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Symbiotic Efficiency of *Bradyrhizobium japonicum*
for Development of a Commercial
Soybean Inoculant for Western Canada

by

Vanessa Kavanagh

A Thesis Submitted to Saint Mary's University, Halifax, Nova Scotia,
in Partial Fulfillment of the Requirements for the
Degree of Masters in Applied Science

November 19, 2007

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DEDICATION

This thesis is dedicated to my amazing mother (Stella) and my sister (Lori-Lynn) and her wonderful family (Matthew, Haley, Kelsey & Patrick) for their unfaltering love, patience and support; and to my late father (Patrick) who is still with me and motivates me to work hard every day.

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ABSTRACT

Symbiotic Efficiency of *Bradyrhizobium japonicum* for Development of a Commercial Soybean Inoculant for Western Canada

By Vanessa Bernadette Kavanagh

Soybean production has increased substantially in western Canada in recent years. Current soybean inoculants (*Bradyrhizobium japonicum*) have not performed the same in western Canada as they have in other regions. *B. japonicum* exhibit genetic change in soil over time and may result in novel rhizobial lines capable of performing better than the original introduced strain. Greenhouse experiments performed on 60 isolates of *B. japonicum* from previously inoculated fields assessed symbiotic efficiency – ability to induce nodulation and fix nitrogen – compared to commercial inoculants 532C and USDA110. A pre-screening stage using one cultivar selected 10 isolates for second stage screening. In second screening using three cultivars, inoculated treatments had higher biomass and tissue nitrogen contents than nitrogen-fertilized and untreated controls confirming the need for inoculation in virgin soils. Five isolates (2-37, 2-38, 3-49, 3-50 & 3-57) resulted in high plant tissue nitrogen contents and biomass and should be tested in a crop setting.

November 19, 2007

1.0 INTRODUCTION

Legumes are plants that bear their seeds in pods. Graham and Vance (2003) name Leguminosae (Fabaceae) as the second most important family to humans; it is also the third largest family of flowering plants (Hirsch *et al.*, 2001). Legumes are important in agriculture; soybeans are especially significant because they carry one of the highest protein contents among seed grains (approximately 35 – 40%), making this species one of the most important agricultural sources of protein in the world (Krishnan *et al.*, 2005). Soybeans and other legumes possess an advantage over cereal grains in that their symbiotic associations with specific bacteria (rhizobia) allow them to fix nitrogen (N) (Alexander, 1984). This process is often efficient enough to eliminate the need for supplemental nitrogen fertilization (Vessey, 2004).

Allorhizobium, Azorhizobium, Bradyrhizobium, Burkholderia, Cupriavidus, Devosia, Ensifer, Herbaspirillum, Mesorhizobium, Methylobacterium, Ochrobactrum, Phyllobacterium and *Rhizobium* are the prokaryote genera that symbiotically perform nitrogen fixation (Weir, 2006) and certain types of rhizobia tend to associate with specific legume species; for example, soybean generally will only efficiently associate with *Bradyrhizobium japonicum* (Sessitsch et al., 2002). The bacterium infects the legume root hair, a specialized structure termed a root nodule forms and it is this structure that the plant supplies with photosynthates for growth and to provide the energy required for processes such as nitrogen fixation (Elkan, 1987; Lum & Hirsch, 2003). It has been shown that application of nitrogen fertilizer actually decreases the effectiveness of symbiosis by inhibiting nodule formation (Streeter, 1998).

The production of soybean [*Glycine max* (L.) Merr.] has increased at an exponential rate in western Canada since the mid -1990's (Manitoba Agriculture, Food, & Rural Initiatives, 2004). This is mainly due to the recent development of early maturing varieties, making it possible to grow the crop further north (Manitoba Trade and Investment, 2006). As soybean is a novel crop to western Canada, it bears great value to farmers by opening up new opportunities. Soper *et al.* (2003) predict that the market for this grain seed will only rise in the future with the increased interest for cleaner burning fuels (bio-diesel) and special diets. In addition to increased market availability, this new introduction provides the opportunity to interrupt disease cycles with more time between rotations. If used as fallow, the nitrogen in soybean residues is efficiently utilized by subsequent crops (Power *et al.*, 1986; Legocki & Bothe, 1997).

Inoculation of soybean with *B. japonicum* is recommended to farmers wishing to grow this crop in order to increase the effective nodulation and nitrogen fixation to maximize production (Vessey, 2003). Commercial *B. japonicum* strains 532 C and USDA 110 are inoculants that perform well over a wide range of temperatures and thus are widely used in Canada and the northern United States (Hume and Shelp, 1990; Lynch and Smith, 1993). However, these strains have not performed as well in western Canada as they have in the regions for which they were originally developed (Ontario and the northern US) and crops have to be inoculated at a rate two to three times higher than normal (10^5 bacteria per seed) (Manitoba Agriculture, Food, & Rural Innovation, unpublished data). It is therefore necessary to find a strain more suited to the regional climate and soil conditions.

Manitoba has a clay-loamy soil and the climate is characterized by hot dry summers and cold winters (Manitoba Agriculture, Food, & Rural Initiatives, 2006). Soil conditions in Ontario (where commercial inoculant 532C was originally developed) generally have lower clay contents than soils in the Red River Valley in Manitoba (Ontario Ministry of Agriculture, Food and Rural Affairs, 2004). Ontario also has greater precipitation and warmer winters than Manitoba (Ontario Ministry of Agriculture, Food and Rural Affairs, 2003). The region where inoculant USDA 110 was developed in Florida, USA (Keyser and Griffin, 1987) has a higher percentage of sand and silt than is found in Manitoba soils (United States Department of Agriculture National Soil Survey Laboratory, 2007). The climate is also much dryer and warmer in Florida than Manitoba (Florida Climate Center, 2007). The differences between regions may be responsible for the inconsistencies observed when using the respective inoculants.

B. japonicum has been shown to exhibit genetic change after introduction to the soil (Santos *et al.*, 1999; Farooq, 2007). While the variation is usually detrimental, it can occasionally result in a higher performance strain (Montasso *et al.*, 2002). This project examines transformed *B. japonicum* isolated from western Canadian and northern United States (US) soils to identify a high performance strain better adapted to the local conditions.

The objectives of this research project were:

1. To assess the symbiotic efficiency of *B. japonicum* isolates from southern Manitoba and northern US soils;

2. To identify an equal or higher performing *B. japonicum* strain(s) as compared to the commercial inoculants 532C and USDA 110 in Manitoba soil for possible marketing in western Canada.

To achieve these objectives, the project industrial partner, Philom Bios Inc. of Saskatoon, Saskatchewan, Canada, and the University of Manitoba collected soybean nodules over two summers from fields with previous inoculation. By collecting nodules from originally uninoculated soybeans found within the fields, selection was already made for strains in the soil that were infective. With the culturable *B. japonicum* isolates recovered from the nodules, five greenhouse trials were conducted in just over one year (2005-2007) to assess symbiotic efficiency of the isolates. The trials tested the resident bacterial isolates against two commercial *B. japonicum* strains, a nitrogen-fertilized control, and an untreated control on different soybean cultivars. Trials were run in two stages, with four First Stage trials testing 60 isolates with controls on the soybean cultivar Gentleman. The 10 most symbiotically effective strains when compared to the commercial inoculant controls progressed on to a Second Stage test using three genetically diverse cultivars: Gentleman, OAC Prudence, and an increasingly used cultivar, Roundup Ready® Apollo. The results are discussed in terms of identifying the most indicative factors for determining elevated plant nitrogen contents and symbiotic efficiency.

2.0 LITERATURE REVIEW

To study the symbiotic efficiency of *B. japonicum* isolates, it is necessary to understand the nitrogen fixation process as it occurs within the legume plant. This review will examine legumes, nitrogen fixation, rhizobia, the rhizobium-legume symbiosis, and inoculant selection.

2.1 Legumes

Legumes are pod-bearing plants and are the third largest family of angiosperms (Hirsch *et al.*, 2001). Legumes have been used in agriculture as a food crop dating as far back as 9,500 BCE in Iran (Cohen, 1977); large scale production began approximately 3,000 years ago in South America and Asia (Hymowitz & Singh, 1987; Kaplan & Lynch, 1999). Legumes were used for crop soil improvement as far back as Roman times, as Varro in 37 BCE noted when he stated that they were to be planted "for good they do to subsequent crops" (Fred *et al.*, 1932).

The great importance of legumes lies in the fact that, unlike other crops that require supplemental N fertilizer, these plants are able to take a proportion of the nitrogen they require from the atmosphere when in association with soil bacteria termed rhizobia (Biederbeck *et al.*, 2005). This provides an inexpensive means of N fertilization at a rate of approximately $2-3 per acre (Vessey, 2004).

2.1.1 Soybeans

Soybean is an important food because it possesses among the highest protein contents among seed grains (approximately 35 – 40%) (Krishnan *et al.*, 2005). This provides key benefits to livestock production as well as to those who choose vegetarian lifestyles or low-fat diets, and to developing countries where large populations may not have access to meat protein on a regular basis. Also, soybean oil is used in formulations from salad dressings to baby foods. In the United States, approximately 80% of the 16 billion pounds of edible oils used each year come from soybean oil (Warner, 2002). Soybean supplies 25% of the world's fats and oils and 75% of the trade in high-protein meals (Keyser & Li, 1992).

Soybeans also carry a high potential as an alternative fuel in the form of biodiesel. Many studies have shown soybean biodiesel to exhibit superior energy returns *versus* other oil-seed biodiesels and corn grain ethanol. Soy biodiesel can yield between 93-200% more energy than is invested in its growth. In contrast, corn grain ethanol yields 25%, gasoline 12%, and petrol-diesel 17% (Hill *et al.*, 2006; Conley & Tao, 2006). Supply of soybean oil for biodiesel remains a problem as the majority of soybean oil is already destined for other markets. If all soybean farming currently underway in the United States were devoted to biodiesel production, it would only offset biodiesel demand in that country by 6% (Conley & Tao, 2006; Tilman, 2007).

Soybeans are also an important addition to crop rotations, providing crop disease disruption. When the same crops are repeatedly planted, pests and diseases often get a chance to establish themselves and this can result in significant losses. Farmers use crop rotation to interrupt such pest and disease cycles, for example substituting wheat for corn

in alternate years. Introducing another crop into the rotation allows more time between repeated crop plantings, decreasing the chance of survival (at least in larger numbers) of the offending pest or pathogen (Abbott & Murphy, 2003).

2.2 Nitrogen Fixation

Nitrogen (in the form of N_2) is a major constituent (79%) of the air we breathe; however, it is often a limiting factor for plant growth. Most organisms are only able to use nitrogen in specific forms, e.g. nitrate (NO_3^-) , ammonium (NH_4^+), or amino acids (R-NH_2). The process of converting atmospheric N_2 to ammonia is called nitrogen fixation.

An alternative source of nitrogen in agriculture is industrial nitrogen fixation. Industrially, nitrogen is produced using the Haber Bosch process, which uses nitrogen, hydrogen and a metal catalyst to produce ammonia. This process is complex and requires a large amount of fossil fuel for production (US National Research Council, 1994). Burns and Hardy (1975) and Hill *et al.* (1979) found that lightning strikes were also a considerable abiotic source of N_2 fixation. Hansen (1994) estimated global lightning N_2 fixation to range from 6 – 50% of total yearly natural N_2 fixed. However, the majority of nitrogen fixation is carried out biologically *via* microbial processes. These occur mainly in terrestrial environments and involve associations between plants and microbes such as rhizobia and *Frankia* (Barimah-Asare, 1992).

In legumes, *Allorhizobium, Azorhizobium, Bradyrhizobium, Burkholderia, Cupriavidus, Devosia, Ensifer, Herbaspirillum, Mesorhizobium, Methylobacterium, Ochrobactrum, Phyllobacterium* and *Rhizobium* are the prokaryote genera that symbiotically perform nitrogen fixation (Weir, 2006). Both the plant and the bacterium

are able to survive independently, but both benefit more from their association. The legume supplies photosynthates for nutrition (and the energy required for the N_2 fixation process) while the microbe in turn provides the plant with usable nitrogen (Elkan, 1987; Lum & Hirsch, 2003). Lindemann & Glover (1998) suggested 4-15 kg N ha^{-1} is fixed by legumes in natural systems, while this number increases to several hundred kg N ha^{-1} in a cropping system. Keyser and Li (1992) reported that under ideal conditions, soybean can fix approximately 300 kg N ha^{-1} annually when in symbiosis with *Bradyrhizobium japonicum*. Typically, soybeans fix 80 – 100 kg N ha-^{1} annually when in association with *B. japonicum* (Warman, 1980).

2.3 Rhizobia

There are several types of legume rhizobia that are identified by their growth rates. The genus *Rhizobium* is comprised of fast-growing rhizobia, whereas *Bradyrhizobium* show slow growth (Summerfield & Roberts, 1985; Höll, 1976). Soybean is generally a non-promiscuous legume, and although a few species of rhizobia can nodulate the roots (e.g. *B japonicum, B. elkanii*) it performs best with the slow-growing species, *Bradyrhizobium japonicum* (Sessitsch *et al.*, 2002; Giller & Wilson, 1991).

2.3.1 Rhizobial Evolution & Survival

Rhizobia can survive for long periods of time in soil. Rao (1995) found that rhizobia can persist without re-application for up to 54 years in very small numbers. Chemining'wa & Vessey (2004) have shown with previous studies of the pea rhizobia

Rhizobium leguminosarum bv. *viciae* that once introduced into the soil, the bacteria begin to change rapidly. They found that in as little as one year, the bacteria showed detectable genetic variation and could persist and continue to transform for as long as 25 years (oldest area tested). In a companion study to this experiment, Farooq (2007) confirmed this property with the soybean rhizobia *B. japonicum*. Bai (2004) examined the capacity to manipulate this fast rate of change, hoping to find strains better suited to various edaphic and climatic conditions by selecting and breeding strains or colonies that performed well at various extremes (e.g. cold or hot temperatures, dry or wet conditions, etc.).

Rhizobia are found in a wide array of climates, from arctic to desert. *B. japonicum* was initially adapted to its host plant in warmer parts of Asia and thus is not well suited for survival in cooler climates (Hymowitz, 1970). Bailey (1989) found that exposing rhizobia living in western Canadian soils to extreme temperatures, thereby mimicking seasonal changes, resulted in poorly performing bacteria showing decreased or no nodulation and poor nitrogen fixation or death. Legros & Smith (1993) and Zhang *et al.* (1995) confirmed these results and found suboptimal temperatures in the rhizosphere decreased nodule formation and function.

Factors other than temperature affect the survival of rhizobial species. Yang *et al.* (2001) found that soil pH affected the ability of rhizobia to induce nodulation. Egamberdiyeve *et al.* (2004) and Dobereiner (1978) found that increased acidity (decreased pH) resulted in poorer nodulation and decreased survival rates.

2.4 Rhizobium-Legume Symbiosis

2.4.1 Symbiotic Efficiency

Symbiotic efficiency of rhizobia strains is defined in two ways: firstly, by their capacity to induce nodulation within the legume roots, and secondly, by their ability to fix nitrogen (Sprent, 2001). Musiyiwa *et al.* (2005) found that these two traits, although related, do not always indicate the function of the other. Hence, a strain that is able to induce nodulation well does not necessarily fix more nitrogen, or *vise versa.*

Efficiency is assessed through a variety of techniques, including genetic and chemical analysis, and field testing. Sarkodie-Addo (1992) identified several genes (i.e. Nif genes) that are potentially useful for the identification of both nodulating and nitrogen- fixing strains. When performing a DNA analysis, the presence or absence of bands representing these genes are used as a means to identify which strains are capable of these functions without actually testing them in the field. There may be a problem however if genes are present but are not expressed (Staples *et al.*, 2007). To be certain of symbiotic capabilities field testing is required.

Field testing is important and necessary stage in identifying symbiotic efficiency. Genetic analysis and growth in laboratory settings are not able to incorporate all the different variables that exist in the natural environment, such as inter-microbial competition (Ferreira & Hungria, 2002); as such, these studies lack ecological validity. Benefits of field testing include the ability to take direct observations and make comparisons of natural growth. Following harvesting, various chemical analyses of the plants can more accurately quantify nitrogen fixing ability. Chen *et al.* (2002) and

Ferreira and Hungria (2002) suggested tissue nitrogen analysis as well as different tissue assays to identify bacterial strains with greater nitrogen fixing capabilities.

2.4.2 Enhancing Growth

Symbiotic nitrogen fixation results in the host plant obtaining nitrogen from the bacteria within its root nodules. In turn, this nitrogen enhances the growth of these plants, and results in significant increasing yields over uninoculated plants (Bai *et al.*, 2002; Dashti *et al.*, 1997). Egamberdiyeva *et al.*, (2004) found that the increase in biomass was not limited to the main bulk of the plant (stem and leaves) but was also reflected on both seed oil and protein content by as much as a 45% increase. The mechanisms by which rhizobia promote plant growth include the following: producing or changing the concentration of specific plant hormones; nitrogen fixation; antagonism against phytopathogenic microorganisms and solubilization of mineral phosphates and other nutrients (Cattelan *et al.*, 1999). The practice of supplying rhizobia to a legume in order to enhance growth and yield is called inoculation, and the bacteria are the inoculant. Vessey (2003) and Herridge *et al.* (2002) found that in most cases it is beneficial to inoculate legumes at each time of planting.

2.4.2 Symbiotic Efficiency and Nitrogen Fertilization

Inoculation with rhizobia is generally recommended to decrease or eliminate the need for supplemental nitrogen fertilization. In some cases, such as that of the common bean (*Phaseolus vulgaris*), rhizobial inoculation alone cannot provide all the nitrogen need by the plant (Food and Agriculture Organization of the United Nations, 1993). In

such cases, a low rate of additional nitrogen application is recommended. Other legumes, such as soybean, are able to supply all the nitrogen the plant requires from symbiotic nitrogen fixation (National Soybean Research Laboratory, 2007; Willis, 1989). Streeter (1988) found that in these situations, additional nitrogen application actually decreased the effectiveness of the symbiosis by reducing and inhibiting nodule formation. Phillips & Dejong (1984) found that available concentrations of nitrogen in the rhizosphere above 2mM decreased soybean nitrogen fixation in symbiosis. Bollman (2004), Heichel & Vance (1979), and Eardly *et al.* (1985) also found nitrogen fertilizer resulted in a decrease in efficiency of the legume-rhizobium symbiosis for other legumes such as alfalfa.

