiological and biochemical aspects of both symbiotic partners. An early phase in the nodulation process involves the selective attachment to and penetration of the plant root by the bacterium. The first step is the release of flavonoid signal molecules that bind the bacterial symbiont and trigger the coordinated expression of a series of bacterial nodulation genes called *nod* genes (Long, 1989, Peters and Verma, 1990). The isoflavones daidzein and genistein are the major components of the soybean root extracts responsible for inducing the *nod* genes of *B. japonicum* (Kosslak et al., 1987). Genistein plays an important role in the establishment of the soybean-*Bradyrhizobium japonicum* nitrogen fixing symbiosis. It is essential to the development of effective root nodules and responsible for inducing the *nod* genes of *B. japonicum* (Kosslak et al., 1987). Zhang et al. (1996c) have shown that the roots of plants germinated and grown at lower RZTs have lower genistein concentrations and contents than plants grown at higher RZTs. The beneficial effect of genistein increased with decreasing RZT (Zhang and Smith, 1995b). At suboptimal RZT (17.5 and 15°C) the most effective concentrations are in the 15 to 20 μM range, whereas at optimal (25°C) RZT 5 μM is most effective.

Studies by Zhang and Smith (1995b) and Zhang et al. (1996d), under controlled environment conditions and under field conditions, have shown that preincubation of *B. japonicum* with genistein hastened the onset of nitrogen fixation, increased the number of nodules produced, increased the size of the nodules, and increased plant growth.

Zhang and Smith (1995b) showed that genistein stimulation of photosynthesis was only seen after the onset of N_2 fixation, while PGPR stimulation of photosynthesis was apparent prior to the onset of N_2 fixation. Thus, while the effects of genistein are through nitrogen fixation, the effects of PGPR are on overall plant vigour (1996a). Since PGPR and genistein seem to work by different mechanisms, their beneficial effects might reasonably be additive. As both PGPR and genistein have been shown to stimulate plant growth and development, but by different mechanisms, a controlled environment experiment was conducted to test the hypothesis that the inclusion of both PGPR and genistein in *B. japonicum* inocula would increase various soybean physiological activities, such as photosynthesis, leading to levels of plant growth at suboptimal RZTs that are greater than is the case with the addition of either PGPR or genistein alone.

9.3. Materials and methods

9.3.1. Experimental design

Two experiments were conducted. Both were arranged in completely randomized designs with three replications. Combinations of *B. japonicum* strains, genistein levels and PGPR strains were used as the treatments at three different RZTs 15, 17.5, and 25°C. At each RZT three PGPR levels (0, *Serratia proteamaculans* 1-102 and *Serratia liquefaciens* 2-68), two *B. japonicum* strains [USDA110 and 532C (Hume and Shelp, 1990)], and two genistein concentrations were factorially combined. The two genistein concentrations tested were 0 and 5 μM, 0 and 15μM and 0 and 20μM at 25, 17.5 and 15°C RZT, respectively (Zhang and Smith, 1995b). The combination of PGPR (*S.*

liquefaciens 2-68), *B. japonicum* (532C), and genistein concentrations (5, 15 and 20 μM at 25, 17.5 and 15°C, respectively), was excluded due to insufficient space. Zhang and Smith (1995b) found that at suboptimal RZTs (17.5 and 15°C) the most effective genistein concentrations were in the 15 to 20 μM range, whereas at an optimal (25°C) RZT 5 μM was most effective.

9.3.2. Plant materials

Seed of the soybean cultivar Maple Glen was surface sterilized in sodium hypochlorite (2% solution containing 4 mL L^{-1} Tween 20) for 5 minutes, then rinsed with distilled water several times (Bhuvaneswari et al., 1980). The seeds were then planted in trays containing a sterilized Turface (an inert calcined clay; Applied Industrial Materials Corp., Deerfield, IL, USA):sand (1:1 vol.) mixture. Two ten-day-old seedlings at the vegetative cotyledonary (VC) stage [unifoliolate leaves unrolled sufficiently that the edges were not touching (Fehr et al., 1971)] were transplanted into each sterilized, 13-cm plastic pot containing the same rooting medium. In experiment 1, the seedlings were grown on a Conviron growth bench (Model GB48, Controlled Environments Ltd., Winnipeg, Canada) at an irradiance of 300 $\mu mol\ m^{-2}\ s^{-1}$ and a 16:8 h (day:night) photoperiod with a constant air temperature of 25°C. Experiment 2, was established in a greenhouse facility where the level of environmental control was comparable to the growth bench used in experiment 1 but with a higher light intensity (800-1200 $\mu mol\ m^{-2}\ s^{-1}$). In both experiments, the pots were sealed to the bottoms of plastic tanks (68 x 42 cm) and RZTs ($\pm$ 0.5°C) were controlled by circulating cooled water around the pots, with eleven pots in each tank. A hole drilled in the bottom of each pot and through the bottom of the tank beneath each pot allowed the pots to drain when watered. Plants were then acclimatized for 24 h prior to inoculation. During the period of plant growth, plants were watered with a modified Hoagland's solution (Hoagland and Arnon, 1950), in which $Ca(NO_3)_2$ and KNO_3 were replaced with $CaCl_2$ (1 mM), K_2HPO_4 (1 mM) and KH_2PO_4 (1 mM), to provide a nitrogen-free solution. Prior to each watering, the added Hoagland's solution was adjusted to the temperature of the treatment RZT.

9.3.3. Inoculum

Two PGPR strains, *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68, were included in this study, based on the reports of Zhang et al. (1996a and 1996b). All PGPR strains were cultured in *Pseudomonas* medium (Polonenko et al., 1987) in 250-mL flasks, shaken at 250 rpm, at room temperature (20 to 23°C). After reaching the stationary phase (36 h), the PGPR were subcultured. When the subculture reached the log phase (24 h), each of the PGPR strains was adjusted spectrophotometrically with distilled water to an A_{420} value giving a cell density of 10^8 cells mL^{-1}. *B. japonicum* strains 532C and USDA110 (Hume and Shelp, 1990) were cultured in yeast extract mannitol broth (Vincent, 1970) in 250 mL flasks shaken at 125 rpm at room temperature. Strain 532C has been shown to perform well over a range of temperatures (Lynch and Smith, 1993). For production of *B. japonicum* with genistein, genistein (4', 5, 7-trihydroxyisoflavone, purity of 98%, Sigma), as a 10 mM stock solution in 100% methanol, was added to the cultures. The final genistein concentrations of the cultures were equal to the treatment requirements. Cultures were incubated at 23°C without shaking for 48h (Halverson and Stacey, 1984). Following incubation, the cell suspensions were pelleted in sterile centrifuge tubes at 7000g for 10 minutes, washed once with distilled water, and resuspended to an A_{620} of 0.08 (approximately 10^8 cells mL^{-1}). The suspension was then mixed with each PGPR (1:1 cell population) strain before inoculation with a final cell density of 10^8 cells mL^{-1}. The inoculum was cooled to the corresponding root temperature and 1 mL was applied by pipette to the rooting medium at the base of the plant.

9.3.4. Harvest and data collection

Leaf photosynthetic rate, plant internal CO_2 concentration and stomatal conductance (LI-6200 portable photosynthesis system, LI-COR Inc., Lincoln, NE, USA) were measured twelve times during the course of experiment 1 and ten times during experiment 2. The first measurement was taken 3 days after inoculation, with subsequent measurements taken every 3 days for experiment 1, and 5 days after inoculation, with subsequent measurements taken every 5 days for experiment 2. All measurements of photosynthesis were made between 14:00 and 17:00 h. Plants from both experiments were harvested at 50 days after inoculation (DAI) and leaf area (Delta-T area meter, Delta-T Devices Ltd., Cambridge, England), leaf number, pod number, plant shoot weight, and root weight were recorded.

9.3.5. Statistical analysis

Results were analyzed by analysis of variance (ANOVA) with SAS (SAS Institute Inc., 1988). When analysis of variance showed significant treatment effects, the least significant difference (LSD) test was applied to make comparisons among the means at the 0.05 levels of significance (Steel and Torrie, 1980).

9.4. Results

9.4.1. Photosynthesis

Plant photosynthetic rates, averaged across the twelve measurement times in experiment 1, and ten measurements in experiment 2 were affected by different combinations of PGPR strains, genistein concentrations, and *B. japonicum* strains in a way that varied with RZT.

9.4.1.1. At 25°C

The photosynthetic rates of plants receiving PGPR 1-102 co-inoculated with 532C increased (35%) in experiment 1 compared with the *B. japonicum* 532C alone (Table 9.1). The photosynthetic rates of plants receiving *B. japonicum* 532C preincubated with 5 μM genistein increased (60 and 23%) over *B. japonicum* 532C alone in experiments 1 and 2, respectively. The combination of *S. proteamaculans* 1-102, 5 μM genistein and *B. japonicum* 532C had (34%) higher photosynthetic rates in experiment 1 than *B. japonicum* 532C preincubated with 5 μM genistein. The same combination increased the photosynthetic rate (60 and 45%) in experiments 1 and 2, respectively, when compared with *B. japonicum* 532C co-inoculated with PGPR 1-102. Co-inoculation of soybean plants with a mixture of *B. japonicum* 532C and PGPR 2-68 increased the photosynthetic rate (79 and 9%) over *B. japonicum* 532C alone in experiments 1 and 2, respectively (Table 9.1).

There was no difference in the photosynthetic rate between the PGPR 1-102 co-inoculated with *B. japonicum* USDA110 treatment and *B. japonicum* USDA110 alone. *B. japonicum* USDA110 preincubated with 5 μM genistein increased the photosynthetic rate (37 and 11%) in experiments 1 and 2, respectively, over *B. japonicum* USDA110 alone (Fig. 9.1). *S. proteamaculans* 1-102, 5 μM genistein and *B. japonicum* USDA110 increased the photosynthetic rate (168 and 52%) in experiments 1 and 2, respectively, when compared with PGPR 1-102 alone. The photosynthetic rate of the same combination was increased (82 and 35%) for experiments 1 and 2 over plants receiving *B. japonicum* USDA110 preincubated with 5 μM genistein.

Co-inoculation of soybean plants with a mixture of *B. japonicum* USDA110 and PGPR 2-68 increased the photosynthetic rate (68 and 10%) in experiments 1 and 2, respectively, over *B. japonicum* USDA110 alone (Fig. 9.1). *S. liquefaciens* 2-68, 5 μM genistein and *B. japonicum* USDA110 had lower photosynthetic rate (9 and 30%) when compared to *B. japonicum* USDA110 preincubated with 5 μM genistein in experiments 1 and 2, respectively. The same combination had lower photosynthetic rates (21 and 25%) than *B. japonicum* USDA110 co-inoculated with PGPR 2-68 alone in experiments 1 and 2, respectively (Table 9.1). The photosynthetic rate of plants receiving *S. proteamaculans* 1-102, genistein 5μM, and *B. japonicum* USDA110 at 25°C RZT were always the highest of all treatments tested.

There were correlations between stomatal conductance and photosynthesis (Figs 9.1 to 9.3), although with relatively low correlation coefficients of 0.253 and 0.244, for experiments 1 and 2, respectively. Leaf CO_2 concentration was not correlated with photosynthesis.

9.4.1.2. At 17.5°C

Plants receiving a mixture of *B. japonicum* 532C and PGPR 1-102 had higher photosynthetic rates (40%) in experiment 1 than *B. japonicum* 532C alone (Fig. 9.2). Pretreatment of *B. japonicum* 532C with 15 μM genistein stimulated plant photosynthetic rates by 31 and 37% in experiments 1 and 2, respectively over *B. japonicum* 532C alone. The combination of *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* 532C decreased the photosynthetic rates (10 and 20%) in experiments

1 and 2, respectively, when compared with *B. japonicum* 532C preincubated with 15 μM genistein. The same combination decreased the photosynthetic rate (21%) in experiment 1 when compared with *B. japonicum* 532C co-inoculated with PGPR 1-102.

Co-inoculation of soybean plants with a mixture of *B. japonicum* 532C and PGPR 2-68 increased the photosynthetic rates (37 and 46%) in experiments 1 and 2, respectively, over *B. japonicum* 532C alone (Fig. 9.2).

