The Abundance, Efficacy, and Diversity of *Rhizobium leguminosarum* bv. *viciae* Populations in Southern Manitoba Soils

BY

GEORGE NDIEMA CHEMINING'WA

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Plant Science
University of Manitoba
Winnipeg, Manitoba

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

THE ABUNDANCE, EFFICACY, AND DIVERSITY OF *Rhizobium leguminosarum* bv. *viciae* POPULATIONS IN SOUTHERN MANITOBA SOILS

BY

George Ndiema Chemining'wa

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

FOREWORD

This thesis is written in a traditional style. It includes an abstract, introduction, literature review, materials and methods, results, discussion, conclusions, references, and terminates with appendices.

DEDICATION

This thesis is dedicated to my wife (Truphenah) and my children (Dennis, Patricia, and Brian) for their selfless love and patience; and to mama Leah and Mzee Francis who inspired my interest in learning.

TABLE OF CONTENTS

Page

TABLE OF CONTENTS

Page

TABLE OF CONTENTS

Page

ABSTRACT

Chemining'wa, George Ndiema, Ph.D., The University of Manitoba, December 2001. The Abundance, Efficacy, and Diversity of *Rhizobium leguminosarum* bv. *viciae* Populations in Southern Manitoba Soils.

Inoculation of field pea is necessary in the absence of compatible rhizobial strains, when the rhizobial populations are low or symbiotically ineffective. The prevalence and efficacy of *Rhizobium leguminosarum* bv. *viciae* (the pea microsymbiont) in southern Manitoba soils is not known. Field experiments were conducted from 1998 to 2000 in multiple sites in southern Manitoba to characterize the abundance, efficacy, and diversity of indigenous *R. leguminosarum* bv. *viciae*. Uninoculated and inoculated (with and without N fertilizer) treatments of pea (*Pisum sativum* L.) were arranged in a randomized complete block design with four replicates in five sites (1999/2000). Pea was established in 21 sites in 1998 to trap indigenous rhizobia. Uninoculated and inoculated plants were evaluated for relative nodulation and growth. Pea and *Lathyrus* sp. isolates and commercial strains of *R. leguminosarum* bv. *viciae* were characterized by plasmid profile and polymerase chain reaction-restriction fragment length polymorphism of 16S-23S rDNA intergenic spacer analyses.

Uninoculated plants were well nodulated in cultivated soils, but not in virgin soils. Inoculation had no effect on nodulation in all sites but one. Inoculated plants accumulated significantly more biomass than uninoculated plants in 1998 and in four of five sites in 2000.

Out of 230 strains of *R. leguminosarum* bv. *viciae* typed, 77 distinct plasmid profiles were established. Plasmid profile diversity index (number of distinct

profiles/number of isolates typed) varied from 0.2 to 0.83 in sites with 10 or more isolates. Few pea isolates from previously inoculated sites had profiles that matched those of the inoculants. Each site shared at least one profile with 3 to18 other sites, but the number of profiles shared was independent of proximity.

Variation of the 16S-23S rDNA IGS regions was observed for all the rhizobial strains but two. Strains with the same plasmid profile did not always have similar chromosomal backgrounds and the converse was the case.

This research has shown that *R. leguminosarum* bv. *viciae* populations in southern Manitoba are abundant, diverse and competitive in nodulation, but may be less effective nitrogen fixers than commercial strains.

ACKNOWLEGEMENTS

I am grateful to Dr. J. Kevin Vessey for his generous support and guidance throughout the duration of this work. He and his family have been very kind to me. I thank Drs. D. Burton, G. Klassen and P. McVetty for serving on my advisory committee and for their valuable support and suggestions. I also thank Dr. M. Hynes for accepting to serve as my external examiner.

I am grateful to Bert Luit for invaluable technical support and friendship, and to the entire "Vessey Lab Gang" (Bert, Carla, Houman, Mavis, Michelle, Miles and Pan) for making life more interesting. Thanks are due to Pat Kenyon for doing nitrogen analysis of my samples, and to the faculty, support staff and graduate students of the Department of Plant Science for their support. I appreciate the support I got from my summer assistants (Jeff, Andrea, Chad, Jeanette, Michelle, Mike, Miles, and Darren) and Ms. Martha Blouw who helped me settle down on my arrival from Kenya. I am also grateful to Dr. Anh Phan for his advice on molecular data analysis, and to Dr. and Mrs. Henry Rempel, my host family, for their hospitality.

I thank the Canadian Commonwealth (my sponsor) and the University of Nairobi (my employer) for their support throughout my tenure in Plant Science Department. I also thank ARDI, Philom Bios (Saskatoon) and the Manitoba Pulse Growers Association for funding this research, as well as the farmers who permitted us to set up experimental plots on their farms.

Last but certainly not least, I thank my wife, Truphenah, and my children, Dennis, Patricia and Brian, for their unswerving love and loyalty throughout the four years we have been apart.

LIST OF TABLES

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF APPENDICES

Page

1.0 INTRODUCTION

The high economic and environmental costs associated with the heavy use of nitrogen fertilizers have prompted interest in biological nitrogen fixation, a microbial process which converts the atmospheric N_2 into a form usable by plants. Biological nitrogen fixation involving host specific symbiotic interactions between root nodulating bacteria, collectively termed rhizobia, and legumes has received the most attention because of its importance in the maintenance of soil fertility. Legume-rhizobial symbioses contribute at least 70 million metric tons of nitrogen annually to terrestrial ecosystems (Brockwell et al., 1995).

Several strategies aimed at enhancing and exploiting symbiotic nitrogen fixation by legumes in agricultural systems have been, and continue to be, pursued. The development of rhizobial inoculants remains, undoubtedly, the major agronomic contribution of scientific research on biological nitrogen fixation. Ideally, inoculation is required in the absence of compatible rhizobia, when the rhizobial population densities are low, or when resident rhizobia are less efficient at N_2 fixation than alternative (commercial) strains (Hansen, 1994). Growers in southern Manitoba are advised to always inoculate pea with compatible and symbiotically effective, commercially available strains with the objective of optimizing crop yields. However, there is evidence that at various low N sites in southern Manitoba pea did not require inoculation to attain the same yield as properly inoculated pea (D. McAndrew, pers. comm.). This observation

begs the question of the prevalence of *Rhizobium leguminosarum* bv. *viciae,* the endosymbiont of pea, in soils of southern Manitoba. *Rhizobium leguminosarum* bv. *viciae* also forms nodules on the roots of lentil, faba bean, *Lathyrus* and vetch. The presence of this bacterium has been well demonstrated in most soils tested in Alberta and British Columbia (Kucey and Hynes, 1989; Rice et al., 1993), but no such tests have been done on southern Manitoba soils. If *R. leguminosarum* bv. *viciae* is present in southern Manitoba, it may have arisen from commercial inoculants introduced at previous times, native strains nodulating native legumes such as vetch and *Lathyrus*, or hybrid descendants of commercial and native strains.

If *R. leguminosarum* bv. *viciae* populations are abundant in southern Manitoba soils, the next relevant question is how efficient they are relative to commercial inoculant strains. Significant numbers of *R. leguminosarum* bv. *viciae* strains in soil do not guarantee fixation of large amounts of atmospheric nitrogen. Strains from native sources may be effective pea nodulators, but inefficient in fixing atmospheric nitrogen (Bottomley, 1992). Previously introduced rhizobial inoculant strains may, after long residence in the soil, lose the very vital agronomic trait of fixing large amounts of N via genetic mutation or genetic exchange with native rhizobial strains or other soil bacteria. Most strains of *R. leguminosarum* bv. *viciae* resident in western Canadian soils appear to be ineffective in fixing nitrogen (Bremer et al., 1988; Rice et al., 1993), but little is known about the efficacy of strains resident in southern Manitoba soils. It is thus worthwhile to evaluate the symbiotic effectiveness of *R. leguminosarum* bv. *viciae* that

may be resident in southern Manitoba soils.

The genome of *R. leguminosarum* bv. *viciae* consists of the chromosome and one or several plasmids, and the genes for nodulation and nitrogen fixation are borne on a large plasmid termed the symbiotic plasmid (*pSym*). Characterizations based on variations in the chromosome and plasmids have shown that diversity of populations of *R. leguminosarum* bv. *viciae* isolated from nodules of pea and other legumes can be vast (Kucey and Hynes, 1989; Laguerre et al., 1992; Handley et al., 1998). However, no such studies have been carried out in southern Manitoba. Information on diversity of rhizobial populations would be useful in developing management strategies to optimize fixation by legumes.

A survey of many sites with varying ecological factors might give a good indication of the influence of the environment on the abundance, efficacy, and diversity of *R. leguminosarum* bv. *viciae* populations in southern Manitoba soils.

The objectives of this research project were:

1. To establish the presence and abundance of *R. leguminosarum* bv. *viciae* in southern Manitoba soils.

2. To establish the efficacy of resident *R. leguminosarum* bv. *viciae* populations in southern Manitoba soils.

3. To establish the genetic diversity of resident *R. leguminosarum* bv. *viciae* populations in southern Manitoba soils.

These objectives were pursued by conducting field studies for three years (1998-

2000) at multiple sites in southern Manitoba to determine the abundance of resident pea rhizobial populations and their symbiotic effectiveness relative to a commercial strain (PBC108, Philom Bios, Saskatoon, SK). Commercial strains of *R. leguminosarum* bv. *viciae* and nodule isolates collected from field sites were characterized for genetic diversity by plasmid profiling and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Only the diversity of resident soil rhizobial populations that formed nodules on pea roots was examined in this study. The results of the investigations are discussed in the context of both the environmental and selection pressures which may be affecting the abundance, diversity, and efficiency of *R. leguminosarum* bv. *viciae* in southern Manitoba soils and what the findings mean for pea production in Manitoba.

2.0 LITERATURE REVIEW

In studying the abundance, efficiency, and diversity of soil rhizobia it is necessary to review the environmental factors affecting rhizobial populations and strategies available to investigate and manage resident rhizobial populations and their symbiosis with host legumes. Hence, this review will include the ecology of rhizobia, the means for introducing rhizobia to the soil, DNA-based markers for characterizing rhizobia, and diversity of resident rhizobial populations and its management for agronomic benefit.

2.1 Ecology of Rhizobia

Groups of bacteria collectively termed rhizobia encompass the genera: *Allorhizobium*, *Azorhizobium, Bradyrhizobium*, *Rhizobium*, *Sinorhizobium,* and *Mesorhizobium*. *Sinorhizobium*, the microsymbiont of alfalfa, and *Mesorhizobium*, the microsymbiont of chickpea and *Lotus*, were recently recognized as separate from *Rhizobium* (de Lajudie et al., 1994; Young and Haukka, 1996; Amarger, 2001). The genus *Rhizobium* includes the *Rhizobium leguminosarum* biovars *viciae* (nodulates pea, lentil, vetch, faba bean), *trifolii* (nodulates clover), and *phaseoli* (nodulates common bean) (Hirsch, 1996). *Azorhizobium* is the microsymbiont for *Sesbania rostrata* (Dreyfus et al., 1988), while *Allorhizobium* is closely related to *Agrobacterium* and nodulates *Neptunia natans* (de Lajudie et al., 1998).

Many abiotic and biotic factors are known to influence both the soil rhizobial populations and the legume-rhizobial symbioses. The abiotic factors include soil pH, osmotic stresses, soil temperature, soil texture, soil carbon content, and soil nitrate-N, while the biotic factors include the presence of the host or non-host plants, the prevalence

of predators, and interactions with other soil microorganisms.

2.1.1 Abiotic Indicators

2.1.1.1 Soil pH

Under acid-soil conditions, resident soil populations of rhizobia are often low and the survival and persistence of inoculant strains are adversely affected. Most rhizobial strains will not grow at pH below 4.5 (Graham et al., 1982; Lowendorf and Alexander, 1983; Karanja and Wood, 1988; Rice et al., 2000a) and fast-growing rhizobia, with the exception of *Rhizobium tropici* which nodulates common bean and *Mesorhizobium loti* (formerly *Rhizobium loti*) which nodulates *Lotus* (Cooper, 1982; Graham, 1992), tend to be less tolerant than slow growing rhizobia. The alfalfa nodulating *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) strains are the most sensitive to soil acidity (Brockwell et al., 1991). Variations in tolerance to low pH occurs both between and within species of rhizobia, and acid tolerant strains of rhizobia have been isolated from root nodules (Graham, 1992). Acid tolerant strains have the capacity to maintain high cytoplasmic pH levels (Booth, 1985) and genetic studies suggest that pH genes may be located on the chromosome rather than on the plasmid (Chen et al., 1991). Studies have shown that rhizobial strains resident in acid-soils are not necessarily acid tolerant and, if they are, may not be more effective than acid sensitive strains in acid soils. Richardson and Simpson (1989) observed that most isolates recovered from an extremely acid soil were not more acid tolerant than laboratory strains and most tended to colonize the less acidic 2 cm top of the soil profile. Hence, the bulk soil pH does not represent the actual pH experienced by the microbes which may reside in specialized microsites (Paul and Clark,

1996).

Low pH may create aluminium toxicity which has the same adverse effects on rhizobial populations as soil acidity. Tolerance to aluminium varies markedly between and within species (Keyser and Munns, 1979; Flis et al., 1993), slow growers being more tolerant (Flis et al., 1993). Acid strains are not necessarily tolerant to aluminium and correlation between aluminium tolerance of strains and their survival in aluminium rich soils is sometimes absent (Lowendorf and Alexander, 1983).

Legumes often fail to nodulate under acid soil conditions, even where viable rhizobial populations can be demonstrated. Nodulation of pea was reported to be 10-fold more susceptible to acidity than either rhizobial multiplication or plant growth (Evans et al., 1980). Low soil pH adversely affects all aspects of rhizobial-legume symbiosis. The early stages in the infection process (e.g., rhizobial attachment to root hairs and root hair curling) which coincide with nod gene induction period are the most pH sensitive (Franco and Munns, 1982; Vargas and Graham, 1988). Rhizobial strains have been shown to differ in their ability to form nodules under acid-soil conditions (McKay and Djordjevic, 1993).

2.1.1.2 Soil Temperature

Rhizobia do not form heat-resistant spores and can not survive extremely high soil temperatures (Hirsch, 1996). The optimum temperature for growth for most rhizobia is in the range of 25°C and 30°C and excessively high temperatures adversely affect growth, survival and populations of soil rhizobia populations (Woomer et al., 1988). Temperature can affect the persistence of inoculant strains in storage and their survival in the soil.

Strains that persist at high soil temperatures may lose their infectiveness (Smith, 1992; Vlassak and Vanderleyden, 1997). Segovia et al. (1991) observed that non-infective isolates outnumbered those that were infective in bean rhizospheres. Curing or partial deletion of the plasmids upon exposure to high temperature have been observed among various rhizobial species (Toro and Olivares, 1986; Karanja and Wood, 1988), but strains of *Rhizobium tropici* IIB are known to be genetically stable (Martinez et al., 1991). The ability of rhizobial strains to withstand high temperatures appear to be favoured by dry rather than moist soil conditions (Graham, 1992) and is greater in soil aggregates than in non-aggregate soils (Ozawa et al., 1988). The specific mechanisms for heat tolerance are not well understood, although cryptic plasmid involvement has been suggested for some strains of *R. leguminosarum* bv. *trifolii* and *Sinorhizobium meliloti*. Elevated temperatures have been shown to induce heat shock proteins in strains of rhizobial species such as *S. meliloti* (Rusanganwa and Gupta, 1993) and *R. leguminosarum* bv. *trifolii* (Sen et al., 1990).

Rhizobia found in temperate and arctic soils survive freezing winter conditions (Lowendorf, 1980). Low-temperature tolerant rhizobia isolated from arctic legumes have a lower minimum temperature for growth (Bordeleau and Prévost, 1994; Drouin et al., 1998). Drouin et al. (1996) demonstrated that some strains of *R. leguminosarum* bv. *viciae* isolated from arctic *Lathyrus* species in Quebec were adapted for growth at low temperatures (5°C). In subsequent studies, cold-adapted strains produced more proteins than cold-senstive strains following cold-shock treatment at 0°C. Strains adapted to very low temperatures would confer an advantage over other rhizobia under climates where the cold periods of the growing season can be limiting for bacterial growth. Selection for

high or low temperature tolerant rhizobial strains may be beneficial for production of symbiotically dependent legumes (Karanja and Wood, 1988; Prevost and Bromfield, 1991; Hungria and Franco, 1993; Hungria *et al.*, 1993).

Extreme soil temperatures delay the early stages of the nodulation process (La favre and Eaglesham, 1986; Lynch and Smith, 1993), while air temperature affects photosynthesis and indirectly nitrogen fixation (Hansen, 1994). Evidence suggests that N_2 fixation is subject to a specific temperature optimum which varies among legume species and their endosymbionts (Graham, 1992).

2.1.1.3 Osmotic Stresses

Osmotic fluctuations associated with drying and wetting have a great impact on soil microbes, including rhizobia. Growth of rhizobia is inhibited under extreme moisture conditions (Mahler and Wollum, 1981; Mary et al., 1994). Mary et al. (1994) reported the death of *S. meliloti* on dessication and subsequent rehydration. Some reports suggest that rhizobia can survive in soil for many years after dessication (reviewed by Lowendorf, 1980). Slow growing rhizobial species have been shown to endure seasonal soil moisture deficits better than fast growing rhizobial species (Chatel and Parker, 1973; Woomer et al., 1988). In adverse soil moisture conditions, rhizobia may be protected in soil microsites (Lowendorf, 1980; Nazih et al., 1993) and the declining rhizobial numbers observed with increasing moisture content may be attributed to increased protozoan predation (Postma et al., 1989).

Osmotic stress due to drought or salt stress reduces nodulation by interfering with root hair growth and root hair curling (Worall and Roughley, 1976; Ikeda, 1994) even in

the presence of high rhizobial numbers. Moisture stress may also induce premature nodule senescence. If nodules lose more than 20% of their maximum fresh weight, nodule activity is permanently terminated (Peña-Cabriales and Castellanos, 1993). The bacterial strain may influence the sensitivity of the symbiosis to water stress. Studies have indicated that rhizobial strains may be more tolerant to elevated salinity than their host legumes (Tu,1981; Zahran and Sprent, 1986).

2.1.1.4 Soil Texture

Rhizobia tend to survive better in fine textured soils than in coarse textured soils. This is attributed partly to the fact that coarse textured soils are prone to water deficits, acidification, and nutrient deficiencies (Mahler and Wollum, 1981; Ozawa, 1988; Bottomley, 1992). Clay-rich soils provide protection for rhizobial populations especially in soils exposed to elevated temperature, severe water stress, and aluminium toxicity or low pH (Mahler and Wollum, 1981; Hartel and Alexander, 1983; England, et al., 1993). In addition, clay microniches can provide protection for rhizobia against predation by protozoa (Heijnen et al., 1991). Strains of *Bradhyrhizobium japonicum* and *R. leguminosarum* bv. *trifolii* have been reported to decline in cell size following prolonged incubation in the soil (Crozat et al., 1982), possibly so as to fit into small clay pores and avoid adverse matric water potential or predation by protozoa (Bottomley, 1992). Strains have been shown to vary in nodule occupancy under different soil types (May and Bohlool, 1983).

2.1.1.5 Soil Nitrate-N

Rhizobia can utilize various compounds of nitrogen for growth, including nitrate, ammonium and amino acids (Elkan and Kwik, 1968; Chakrabarti et al., 1981). In a free-living state, rhizobia are not capable of utilizing atmospheric nitrogen, except *Azorhizobium caulinodans* which forms stem nodules on *Sesbania rostrata* (Dreyfus et al., 1988). The ability to utilize various nitrogen sources varies between and within species of rhizobia. For example, whereas the amino acid glutamate can be utilized as a nitrogen source by most rhizobia, only a few rhizobial strains can utilize glycine (Elkan and Kwik, 1968; Chakrabarti et al., 1981).