In certain circumstances other than in the common bean (*Phaseolus vulgaris*), a "starter" nitrogen application is recommended at planting. It was shown that in nitrogen-depleted soils, the initial nitrogen application could be stimulatory and possibly increases plant growth before the biological nitrogen fixation process initiated (Giller and Cadisch, 1995). However, Cowell *et al.* (1989) showed that nitrogen application at planting had no effect on the growth of pea in Saskatchewan soils.

2.5 Inoculant Selection

2.5.1 Inoculation Industry

Legume inoculation has been practiced for centuries, however the first commercial inoculants were not produced until the end of the 1800s (Brockwell & Bottomly, 1995). Inoculants are available in many formulations, including non-sterilized/sterilized peat, peat pellets, coal, powder and liquid. These formulations vary

in bacterial holding capacity and also differentially affect bacterial survival (Herridge *et al.*, 2002). A good commercial inoculant has to possess both a good carrier for the bacteria and a high-performing rhizobia. Keyser & Li (1992) and Stephens & Rask (2000) set out the properties that make for a good carrier and rhizobia mixture. Some properties of a good carrier include a high water-holding capacity, non-toxicity to rhizobia, easy sterilization, a pH buffering capacity and good adhesion to the seed. Properties of suitable rhizobia include the ability to form nodules and fix nitrogen across a wide range of host genotypes and soil types, competiveness with rhizobia already present in the soil, and the ability to grow in artificial media (i.e. the inoculant carrier). Also, a good commercial-grade rhizobial inoculant should migrate from the initial site of inoculation and persist in the soil. The inoculation industry is continuously trying to improve inoculant formulations and carry out ongoing inoculant assessment programs.

2.5.2 Inoculant Assessment

Techniques to assess potentially valuable resident strains of rhizobia vary, but all contain a laboratory component and a greenhouse and/or field test component. The laboratory primarily tests for culturability and stability of colonies at high densities (Brockwell & Bottomly, 1995). Although the bacteria may thrive in soil, reproducing these conditions in a laboratory setting is difficult and they often do not grow. Isolation from nodules is problematic, and developing a working protocol is important from the beginning. If growth is achieved, the ability to sustain adequate numbers for commercial inoculant use has to be determined (Stephens & Rask, 2000). Greenhouse testing can be carried out using a variety of media, including sterile vermiculite, sand and different

soils. A major benefit of greenhouse trials is the ability to control conditions, from the ideal to specific stresses. The greatest disadvantage of this method is the inability to entirely mimic natural conditions. This can result in greenhouse findings that are inapplicable in the field. Field testing in a crop system is the most crucial component in inoculant assessment. Here, results are most comparable to those expected from standard farming techniques, although conditions can vary depending on the location (Herridge *et al.*, 2002).

2.5.3 Isolation Methodologies

Currently, there are several methods available for isolating rhizobia from nodules, including a complex multi-stage genetic and chemical analysis tailored for specific strain identification (Cook, 2001), and a fast isolation technique developed by Beattie and Handelsman (1989). The biggest problem however, remains the high level of contamination caused by the convoluted surface of the nodule. Often, this contamination results in the need for re-isolation from the nodules that may be limited in availability. Rice & Olsen (1993) suggested adding fungicides to the bacterial growth media such as cyclohexamide to help deal with this problem, or the addition of Congo red dye or antibiotics to the agar to assist in identification. Chemining'wa (2002) modified the technique by Rice & Olsen by adjusting the storage and sterilization procedures and this method is currently the preferred isolation technique. Proper isolation methods are crucial, as there are often a limited number of nodules from each area, and with increased distance (spatial or temporal) there is an increased opportunity for genetic change to occur.

2.5.3 Rhizobia Strain Selection Strategies

Rhizobia strain selection is primarily based on the criteria laid out by Keyser & Li (1992) and Stephens & Rask (2000) as indicated in section *2.5.1.* To assess nodulation capacity, nodule counts are made at the late-flowering to early podfill stages of soybean development, when nitrogen fixation is generally at its peak (Keyser & Li, 1992; Ismande, 1989). Symbiotic effectiveness is most often determined by total plant or shoot nitrogen content as well as total yield or biomass (Hungria *et al.*, 2001; Musiyiwa *et al.*, 2005; Ferreira & Hungria, 2002). Stephens and Rask (2000) correctly suggest the legume inoculant end user is concerned with crop productivity; however, bacterial efficiency will mostly be reflected in nitrogen content. In most cases, growth is limited by nitrogen supply.

2.6 Conclusion

Legume crops are an essential part of agriculture. They are a means of providing low cost/high return options to farmers by taking advantage of expanding markets and efficiently using rotations. Symbiotic nitrogen fixation is a desirable addition to farming in its ability to decrease the monetary and environmental costs of chemical nitrogen application. *B. japonicum* strains that are best adapted to western Canadian conditions have yet to be identified; however, given the proven rapid rate of genetic change within these bacteria, the prospects in identifying better adapted strains are promising.

3.0 MATERIALS AND METHODS

3.1 Nodule collection

Nodules were collected in the summers of 2004 and 2005 by personnel from the industrial partner, Philom Bios Inc., and the University of Manitoba. The nodules were taken from 29 fields, five of which had never grown the crop (Tables 3.1 & 3.2). Additional information of site locations, please see Appendices 8.1 – 8.3. Nodules were collected from trap plots set up in 1 m^2 sections where uninoculated soybeans were planted, or collected from soybean plants that grew randomly from seed left in the soil from previous years as opposed to having been deliberately planted. Workers selected late-flowering soybeans and confirmed the presence of nodules on the roots. Using forceps and scissors, roots were severed 0.5 cm above and below the site of nodule attachment. Nodules from individual plants were placed in a small container with clean water and agitated to remove excess soil debris. The nodules were then lightly dried with a paper towel and placed in 2-mL microfuge tubes containing 1.5 mL 20% glycerol in water. The tubes were vortexed for one minute and then completely filled with 20% glycerol. Samples were stored on ice until they were returned to the lab and frozen at -80°C. The procedure was repeated for a total of five samples per site. No samples were kept frozen for longer than a year.

Table 3.1 2004 collection site information as adapted from Farooq (2007) including site name (if available), crop history for the last two years (if available), year since last soybean crop grown, soil texture, soil drainage and if inoculant(s) was used.

Site #	Crop History (current crop/last yr)	Year since soybean grown	Soil texture	Drainage	Inoculant used
1	Unknown	Never	Silt clay	Poor	No
2	Soybean/Wheat	Current	Silt loam	Poor	No
3	Unknown	Never	Silt clay	Poor	No
4	Corn/Soybean	One	Loamy	Well	Unknown
5	Corn/Unknown	One	Silt clay loam	Very poor	Unknown
6	Oats/Soybean	One	Loamy	Imperfect	Yes
7	Canola/ Unknown	One	Unclassified	Unclassified	Unknown
8	Barley/Soybean	One	Unclassified	Unclassified	Yes
9	Oat/Soybean	One	Marsh	Imperfect	Yes
10	Corn/Soybean	One	Unclassified	Unclassified	Yes
11	Corn/Soybean	One	Unclassified	Unclassified	Unknown
12	Wheat/Soybean	One	Coarse loamy	Imperfect	Unknown
13	Wheat/Soybean	One	Eroded slope	Imperfect	Unknown
14	Clover/Soybean	One	Unclassified	Unclassified	Unknown

Table 3.2 2005 collection site information as adapted from Farooq (2007) including crop history for the last two years (if available), year since last soybean crop grown, soil texture, soil drainage and if inoculant(s) was used.

Site #	Crop History (current crop/last yr)	Year since soybean grown	Soil texture	Drainage	Inoculant used
1′	Corn/Soybean	Two	Silt clay loam	Poor	Yes
2′	Corn/Soybean	Two	Silt clay loam	Very poor	Yes
3′	Corn/Soybean	Two	Silt clay loam	Well	Yes
4′	Corn/Soybean	Two	Sioux loam	Well	No
5′	Corn/Soybean	Two	Clay loam	Well	No
6′	Corn/Sod (30 yrs)	Two	Silt loam	Well	No
7′	Corn/Soybean	Never	Silt loam	Well	No
8′	Corn/Soybean	Two	Loam	Well	No
9′	Corn/Soybean	Two	Silt clay loam	Very poor	No
10′	Corn/Soybean	Two	Unclassified	Unclassified	No
11′	Corn/Soybean	Two	Unclassified	Unclassified	No
12′	Corn/Soybean	Two	Clay loam	Well	No
13′	Unknown/Soybean	One	Sandy loam	Unclassified	Unknown
14′	No soybean	Never	Clay	Imperfect	Unknown
15′	Unknown	Never	Unclassified	Unclassified	No

3.2 Laboratory Experiments

3.2.1 Isolation of *Bradyrhizobium japonicum* from nodules

Isolation of *B. japonicum* was performed following the protocols of Chemining'wa (2002) and Rice & Olson (1993) with minor modifications. Samples were allowed to thaw and one nodule from each tube was removed with sterilized forceps and placed in a clean, 2 mL microcentrifuge tube. In each tube, 1.5 mL of sterile water was added and the tubes were vortexed for approximately 20 s, after which the water was removed with a pipet. The wash was repeated four times to remove excess soil and debris that adhered to the surface of the nodule. Following washing, 0.5 mL of 95% ethanol was added to the tubes and allowed to sit for 15 s. The ethanol was then removed from the tube with a pipette. To ensure sterilization, 1 mL of 1% mercuric chloride ($HgCl_2$) was added, followed by vortexing for five minutes. The mercuric chloride was pipetted out and the nodules were rinsed four more times with sterilized water.

After the sterilization treatment, 100 µL of sterile yeast mannitol broth was added to each tube. The nodules were crushed within the tubes using sterilized forceps and mixed to form a homogenous suspension. Using a sterile loop, a drop of each suspension was streaked into quadrants on yeast mannitol agar plates supplemented with Congo red as described by Thiel (1999). Congo red dye was used as a contra-indicator for rhizobia because, unlike most bacteria, they do not readily absorb the dye (Hahn, 1966). The inoculated plates were sealed with parafilm, inverted, and incubated without light at 28°C for 10-14 days. Plates with colonies that all turned red or did not grow were discarded. White colonies on plates with both white and red colonies present were re-streaked on a fresh agar plate to eliminate contamination. One colony was selected from

each plate. Nodules have been reported to contain more than one strain of rhizobia, so it was presumed each colony represented a single strain, or if more than one strain was present, only the most abundant were sampled (Moawad & Schmidt, 1987). The selected colonies were re-plated onto a yeast mannitol agar plate and incubated at 28°C for 10-14 days. One colony from each plate was placed in a 2 mL microcentrifuge tube containing 1.5 mL of 20% glycerol and stored at -80°C. The agar plates were stored at 4°C in a refrigerator.

3.2.2 Quantification of *B. japonicum* isolates

For Second Stage Testing (see section 3.3. below), isolates were quantified to normalize the rate of application. The rhizobia were taken from -80°C and thawed. Sterilized 125 mL Erlenmeyer flasks were filled with 30 mL of yeast mannitol broth. In each flask 0.1 mL of the bacterial suspension was added. The flasks were placed on an orbital shaker set at 200 RPM and 28°C. The optical density (OD) was measured on a daily basis starting on day four of incubation with a spectrophotometer set at 600 nm. Commencing when samples reached an OD of 0.3, serial dilutions on the cultures were performed to enumerate the number of colony forming units (CFUs) in the culture (Hartel & Bouton, 1989). The dilution was plated on yeast mannitol agar plates and counted. Serial dilutions were carried out on each culture as the cultures reached OD's of 0.30, 0.40, 0.50, 0.60, and 0.70 (±0.02) to enable manufacture of a growth curve (Figure 3.1) and to determine relative growth rates (Table 3.3). Relative growth rates were calculated as $[\log \text{CFUs } (t_2) - \log \text{CFUs } (t_1)]/\Delta t$. From the curve, the desired OD for each isolate

was determined to ensure an approximate application rate of 10^4 bacteria per seed. This was slightly below the application rate set out by the Canadian Food Inspection Agency (CFIA) of 10^5 bacteria per large-seeded legume (including soybean) (CFIA, 1997).

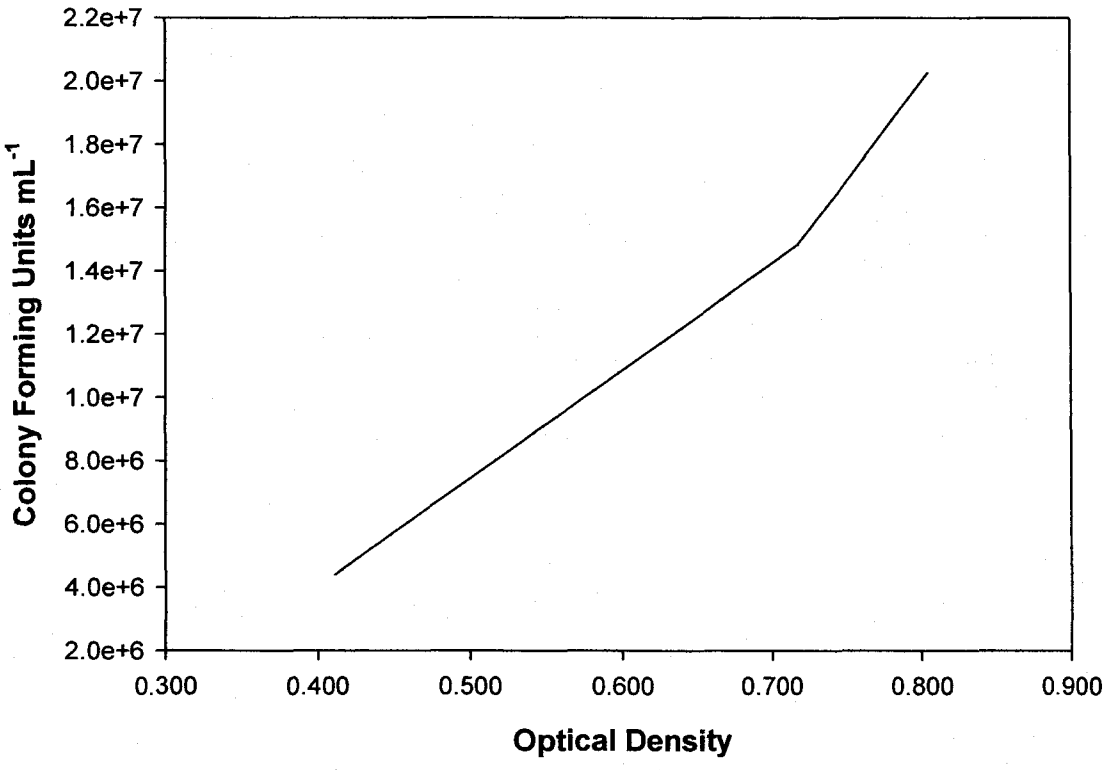

Figure 3.1 Example of growth curve obtained from quantification procedure. Curve is representative of isolate 73.

Table 3.3 Relative growth rates of commercial controls 532C and USDA 110 and ten isolates assessed in Second Stage Testing. Growth rates are reported as colony forming units (CFU) produced per colony forming unit per day (CFU/CFU/day).

Inoculant Treatment	Relative Growth Rate (CFU/CFU/day)
532C	0.07
USDA 110	0.22
1-2	0.14
1-15	0.10
1-17	0.11
2-33	0.18
2-32	0.04
2-37	0.21
2-38	0.10
3-49	0.23
3-50	0.04
3-57	0.07

3.3 Greenhouse Experiments

3.3.1 Experimental Design

Greenhouse trials were conducted in two stages. First Stage Testing identified potentially promising isolates using a single soybean cultivar. In Second Stage Testing, the best performers from the First Stage were tested using three soybean cultivars. Both stages included controls namely, untreated soybean plants, plants inoculated with commercial strain 532C, plants inoculated with commercial strain USDA 110 (not all

trials), and plants that were nitrogen-fertilized but uninoculated. The fertilized plants had ammonium nitrate (NH_4NO_3) applied at a rate of 100 Kg N ha^{-1}. The treatments were arranged in a randomized complete block design with six replicates each. The location of each treatment was rotated weekly to compensate for the variation in greenhouse temperature and/or light.

Standard 15 cm diameter pots were filled with 1.5 L of soil collected from a field at the Carman Research Station, University of Manitoba. Soil was analyzed and by Norwest Labs, Calgary, Alberta, Canada, and is described in Table 3.2. The soil had no previous history of soybean cultivation and had been watered for at least two days to encourage normal microbial activity. In each pot, three seeds of the desired cultivar were planted at a depth of approximately 1.3 cm. Inoculant was applied at different rates depending on the stage of testing (see below). Photoperiod of supplemental lighting was set to 14 hours on and 10 hours off at an intensity between 200-340 $\mu mol/m^{-2}/s^{-1}$. Plants were watered every two days or as needed to keep the surface soil moist. Care was taken during watering to ensure no cross-contamination. When plants emerged, plantings were reduced to one seedling per pot.

After plants reached mid-flowering stage, shoots were cut at soil level and placed in paper bags. The roots were carefully cleaned of excess soil and placed in a labeled plastic bag and stored at 4°C until nodule counting. The shoots were dried in an 80°C drying oven and weighed after a week.

Table 3.4 Nutrient analysis of soil used for greenhouse trials. Nutrient contents are reported in parts per million (PPM) and were categorized by Norwest Labs as deficient (D), marginal (M), optimum (O), or excess (E). Nitrogen and sulfur are reported as nitrate and sulfate respectively.