The photosynthetic rates of plants co-inoculated with *S. proteamaculans* 1-102 and *B. japonicum* USDA110 were increased 6 and 55% over those receiving *B. japonicum* USDA110 alone in experiments 1 and 2, respectively. The photosynthetic rates of plants receiving 15 μM genistein and *B. japonicum* USDA110 were increased (24 and 40%) over those receiving *B. japonicum* USDA110 alone in experiments 1 and 2, respectively (Fig. 9.2). *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* USDA110 stimulated plant photosynthesis by 30 and 14% over plants receiving *B. japonicum* USDA110 co-inoculated with PGPR 1-102 alone, and 15 and 26% over plants receiving *B. japonicum* USDA110 preincubated with 15 μM genistein alone in experiments 1 and 2, respectively.

Co-inoculation of soybean plants with a mixture of *B. japonicum* USDA110 and PGPR 2-68 increased photosynthetic rates by 45% over *B. japonicum* USDA110 alone in experiment 1 (Table 9.2). The combination of *S. liquefaciens* 2-68, 15 μM genistein and *B. japonicum* USDA110 decreased photosynthetic rates by 26% in experiment 1, when compared to *B. japonicum* USDA110 co-inoculated with PGPR 2-68. The same combination decreased photosynthetic rates by 10 and 18% in experiments 1 and 2, respectively, when compared to *B. japonicum* USDA110 preincubated with 15 μM genistein.

At 17.5°C the photosynthetic rate was highest for plants receiving *S. proteamaculans* 1-102, genistein 15μM, and *B. japonicum* USDA110 (Figs 9.4 and 9.5), except at the third measurement in experiment 1 where the photosynthetic rate was highest for plants receiving *S. proteamaculans* 1-102 and *B. japonicum* USDA110, and the 7th measurement where the photosynthetic rate was highest for plants receiving genistein 15μM and *B. japonicum* USDA110. There was a correlation between stomatal conductance (correlation coefficient 0.552) and leaf internal CO_2 concentration (correlation coefficient of -0.223) in experiment 2 (Figs 9.1 to 9.3). In experiment 1 there was no such correlation.

9.4.1.3. At 15°C

Plants co-inoculated with *B. japonicum* 532C and PGPR 1-102 had higher photosynthetic rates (28 and 7%) in experiments 1 and 2, respectively, than those inoculated with *B. japonicum* 532C alone (Fig. 9.3). Preincubation of *B. japonicum* 532C with 20 μM genistein, stimulated plant photosynthetic rates by 98 and 10% in experiments 1 and 2, respectively, over *B. japonicum* 532C alone. The combination of *S. proteamaculans* 1-102, 20 μM genistein and *B. japonicum* 532C decreased the photosynthetic rates (33 and 27%) in experiments 1 and 2, respectively, when compared with *B. japonicum* 532C preincubated with 20 μM genistein. The same combination also decreased the photosynthetic rates (21%) in experiment 2 when compared with *B. japonicum* 532C co-inoculated with PGPR 1-102.

Co-inoculation of soybean plants with a mixture of *B. japonicum* 532C and PGPR 2-68 increased photosynthetic rates (24%) in experiment 1 over *B. japonicum* 532C alone (Fig. 9.2).

The photosynthetic rates of plants receiving *S. proteamaculans* 1-102, *B. japonicum* USDA110 increased 140 and 106% over those receiving *B. japonicum* USDA110 alone in experiments 1 and 2, respectively. Photosynthetic rates of plants receiving 20 μM genistein and *B. japonicum* USDA110, were stimulated by 82 and 87% compared to *B. japonicum* USDA110 alone in experiments 1 and 2, respectively (Fig. 9.3). *S. proteamaculans* 1-102, 20 μM genistein and *B. japonicum* USDA110 decreased photosynthetic rates by 65 and 13% over plants receiving *B. japonicum* USDA110 co-inoculated with PGPR 1-102 in experiments 1 and 2, respectively, and by 51 and 13% over plants receiving *B. japonicum* USDA110 preincubated with 20 μM genistein in experiments 1 and 2, respectively.

Co-inoculation of soybean plants with a mixture of *B. japonicum* USDA110 and PGPR 2-68 increased photosynthetic rates by 24 and 20% over *B. japonicum* USDA110 alone in experiments 1 and 2, respectively (Fig. 9.3). The combination of *S. liquefaciens* 2-68, 20 μM genistein and *B. japonicum* USDA110 increased photosynthetic rates by 27 and 47% in experiment 1 and 2, respectively, when compared to *B. japonicum* USDA110 co-inoculated with PGPR 2-68. The same combination decreased photosynthetic rates by 31 and 10% in experiments 1 and 2, respectively, when compared to *B. japonicum* USDA110 preincubated with 20 μM genistein.

The change in plant photosynthetic rates across the ten measurements in experiment 1, and twelve measurements in experiment 2, was calculated for the most stimulatory PGPR strain, genistien concentration, and *B. japonicum* strain combinations for each RZT. In both experiments 1 and 2, at the 15°C RZT, the photosynthetic rates for the combination of *S. proteamaculans* 1-102 and *B. japonicum* USDA110 were highest over time. In experiment 1, for the first five measurements there was no difference between this treatment and *B. japonicum* USDA110 receiving 20 μM genistein, but photosynthetic rates thereafter were higher for the three-way combination than the *B. japonicum* USDA110-genistein treatment.

There were correlations between photosynthesis and stomatal conductance (correlation coefficient of 0.402) and between photosynthesis and leaf internal CO_2 concentration (correlation coefficient of -0.349) in experiment 2 (Figs 9.1 to 9.3). In experiment 1 there were no such correlation.

9.4.2. Plant leaf development and dry weight conductance

9.4.2.1. At 25°C

There was no effect of PGPR 1-102 co-inoculated with 532C on any of the soybean plant variables measured when compared with the *B. japonicum* 532C alone in either experiment (Table 9.1). *B. japonicum* 532C preincubated with 5 μM genistein increased leaf area (28 and 19%), pod number (54 and 96%), and total plant weight (19 and 10%) over the *B. japonicum* 532C alone in experiments 1 and 2, respectively

(Table 9.1). There was no difference between the combination of *S. proteamaculans* 1-102, 5 μM genistein, *B. japonicum* 532C and *B. japonicum* 532C preincubated with 5 μM genistein in either experiment 1 or 2. The same combination increased pod number (67%) and total plant dry weight (15%) in experiment 1 and increased leaf number (9%), leaf area (22%), pod number (94%), and total plant weight (13%) in experiment 2 when compared with *B. japonicum* 532C co-inoculated with PGPR 1-102. Co-inoculation of soybean plants with a mixture of *B. japonicum* 532C and PGPR 2-68 increased leaf area (26 and 18%) and total plant weight (10 and 2%) over *B. japonicum* 532C alone in experiments 1 and 2, respectively (Table 9.1).

PGPR 1-102 co-inoculated with *B. japonicum* USDA110 increased total plant weight (12%) in experiment 1 and increased leaf area (20%) and total plant weight (10%) in experiment 2 over the *B. japonicum* USDA110 alone. *B. japonicum* USDA110 preincubated with 5 μM genistein increased leaf number (12%) and total plant dry weight (18%) in experiment 1, while it increased leaf number (18%), leaf area (46%), pod number (110%) and total plant dry weight (17%) in experiment 2 over *B. japonicum* USDA110 alone (Table 9.1). *S. proteamaculans* 1-102, 5 μM genistein and *B. japonicum* USDA110 increased leaf number (17%) and plant total dry weight (3%) in experiment 1, and increased leaf number (31%), leaf area (40%), pod number (68%) and plant total dry weight (4%) in experiment 2, when compared with *B. japonicum* USDA110 co-inoculated with PGPR 1-102. The same combination increased leaf area (20 and 15%), in experiments 1 and 2, respectively, when compared with *B. japonicum* USDA110 preincubated with 5 μM genistein.

Co-inoculation of soybean plants with a mixture of *B. japonicum* USDA110 and PGPR 2-68 increased leaf number (15 and 24%), leaf area (45 and 61%) and total plant dry weight (33 and 29%) over *B. japonicum* USDA110 alone in experiments 1 and 2, respectively (Table 9.1). *S. liquefaciens* 2-68, 5 μM genistein and *B. japonicum* USDA110 decreased leaf number (12 and 13%), leaf area (19 and 32%), pod number (84 and 91%) and total plant dry weight (12 and 11%) when compared to *B. japonicum* USDA110 preincubated with 5 μM genistein in experiments 1 and 2, respectively. The same combination decreased leaf number (15 and 18%), leaf area (37 and 38%), pod number (83 and 89%) and total plant dry weight (22 and 20%) when compared to *B. japonicum* USDA110 co-inoculated with PGPR 2-68 in experiments 1 and 2, respectively (Table 9.1).

9.4.2.2 At 17.5 °C

Plants co-inoculated with *B. japonicum* 532C and PGPR 1-102 had increased total plant dry weight (11%) in experiment 1 and increased leaf number (16%), and leaf area (24%) in experiment 2 over *B. japonicum* 532C alone (Table 9.2). *B. japonicum* 532C preincubated with 15 μM genistein increased total plant weight (14%) in experiment 1 and increased leaf number (32%), leaf area (47%) and total plant weight (11%) in experiment 2 over *B. japonicum* 532C alone. The combination of *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* 532C increased total plant weight (9%) in experiment 1, when compared with *B. japonicum* 532C preincubated with 15 μM genistein. The same combination increased leaf number (40 and 12%), leaf area (36 and 12%) and total plant weight (12 and 13%) in experiments 1 and 2 when compared with *B. japonicum* 532C co-inoculated with PGPR 1-102.

Co-inoculation of soybean plants with a mixture of *B. japonicum* 532C and PGPR 2-68 increased total plant weight (11%) over *B. japonicum* 532C in experiment 1 (Table 9.2).

Soybean plants co-inoculated with a mixture of *B. japonicum* USDA110 and PGPR 1-102 increased leaf number (14%) and total plant dry weight (13%) over *B. japonicum* USDA110 alone in experiment 1 and increased leaf number (29%), leaf area (13%) and total plant dry weight (6%) in experiment 2 (Table 9.2). *B. japonicum* USDA110 strains preincubated with 15 μM genistein increased leaf number (52 and 50%), leaf area (66 and 99%) and total plant dry weight (31 and 24%) over *B. japonicum* USDA110 alone in experiments 1 and 2, respectively, (Table 9.2). *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* USDA110 increased leaf number (32 and 16%), leaf area (69 and 71%) and total plant weight (14 and 15%) in experiments 1 and 2, respectively, when compared with PGPR 1-102 alone (Table 9.2).

There was no difference between *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* USDA110 preincubated with 15 μM genistein (Table 9.2).

Co-inoculation of soybean plants with a mixture of *B. japonicum* USDA110 and PGPR 2-68 increased leaf number (15%), and total plant weight (8%) over *B. japonicum* USDA110 alone in experiment 1 (Table 9.2). The combination of *S. liquefaciens* 2-68, 15 μM genistein and *B. japonicum* USDA110 increased total plant weight (7 and 5%) in experiments 1 and 2, repectively, when compared to PGPR 2-68 alone. The same combination decreased leaf number (15 and 48%), leaf area (32 and 52%) and total plant weight (11 and 20%) in experiments 1 and 2, respectively, when compared to *B. japonicum* USDA110 preincubated with 15 μM genistein.

9.4.2.3. At 15 °C

Plants co-inoculated with a mixture of *B. japonicum* 532C and PGPR 1-102 had increased leaf number (62 and 52%) and total plant weight (22%) in experiment 2 over *B. japonicum* 532C alone (Table 9.3). *B. japonicum* 532C preincubated with 20 μM genistein had increased leaf number (61 and 49%) and total plant weight (62 and 17%) in experiments 1 and 2, respectively, over *B. japonicum* 532C alone. The combination of *S. proteamaculans* 1-102, 20 μM genistein and *B. japonicum* 532C decreased leaf number (20 and 27%) and total plant weight (53 and 9%) in experiments 1 and 2, when compared with *B. japonicum* 532C co-inoculated with PGPR 1-102. The same combination deceased leaf number (20 and 25%) and total plant weight (39 and 5%) in experiments 1 and 2 when compared with *B. japonicum* 532C co-inoculated with 20 μM genistein.