It is widely acknowledged that inorganic forms of nitrogen, especially nitrate, interfere with nodulation and nitrogen fixation processes in legumes. There have been reports of reduced number of nodules (Rai, 1992), delayed nodulation (Herridge et al., 1984), and even complete cessation of nodulation in the presence of nitrate (da Silva et al., 1993). The mode of action of nitrate in reducing nodulation is not clearly understood, but carbohydrate deprivation in the nodules as a result of the energy required for nitrate reduction is one of the major explanatory hypotheses (Havelka et al., 1982). Nitrate tolerance in legumes occurs naturally (Herridge and Betts, 1988) and can be induced by genetic manipulation. Ethyl methyl sulphonate mutagenesis has been used to generate nitrate tolerance (supernodulation) in crops such as soybean (Carrol et al., 1985). In subsequent studies, these soybean cultivars were shown to be partially tolerant and to have reduced mineral uptake (Eskew et al., 1989).

In nitrogen depleted soils, especially in the tropics, a moderate dose of fertilizer N at planting may have a stimulatory effect on legume nodulation and N-fixation. This

"starter" N would promote growth in the period between root emergence and the onset of active N-fixation (Giller and Cadisch, 1995). The use of "starter" N may not apply to the Canadian prairie soils which have moderate residual soil N levels. Pea farmers in southern Manitoba are advised not to apply N-fertilizer if they have inoculated their crops with the expectation that the residual soil N and the atmospheric fixed N would meet the crop N-needs. Waterer et al. (1994) reviewed several experiments on yield response to N-fertilizer application and noted positive responses in only 12 of 81 site years of pea. Experiments carried out with 4 site years of pea in Saskatchewan showed no response to N-fertilizer (Cowell et al., 1989); however, a previous study by Sosulki and Buchan (1978) had shown an increase in pea yield with N-fertilizer application. Similar studies in Alberta showed no yield response by pea to N-fertilizer application (Izaurralde et al., 1990).

2.1.1.6 Pesticides

Since pesticide use has become an integral part of intensive agriculture, there are concerns about the possible ecological consequences of this practice on beneficial soil microorganisms such as the root nodule bacteria (Castro et al., 1997). It is not common under field conditions for herbicide and post-emergence agrochemical applications to deleteriously affect rhizobial populations. However, certain fungicides, employed as chemical seed protectants in legumes, have been shown to markedly inhibit nodulation and the growth of rhizobia. These fungicides include carbendazim, carboxin, mancozeb, chloranil, hymexazol, iprodione, metalaxyl, and thiram (Rennie and Dubetz, 1984; Revellin et al., 1993; Castro et al., 1997). Castro et al. (1997) reported that mancozeb

reduced growth of *Rhizobium* sp USDA 3187 by 50% in pure culture, but there was no effect on yield of peanut under field conditions, suggesting that the soil environment ameliorated the adverse effect of mancozeb on bacterial growth. Several attempts to find effective rhizobia resistant to chemical seed protectants have not been successful, although Andrés et al. (1998) have recently reported isolating effective thiram-resistant soybean *Bradyrhizobium japonicum* strains. Soil fumigants such as formaldehyde (Sreenivasa and Bagyaraj, 1989) and Dazomet^{R} (Hirsch, 1996) have also been reported to reduce rhizobial populations.

2.1.1.7 Carbon Sources

Rhizobial populations generally flourish in fertile, carbon-rich environments. Studies have demonstrated that soil microbial biomass increases in proportion to soil organic carbon (Jenkinson and Ladd, 1981; Paul and Clark, 1996) and so does rhizobial biomass. Thies et al. (1992) reported an inverse relationship between nodule occupancy by inoculants on leguminous crop hosts and soil organic matter. The carbon-rich soils may have had large populations of resident rhizobia which numerically out-competed the inoculant strains.

Rhizobia are saprophytic microbes able to utilize sugars, sugar alcohols (glycerol, mannitol, dulcitol), organic acids (malate), and aromatic compounds. Malate is considered the main carbon and energy source which supports nitrogen fixation (Hansen, 1994) and *Bradhyrhizobium* species can not utilize citrate unlike other rhizobial species. Differences in enzyme activity account for the variations in utilization of carbon. Rhizobia that efficiently utilize a broad range of carbon sources would be advantaged

under carbon-limited environments (Parker at al., 1977; Vlassak and Vanderleyden, 1997).

2.1.2 Biotic Indicators

2.1.2.1 Presence of Host legumes and Other Crops

Growth of rhizobia is preferentially stimulated in the rhizospheres of legumes compared to those of the non-legumes. This is attributed to the large number of substances released into the rhizosphere, consisting primarily of sugars, amino acids, and vitamins such as biotin and pantothenic acid (Rao, 1995). Host plants produce micromolar concentrations of secondary metabolites such as trigonelline (Boivin et al., 1991), stachydrine, (Sande et al., 1995), homoserine (van Egeraat, 1975), and flavones (Hartwig et al., 1991). Rhizobia that have evolved catabolic functions to benefit from these secondary metabolites have a nutritional advantage in both the rhizosphere and in the infection thread within nodules (Vlassak and Vanderleyden, 1997). Hence, legumes have been shown to stimulate more proliferation of rhizobia that infect them than those that do not (Kucey and Hynes, 1989; Bushby, 1993; Thies et al., 1995). Host pea plants produce homoserine which is a selective substrate for *R. leguminosarum* bv. *viciae* (van Egeraat, 1975), while *S. meliloti* strains (Sande et al., 1995) have the capacity to use stachydrine as an energy source.

Two inositol-based carbohydrates, *L-3-O*-methyl-*scyllo*-inosamine, and *scyllo*-inosamine, termed rhizopines have been discovered (Murphy et al., 1987; Saint et al., 1993). Rhizopines are synthesized in bacteroids within nodules and are, therefore, not under the control of the host plant like the secondary plant metabolites mentioned earlier.

These compounds can be specifically utilized as the sole source of nitrogen or carbon by the rhizobia strains that produce them. Metabolism of rhizopines has been demonstrated in some strains of *S. meliloti* and *R. leguminosarum* bv. *viciae* (Saint et al., 1993; Murphy et al., 1995; Wexler et al., 1995). A survey showed that only 10% of *S. meliloti* and 14% of *R. leguminosarum* bv. *viciae* were capable of catabolizing rhizopines (Murphy et al., 1995). Strains with the capacity to metabolize rhizopines have been reported to persist in the soil for long and to be highly competitive for nodule occupancy (Murphy et al., 1995; Wexler et al., 1995). This could be attributed to the ability of these strains to proliferate in the nodules and in the soil by catabolizing the rhizopines in the infection threads and the rhizopines released into the soil upon nodule senescence (Hansen, 1994; Murphy et al., 1995; Wexler et al., 1995).

In the absence of the host legumes, soil rhizobial populations decline but they can survive for 19 to 54 years (Rao, 1995). Different species and biovars within the genus *Rhizobium* can coexist in low densities in soil for many years (Kucey and Hynes, 1989). However, it has been shown that the presence of the host changes the population dynamics of rhizobia in the soil (Kucey and Hynes, 1989; Thies et al., 1995). In studies in southern Alberta, Kucey and Hynes (1989) reported 10 to 100 times more *R. leguminosarum* bv. *viciae* in pea fields than in common bean or wheat fields. Similarly, *R. leguminosarum* bv. *phaseoli* populations were 100 to 1000 times higher in common bean fields than in pea or wheat fields. The enrichment of the rhizobial populations by the homologous host legumes can be attributed to proliferation in the homologous host rhizosphere, but the bulk of the increase is likely due to the bacteria released from senescing nodules (Bottomley, 1992; Thies et al., 1995; Hirsch, 1996). The root nodules

provide a conducive environment, akin to pure culture, in which rapid multiplication of rhizobia occurs (Brockwell et al., 1995). The rhizobia cells which differentiate into bacteroids in the root nodule appear to be non-viable, but the cells that remain undifferentiated in the nodules are viable. Viable bacterial cell number per nodule can reach 10^6 for *Rhizobium* species and above 10^7 for *B. japonicum* (Tsien et al., 1977; Hirsch, 1996). Once the nodules senescence, these large numbers of viable bacterial cells are released into the soil and usually become a persistent component of the soil microbial population (Kuykendall, 1989; Thies et al., 1995). Kuykendal (1989) reported 10 times more inoculant cells in soils that nodulated plants had been left in the field for six months (for nodules to senesce) after soybean maturity than in soils in which soybean plants had been uprooted. It is worth noting that the ability of senescing nodules to drastically change the rhizobial sub-population structure may be limited under carbon-rich pasture and minimum tillage systems which have the potential to maintain larger rhizobial populations than conventionally tilled soils (Bottomley, 1992)

Significant numbers of compatible rhizobia may still be found in soils that have no history of cultivation of the target legume host. This may be attributed to previous vegetation patterns that may have included specific host legumes, or rhizobia invading from nearby sites through seeds and airborne dust (Hirsch, 1996; Parker et al., 1977). It is conceivable that cultivation implements may spread rhizobia from one field to another.

2.1.2.2 Microbial Antagonism and Parasitism

2.1.2.2.1 Bacteriocin Production

Bacteriocins are protein-based antimicrobial compounds of microbial origin whose activity is often restricted to closely related strains. Bacteriocin producing strains of rhizobia are commonly found in soils and have been reported for *R. leguminosarum* biovars *viciae*, *phaseoli* and *trifolii*, *Rhizobium lupini*, and *Bradhyrhizobium japonicum* (Gross and Vidaver, 1978; Johnston et al., 1978; Hirsch, 1979; Joseph et al., 1983; Oresnik et al., 1999; Venter et al., 2001; Wilson et al., 1998). Many reports suggest that rhizobial strains produce bacteriocins in order to relieve pressure of competition for identical but limited soil resources with closely related strains (Hirsch, 1979; Hogson et al., 1985; Wilson, 1998). Wilson et al. (1998) found that all the isolates of *R. leguminosarum* bv. *viciae* from one particular site were of the same genotype based on plasmid and RAPD profiles and they produced a potent bacteriocin to which most of the strains from the other sites were sensitive. When sampling was done 1 and 2 years later, the bacteriocin producing strain was absent but the new isolates were not affected by the bacteriocin produced by the first strain. The authors believed that the bacteriocin produced by the first strain was responsible for its dominance in that year and that the other bacteria responded with subsequent proliferation of resistant strains. In *R. leguminosarum bv. viciae*, the genes coding for bacteriocin production have been recorded on plasmids (Johnston et al., 1978; Hirsh, 1979; Venter et al., 2001). Trifolitoxin, produced by *R. leguminosarum* bv. *trifolii* T24, is the best characterized bacteriocin and has been shown to inhibit all species of *Rhizobium* species tested (Maier and Triplett, 1996).

2.1.2.2.2 Protozoan Predation

Laboratory studies have demonstrated that protozoa cause large declines in populations of rhizobia introduced into the soil (Chao and Alexander, 1981; Lennox and Alexander, 1981). However, protozoa do not eliminate all the rhizobia, instead they leave a low but stable population of cells similar to rhizobial numbers encountered in non-sterilized soils (Danso and Alexander, 1975). Addition of clay (e.g., bentonite) improves the survival of rhizobia, possibly because clay particles provide refuge for rhizobia to which protozoa have limited access (Heijnen et al., 1991). In contrast, high moisture content makes protozoa to be more mobile and rhizobia, therefore, become more available for protozoan predation (Postma et al., 1989). Nodule occupancy by inoculant strains in the presence of resident strains might be greatly diminished in protozoa-rich soils. Rhizobial inoculants applied to soils or seeds would be reduced rapidly by protozoan predation, eliminating the advantage of the inoculant strain (Vlassak and Vanderleyden, 1997). However, Bottomley (1992) cautioned that protozoan predation of both indigenous and introduced rhizobia needs to be examined in the bulk soil and in rhizospheres, because the soil distribution of indigenous and introduced rhizobia is different.

2.1.2.2.3 Bacteriophages

Scientific interest in bacteriophages infecting *Rhizobium* species has been prompted mainly by their potential role in the ecology of their hosts and their ability to perform genetic exchange between host strains (Kankila and Lindstöm, 1994). The lytic phases of rhizobiophages have been shown to reduce rhizobial populations, but high

phage concentrations would be required to reduce nodulation (Evans et al., 1979). In fact, Lawson et al. (1987) observed that rhizobiophage counts in clover fields were positively correlated with rhizobial counts and vegetation height. Differences in susceptibilities to rhizobiophages exist in rhizobia. Phages may reduce populations of the susceptible strains of rhizobia giving an advantage to phage resistant strains which end up occupying the nodules (Evans et al., 1979; Hashem and Angle, 1988). Thus, the significance of rhizobiophages in the field most probably lies in the competitiveness of rhizobial strains. Although the level and importance of phage-mediated genetic exchange (transduction) in resident populations of rhizobia is not known, it appears that this phenomenon is common in temperate *Sinorhizobium meliloti* strains (Kankila and Lindstöm, 1994).

2.1.2.2.4 Mycotoxins

Indigenous soil fungi of the genera *Aspergillus*, *Fusarium*, *Paecilomyces*, and *Penicillium* have been shown to suppress rhizobial growth (Chhongai and Subba-Rao, 1966; Sethi and Subba-Rao, 1968). These antagonistic effects on rhizobia have been attributed principally to mycotoxin action, but competition, predation, and parasitism may also be involved (Mahmoud and Abd-Alla, 1994). Mycotoxins are produced by indigenous soil fungi capable of degrading a wide range of organic compounds (Bell and Crawford, 1967), and may be introduced into the soil by contaminated leguminous seeds and crop residues (Saber, 1992; Mahmoud and Abd-Alla, 1994). Mahmoud and Abd-Alla (1994) found aflatoxins in 11 out of 100 faba bean (*Vicia faba* L.) seed samples tested and application of aflatoxins to pot soils sown to faba bean (100 or 200 $\mu g\ Kg^{-1}$ soil) caused a decline in nodulation and nitrogenase activity. However, this experiment used

more than three times the aflatoxin concentration found on the faba bean seeds which perhaps would not have affected nodule number and function. Mycotoxin-resistant rhizobia could possibly have an advantage in occupying nodules in mycotoxin-rich soils or in cases where the seeds are contaminated with mycotoxins (Vlassak and Vanderleyden, 1997).

2.1.2.3 Microbial Synergism

2.1.2.3.1 *Penicillium bilaii*

Penicillium bilaii (formerly *P. bilaji)* is a naturally occurring rhizospheric fungus first isolated from southern Alberta and has been demonstrated to solubilize calcium phosphate (Kucey, 1983; Kucey et al., 1989) and improve P uptake by plants. The phosphorous solubilization ability of this fungus has been attributed to its production of organic acids (oxalic acid and citric acid) (Kucey et al., 1989), but the mechanism(s) by which it improves P uptake is not well understood. Recent studies suggest that it may have an effect on root architecture (Gulden and Vessey, 2000; Vessey and Heisinger, 2001). *P. bilaii* can survive under field conditions, even during winter, and colonizes mainly the top 10 cm of the soil profile (Keyes, 1990).

Inoculation of field pea and lentil with a commercial inoculant of *P. bilaii* (PB-50), available in Canada since 1993 (Rice et al., 2000b), has been shown to increase nodulation and N uptake (Gleddie, 1993). *P. bilaii* has successfully been co-cultured with *S. meliloti*, demonstrating that the fungus is not antagonistic to rhizobia (Rice et al., 1994). Rice et al. (2000b) reported increased nodule occupancy with dual inoculation with *S. meliloti* strain NRG-34 and *P. bilaii* PB-50. The response to inoculations with

separate NRG-34 and PB-50 inoculant products was the same as the response to the co-cultured inoculants. They concluded that use of a single inoculation culture would provide the ultimate convenience to farmers.

2.1.2.3.2 Vesicular-Arbuscular Mycorrhizae (VAM)

Vesicular-arbuscular mycorrhizae are symbiotic associations between plants and fungi that colonize the intracellular cortical tissues of roots during periods of active growth (Sylvia, 1998). The VAM symbioses are capable of improving plant growth by acquiring nutrients in nutrient-poor or moisture-deficient soils (Brockwell et al., 1995; Paul and Clark, 1996). Almost all the legumes are mycorrhizal, and the interaction between VAM fungi and legume-rhizobial symbioses has been reported to be synergistic. The infection of legume roots by rhizobia and VAM fungi occurs at the same time, but the two do not appear to compete for infection sites on legume roots (Barea and Azcón-Aguilar, 1983). Dual inoculation of legumes with rhizobia and mycorrhizae has been reported. Thiagarajan and Ahmad (1993) demonstrated that introduced *Bradyrhizobium* species occupied more nodules than indigenous rhizobia in the presence of VAM fungi. The mechanism for this alteration in nodule occupancy is not clear.

2.1.2.3.3 Plant Growth Promoting Rhizobacteria

Plant growth-promoting rhizobacteria (PGPR) refers to free living bacteria known to enhance plant growth when applied to seeds, tubers, or roots (Glick, 1995). Some of the genera with known PGPR include *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Clostridium*, *Enterobacter*, and *Pseudomonas* (Kapulnik, 1991; Glick, 1995). There are

two mechanisms by which PGPR are thought to act on plant growth (Glick, 1995). One, PGPR may suppress the deleterious effects of soil phytopathogens through production of siderophores and antibiotics or by aggressive competition for limited soil resources. Two, PGPR may act directly by synthesizing plant growth promoting substances or by enhancing uptake of specific nutrients. There have been reports of positive effects of PGPR on nodulation and growth of legumes. Nodule occupancy by *Bradyrhizobium japonicum* USDA110 (Fuhrmann and Wollum, 1989) was shown to increase in the presence of fluorescent *Pseudomonas* species. This was attributed to improved iron availability as a result of siderophore production. Li and Alexander (1988) observed that nodulating *Sinorhizobium meliloti* and *B. japonicum* with antibiotic-producing *Bacillus* and *Pseudomonas* greatly improved nodulation relative to the rhizobial strains alone. They attributed this to the elimination of rhizosphere microorganisms that were antagonistic to *S. meliloti* and *B. japonicum* strains. Buss (1998) showed that *Bacillus cereus* UW85 stimulated nodulation in soybean indirectly by stimulating root growth. *Azospirillum* has also been shown to stimulate rhizobial nodulation (Yahalom et al., 1990).

2.2 Inoculation

The major agronomic benefit accruing from scientific research on biological nitrogen fixation has been the development of rhizobial inoculants (Giller and Cadisch, 1995). Inoculation of legumes with rhizobial inoculants is necessary in the absence of compatible rhizobia or where the rhizobial population density is very low, and if the resident rhizobia are less effective than alternative (commercial) strains (Hansen, 1994;

Brockwell et al. 1995; Giller and Cadisch, 1995). Soils lacking in compatible rhizobia are found in areas where indigenous related legumes are absent or where levels of pH, osmotic stress, high temperature, and heavy metals are detrimental to rhizobial populations (Catroux et al., 2001).

Inoculants are commercially available in powder, liquid, and granular formulations. Application of peat-based inoculants to the seed surface prior to planting is the most common means of inoculation. However, this method may not be efficient in the presence of seed coat toxicity, when it is too dry, or when the seed is dressed with incompatible pesticides (Kremer et al., 1982; Salema et al., 1982; Materon and Weaver, 1984; Smith, 1992; Brockwell et al., 1995). An inoculation study on lupin showed that 95% of the original bacteria present in peat inoculant (with seed adhesives) died during inoculation and sowing and further 83% of the remaining bacteria lost viability during the following day in the soil (Roughley et al., 1993). Brockwell et al. (1995) observed that the likelihood of nodulation following rhizobial inoculation of a legume seed can be greatly improved by reducing the inoculant mortality rate, increasing the rate of inoculant application or decreasing the time to germination. Seed pelleting, which involves applying peat inoculant on the seed and then coating with lime or rock phosphate, may provide better survival of rhizobia especially in acid soils. In addition, this method provides the ultimate in convenience to the farmer who can buy preinoculated seed (Hansen, 1994).

In contrast to seed inoculation, soil applied inoculants allow a substantially greater delivery rate of rhizobia, eliminate potentially detrimental seed mixing, and are characterised by a greater resistance to low moisture conditions (Hansen,1994). Granular

inoculation is particularly useful when seeds are dressed with pesticides which are toxic to rhizobia (Ramos and Ribeiro, 1993). Soil applied inoculants are usually delivered into the seed furrow directly, but rhizobia may also be successfully introduced into the soil via irrigation water. Ciafardini et al. (1992) demonstrated increased nodulation and nitrogen fixation in soybean by seed inoculation followed by cover inoculation through irrigation water when the plants were at the three-node phenological stage. They attributed this to improved mobility of the rhizobial strain, resulting in more infections and nodulation on secondary roots of soybean plants.