	N*	P	K	S**	Ca	Mg	Fe	Cu	Zn	B	Mn	Cl
Content (PPM)	11	24	341	4	4530	555	21.4	1.47	3.34	1.4	17.5	5.5
Category	D	O	O	M	E	E	O	O	O	O	O	M

** Nitrate-N* *** Sulphate-S*

3.3.1.1 First Stage Testing

Sixty *B. japonicum* isolates were tested during First Stage Testing using the soybean cultivar Gentleman. Testing was conducted over four trials, with the first trial conducted at the greenhouse at the Nova Scotia Agricultural College in Truro, Nova Scotia, Canada, and the three remaining trials at Dalhousie University in Halifax, Nova Scotia, Canada. Twenty isolates were assessed in each trial in addition to the controls as described in the above section. In the first two trials, commercial strain USDA 110 was not available as a control.

The tubes of rhizobial culture were taken from -80°C freezer and thawed. From each tube, 0.1 mL of culture was removed and placed in 125 mL Erlenmeyer flasks containing 30 mL of yeast mannitol broth. The flasks were shaken at 200 RPM in an orbital shaker and incubated at 28°C until each reached an OD of 0.60 ± 0.01. Three seeds were planted per pot at a depth of 1.3 cm and one mL of inoculant was applied

directly to each seed. The ammonium nitrate was applied by adding 1.5 ml of a 2.4 M NH_4NO_3 solution to the water before watering. Based upon the volume of soil in the pot, this provided fertilizer nitrogen at a rate equivalent to 100 Kg N ha^{-1}. Extra seedlings were removed between week 2 and 3 to leave one plant per pot.

Plants were harvested in the mid-late flowering stage, which varied in duration by trial from 4.5-6 weeks. Roots were cleaned on-site and nodules were counted the following week. Both mature and immature nodules were counted and the total number recorded. Symbiotic performance was assessed by comparing specific nodulation rates (plant nodules per plant/g root dry weight per plant). In the absence of statistical differences, the ten treatments with the highest values were selected for Second Stage Testing.

3.3.1.2 Second Stage Testing

The 10 isolates selected from the First Stage Testing were tested again in the greenhouse at Saint Mary's University, Halifax, Nova Scotia, Canada. For this stage of testing a new supply of soil was collected from the same location as the previous soil source (Carmen Research Station). The soil was dried and diluted by 50% with sterile fine grain silica sand to decrease the availability of nitrogen in the rooting media. During this stage, three genetically diverse soybean cultivars were used to reflect the diversity of soybean cultivars used in western Canada. The cultivars were Gentleman, OAC Prudence, and Round-up Ready™ Apollo. Seeds were treated with a 10% bleach solution and rinsed prior to planting. Promising isolates were taken from -80°C, thawed,

and prepared for orbital shaking as described in section 3.3.1.1. Isolates were ready when the cultures reached an OD determined during the quantification stage (section 3.2.2) to equal a bacterial concentration of approximately 10^4 CFUs per mL. The inoculation rate was 1.5 mL per seed. Treatments were grouped together in blocks, each testing one inoculant or one control. A block consisted of the three different cultivars with six replicate pots per cultivar for a total of 18 pots per block.

During the third week of growth, there was a pest problem due to a thrips infestation (*Frankiniella occidentals*) that required a single pesticide application. Details can be found in Section 3.3.1.3 below. A 0.2% pyrethrin-based pesticide was applied as a foliar spray.

Plants were harvested after six weeks. Shoots were removed, placed in paper bags, dried and weighed. The roots were washed on-site, placed into plastic bags and chilled at 4°C to maintain integrity and prevent rotting. Nodule numbers were recorded and nodules were removed, dried, and weighed in Second Stage Testing. For nitrogen analysis, plant tissues were recombined (root, shoot and nodules) and coarsely ground in a domestic coffee grinder. Additional grinding with a ToothMaster (Racine, Wisconsin, USA) Hi-speed Amalgamator dental amalgam mixer (model # 7-HB) was used to reduce coarseness. The ground material was placed in 1.5 mL microcentrifuge tubes and sent to the Nitrogen Analysis Lab, Department of Plant Sciences, University of Manitoba,Winnipeg, Canada.

Criteria for selection at this stage included g nitrogen per plant (described in the following section), nodules/g root dry weight, and biomass. Isolates that performed well

using all three cultivars when compared to the commercial controls were sent to Philom Bios Inc., Saskatoon, Saskatchewan, Canada for field testing.

3.3.1.3 Pest Problems

In Second Stage Testing thrips were discovered in the Saint Mary's University greenhouse. A pre-mixed, pyrethrin-based pesticide was used at a concentration of 0.2% that was applied as a light foliar spray. Care was taken to spray closer to the end of the photoperiod to lessen the chance of leaf burn as directed on the label. The following day, it was discovered that some pesticide damage did occur on approximately 10% of the leaves. The application was successful however, and the pest was sufficiently reduced to enable the trial to continue without reapplication. The damage was consistent across all treatments and did not interfere with growth or interpretation of the results.

3.3.1.4 Data Collection

Data collected for isolate analysis during greenhouse trials include nodule numbers, root dry weight (DW), shoot DW, total nodule DW (2^{nd} stage), nodules/g root DW, total plant biomass, plant nitrogen concentration (%) (2^{nd} stage), and plant tissue nitrogen content (mg N $plant^{-1}$) (2^{nd} stage). Sampling was done between the late flowering and early pod-filling stages. Plants were cut level with the top of the pot (at the collar level) to ensure equal amounts of stalk for each root. Shoot samples were placed in labeled paper bags and dried in an 80°C dryer oven for a week. Shoots were weighed using an analytical balance. Roots were washed on-site immediately following harvesting

and chilled at 4°C until counting. Total nodule numbers were recorded. In the Second Stage, nodules were removed and weighed separately. Total plant biomass was measured by adding together shoot DW, root DW, and nodule DW (when applicable) per plant. Nodules/g root DW was a simple but informative equation that divided the total nodule number by the root DW. The calculation helped normalize the data and compensated for plant growth variations. The final number of mg N plant^{-1} was obtained by multiplying the percent N content for each plant by its biomass x 1000.

3.3.1.5 Data Analysis

First Stage data was analyzed using one-way analysis of variance tests (ANOVAs) using JMP software (SAS Institute, Cary, NC, USA). ANOVAs were performed on nodule number, specific nodulation, shoot DW, root DW, and total plant biomass. When the F-test was significant, treatment means of each parameter were separated by the least significant difference (LSD) test. Second Stage Testing results involved two analyses which utilized both one-way and two-way ANOVAs. A One-way ANOVA was performed on nodule number, specific nodulation, nodule DWs, total plant biomass, and nitrogen contents (mg N plant^{-1}). A two-way ANOVA were used to assess cultivar, isolate, and cultivar x isolate interactions. Treatment means of Second Stage Testing parameters were separated using LSD tests when the F-test was significant. The significance level was set at the 0.05 probability level for both First and Second Stage Testing. Each greenhouse trial was treated as a separate test and was not compared to other trials.

4.0 RESULTS

4.1 First Stage Testing

First Stage Testing assessed the performance of *B. japonicum* isolates from nodules collected from soybeans in the field in a single cultivar (Gentleman) under greenhouse conditions. Tests were conducted over three trials, 20 isolates were tested in each trial. Controls for First Stage trials included an untreated soybean control, soybeans inoculated with commercially available inoculants (532C & USDA 110), and nitrogen-fertilized soybeans. There was one commercial inoculant control (532C) for the first two trials, and a second commercial inoculant control (USDA 110) was added in the last two trials. The titre of test isolates and inoculant controls in First Stage Testing was determined by optical density (0.6). Data analysis compared the performance of the test isolates to the controls. Statistical differences are discussed.

4.1.1 First Trial

The first trial was conducted at the Nova Scotia Agricultural College, Truro, Nova Scotia, Canada in Juuly and August, 2005. Conditions were suboptimal with large temperature variations (18 to 41°C) and uneven light distribution on the greenhouse growth benches. Plants were moved bi-weekly to minimize the irradiance differences, but plants were still elongated and grew more like vines rather than the bushier growth habit expected if planted in the field.

4.1.1.1 Nodule Number

In the first trial, no nodulation occurred in the untreated and nitrogen-fertilized controls (Tables 4.1). Isolates that showed very low to no nodules showed no difference from these two controls, as was expected. The range of nodule numbers in Trial 1 was 0 – 53.8 (Table 4.1). Five isolates showed low or no nodulation in this trial and no isolates displayed higher nodule numbers than the commercial 532C control, although several isolates yielded lower numbers. Five isolates were equal to the commercial control in nodule numbers. (ANOVA, $P<0.0001$).

In this first trial of First Stage Testing, isolates 1-1, 1-5, 1-7, 1-9, 1-14 and 1-19 exhibited no difference to the non-nodulating controls; isolates 1-2, 1-8, 1-10, 1-15 and 1-17 showed no difference to the commercial 532C control. The remaining isolates showed lower nodulation rates when compared to inoculant 532C.

4.1.1.2 Shoot Dry Weight

Shoot dry weights (DW) were similar between treatments in the first trial, with a shoot DW range of 0.95 – 1.43 g (Table 4.1). The untreated and nitrogen-fertilized treatments had the highest shoot DW values. Two isolates (1-8 and 1-17) equaled these controls in shoot DWs, the remaining had lower DWs. Four isolates' (1-5, 1-6, 1-14 and 1-18) shoot DWs differed from the commercial inoculant control (all lower). ANOVA for the first trial shoot DW was $P<0.0001$. No other differences were noted.

Table 4.1 First Stage Testing, Trial 1: results of 20 *B. japonicum* isolates run against untreated, nitrogen-fertilized, and commercial inoculant 532C controls. Mean plant nodule number, shoot DW (g), root DW (g), specific nodulation, and total biomass (g) are reported. Isolates which were significantly different than the 532C control at $P \leq 0.05$ are indicated in bold with an asterisk (*). Standard errors are reported in brackets.

Treatment	Nodule Number	Shoot DW (g)	Root DW (g)	Specific Nodulation [nodules (g root DW^{-1})]	Total Plant Biomass (g DW)
Untreated	0.0	1.43 (0.08)	0.18 (0.01)	0.00	1.61 (0.09)
NH_4NO_3	0.0	1.40 (0.06)	0.18 (0.01)	0.00	1.59 (0.07)
532C	53.8 (5.9)	1.18 (0.05)	0.17 (0.01)	318.2 (34.6)	1.35 (0.06)
1-1	**5.7* (1.7)**	1.05 (0.05)	**0.14* (0.00)**	**42.2* (12.9)**	1.19 (0.05)
1-2	49.8 (8.2)	1.11 (0.06)	0.14 (0.01)	339.6 (49.3)	1.25 (0.06)
1-3	**26.8* (5.8)**	1.14 (0.05)	0.15 (0.01)	**172.0* (35.8)**	1.29 (0.06)
1-4	**35.5* (5.1)**	1.12 (0.03)	0.16 (0.01)	217.3 (27.0)	1.28 (0.03)
1-5	**13.5* (12)**	**1.01* (0.06)**	**0.12* (0.01)**	**101.6* (88.6)**	**1.13* (0.07)**
1-6	**20.5* (3.6)**	**0.96* (0.05)**	**0.13* (0.01)**	**157.0* (24.1)**	**1.09* (0.06)**
1-7	**0.2* (0.2)**	1.15 (0.04)	0.14 (0.01)	**1.5* (1.5)**	1.29 (0.04)
1-8	43 (9.2)	1.31 (0.06)	0.16 (0.01)	268.7 (60.5)	1.47 (0.06)
1-9	**0.0***	1.19 (0.04)	**0.13* (0.01)**	**0.0***	1.32 (0.04)
1-10	40.5 (10.8)	1.05 (0.08)	**0.13* (0.02)**	293.6 (60.1)	1.18 (0.08)
1-11	**16.8* (2.7)**	1.03 (0.06)	**0.13* (0.01)**	**132.8* (20.7)**	**1.16* (0.07)**
1-12	**26.2* (4.9)**	1.03 (0.06)	**0.14* (0.01)**	**190.2* (31.5)**	**1.17* (0.06)**
1-13	**22.5* (4.6)**	1.07 (0.05)	**0.14* (0.01)**	154.8* (16.3)	1.21 (0.06)
1-14	**0.0***	**0.95* (0.02)**	**0.10* (0.01)**	**0.0***	**1.05* (0.03)**
1-15	**41.7 (9.6)**	1.02 (0.05)	**0.13* (0.01)**	376.0 (128.7)	1.15 (0.06)
1-16	**25.2* (4.3)**	1.07 (0.03)	**0.12* (0.01)**	222.0 (36.5)	1.19 (0.04)
1-17	56.3 (3.3)	1.32 (0.05)	0.15 (0.01)	376.5 (35.6)	1.47 (0.05)
1-18	**28.0* (3.1)**	**1.00* (0.04)**	**0.13* (0.01)**	225.2 (30)	**1.13* (0.04)**
1-19	**0.8* (0.5)**	1.04 (0.05)	**0.12* (0.01)**	**6.7* (3.9)**	**1.15* (0.05)**
1-20	**30.3* (3.4)**	1.12 (0.12)	**0.12* (0.02)**	276.1 (27.6)	1.24 (0.13)

4.1.1.3 Root Dry Weight

Root DWs did not show many significant differences between test isolates. The root DW range was between 0.10 – 0.18 g per root (Table 4.1). The untreated and nitrogen-fertilized treatment had the highest root DWs; only two test isolates equaled them (1-4 and 1-8). The remaining isolates had lower root DWs. Five isolates (1-2, 1-3, 1-4, 1-7, 1-8 and 1-17) did not differ in root DW from the commercial 532C inoculant, while fifteen isolates had lower root DWs when compared to the commercial inoculant. The ANOVA of root DW for Trial 1 was $P<0.0001$.

4.1.1.4 Specific Nodulation

Specific nodulation takes into account the effect of root size on nodulation [nodule number (g root DW)$^{-1}$]. The range of specific nodulation rates varied between 0 – 376.0 nodules(g root DW)$^{-1}$ (Table 4.1). The untreated, nitrogen-fertilized and test isolate treatments that resulted in little to no nodulation also had low specific nodulation numbers [0 – 6.7 nodules(g root DW)$^{-1}$]; isolates 1-1, 1-5, 1-7, 1-9, 1-14 and 1-19 showed no difference from the non-inoculated and nitrogen-fertilized controls; these isolates were not expected to show a difference as they exhibited no nodulation. No isolates had higher specific nodulation rates than the commercial control; nine isolates (1-2, 1-4, 1-8, 1-10, 1-15, 1-16, 1-17, 1-18 and 1-20) showed no significant difference compared to the commercial control. The remaining eleven isolates showed lower specific nodulation rates than the 532C inoculant (ANOVA, $P<0.0001$).

4.1.1.5 Total Plant Biomass

Trial 1 total plant biomass was calculated as a combination of both shoot and root DWs. The biomass range recorded in this trial was 1.05 – 1.61 g (Table 4.1). The untreated and nitrogen-fertilized controls had the highest biomass accumulation values in the trial; all other isolates tested showed lower biomass with the exception of two isolates (1-8 and 1-17). No isolates exhibited higher biomasses when compared to commercial control 532C; eight isolates (1-5, 1-6, 1-11, 1-12, 1-14, 1-15, 1-18 and 1-19) displayed lower biomass than the commercial control. The ANOVA P-value was $P<0.0001$.

4.1.2 Second Trial

The second trial was completed at Dalhousie University in Halifax, Nova Scotia, Canada in December, 2005. Conditions more consistent than the first trial, however, temperatures were still high and at one point reached 31°C.

4.1.2.1 Nodule Number

The range of nodule numbers for the second trial was 0 – 86 (Table 4.2). Six isolates (2-23, 2-24, 2-25, 2-26, 2-27 and 2-40) showed little to no nodulation. The commercial inoculant 532C had a low nodule number in this trial and nine isolates (2-22, 2-29, 2-30, 2-32, 2-33, 2-34, 2-35, 2-37 and 2-38) produced higher nodule numbers than the commercial control. Six isolates, including 2-23, 2-24, 2-25, 2-26, 2-27 and 40, showed lower nodule numbers than the commercial control. The ANOVA P-value was $P<0.0001$.

4.1.2.2 Shoot Dry Weight

The range of shoot DWs for trial 2 was 0.40 – 0.73 g (Table 4.2). Two isolates (2-30 and 2-31) exhibited a higher shoot DW than the untreated and nitrogen-fertilized controls. Only three isolates (2-24, 2-25 and 2-38) had similar shoot DWs to the commercial inoculant 532C control. The remaining isolates had higher shoot DWs than inoculant 532C. The ANOVA P-value was P=0.0047.

4.1.2.3 Root Dry Weight

The range of root DW for trial 2 was 0.15 – 0.26 g (Table 4.2). Six isolates (2-30, 2-31, 2-32, 2-34, 2-35 and 2-36) had higher root DWs than the untreated and nitrogen-fertilized controls. The commercial control 532C had the lowest root DW value. Twelve isolates (2-21, 2-22, 2-23, 2-29, 2-30, 2-31, 2-32, 2-34, 2-35, 2-36, 2-39 and 2-40) had higher root DWs than the commercial control. There were no other differences detected. The ANOVA had a P-value of P=0.0001.

4.1.2.4 Specific Nodulation

The range of specific nodulation rates for trial 2 was 0.0 – 363.4 nodules (g root DW)$^{-1}$ (Table 4.2). The six isolates with low nodule numbers (2-23, 2-24, 2-25, 2-26, 2-27 and 2-40) also had the lowest specific nodulation rates, as did isolate 2-39. Two isolates (2-37 and 2-38) exhibited higher specific nodulation rates than the commercial 532C control. The remaining isolates were not significantly different from the 532C control. The P-value of the specific nodulation ANOVA was P<0.0001.

Table 4.2 First Stage Testing Trial 2, results of 20 *B. japonicum* isolates against untreated, nitrogen-fertilized, and commercial inoculant 532C controls. Mean plant nodule number, shoot DW (g), root DW (g), specific nodulation, and total biomass (g) are reported. Isolates which were significantly different than the 532C control at $P \leq 0.05$ are indicated in bold with an asterisk (*). Standard errors are reported in brackets.