Co-inoculation of soybean plants with a mixture of *B. japonicum* 532C and PGPR 2-68 increased leaf number (58 and 99%) in experiments 1 and 2, respectively, and increased total plant weight (9%) over *B. japonicum* 532C alone in experiment 2 (Table 9.3). Soybean plants co-inoculated with a mixture of *B. japonicum* USDA110 and PGPR 1-102 had increased leaf number (133 and 125%) in experiments 1 and 2, respectively, and total plant dry weight increased almost 3 times in experiment 1over *B. japonicum* USDA110 alone. *B. japonicum* USDA110 preincubated with 20 μM genistein increased leaf number (64 and 93%) and total plant dry weight (29 and 11%) over *B. japonicum* USDA110 alone in experiments 1 and 2, respectively, (Table 9.3). *S. proteamaculans* 1-102, 20 μM genistein, and *B. japonicum* USDA110 decreased leaf number (19 and 21%) and total plant dry weight (10 and 10%) in experiments 1 and 2, respectively, when compared with *B. japonicum* USDA110 co-inoculated with PGPR 1-102. *S. proteamaculans* 1-102, 20 μM genistein, and *B. japonicum* USDA110 decreased plant total dry weight (52%) in experiment 1 when compared with *B. japonicum* USDA110 preincubated with 20 μM genistein.

Co-inoculation of soybean plants with a mixture of *B. japonicum* USDA110 and PGPR 2-68 increased leaf number (39 and 80%), and total plant weight (66 and 5%) over *B. japonicum* USDA110 alone in experiments 1 and 2 (Table 9.3). The combination of *S. liquefaciens* 2-68, 20 μM genistein and *B. japonicum* USDA110 decreased leaf number (5 and 27%) and total plant weight (17 and 2%) in experiment 1 and 2, respectively, when compared to PGPR 2-68 alone. The same combination decreased leaf number (20%) in experiment 1, while it decreased leaf number (23%) and total plant weight (8%) in experiment 2 when compared to *B. japonicum* USDA110 preincubated with 20 μM genistein.

9.5. Discussion

Inoculation of soybean plants with PGPR strains produced a wide range of effects on photosynthesis and growth variables which varied among strains of PGPR and over RZTs. PGPR strains *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68 were reported to stimulate plant growth, development, and plant physiological activities (Zhang et al., 1996a). At optimal RZT (25°C), *S. liquefaciens* 2-68 was reported to increase plant leaf development and dry matter accumulation, while at 15°C RZT *S. proteamaculans* 1-102 increased plant dry matter accumulation. *S. proteamaculans* 1-102 was reported to perform very poorly at 25°C (Zhang et al., 1996a,b). Those results indicated that the physiological effects of PGPR on soybean plants are affected by soil temperature. The same patterns of results were observed in both experiments 1 and 2 of the current work. At an optimal RZT (25°C), *S. liquefaciens* 2-68 increased plant leaf number, leaf area, pod number, and total dry matter accumulation, while at 15°C RZT *S. proteamaculans* 1-102 increased leaf number and plant dry matter accumulation. These results confirmed the previous observations that the physiological effects of PGPR on soybean plants are affected by soil temperature.

Genistein (the most effective plant-to-bacterium signal in the soybean-*B. japonicum* N_2 fixing symbiosis) has been reported to increase soybean nodulation at suboptimal RZTs, with the effects increasing with decreasing RZT (Zhang and Smith, 1995b). At suboptimal RZT (17.5 and 15°C) the most effective concentrations are in the 15 to 20 μM range, whereas at optimal (25°C) RZT 5 μM was most effective.

Our data agrees with these results since at an optimal (25°C) RZT *B. japonicum* USDA110 preincubated with 5 μM genistein increased leaf number in experiment 1, and increased leaf number, leaf area and pod number in experiment 2. At suboptimal RZT (17.5°C) *B. japonicum* USDA110 preincubated with 15 μM genistein increased leaf number, leaf area and total plant dry weight in experiment 1 compared to *B. japonicum* USDA110 alone, while in experiment 2, either *B. japonicum* USDA110 or 532C co-inoculated with 15 μM genistein increased leaf number, leaf area, pod number, and total plant dry weight compared to either *B. japonicum* USDA110 or 532C (Table 9.2). At 15°C *B. japonicum* USDA110 or 532C preincubated with 20 μM genistein increased leaf and total plant dry weight in experiment 1. In experiment 2, *B. japonicum* USDA110 co-inoculated with 20 μM genistein increased leaf number while *B. japonicum* 532C co-inoculated with 20 μM genistein increased leaf number and total plant dry weight (Table 9.3).

Zhang and Smith (1995b) showed that genistein effects on photosynthesis were only seen after the onset of nitrogen fixation, while the effects of PGPR were seen prior to the onset of nitrogen fixation (Zhang et al.,1996b). The changes in photosynthetic rate over time showed that plant photosynthesis was increased by some PGPR strain, genistein, and *B. japonicum* strain combinations over a wide range of plant growth stages (Figs 9.4 and 9.5). As photosynthesis (Figs 9.4 and 9.5), was increased by stimulatory strain combinations before the onset of nitrogen fixation, the improvements in plant growth, development and physiological activities must have been through an effect of PGPR on overall plant physiology followed by a genistein effect on nitrogen fixation. Our findings agree with those results and, since PGPR and genistein stimulations appear to take place by different mechanisms, they might reasonably be additive.

A wide range of possibilities have been postulated for the mechanisms of PGPR, including an increase in mobilization of insoluble nutrients and subsequent enhancement of uptake by the plants (Lifshitz et al., 1987), production of antibiotics toxic to soil-borne pathogens (Li and Alexander, 1988), production of plant growth regulators that stimulate plant growth (Gaskins et al., 1985), and associative nitrogen fixation (Chanway and Holl, 1991a). Given that our plants were growing in nutrient solution and without pathogen pressure, and that *Serratia* species have not been shown to fix nitrogen, our results suggests the third possibility. PGPR applied to legume crops have also been demonstrated to positively affect symbiotic nitrogen fixation by enhancing root nodule number or mass (Grimes and Mount, 1984; Polonenko et al., 1987; Yahalom et al., 1987; Zhang et al., 1996b) and increase nitrogenase activity (Iruthayathas et al., 1983; Alagawadi and Gaur, 1988).

Genistein (the most effective plant-to-bacterium signal in the soybean-*B. japonicum* N_2 fixing symbiosis) was reported to increase soybean nodulation and at suboptimal RZTs. The effect of genistein increased with decreasing RZT (Zhang and Smith, 1995b). Zhang and Smith, 1996c) showed that at low RZT soybean roots produce less genistein than at higher RZT. Thus, it seems that the observed increases in plant growth and development due to PGPR and genistein addition may be explained as : 1) being from improvement of plant physiological activities such as photosynthesis and growth during very early development leading to improved nodulation and nitrogen fixation during subsequent growth, and 2) more rapid infection and nodule development due to genistein addition leading to a further acceleration of nodule development and onset of N_2 fixation.

Some PGPR and genistein combinations showed additive effects. For instance, the combination of PGPR 1-102, *B. japonicum* USDA110 and 5μM genistein had additive effects for leaf number, leaf area, pod number and plant dry weight at 25°C RZT. The same combination resulted in a higher average total plant dry weight than *B. japonicum* USDA110 and either PGPR or genistein alone at 25°C. In the same way, at 17.5°C, the combination of PGPR 1-102, *B. japonicum* USDA110, and 15μM genistein produced higher leaf number and total plant dry weight values than *B. japonicum* USDA110 and either PGPR or genistein alone treatment. One exception was the combination of PGPR 2-68, 5μM genistein concentrations and *B. japonicum* USDA110 for which the PGPR and genistein showed antagonistic effects at 25°C when compared to genistein or PGPR alone. Also, PGPR strains and genistein combinations with either *B. japonicum* strain showed antagonistic effects at the lowest RZT (15°C). For example, the combination of PGPR 2-68, 20μM genistein concentrations and *B. japonicum* USDA110 had lower values for most variables than was the case when either genistein or PGPR were added with *B. japonicum* USDA110.

The proportional increases in nodulation and N_2 fixation (section 8) were larger at lower than higher RZTs. The degree of the increase was much larger for nodulation and nitrogen fixation variables (section 8) than for photosynthesis (Figs 9.1 to 9.3) and dry weight (Tables 9.1 to 9.3). This was probably due to lower nodule effeciencies of the extra nodule mass present due to PGPR and/or genistein. In a similar way, the larger degrees of relative increase in N_2 fixation (section 8) than in dry weight production (Tables 9.1 to 9.3) indicates less effective use of the available N, in terms of dry matter production. This was demonstrated by higher N concentrations for a plant receiving PGPR and/or genistein treatments (data not shown).

Photosynthesis was more sensitive to the application of PGPR at both the optimal and suboptimal RZTs than were either leaf internal CO_2 concentration or stomatal conductance (Figs 9.1 to 9.3). Within each RZT, comparisons of the photosynthetic rate increases by the most stimuatory PGPR strain in both experiments showed that the suboptimal RZT of 17.5°C , *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* USDA110 stimulated plant photosynthesis the most. This combination increased photosynthetic rates 40 and 73% over plants receiving *B. japonicum* USDA110 alone. At the lowest RZT (15°C), the photosynthetic rates of plants receiving *S. proteamaculans* 1-102, *B. japonicum* USDA110 were increase 140 and 106% over those receiving *B. japonicum* USDA110 alone in experiments 1 and 2, respectively. This was accompained with an increase in the stomatal conductance. Wilson and McMurdo (1981) reported that plant photosynthesis was more sensitive to low temperature effects than was transpiration, which has a direct relationship with stomatal conductance under uniform conditions. Therefore, PGPR may have increased plant photosynthetic rates more at low RZTs, relative to plants growing at on optimal RZT, because the increased photosynthetic rate at the low RZTs was a part of a reduction of the direct adverse effects of low RZT on photosynthesis. The larger changes, due to the applied treatments, for stomatal conductance in experiment 2 than in experiment 1 (Figs 9.1 to 9.3) were probably due to the higher light intensity for experiment 2.

The combination of *S. liquefaciens* 2-68, 20μM genistein and *B. japonicum* USDA110 increased photosynthesis rates by 27 and 47% in experiments 1 and 2, repectively, when compared to *B. japonicum* USDA110 co-inoculated with PGPR 2-68, but did not result any increase in the amount of nitrogen fixed. Zhang et al. (1996b) reported that the effect of PGPR on photosynthesis were seen prior to the onset of nitrogen fixation. PGPR appear to stimulate overall plant growth including photosynthesis, which may effect lead to increased nitrogen fixation. The observed increase in the photosynthesis in the absence of any increase in nitrogen fixation was probably due to a PGPR effect.

The stomatal conductance changes showed relationships with changes in photosynthetic rate. When PGPR strain and/or genistein treatments caused increases in photosynthesis, stomatal conductance generally increased, and the internal CO_2 concentration generally decreased. Thus increases in leaf photosynthesic activity were due to both decreased resistance to the entry of CO_2 into the leaves and were rapid uptake of CO_2 because of increased photosynthetic activity inside the leaves.

Photosynthetic rate showed correlations with stomatal conductance in experiments 1 and 2 and leaf internal CO_2 concentration (experiment 2 only) that were more frequent and stronger for stomatal conductance than leaf internal CO_2 concentration. Thus increases in photosynthesis activity, due to the addition of PGPR, genistein or both, were related to both stomatal conductance and leaf internal CO_2 concentration but more to stomatal conductance.

Some PGPR and genistein combinations showed additive effects on the photosynthetic rates at 25 and 17°C RZTs. The combination of PGPR 1-102 with either *B. japonicum* USDA110 or 532C and 5μM genistein increased the photosynthetic rate additively compared to *B. japonicum* USDA110 or 532C, and PGPR 1-102 or *B. japonicum* USDA110 or 532C and 5μM genistein. On the other hand, at 15 °C RZT, PGPR and genistein were antagonistic as the photosynthetic rate decreased relative to *B. japonicum* plus PGPR or genistein individually. The combination of PGPR 1-102, *B. japonicum* USDA110 and 20μM genistein even decreased the photosynthetic rate compared to soybean plants which received *B. japonicum* USDA110 alone.

In summary, the results of this study indicated that 1) some PGPR strains combined with genistein can stimulate aspects of soybean growth and development, 2) PGPR *S. proteamaculans* 1-102 which was reported to perform poorly at 25°C (Zhang et al., 1996a,b) was shown in this study to be effective at 25°C when combined with 5 μM genistein, 3) the stimulations due to combinations of PGPR and genistein were probably due to PGPR stimulation prior to the onset of N_2 fixation on PGPR plus genistein effects after the onset of nitrogen fixation, 4) at 25°C, some PGPR strains, genistein concentrations, and *B. japonicum* strain showed additive effects relative to *B. japonicum* alone, while at 15°C some showed antagonistic effects. Overall these findings confirm that adding PGPR or genistein alone with *B. japonicum* to soybean roots each accelerates nitrogen fixation leading to increased growth and shows, in addition, that adding the two together caused stimulations of N_2 fixation and growth that, in some cases, were greater than those caused by either PGPR or genistein alone.