To optimize agronomic benefits from inoculation, high quality standards for the manufacture of inoculants are needed. The quality of inoculants is determined by enumeration of viable rhizobia by plate counts or determination of the most probable number on plants (Vincent, 1970). Catroux et al. (2001) observed that the quality of inoculants sold on the market worldwide remains poor yet existing technologies are able to produce high quality inoculants with a shelf-life of more than one year. However, the authors noted that good quality inoculants are available for use by farmers in North America, Europe and a few other countries.

2.3 DNA-based Markers for Characterizing Rhizobia

2.3.1 Plasmids

The genomes of *Rhizobium* and *Sinorhizobium* consist of the chromosome and circular extra-chromosomal elements known as plasmids. Large molecular weight plasmids, and even extra-large plasmids (megaplasmids), can be easily visualized by electrophoresis of cell lysates on Eckhardt agarose gels (Hynes et al., 1986; Wheatcroft et

al., 1990). *Rhizobium* and *Sinorhizobium* strains harbour 1 to 10 plasmids (Amarger 2001) ranging in size from less than 100 kb to 1500 kb (Sutton, 1974; Martínez-Romero and Caballero-Mellado, 1996). Based on assumed chromosome sizes, plasmid DNA can constitute up to 50 % of the cell genome (Prakash and Atherly, 1986; Sobral et al., 1991). Most genes involved in host recognition, nodulation and nitrogen fixation in *Rhizobium* and *Sinorhizobium* are located primarily on a single plasmid, the symbiotic plasmid or *pSym* (Banfalvi et al., 1981; Lamb et al., 1982; Hirsch, 1996; Mazurier and Laguerre, 1997). The *pSyms* in *R. leguminosarum* bv. *viciae* strains range in size from about 160 to 900 kb (Hynes et al., 1989; Laguerre et al., 1992). The non-symbiotic plasmids are termed cryptic plasmids.

Plasmids are rare in *Bradyrhizobium* species and, if present, do not the carry genes essential in symbiotic function. Kündig et al. (1993) have mapped the symbiotic genes of *B. japonicum* to a 400 kb region within the bacterial chromosome. Among the *Mesorhizobium* species, symbiotic genes are chromosomal in *Mesorhizobium loti* and plasmidic in *Mesorhizobium plurifarium* and *Mesorhizobium huakaii* (Sullivan et al., 1995; Amarger, 2001). Using symbiotic probes, Mazurier and Laguerre (1997) did not find symbiotic plasmids in wild strains of *R. leguminosarum* bv. *viciae* that nodulated vetch. They concluded that either these strains carried symbiotic genes on the chromosome or on a plasmid larger than the *pSyms* previously described. Nonsymbiotic rhizobia lack symbiotic plasmids, but are taxonomically closely related to the populations of symbiotic bacteria (Segovia et al., 1991).

Symbiotic plasmids play other essential roles apart from nodulation and nitrogen fixation. Genes for catabolism of homoserine in *R. leguminosarum* bv. *viciae* (Johnston et

al., 1988) are encoded on the *pSym*s, demonstrating that *pSym*s can contribute to the saprophytic competence of rhizobia in the soil (Hirsch, 1996). Since cryptic plasmids often constitute a relatively larger part of the extrachromosomal DNA pool than the symbiotic plasmid, questions about whether they carry essential functions or whether they are just a burden to the bacterial cell have been of interest to rhizobiologists. Studies have shown that *R. leguminosarum* bv. *viciae* cryptic plasmids are essential for the formation of functional nodules (Hynes and McGregor, 1990). Similarly, plasmid-cured strains of *R. leguminosarum* bv. *trifolii* were demonstrated to lose the ability to catabolize various carbon substrates (Baldani et al., 1992).

Plasmid profile analysis has been utilized to examine strain heterogeniety in field rhizobial isolates obtained from pea, clovers, faba bean, lentil, common bean, alfalfa, and other leguminous plants (Harrison et al., 1989; Kucey and Hynes, 1989; Hynes and O'Connell, 1990; Laguerre et al., 1992; Kuykendall et al., 1996) . This method is considered fast and reliable in characterising rhizobia at the subspecies level.

2.3.2 Restriction Fragment Length Polymorphisms (RFLPs)

Restriction endonucleases that recognize specific 4 to 6 bp sequences and cleave DNA at these sites have played a prominent role in genomic DNA analysis (Pepper and Josephson, 1998). Variations in the genomic DNA sequences create restriction fragment length polymorphisms (RFLPs) which show up on an ethidium bromide-stained agarose gels as different restriction patterns. The similarities between the restriction patterns indicate the relatedness of isolates. Total DNA restriction profiles have been used to distinguish rhizobial isolates (Hynes and O'Connell, 1990; Hartman and Amarger, 1991;

Laguerre et al., 1992), but the use of this method is limited because it generates complex banding patterns.

Restriction enzymes used in conjunction with DNA probes can reveal divergence in DNA sequences of organisms. Hybridization of the Southern blotted total DNA fragments with specifically labelled chromosomal or symbiotic DNA probes produces simple restriction patterns and reveals information about specific DNA regions. Southern RFLPs have been used to type rhizobial strains (Engvild et al., 1990; Demezas et al., 1991; Laguerre et al., 1994). The use of this technique has, however, diminished as more rapid and economical polymerase chain reaction (PCR)-based techniques have been developed. Navarro et al. (1992) observed that traditional RFLP analyses were not appropriate for routine identification of bacterial isolates. The PCR-based techniques include random amplified polymorphic DNA (RAPD), repetitive (Re)-PCR, and PCR-RFLP. These techniques are very sensitive, require small quantities of DNA, and do not involve probe labelling and hybridization procedures.

2.3.3 PCR-based Methods

2.3.3.1 Rep-PCR

Repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) elements are subclasses of families of short (<200 bp) intergenic repetitive sequences interspersed throughout prokaryotic genomes. The ERIC and REP elements, also known as palindromic units and intergenic repeat units, respectively, contain highly conserved palindromic inverted repeat sequences (de Bruijn, 1992; Judd et al., 1993). Pairs of specific oligonucleotide primers (two 20 bp each) designed from these

sequences and the PCR reaction can be used to amplify defined segments between copies of ERIC and REP. Agarose gel electrophoresis of the multiple amplified fragments results in DNA fingerprints specific for the target prokaryote (de Bruijn, 1992; Pepper and Josephson, 1998). de Bruijn (1992) demonstrated that PCR-based DNA fingerprinting using REP and ERIC primers was effective in the identification and classification of bacterial strains, including various strains of rhizobia. The usefulness of rep-PCR finger printing in characterizing rhizobial populations has since been well established (Madrzak et al., 1995; Niemann et al., 1997; Del papa et al., 1999; Santamaria et al., 1999). Rep-PCR fingerprinting permits rapid processing of large samples and its ability to discriminate isolates is comparable to the RAPD technique.

2.3.3.2 RAPD

The random amplified polymorphic DNA (RAPD) technique uses short arbitrary oligonucleotide primers to amplify DNA fragments of variable lengths throughout the genome. The randomly amplified fragments are separated on an agarose gel based on their size and the sequence variations revealed by the number and length of the amplified products. The generated DNA finger prints are used to determine similarities among isolates using numerical analytical techniques (Young and Cheng, 1998). The RAPD technique has been shown to effectively differentiate genomes of a diverse collection of *S. meliloti*, *R. leguminosarum* bv. *trifolii*, *R. leguminosarum* bv. *viciae*, *Sinorhizobium fredii*, and *B. japonicum* (Harrison et al., 1992; Richardson et al., 1995; Handley et al., 1998; Young and Cheng, 1998). The quality of phylogenetic information obtained from RAPDs is similar to that obtained from conventional RFLP techniques, but the RAPD

technique avoids fastidious DNA extraction and hybridization and prior knowledge of the target sequences is not necessary (Harrison et al., 1992). One of the criticisms of the RAPD technique is the frequent appearance of non-specific bands in PCR amplification. This problem can be minimized by repeating specific amplifications in order to obtain valid interpretations about similarity levels among isolates.

2.3.3.3 PCR-RFLP

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, which involves amplifying defined regions with specific primers and digesting the amplified products with restriction endonucleases, has become an increasingly important tool for differentiating rhizobial isolates as more DNA sequences are defined (Laguerre et al., 1994; Nour et al., 1994; Laguerre et al., 1996). Ribosomal DNA sequences are conserved and can be used to design primers, but they also have variable regions that can be utilized to infer phylogenies and to differentiate closely related bacterial isolates. Variation in 16S rRNA genes can be determined by RFLP analysis of 16S rDNA amplified by PCR. This method is commonly used to differentiate between species of root nodule bacteria (Laguerre et al., 1994). The RFLP analysis of amplified symbiotic *(nod* and *nif)* gene regions has been demonstrated to effectively differentiate *Rhizobium* isolates within and between species (Laguerre et al., 1996; Haukka et al., 1998).

Sequences for 16S rDNA are highly conserved among proteobacteria and analysis of genetic variations in these regions can not effectively differentiate isolates at the intraspecies level (Woese, 1987). However, the intergenic spacer (IGS) sequences

between 16S rDNA and 23S rDNA are highly variable and can, thus, be used to determine chromosomally encoded genetic variations among strains of the same species (Barry et al., 1991, cited by Laguerre et al., 1996). RFLP analysis of 16S-23S rDNA IGS sequences has been used to discriminate strains within various rhizobial species, including pea *R. leguminosarum* bv. *viciae* (Laguerre et al., 1996) and chickpea *Mesorhizobium ciceri* (Nour et al., 1994). This technique has the same discriminating power as total DNA restriction analysis and RFLP analysis using chromosomal and symbiotic gene probes.

2.4 Diversity of Rhizobial Populations as Revealed by DNA Markers

Different DNA techniques have been used to characterize rhizobial populations under field soil conditions. Analyses of plasmid profiles (Laguerre et al., 1992; van Berkum et al., 1995; Handley et al., 1998), total DNA restriction profiles (Laguerre et al., 1992), Southern RFLPs of plasmid-encoded symbiotic regions (Laguerre et al., 1993), RAPD profiles (Handley et al., 1998), rep-PCR profiles (de Bruijn, 1992; Laguerre et al. 1996), and RFLP analysis of 16S-23S rDNA IGS (Nour et al., 1994; Laguerre et al., 1996) have demonstrated rich genetic diversity among rhizobial populations.

The level of diversity of rhizobial populations can vary within and among sites. Handley et al. (1998) characterised numerous isolates of *Rhizobium leguminosarum* from 15 different sites in Britain by plasmid profile and RAPD-PCR analyses and observed large variations in diversity between the sites, including those in very close proximity. The diversity index (number of RAPD profiles/total number of isolates typed) varied from less than 0.1 to above 0.65. This index was used to indicate the level of diversity of

rhizobial isolates at each site. The diversity of isolates varied with sampling time since it was rare to reisolate the same profiles in subsequent years. DNA fingerprinting and plasmid profile analysis of *Rhizobium etli* isolates obtained from wild beans showed huge diversity within plants and among and within sites in northwestern Argentina (Aguilar et al., 1998). Variations in the diversity of populations among sites has also been reported for rhizobia nodulating clover (Hagen and Hamrick, 1996).

The composition of rhizobial populations may be modified by soil ecological factors. Anyango et al. (1995) reported that thirty out of 35 isolates from *Phaseolus vulgaris* L. grown in a low pH soil in Kenya comprised the acid tolerant *Rhizobium tropici* strains but this species of *Rhizobium* was almost absent in a near neutral pH soil where *R. etli* and *R. leguminosarum* bv. *phaseoli* predominated (40/41). Brockman and Bezdicek (1989) studied the effects of topography on the diversity of pea rhizobia isolates using serology and plasmid profiling. The distribution of serogroups and plasmid profiles among isolates obtained from pea grown on the south slope and pea grown on the bottomland was different. The authors speculated that the differences in soil microclimate between the two topographical positions, cooler and wetter on the bottomlands, created the observed variations in the rhizobial populations. Labes et al. (1996) used ERIC-PCR technique to confirm that slope position was a major factor influencing the composition of the populations of *R. leguminosarum* bv. *viciae* nodulating pea. The treatment of the soils with polluted or non polluted slurry led to changes in rhizobial populations; ERIC-PCR profiles (i.e. strains) found in non-polluted soils were absent in the polluted soils and new profiles (i.e. strains) appeared in the slurry-polluted soil. N-fertilizer application has been shown to limit the genetic diversity among isolates of some common bean

cultivars in Mexico (Caballero-Mellado and Martinez-Romero, 1999).

Host species, cultivars, and even individual plants within cultivars may show preferences for certain rhizobial strains. Pea and faba bean exhibited strong preference for certain specific plasmid profiles of *R. leguminosarum* bv. *viciae* present in two Alberta soils (Hynes and O'Connell, 1990). The plasmid profile group that formed most nodules on pea did not form nodules on faba bean and the converse was true, whereas lentil appeared to be less selective for the plasmid genotypes. Detailed sampling of isolates from different host species (*Vicia faba*, *Lens esculenta*, *Lathyrus odoratus*, and *Lathyrus sylvestris*) grown in the same soils by Handley et al. (1998) showed that RAPD and plasmid types of *R. leguminosarum* bv. *viciae* were not randomly distributed among the species and within individual plants in the same species. Selection of different strains by different crop species or cultivars has also been reported for clover (Leung et al., 1994) and *Medicago sativa* (Bromfield et al., 1995). These findings suggest that the abundance of rhizobial genotypes in the nodules may not reflect that in the soil population. Nitrogen status of the soil may also influence the type of strains 'trapped' by leguminous plants. Bushby (1993) observed that N level improved the success of the soybean inoculant strain.

2.5 Genetic Stability and Exchange in the Field

Most soil resident rhizobia are less effective than commercially available inoculant strains (Hagedorn, 1978; Thurman and Bromfield, 1988), mostly due to symbiotic 'mismatches' with introduced crop varieties. However, poor performance by resident rhizobia could also be explained by the loss of the effectiveness trait in the

inoculant strain after several years in the soil (Bottomley, 1992). Genetic instability of the symbiotic characteristics of isolates has been demonstrated in rhizobia nodulating common bean (Flores et al., 1988) and clovers (Gibson et al., 1975; Hirsch et al., 1993). Some reports suggest that inoculant strains can remain genetically stable (in symbiotic characteristics) for long periods under field soil conditions. Populations of *R. leguminosarum* bv. *viciae* inoculants released in Rothamstead soils containing indigenous rhizobia were maintained at the same level as the indigenous rhizobial population for several years (Hirsch and Spokes, 1994; Hirsch, 1996). A symbiotic plasmid-cured strain of *R. leguminosarum* bv. *viciae* also persisted several years after being released into the soil (Hirsch, 1996).

There are many indications that genetic exchange occurs in rhizobial populations. Populations of rhizobia that were different from the original inoculant, previously introduced in the soil in the presence (Kucey and Hynes, 1989) and absence (Sullivan et al., 1995) of indigenous rhizobia have been reported. The new strains in the experiments by Kucey and Hynes (1989) were different from the indigenous strains. The authors speculated that the new strains were possibly inoculant strains whose plasmid profiles had been altered. Based on RFLP analysis, the same *pSym* was found in chromosomally different rhizobial isolates (Schofield et al., 1987), and isolates with similar chromosomal backgrounds had divergent *pSym*s (Schofield et al., 1987; Laguerre et al., 1993). Sullivan et al. (1995) recently provided evidence of lateral transfer of chromosomal symbiotic genes from a *Mesorhizobium loti* inoculant strain to indigenous *M. loti* populations that could not previously nodulate *Lotus corniculatus*. The extension of these studies eventually led to the transfer of a 500-kb symbiosis island from a strain of *M. loti* to at

least three nonsymbiotic *Mesorhizobium* species in laboratory matings (Sullivan et al., 1996). Amarger (2001) cited the rich diversity of populations of soybean *B. japonicum*, introduced less than a century ago, as an indicator that genetic exchange has been occurring between inoculant strains and naturalized bradyrhizobia. Deng et al. (1995) provided evidence for horizontal DNA transfer from *Sinorhizobium meliloti* to *Agrobacterium tumafaciens* when they isolated similar insertion sequences in both bacterial species.

Plasmid loss or gain could be the main determinants for the level of genetic stability and genetic exchange among *Rhizobium*, *Sinorhizobium*, and some *Mesorhizobium* strains. Conjugal transmission of plasmids among rhizobial strains has been well demonstrated in the laboratory (Kondorosi et al., 1982; Rao et al., 1994) and symbiotic plasmids have been transferred to symbiotic plasmid cured strains in sterile and non-sterile soils (Kinkle and Schmidt, 1991; Rao et al., 1994). The instability of rhizobial genomes could also be attributed to the presence of widespread reiterated DNA sequences. These repeated elements act as potential sites for homologous recombination which may provoke events such as translocation, deletion, and amplification of specific sequences (Romero et al., 1995). The root nodule is potentially an excellent microsite for genetic exchange (Bottomley, 1992) since it is well known that root nodules can be extensively co-occupied by multiple strains (May and Bohlool, 1983; Demezas and Bottomley, 1986).

2.6 Managing Diversity of Rhizobia in the Field

The huge diversity of rhizobial populations in agricultural soils is well acknowledged. Most of these diverse populations are less efficient than the inoculant strains, but they are effective in locking out most inoculant strains from occupying the nodules (Streeter, 1994). One of the approaches used to establish a new strain of rhizobia among indigenous rhizobia is to apply a heavy dose of an effective, persistent inoculant strain. The main drawback of this strategy is that the inoculant levels required to displace indigenous rhizobia from nodules are not practical (Weaver and Frederick, 1974).

Placement of the inoculant may also influence the success of nodulation by an inoculant strain. Inoculant bacteria applied to the seed have been shown to contribute little to nodule formation on lateral roots (McDermott and Graham, 1989). Inoculation with liquid inoculants would provide better distribution of the inoculant strains in the soil (Zablotowicz et al., 1991). Post-emergence application of the inoculant through irrigation water has been shown to improve infections and nodulation of the lateral roots of the legume crop by the inoculant strain (Ciafardini et al., 1992). Inoculant formulations which prevent excessive mortality of rhizobia while still on the seed or once introduced into the soil would be desirable (Roughley et al., 1993; Streeter, 1994; Brockwell et al., 1995; Rice et al., 2000a). Rice et al. (2000a) reported that soil applied granular inoculant was more effective in establishing nodules on pea than seed-applied liquid and powdered peat inoculants in an acid-soil (pH 4.4) from north western Canada.

One of the more attractive strategies is to use bacteriocidal agents in conjunction with resistant inoculant strains. This would improve the competitiveness of the inoculant strain and at the same time prevent the resident rhizobia from nodulating (Streeter,1994;

Vlassak and Vanderleyden, 1997). Strains resistant to fungicides (Ramirez and Alexander, 1980), antibiotics (Li and Alexander, 1990), and bacteriocins (Triplett, 1990) have shown promise in improving nodule occupancy by inoculant strains. The major challenge would be whether toxins like antibiotics and bacteriocins would be synthesized or persist long enough in non-sterile soils. It is known that antibiotic resistance may not be stable (McGloughlin et al., 1990) and spontaneous resistance can occur among rhizobia in the soil (Streeter, 1994). Genetic construction (or selection among naturally occurring populations) of rhizobial strains that would utilize specific secondary metabolites such as trigonelline (Boivin et al., 1990), stachydrine (Sande et al., 1995), and rhizopines (Murphy et al., 1995; Wexler et al., 1995) might also be a good avenue for improving the competitiveness and persistence of rhizobial inoculants.

It has previously been mentioned that legume hosts appear to have preferences for certain strains of rhizobia. A promising strategy which has been tried in soybean-*Bradhyrhizobium* symbiosis is to select legume genotypes that restrict nodulation by indigenous strain and subsequently develop a superior strain capable of nodulating the unique plant genotype (Cregan and Keyser, 1986; Weiser et al., 1990). In glass house experiments, Fobert et al. (1991) were able to pre-empt competition from soil rhizobia against inoculant strains by exploiting the specificity between a symbiotic gene in pea and a nodulation gene in *R. leguminosarum* bv. *viciae*. Brockwell et al. (1995) have suggested that naturalized rhizobial populations could be more efficiently utilized by plants with enhanced capacity for nodulation and nitrogen fixation (supernodulators). Alternatively, symbiotically promiscuous lines that nodulate and fix N vigorously with diverse resident rhizobial populations could be developed. Such promising lines have been reported for

soybean (Thompson et al., 1991).