Treatment	Nodule Number	Shoot DW (g)	Root DW (g)	Specific Nodulation [nodules (g root DW^{-1})]	Total Plant Biomass (g DW)
Untreated	0.0	0.57 (0.05)	0.18 (0.02)	0.0	1.31 (0.07)
NH_4NO_3	0.0	0.63 (0.05)	0.17 (0.01)	0.0	0.80 (0.05)
532C	36.4 (10.6)	0.40 (0.09)	0.15 (0.04)	238.2 (58.1)	0.55 (0.15)
2-21	62.0 (17)	**0.59* (0.06)**	0.21 (0.02)	284.4 (61.6)	**0.80* (0.08)**
2-22	**64.2* (11.5)**	**0.65* (0.05)**	**0.21* (0.01)**	293.2 (32.9)	**0.86* (0.07)**
2-23	**12.3* (4.3)**	**0.66* (0.03)**	**0.21* (0.01)**	**57.6* (18.4)**	**0.87* (0.04)**
2-24	**0.0***	0.53 (0.04)	0.17 (0.01)	**0.0***	0.70 (0.06)
2-25	**10.2* (3)**	0.52 (0.05)	0.17 (0.02)	**65.5* (23.0)**	0.69 (0.06)
2-26	**1.8* (0.8)**	**0.65* (0.03)**	0.20 (0.02)	**9.6* (4.3)**	**0.85* (0.03)**
2-27	**11.3* (3.8)**	**0.60* (0.02)**	0.17 (0.02)	**68.4* (25.9)**	**0.77* (0.03)**
2-28	35.5 (5.9)	**0.56* (0.05)**	0.16 (0.02)	217.2 (25.9)	**0.72* (0.06)**
2-29	**69.7* (11.6)**	**0.63* (0.05)**	**0.22* (0.02)**	313.4 (29.6)	**0.85* (0.05)**
2-30	62.3 (3.9)	**0.73* (0.02)**	**0.25* (0.02)**	255.2 (23.8)	**0.98* (0.04)**
2-31	57.7 (11.8)	**0.70* (0.04)**	**0.25* (0.01)**	229.0 (43.3)	**0.95* (0.05)**
2-32	**86.0* (14.3)**	**0.64* (0.06)**	**0.26* (0.03)**	330.3 (49.2)	**0.90* (0.08)**
2-33	**67.2* (14.0)**	**0.60* (0.05)**	0.20 (0.03)	342.4 (56.5)	**0.80* (0.08)**
2-34	**77.3* (6.2)**	**0.65* (0.04)**	**0.26* (0.02)**	305.3 (28.1)	**0.90* (0.06)**
2-35	63.2 (6.1)	**0.68* (0.05)**	**0.23* (0.01)**	268.3 (18.8)	**0.91* (0.05)**
2-36	39.5 (8.2)	**0.69* (0.04)**	**0.23* (0.02)**	174.5 (36.9)	**0.93* (0.06)**
2-37	65.0 (11.3)	**0.57* (0.06)**	0.15 (0.03)	**448.7* (57.4)**	0.73 (0.09)
2-38	64.0 (11.1)	0.54 (0.06)	0.18 (0.02)	**363.4* (68.5)**	0.72 (0.08)
2-39	25.0 (8.7)	**0.64* (0.05)**	0.21 (0.02)	**106.1* (27.5)**	**0.85* (0.07)**
2-40	**5.8* (1.8)**	**0.70* (0.06)**	**0.22* (0.02)**	**26.9* (8.3)**	**0.92* (0.07)**

4.1.2.5 Total Plant Biomass

The range of total plant biomass in trial 2 was 0.55 – 1.31 g (Table 4.2). All isolates had a lower biomass than the untreated control. Two isolates (10 and 11) had higher biomasses recorded than that for the nitrogen-fertilized control. The commercial inoculant 532C control had the lowest biomass. Fifteen isolates had higher biomasses than the commercial control and only five isolates (2-24, 2-25, 2-28, 2-37 and 2-38) did not show higher biomass than the 532C. The ANOVA P-value was P=0.0021.

4.1.3 Third Trial

The third trial was completed at Dalhousie University, Halifax, Nova Scotia, Canada in February, 2006. Conditions were similar to the second trial, however, an outbreak of thrips stressed the plants. The outbreak was only in its beginning stages by the completion of the trial and minimal damage to leaves was sustained (10% or less).

4.1.3.1 Nodule Number

The range of nodule numbers in the third trial was 0 – 133.5 (Table 4.3). Eight isolates (3-41, 3-45, 3-52, 3-53, 3-55, 3-56, 3-58 and 3-60) exhibited low nodulation that were not significantly different from the untreated and nitrogen-fertilized controls. The remaining isolates had similar nodule numbers. Two isolates (3-44 and 3-54) had a lower nodule number than the commercial inoculant controls 532C and USDA 110. Only one isolate (3-50) showed a higher nodule number than both commercial inoculant controls. The ANOVA P-value was P<0.0001.

Table 4.3 Trial 3, results of First Stage Testing. The mean plant nodule number, shoot DW (g), root DW (g), total plant biomass (g), and specific nodulation are reported. Isolates which were significantly different from the 532C and USDA 110 controls at $P \leq 0.05$ are indicated in bold with an asterisk (*) and plus sign (+) respectively. Standard errors are reported in brackets.

Treatment	Nodule Number	Shoot DW (g)	Root DW (g)	Specific Nodulation [nodules (g root DW^{-1})]	Total Plant Biomass (g DW)
Untreated	0.0	0.66 (0.06)	0.25 (0.02)	0.0	0.90 (0.07)
NH_4NO_3	0.0	0.57 (0.07)	0.24 (0.02)	0.0	0.80 (0.08)
532C	72.2 (15.2)	0.59 (0.04)	0.30 (0.02)	236.9 (31.7)	0.88 (0.06)
USDA110	66.5 (16.5)	0.65 (0.05)	0.25 (0.02)	309.0 (69)	0.91 (0.07)
3-41	**13.0*+ (2.22)**	0.61 (0.09)	**0.23* (0.02)**	**55.7*+ (4.7)**	0.83 (0.11)
3-42	53.0 (13.4)	0.66 (0.04)	0.25 (0.01)	213.2 (48.7)	0.90 (0.04)
3-43	49.2 (9.4)	0.66 (0.06)	0.24 (0.02)	210.1 (41.1)	0.90 (0.08)
3-44	**32.7*+ (6.5)**	0.55 (0.04)	**0.21* (0.01)**	**150.2+ (26.8)**	0.76 (0.05)
3-45	**0.0*+**	0.73 (0.05)	**0.22* (0.01)**	**0.0*+**	0.95 (0.05)
3-46	55.7 (8.8)	0.58 (0.04)	**0.21* (0.01)**	265.0 (35.8)	0.79 (0.06)
3-47	62.8 (8.6)	0.59 (0.04)	**0.23* (0.02)**	279.4 (26.7)	0.82 (0.06)
3-48	77.5 (16.4)	0.69 (0.05)	0.24 (0.02)	314.3 (46.6)	0.92 (0.07)
3-49	82.0 (15.6)	0.59 (0.06)	**0.19*+ (0.02)**	**416.8*+ (65.5)**	0.78 (0.07)
3-50	**133.5*+ (23.4)**	0.67 (0.03)	0.25 (0.02)	**528.8*+ (75.1)**	0.92 (0.04)
3-51	65.0 (4.3)	0.64 (0.04)	0.26 (0.02)	264.8 (32.7)	0.89 (0.06)
3-52	**0.0*+**	0.75 (0.06)	0.26 (0.03)	**0.0*+**	1.02 (0.08)
3-53	**25.5*+ (5.5)**	0.45 (0.05)	**0.16*+ (0.01)**	**162.3+ (42)**	0.61 (0.05)
3-54	**42.0* (13)**	0.65 (0.06)	0.26 (0.02)	**155.9*+ (38.4)**	0.91 (0.09)
3-55	**4.2*+ (2.9)**	0.65 (0.04)	**0.23* (0.02)**	**15.1*+ (0.02)**	0.88 (0.06)
3-56	**9.3*+ (3.4)**	0.60 (0.08)	**0.21* (0.04)**	**39.7*+ (15)**	0.81 (11.4)
3-57	73.0 (10.4)	0.63 (0.08)	**0.22* (0.02)**	336.9 (38.2)	0.85 (0.10)
3-58	**28.0*+ (10.3)**	0.47 (0.06)	**0.18*+ (0.02)**	**141.0+ (40.8)**	0.64 (0.09)
3-59	68.2 (4.2)	0.60 (0.07)	**0.23* (0.02)**	312.3 (34.7)	0.83 (0.09)
3-60	**23.8*+ (5.8)**	0.57 (0.03)	**0.21* (0.01)**	**112.8*+ (27.2)**	0.79 (0.04)

4.1.3.2 Shoot Dry Weight

No differences were observed between the controls and the isolates for shoot DW. The ANOVA P-value was P=0.0520.

4.1.3.3 Root Dry Weight

In the third trial, the range of root DWs was 0.16 – 0.26 g (Table 4.3). Two isolates (3-53 and 3-58) had lower root DWs than the untreated controls, and only isolate 3-53 was lower than the nitrogen-fertilized controls. The commercial control 532C had the highest root DW value. Seven isolates (3-42, 3-43, 3-48, 3-50, 3-51, 3-52 and 3-54) were equivalent to 532C in root DW performance; the remaining isolates had lower root DWs. Three isolates (3-49, 3-53, and 3-58) had lower root DWs than the commercial inoculant USDA 110 control. The ANOVA P-value was P<0.0201.

4.1.3.4 Specific Nodulation

The range for specific nodulation rates in the third trial was 0.0 – 528.8 nodules (g root DW) $^{-1}$ (Table 4.3). Five isolates (3-41, 3-45, 3-52, 3-55 and 3-56) had low specific nodulation rates similar to the untreated and nitrogen-fertilized controls. Ten isolates (3-41, 3-44, 3-45, 3-52, 3-54, 3-55, 3-56, 3-58 and 3-60) had lower specific nodulation rates than the commercial USDA 110 control. Six isolates (3-41, 3-45, 3-52, 3-55, 3-56 and 3-60) displayed lower rates than the 532C control. Two isolates (3-49 and 3-50) showed higher specific nodulation rates than both commercial controls. The P-value for the ANOVA was P<0.0001.

4.1.3.5 Total Plant Biomass

The ANOVA performed on biomass in the third trial was not significant with a P value of 0.0639. This meant there were no statistical differences detected.

4.2 Second Stage Testing

Second Stage Testing was carried out on isolates selected from First Stage Testing that showed the highest specific nodulation values when compared to the commercial controls 532C and USDA 110 (Table 4.4). Performance was assessed using three different soybean cultivars: Gentleman, Apollo, and OAC Prudence. An important additional parameter considered in this stage of testing was nitrogen concentration per plant (%) and plant tissue nitrogen content (mg N per plant). As well, the nodules were removed from the plants, dried in an oven and total nodule DW was recorded. Also different in this stage was the titre of inoculant for each isolate to be tested, which was determined prior to planting to ensure a consistent rate of 10^4 rhizobia per mL. Each cultivar was treated as a separate test except for effects analysis (cultivar, isolate, cultivar x isolate effects) where results from all three cultivars were combined. Statistical differences are discussed.

Table 4.4 Strains selected for second stage testing. The first stage trial number is listed along with mean plant nodule number, shoot DW (g), root DW (g), total plant biomass (g), and specific nodulation [nodules (g root DW)$^{-1}$] . Δ Specific nodulation is reported relative to the 532C control in the relevant trial. Values bolded and marked with an asterisk were significantly higher than the 532C control.

Isolate #	Nodule Number	Shoot DW (g)	Root DW (g)	Total Plant Biomass (g DW)	Specific Nodulation [nodules(g root DW)$^{-1}$]	Δ Specific Nodulation
1-2	49.8	1.11	0.14	1.25	339.6	21.4
1-15	41.7	1.02	0.13	1.15	376.0	57.8
1-17	56.3	1.32	0.15	1.47	376.5	58.3
2-32	**86.0***	**0.64***	**0.26***	**0.90***	330.3	92.1
2-33	**67.2***	**0.60***	0.20	**0.80***	342.4	104.2
2-37	65.0	**0.57***	0.15	0.73	**448.7***	**210.5***
2-38	64.0	0.54	0.18	0.72	**363.4***	**125.2***
3-49	82.0	0.58	**0.19***	0.78	**416.8***	**179.9***
3-50	**133.5***	0.67	0.25	0.92	**528.8***	**291.9***
3-57	73.0	0.63	**0.22***	0.85	336.9	100.0

4.2.1 Soybean Cultivar Apollo

4.2.1.1 Nodule Number

The range of nodule numbers using the Apollo cultivar was 26.3 – 125.3 nodules. Most isolates tested on the Apollo cultivar had similar nodulation rates (Table 4.5). One isolate (1-2) had a higher nodule number than isolates 1-15, 1-17, 2-38 and 3-50, as well a higher number than both commercial controls; all isolates tested showed higher values than isolate 1-17. Isolates 2-33, 2-37, 3-49 and 3-57 had higher nodule numbers than 1-15, 2-38 and 3-50, whereas isolates 1-15, 2-32, 2-38 and 3-50 had higher nodule numbers than isolate 1-17. The ANOVA produced a P-value of $P<0.0001$.

4.2.1.2 Specific Nodulation

The range of specific nodulation rates using the Apollo cultivar was 70.7 – 264.7 nodules (Table 4.5). Only one isolate (1-17) had a lower rate than the commercial 532C and USDA 110 controls; the remaining isolates showed equivalent specific nodulation rates to the commercial controls. All isolates had higher specific nodulation rates than isolate 1-17 except for 2-38 and 3-50. Isolate 2-32 showed the highest specific nodulation rate statistically different from 1-2, 1-15, 1-17, 2-37, 2-38 and 3-50, whereas isolate 3-57 showed a higher rate than 1-2, 1-17, 2-38 and 3-50. Finally, isolates 2-33 and 3-49 had higher rates than isolates 1-17, 2-38 and 3-50. The P-value of the ANOVA was $P<0.0001$.

4.2.1.3 Total Nodule Dry Weight

The total nodule DW range using the Apollo cultivar was 0.06 – 0.16 g (Table 4.5). Three isolates (2-37, 3-49 and 3-57) had higher nodule DWs than the commercial inoculants 532C and USDA 110. One isolate (2-33) had a higher nodule DW than the commercial USDA inoculant only. Isolate 3-49 showed the highest total nodule DW relative to isolates 1-2, 1-15, 1-17, 2-32, 2-38 and 3-50, whereas isolates 2-37 and 3-57 exhibited higher total nodule DW than isolate 2-32. The ANOVA P-value was $P=0.0001$.

4.2.1.4 Total Plant Biomass

Biomass was measured as a combination of shoot DW, root DW and nodule DW. Nodule DW was not applicable for the untreated and nitrogen-fertilized controls. The

Table 4.5 Second stage testing of *B. japonicum* isolates using soybean cultivar Apollo. Mean total nodule number, specific nodulation, total plant biomass (g), N concentration (%) and plant tissue N content (mg N plant^{-1}) are reported. Values followed by the same letter in each column are not statistically different at the $P \leq 0.05$ level. Standard errors are given in brackets.

Treatment	Nodule Number	Specific Nodulation [nodules(g root DW)$^{-1}$]	Total Nodule DW (g)	Total Plant Biomass (g)	N Concentration (%)	Tissue N Content (mg N plant^{-1})
Untreated	0.0	N/A	N/A	2.31^{bc} (0.09)	1.6^{f} (0.1)	36.60^{fgh} (2.0)
NH_4NO_3	0.0	N/A	N/A	1.18^{f} (0.11)	2.8^{abc} (0.1)	33.23^{gh} (3.6)
532C	88.2^{bc} (11.8)	195.3^{abcd} (24.9)	0.07^{cd} (0.01)	2.23^{bcd} (0.21)	2.1^{e} (0.1)	47.59^{efg} (5.9)
USDA110	82.3^{bc} (8.7)	202.9^{abcd} (8.7)	0.06^{d} (0.01)	2.09^{cde} (0.13)	2.2^{e} (0.1)	45.50^{efg} (4.5)
1-2	125.3^{a} (9.3)	171.3^{cd} (62.0)	0.10^{bcd} (0.01)	2.61^{ab} (0.11)	2.4^{cde} (0.1)	63.81^{cd} (4.4)
1-15	69.0^{c} (5.3)	190.6^{bcd} (20.2)	0.08^{bcd} (0.01)	2.15^{cde} (0.23)	2.3^{de} (0.1)	50.43^{def} (5.7)
1-17	26.3^{d} (7.3)	70.7^{e} (21.2)	0.09^{bcd} (0.05)	1.83^{de} (0.15)	2.2^{e} (0.1)	41.01^{fg} (3.9)
2-32	93.5^{abc} (10.4)	278.0^{a} (46.3)	0.07^{cd} (0.01)	1.76^{e} (0.15)	1.4^{f} (0.1)	24.31^{h} (2.3)
2-33	104.8^{ab} (8.9)	239.3^{abc} (24.4)	0.11^{abc} (0.01)	2.38^{abc} (0.09)	2.7^{bcd} (0.0)	63.97^{bcd} (3.2)
2-37	109.0^{ab} (20.7)	187.8^{bcd} (30.1)	0.12^{ab} (0.01)	2.61^{ab} (0.17)	3.0^{ab} (0.1)	79.43^{ab} (6.1)
2-38	62.8^{c} (7.1)	125.1^{de} (6.6)	0.11^{bcd} (0.02)	2.80^{a} (0.20)	2.3^{de} (0.2)	64.53^{bcd} (7.5)
3-49	108.2^{ab} (19.1)	215.0^{abc} (27.7)	0.16^{a} (0.02)	2.58^{ab} (0.19)	3.1^{a} (0.2)	80.30^{a} (8.1)
3-50	63.5^{c} (11.5)	125.8^{de} (22.5)	0.08^{bcd} (0.02)	2.25^{bcd} (0.12)	2.7^{bcd} (0.3)	60.96^{cde} (8.3)
3-57	113.0^{ab} (14.3)	264.7^{ab} (30.0)	0.12^{ab} (0.02)	2.29^{bc} (0.09)	3.0^{ab} (0.2)	69.73^{abc} (6.8)

range of total plant biomass using the Apollo cultivar was 1.18 – 2.80 g (Table 4.5). Two isolates had a lower biomass than the untreated control. One isolate (2-38) had a higher total plant biomass than isolates 1-15, 1-17, 2-32, 3-50, 3-57 and all controls. All isolates had higher biomasses than the nitrogen-fertilized control. Most isolates, with the exception of isolates 1-15 and 3-50, showed higher biomass values than isolates 1-17 and 2-32. Isolates 1-2, 2-37, and 3-49 exhibited higher biomass than isolates 1-15, 1-17, 2-32 and the nitrogen-fertilized control. One isolate (2-38) had a higher biomass than both commercial inoculants 532C and USDA 110. The P-value for the biomass ANOVA was $P<0.0001$.