Table 9.1. Effects of PGPR application on soybean growth at 25° C RZT

PGPR	*B. japonicum*	Genistein (μ M)	leaf(plant^{-1}) number	leaf(plant^{-1}) area(cm^2)	Pod no	plant dry weight (mg) root	plant dry weight (mg) shoot	plant dry weight (mg) total (plant^{-1})
Experiment 1								
1-102	USDA110	0	40.21	409.9	3.13	888.6	4126.6	5015.2
		5	47.22	460.7	4.27	869.8	4304.4	5174.2
	532C	0	40.69	402.4	2.67	875.7	3622.6	4498.3
		5	43.23	450.3	4.47	862.8	4296.6	5159.4
2-68	USDA110	0	45.55	489.3	3.80	1365.3	4595.5	5960.7
		5	38.79	309.3	0.63	834.8	3823.2	4657.9
	532C	0	44.05	460.2	2.67	704.5	4151.9	4856.5
Control	USDA110	0	39.54	337.8	2.90	635.0	3858.3	4493.3
		5	44.13	383.9	4.00	803.7	4512.7	5316.5
	532C	0	42.22	365.7	3.00	660.3	3758.0	4418.3
		5	45.45	467.6	4.63	948.8	4313.4	5262.2
$LSD_{0.05}$			4.08	78.6	1.28	86.6	60.3	108.6
Experiment 2								
1-102	USDA110	0	35.70	416.0	2.93	1033.3	4448.8	5482.1
		5	46.67	583.8	4.93	1025.8	4649.4	5675.2
	532C	0	40.64	405.8	2.67	1031.7	3967.6	4999.3
		5	44.21	494.8	5.17	1018.8	4641.6	5660.4
2-68	USDA110	0	46.00	557.3	4.67	1521.3	4940.5	6461.7
		5	37.89	346.2	0.53	990.8	4168.2	5158.9
	532C	0	43.64	529.7	3.60	860.5	4496.9	5357.5
Control	USDA110	0	37.00	346.2	2.70	791.7	4203.3	4995.0
		5	43.67	506.0	5.67	961.6	4857.8	5819.4
	532C	0	42.22	449.7	2.97	1124.9	4103.0	5227.9
		5	44.00	534.0	5.83	1104.8	4658.4	5763.2
$LSD_{0.05}$			3.35	55.3	1.44	88.3	61.3	109.7

Means within the same column an experiment were analyzed b ANOVA protected LSD test.

Table 9.2. Effects of PGPR application on soybean growth at 17.5°C RZT.

			leaf (plant^{-1})		plant dry weight (mg)		
PGPR	*B. japonicum*	Genistein μM)	number	area(cm^{2})	root	shoot	total (plant^{-1})
Experiment 1							
1-102	USDA110	0	18.17	107.00	341.1	1031.9	1373.0
		15	24.00	181.17	394.2	1173.3	1567.5
	532C	0	16.17	109.67	289.6	989.9	1279.6
		15	22.67	148.67	335.3	1104.3	1437.0
2-68	USDA110	0	18.33	117.50	342.6	975.6	1318.2
		15	20.67	128.33	347.7	1064.7	1412.3
	532C	0	16.94	109.10	266.4	1012.1	1278.5
Control	USDA110	0	16.00	114.50	311.7	905.2	1216.9
		15	24.28	190.00	351.b	1244.0	1595.7
	532C	0	18.83	155.33	289.3	858.4	1156.7
		15	20.33	132.17	234.8	985.6	1320.5
$LSD_{0.05}$			1.80	33.82	12.4	39.4	45.3
Experiment 2							
1-102	USDA110	0	20.67	129.17	398.6	1280.1	1678.7
		15	24.00	220.25	489.5	1442.4	1931.9
	532C	0	20.33	132.00	388.3	1125.3	1513.6
		15	22.67	148.33	407.7	1298.3	1706.0
2-68	USDA110	0	16.94	109.27	362.1	1127.5	1489.6
		15	16.17	110.33	370.1	1192.8	1562.9
	532C	0	18.33	117.50	393.1	1210.1	1603.2
Control	USDA110	0	16.00	114.33	361.3	1218.2	1579.5
		15	23.94	227.50	491.5	1462.2	1953.7
	532C	0	17.50	106.33	352.1	1239.5	1591.6
		15	23.17	156.33	428.8	1344.9	1773.7
$LSD_{0.05}$			1.96	8.45	12.9	16.5	24.7

Means the same column an experiment were analyzed by an ANOVA protected LSD test.

Table 9.3. Effects of PGPR application on soybean growth at 15°C RZT.

PGPR	*B. japonicum*	Genistein (μ M)	leaf number	Plant dry weight (mg) root	shoot	total (plant^{-1})
Experiment 1						
1-102	USDA110	0	6.77	534.1	382.3	916.5
		20	5.50	510.4	314.7	825.1
	532C	0	4.17	205.3	192.9	398.1
		20	3.33	487.0	363.2	850.2
2-68	USDA110	0	4.03	279.7	231.0	510.7
		20	3.83	209.0	162.9	371.9
	532C	0	4.07	181.6	225.6	406.9
Control	USDA110	0	2.90	194.4	113.2	307.9
		20	4.77	234.2	161.9	396.1
	532C	0	2.57	188.0	216.4	404.4
		20	4.13	358.3	298.8	657.0
$LSD_{0.05}$			0.94	15.8	28.7	32.2
Experiment 2						
1-102	USDA110	0	6.55	407.2	453.0	860.2
		20	5.17	363.9	412.2	776.2
	532C	0	3.92	386.6	421.3	807.9
		20	2.88	367.1	405.4	772.5
2-68	USDA110	0	5.23	337.5	383.9	721.4
		20	4.33	332.8	374.4	707.2
	532C	0	5.13	345.2	378.9	724.2
Control	USDA110	0	2.9c	318.6	367.9	686.5
		20	5.60	352.9	412.1	764.9
	532C	0	2.57	308.4	353.4	661.8
		20	3.83	339.5	392.7	732.2
$LSD_{0.05}$			1.08	10.2	9.9	11.3

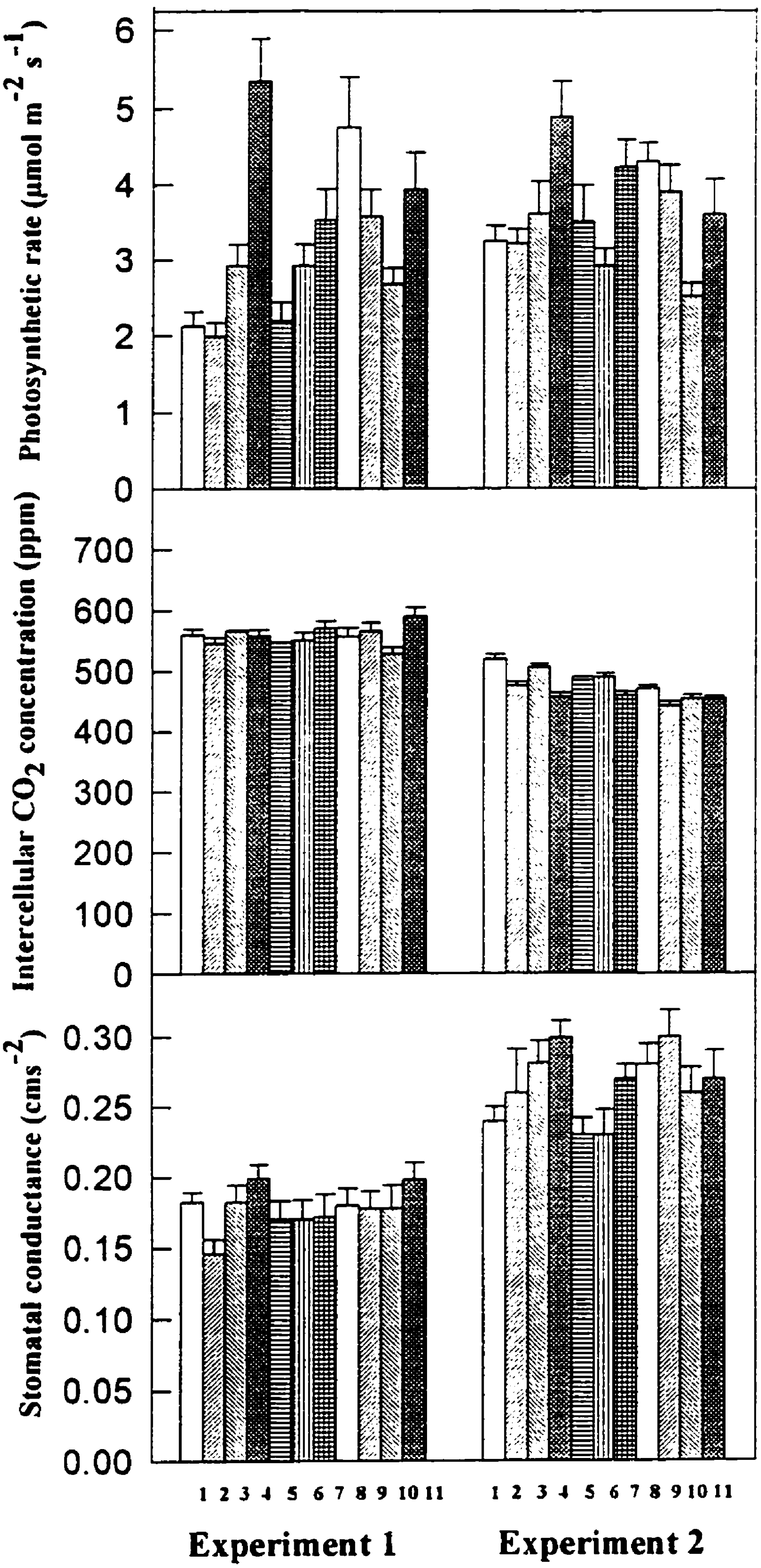
Means the same column an experiment were analyzed by an ANOVA protected LSD test.

9.1. Effects of PGPR, genistein, and *B. japonicum* on photosynthesis,internal CO_2 concentration, and stomatal conductance for plants grown at 25°C.

Vertical lines on top of each bar indicated one standard error unit (n = 36 for exp 1) (n = 30 for exp 2).