2.7 Conclusion

The soil environment and its microhabitats are highly variable and, together with genetic exchange that appears common among rhizobia and other bacteria, generate tremendous diversity in soil rhizobial populations which limits the success of introduced strains. Reviews of this subject (e.g., Lowendorf, 1980; Bottomley, 1992; Brockwell et al., 1995; Vlassak and Vanderleyden, 1997) suggest that there is no simple fix for the failure of inoculant strains to overcome competition from resident rhizobia. A better understanding of the ecology of rhizobial-legume symbiosis, development of practical tools to assess genetic diversity and knowledge of the frequency of genetic exchange among rhizobia is necessary to address this agronomic problem. Meanwhile, relief of environmental stress through sound agronomic practices will ensure that the advances made in terms of improved inoculants and inoculation techniques and manipulation of rhizobial populations in the soil are realized (Brockwell et al., 1995).

3.0 MATERIALS AND METHODS

3. 1 Field Experiments

3.1.1 Experimental Sites

Multi-site field experiments were conducted for three years (1998-2000) to evaluate the abundance and efficacy of resident *Rhizobium leguminosarum* bv. *viciae* populations in southern Manitoba soils. In 1998, "trap" plots for rhizobia were set up in 21 sites across southern Manitoba. The sites were identified with the help of the Manitoba Pulse Growers Association and field managers of Fort Garry Campus, Glenlea, and Carman Research Stations of the University of Manitoba. Fields were selected for the trials only if they were known to have been planted to pea or lentil in the previous ten years. In addition, information on the brands of commercial inoculants used in previous pea or lentil inoculations was needed and the farmers had to guarantee non-interference with the plots throughout the experimental period. Table 3.1 is the list of the sites where the experimental trap plots were established. There were four sites at the Carman Research Station, two sites at Arborg, Glenlea, Morris, Souris, Teulon, and Westbourne, and only one site at Fort Garry Campus, Letellier, and Stuartburn. Sites located in the same area were on a single producer's farm (or research farm as was the case with Carman), and the physical distance between any two such sites ranged from 50 m to 5 Km. Carman 4, Teulon 1, Arborg 1, and Fort Garry Campus were cultivated sites that had not seen pea in at least 15 years, while Westbourne 2 (located adjacent to an arable field) and Stuartburn (located on a prairie preserve) had not been cultivated for over 25 and 50 years, respectively. The rest of the sites had been planted to pea or lentil in the previous one to four years.

Table 3.1 Field site information (1998).

Site	Site code	Last host crop*	Commercial inoculant brand
Carman 1**	S1	Lentil 1996	Nitragin
Carman 2	S2	Lentil 1995	Nitragin
Carman 3	S3	Pea 1997	Nitragin
Carman 4	S4	-	-
Fort Garry Campus	S5	-	-
Letellier**	S6	Pea 1995	-
Morden 1	S7	Pea 1995	Cell-Tech
Arborg 1	S8	-	-
Arborg 2	S9	Pea 1995	Unknown
Teulon 1	S10	-	-
Teulon 2	S11	Pea 1993	Selfstick
Morden 2**	S12	Pea 1996	Nitragin
Westbourne 1**	S13	Pea 1990/94	Nitragin
Westbourne 2	S14	-	-
Morris 1	S15	Pea 1995	Nitragin
Morris 2	S16	Pea 1994	Nitragin
Souris 1	S17	Lentil 1996	Biorhizoliquid
Souris 2**	S18	Pea 1995	Biorhizoliquid
Stuartburn	S19	-	-
Glenlea 1	S20	Pea 1993	Nitragin
Glenlea 2	S21	Pea 1997	Nitragin

* Crop and year site last planted to pea or lentil in previous 10-year period.

** Sites selected for 1999 and 2000 experiments.

Prior to planting, soils in all the sites were sampled at a depth of 30 cm and analysed for percent organic matter, electrical conductivity, pH, nitrate-N, and available P at Norwest Labs (Winnipeg). Soil texture was also determined for all the sites using the hand-feel method. The soil test results are summarized in Table 3.2.

In both 1999 and 2000, field experiments were conducted in only five of the 21 sites established in 1998. These sites were Carman 1, Letellier, Morden 2, Souris 2, and Westbourne 1. Prior to 1999, Carman 1, Letellier, and Souris 2 were under wheat while Westbourne 1 and Morden 2 were under barley and canola, respectively. The 2000 Carman 1 site was under fallow the previous year, while Souris 2 and Letellier were under common bean, Morden 2 under wheat, and Westbourne 1 under canola. Morden 2 and Souris 2 had been zero tilled in 1999. Tables 3.3 and 3.4 summarize the physicochemical characteristics of the soils at the experimental sites in 1999 and 2000.

Table 3.2 Soil physicochemical characteristics at experimental sites in 1998.

Site	Soil attribute					
	pH	%SOM	EC (ds/m)	NO^-_3-N (ppm)	P (ppm)	Soil texture
Carman 1	6	5.6	0.8	24	30	Clay loam
Carman 2	6.4	7	0.6	38	46	Clay loam
Carman 3	6	8.4	0.8	64	>60	Clay loam
Carman 4	7	7.5	0.6	36	>60	Clay
Fort Garry	7.5	6.4	1	28	52	Clay
Letellier	8	6.6	1.2	55	27	Clay
Morden 1	7	8.5	1	60	15	Silty clay loam
Arborg 1	8.3	3.8	0.6	28	11	Silty clay
Arborg 2	8.3	3.2	0.6	28	15	Silty clay
Teulon 1	8.3	3.9	0.8	30	57	Fine sandy clay
Teulon 2	7.8	6.9	1	38	8	Silty clay loam
Morden 2	6.6	9.6	1	>80	16	Silty clay loam
Westbourne 1	7.9	6.7	3.3	31	20	Clay
Westbourne 2	8.3	8.1	0.8	8	8	Clay
Morris 1	8.1	4.7	1.9	>80	25	Clay
Morris 2	7.7	4.3	0.8	20	25	Silty clay
Souris 1	6.1	1.9	0.2	16	16	Loamy sand
Souris 2	8.1	2.9	0.4	13	24	Sandy clay loam
Glenlea 1	7.7	4.3	0.8	15	7	Silty clay
Glenlea 2	6.9	5	0.6	14	16	Silty clay
Stuartburn	8.2	5.4	0.8	4	6	Sandy clay loam

Table 3.3 Soil physicochemical characteristics at experimental sites in 1999.

Site	Soil attribute					
	pH	%SOM	EC (ds/m)	NO^-_3-N (ppm)	P (ppm)	Soil texture
Carman 1	6.0	3.6	0.29	15	15	Clay loam
Letellier	7.7	6.7	1.33	28	38	Clay
Morden 2	7.2	7.5	0.96	45	13	Clay
Souris 2	7.2	4.1	0.81	16	19	Sandy clay loam
Westbourne 1	7.8	5.5	1.4	36	8	Clay

Table 3.4 Soil physicochemical characteristics at experimental sites in 2000.

Site	Soil attribute					
	pH	%SOM	EC (ds/m)	NO^-_3-N (ppm)	P (ppm)	Soil texture
Carman 1	6.0	3.2	0.49	23	14	Clay loam
Letellier	7.9	5.7	4.03	69	17	Clay
Morden 2	7.8	7.5	1.33	34	11	Clay
Souris 2	7.2	3.2	0.81	20	11	Sandy clay loam
Westbourne 1	8	6.6	1.61	41	11	Clay

3.1.2 Experimental Design and Crop Husbandry

The objectives of the study were addressed via a simple, three treatment experiment. The treatments in all the experimental years comprised: (1) uninoculated field pea; (2) field pea inoculated with a *R. leguminosarum* bv. *viciae* strain PBC108 (provided by Philom Bios, Saskatoon, SK); and (3) field pea inoculated with PBC108, and supplied with 100 Kg N ha^{-1}. In addition, a non N-fixing reference crop was included to determine the amount of nitrogen fixed by pea plants. The 1998 study was a survey with 21 sites over a broad geographical range of southern Manitoba. Five sites were selected from the 1998 sites for more detailed study in1999 and 2000. The selected sites were representative of most geographical areas of southern Manitoba, were less susceptible to crop failure due to waterlogging, and the owners were willing to have our experimental plots on their farms. The treatments were arranged in a randomized complete block design (RCBD) with four replicates in 1999 and 2000. The RCBD with 19 replicates was used in 1998, each site representing one replicate. Only the uninoculated treatment was performed at Westbourne 2. Experiments in all the three years were performed with field pea (*Pisum sativum* cv. Express). A Nod^- line R251 of 'Juneau' pea (Shelp et al., 1998) was used as a non N-fixing reference crop in 1999 and 2000, replacing barley (*Hordeum vulgare* cv. Argyle) which was the reference crop for the 1998 trials.

The experimental plots measured 1.00 m X 1.05 m and the spacing between them was 0.75 m. Replicates were spaced 1 m apart and a buffer zone of at least 3 metres maintained between the experimental plots and the farmer's or Research Station's crop. The experimental plots were tilled with a rotavator and by hand hoeing to achieve a

moderate tilth in the seed bed. Ten furrows, 15 cm apart, were made with a wooden stick, and one single pea seed placed at 10 cm intervals within each furrow. Field pea was planted to an approximate depth of 4.0 cm in all cases at a seeding rate of 10 viable seeds per metre of seed row. Phosphorous was uniformly applied to all experimental plots as monobasic potassium phosphate ($KHPO_4$) at the rate of 40 Kg P_2O_5 ha^{-1}, whereas nitrogen was applied only to inoculation plus N fertilizer treatment plots in the form of ammonium nitrate (NH_4NO_3) at the rate of 100 Kg N ha^{-1}. Monobasic potassium phosphate and NH_4NO_3 were separately dissolved in water and sprayed on the seedbed after planting. In all cases, each experimental plot received about 8 litres of water at planting. Farmers were provided with plastic tarps to cover the experimental plots when spraying their crops to avoid pesticide drift.

Field pea seed was inoculated with *R. leguminosarum* bv. *viciae* strain PBC108 (N-prove™) on the morning of the planting day at the rate recommended by the manufacturer (Philom Bios, Saskatoon, SK). The seed was dusted with the peat based inoculant in a flask and wetted with tap water in the ratio 4 L H_20/ 2.2 Kg inoculant/1360 Kg pea seed. The flasks containing seeds were kept cool while in transit to the planting site to prevent excessive drying of the inoculant. To prevent contamination of the uninoculated seeds with the inoculant, inoculated and uninoculated seeds were handled and stored separately. Hands were washed with 70% alcohol and 20% v/v Javex™ solution (5.25% sodium hypochlorite) after each inoculation episode. After planting, all the tools (e.g., rota-tiller, rakes, one metre rulers, watering cans, and hoes) were cleaned with water from a power spray pump and thoroughly washed with 20% v/v Javex solution. Hands and boots were also washed with Javex solution after planting.

Uninoculated plots were always planted before the inoculated plots.

Seedling emergence was good in most sites and there were no differences among treatments, though the mutant pea had slightly lower emergence rate. Hand pulling and inter-row tillage with a hoe were used for post-emergence weed control. There were no serious cases of insect and plant pathogen attacks, although green aphids were noticed during early pod-filling stages in 2000 experiments.

3.1.3 Data Collection

Sampling for shoot biomass, nodule number, nodule weight, and shoot fixed N was done at early pod-filling stage (8 weeks post-planting) in all the experiments. In 1998, aboveground biomass was harvested from 20 plants per experimental plot (5 plants each randomly harvested from 4 rows) by cutting at the base of the stems. Shoot samples were initially air dried and later oven dried at 70°C until daily checks indicated no further decreases in weight. All dried samples were weighed, coarsely ground in a Wiley mill using a 2.00 mm screen, and finely ground with a coffee grinder. Subsamples of the ground material were analysed for N concentration by a combustion technique using Leco N analyzer (Model FP 428; Leco Corp., Mississauga, ON). Shoot samples for 1999 and 2000 were processed in the same manner as for 1998 except that aboveground biomass was harvested from an inner plant row area of 0.525 m^2. The amount of biologically fixed shoot N was determined by the N-difference method.

After shoot harvest, 12 pea root cores per experimental plot were randomly taken using a soil corer (6.5 cm diameter, 15 cm depth) from four inner rows(3 root cores each) of pea plants in 1998. In 1999 and 2000, three pea root cores were randomly taken from

each experimental plot, excluding mutant pea plots. Before taking the root cores, it was made sure that the root crown was positioned at the centre of the soil corer. The root cores of each treatment were placed in separate, clearly labelled plastic bags and transported to the lab. Nodules were picked from representative plants in both inoculated and uninoculated plots in 1998 and 1999 for bacteria isolation. In 2000, however, nodules for bacterial isolation were picked only from inoculated (with no fertilizer N) plants. We also picked nodules from root cores of *Lathyrus* sp. plants growing on the roadsides or ditches close to the Arborg, Glenlea, Morden, and Teulon sites. Root cores were stored at -17°C for weeks or months after which they were thawed and nodules picked, counted, freeze-dried, and weighed.

In 2000, at the time of full maturity, pods were harvested from all the remaining plants occupying 0.475 m^2 of the experimental plots. It is worth noting that after shoot samples were taken, most of the pea plants left behind especially in the inoculated plots were highly lodged because of excessive intertwining. Hence, differences in seed yield between inoculated and uninoculated treatments were likely minimized. The harvested pods were air dried for a few weeks and shelled by hand. The seed was cleaned and weighed. Seed yield was then calculated on a per m^2 basis. Seed samples were finely ground with a coffee grinder and subsamples analysed for N concentration using Leco N analyser (ModelFP 428; Leco Corp., Missisauga, ON). The weight of 1000 pea seeds was determined by weighing a subsample of 250 seeds from each experimental plot and multiplying the weight by 4. Seed yield was not determined in 1998 and 1999 experiments.

3.1.4 Data Analysis

Analysis of variance (ANOVA) was performed on shoot biomass, nodule number, nodule dry weight, seed yield, and shoot and seed fixed N using SAS. When the F test was significant, treatment means for these parameters were separated by the least significant difference (LSD) test at 0.05 probability level (unless indicated otherwise). In 1998, each site was treated as a replicate and treatments arranged in a randomized complete block design. Since location by treatment interactions were significant for all the parameters in 1999 and 2000, mean separation of treatments was performed for each site. Location means were also separated using LSD.

3.2 Laboratory Experiments

3.2.1. Isolation of *Rhizobium* Strains from Nodules

Isolation of *Rhizobium* strains was carried out according to the protocols by Beattie and Handelsman (1989) and Rice and Olsen (1993) with a few modifications. Nodules were removed from pea and *Lathyrus* sp. plant roots with forceps and placed in 2 mL microfuge tubes with 1.5mL of 20% glycerol. The microfuge tubes were vigorously vortexed for 1 min, filled with 20% glycerol , and stored at -17°C . The microfuge tubes were thawed out after 3-10 weeks and the nodules were transferred to tubes with 1.5 mL of 3% H_2O_2. The tubes were vortexed for 1 min and H_2O_2 emptied. Another fresh sample of 3% H_2O_2 was added, the tubes vortexed for 1 min, and H_2O_2 pipetted out. Nodules were washed at least three times with sterile water and allowed to sit in sterile water for 15 min. Each nodule was placed in an individual well of a sterile 96-well microtitre plate holding 120 µl of sterile yeast mannitol broth. The multiple crusher/inoculator apparatus

containing 24 bolts, developed by Beattie and Handelsman (1989), was dipped into 95% ethanol, flame sterilized, then dipped into the wells and rocked to crush the nodules. The crushed nodule suspensions carried on the bolts were dropped onto yeast mannitol agar (Rice and Olsen, 1993) plates and incubated in an inverted position at 28°C. After two or more days, rhizobia growing in each spot were streaked onto yeast mannitol agar plates and incubated at 28°C for two to three days. Single colonies were then picked and transferred to new yeast mannitol agar plates. We assumed that each single colony represented a single strain and that each nodule contained a single strain, or if more than one strain existed per nodule, only the most abundant was sampled. It was noted that the recovery of viable rhizobia from nodules was high (above 80%), even after the nodules had been stored at -17°C (fully immersed in 20% glycerol) for more than six months. The recovery of viable rhizobial strains from small, white nodules that was characteristic of nodules from native *Lathyrus* sp. plants was relatively low. Surface sterilization of the nodules with H_2O_2 and ethanol (and flaming) sterilization of the inoculator apparatus was effective in reducing contamination of the colonies. The cultures were maintained in yeast mannitol agar plates at 4°C.

3.2.2 Plasmid Profiling

3.2.2.1 *Rhizobium* Strains

Analysis of plasmid profiles was used to characterize rhizobial strains from nodules of pea and *Lathyrus* sp. plants, as well as commercial strains. Note that we examined only the resident populations of *Rhizobium leguminosarum* bv. *viciae* that were able to form nodules on roots of pea or *Lathyrus* sp. Plasmid profiles of 230 strains were

examined. The strains included: two hundred and seven isolates from field pea root nodules (mainly uninoculated plants, although all the isolates from Glenlea 1 were from non-N fertilized inoculated plants because uninoculated root cores had been inadvertently discarded); seven native roadside *Lathyrus* sp. isolates; and 16 commercial strains provided by respective manufacturers. The commercial inoculant strains of *R. leguminosarum* bv. *viciae* were 128C56G, C1, RGP4, RGP2, 175G10B, 99AA1, RP212-2, RP212-19, RP212-13, RP212-37, RP213-5, RGL4, RGFP, PEA082, PBC108, and RGAA1 (Table 3.5), while the native *Lathyrus* sp. isolates comprised four isolates from Teulon, two from Morden, and one from Glenlea.

Table 3.5 List of commercial inoculant strains typed by plasmid profiling.

Strain	Commercial source
C1	MicroBioRhizogen
RGP2	MicroBioRhizogen
RGP4	MicroBioRhizogen
RGL4	MicroBioRhizogen
RGAA1	MicroBioRhizogen
RGFP	MicroBioRhizogen
RP212-2	Urbana Laboratories
RP212-13	Urbana Laboratories
RP212-19	Urbana Laboratories
RP212-37	Urbana Laboratories
RP213-5	Urbana Laboratories
PEA082	Urbana Laboratories
128C56G	Liphatech
175G10B	Liphatech
99AA1	Agrium
PBC108	Philom Bios

3.2.2.2 Sample Treatment and Gel Electrophoresis

Plasmid profiles of isolates were examined using a modified Eckhardt (1978) rapid visualization technique as described by Hynes et al. (1986) with a few modifications. PTY (8 g peptone, 1.0 g tryptone, 1.0 g yeast extract, 0.4 g $CaCl_2$, 0.4 $MgSO_4$, 30g agar, 1 L water) plates were streaked with *Rhizobium* isolates growing on YMB plates and incubated at 28°C for 3 or more days, depending on the growth rate of the strain. Most field isolates grew fast relative to the commercial strains. A loop of the culture in a PTY plate was transferred to a test tube containing 5 mL of PTY liquid medium (8 g peptone, 1.0 g tryptone, 1.0 g yeast extract, 0.4 g $CaCl_2$, 0.4 $MgSO_4$, 1 L water) and incubated at 28°C for 18 h. A sample of the bacterial culture with an optical density of about 0.4 was then transfered to an Eppendorf tube on ice and allowed to sit for 20 minutes. One mL of 0.6% cold sarkosyl (N-lauroylsarcosine) solution was added, mixed by gently inverting the Eppendorf tube, and allowed to sit on ice for 15 min. The tube was centrifuged for 10 minutes at 14,000 rpm, the supernatant was removed, and the tube centrifuged again at 14,000 rpm for 20 s. After most of the supernatant was removed, the pellet was suspended in 20 µL of lysis solution made up of sucrose (0.1 g/mL), RNase (10 µg/mL) and lysozyme (100 µg/mL) in TBE (89 mM Tris, 89mM boric acid, 2 mM EDTA, pH 8.0) and allowed to sit on ice for 20 min. The RNase and lysozyme were purchased from Sigma Chem., St. Louis, Mo. The contents of the tube were loaded onto 1% agarose wells (containing 10% SDS) and the gel was run at 82 V for 4.5 h. The gel was stained over night in an aqueous ethidium bromide solution (0.5 mg/L), rinsed in de-ionized water and imaged by the Fluor-S™ Multi-Imager. *Rhizobium etli* CE3 (from David Romero, University of Mexico) with five well characterized

plasmids (630, 510, 390, 270, and 175 kb) was included in each gel as the reference for determining the approximate molecular weights of plasmids of test strains. The Eagle Eye software program (Strategene, La Jolla, California) and visual observation were used to determine plasmid band sizes.