4.2.1.5 Nitrogen Concentration (%)

In Second Stage Testing nitrogen contents of the plants were measured in two ways. One way was total plant percent nitrogen concentration. The range of nitrogen concentration using the cultivar Apollo was 1.4 – 3.1% (Table 4.5). The untreated control plants had the lowest percent nitrogen concentrations that were lower than all other treatments with the exception of plants inoculated with isolate 2-32 which also was lower than all other treatments. Both commercial inoculant controls 532C and USDA 110 had similar plant nitrogen concentrations that were also similar to plants inoculated with isolates 1-2, 1-15, 1-17 and 2-38. Plants inoculated with isolates 2-33, 2-37, 3-49, 3-50, 3-57 and the nitrogen-fertilized control had similar nitrogen concentrations that were higher than plants inoculated with the commercial controls. Plants inoculated with isolate 3-47 had the highest nitrogen concentrations that were similar to plants treated

with isolates 2-37 and 3-57 and the nitrogen-fertilized treatment. The P-value of the ANOVA was $P<0.0001$.

4.2.1.6 Plant Tissue Nitrogen Content

Nitrogen content was measured as mg N per plant. The range of nitrogen contents in the soybean cultivar Apollo was between 24.31 – 80.30 mg N per plant (Table 4.5). Seven isolates had higher nitrogen contents than the untreated and nitrogen-fertilized controls. Six isolates (1-2, 2-33, 2-37, 2-38, 3-49 and 3-57) exhibited higher nitrogen contents than both of the commercial inoculants 532C and USDA 110. One isolate (2-32) showed a lower nitrogen content than the commercial controls. All isolates had higher nitrogen contents than isolate 2-32, whereas isolate 3-49 possessed the highest nitrogen content with the exception of isolates 2-37, which had a higher nitrogen content than isolates 1-2, 1-15, 1-17, 2-32 and 3-50 and all controls, and 2-57, which showed higher nitrogen values than 1-15, 1-17, 2-32 and the controls. Isolate 3-50 also had a higher nitrogen content than isolates 1-17 and 2-32 but only outperformed the untreated and nitrogen-fertilized controls in nitrogen content, whereas isolates 1-2, 2-33 and 2-38 ranked higher in nitrogen content than 1-17, 2-32 and the controls. The P-value of the ANOVA was $P<0.0001$.

4.2.2 Soybean Cultivar Gentleman

4.2.2.1 Nodule Number

Nodulation responses in the soybean cultivar Gentleman showed some differences, it ranged between was 68.8 – 130.3 (Table 4.6). Three isolates (1-15, 1-17

Table 4.6 Second stage testing of *B. japonicum* using soybean cultivar Gentleman. Mean total nodule number, specific nodulation, total plant biomass (g), N concentration (%) and plant tissue N content (mg N plant^{-1}) are reported. Values followed by the same letter in each column are not statistically different at the P≤0.05 level. Standard errors are given in brackets.

Treatment	Nodule Number	Specific Nodulation [nodules(g root DW)$^{-1}$]	Total Nodule DW (g)	Total Plant Biomass (g)	N Concentration (%)	Tissue N Content (mg N plant^{-1})
Untreated	0.0	N/A	N/A	2.00^{abcd} (0.13)	1.4^{h} (0.1)	28.41^{efg} (2.4)
NH_4NO_3	0.0	N/A	N/A	1.34^{f} (0.06)	1.9^{fg} (0.2)	24.94^{g} (1.7)
532C	130.3^{a} (9.1)	280.0^{bc} (11.4)	0.11^{def} (0.01)	2.32^{ab} (0.18)	2.6^{cde} (0.1)	61.59^{bc} (6.3)
USDA110	81.2^{cd} (6.4)	192.1^{c} (15.5)	0.09^{ef} (0.01)	1.88^{cde} (0.11)	2.3^{ef} (0.1)	42.26^{def} (2.4)
1-2	111.0^{abc} (23.2)	316.1^{ab} (62.9)	0.10^{ef} (0.02)	1.82^{de} (0.14)	2.9^{bc} (0.2)	54.52^{bcd} (7.8)
1-15	78.2^{cd} (15.6)	254.1^{bc} (51.1)	0.08^{f} (0.01)	1.87^{cde} (0.15)	2.3^{ef} (0.1)	43.52^{d} (4.4)
1-17	68.8^{d} (5.6)	196.3^{c} (20.5)	0.10^{ef} (0.02)	1.87^{cde} (0.17)	2.7^{cde} (0.1)	49.88^{cd} (3.9)
2-32	120.7^{ab} (14.3)	403.7^{a} (44.3)	0.08^{ef} (0.01)	1.50^{ef} (0.11)	1.8^{gh} (0.2)	27.19^{g} (3.2)
2-33	98.3^{abcd} (20.7)	235.8^{bc} (47.4)	0.12^{cde} (0.02)	1.97^{abcd} (0.12)	2.5^{de} (0.2)	48.74^{cd} (6.0)
2-37	107.2^{abc} (13.7)	255.8^{bc} (34.2)	0.11^{def} (0.02)	2.07^{abcd} (0.18)	3.2^{ab} (0.2)	67.11^{ab} (7.0)
2-38	91.0^{bcd} (7.8)	225.9^{bc} (20.1)	0.17^{ab} (0.02)	2.40^{a} (0.26)	2.9^{bcd} (0.1)	68.88^{ab} (7.6)
3-49	116.3^{abc} (15.7)	317.9^{ab} (19.6)	0.16^{bc} (0.03)	1.90^{bcde} (0.18)	3.6^{a} (0.1)	69.43^{ab} (7.5)
3-50	95.2^{abcd} (5.6)	200.6^{c} (12.8)	0.14^{bcd} (0.01)	2.34^{ab} (0.12)	3.0^{bc} (0.2)	68.45^{ab} (2.6)
3-57	119.7^{ab} (10.3)	304.0^{ab} (38.3)	0.21^{a} (0.03)	2.29^{abc} (0.16)	3.5^{a} (0.2)	81.77^{a} (10.3)

and 2-38) had a lower nodule number than the commercial 532C control. Two isolates (2-32 and 3-57) had a higher nodule number than the commercial USDA 110 control. Isolates 1-2, 2-32, 2-37, 3-49 and 3-57 showed higher nodule numbers than 1-17, 1-17 and the USDA 110 commercial control. There were no other statistical differences observed between the isolates and commercial controls. The ANOVA P-value was $P<0.0001$.

4.2.2.2 Specific Nodulation

The range with the cultivar Gentleman was between 192.1 – 403.7 nodules (g root DW)$^{-1}$ (Table 4.6). The isolates from the Gentleman cultivar showed little differences in specific nodulation rates. One isolate (2-32) had a higher specific nodulation rate than the commercial 532C control. Four isolates (1-2, 2-32, 3-49 and 3-57) showed higher nodulation rates than the commercial USDA 110 control. Isolates 1-2, 3-49 and 3-57 also had higher rates than isolates 3 and 9, and isolate 5 had a higher rate than isolates 1-15, 1-17, 2-33, 2-37, 2-38 and 3-50. The P-value of the ANOVA was $P<0.0001$.

4.2.2.3 Total Nodule Dry Weight

The soybean cultivar Gentleman total nodule DW results are reported in Table 4.6. The range of nodule DWs was 0.08 – 0.21 g. Three isolates (2-38, 3-49 and 3-57) had higher total nodule DWs than the commercial 532C and USDA 110 controls. One isolate (3-50) had a higher total nodule DW than the USDA 110 control only; this isolate also had higher nodule DWs than isolates 1-2, 1-15, 1-17 and 2-32. Isolate 3-57 outperformed all isolates and controls in nodule DWs except for 2-38, which showed

equivalent DWs. Isolate 2-38 also had higher nodule DWs all other isolates except 3-49 and 3-50. Isolate 3-49 showed higher nodule DWs than 1-2, 1-15, 1-17, 2-32, 2-37 and both commercial inoculant controls, while isolate 2-33 outperformed isolate 1-15. The total nodule DW ANOVA P-value was $P<0.0001$.

4.2.2.4 Total Plant Biomass

The range of total plant biomass differences detected in cultivar Gentleman was between 1.34 – 2.40 g (Table 4.6). The nitrogen-fertilized treatment had the lowest biomass. One of the isolates (2-32) had a lower biomass than the untreated control. Four isolates (1-2, 1-15, 1-17, and 2-32) had lower biomasses than the commercial 532C control. Two isolates (2-38 and 3-50) had higher biomasses than the USDA 110 control; isolate 2-38 had resulted in a higher biomass than isolates 1-2, 1-15, 1-17, 2-32 and 3-49, whereas isolate 3-50 showed higher biomass values than isolates 1-2, 1-15, 1-17 and 2-32. Both 2-38 and 2-50 exhibited similar biomasses to each other. Isolate 2-57 exhibited higher biomass than 1-2 and 2-32, and isolates 2-33 and 2-37 also outperformed 2-32 in biomass. The ANOVA P-value was $P<0.0001$.

4.2.2.5 Nitrogen Concentration (%)

The nitrogen concentrations in the cultivar Gentleman ranged from 1.4 – 3.6% (Table 4.6). The untreated control had the lowest nitrogen concentration (1.4%) that was not different than plants inoculated with isolate 2-32. The nitrogen-fertilized control had a low nitrogen concentration that was similar to plants using isolates 1-15, 2-32 and the commercial USDA 110 control. Plants inoculated with isolates 3-49 and 3-50 had the

highest nitrogen concentrations that were higher than the controls and all other isolates with the exception of isolate 2-37. Isolate 2-37 also had a similar nitrogen concentration to isolates 1-2, 2-38 and 2-50. Six isolates (1-2, 1-15, 1-17, 2-33, 2-38 and 2-50) and the commercial USDA 110 inoculant had similar concentrations to the commercial 532C inoculant. Isolates 1-15, 1-17 and 2-33 resulted in similar nitrogen concentrations to the USDA 110 inoculated plants. The ANOVA P-value was $P<0.0001$.

4.2.2.6 Plant Tissue Nitrogen Content

The tissue nitrogen content of the Gentleman cultivar was measured as mg N plant^{-1}. The range of nitrogen contents for this variety was 24.94 – 81.77 mg N plant^{-1} (Table 4.6). The untreated and nitrogen-fertilized treatments had low nitrogen contents. Only one isolate (2-32) had an equivalent nitrogen content. One isolate (2-32) had a lower nitrogen content than the commercial 532C control and one isolate (3-57) had a higher content. Five isolates (2-37, 2-38, 3-49, 3-50 and 3-57) had higher nitrogen than the USDA control, and only one (2-32) had a lower content. Isolate 3-57 resulted in a higher nitrogen tissue content than plants inoculated with isolates 1-2, 1-15, 1-17 and 2-33, while isolates 2-37, 2-38, 3-49 and 3-50 showed higher nitrogen content values than isolates 1-15, 1-17 and 2-33. The P-value for this ANOVA was $P<0.0001$.

4.2.3 Soybean Cultivar OAC Prudence

4.2.3.1 Nodule Number

No differences were observed in OAC Prudence nodule numbers (Table 4.7). The ANOVA had a P-value of $P=0.8486$.

4.2.3.2 Specific Nodulation

Little variation was detected in OAC Prudence specific nodulation rates. The range of rates in this variety was 115.3 – 241.1 nodules(g root DW)$^{-1}$ (Table 4.7). Two isolates (2-32 and 3-57) showed higher specific nodulation rates than the commercial 532C control. Isolate 2-32 also outranked isolates 1-2, 1-17 and 2-33, whereas isolate 3-57 outranked isolates 1-2, 1-17, 2-33 and 2-37. Isolates 1-15 and 3-49 had a higher rate than isolate 1-17. There were no differences between the isolates and the commercial USDA 110 control. The ANOVA P-value was $P<0.0001$.

4.2.3.3 Total Nodule Dry Weight

The range of total nodule DWs using the OAC Prudence cultivar was 0.08 – 0.25 g (Table 4.7). There were no differences observed between the isolates and control 532C. The commercial control USDA 110 had the lowest total nodule DW value recorded. Four isolates (1-15, 1-17, 2-33 and 2-38) showed higher DWs than USDA 110. Isolate 2-38 also showed a higher DW than isolates 2-32 and 2-37. The ANOVA P-value was $P<0.0001$

4.2.3.4 Total Plant Biomass

There were no differences between isolates regarding total plant biomass using the OAC Prudence cultivar (Table 4.7). The ANOVA P-value was $P=0.1041$.

Table 4.7 Second stage testing of *B. japonicum* using soybean cultivar OAC Prudence. Mean total nodule number, specific nodulation, total plant biomass (g), N concentration (%) and plant tissue N content (mg N plant^{-1}) are reported. Values followed by the same letter in each column are not statistically different at the P≤0.05 level. Standard errors are given in brackets.

Treatment	Nodule Number	Specific Nodulation [nodules(g root DW)$^{-1}$]	Total Nodule DW (g)	Total Plant Biomass (g)	% N Content	Tissue N Content (mg N plant^{-1})
Untreated	N/A	N/A	N/A	2.27 (0.38)	1.6^{e} (0.2)	34.41^{b} (4.2)
NH_4NO_3	N/A	N/A	N/A	1.57 (0.04)	1.6^{e} (0.1)	25.42^{b} (0.65)
532C	82.0 (5.5)	137.9^{cd} (10.1)	0.14^{abc} (0.02)	3.12 (0.38)	3.1^{abcd} (0.1)	94.24^{a} (9.7)
USDA110	95.0 (11.1)	175.5^{abcd} (17.8)	0.08^{c} (0.01)	2.40 (0.25)	2.6^{cd} (0.2)	64.47^{a} (9.8)
1-2	86.8 (17.2)	134.3^{cd} (21.9)	0.17^{abc} (0.05)	2.87 (0.51)	3.2^{ab} (0.2)	92.57^{a} (18.2)
1-15	90.7 (7.3)	185.1^{abc} (12.0)	0.18^{ab} (0.03)	2.56 (0.18)	2.8^{abcd} (0.1)	72.40^{a} (7.2)
1-17	60.8 (12.3)	115.3^{d} (31.3)	0.19^{ab} (0.03)	2.65 (0.24)	2.6^{d} (0.2)	69.07^{a} (9.0)
2-32	99.5 (16.5)	233.0^{ab} (28.5)	0.11^{bc} (0.02)	1.71 (0.31)	1.6^{e} (0.1)	25.82^{b} (4.7)
2-33	101.0 (6.6)	156.2cd (10.5)	0.20ab (0.04)	2.81 (0.37)	2.8^{bcd} (0.2)	81.13a (14.0)
2-37	85.3 (11.8)	164.9^{bcd} (24.0)	0.13^{bc} (0.01)	3.02 (0.27)	3.2^{ab} (0.1)	98.61^{a} (10.8)
2-38	99.0 (28.1)	177.2^{abcd} (34.9)	0.25^{a} (0.06)	2.81 (0.40)	3.2^{ab} (0.2)	89.93^{a} (13.2)
3-49	93.7 (16.7)	221.6^{abc} (40.7)	0.13^{abc} (0.01)	2.26 (0.05)	3.3^{ab} (0.1)	73.98^{a} (0.51)
3-50	98.5 (21.2)	179.0^{abcd} (20.6)	0.14^{abc} (0.04)	3.18 (0.33)	3.1^{abc} (0.1)	97.95^{a} (11.9)
3-57	94.2 (13.3)	241.1^{a} (26.3)	0.18^{abc} (0.05)	2.32 (0.48)	3.3^{a} (0.2)	79.98^{a} (19.1)

4.2.3.5 Nitrogen Concentration (%)

The nitrogen concentrations using the cultivar OAC Prudence ranged from 1.6 – 3.3% (Table 4.7). The untreated and nitrogen-fertilized controls and isolate 2-32 had similar nitrogen concentrations that were lower than all other treatments. No other isolates had different nitrogen concentrations than the commercial 532C control with the exception of isolate 2-17 which was lower. Five isolates (1-2, 2-37, 2-38, 3-49 and 3-57) had higher nitrogen concentrations than the commercial USDA 110 control and isolate 2-17. Isolate 3-57 had a higher nitrogen concentration than isolates 1-17 and 2-33 as well as the commercial USDA 110 control but was similar to the remaining treatments. The ANOVA P-value was $P<0.0001$.

4.2.3.6 Plant Tissue Nitrogen Content

The range of plant tissue nitrogen contents using the cultivar OAC Prudence was between 25.42 – 98.61 mg N $plant^{-1}$ (Table 4.7). All but one isolate (2-32) had higher nitrogen contents than the untreated and nitrogen controls and all isolates tested had higher contents than isolate 2-32 (Table 4.7). This same isolate showed a lower tissue nitrogen content than the commercial inoculant controls. The P-value for this ANOVA was $P=0.0001$.

4.3 Host by Strain Performance

Because testing of all cultivars occurred at the same time and under the same conditions, performance of each test isolate in relation to the three soybean cultivars (Apollo, Gentleman, and OAC Prudence) can be assessed using two-way ANOVAs

(Table 4.8). The effects tested included cultivar (host) effects where isolates may perform differently depending on the cultivar used; isolate (strain) effects where isolates perform differently from each other using the same cultivar(s), and cultivar (host) x isolate (strain) effects where an isolate may prefer one cultivar of soybean over another (Table 4.8).