In order fron left to right

- PGPR 0 *B. japonicum* USDA110 Genistein 0μ
- PGPR1-102 *B. japonicum* USDA110 Genistein 0μ
- PGPR 0 *B. japonicum* USDA110 Genistein 5μ
- PGPR1-102 *B. japonicum* USDA110 Genistein 5μ
- PGPR 0 *B. japonicum* 532 Genistein 0μ
- PGPR1-102 *B. japonicum* 532 Genistein 0μ
- PGPR 0 *B. japonicum* 532 Genistein 5μ
- PGPR1-102 *B. japonicum* 532 Genistein 5μ
- PGPR2-68 *B. japonicum* USDA110 Genistein 0μ
- PGPR2-68 *B. japonicum* USDA110 Genistein 5μ
- PGPR2-68 *B. japonicum* 532 Genistein 0μ



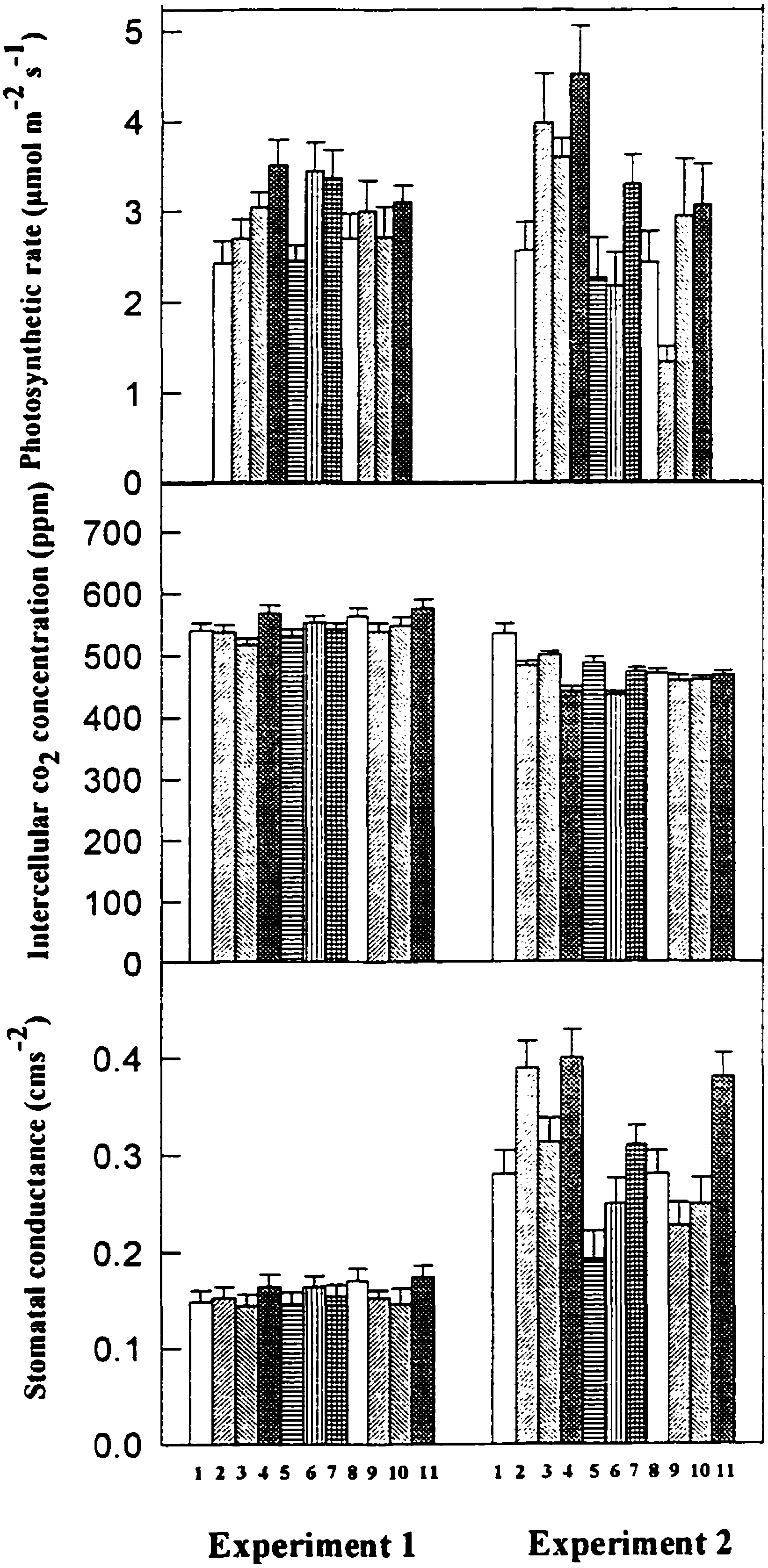
Photosynthetic rate (µmol m^{-2} s^{-1})
6
5
4
3
2
1
0
Intercellular CO_2 concentration (ppm)
700
600
500
400
300
200
100
0
Stomatal conductance (cms^{-2})
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11
Experiment 1
Experiment 2

Fig. 9.2. Effects of PGPR, genistein, and *B. japonicum* on photosynthesis,internal CO_2 concentration, and stomatal conductance for plants grown at 17.5°C.

Vertical lines on top of each bar indicated one standard error unit (n = 36 for exp 1) (n = 30 for exp 2).

In order fron left to right

PGPR 0 *B. japonicum* USDA110 Genistein 0µ
PGPR1-102 *B. japonicum* USDA110 Genistein 0µ
PGPR 0 *B. japonicum* USDA110 Genistein 15µ
PGPR1-102 *B. japonicum* USDA110 Genistein 15µ
PGPR 0 *B. japonicum* 532 Genistein 0µ
PGPR1-102 *B. japonicum* 532 Genistein 0µ
PGPR 0 *B. japonicum* 532 Genistein 15µ
PGPR1-102 *B. japonicum* 532 Genistein 15µ
PGPR2-68 *B. japonicum* USDA110 Genistein 0µ
PGPR2-68 *B. japonicum* USDA110 Genistein 15µ
PGPR2-68 *B. japonicum* 532 Genistein 0µ



Photosynthetic rate (μmol m^{-2} s^{-1})
0
1
2
3
4
5
Intercellular co$_2$ concentration (ppm)
0
100
200
300
400
500
600
700
Stomatal conductance (cms^{-2})
0.0
0.1
0.2
0.3
0.4
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11
Experiment 1
Experiment 2

Fig. 9.3. Effects of PGPR, genistein, and *B. japonicum* on photosynthesis,internal CO_2 concenration, and stomatal conductance for plants grown at 15°C.

Vertical lines on top of each bar indicated one standard error unit (n = 36 for exp 1) (n = 30 for exp 2).
In order fron left to right

- PGPR 0 *B. japonicum* USDA110 Genistein 0µ
- PGPR1-102 *B. japonicum* USDA110 Genistein 0µ
- PGPR 0 *B. japonicum* USDA110 Genistein 20µ
- PGPR1-102 *B. japonicum* USDA110 Genistein 20µ
- PGPR 0 *B. japonicum* 532 Genistein 0µ
- PGPR1-102 *B. japonicum* 532 Genistein 0µ
- PGPR 0 *B. japonicum* 532 Genistein 20µ
- PGPR1-102 *B. japonicum* 532 Genistein 5µ
- PGPR2-68 *B. japonicum* USDA110 Genistein 0µ
- PGPR2-68 *B. japonicum* USDA110 Genistein 20µ
- PGPR2-68 *B. japonicum* 532 Genistein 0µ

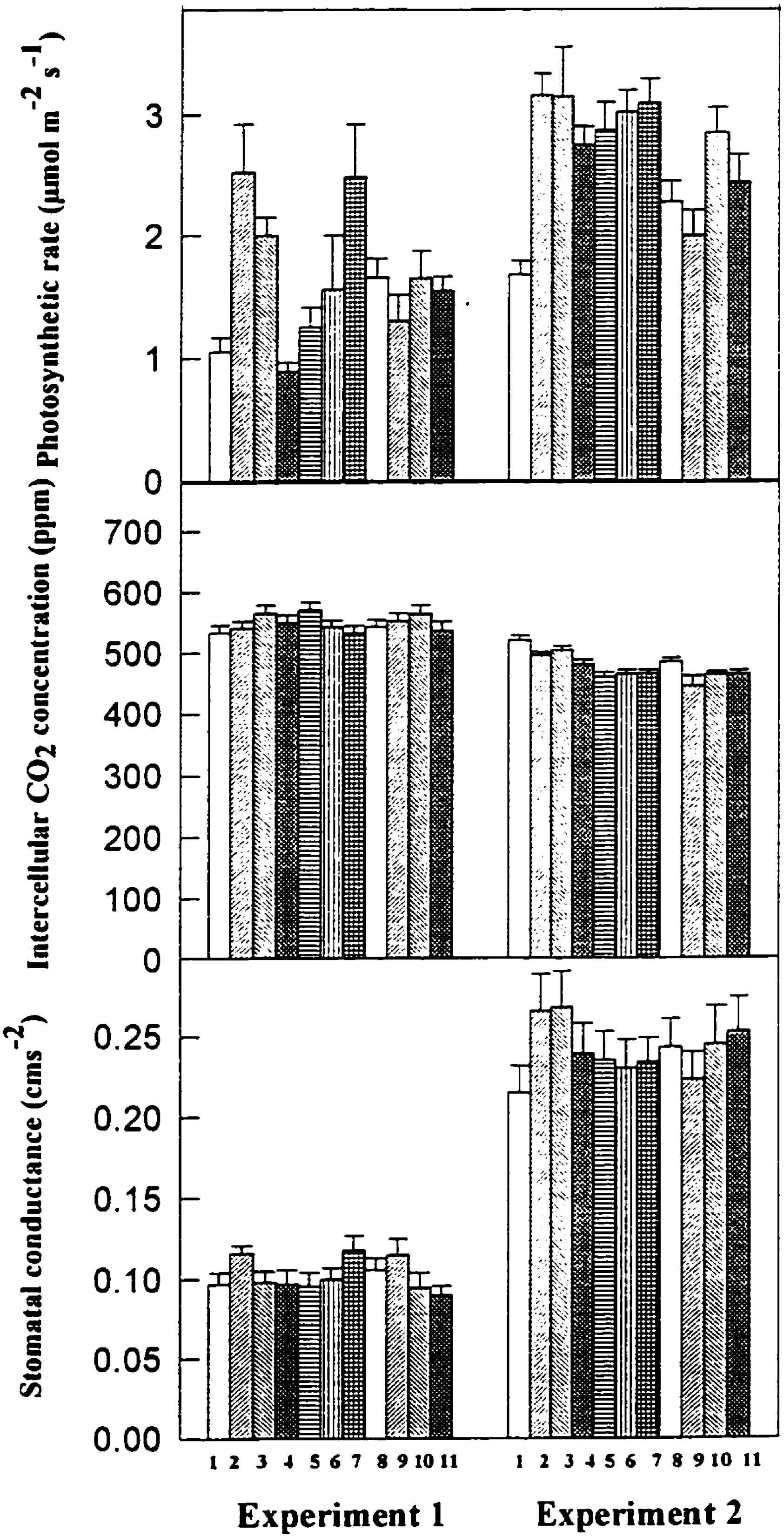

Photosynthetic rate (μmol m-2 s-1)
Intercellular CO2 concentration (ppm)
Stomatal conductance (cms-2)
0
1
2
3
0
100
200
300
400
500
600
700
0.00
0.05
0.10
0.15
0.20
0.25
1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11
Experiment 1
Experiment 2

Fig. 9.4. Photosynthetic rate of four treatments over time at three temperatures (results from experiment 1). Vertical lines on top of each bar indicated one standard error unit (n = 36 for exp. 1, n = 30 for exp. 2)

—○— *B. japonicum* USDA110, Genistein, PGPR1-102

—■— *B. japonicum* USDA110, Genistein, no PGPR

—▲— *B. japonicum* USD110, no Genistein, PGPR 1-102

—◇— *B. japonicum* USDA110, no Genistein, no PGPR

for each RZT, when applied the Genistein concentrations were:20 μM at 15°C, 15 μM at 17.5°C, and 5 μM at 25°C.