3.2.2.3 Plasmid Profile Data Analysis

Isolates were placed in different plasmid profile groups, based on the number and size of plasmid bands. Isolates that were identical in number and size of plasmid bands were considered to belong to the same plasmid profile group. The use of molecular weights generated by the Eagle Eye program was supplemented by close eye inspection of the profiles by two individuals. Each plasmid group was given a label. Diversity in plasmid profiles within each site was expressed in terms of plasmid profile diversity index (PPDI) which we defined as the number of distinct plasmid profiles divided by the total number of isolates typed.

3.2.3. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of 16S-23S rDNA Intergenic Spacer (IGS) Sequences

3.2.3.1 Rhizobial Strains

Twenty eight *Rhizobium* isolates were selected for PCR-RFLP analysis to relate plasmid profiles to chromosomal backgrounds and to evaluate the diversity of isolates at the chromosomal level. The selection was carried out to include: (1) strains from the same site with identical or different plasmid profiles; (2) strains from different

geographical distances exhibiting identical or different plasmid profiles; (3) a field strain with the same plasmid profile as an inoculant strain; (4) commercial strains from different suppliers with the same or different plasmid profiles; and (5) a strain of *Rhizobium leguminosarum* bv. *phaseoli*. As shown in Table 3.6 , 12 commercial and 15 field strains of *R. leguminosarum* bv. *viciae*, plus one *R. leguminasarum* bv. *phaseoli* strain (H441) obtained from France, were examined. The nomenclature adopted for the isolates: all field pea isolates start with S followed by the number of the site and a capital letter A, B or C if there were more than two isolates from the same site. Strain RGFP is the commercial strain recommended for *Vicia faba* L. (faba bean) inoculation while RGL4 is recommended for lentil inoculation. *Phaseolus vulgaris* L (common bean) is the host for H441.

Table 3.6 Rhizobial strains used in PCR-RFLP of 16S-23S rDNA IGS study.

Strain	Host plant	Source/site of origin
C1	Pea	Commercial
RGP4	Pea	Commercial
128C56G	Pea	Commercial
RGL4	Lentil	Commercial
RGFP	Faba bean	Commercial
RGP2	Pea	Commercial
RP212-19	Pea	Commercial
RP212-37	Pea	Commercial
RP213-5	Pea	Commercial
PEA082	Pea	Commercial
PB108	Pea	Commercial
99AA1	Pea	Commercial
H441	Common Bean	France
S1A	Pea	Carman 1
S1B	Pea	Carman 1
S5A	Pea	Fort Garry Campus
S5B	Pea	Fort Garry Campus
S10	Pea	Teulon 1
S12	Pea	Morden 2
S13	Pea	Westbourne 1
S15A	Pea	Morris 1
S15B	Pea	Morris 1
S15C	Pea	Morris 1
S16	Pea	Morris 2
S17	Pea	Souris 1
S18	Pea	Souris 2
S20A	Pea	Glenlea 1
S20B	Pea	Glenlea 1

3.2.3.2 Extraction of DNA

Genomic DNA was isolated from the aforementioned strains following the method described by Laguerre et al. (1992) with modifications. *Rhizobium* strains were transferred from YMB plates onto 5 mL of TY liquid medium (Beringer, 1974) and incubated at 28°C for about 18 h. The samples were centrifuged at 10,000 rpm for 20 min and the pellet washed in 1 mL 100 mM Tris-HCl (pH 8.0). The culture was pelleted again for 2 min at 14,000 rpm and the pellet resuspended in 400 µL TES buffer (20 mM Tris, 50 mM, 150 mM NaCl, pH 8.0). The mixture was treated with 100 µL of Lysozyme (5 mg/mL) and incubated at 37°C for 1.5 h. Then 60 µL of SDS (5% w/v) was added, the mixture incubated at 65°C for 20 min and transferred to a 37°C incubator. Once a clear lysate was formed, 5 µL of RNAse A (10 mg/mL) was added and the mixture incubated for 1.5 h. Forty microlitres of pronase E (25 mg/mL) were added to the lysate and left to incubate overnight at 37°C. Note that the lysozyme, RNAse and pronase E were purchased from Sigma Chem., St. Louis, Mo. DNA was initially extracted with a phenol/chloroform isoamyl alcohol mixture in the ratio 50/48/2 followed by several extractions with chloroform/isoamyl (24/1). The DNA was precipitated with isopropanol (v/v) and centrifuged at 14,000 rpm for 10 min to pellet DNA. The supernatant was discarded carefully, the DNA pellet dissolved in 500 µL TE (10 mM Tris, 1 mM EDTA, pH 8) and re-precipitated with 1 volume isopropanol in the presence of 50 µL of 3 M sodium acetate. The DNA precipitate was again pelleted by centrifuging at 14,000 rpm for 10 min and the supernatant carefully discarded. The DNA pellet was washed twice with 70% ethyl alcohol, air dried on the lab bench, and dissolved in 100 TE buffer. The DNA sample was stored at 4°C for use within a few days. Purity and concentration of

DNA was determined using UV-spectrophotometry.

3.2.3.3 PCR Oligonucleotide Primers

Oligonucleotides primers used to amplify the IGS sequences between 16S and 23S rRNA genes were 5'-GCCCGGCTACTTGCAGAGATGGAAGGTTCCC-3' (16SR11) and 5'-CCGGGTTTCCCCATTCGG-3' (FGPL132'). Forward primer 16SR11corresponds to an oligonucleotide at the 3' part of the 16S rDNA which permits the specific amplification of *R. leguminosarum* and *Rhizobium etli* 16S-23S rDNA IGS when used with the universal FGPL132' as the reverse primer (Laguerre et al., 1996). FGPL132' corresponds to the 5' part of the 23S rDNA right next to the IGS (Navorro et al., 1992). These oligonucleotides were synthesized by Canadian Life Technologies, Burlington, ON.

3.2.3.4 PCR Amplification

DNA amplification was carried out as described by Laguerre et al. (1996) with minor modifications. PCR reactions were performed in a final volume of 150 µL containing: approximately 0.5 µg template genomic DNA (in 10 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% w/v gelatin, 20 µM each of dATP, dCTP, dTTP, and dGTP, 0.1 µM of each primer, and 5 units of *Taq* polymerase). The supplies, including *Taq* polymerase and dNTPS, were purchased from Canadian Life Technologies, Burlington, ON. Amplification reactions were performed in a thermal cycler with the following standard temperature profile: initial denaturation at 95°C for 3 min, 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C), and extension (2 min at 72°C)

and then a final extension (3 min at 72°C). The reaction samples were stored at -20°C after the amplification process. Note that PCR reactions were run more than once for a number of the strains to ensure repeatability of the results. The amplified DNA fragments were analysed by horizontal gel electrophoresis in 1% agarose gels (containing 0.5 mg /L ethidium bromide) in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Electrophoresis was carried out at 90 V for 1 h using 6 cm by 8 cm standard gels. Ethidium bromide (0.5 mg /L) was added to the electrophoresis buffer before each run. The gels were imaged using the Fluor-S™ MultiImager. The sizes of the PCR products were estimated by the Eagle-Eye program based on a molecular weight standard (Norgen Biotek, St. Catharines, ON).

3.2.3.5 Restriction Enzyme Digestion of Amplified DNA Fragments

Seven restriction endonucleases, *Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Msp*I, *Nde*II, and *Taq*I (Canadian Life Technologies, Burlington, ON.), were used to digest the amplified DNA. Ten microlitres of the PCR products were digested with 12-15 units of enzyme. The buffer and incubation temperatures for each enzyme were those recommended by the manufacturer. The reactions with *Taq*I were performed in a 65°C water bath, while the reactions with all the other enzymes were carried out at 37°C. The restriction fragments were analyzed by horizontal gel electrophoresis in TBE buffer on 4% NuSieve 3:1 agarose (FMC, Maine, USA) gels containing 0.5 mg/L ethidium bromide. The gels were run at 82 V for 4 hours and imaged using the Fluor-S multi-Imager. The approximate sizes of the restriction fragments were determined by the Eagle-Eye Software program based on a 100 bp molecular weight standard (Norgen Biotek, St. Catharines, ON.).

3.2.3.6 Restriction Data Analysis

Restriction fragment bands smaller than 100 bp were not included in the analysis because they were generally not well resolved on 4% NuSieve 3:1 agarose gels. Restriction bands produced by each endonuclease across all 28 *Rhizobium* strains were assigned a number (1, 2, 3,.....n) according to decreasing molecular weights. Restriction bands of different strains that had similar molecular weights when cut by the same endonuclease were assigned the same number, while bands of similar molecular weight produced by different endonucleases were considered different. Each band was treated as a unit character, and the strain scored for the presence or absence of a band and coded as 1 or 0, respectively. Statistical analyses were performed by the NTSYSpc analysis package (version 2.0, Exeter software, Setauket, N.Y.). Genetic similarity between a pair of strains was evaluated as a simple matching coefficient (S) (Sokal and Michener, 1958), where similarity between individual pairs i and j was:

Sij = (a +d)/(a + b+ c+d), where a = number of 1-1 matches, b = number of 1-0 matches, c = number of 0-1 matches, and d = number of 0-0 matches. The S matrix was used to construct phenograms based on the unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) and the neighbour-joining method (Saitou and Nei, 1987). The UPGMA method uses a sequential clustering algorithm in which topological relationships between operational taxonomic units (OTUs), i.e, strains in our case, are inferred in order of decreasing similarity. Genetic similarity among the OTUs was further examined by nonmetric multidimensional scaling (MDS), a technique designed to construct a 'map' from data measuring the relationships between pairs of objects (Manly, 1994). Multidimensional scaling analysis has previously been used to reveal relationships

between strains based on molecular data (Nienhuis et al., 1994). The 28 X 28 matrix of simple matching coefficient values was reduced to two dimensions and displayed as an MDS plot.

3.2.4 Nodule Occupancy

Isolates were obtained from plants inoculated with PBC108 strain (with no N-fertilizer) at Morden 2 in 2000. Root cores of 4 plants (one plant per replicate) were taken with a 6.5 cm diameter soil auger and 8 nodules were picked from the root cores of each plant close to the base of the stem and bagged separately. The base of the stem was positioned at the centre of the soil corer before taking root cores. A 2 cm wide slice of soil was taken from the wall (facing the plants in the next row) of each of the four holes where root cores had been taken and 8 nodules picked from each of the soil samples and bagged separately. The plant and the root region from which each nodule sample was obtained were recorded. The nodules were placed in Eppendorf tubes, filled with 20% glycerol, thoroughly vortexed, and stored at -17°C for about 10 months. Rhizobia were isolated and cultured as previously described.

Analysis of plasmid profiles was used to determine the proportion of the proximal (borne on the tap root near the base of the stem) and distal (borne on lateral roots far from the base of the stem) nodules occupied by the inoculant strain. Inoculant strain PBC108 was run in every gel as the reference strain. Strains that had identical plasmid profiles as the inoculant strain were considered to have a PBC108 plasmid profile. Relative plasmid profile diversity between proximal and distal nodule isolates was determined by calculating plasmid profile diversity index.

4.0 RESULTS

4.1 Field Experiments

4.1.1 Nodulation

Relative nodulation levels of uninoculated and non-N fertilized inoculated plants were used to indicate the presence and abundance of agronomically important populations of *Rhizobium leguminosarum* bv. *viciae* in southern Manitoba soils. In a survey of 21 field sites in 1998 good levels of nodulation were observed in uninoculated pea plants growing in all arable soils, including those that had not seen pea or lentil in recent time. In contrast, uninoculated pea plants grown in two sites with no recent history of cultivation (Stuartburn and Westbourne 2) were poorly nodulated, especially at the Stuartburn prairie preserve site where only two small nodules were found out of 70 uninoculated pea plants sampled. Nodules were few or absent in *Lathyrus* sp. and vetch (*Vicia* sp.) plants sampled on roadsides close to Arborg, Carman, Glenlea, Souris, Teulon, and Westbourne sites, and most of the nodules were small and white.

Tables 4.1, 4.2 and 4.3 show the effect of inoculation on nodulation. Inoculation (with no fertilizer N) did not significantly increase nodules per plant in 1998 and in all the five sites in 1999. A similar trend was reflected in all the five sites in 2000 (Table 4.3), with the exception of Carman where non-fertilized inoculated plants exhibited significantly more nodules than uninoculated pea plants. Nitrogen fertilizer application significantly depressed nodules per plant relative to inoculation alone in 1998, 1999 (at Carman, Souris, and Westbourne) and 2000 (at Carman, Morden, Souris, and Westbourne). Uninoculated plants had similar nodule numbers as N-fertilized plants in all the experimental years except at Morden in 2000 and Westbourne in both 1999 and

Table 4.1 Nodule number and shoot dry matter (DM) of pea plants as affected by *Rhizobium* inoculation with and without N-fertilization at 17-19 sites in southern Manitoba in 1998.

Treatment	Plant parameter		
	Nodule number plant^{-1}	Nodule dry weight (mg) plant^{-1}	Shoot DM (g) plant^{-1}
Uninoculated	33.1 ab	111.4 a	6.2 b
Inoculated	40.5 a	115.1 a	7.4 a
Inoculated + N	31.7 b	70.4 a	8.3 a
LSD	7.8	47	1.1

Means followed by the same letter within a column are not significantly different using LSD (P=0.05).

Table 4.2 Number of pea nodules plant^{-1} as affected by *Rhizobium* inoculation with and without N-fertilization in 1999.

Treatment	Site				
	Carman	Letellier	Morden	Souris	Westbourne
Uninoculated	25.3 ab	59.3 a	50.0 a	47.2 ab	78.4 a
Inoculated	33.8 a	62.7 a	49.1 a	49.8 a	67.2 a
Inoculated + N	15.6 b	47.8 a	40.0 a	29.0 b	42.8 b
LSD	10.9	17.9	24.1	19.4	17.5

Means followed by the same letter within a column are not significantly different using LSD (P=0.05).

Table 4.3 Number of pea nodules plant^{-1} as affected by *Rhizobium* inoculation with and without N-fertilization in 2000.

Treatment	Site				
	Carman	Letellier	Morden	Souris	Westbourne
Uninoculated	18.9 b	64.8 a	78.9 a	72.9 ab	59.1 a
Inoculated	37.8 a	63.0 a	80.9 a	84.1 a	52.6 a
Inoculated + N	21.8 b	54.5 a	46.8 b	62.6 b	38.6 b
LSD	6.8	20.9	19	14.4	10.4

Means followed by the same letter within a column are not significantly different using LSD (P=0.05).

2000. In these three cases, uninoculated plants had significantly more nodules than N-fertilized plants.

Location had a significant effect on nodules per plant in both 1999 and 2000 (Tables 4.4 and 4.5). Carman registered significantly the least nodules per plant compared to the rest of the sites in both years. There was no difference in nodules per plant between Morden and Souris sites in both years, and between Letellier and Westbourne in 1999. Pea plants at Westbourne had significantly more nodules than pea plants at Morden and Souris in 1999, but the converse was the case in 2000.

Table 4.4 Location effects on nodule number, nodule DM, shoot DM, and shoot fixed-N of pea plants in 1999.

Location	Plant parameter			
	Nodules plant^{-1}	Nodule DM (g plant^{-1})	Shoot DM (g m^{-2})	Shoot fixed-N (g m^{-2})
Carman	24.9 d	28.4 c	609.7 a	15.1 a
Letellier	56.6 a	175.4 b	468.9 c	6.0 c
Morden	46.4 c	161.9 b	412.4 d	8.6 b
Souris	42.0 c	294.4 a	425.5 cd	4.6 c
Westbourne	62.8 a	253.4 a	544.0 b	9.8 b
LSD	8	53.8	49.2	1.4

Means followed by the same letter within a column are not significantly different using LSD (P=0.05).

Table 4.5 Location effects on nodule number, nodule DM, shoot DM, seed yield, 1000-seed WT, shoot fixed-N, and seed fixed-N of pea plants in 2000.

Location	Plant parameter						
	Nodules plant^{-1}	Nodule DM (mg plant^{-1})	Shoot DM (g m^{-2})	Seed yield (g m^{-2})	1000-seed WT (g)	Shoot fixed-N (g m^{-2})	Seed fixed-N (g m^{-2})
Carman	26.2 d	40.1 d	438.8 ab	866.9 a	211.8 a	6.7 c	13.8 b
Letellier	60.8 b	201.3 c	283.4 c	357.3 e	192.5 b	3.2 d	5.6 d
Morden	68.9 a	260.4 b	420.9 b	466.6 d	191.2 b	9.0 b	5.3 d
Souris	73.2 a	346.6 a	461.7 a	534.7 c	190.2 b	9.5 b	11.0 c
Westbourne	50.1 c	340.5 a	449.4 ab	764.0 b	211.5 a	12.6 a	26.1 a
LSD	6.6	41.5	37	50.8	6.4	1.1	1.8

Means followed by the same letter within a column are not significantly different using LSD (P=0.01).

4.1.2 Nodule Biomass

Inoculated plants (with no N fertilizer) produced significantly more nodule biomass than uninoculated plants at Letellier and Carman in 2000 (Table 4.7). No difference in nodule biomass between inoculated (with no N fertilizer) and uninoculated plants was observed in all sites in 1999 (Table 4.6) and at Morden, Westbourne, and Souris in 2000. Nodule dry weights of N-fertilized plants were significantly lower than those of inoculated but non-N fertilized plants across all the five sites in both years except at Souris in 1999 where there was no difference between the two treatments. Uninoculated plants accumulated significantly more biomass in the nodules than N-fertilized plants at Letellier and Westbourne in 1999, Souris in 2000, and Morden in both 1999 and 2000. Treatments had no significant effect on nodule dry weight in 1998 (Table 4.1).

Nodule dry weights per plant were significantly influenced by location in 1999 and 2000 (Tables 4.4 and 4.5). Pea plants at Carman had significantly the lowest nodule biomass compared to pea plants at all the other four sites. Nodule biomass in the latter sites ranged from 5.0 to 10.4 times that at Carman. In 1999, there was no difference in nodule dry weights between Letellier and Morden and between Souris and Westbourne. Nodule dry weights were significantly higher at Souris and Westbourne than at Morden and Letellier. Similar results were found in 2000, although pea plants at Morden accumulated more nodule dry mass than pea plants at Letellier.

Table 4.6 Pea nodule dry weight (mg plant^{-1}) as affected by *Rhizobium* inoculation with and without N-fertilization in 1999.

Treatment	Site				
	Carman	Letellier	Morden	Souris	Westbourne
Uninoculated	27.8 ab	188.3 a	188.3 a	290.3 a	323.8 a
Inoculated	34.8 a	212.2 a	217.5 a	335.0 a	335.8 a
Inoculated + N	22.4 b	125.8 b	79.8 b	258.0 a	100.8 b
LSD	7.3	35.7	82.8	253.8	73.5

Means followed by the same letter within a column are not significantly different using LSD (P=0.05).

Table 4.7 Pea nodule dry weight (mg plant^{-1}) as affected by *Rhizobium* inoculation with and without N-fertilization in 2000.

Treatment	Site				
	Carman	Letellier	Morden	Souris	Westbourne
Uninoculated	30.2 b	169.3 b	298.1 a	399.3 a	322.9 ab
Inoculated	72.6 a	309.3 a	324.4 a	437.5 a	456.9 a
Inoculated + N	17.6 b	125.4 b	158.8 b	203.0 b	241.3 b
LSD	13.5	87.9	74.8	59.6	171.6

Means followed by the same letter within a column are not significantly different using LSD (P=0.05).