Table 4.8 Probability (P) values of two-way ANOVAs for cultivar, isolate, and cultivar x isolate effects on nodule number, specific nodulation rates, nodule DW, biomass, nitrogen concentrations (%) and tissue nitrogen contents of test plants.

Effect	Nodule #	Specific Nodulation	Nodule DW	Biomass	% N	Tissue N Content
Cultivar	0.2667	<0.0001	<0.0001	<0.0001	0.2331	<0.0001
Isolate	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Cultivar x Isolate	0.0001	<0.0001	<0.0001	<0.0001	0.0005	0.0020

Table 4.9 Mean nodule numbers, specific nodulation rates, nodule DWs, biomass (g), nitrogen concentration (%), and plant tissue nitrogen contents (mg N plant $^{-1}$) of cultivars Apollo, Gentleman, and OAC Prudence. Values followed by the same letter in each column are not statistically different at the $P \leq 0.05$ level and are indicative of cultivar effects. Standard errors are reported in brackets.

Cultivar	Nodule #	Specific Nodulation	Nodule DW (g)	Biomass (g)	N Concentration %	Tissue N Content mg N plant^{-1}
Apollo	87.2^{a}	188.8^{b}	0.10^{b}	2.22^{ab}	2.42^{a}	54.37^{b}
	(25.2)	(32.5)	(0.02)	(0.59)	(0.65)	(8.51)
Gentleman	101.5^{a}	265.2^{a}	0.12^{b}	1.97^{b}	2.62^{a}	52.62^{b}
	(29.3)	(36.6)	(0.01)	(0.22)	(0.70)	(7.12)
OAC Prudence	90.5^{a}	176.8^{b}	0.16^{a}	2.54^{a}	2.71^{a}	71.4^{a}
	(26.1)	(41.0)	(0.02)	(0.33)	(0.72)	(8.10)

Table 4.10 Mean nodule numbers, specific nodulation, nodule DW (g), biomass (g), nitrogen concentrations (%) and tissue nitrogen contents (mg N plant^{-1}) of treatments in Second Stage Testing using the three soybean cultivars Apollo, Gentleman, and OAC Prudence. Values followed by the same letter in each column are not statistically different at the P≤0.05 level and are indicative of isolate effects.

Treatment	Nodule #	Specific Nodulation	Nodule DW (g)	Biomass (g)	N Concentration %	Tissue N Content mg N plant^{-1}
Untreated	0.0	0.00	0.0	2.27^{a}	1.5^{g}	30.14^{d}
NH_4NO_3	0.0	0.00	0.0	1.36^{b}	2.1^{ef}	30.86^{d}
532C	100.2^{ab}	204.4^{bcd}	0.11^{bc}	2.23^{a}	2.6^{bcde}	67.81^{abc}
USDA110	86.2^{ab}	190.2^{bcd}	0.08^{c}	2.12^{a}	2.4^{de}	50.74^{bcd}
1-2	107.7^{a}	207.2^{bcd}	0.12^{abc}	2.43^{a}	2.8^{abcd}	70.30^{abc}
1-15	79.3^{b}	193.0^{bcd}	0.11^{abc}	2.19^{a}	2.5^{cde}	55.45^{abcd}
1-17	52.0^{c}	150.7^{d}	0.13^{abc}	2.12^{a}	2.5^{cde}	53.32^{bcd}
2-32	105.1^{ab}	304.9^{a}	0.11^{abc}	2.02^{ab}	1.6^{fg}	46.18^{cd}
2-33	100.9^{ab}	196.8^{bcd}	0.12^{abc}	2.02^{ab}	2.7^{bcd}	44.21^{cd}
2-37	95.1^{ab}	199.9^{bcd}	0.12^{abc}	2.57^{a}	3.1^{ab}	81.88^{a}
2-38	92.7^{ab}	172.0^{cd}	0.18^{a}	2.67^{a}	2.8^{abcd}	74.45^{ab}
3-49	106.1^{a}	251.5^{abc}	0.15^{ab}	2.25^{a}	3.3^{a}	74.51^{ab}
3-50	85.7^{ab}	168.5^{cd}	0.12^{abc}	2.59^{a}	2.9^{abc}	75.79^{ab}
3-57	109.0^{a}	269.9^{ab}	0.17^{ab}	2.30^{a}	3.3^{a}	77.06^{ab}

4.3.1 Nodule Numbers

Assessment of nodule numbers confirmed isolate effects and cultivar x isolate effects on nodule numbers; however, no cultivar effects could be determined (Tables 4.8). Isolate effects were observed between nodule numbers when the results of the three cultivars were combined (Table 4.10). Isolates 1-2. 3-49, and 3-57 resulted in higher nodule numbers than isolates 1-15 and 1-17. Isolate 1-15 resulted in a higher nodule number than isolate 1-17. The P-value of the isolate effect was $P<0.0001$. Cultivar x isolate effects were observed when using isolates 2-38 and 3-50, both having resulted in higher nodule numbers in the cultivar Gentleman than in cultivar Apollo (Figure 4.1). Isolate 1-17 resulted in higher nodule numbers in Gentleman and OAC Prudence than in Apollo. The P-value of the cultivar x isolate analysis was $P=0.0001$

4.3.2 Specific Nodulation

Specific nodulation rates were affected by isolate, cultivar and cultivar x isolate effects (Table 4.8). Cultivar effects were observed between the specific nodulation rates of Gentleman and Apollo plants, where cultivar Gentleman had higher specific nodulation rates than cultivars Apollo and OAC Prudence (Table 4.9, Figure 4.2). The P-value for the cultivar effect test was $P<0.0001$. Isolate effects were observed when comparing specific nodulation rates (Table 4.10). Isolate 2-32 resulted in a higher specific nodulation rate than all other isolates with the exception of isolates 3-49 and 3-57. Isolate 3-57 resulted in a higher specific nodulation rate than isolates 1-17, 2-38, and 3-50. Isolate 3-49 resulted in a higher specific nodulation rate than isolate 1-17. The P-value for the isolate effect was $P<0.0001$. Cultivar x isolate effects were observed when

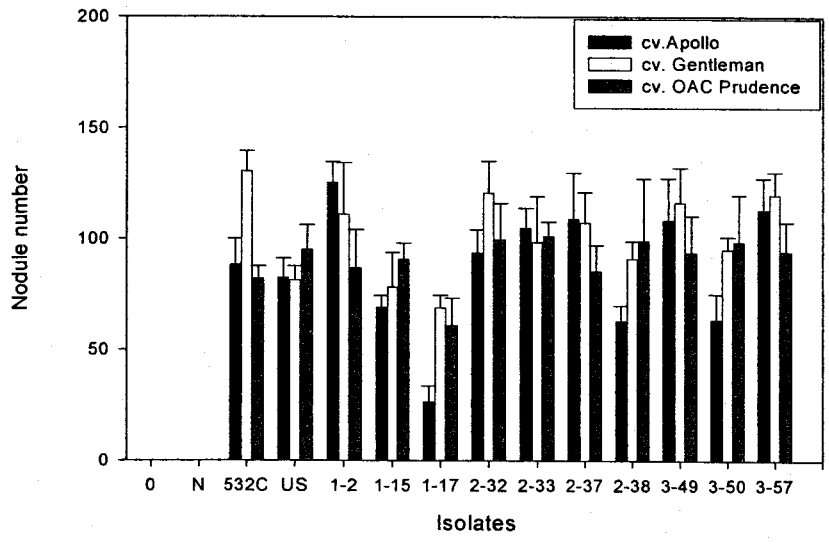

Figure 4.1 Mean nodule numbers in the three soybean cultivars Apollo, Gentleman, and OAC Prudence inoculated with 10 different isolates. Bars indicate standard error.

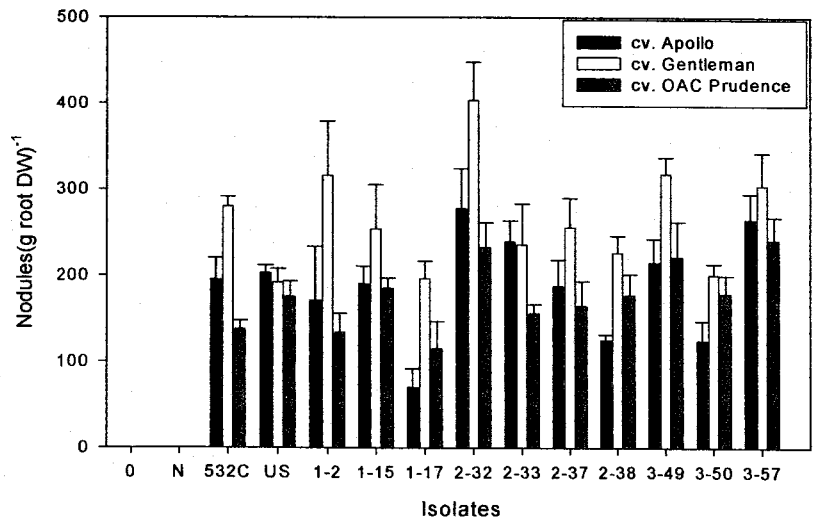

Figure 4.2 Mean specific nodulation rates in the three soybean cultivars Apollo, Gentleman, and OAC Prudence inoculated with 10 different isolates. Bars indicate standard error.

using isolates 1-2, 1-17 and 2-32 where Gentleman plants exhibited higher specific nodulation rates than both OAC Prudence and Apollo plants. Isolates 2-38, 3-49 and 3-50 resulted in a higher specific nodulation rate when used on the cultivar Gentleman than when tested on cultivar Apollo (Figure 4.2). The P-value for cultivar x isolate effect was $P<0.0001$.

4.3.3 Total Nodule Dry Weight

Total nodule DWs showed all three effects of cultivar, isolate, and cultivar x isolate with ANOVA P values of $P<0.0001$ (Table 4.8). Cultivar effects were observed between the cultivars OAC Prudence and Apollo (Table 4.9). OAC Prudence had higher total nodule DWs than Apollo (Figure 4.3). Isolate effects were observed when comparing nodule dry weight using all three cultivars (Table4.10). Isolate 2-38 resulted in a higher nodule DW than the commercial inoculant controls 532C and USDA 110. Isolates 3-49 and 3-57 resulted in higher nodule DWs than the commercial inoculant USDA 110. No other differences were detected between isolates. The ANOVA P-value for the isolate effect was $P<0.0001$. Cultivar x isolate effects were observed when using test isolates 1-15, 1-17, and 2-33, where total nodule DWs were higher in the cultivar OAC Prudence than in cultivars Gentleman or Apollo (Figure 4.3). Isolates 2-32, 2-38, and 3-50 resulted in higher total nodule DWs in the cultivar OAC Prudence than in the Apollo cultivar. When tested in the cultivar Gentleman, isolate 3-57 resulted in a higher total nodule DW than when tested in the cultivar Apollo.

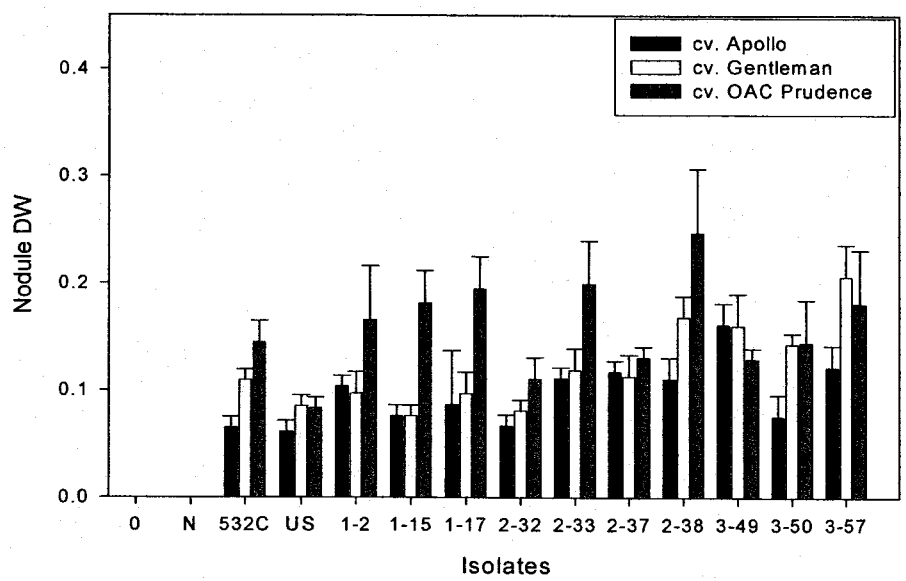

Figure 4.3 Mean total nodule DWs in the three soybean cultivars Apollo, Gentleman, and OAC Prudence inoculated with 10 different isolates. Bars indicate standard error.

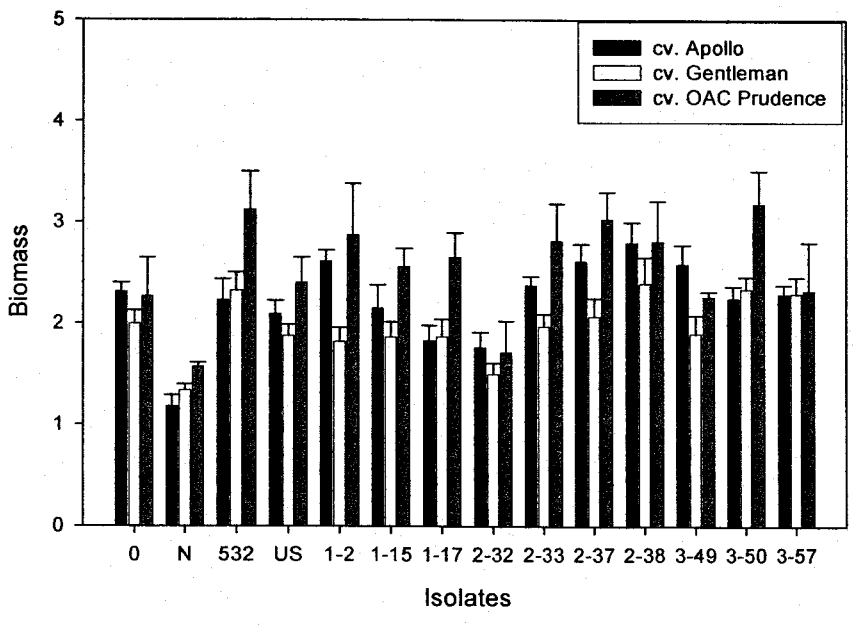

Figure 4.4 Mean biomass values in the three soybean cultivars Apollo, Gentleman, and OAC Prudence inoculated with 10 different isolates. Bars indicate standard error.

4.3.4 Total Plant Biomass

Analysis of cultivar, isolate, and cultivar x isolate effects on biomass produced differences in all three categories with ANOVA P values of $P<0.0001$ (Table 4.8). Cultivar effects were observed using the cultivar OAC soybean where that cultivar generally showed a higher total plant biomass than the cultivar Gentleman (Table 4.9, Figure 4.4). Isolate effects were observed when comparing biomass using all three cultivars (Table 4.10). The nitrogen-fertilized control had a lower biomass than all other isolates with the exception of isolates 2-32 and 2-33. The isolate effect ANOVA P-value was $P<0.0001$. Cultivar x isolate effects on biomass were observed when using isolates 1-17 and 3-50, which resulted higher biomasses in OAC plants than in Gentleman and Apollo plants. Test isolates 1-2, 1-15, 2-33, and 2-37 resulted in higher biomasses in OAC Prudence plants than in Gentleman plants (Figure 4.4); isolates 1-2 and 2-37 resulted in higher biomasses in the cultivar Apollo versus the cultivar Gentleman ($P<0.0001$).

4.3.5 Nitrogen Concentration (%)

Isolate and cultivar x isolate effects were detected in plant nitrogen concentrations with two-way ANOVA values of $P<0.0001$ (Table 4.8). Isolate effects were observed when comparing nitrogen concentrations using data from all three cultivars (Table 4.10). Isolates 3-49 and 3-57 resulted in higher nitrogen concentrations than plants using all other isolate with the exception of isolates 1-2, 2-37, 2-38, and 3-50. Isolate 2-37 resulted in a higher nitrogen concentration than all other isolates with the exception of plants inoculated with isolates 1-2, 2-38, and 3-50. Isolate 3-50 resulted in a higher

nitrogen concentration than isolate 2-32, the commercial inoculant control USDA 110, the nitrogen-fertilized control and untreated control. Isolates 1-2, 2-33, and 2-38 resulted in higher nitrogen concentrations than isolate 2-32 and the nitrogen-fertilized and untreated controls. Isolate 2-32 and the untreated control resulted in lower nitrogen concentrations than all other isolates and commercial inoculant controls. The isolate effect P value was $P<0.0001$. Cultivar x isolate effects were observed when comparing nitrogen concentrations. Two isolates (1-2 and 1-17) resulted in higher nitrogen concentrations when using cultivar Gentleman than when using cultivar Apollo (Table 4.9, Figure 4.5). Isolate 3-49 resulted in a higher nitrogen concentration when using cultivar Gentleman than when using cultivar Apollo or cultivar OAC Prudence. One isolate (1-15) resulted in a higher nitrogen concentration when using cultivar OAC Prudence than when using cultivar Apollo or Gentleman. Isolate 2-38 resulted in higher nitrogen concentrations when using cultivars Gentleman and OAC Prudence than when using cultivar Apollo.

4.3.6 Plant Tissue Nitrogen Content

Using a two-way ANOVA to assess plant tissue nitrogen content, the cultivar, isolate and cultivar x isolate effects were all detected (Table 4.8). Cultivar effects were observed between the three cultivars (Table 4.9, Figure 4.5). OAC Prudence had a higher N content than cultivar Apollo or cultivar Gentleman.