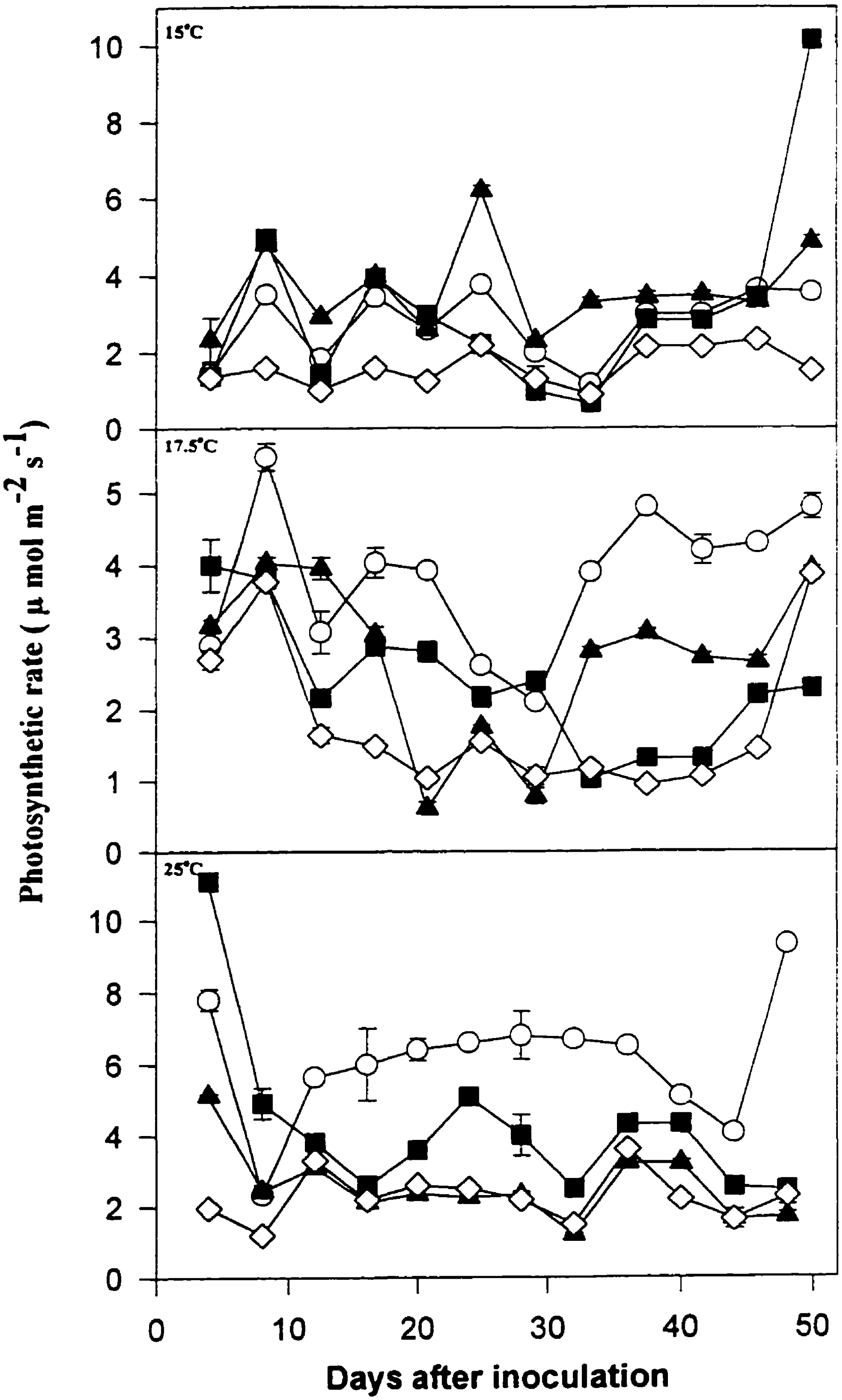

15°C
17.5°C
25°C
Photosynthetic rate (μ mol m-2 s-1)
Days after inoculation
0
10
20
30
40
50
2
4
6
8
1
3
5

Fig. 9.5. Photosynthetic rate of four treatments over time at three temperatures (results from experiment 2). Vertical lines on top of each bar indicated one standard error unit (n = 36 for exp. 1, n = 30 for exp. 2)

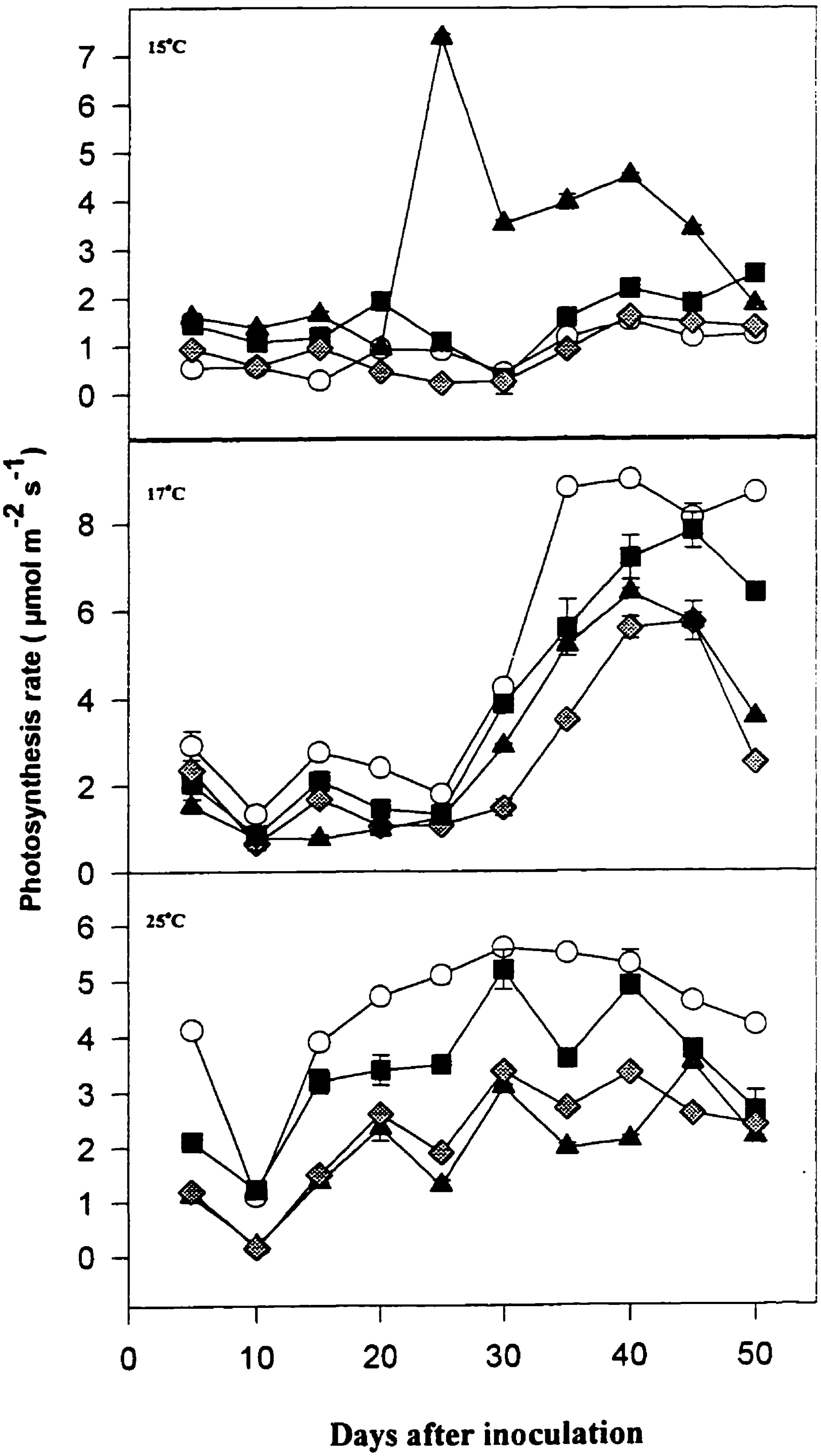
—○— *B. japonicum* USDA110, Genistein, PGPR1-102

—■— *B. japonicum* USDA110, Genistein, no PGPR

—▲— *B. japonicum* USD110, no Genistein, PGPR 1-102

—◇— *B. japonicum* USDA110, no Genistein, no PGPR

for each RZT, when applied the Genistein concentrations were:20 μM at 15°C, 15 μM at 17.5°C, and 5 μM at 25°C.


15°C
17°C
25°C
Photosynthesis rate (µmol m-2 s-1)
Days after inoculation

Section 10

GENERAL DISCUSSION

10.1. Application of PGPR to *B. japonicum* reduced the inhibition of suboptimal RZTs

Plant growth promoting rhizobacteria can increase plant growth, development, and yield in non legume crops such as potato, radish, sugar beet, wheat and canola (Gaskins et al., 1985; Polonenko et al., 1987). Co-inoculation studies with PGPR and *B. japonicum* have demonstrated increased plant root and shoot weight, seed yield, plant vigour, nodulation, and N_2 fixation (Verma et al., 1986; Li and Alexander, 1988). However, effects of PGPR strains on soybean plant growth, some physiological activities, and the N_2 fixation symbiosis were shown to vary with RZT. Previous work by Zhang et al. (1996a and 1996b) showed that some PGPR increase plant growth, photosynthesis, amount of fixed N and number of nodules formed when co-inoculated with *B. japonicum*, although the stimulatory strains varied with RZT. The most stimulatory strains at each temperature were: 15°C - *Serratia proteamaculans* 1-102, 17.5°C - *S. proteamaculans* 1-102, *Aeromonas hydrophila* P73, *S. liquefaciens* 2-68, and 25°C - *S. liquefaciens* 2-68. It was therefore reasonable to test the effects of PGPR on soybean plants under field conditions. The results of sections 3 and 4 showed that co-inoculation of *B. japonicum* with PGPR accelerates soybean nodule development and N_2 fixation under cool early spring field conditions. These field experiments showed that the onset of N_2 fixation by soybean plants receiving *B. japonicum* preincubated with PGPR was two to three days earlier than those receiving no PGPR treatment at both the unsterilized and sterilized sites. PGPR application also increased the number of nodules per plant at the unsterilized site. Since nodule dry weight per plant was increased and the onset of nitrogen fixation was hastened by *B. japonicum* co-inoculation with PGPR, total fixed nitrogen and nitrogen yield per plant were increased. Sprent (1979) postulated that an increase of ten percent in the period of nodule activity of a grain legume, particularly between the onset of N_2 fixation and the achievement of maximum fixation, could double the seasonal level of nitrogen fixed.

Our data (section 3) showed that the period of nodule function was about 70 days (late-June to early-September). Because *B. japonicum* co-inoculated with PGPR resulted in a two to three day increase in the duration of N_2 fixation, the total amount of N fixed throughout the whole season was increased. Therefore, it seems likely that some of the increase in total fixed N was due to an earlier onset of N_2 fixation due to PGPR application under conditions where soils were stressfully cool in the early growing season, with the remainder of the increase being due to the increased plant nodule mass.

As nodule number and seasonal fixed N were increased by PGPR application, plant dry matter and total protein production, and grain and grain protein yield were increased. Direct application of PGPR onto seeds and soil in the furrow at the time of planting also improved plant nodulation and N_2 fixation. At the unsterilized site, although both of the PGPR tested did not increase plant nodule number, they both increased weight per nodule, total fixed nitrogen and N yield. At the sterilized site, both PGPRs increased nodule number, total nitrogen fixed, and N yield.

The average proportional increases in nodule number per plant, nodule weight per plant, total fixed nitrogen, N yield, final grain, and protein yield when *B. japonicum* was co-inoculated with PGPR were generally larger than when PGPR were directly applied onto seeds and soil in the furrow at the time of planting. There are two possible explanations for this observation. First, PGPR were preincubated with *B. japonicum* for a period of at least 30 minutes before inoculation, during which PGPR-*B. japonicum* interactions might have occurred. Plant growth promoting rhizobacteria produce many phytohormones and signal molecules (Burr and Caesar, 1984; Davison, 1988; Kapulnik, 1991), such as genistein, the plant-to-bacteria signal involved in the soybean nodule infection and formation processes. Therefore, inoculation of soybean plants with *B. japonicum* and PGPR could have resulted in higher relative increases in nitrogen fixation and subsequent soybean growth and yield than *B. japonicum* or PGPR alone. Second, the PGPR may have stimulated overall plant growth, leading to greater nitrogen demand by the developing soybean plants, leading, in turn, to greater nodulation and nitrogen fixation. The data of Zhang et al. (1996b), showing improved soybean photosynthesis and growth due to PGPR prior to the onset of nitrogen fixation, argue against the former of these two possibilities.

10.2. PGPR growth and survival under field conditions

Co-inoculation of *B. japonicum* with plant growth promoting rhizobacteria (PGPR) has been shown to increase soybean nodulation, nitrogen fixation and growth compared to the non-treated controls, in areas with low spring soil temperatures (section 3). The survival and growth of rhizosphere populations of two PGPR *Serratia liquefaciens* 2-68 or *Serratia proteamaculans* 1-102 inoculated on soybean were examined under short season conditions. Colonization of soybean plants varied among PGPR strains and soil conditions. At an unfumigated site, PGPR 2-68 colonized soybean plants roots more efficiently than PGPR 1-102 in the first sampling, while there was no difference by the second sampling which indicated that PGPR 2-68 was able to grow and colonize the soybean root more effectively initially, but over time PGPR 2-68 was able to grow and colonize soybean roots as effectively as PGPR 1-102. PGPR 2-68 was able to proliferate successfully in the soil at both samplings. The population density of both PGPR 68 and 1-102 with the different combinations of *B. japonicum* strains and soybean cultivars decreased over time except for the combination of PGPR 2-68, *B. japonicum* USDA110 and cultivar AC Bravor where the population density increased over time at the unfumigated site. These observations indicated that PGPR 2-68 can survive and colonize the roots of the soybean plants effectively in the presence of other microflora.

At the fumigated site, where we assumed, no other microflora competed with the PGPR, PGPR 2-68 showed the same pattern as at the unfumigated site. Also, the population density of both PGPR increased over time. These observations suggest that both PGPR were able to survive and increase in number in the absence of other microflora elements.