4.1.3 Shoot Dry Weight

In 1998, inoculation significantly increased shoot biomass (Tables 4.1).Within the inoculated treatments, there was no difference in this parameter between the N-fertilized and the non-N fertilized plants. In 2000, the treatment effect on shoot biomass was significant at all the sites, except at Westbourne (Table 4.9). Plants inoculated without fertilizer N accumulated significantly more shoot biomass than the uninoculated plants at Carman and Letellier, but there was no difference in all the other sites. N-fertilized plants exhibited more shoot dry matter than the uninoculated plants at Carman, Morden and Souris, but did not out-perform non-N fertilized inoculated plants. The uninoculated plants attained 71.4, 76.7, and 82.5% of the N fertilized plant shoot biomass at Carman, Morden and Souris, respectively. The shoot dry matter of the uninoculated plants was 85.1% that of N-fertilized plants at Letellier and Westbourne. In 1999, the treatments did not significantly influence shoot biomass at four of the five sites (Table 4.8). N-fertilized plants had significantly more shoot biomass than both the non-N fertilized inoculated plants and the uninoculated plants at the Souris site. Uninoculated plants had 93.1, 88.2, 84, 83 and 73.3 % of the biomass accumulated by N-fertilized plants at Carman, Westbourne, Letellier, Morden and Souris, respectively.

Location had a significant effect on shoot dry matter in both years (Tables 4.4 and 4.5). In 1999, shoot biomass for pea plants at Carman was significantly higher than for pea plants at the rest of the sites. Shoot biomass for pea plants at Westbourne was significantly higher than for pea plants at Letellier, Morden, and Souris. The latter site was not different from Letellier and Morden in pea biomass, but Letellier had significantly more pea biomass than Morden. Differences in shoot biomass of pea plants

Table 4.8 Shoot dry matter (g m^{-2}) of pea plants as affected by *Rhizobium* inoculation with and without N-fertilization in 1999.

Treatment	Site				
	Carman	Letellier	Morden	Souris	Westbourne
Uninoculated	583.8 a	416.0 a	364.8 a	379.8 b	517.8 a
Inoculated	618.5 a	495.3 a	433.1 a	378.4 b	528.2 a
Inoculated + N	626.9 a	495.4 a	439.4 a	518.3 a	586.8 a
LSD	82.3	126.8	77.4	101.1	161.3

Means followed by the same letter within a column are not significantly different using LSD (*P*=0.05).

Table 4.9 Shoot dry matter (g m^{-2}) of pea plants as affected by *Rhizobium* inoculation with and without N-fertilization in 2000.

Treatment	Site				
	Carman	Letellier	Morden	Souris	Westbourne
Uninoculated	357.0 b	246.5 b	373.7 b	402.2 b	423.9 a
Inoculated	459.8 a	314.2 a	401.5 ab	475.7 ab	426.1 a
Inoculated + N	499.7 a	289.5 ab	487.5 a	507.3 a	498.2 a
LSD	58.1	45.9	111.2	93.9	100.9

Means followed by the same letter within a column are not significantly different using LSD (*P*=0.05).

at different sites were less pronounced in 2000 than in 1999. Pea plants at Letellier produced significantly the least amount of shoot biomass compared to pea plants at the rest of the sites. Carman, Souris, and Westbourne registered similar pea biomass levels, as did Morden, Carman, and Westbourne. However, pea plants at Souris produced more biomass than pea plants at Morden.

4.1.4 Seed Yield

Seed yield determined only in 2000 is shown in Table 4.10. No significant differences in seed yield were observed among all the treatments at Westbourne, Souris, Letellier and Morden. At Carman, however, inoculated (with no fertilizer N) plants significantly out-yielded uninoculated plants. The seed yield of non-N-fertilized inoculated plants was above 15% more than that of uninoculated plants. Inoculation plus N-fertilizer treatment was not different from either of the other two treatments.

Location significantly affected seed yield (Table 4.5). Seed yield was significantly higher at Carman than at any of the other sites, while Letellier exhibited significantly the lowest seed yield. Westbourne registered significantly higher seed yield than Souris which, in turn, significantly out-performed Morden.

4.1.5 1000-Seed Weight

Table 4.11 depicts 1000 seed weight of pea determined at five sites in 2000. There were no differences among the treatments in all the five sites with respect to this plant attribute.

As shown in Table 4.5, location significantly affected 1000-seed weight. Carman and Westbourne, which had similar 1000-seed weight, had significantly heavier seeds than Letellier, Morden, and Souris. There was no difference in 1000-seed weight in the latter three sites.

Table 4.10 Seed yield (g m^{-2}) of pea plants as affected by *Rhizobium* inoculation with and without N-fertilization in 2000.

Treatment	Site				
	Carman	Letellier	Morden	Souris	Westbourne
Uninoculated	792.9 b	356.4 a	463.7 a	520.3 a	732.8 a
Inoculated	933.7 a	339.3 a	469.7 a	523.1 a	777.7 a
Inoculated + N	874.2 ab	376.3 a	466.4 a	560.7 a	781.5 a
LSD	110.2	43.2	142	81.2	149.4

Means followed by the same letter within a column are not significantly different using LSD (*P*=0.05).

Table 4.11 Weight (g) of 1000 pea seeds as affected by *Rhizobium* inoculation with and without N-fertilization in 2000.

Treatment	Site				
	Carman	Letellier	Morden	Souris	Westbourne
Uninoculated	213.6 a	198.6 a	185.6 a	181.4 a	206.8 a
Inoculated	213.5 a	191.0 a	186.0 a	198.0 a	218.1 a
Inoculated + N	208.2 a	187.8 a	202.0 a	191.2 a	209.6 a
LSD	16.5	13.6	17.8	18.5	19.1

Means followed by the same letter within a column are not significantly different using LSD (*P*=0.05).

4.1.6 Biologically-Fixed Shoot N

The N-difference method, using a non-N fixing pea as the reference crop, was used to determine the amount of nitrogen in shoot biomass that could be attributed to symbiotic nitrogen fixation. Inoculation had a significant effect on biologically fixed shoot N only at Carman in both 1999 and 2000 (Tables 4.12 and 4.13). At this site, inoculated plants fixed significantly more nitrogen from the air than uninoculated plants. There was generally a non-significant increase in shoot-fixed N in most sites. At Letellier, inoculated plants accumulated 60.9 and 78.3 % more fixed shoot N than uninoculated plants in 1999 and 2000, respectively. In 1999, inoculated plants at Morden and Westbourne fixed 11.1% and 13% more nitrogen than uninoculated plants, respectively. Numerical values for shoot fixed N were 6.9% and 22.4 % higher for inoculated plants than for uninoculated plants in 2000 at Morden and Souris, respectively.

Shoot fixed N was significantly influenced by location in both years (Tables 4.4 and 4.5). Carman had significantly more shoot fixed N than all the other sites in 1999, while Westbourne and Morden had significantly more shoot fixed N than Souris and Letellier which were similar in this attribute. There was no difference in shoot fixed N between Morden and Westbourne. In 2000, pea plants at Letellier and Westbourne, respectively, fixed the least and most N in the shoot compared to pea plants at the other sites. Morden and Souris pea plants fixed similar amounts of N, but significantly more than pea plants at Carman.

Table 4.12 Shoot fixed-N (g m^{-2}) of uninoculated and inoculated (with zero N-fertilizer) pea plants in 1999.

Treatmen	Site				
	Carman	Letellier	Morden	Souris	Westbourne
Uninoculated	14.3 b	4.6 a	8.1 a	4.5 a	9.2 a
Inoculated	15.9 a	7.4 a	9.0 a	4.6 a	10.4 a
LSD	1.45	5	2.8	1.9	7.3

Means followed by the same letter within a column are not significantly different using LSD (*P*=0.05).

Table 4.13 Shoot fixed-N (g m^{-2}) of uninoculated and inoculated (with zero N-fertilizer) pea plants in 2000.

Treatment	Site				
	Carman	Letellier	Morden	Souris	Westbourne
Uninoculated	5.3 a	2.3 a	8.7 a	8.5 a	12.4 a
Inoculated	8.0 b	4.1 a	9.3 a	10.4 a	12.7 a
LSD	1.5	2.9	3.4	5.2	3

Means followed by the same letter within a column are not significantly different using LSD (*P*=0.05).

4.1.7 Seed-Fixed N

Table 4.14 shows the amount of N fixed in seeds of pea plants in five sites in 2000. Seed fixed N did not significantly increase with inoculation except at Carman in 2000 where inoculated pea plants had significantly more fixed-N than uninoculated pea plants.

Seed fixed N was significantly influenced by location. As shown in Table 4.5, pea plants at Westbourne fixed significantly more N in the seed than pea plants at all the other sites. Pea plants at Letellier and Morden fixed similar levels of N in the seed, but significantly lower than those of pea plants at Carman and Souris. Carman pea plants fixed more N in the seed that pea plants at Souris.

Table 4.14 Seed fixed-N (g m^{-2}) of uninoculated and inoculated (with zero N-fertilizer) pea plants in 2000.

Treatment	Site				
	Carman	Letellier	Morden	Souris	Westbourne
Uninoculated	10.4 b	5.5 a	5.2 a	10.7 a	23.2 a
Inoculated	17.2 a	5.7 a	5.3 a	11.3 a	28.9 a
LSD	3.7	3.7	6.9	3.8	7.8

Means followed by the same letter within a column are not significantly different using LSD (P=0.05).

4.2 Plasmid Profiles (1998 Survey)

Rhizobial strains were isolated from nodules of pea plants grown in 20 sites in southern Manitoba and native *Lathyrus* sp. plants growing on roadsides adjacent to the Morden, Glenlea, and Teulon experimental sites. It was not possible to isolate strains in most cases where the nodules were small and white, a feature that was typical of *Lathyrus* sp. root nodules. Rhizobial strains obtained from pea plants in various parts of southern Manitoba appeared to grow faster than commercial inoculants and also produced more mucoid material. The field isolates and commercial inoculant strains of *R. leguminosarum* bv. *viciae* were typed by examining their plasmid profiles in agarose gels. It was relatively difficult to type field isolates that produced a lot of mucoid material and those that developed intense pigmentation on being transferred from yeast mannitol to PTY agar plates. Most isolates from Carman, for example, developed high levels of pigmentation on PTY plates.

Two hundred and seven field pea isolates, 7 *Lathyrus* sp. isolates, and 16 inoculant strains of *R. leguminosarum* bv. *viciae* were typed. Field pea isolates typed were obtained primarily from uninoculated plant nodules except isolates from Glenlea 1 all of which were obtained from inoculated plant nodules. Isolates were placed in distinct plasmid profile types based on size (determined by the Eagle-Eye software program using *R. etli* CE3 as the reference strain) and number of plasmid bands. Isolates deemed identical in number and size of plasmid bands were placed in the same profile type. Figure 4.1 shows a typical agarose gel of plasmid profiles of inoculant *R. leguminosarum* bv. *viciae* strains.

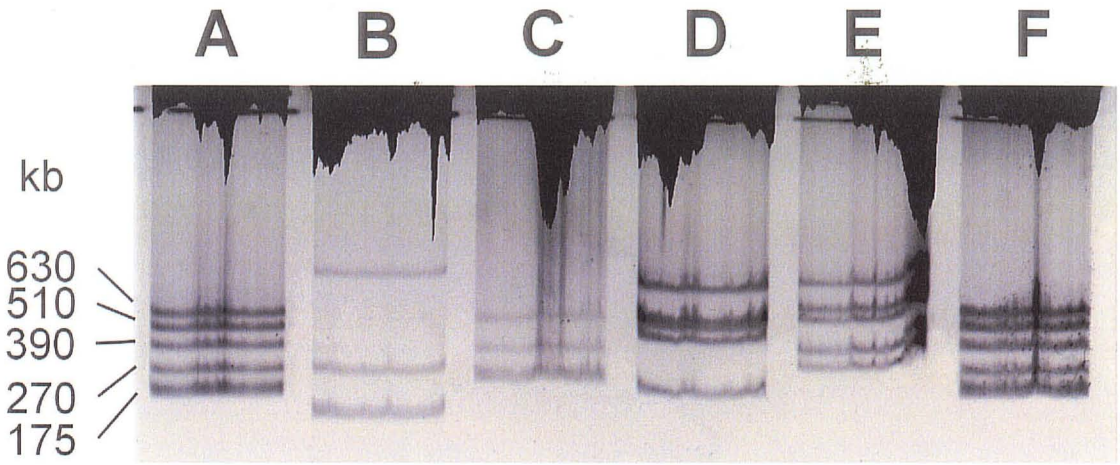

Figure 4.1 Plasmid profiles of inoculant strains RGAA1, RGP2, RGP4, and C1 (lanes B, C, D, E, respectively) and reference strain CE3 (lanes A and F) in 1% agarose gel.

Strains varied in number of plasmid bands from one to eight. Most field isolates exhibited one to five plasmid bands, while inoculant strains had only three to five plasmid bands. Single- and four-plasmid band isolates were the most common, comprising 27.8% and 27.4% of all the isolates (Table 4.15). The percentage of isolates with two, three, and five bands was 6.1, 14.3, and 19.1, respectively; isolates with six to eight plasmid bands constituted just about 5% of the total isolates. Strains varied in plasmid size from less than 50 kb to more than 1000 kb. Most isolates with more than two plasmids had at least one plasmid of more than 600 kb. All the distinct profiles with six, seven, and eight plasmids had plasmid band sizes of above 600 kb. In contrast, 30, 44.4, 70, 90.5, and 89.5% of the profiles with one, two, three, four and five plasmids, respectively, exhibited plasmid bands of above 600 kb.

Out of 230 strains typed, 77 distinct plasmid profiles were established (Appendix 8.4). Most of the distinct profiles were shared by at least two isolates, as only 35 strains had unique plasmid profiles. Table 4.16 shows some of the profiles that were isolated more than once. No single profile was dominant across sites. Profiles 1a-3 and 1a-4 had single bands and were the most common, each constituting 7% (16 out of 230) of all the isolates typed. Profile 3a-2 had three bands and was isolated 15 times. Profile 4a-11 with four bands was isolated 8 times, while profile 5a-5 with five plasmid bands was isolated 11 times. Profile 3a-2 was found in mores sites (nine sites) than all the profiles, followed by 1a-3 and 5a-5 which were isolated from seven and six sites, respectively. Some isolates appeared to be dominant at some sites. Nine out of 10 (90%) of the isolates typed at Teulon 2 exhibited profile 1a-4, while seven out of nine (78%) isolates typed at Morden 1 had profile 1a-3.

Table 4.15 Plasmid content of *Rhizobium* strains in 1998 survey.

Plasmid content	Number of Isolates	Number of distinct profiles	Profiles with plasmids over 600 kb
One plasmid band	64	10	3
Two plasmid bands	14	9	4
Three plasmid bands	33	10	7
Four plasmid bands	63	21	19
Five plasmid bands	44	19	17
Six plasmid bands	6	4	4
Seven plasmid bands	5	3	3
Eight plasmid bands	1	1	1

Table 4.16 Examples of profiles isolated more than once from uninoculated plants across southern Manitoba in 1998.

Profile	Number of isolations	Sites with the same profile	Observations
1a-3	16	S1, S7, S8, S13, S15, S16, S18	Major profile at S1 and S7
1a-4	16	S10, S12, S14, S19, S21,	Major profile at S10
1a-5	10	S6, S15, S12, S9	
3a-2	15	S1, S2, S3, S4, S12, S13, S15, S18, S20	Found in all Carman sites
4a-3	4	S1, S12, S17	Same as for RGP2 (inoculant)
4a-6	3	S12, S21	Same as for RGP212-2 and 99AA1 (inoculants)
4a-11	8	S16, S17, S18, S20, S21	
5a-5	11	S7, S12, S15, S16, S21, S17	
6a-3	3	S21, S20	
7a-1	2	S10, S15	

The number of isolates typed varied from one at Stuartburn to 33 at Morden, as shown in Table 4.17. Considerable diversity in plasmid profiles was demonstrated in most sites. Plasmid profile diversity index (PPDI), which we defined as the number of distinct profiles divided by the total number of isolates typed, for the 19 sites which had two or more isolates typed was about 0.6. The PPDI was considered to be zero in the sites where all the isolates had the same plasmid profile. Isolates obtained from pea plants at Teulon 1 and Westbourne 2, sites that had not seen pea or lentil in over 15 yr, registered low PPDIs. Out of 10 isolates profiled at Teulon only two distinct profiles were established (PDDI=0.2), while all the four isolates from Westbourne 2 had an identical profile. In contrast, Carman 4 site which had also not seen pea in at least 15 yr had two distinct profiles out of three isolates (PPDI=0.67). Among the sites with 10 or more isolates, the PPDIs varied from 0.2 at Teulon 1 to 0.83 at both Glenlea sites (S20 and S21). With the exception of Teulon 1, the PPDIs of the other sites were above 0.5. Plasmid profile diversity indices of sites within the same area appeared to be close but not identical. Isolates from native *Lathyrus* sp. were also diverse. Out of four isolates obtained from *Lathyrus* sp. plants at Teulon 1, three distinct profiles were recorded. The two isolates obtained from *Lathyrus* sp. plants at the Morden area had an identical profile, while the only *Lathyrus* sp. isolate from the Glenlea area had a unique plasmid profile.

Pearson correlation coefficients, relating PPDI to soil pH, soil organic matter, soil electrical conductivity, soil nitrate-N, and soil P content, demonstrated non-significant negative correlations (data not shown). A small, but significant, negative correlation ($r = -0.57$, $P=0.05$) was demonstrated between PPDI and number of years since previous pea or lentil cultivation (Figure 4.2). However, no significant correlation (r=-36) was noted

Table 4.17 Isolates typed from 20 different sites (1998 samples).

Site/source	Number of Isolates	Number of distinct profiles	Plasmid profile diversity index	Sites with at least one similar profile
Carman 1	5	3	0.6	12
Carman 2	3	3	1	8
Carman 3	3	2	0.67	9
Carman 4	3	2	0.67	9
Fort Garry Campus	2	1	0	3
Letellier	4	3	0.75	3
Morden 1	9	3	0.33	9
Morden 2	33	17	0.52	18
Arborg 1	5	5	1	9
Arborg 2	3	2	0.67	4
Teulon 1	10	2	0.2	5
Westbourne 1	22	13	0.59	15
Westbourne 2	4	1	0	4
Morris 1	14	11	0.79	16
Morris 2	22	15	0.68	12
Souris 1	18	13	0.72	10
Souris 2	10	7	0.7	12
Glenlea 1	12	10	0.83	13
Glenlea 2	24	20	0.83	13
Stuartburn	1	1	-	4
Lathyrus sp. isolates	7	5	-	0
Inoculant strains	16	13	-	7
Total	230			

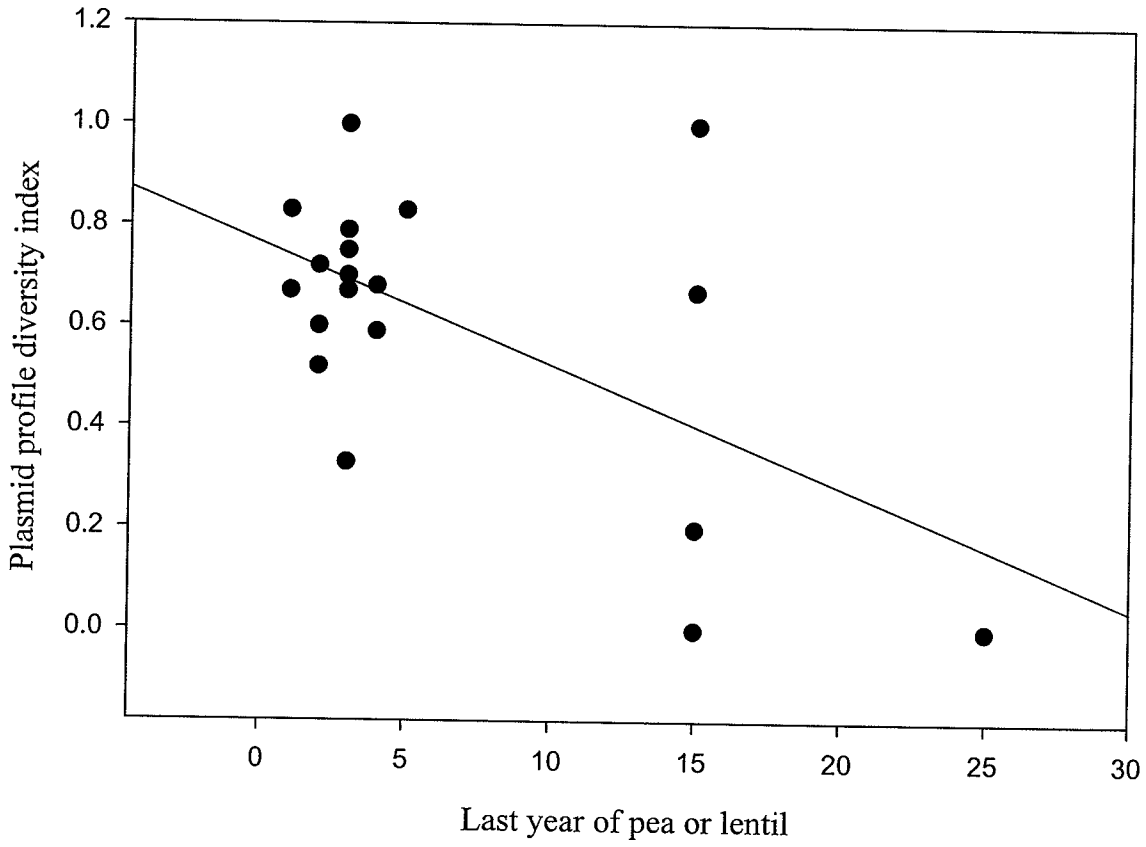

Figure 4.2 The relationship between diversity of pea nodule isolates, as measured by plasmid profile diversity index, from 19 sites (with at least two isolates each) and the last year of pea or lentil cultivation.

between these two attributes when Westbourne 2 which had not been grown to pea or lentil in the previous 25 yr was excluded in the analysis.