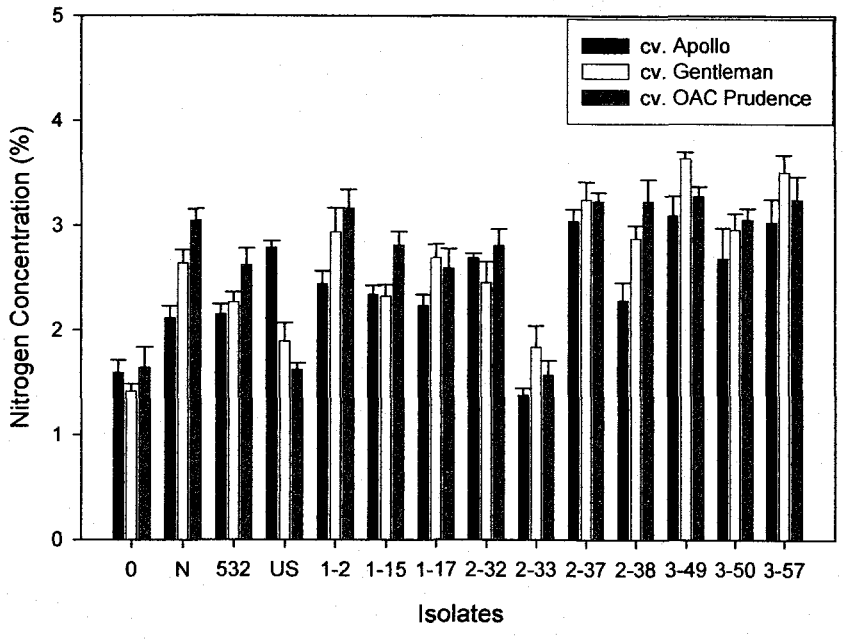

Figure 4.5 Mean nitrogen concentrations (%) in the three soybean cultivars Apollo, Gentleman, and OAC Prudence inoculated with 10 different isolates. Bars indicate standard error

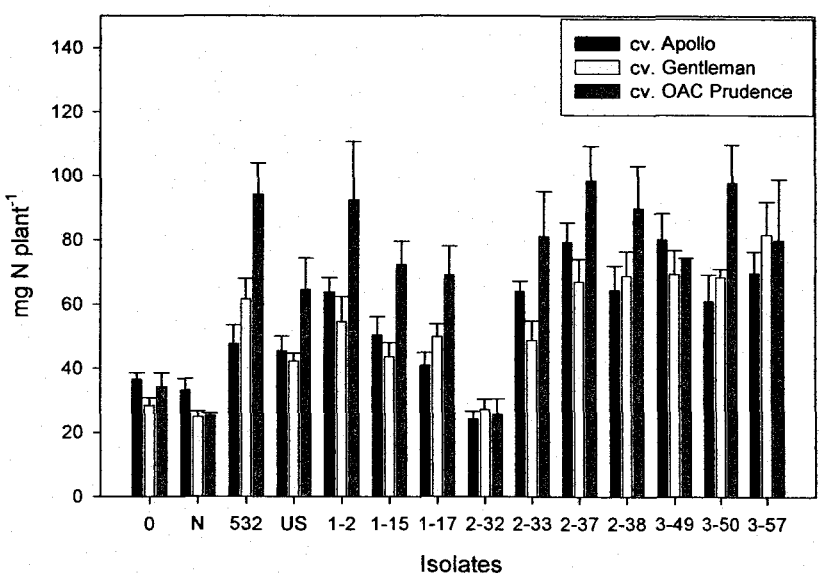

Figure 4.6 Mean plant tissue nitrogen contents in the three soybean cultivars Apollo, Gentleman, and OAC Prudence inoculated with 10 different isolates. Bars indicate standard error.

The P-value of the cultivar analysis was P<0.0001. Isolate effects were observed when comparing plant tissue nitrogen contents using all three cultivars (Tables 4.10). Isolate 2-37 resulted in a higher tissue nitrogen content than isolates 1-17, 2-32, 2-33, the commercial inoculant USDA 110 control, and the untreated and nitrogen-fertilized controls. Isolates 2-38, 3-49, 3-50, and 3-57 resulted in a higher tissue nitrogen content than isolates 2-32 and 2-33 and the untreated and nitrogen-fertilized controls. The untreated and nitrogen-fertilized controls had a lower tissue nitrogen content than isolates 1-2, 2-37, 2-38, 3-49, 3-40 and isolate 3-57. The ANOVA P-value of the isolate effect analysis was P<0.0001. Cultivar x isolate effects were observed when using isolates 1-2, 1-15, 1-17, and 3-50, which resulted in higher biomasses in OAC Prudence plants than in the Gentleman and Apollo plants (Figure 4.5). Testing of isolates 2-33 and 2-37 resulted in higher nitrogen contents in the cultivar OAC Prudence than in the cultivar Gentleman. The ANOVA P-value for the cultivar x isolate effect was P=0.0020.

4.4 Performance Correlation

Several different parameters were measured in Second Stage Testing to better describe performance including nitrogen content and total nodule dry weights. Using data from inoculated treatments in all three cultivars, a Pearson Correlation Matrix was performed to see if any one of these parameters was a better indicator of overall superior symbiotic performance, i.e. which parameter correlated best with the category mg N plant^{-1} (Appendix 8.4). The parameters most closely associated with total mg N plant $^{-1}$ were biomass at 0.794 and total nodule dry weight at 0.765.

5.0 DISCUSSION

5.1 FIRST STAGE TESTING

5.1.1 Assessment of Resident *B. japonicum*

First Stage Testing assessed the performance of *B. japonicum* isolates using a single cultivar (Gentleman) under greenhouse conditions. Testing occurred in three trials in which 20 isolates were tested per trial for a total of 60 isolates. First Stage trials included an untreated control, controls inoculated with commercially available inoculants (532C & USDA 110), and a nitrogen-fertilized control. There was only one commercial inoculant (532C) available for the first two trials. The second commercial inoculant (USDA 110) was used as an additional control for the last trial. Analysis compared the performance of the test isolates to the controls. The titre of the inoculants used in First Stage Testing were not determined, instead inoculants were cultured to an optical density of approximately OD 0.6.

5.1.1.1 Biomass

Inoculation has been recommended for legumes planted in nitrogen deficient soils to increase the productivity of the crop. The majority of previous studies (e.g. Ferreira and Hungria, 2002; Hungria *et al.*, 2001) use biomass, either as shoot dry weight (DW) alone or as complete plant DW, as a performance indicator of superior *B. japonicum*.

From the First Stage Testing results, biomass was not very informative. When looking at shoot DW, there was little increase of shoot DW between the inoculated and nitrogen-fertilized treatments, or the untreated and inoculated treatments. This is contrary to the findings of Seneviratne *et al.* (2000). More often in trials 1 and 3 (Tables

4.1 & 4.3), inoculated treatments – including the commercial inoculant control – showed a lower shoot DW than the untreated and nitrogen controls. Fewer test inoculants exhibited decreased root DW than the untreated and nitrogen controls, but none showed an increased root DW. This trend may be indicative of the high energy costs of biological nitrogen fixation (BNF). To convert atmospheric nitrogen to NH_4^+ large amounts of ATP are required (Halbleib and Ludden, 2000). Under normal conditions, these requirements are easily met but in times of stress, BNF may negatively impact plant growth (Elmore, 2001). For example, excessive heat, low root zone temperatures, or high acidity can delay nodule development and/or nitrogen fixation (Bailey, 1989; Zhang *et al.*, 1995; Egamberdiyeve *et al.*, 2004). This delay can lead to poorer growth because energy from the plant can still be diverted to nodule formation however without further nodule development or nitrogen fixation, nitrogen may not be supplied to the plant. In each trial, there were stressors present that could account for the negative growth trend. In the first trial, abnormal growth in test plants was a result of poor temperature regulation in the greenhouse, resulting in excessive heat and humidity. In the second and third trials, again there were temperature concerns, despite trials being conducted at another greenhouse (Dalhousie University). Also in the third trial, there was an outbreak of thrips that damaged the plants. It is expected that the results obtained by Seneviratne *et al.* (2000) showing increased biomass would have been reflected in this experiment had the stressors not been so extreme and if trials were conducted in the field. Conversely, the trend might have been more pronounced if plants had been left to grow through the pod-filling stage, as this is the stage that requires the most energy and where the rhizobia may not have been able to keep up with demand (Salon *et al.*, 2001).

5.1.1.1.1 Untreated Controls

During the First Stage Testing there was little difference observed between the untreated soybeans and soybeans treated with the other controls or isolates although it was expected that there would be much less biomass because there should have been less nitrogen available for growth in these treatments. There may be several reasons for this discrepancy. It is possible that although the nitrogen content of the soil tested as deficient, there was still enough to sustain the plant to the early pod-filling stage; also, the soil that was used for First Stage Testing was collected during 2004 and portions were stored for over a year before use increasing the possibility of mineralization. While initial nitrogen analysis of the soil (Table 3.2) indicated the soil was nitrogen deficient, it is possible that the drying and rewetting of the soil during storage and growth trials increased the mineralization of organic compounds within the soil, increasing nitrogen content. Cattanio (2002) found that in trials, mineralization doubled the nitrogen content in sandy soil after 64 days. Mineralization could have increased the nitrogen content sufficiently to provide the untreated controls with the nitrogen they required for growth up to that stage of development. It is unclear if the nitrogen content would have been sufficient to sustain the plants during the more nitrogen-consuming pod-filling stage. Other studies use soil diluted with another benign media such as sterilized silica sand to ensure no confounding nitrogen (or other nutrient) effects (Jacobi *et al*, 1998). During Second Stage Testing, rooting medium was mixed in a 1:1 sterilized silica sand to soil ratio, and growth differences were easy to detect with the unaided eye, i.e. untreated soybeans were yellowish, indicating a nitrogen deficiency (Berry, 2006).

5.1.1.2 Nodule Numbers & Specific Nodulation

Nodule numbers and specific nodulation rates in First Stage Testing showed larger statistical differences than the shoot and root DWs and total plant biomass. In the first stage it was unknown what isolates could induce nodulation and nodule numbers ranged from zero to 133.5. This large difference in nodule numbers was also reflected in the specific nodulation rates. To compare nodule numbers, specific nodulation [nodules (gram root DW)$^{-1}$] was an important parameter to use because it takes into account root size when comparing nodule numbers (Vessey and Luit, 1999). It is also useful as a selection tool for Second Stage Testing because increased specific nodulation can represent the potential for increased nitrogen fixation. A drawback of using this criterion is that it does not consider ineffective nodulation. An isolate may induce high rates of nodulation but may not be capable of fixing nitrogen. This was the case with isolate 2-32 in Second Stage Testing. In First Stage Testing, three isolates (2-37, 3-49 & 3-50) showed a statistical specific nodulation rate increase when compared to the commercial control(s); these isolates were selected to advance to Second Stage Testing. The remaining seven isolates selected to progress to the second screening (1-2, 1-15, 1-17, 2-32, 2-33, 2-38, & 3-57) had equivalent specific nodulation rates to the commercial inoculant control(s). This indicated that these isolates had the potential to perform at a commercial inoculant level; nitrogen analysis carried out during Second Stage screening would confirm this potential.

5.1.2 Effectiveness of First Stage Testing

First Stage Testing was an important factor in this experiment. It was able to identify strains that performed as well as the commercial inoculants for specific nodulation rates and possibly in nitrogen fixation capacity (nitrogen content). Only one of the studies reviewed for this experiment (Mostasso *et al.*, 2001) included a pre-screening stage such as First Stage Testing. The main difference between the above-mentioned experiment and the other experiments reviewed is the number of isolates tested. The studies without pre-screening tested 40 isolates or less, whereas the one study that did include pre-screening tested over 100 isolates. The main benefit of pre-screening is the ability to narrow down the number of isolates used for the more thorough assessment that follows. In Second Stage Testing, more materials were required to assess *B. japonicum* isolates, including the addition of two other cultivars to the one cultivar in First Stage Testing, and the 6 replicates required for each cultivar added. By narrowing down the number of isolates from 60 to 10, considerable resources (i.e. soil, pots, seeds, and space) were conserved. Although it appears more time is required to include pre-screening, greenhouse space restrictions in this experiment would have reduced the number of isolates per trial considerably if three cultivars had been used from the beginning. This would negate the benefits of time saved by omitting the pre-screening protocol. The economic benefits derived from a pre-screening stage include the harvesting of less soil to dry and transport, and fewer supplies (i.e. pots) to purchase. It is also true that by limiting the nitrogen analysis to Second Stage Testing, considerable funds were conserved. It would seem to be beneficial to employ pre-screening in any experiment involving more than 20 isolates.

5.2 SECOND STAGE TESTING

Second Stage Testing was completed in one trial at the Saint Mary's University greenhouse in February, 2007. The ten isolates selected from the previous screening stage (exhibiting the highest specific nodulation values) were tested again on three cultivars of soybean: Apollo, Gentleman, and OAC Prudence. The titre of the test inoculants used was determined to be 10^4 rhizobia per seed in Second Stage Testing instead of using a more rudimentary OD value from First Stage Testing. In addition to parameters measured in the First Stage, total nodule DW, nitrogen concentration (%) and plant tissue nitrogen content (mg N $plant^{-1}$) were added.

5.2.1 Diversity of Resident *B. japonicum*

Farooq (2007) investigated the genetic diversity of the *B. japonicum* isolates used in Second Stage Testing using molecular analytical techniques (REP and ERIC PCR). He confirmed that isolates remaining in soil for extended periods of time exhibit genetic change from the original introduced strain. In this experiment, the performance differences between isolates and the commercial inoculants bolster Farooq's findings. From figures 4.1 – 4.6 and Table 4.10 it is easy to observe differences between isolate performances as well as between isolate performances and the two most commonly used commercial inoculants 532C and USDA110. Two isolates (2-38 and 3-49) tested in Second Stage were isolated from nodules taken from the same field and had different performances (Table 4.5-4.6). These results coupled with the profiles obtained by Farooq (2007) confirms similar findings from Brockwell *et al.*, (1995) that the changes are not necessarily uniform nor are they always transferred to other bacteria within the same

location. One reason for this is that within the same location, the nutrient supply and conditions can vary greatly creating different microhabitats for the change to occur.

5.2.2 Inoculated and Nitrogen-Fertilized Treatments

In Second Stage Testing nitrogen-fertilized treatments resulted in significantly lower plant biomass despite the fact that nitrogen was applied at the rate recommended to farmers growing soybean (100 kg N ha^{-1}). A possible reason for this is that nitrogen supply alone may not be responsible for the positive growth response observed in the inoculated treatments. Rhizobia have been shown to produce several molecules and compounds that assist in plant growth. *B. japonicum* produce molecules called Nod factors that assist in seed germination and seedling development, increase photosynthetic rates, and induce the expression of flavonoid genes within the plant (Zhang and Smith, 2001; Smith et al. 2002). Souleimanov *et al.* (2002) investigated the effects of Nod factor from *B. japonicum* on soybean and corn and found that it significantly increased root length and promoted dry mass accumulation. Some of the accumulation in dry mass can be attributed to the increased root surface area that provided larger areas for nutrient absorption. In addition to Nod factors, rhizobia also produce riboflavin which is a vitamin that can be used for both the plant and the bacteria. The riboflavin is converted to lumichrome that has been shown to stimulate seedling growth and production of CO_2 in the roots (Phillips *et al.*, 1999). Other plant growth promoters produced include indole acetic acid (IAA), gibberellins and cytokinins (Lynch and Clark, 1984; Law and Strijdom, 1989). Siderophores produced assist in plant uptakes of minerals from the soil and have been shown to increase mineral concentrations in plants (Howell, 1987).

Rhizobia produce numerous other vitamins and amino acids that assist in growth but will not be discussed here. All of the plant growth promoters produced by the rhizobia can affect the development of the host soybean plant and may be responsible for the large differences observed between the test isolates and the nitrogen-fertilized controls.

5.2.3 Responsiveness of Nitrogen-fertilized Treatments

The nitrogen-fertilized treatments in Second Stage Testing exhibited no benefit over inoculated treatments or even untreated plants in reference to biomass (Figure 4.4) or plant tissue nitrogen contents (Table 4.6). The overall nitrogen concentration however is comparable to other isolates and higher than the untreated control (Table 4.5). This suggests that the nitrogen accumulated was not effectively utilized for growth. Although the percentage of nitrogen was high, the plants were smaller and therefore the actual nitrogen accumulation was much less than plants with high nitrogen percentages and high biomasses. A possible reason for the unresponsiveness of the nitrogen-fertilized treatments could be the low level of sulfur in the soil (Table 3.4). Sinclair (1993) and Heatherly and Hodges (1999) found that sulfur deficiencies mimic nitrogen deficiencies in plants due to the inability to produce several amino acids. Also, the low sulfur levels lead to accumulation of nitrate within plant tissues and could lead to yellowing. Yellowing was not observed in the nitrogen-fertilized plants possibly because the sulfur was not at a low enough level. However the levels may have been low enough to inhibit growth. The inoculated treatments may not have been sensitive to the low levels of sulfur due to the array of compounds produced by the rhizobia (Section 5.2.2). Interestingly, untreated control plants had higher biomasses than the nitrogen-fertilized controls despite

that they were grown in the same low-sulfur soil and did not benefit from rhizobial inoculation. This difference between untreated and nitrogen-fertilized plants may have been caused again by the low sulfur levels, which cause nitrate accumulation in the tissues. Zhou *et al.* (2006) have shown that increased nitrogen accumulation in soybean tissue leads to the impairment of electron transport and can damage photosynthetic mechanisms. This may be responsible for the unresponsiveness of the nitrogen-fertilized treatment.

5.2.4 Cultivar and Strain Interactions

5.2.4.1 Cultivar effects

This experiment was able to positively identify cultivar effects between the isolates and the three cultivars. Generally, there was a trend of increased nitrogen accumulation, total nodule DW and total plant biomass in cultivar OAC Prudence, regardless of the isolate tested. This is an indication of cultivar effects rather than cultivar x rhizobial strain effects (Skøt, 1983). Interestingly, the cultivar Gentleman had the highest specific nodulation rates, which can also indicate superior biomass and nitrogen content due to the potential for increased nitrogen fixation. One reason for this disconnect could be that in cultivar Gentleman, low numbers of larger, more mature nodules were more efficient at nitrogen fixation than greater numbers of smaller nodules. The total nodule DW values for Gentleman are generally lower than the OAC Prudence cultivar, supporting this hypothesis. Also supportive are the results of cultivar Apollo. Apollo plants had similar specific nodulation rates to OAC Prudence, but had lower total nodule DWs that were very similar to the Gentleman total nodule DWs. Consequently,

the plant tissue nitrogen contents of Apollo plants were lower than those of OAC Prudence plants, but similar to the Gentleman cultivar's nitrogen contents. This is similar to results found by Zhang *et al.* (2003) that isolates with greater nodule DWs lead to greater plant tissue nitrogen contents.