Colonization of plant roots by microorganisms is not well understood. Several factors have been suggested as affecting colonization, including the ability of certain microorganisms to adhere to the root. The presence of polysaccharides on the cell surface seems to play a role in plant-microbe associations such as crown gall by *Agrobacterium tumefaciens* (Douglas et al., 1982, 1985; Matthysse et al., 1981; Thomashow et al., 1987) and the nodulation of legumes by *(Brady)Rhizobium* species (Cangelosi et al., 1987; Dazzo et al., 1984; Leigh et al., 1985; Smit et al., 1987).

Many of the PGPR strains which stimulate the growth of potatoes have been identified as fluorescent pseudomonads (Bahme and Schroth, 1987; Baker, 1968; Brown, 1975; Burr and Caeser, 1984; Xu and Gross, 1986). These bacteria have the ability to establish high population densities in the rhizosphere (Bahme and Schorth, 1987; Suslow, 1982), a characteristic suggested to be an essential factor for the production of consistent plant growth responses (Bahme and Schroth, 1987; Klein et al., 1990; Kloepper et al., 1980a and 1985; Kloepper et al., 1991; Parke, 1991; Suslow, 1982).

Studies on rhizosphere colonization have been reviewed by van Elsas and Heijnen (1990), Kloepper and Beauchamp (1992) and Kluepfel (1993). Lack of consistent effectiveness of the inoculant was found to be the major problem preventing successful application of PGPR strains to soils (van Elsas and Heijnen, 1990). This has always been caused by ineffective colonization of the plant, as well as poor survival and/or low activity of the introduced population. Xu and Gross (1986b) and Bull et al. (1991), demonstrated a positive relationship between root colonization by a PGPR strain and disease control, suggesting that methods applied which improve root colonization may also improve the establishment of a PGPR strain in soil. The extent and amount of root colonization required by a PGPR strain to increase plant growth depends on numerous interrelated factors. The choice of methods used to try to increase rhizosphere colonization and plant growth should take these factors into account (Stephens, 1994b).

Previous studies have suggested that the fitness of a bacterial strain in the rhizosphere may be dependent upon the plant species (van Peer and Schippers, 1989, Beauchamp et al., 1991) and even plant cultivar (Weller, 1986) One method of increasing rhizosphere colonization by certain PGPR strains may be through maximising the bacterial inoculum load on the seed. Hebbar et al. (1992) reported that the colonization and spread of *Pseudomonas cepacia* (which acts as a bio-control agent against *Fusarium moniliforme*) on the roots and in the rhizosphere of maize correlated with the amount of inoculum on the seed. However, the dependence of final colonization level on the initial inoculum level has not been a universal observation. For example, the colonization of introduced pseudomonad strains on maize (Scher et al., 1984) and wheat (Juhnkle et al., 1989) has been shown to be independent of the initial inoculum level. It is apparent that, under certain conditions, increasing the level of inoculum may increase the rhizosphere competence of some, but not all, bacteria.

Beneficial bacteria which are introduced into the rhizosphere are involved in a complex of biological interactions with the host plant. The introduced bacteria are nourished by root exudates and are thus dependent on the host plant. At the same time, the introduced bacteria may affect the host plant by inducing physiological changes (Kloepper et al, 1988b). The genetic marking of bacteria with antibiotic resistance for identification purposes allows the study of population dynamics of soil-inhabiting bacteria. Specific PGPR that cause marked increases in plant growth and yield have been marked to follow their populations during the various stages of plant development (Polonenko et al., 1987).

We found that co-inoculation with PGPR and *B. japonicum* improved plant growth, development, yield components, and final grain and protein yield under field conditions at both fumigated and unfumigated sites. Also, application of PGPR with the *B. japonicum* directly onto the seeds in the furrow at the time of planting also improved plant growth and increased grain and protein yield at the fumigated site. The effects of PGPR *S. liquefaciens* 2-68 and *S. proteamaculans* 1-102 on plant growth, development, and final protein yield were shown to be not different, which was attributed to variations in field soil temperature during the entire soybean growing season. In addition, co-inoculation of PGPR and *B. japonicum* accelerated soybean nodulation and the onset of nitrogen fixation under short-season conditions. This study indicated that PGPR 2-68 was able to grow and survive better than PGPR 1-102 under short season conditions. Inoculation with PGPR 2-68 generally increased soybean nodulation, nitrogen fixation, growth and yield more than PGPR 1-102 (sections 3 and 4). These findings suggest that there is a direct relationship between the ability of these PGPR to colonize the roots of the soybean plants and their ability to stimulate soybean nodulation, nitrogen fixation, plant growth and physiological activities under short season conditions.

10.3. Effects of suboptimal RZTs on PGPR growth and survival

Some PGPR strains are able to colonize soybean root plants more efficiently than others, while others proliferated more successfully in the rooting medium. At 15°C RZT, PGPR *Serratia proteamaculans* (1-102) had a higher population density associated with the soybean roots while its population density was reduced in the rooting medium. The same pattern was seen for the PGPR *Serratia liquefaciens* (2-68) at its more appropriate RZT, 25°C. These results indicate that the colonization of soybean roots and the rhizosphere by PGPR is altered by temperature.

Zhang et al. (1996a,b) have shown that co-inoculation of *B. japonicum* with some PGPR strains increased soybean nodulation and nitrogen fixation and increased soybean growth and development, but the stimulatory effect varied with the RZT. The ability of the PGPR to colonize roots effectively is probably a prerequisite to the stimulation of soybean growth, nodulation and nitrogen fixation.

Root zone temperature exert a clear effect on the ability of PGPR to colonize soybean roots and this probably explains at least part of the differences in the colonization and plant growth stimulating abilities of PGPR 2-68 and 1-102 noted in sections (3, 4 and 5). Elements of the soil flora and fauna and aspects of the soil chemistry may also have contributed to the differences in performance of the two PGPR tested.

10.4. Effect of PGPR on root hair infection and early nodule development

All stages of symbiotic establishment investigated to date, such as root hair curling, infection thread formation and penetration, and nodule development and function, are inhibited by suboptimal RZTs (Lie, 1981; Zhang and Smith, 1994). Gibson (1971) suggested that low temperatures retard root hair infection more than nodule initiation, nodule development, and N assimilation in the legume-*(Brady)Rhizobium* symbiosis. Studies on the effects of suboptimal RZTs on soybean suggested that such conditions decrease N_2 fixation activity by directly decreasing the activity of the nitrogenase enzyme complex (Layzell et al., 1984a) and by suppressing and/or delaying root infection and nodulation (Walsh and Layzell, 1986). Results from microscopic observations of co-inoculation of soybean plants with plant growth promoting rhizobacteria at optimal (25°) and suboptimal (17.5 and 15°C) RZTs have indicated that at 25 and 17°C, application of the PGPR *Serratia liquefaciens* (2-68) shortened the time elapsed from the beginning of root hair curling until the infection thread reached the base of the root hair. At 15°C PGPR *Serratia proteamaculans* (1-102) shortened the duration of root hair curling, infection thread initiation and infection thread growth to the base of the root hair.

The negative effects of low RZT on soybean nodulation have been shown to be particularly extreme during the first stages of root hair infection (Zhang and Smith, 1994). Low RZTs of these types are typical of field conditions in Eastern Canada (Zhang et al., 1995a). The observation that PGPR accelerate the earliest stages of soybean root hair infection helps to explain their beneficial effects at low RZT (Zhang et al., 1996a,b) and under eastern Canadian field conditions (sections 3 and 4).

At both 15 and 25°C treatment of the soybean plants with PGPR supernatant at inoculation only or through daily applications reduced the time required for root hair curling, infection thread initiation, and the infection thread to reach the base of the root hair. The frequency of the occurance of the different stages was the highest at both 15 and 25°C for soybean plants treated daily with PGPR supernatant. These results suggest that both PRPR *S. proteamaculans and S. liquefaciens* produce growth promoting substances and possibly that PGPR *S. proteamaculans* (1-102) does it well at high temperatures (25°C) and PGPR *S. liquefaciens* (2-68) at lower temperatures.

10.5 Co-inoculation of PGPR and *B. japonicum* with genistein addition under a range of RZTs

Co-inoculation of soybean plants with PGPR produced a wide range of effects which varied among strains of PGPR and over RZTs. *S. proteamaculans* 1-102 and *S. liquefaciens* 2-68 were reported to stimulate soybean plant growth, development, plant physiological activities, and enhance nodulation and nitrogen fixation at suboptimal root zone temperatures (Zhang et al., 1996b). At an optimal RZT (25°C), *S. liquefaciens* 2-68 was reported to increase plant leaf development, dry matter accumulation, nodule size and nitrogen fixation, while *S. proteamaculans* 1-102 had the same effect but at a lower RZT (15°C) . *S. proteamaculans* 1-102 was reported to perform very poorly at 25°C (Zhang et a., 1996a,b). In this study (sections 8 and 9) it was observed that at an optimal RZT (25°C), *S. liquefaciens* 2-68 increased plant leaf number, leaf area, pod number, total dry matter accumulation, nodule dry weight per plant, nodule size and total nodule weight per plant, while at 15°C RZT *S. proteamaculans* 1-102 increased leaf number plant dry matter accumulations, nodule number, nodule dry weight per plant, nodule size and total nodule weight per plant. These results confirmed that the physiological effects of PGPR on soybean plants are affected by RZT.

The change in photosynthetic rate over time showed that plant photosynthesis was increased by some PGPR strains, genistein, and *B. japonicum* strain combinations over a wide range of plant growth stages. As photosynthesis was increased by stimulatory strain combinations before the onset of nitrogen fixation, the improvement of plant growth, development and physiological activities must have been through an effect of PGPR on overall plant physiology followed by a genistein effect on nitrogen fixation. Also, application of PGPR 2-68 shortened all early infection stages (section 7).

Zhang and Smith (1995b) showed that genistein effects on photosynthesis were only seen after the onset of N_2 fixation, while the effect of PGPR was seen prior to the onset of nitrogen fixation (Zhang 1996b). Also, (Zhang 1996 a,b) showed that PGPR strain *S. proteamaculans* 1-102 performed very poorly alone at 17 and 25°C. Our findings agree with those results, and since both PGPR and genistein seem to work by different mechanisms, their effects might reasonably, be additive. A wide range of possibilities have been postulated to illustrate the mechanisms of PGPR, including an increase in mobilization of insoluble nutrients and subsequent enhancement of uptake by the plants (Lifshitz et al., 1987), production of antibiotics toxic to soil-borne pathogens (Li and Alexander, 1988), associative N_2 fixation (Chanway and Holl., 1991a) and production of plant growth regulators that stimulate plant growth (Gaskins et al., 1985). Given that our plants were growing in nutrient solution and without pathogen pressure, our results suggest the fourth possibility. PGPR applied to legume crops have also been demonstrated to positively affect symbiotic nitrogen fixation by enhancing root nodule number or mass (Grimes and Mount, 1984; Polonenko et al., 1987; Yahalom et al., 1987; Zhang et al., 1996b) and increase nitrogenase activity (Iruthayathas et al., 1983; Alagawadi and Gaur, 1988). Genistein (the most effective plant-to-bacterium signal in the soybean-*B. japonicum* N_2 fixing symbiosis) was reported to increase soybean nodulation and at suboptimal RZTs. The effect of genistein increased with decreasing RZT (Zhang and Smith, 1995b). Therefore, the observed increases in plant growth and development due to PGPR and genistein addition may be explained as : 1) from improvement of plant physiological activities such as photosynthesis and growth leading to improved nodulation and nitrogen fixation after the onset of N_2 fixation, and 2) more rapid in infection and nodule development due to genistein addition leading to a further acceleration of nodule development and onset of N_2 fixation.

Some PGPR and genistein combinations showed additive effects. The combination of PGPR 1-102, *B. japonicum* USDA110 and genistein had an additive effect on the nodule dry weight per plant at 25 but not 15°C. At 17.5°C, the combination of PGPR 1-102, *B. japonicum* USDA110, and 15μM genistein has an additive effect on the nodule number and nodule size. At 15°C PGPR 1-102 performed better by itself than with genistein.