Each site shared at least one plasmid profile with 3 to 18 other sites (Table 4.17). Fort Garry Campus and Letellier sites shared at least one similar profile with three other sites, whereas Morden 2 had at least one common profile with 18 other sites. All sites within the same area, except at Arborg, had at least one plasmid profile in common. Table 4.18 shows a matrix of number of distinct plasmid profiles shared by eight sites that had 10 or more isolates typed. The proximity between sites did not appear to appreciably influence the number of distinct profiles they had in common. Any pair of the eight sites shared at least one distinct profile. Whereas Morris sites (S15 and S16) shared six distinct profiles, Souris sites (S17 and S18) had only one distinct profile in common. Glenlea 2 and Souris 1 sites (located far apart) shared five distinct profiles. Morden 2 site (S12) shared more distinct profiles with the farther Westbourne 1 (S13) and Souris 1 (S17) sites than with the nearer Glenlea 1 site.

Plasmid profiles identical to those of commercial strains were isolated from pea plants at some sites (Appendix 8.4). Inoculant strain RGP2 had the same plasmid profile as two isolates from Morden 2 and one isolate each from Carman 1 and Souris1. Strain RP212-37 had the same profile as one isolate of Morris 2 and two isolates of Morris 1, while 99AA1 and RP212-2 had the same profile as one isolate from Glenlea 2 and two isolates from Morden 2. Two isolates from Morden 1 and one isolate from Westbourne 2 shared the same distinct profile with RP212-19. In the few cases where isolates from inoculated plants were typed, the plasmid profile of the strain used in the inoculant was not always found. For example, none of the twelve Glenlea 1 isolates, all of which were

obtained from inoculated pea plants, had the same profile as the PBC108 inoculant strain. It was also found out that none of the native *Lathyrus* sp. isolates had profiles identical to those of field isolates. Commercial isolates C1, RP213-5, and 128C569G had the same profile, as did RP212-2 and 99AA1. The rest of the commercial strains (RGAA1, RGP4, RGL4, PEA082, 175G10B, RGFP, RP212-13) had unique profiles.

Table 4.18 A matrix showing the number of distinct plasmid profiles shared by pairs of sites (1998 survey) that had more than 10 isolates typed.

Site	Site							
	S12	S13	S15	S16	S17	S18	S20	S21
S12								
S13	3							
S15	3	3						
S16	3	3	6					
S17	4	2	3	5				
S18	1	3	2	3	1			
S20	2	1	2	2	2	2		
S21	4	1	2	4	5	2	2	

4.3 PCR Amplification of the IGS of 16S-23S rDNA Regions

Chromosomal DNA samples of 28 isolates were amplified with 16S and 23S rRNA primers designated as FGPL132' and 16SR11. Forward primer 16SR11 (corresponding to sequences at the 3' part of the 16S rRNA gene) when used with the universal primer FGPL32' (corresponding to the sequences at 5' end of 23S rRNA gene right next to the IGS) allows specific amplification of 16S-23S rDNA IGS of *R. leguminosarum* and *R. etli*. Electrophoretic analysis of uncut amplified products showed that all isolates but one produced single bands ranging in size from 1700 to 2000 bp as estimated by the Eagle-eye Program (Figure 4.3). Strain RGFP, a commercial inoculant strain prepared for faba bean, had a smaller PCR product (approximately 1700 bp) than all the other strains. Strain RGL4, a commercial strain prepared for lentil inoculation, had one band at 2000 bp and an extra one at about 1700 bp. The size range of the 16S-23S rDNA IGS regions for all the isolates corresponded to what was expected based on the primer sequence positions.

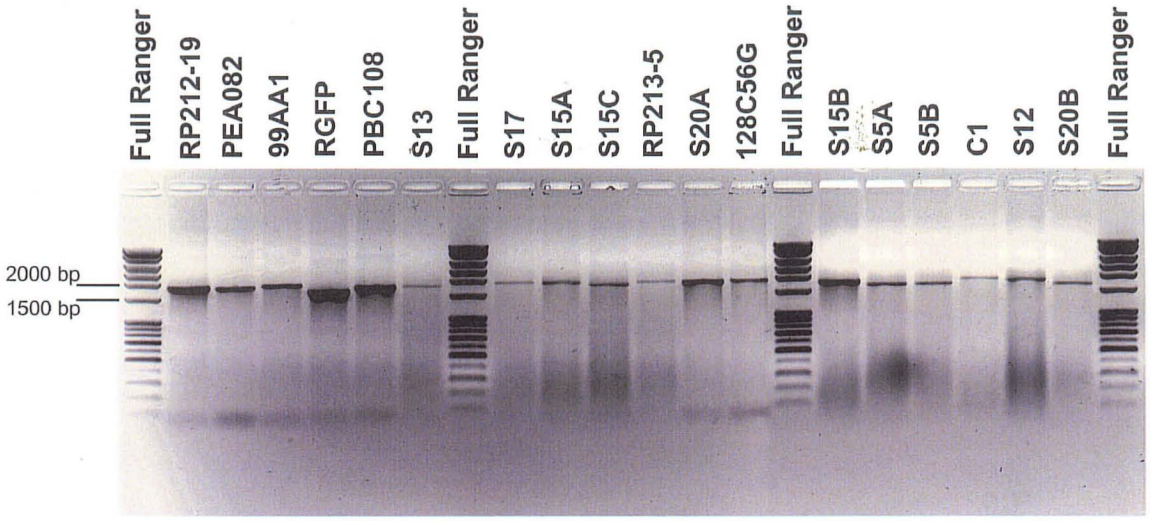

Figure 4.3 PCR-amplified fragments of 16S-23S rDNA IGS regions of field pea isolates and inoculant strains in a 1% agarose gel. The wells labeled F were loaded with a 100 bp molecular weight ladder.

4.4 RFLP Analysis of Amplified IGS of 16-23S rDNA

The RFLP technique was used to detect polymorphisms in the intergenic spacer sequences between genes coding for 16S and 23S rRNA of *Rhizobium* strains. Seven 4-base cutting restriction endonucleases (*Alu*I, *Cfo*I, *Dde*I, *Hae*III, *Msp*I, *Nde*II, *Taq*I) digested the amplified 16S-23S IGS rDNA fragments of all the 28 strains, yielding a variety of distinct restriction band patterns. Figure 4.4 is an electrophoresis gel showing restriction patterns of rDNA IGS digested with *Alu*I, *Hae*III, and *Msp*I enzymes. Bands shorter than 100bp were not scored (for all the enzymes) because they were, in most cases, not well resolved in NuSieve 3:1 agarose gels. Hence, the size of the amplified fragments estimated by summing the molecular weights of the restricted fragments was mostly smaller than or equal to the estimated sizes of the undigested PCR products. Table 4.19 shows the restriction patterns, for all the isolates, produced by the tested enzymes. The number of distinct restriction patterns produced by the individual restriction endonucleases ranged from 14 to 23. *Alu*1 was the least discriminating endonuclease, yielding 14 distinct profiles among the 28 isolates tested, while *Dde*I was the most discriminating, producing 23 distinct profiles. Enzymes *Hae*III and *Nde*II had each 16 different profiles, whereas *Msp*I, *Cfo*1, and *Taq*I had each 17 different profiles. Two to 8 bands per restriction pattern were obtained with each restriction enzyme. Restriction with *Alu*I and *Taq*I produced more restriction fragments compared to restriction with other enzymes, while *Cfo*1 exhibited the least number of restriction fragments. The following groups of strains were distinguished only when the amplified products were digested with *Dde*I: C1, 128C56G, and 99AA1; RGP2, S15A, S15C, S17; and S5A/S5B and S12. Whereas RP212-19, RGFP, S1B, S20B, S10, RGL4, H441, RP212-37 and S16 exhibited

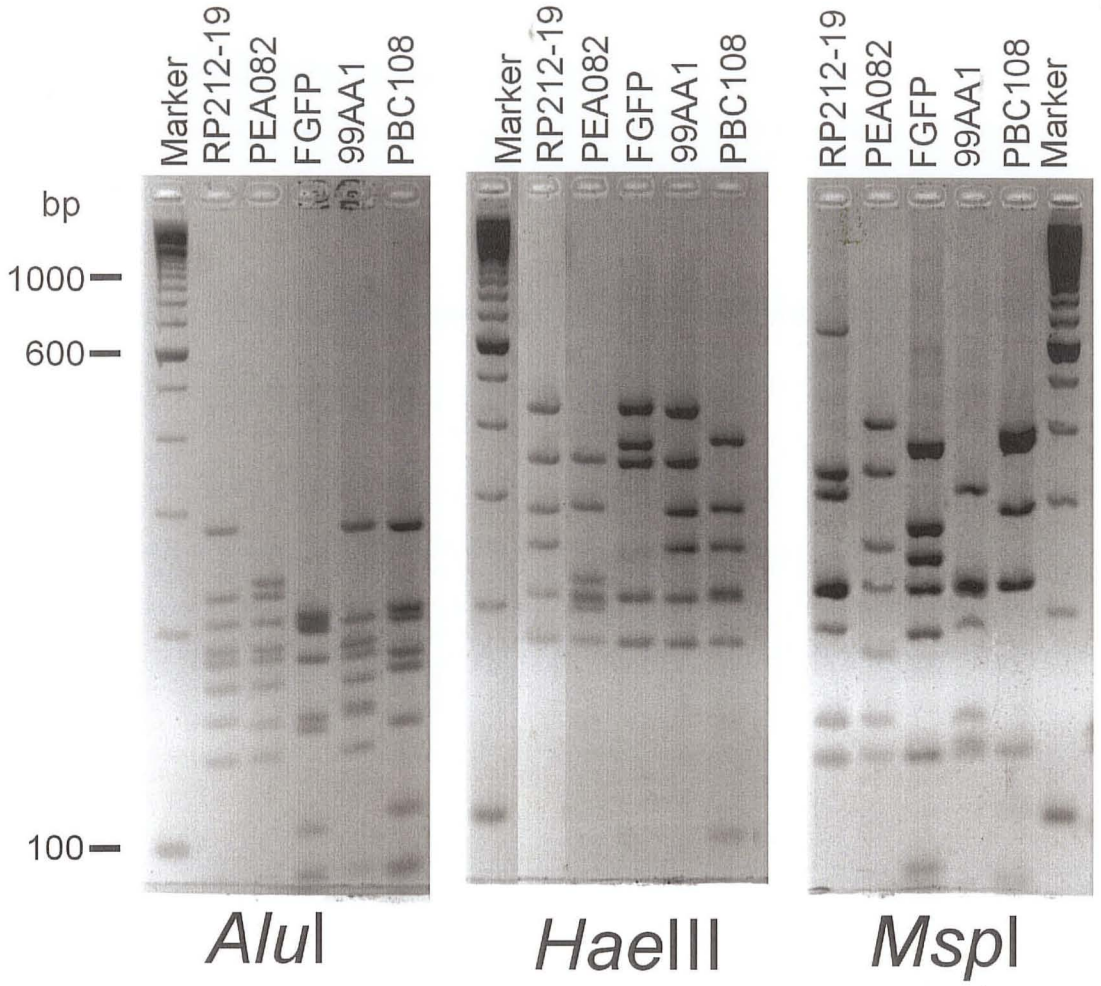

Figure 4.4 Restriction patterns of PCR-amplified 16S-23S rDNA IGS regions of inoculant strains (RP212-19, FGFP, PEA082, 99AA1, PBC108) digested with *Alu*I, *Hae*III, and *Msp*I in a 4% agarose (NuSieve 3:1) gel.

Table 4.19 The 16S-23S rDNA IGS restriction patterns of 28 *Rhizobium leguminosarum* bv. *viciae* strains obtained with seven restriction enzymes.

	Restriction pattern type of 16S-23S rDNA IGS digested with:						
Strain	*Alu*I	*Cfo*I	*Dde*I	HaeIII	*Msp*I	*Nde*II	*Taq*I
RP212-19	A1	C1	D1	H1	M1	N1	T1
PEA082	A2	C2	D2	H2	M2	N2	T2
S15B	A2	C2	D3	H2	M2	N3	T3
RGP4	A2	C2	D3	H2	M2	N2	T3
RGFP	A3	C3	D4	H3	M3	N4	T4
99AA1	A4	C4	D5	H4	M4	N1	T5
128C56G	A4	C4	D6	H4	M4	N1	T5
C1	A4	C4	D7	H4	M4	N1	T5
RGP2	A4	C5	D8	H4	M5	N1	T5
S1A	A4	C6	D5	H4	M5	N1	T5
S15A	A4	C5	D9	H4	M5	N1	T5
S15C	A4	C5	D10	H4	M5	N1	T5
S17	A4	C5	D5	H4	M5	N1	T5
S1B	A5	C7	D11	H5	M6	N5	T6
PBC108	A6	C8	D12	H6	M7	N6	T7
S20A	A7	C9	D13	H7	M8	N3	T8
S20B	A8	C10	D14	H8	M9	N7	T9
S10	A9	C11	D15	H9	M10	N8	T10
S12	A10	C12	D16	H10	M11	N9	T11
S5A	A10	C12	D17	H10	M11	N9	T11
S5B	A10	C12	D17	H10	M11	N9	T11
S13	A6	C13	D18	H6	M12	N10	T12
S18	A10	C14	D19	H11	M13	N11	T13
RP213-5	A7	C9	D13	H12	M8	N12	T13
RGL4	A11	C15	D20	H13	M14	N13	T14
H441	A12	C16	D21	H14	M15	N14	T15
RP212-37	A13	C17	D22	H15	M16	N15	T16
S16	A14	C13	D23	H16	M17	N16	T17
Restriction types	14	17	23	16	17	16	17
Bands per profile	5 - 8	2 - 6	4 - 6	3 - 6	3 - 7	4 - 8	3 - 6

Note: All the field strains begin with capital S, followed by site number and capital A , B or C if the site was represented by two or more isolates; the rest are commercial isolates. The letters for the restriction pattern data are the abbreviations of the enzymes used followed by numbers representing rDNA restriction pattern.

unique restriction patterns with all the enzymes tested, restriction patterns for strains S5A and S5B were not differentiated by any of the enzymes tested. All the restriction enzymes differentiated strain S15B from sister isolates S15A and S15C. Inoculant strain PBC108 was not distinguished from strain S13 when tested with *Alu*I and *Hae*III, while RP213-5 was differentiated from S20A only when respective amplified rDNA IGS regions were digested with *Hae*III, *Nde*II, and *Taq*I.

Each isolate was scored for the presence and absence of a restriction band and coded as 1 or 0, respectively. A total of 148 polymorphic bands were scored for all the enzymes, resulting in a 28 by 148 matrix. The highest number of bands (24 bands) were scored for *Taq*I enzyme, while *Cfo*I had the least number of scored bands (17 bands). The scored bands for the remaining enzymes were: 20 for *Dde*I, 21 each for *Alu*I and *Hae*III, 22 for *Nde*II, and 23 for *Msp*I. The 28 by 148 matrix was used to generate a simple matching coefficient (SMC) matrix (28 by 28). The SMC matrix was used to generate a phenogram, depicting similarity in the rDNA IGS regions of *Rhizobium* strains, using the unweighted pair group method with an arithmetic mean (UPGMA). Calculation of the SMC and development of the phenogram was performed with an NTSYSpc package (Rohlf, 1997). The SMC ranged from 0.58 to 1 (data not shown). Fort Garry Campus strains S5A and S5B had a SMC of 1.00, while H441 and S10, like RP212-37 and PEA082, had an SMC of 0.581.