5.2.4.2 Isolate effects

Differences occurring between isolates within the same cultivar were indicative of rhizobial variation (isolate) effects (Martensson and Rydberg, 1996) and were further confirmation of the genetic differences identified by Farooq (2007). Isolate effects were readily observed in the Second Stage trials (Table 4.10). Isolates often performed differently from each other depending on which parameter was measured. It is interesting to note that the performances were not linked to growth rate of the isolate in culture, in that isolates with a faster growth rate in culture (Table 3.3) did not translate to increased nodulation or nitrogen accumulation when tested on soybean plants.

An interesting isolate difference that arose in Second Stage Testing involved isolate 2-32. Isolate 2-32 resulted in plants with the highest specific nodulation rate across two trials and was second in another trial. Despite the increased number of nodules per gram of root dry weight, isolate 2-32 resulted in the lowest plant tissue nitrogen content when compared to the other isolates used in all three trials (Table 4.10). This was an indication that the nodules were not efficiently fixing nitrogen and instead were draining important plant resources for nodule development. When nodules are demanding more carbon than they are providing benefit to the plant in nitrogen, they are

sometimes called parasitic (Denison & Kiers, 2004; Hansen *et al.*, 1992). This could be significant to the experimental design of this project, as the criterion for strain selection for Second Stage Testing was specific nodulation without additional nitrogen content information. However, when examining the results of the other nine strains that were selected for Second Stage Testing (Figure 5.1) and comparing them to the commercial controls, the parameter retains its value. Most isolates either equal or surpass the controls in plant tissue nitrogen content.

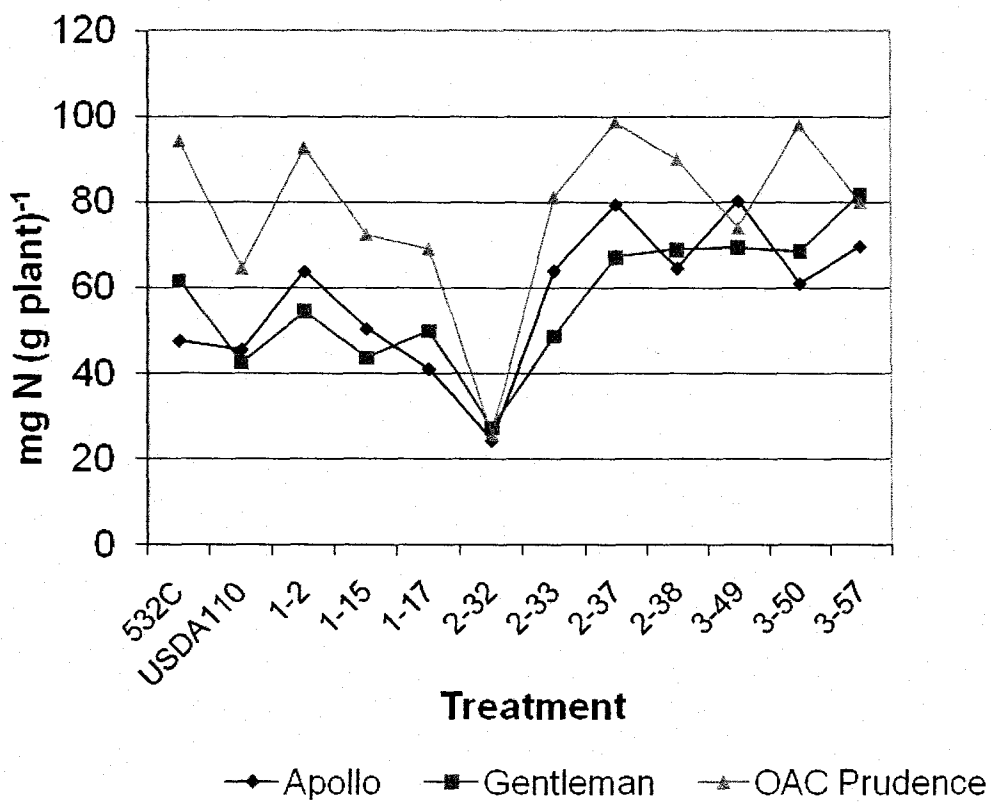

Figure 5.1 Graph indicating plant tissue nitrogen contents of inoculated treatments in Second Stage Screening using cultivars Apollo, Gentleman, and OAC Prudence.

5.2.4.3 Cultivar x Isolate Effects

When looking at the isolate differences, it is interesting to note that isolates performed differently in relation to each other depending on which cultivar was used. For example, isolate 2-38 had different nodule numbers than isolates 2-37 and 3-49 in the Apollo cultivar, but not when used in the Gentleman and OAC Prudence cultivars. This is evidence that there are more factors involved than simple isolate differences. A cultivar x isolate effect occurs when an isolate 'prefers' a cultivar (shows higher values when used on a particular cultivar versus other cultivars) (Ahmad, 1996). It can be difficult to discern true cultivar x isolate effects. Much of the cultivar x isolate effects detected in this experiment also followed the cultivar effect trend. For example, the cultivar effects of specific nodulation rates showed a trend of higher rates in the cultivar Gentleman than in the cultivars Apollo and OAC Prudence. Similarly, cultivar x isolate effects showed isolates 1-2, 1-17, and 2-32 displaying higher specific nodulation rates when used on the cultivar Gentleman than when used on cultivars Apollo and OAC Prudence. Also, isolates 2-38, 3-49 and 3-50 showed higher rates in the cultivar Gentleman cultivar than in the cultivar Apollo. It is understandably difficult to decide if the true effect observed in the examples above is the cultivar effect or the cultivar x isolate effect. In other cases, i.e. total nodule DWs, there were instances of cultivar x isolate interactions that did not follow the cultivar trend. For example, in nodule DWs there were no cultivar differences between Apollo and Gentleman. Here, isolate 3-57 had a higher nodule DW in the Gentleman cultivar than in the Apollo cultivar and so it is easier to make a statement about cultivar x isolate effects in this case.

5.3 Indicators of Superior Symbiotic Effectiveness

To assist in performance assessment, a correlation matrix was constructed on the parameters measured in Second Stage Testing (nodule number, specific nodulation, total nodule DW, total biomass, nitrogen concentration and plant tissue nitrogen content) to indicate which parameters were more closely associated with nitrogen content. This may be helpful when performing other experiments like this one, especially with a prescreening stage that does not include nitrogen analysis. Biomass and total nodule DW were found to be the parameters most closely associated with nitrogen content. Biomass was expected to be highly correlated with nitrogen content, as a deficiency in nitrogen would result in lower dry weights. Biomass has also been identified by Relic *et al.* (1994) as an important indicator of symbiotic performance. The high correlation value of total nodule DW with nitrogen content confirms the importance of the legume-rhizobia symbiosis. Mayz *et al.* (2003) found a similar correlation with nodule DW and nitrogen contents in pea (*Pisum sativum*). Pimrach *et al.* (2004) confirmed a nodule DW and nitrogen content relationship with peanut (*Arachis hypogaea* L.) It may be inferred that higher total nodule DWs lead to higher nitrogen contents, which in turn result in higher biomass values.

6.0 CONCLUSIONS

This research has shown that populations of *B. japonicum* remaining in the soil after soybean inoculation events in western Canada are viable and diverse. As confirmed by First Stage Testing, many of these populations are not as symbiotically efficient as the original commercial inoculants. This means that most of the test isolates were not able to induce nodulation or fix nitrogen as well as the commercial controls. From this, we can infer the genetic changes that take place in *B. japonicum* residing in the soil as detected by Farooq (2007) are generally negative in terms of symbiotic efficiency. These findings bolster the recommendation that soybean be inoculated in fields regardless of inoculation history. However, some transformed strains are capable of nodulating soybeans and fixing nitrogen as well as, or better than, the established commercial strains. This experiment successfully identified several strains that may be desirable to develop as market-grade inoculants.

Plants inoculated with commercial and test isolates in Second Stage Testing had higher plant tissue nitrogen contents and total plant biomass than the untreated and nitrogen-fertilized treatments with the exception of isolate 2-32. These results underline the importance of the legume-rhizobia symbiosis for growth and also underscore the recommendation that soybean be inoculated when planted in fields with no previous history of soybean cultivation. This also suggests that nitrogen application at the recommended application rate of 100 kg N ha^{-1} is inferior to inoculant application in previously uninoculated soils.

Although not a focus of this study, the cultivar effect identified in this experiment was pronounced. OAC Prudence plants accumulated more nitrogen and biomass when inoculated than the Apollo and Gentleman soybean cultivars tested. This indicates the importance of cultivar selection when growing soybean. More emphasis can be placed on cultivar selection when inoculating, rather than selecting cultivars based on regional suitability. If equivalent in field performance and other cultivar selection factors (i.e. cost, disease and pest resistance) cultivar OAC Prudence could be recommended to farmers over other varieties of soybean when using the commercial inoculants 532C and USDA 110.

Isolate differences were easy to detect between isolates within the same cultivar. The diversity of the isolates tested was reflected in their performance. The best performers were assessed to have consistently high numbers across all three cultivars. Most isolates performed similarly to the commercial inoculant controls. All isolates in Second Stage Testing, with the exception of isolate 1-17, were good nodulators and showed good specific nodulation rates. The isolates with the highest biomass and plant tissue nitrogen contents show the most potential, and should be further tested in field trials to assess their performance in a crop setting. In this experiment, isolates 2-37, 2-38, 3-49, 3-50 and 3-57 had consistently high biomass and tissue nitrogen contents and qualify for further field testing. It is anticipated that Philom Bios Inc. will perform these field tests in the summer of 2008.

Some possible future directions from this study include the following: Firstly, longer growth trials to determine if nitrogen fixation is sufficient for the more energy-

taxing pod-filling stage. Secondly, deployment of multiple inoculation events at 0 and 3 weeks and assessment of nitrogen accumulation to determine if new nodulation increases the energy available for the pod-filling stage. Thirdly, assessment of different inoculation techniques on infectivity of different isolates and finally, evaluation of competitive ability through the use of multiple inoculant blends; this last suggestion would also involve genomic fingerprinting techniques to determine nodule occupancy.

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8.0 APPENDICES

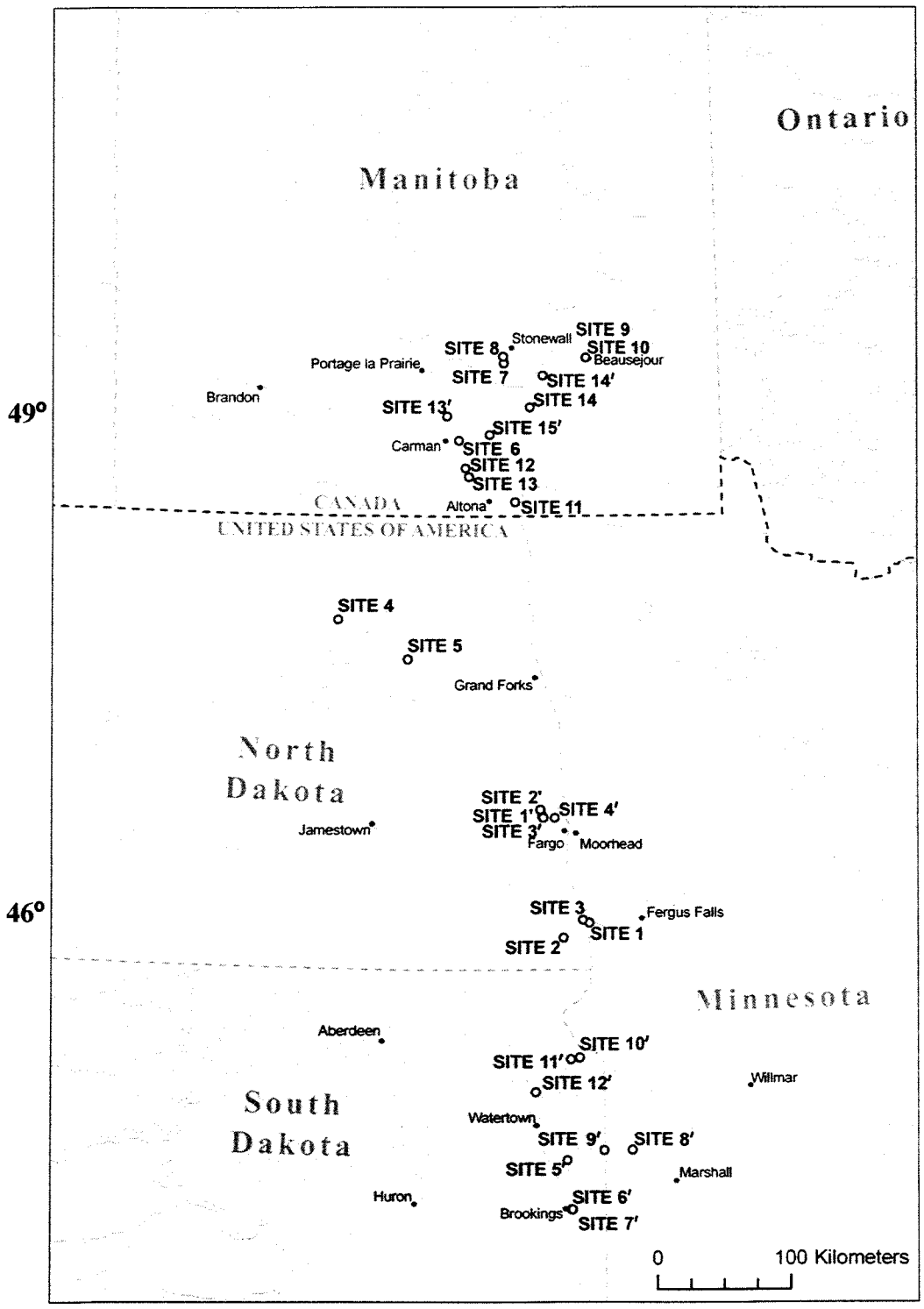
Appendix 8.1 Location information of 2004 nodule collection sites as adapted from Farooq (2007) including site name (if available), GPS coordinates, geographical location, and country.

Site #	Site name	GPS	Geographical location	Country
1	Breckenridge	46° 15.257' N 96° 34.421' W	Minnesota	US
2	Great Bend	46° 09.219' N 96° 49.794' W	North Dakota	US
3	Richland County	46° 16.364' N 96° 38.183' W	North Dakota	US
4	Ramsey	48° 17.781' N 99° 03.849' W	North Dakota	US
5	Lacota	48 ° 02.000' N 98° 22.000' W	North Dakota	US
6	Unnamed	49° 30.394' N 97° 51.547' W	Manitoba	Canada
7	Unnamed	50° 02.500' N 97° 24.181' W	Manitoba	Canada
8	Unnamed	50° 04.466' N 97° 24.497' W	Manitoba	Canada
9	Unnamed	50° 10.488' N 96° 38.048' W	Manitoba	Canada
10	Unnamed	50° 04.088' N 96° 32.382' W	Manitoba	Canada
11	Unnamed	49° 05.487' N 97° 17.203' W	Manitoba	Canada
12	Plum Coulee1	49° 19.324' N 97° 47.528' W	Manitoba	Canada
13	Plum Coulee2	49° 15.586' N 97° 45.471' W	Manitoba	Canada
14	Agra seeds	49° 04.414' N 97° 08.118' W	Manitoba	Canada

Appendix 8.2 Location information of 2005 nodule collection sites as adapted from Farooq (2007) including GPS coordinates, geographical location, and country.

Site #	GPS	Geographical location	Country
1′	46° 58.638' N 97° 01.826' W	Fargo, North Dakota	US
2′	47° 01.931' N 97° 02.952' W	Fargo, North Dakota	US
3′	46° 57.848' N 97° 01.189' W	Fargo, North Dakota	US
4′	46° 57.821 'N 96° 54.419' W	Fargo, North Dakota	US
5′	44° 39.261' N 96° 48.283' W	Brandt, South Dakota	US
6′	44° 19.407' N 96° 46.051' W	SDSU, South Dakota	US
7′	44° 19.293' N 96° 45.625' W	Brookings, South Dakota	US
8′	44° 43.169' N 96° 11.251' W	Yellow Medicine, Minnesota	US
9′	44° 43.132' N 96° 27.210' W	Gary, South Dakota	US
10′	45° 21.095' N 96° 08.461' W	Corona, South Dakota	US
11′	45° 20.174' N 96° 09.288' W	Corona, South Dakota	US
12′	45° 06.846' N 97° 06.211' W	Pioneer field trial, South Dakota	US
13′	45° 09.774' N 93° 24.682' W	Manitoba	Canada
14′	49° 57.036' N 97° 00.068' W	Manitoba	Canada
15′	49° 32.998' N 97° 33.000' W	Manitoba	Canada

Appendix 8.3 Map showing location information for nodule collection sites from 2004 and 2005 as adapted from Farooq (2007).



Appendix 8.4 Pearson Correlation matrix results to determine parameter association with plant tissue nitrogen content (mg N plant^{-1}). Nodule number, total nodule DW, shoot DW, root DW, specific nodulation [nodules(g root DW)$^{-1}$], nitrogen concentration and total plant biomass were the parameters assessed.

Parameter	Nitrogen Content (mg N plant^{-1}) Correlation Coefficient
Nodule number	0.542
Nodule Dry Weight	0.765
Specific Nodulation [nodules(g root dry weight)$^{-1}$]	0.268
Nitrogen Concentration	0.699
Total Plant Biomass	0.794

Appendix 8.5 Original labels of samples used for Second Stage Testing. Labels are originally from Philom Bios Inc. and the University of Manitoba.

Isolate Label	Original label
1-2	V1-1/08/06
1-15	U4X3
1-17	U8X5
2-32	U6X1
2-33	U7X3
2-37	U52180705
2-38	U62190705
3-49	U63190705
3-50	U73190705
3-57	U125200705