Previous studies have shown that co-inoculation of *Pseudomonas* sp. (including *P. fluorescens* and *P. putida*) with *B. japonicum* increased nodule formation and weight by soybean plants in different rooting media (soil and perlite mix) (Polonenko et al., 1987); in the present study both *Serratia* species increased these two variables. The enhancement of nodule size by PGPR was always related to increased nitrogen fixation. Photosynthesis was more sensitive to the application of PGPR, at both the optimal and suboptimal RZTs, than transpiration or stomatal conductance. Within each RZT, comparisons of the photosynthetic rate increases by the best stimulatory PGPR strain in both experiments showed that at suboptimal RZT (17.5°C), *S. proteamaculans* 1-102, 15 μM genistein and *B. japonicum* USDA110 stimulated plant photosynthesis the most. This combination increased photosynthetic rates by 38 and 76% compared to the no PGPR, 0 μM genistein treatment. At the lowest RZT (15°C), *S. proteamaculans* 1-102 increased leaf photosynthetic rate by 100 and 86% when compared to the no PGPR, 0 μM genistein treatment. Wilson and McMurdo (1981) reported that plant photosynthesis was more sensitive to low temperature effects than was transpiration. Therefore, PGPR may have increased plant photosynthetic rates more at low RZTs, relative to plants growing at the optimal RZT, because the increased photosynthetic rate at the low RZTs was a part of a reduction of the direct adverse effects of low temperature on photosynthesis.

Some PGPR and genistein combinations showed additive effects in the photosynthetic rate at 25 and 17°C RZTs. The combination of PGPR 1-102 with either *B. japonicum* USDA110 or 532C and 5μM genistein increased the photosynthetic rate additively compared to *B. japonicum* USDA110, 532C, PGPR 1-102 or 5μM genistein alone. On the other hand, at 15 °C RZT, PGPR and genistein combinations showed antagonistic effects as the photosynthetic rate was decreased relative to PGPR plus *B. japonicum* or genistein plus *B. japonicum* treatments. The combination of PGPR 1-102, *B. japonicum* USDA110 and 20μM genistein decreased the photosynthetic rate compared to *B. japonicum* USDA110 alone.

Zhang et al. (1995a) demonstrated the existence of a linear relationship between RZT and the time from inoculation with *B. japonicum* to the onset of nitrogen fixation between 25 and 17°C RZTs. In this RZT range the time between inoculation and the onset of nitrogen fixation was delayed by 2 days for each degree decrease in RZT. However, the onset of nitrogen fixation was delayed by one week per °C as the RZT decreased from 17 to 15°C. Our results showed that the nitrogen concentrations of plant shoots and whole plants grown at 15°C were lower than those at 17.5 and 25°C. These results agreed with those of a previous study (Zhang et al., 1995a). Co-inoculation of *B. japonicum* with *S. proteamaculans* 1-102 was shown to lessen the decrease in nitrogen concentration of plant shoots at 15°C RZT. The results of this study indicated that some PGPR strains combined with genistein can enhance soybean nodulation and nitrogen fixation. PGPR *S. proteamaculans* 1-102 which was shown to perform poorly at 25°C was shown in this study to be effective at 25°C when combined with 5 μM genistein. The stimulations due to combinations of PGPR and genistein were probably due to PGPR stimulation prior to the onset of N_2 fixation, and PGPR plus genistein effects after the onset of nitrogen fixation. Overall these findings confirm that adding PGPR or genistein alone with *B. japonicum* to soybean roots accelerates nitrogen fixation and that adding the two together caused stimulations of N_2 fixation that, in some cases, were greater than those cause by either PGPR or genistein alone.

Section 11

SUMMARY AND CONCLUSIONS

Based on the findings of this thesis, a range of general and specific conclusions may be drawn. These have been placed together in five groups, related to specific aspects of soybean biology.

1. RZTs and PGPR effects

1.1. Some PGPR strains are able to grow and multiply effectively and colonize soybean roots effectively at low RZTs, while others are able to grow and multiply and colonize the soybean roots effectively at higher RZTs, and in its optimum temperature range an effective PGPR will be heavily present on the root, but be relatively less present in the surrounding rooting medium, while outside the optimum RZT range the reverse is true.

1.2. The ability of PGPR to colonize the root effectively, at least among the PGPR tested in this work, is a prerequisite to the stimulation of growth, nodulation and nitrogen fixation of soybean plants.

2. PGPR and soybean infection by *B. japonicum*

2.1. At 25 and 17°C, application of PGPR 2-68, which performed better at higher RZT, shortens the time elapsed from the beginning of root hair curling until the infection thread reached the base of the root hair, while at 15°C PGPR 1-102, which performed better at lower RZT, shortens the duration of the root hair curling, infection thread initiation and infection thread growth to the base of the root hair.

2.2. At both 15 and 25°C treatment of the soybean plants with PGPR supernatant at inoculation only or through daily applications reduces the time required for root hair curling, infection thread initiation, and the infection threads to reach the base of the root hairs, and increased the frequency of each of these stages, with the frequency being highest at both 15 and 25°C when soybean plants were treated daily with PGPR supernatant.

2.3. Both PGPR 1-102 and 2-68 produce diffusable growth promoting substances.

2.4. Since addition of the PGPR supernatant results in stimulation which is strain specific and temperature dependent, each PGPR probably releases a different growth stimulating substance. Although, given that they have similar effects on plant growth, they may be similar molecules.

3. PGPR effects on soybean N_2 fixation and growth under field conditions

3.1. Co-inoculation of *B. japonicum* with PGPR improves plant growth, development, yield components, grain yield, protein yield, and increased soybean nodulation and nitrogen fixation.

3.2. Application of PGPR to *B. japonicum* inoculum accelerates the beginning of N_2 fixation by 2 to 3 days during the early portion of the soybean growing season, when the soils are still cool. This is especially true for the earlier-seeded (one week before the normal planting date) soybean plants.

3.3. Total fixed nitrogen, fixed nitrogen as a percentage of total plant nitrogen, and total nitrogen yield were all increased in at least some cases due to PGPR application.

4. PGPR proliferation and root colonization under field conditions

4.1. PGPR 2-68 is able to grow and survive better than PGPR 1-102 under short season conditions.

4.2. There is a relationship between the ability of the tested PGPR to colonize the roots of the soybean plants and their ability to stimulate soybean nodulation, nitrogen fixation, plant growth and physiological activities under short season conditions.

5. PGPR plus genistein effects on soybean N_2 fixation and growth

5.1. Some PGPR strains combined with genistein can enhance soybean nodulation and nitrogen fixation

5.2. PGPR *S. proteamaculans* 1-102, which was shown to perform poorly at 25°C when added alone, is effective at 25°C when combined with 5 μM genistein.

5.3. Some PGPR strains combined with genistein has additive effects of soybean growth and development at 17 and 25°C, while others showed antagonistic effects, and antagonism is the rule at 15°C RZT.

Section 12

ACCEPTANCE OR REJECTION OF HYPOTHESIS

Hypothesis 1:

PGPR accelerate nodulation, leading to increased nitrogen fixation and improved yield by soybean in areas with cool spring temperatures.

Results related to hypothesis 1: In this study, preincubation of *B. japonicum* with PGPR increased soybean nodulation and nitrogen fixation and subsequent soybean growth and yield in the early part of the growing season when soil temperatures were low (sections 3 and 4). Thus, we **accept hypothesis 1.**

Hypothesis 2:

The ability of PGPR to survive, grow and multiply in the field under short-season conditions is related to their ability to stimulate soybean nitrogen fixation, growth and yield under field conditions.

Results related to hypothesis 2: PGPR 2-68 *Serratia liquefaciens* was able to grow and survive better than PGPR 1-102 *Serratia proteamaculans* under short season conditions. A previous study (unpublished data), has shown that the combination of PGPR 2-68 with AC Bravor plants increased leaf area, seed number, and grain protein yield, suggesting that there is a direct relationship between the ability of these PGPR to colonize the roots of the soybean plants and their ability to stimulate soybean nodulation, nitrogen fixation, plant growth and physiological activities under short season conditions (section 5). Thus, we **accept hypothesis 2.**

Hypothesis 3:

The ability of PGPR to colonize soybean roots is related to their ability to stimulate soybean N_2 fixation and growth, and this is affected by RZT.

Results related to hypothesis 3: The ability of PGPR strains to grow, multiply, and survive is strain specific and temperature dependent. Some PGPR strains are able to multiply and colonize the roots effectively at low RZTs. Others are able to multiply and colonize the roots effectively at higher RZTs. It was shown that in the optimum temperature range an effective PGPR will be heavily present on the root, but be relatively less present in the surrounding rooting medium, while outside the optimum RZT range the reverse was true (section 6). Thus, we **accept hypothesis 3**.

Hypothesis 4:

Early stages of soybean nodulation are affected by PGPR.

Results related to hypothesis 4: Application of PGPR 2-68 shortened the time elapsed from the beginning of root hair curling until the infection thread reached the base of the root hair at 25 and 17°C, while at 15°C PGPR 1-102 shortened the time until the onset of root hair curling, infection thread initiation and infection thread growth to the base of the root hair. Thus, we **accept hypothesis 4.**

Hypothesis 5:

PGPR enhance nodule development of soybean at low RZT through production of diffusible compound.

Results related to hypothesis 5: At both 15 and 25°C RZT, soybean plants treated with PGPR supernatant at inoculation only or through daily applications reduced the time required for root hair curling, infection thread initiation, and the infection thread to reach the base of the root hair, and the frequency of occurance of the different stages was the highest at both 15 and 25°C for soybean plants treated daily with PGPR supernatant. This demonstrates that both PGPR 1-102 and 2-68 produce growth promoting substances (section 7). Thus, we **accept hypothesis 5**.

Hypothesis 6:

Addition of genistein plus PGPR will stimulate soybean nodulation, N_2 fixation and growth more than the addition of PGPR alone.

Results related to hypothesis 6: Some PGPR strains combined with genistein stimulated some aspects of soybean growth and development. Adding the two together caused stimulations of soybean plants nodulation, N_2 fixation and growth that were greater than those caused by either PGPR or genistein alone at 25 and 17°C RZT with most combinations of *B. japonicum* strains, PGPR strain and genistein (sections 8 and 9). Thus, as stated we can **neither accept non reject hypothesis 6.**

Section 13

CONTRIBUTIONS TO KNOWLEDGE

The following are considered to be original contributions to knowledge developed from the work in this thesis:

1. Co-inoculation of PGPR and *B. japonicum* increased soybean nodulation, N_2 fixation, protein production and seed yield under field conditions.

2. Under field conditions, the PGPR best to colonize soybean roots are most stimulatory to soybean growth.

3. In an effective RZT range a PGPR will colonize soybean roots effectively and be less present in the rhizosphere. Out side an effective RZT the reverse is true.

4. At least part of the reason RZT affect the efficiency of PGPR is by affecting their ability to colonize soybean roots.

5. Within an effective temperature range the PGPR can shorten the early stages of nodule development.

6. The PGPR tested exert their beneficial effects on soybean plants via a diffusible compound or compounds produced in the presence or absence of soybean roots.

7. At higher RZTs (17.5 and 25°C) the effect of PGPR plus genistein can be greater than those of either alone.

8. At 15°C RZT PGPR and genistein are antagonistic.

Section 14

SUGGESTIONS FOR FUTURE RESEARCH

To expand on the work reported here and elucidate the role of PGPRs in increasing the nodulation of legumes at suboptimal RZTs, the following research remains to be done.

1. Determination of the mechanism by which PGPR stimulate soybean plant nodulation and nitrogen fixation.

As we found that PGPR can stimulate soybean plant nodulation and nitrogen fixation, it is important to determine the mechanism by which PGPR do this. The findings that PGPR produce diffusible compounds raises the possibility of purifying and identifying the compound (s).

2. Determine whether or not *Serratia* strain has effects on other crops grown under short season conditions.

The studies contained in this thesis have focused on soybean and *Serratia* strains. The tested *Serratia* have shown effects on soybean plants so futher research to test these PGPRs on other crops grown under short season conditions, such as corn, is recommended

3. Testing a wider range of *Serratia* strains.

The genus *Serratia* has been little investigated in the capacity of a PGPR. Arange of *Serratia* species and strains should be assembled and tested.

4. Testing the additive effects of PGPR and genistein under field conditions.

As PGPR and genistein show additive effects under some conditions in green house environment, testing their ability to increase plant nodulation and nitrogen fixation under field condition is required.

5. The interactions between genistein and PGPR should be further characterized so that the mechanisms can be investigated.

As there is no current information regarding the genistein-PGPR antagonism at lower RZT, this needs further investigation.

6. The two tested soybean cultivars responded differently to PGPR; a wider range of cultivars should be tested in order to determine the norms and variability.

It would be of value to know if most soybean cultivars will respond strongly or weakly to PGPR applications.

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