Clustering of the SMCs using UPGMA placed the isolates in different groupings (Figure 4.5). The different clusters mirrored the similarity in restriction patterns among the isolates demonstrated in Table 4.19. The tree had three major groupings: the first

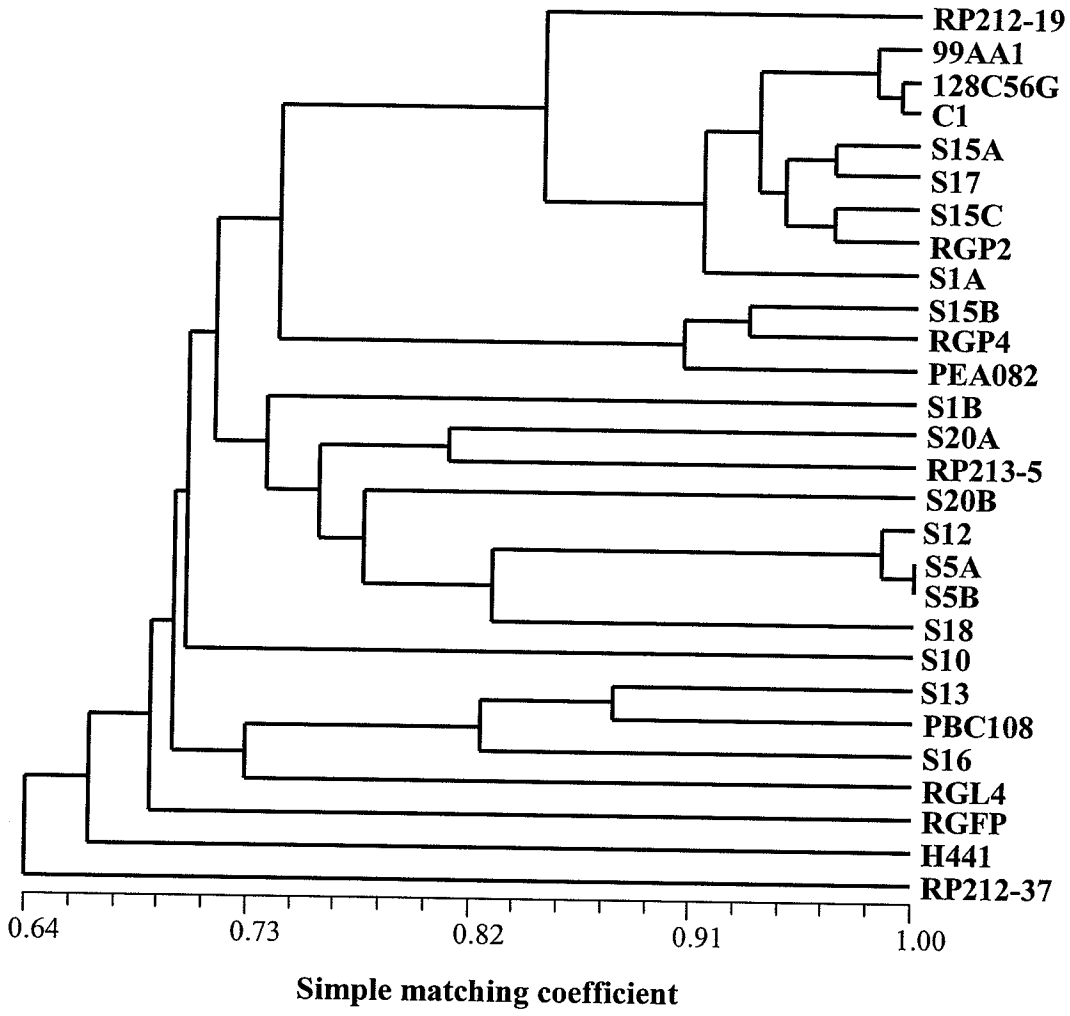

Figure 4.5 Phenogram (UPGMA) depicting genetic similarities among strains of *Rhizobium* strains revealed by PCR-RFLP analysis of their 16S-23S rDNA IGS regions. Simple matching coefficients were generated for cluster analysis.

grouping had 4 strains (RGL4, S16, PBC108, S13), the second had 8 strains (from S18 to S1B), and the third had 12 strains (from PEA082 to RP212-19). Isolate S10 from Teulon 1 was placed in its own unique group as were H441 (*R. leguminosarum* bv. *phaseoli* strain obtained from France), RGFP (inoculant strain for faba bean) and RP212-37. There were further subdivisions in the second and third groupings. Isolates from Fort Garry Campus (S5A and S5B) had identical plasmid profiles and were not differentiated by UPGMA analysis. These two strains were clustered very close to Morden S12 strain (SMC= 0.986), although the latter had exhibited a different plasmid profile (Appendix 8.4). Commercial strains C1 and 128C56G had identical plasmid profiles and were very close to each other (SMC=0.993), but far from RP213-5 which had the same plasmid profile. Similarly, isolates S15B (Morris 1) and S1B (Carman 1) had the same plasmid profile but were not close chromosomally. Isolates S20A and S20B (SMC=0.777) from Glenlea had different plasmid profiles and were placed in the same main grouping; however, relative to each other, S20A was closer to RP213-5 (SMC=0.811) while S20B was closer to S12 , S5A, and S5B (SMC=0.784). Isolates S1A and S1B (SMC=0.696) from Carman 1 had different plasmid profiles and were clustered in two divergent groups. Isolates S15A, S15B, and S15C from Morris 1, each with a distinct plasmid profile, belonged to the same main grouping, but S15A and S15C were much closer to each other than they were to S15B. Isolate S17 from Souris had a unique plasmid profile, but UPGMA analysis showed it to be chromosomally very close to S15A. Strain S15C was the field isolate that exhibited the highest level of genetic similarity with an inoculant strain; S15 C was very close to inoculant strain RGP2 (SMC=0.966), although they had demonstrated different plasmid profiles. Strains S1A and RGP2 (SMC=0.919) had the

same plasmid profile and were clustered in the same major subgroup (but not in the same sub-subgroup) by UPGMA analysis. However, strain RGP2 was demonstrated to be actually closer to five other strains (from S15C to 99AA1, SMC= >0.925) with which they differed in plamid profiles. Isolate S16 from Morris 2 was much more similar to isolate S13 (SMC= 0.824) from Westbourne 1 than it was to isolates S15A, S15B and S15C (SMC= 0.736, 0.689, 0.716, respectively) from the neighbouring Morris 1 site.

The neighbour joining method (NJ) and an ordination technique (multidimensional scaling) were also used to depict genetic similarity of isolates. The 28 by 28 SMC matrix was used to generate a phenogram based on the NJ method using NTSYpc software program (Rohlf, 1997). The similarity tree generated by this method is depicted in Figure 4.6. The NJ method clustered the strains in almost the same way as UPGMA, with a few glaring exceptions. S1A was not distinguished from S17, although they were clearly differentiated based on rDNA IGS restriction pattern profiles (Table 4.19) and the SMC (0.953). On the other hand, S5A and S5B were clustered close together, but they remained distinct from each other contrary to the restriction patterns (Table 4.19) and the SMC(1.000) which showed them to be the same. The NJ method placed S20 B and RGFP in the same cluster, unlike the UPGMA which placed RGFP in a unique position. Strains S20 and RGFP had a SMC of 0.76. Strain RGL4 was placed in its own unique group by the NJ method as were S10, S1B, and H441.

Using the NTYSpc package, the 28 by 28 SMC matrix was reduced to two dimensions and displayed as a multi-dimensional scaling (MDS) plot (Figure 4.7). The MDS 'map' showed relationships among isolates that were largely similar to those generated by UPGMA and NJ clustering methods. Strains RGL4 and H441 are placed in

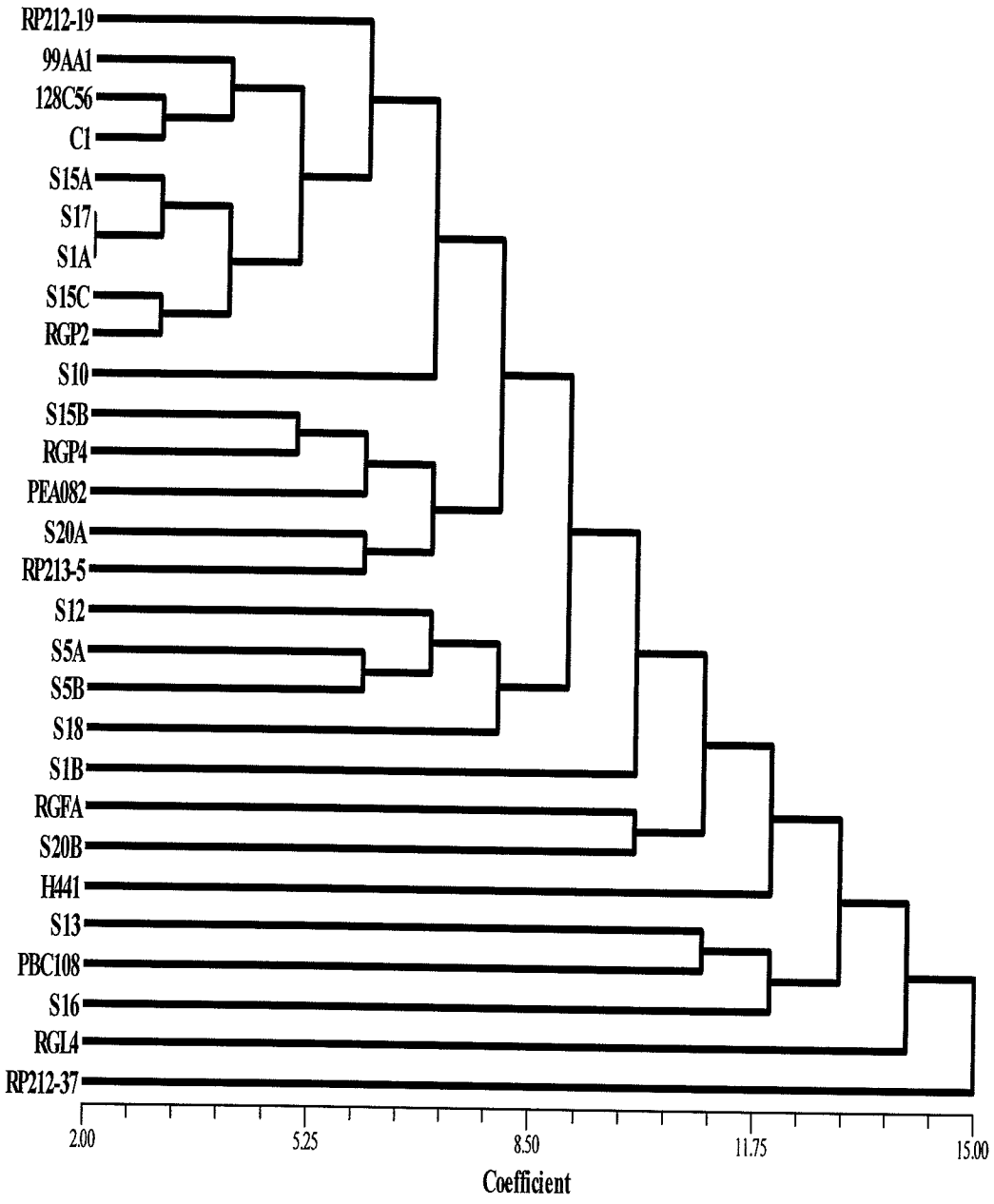

Figure 4.6 Phenogram (NJ method) of genetic similarities among *Rhizobium* strains revealed by PCR-RFLP analysis of their 16S-23S rDNA IGS regions. Simple matching coefficients were generated for cluster analysis.

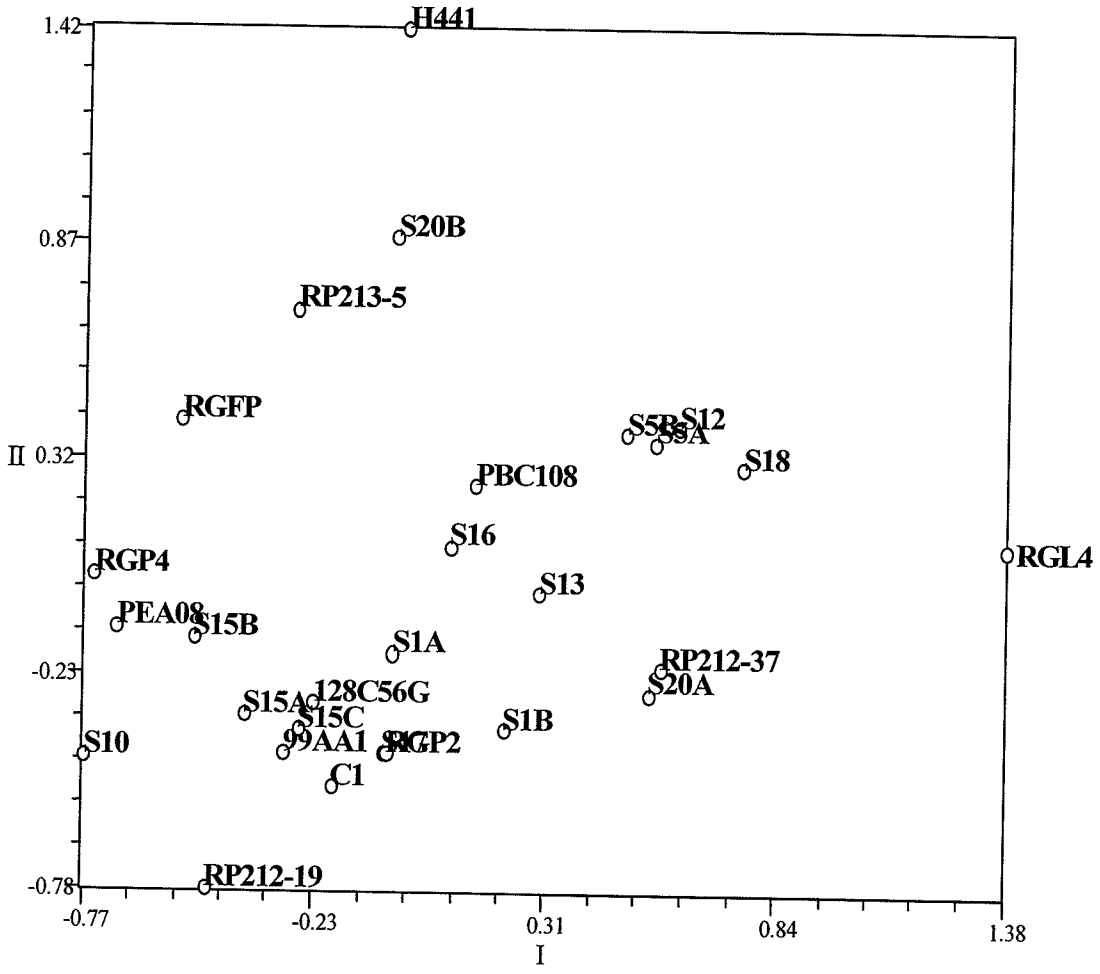

Figure 4.6 Multidimensional scaling (MDS) plot of the genetic relationships among *Rhizobium* strains based on PCR-RFLP analysis of their 16S-23S rDNA IGS regions. Simple matching coefficients were used to generate the plot.

unique individual positions on the plot. Looking at the MDS plot from both dimensions, the following groupings could be discerned: S15A, 128C56G, 99AA1, S15C, S17, RGP2, C1 and S1A; RGP4, PEA082 and S15B; PBC108, S13, and S16; and S5A, S5B, and S12. These groupings were similar to groupings generated by the clustering methods, although there were slight variations. Field isolate S20A and inoculant strain RP212-37 were placed very close together on the plot (in both dimensions), although each had distinct restriction patterns with all the tested endonucleases (Table 4.19). The SMC between the two strains was only 0.615 and clustering methods had shown little similarity in the rDNA IGS regions of the two isolates. Looked at in both dimensions, the MDS plot was not able to separate RGP2 from S17. These two strains were very similar but not identical in restrictions patterns (Table 4.19), and clustering methods showed them to be similar but distinguishable (Figures 4.5 and 4.6). Strains RP213-5 and S20B were placed close on MDS plot.

4.5 Nodule Occupancy

Plasmid profiles of 64 *Rhizobium* isolates obtained from nodules occupying proximal and distal root regions of inoculated pea plants grown at Morden 2 in 2000 were examined. Figure 4.8 shows an agarose gel image of plasmid profiles of inoculated field pea isolates. The profiles of 22% of the proximal isolates (7 out of 32 isolates) were identical to that of the inoculant strain, while only 6% of the distal isolates (2 out of 32 isolates) had the same profile as the inoculant strain. There was no single profile that dominated pea nodules. The most common profile (M7) was found in 18.8% of all the 64 isolates, representing 34.4% of all the isolates obtained from distal root nodules (Table 4.20). Isolates obtained from proximal and distal root regions of pea plants appeared to have different profile types. For example, profiles M1 and M2 were isolated thrice and seven-fold from distal root nodules but none were isolated from proximal root nodules. In contrast, profile M10 was absent in distal isolates but it was isolated four times from proximal isolates. Some profiles appeared to be associated more with some plants than with others. For example, profile M12 was isolated three times from plant 2 but none in the other plants, while profile PBC108 was isolated three times from plant 1, once in plant 2, four times from plant 3 and once in plant 4 (Table 4.21).

Figure 4.9 shows the plasmid profile diversity index (PPDI) of proximal and distal root region isolates. Proximal isolates had a higher PPDI than the distal isolates. The PPDI for proximal isolates (0.53) was about twice that of distal isolates (PPDI=0.25) when the inoculant strain was included in the analysis. However, in case the inoculant strain was excluded from the analysis, the PPDI of proximal isolates (0.64) increased to close to three times that of distal isolates (PPDI= 0.23).

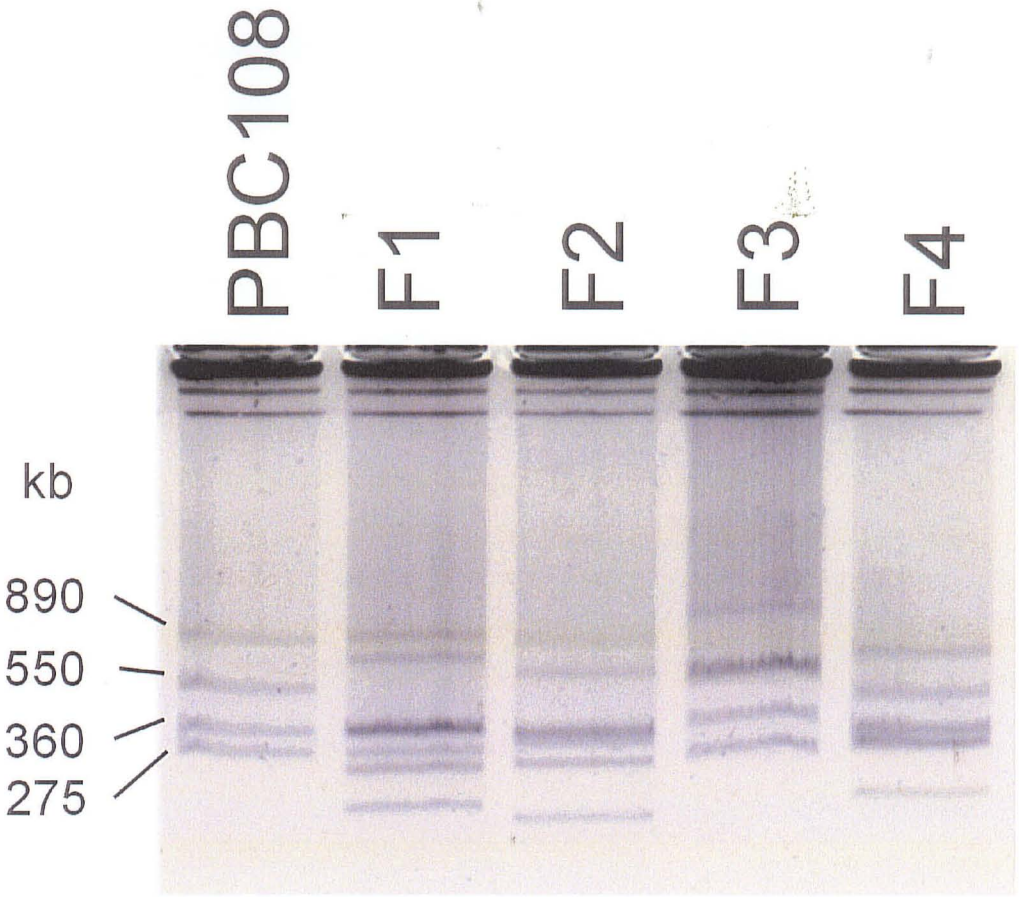

Figure 4.8 Plasmid profiles of field isolates (F1, F2, F3, F4) obtained from nodules of inoculated pea plants at Morden 2 in 2000 and kilobase size markers of PBC108 (PBC).

Table 4.20 Distribution of plasmid profiles (isolated more than once) between proximal and distal root regions of inoculated pea plants at Morden 2 site in 2000.

Profile	Nodule source	Plant 1	Plant 2	Plant 3	Plant 4	Total	% of isolates
M1	Proximal	0	0	0	0	0	0
M1	Distal	0	1	2	0	3	9.4
M2	Proximal	0	0	0	0	0	0
M2	Distal	2	3	1	1	7	21.9
M7	Proximal	1	0	0	0	1	3.1
M7	Distal	2	1	4	4	11	34.4
M10	Proximal	1	0	3	0	4	12.5
M10	Distal	0	0	0	0	0	0
M12	Proximal	0	0	0	0	0	0
M12	Distal	0	3	0	0	3	9.4
M13	Proximal	0	0	1	0	1	3.1
M13	Distal	0	1	0	2	3	9.4
M14	Proximal	0	0	1	0	1	3.1
M14	Distal	3	1	0	0	4	12.5
M15	Proximal	0	1	1	3	5	15.6
M15	Distal	0	0	0	0	0	0
PBC108	Proximal	3	1	2	1	7	21.9
PBC108	Distal	0	0	2	0	2	6.3

Example: Profile M1 was isolated from distal root nodules once in plant 1, twice in plant 2; none was isolated from proximal nodules. Profile PBC108 was isolated from proximal nodules three times in plant 1, once in plant 2, twice in plant 3, and once in plant 4, but was isolated from distal nodules of plant 3 only (twice).

Table 4.21 Distribution among individual inoculated plants of profiles isolated more than once from the Morden 2 site in 2000.

Profile	Plant 1	Plant 2	Plant 3	Plant 4	Total	% of all isolates
M1	0	1	2	0	3	4.7
M2	2	3	1	1	7	10.9
M7	3	1	4	4	12	18.8
M10	1	0	3	0	4	6.3
M12	0	3	0	0	3	4.7
M13	0	1	1	2	4	6.3
M14	3	1	1	0	5	7.8
M15	0	1	1	2	4	6.3
PBC108	3	1	4	1	9	14.1

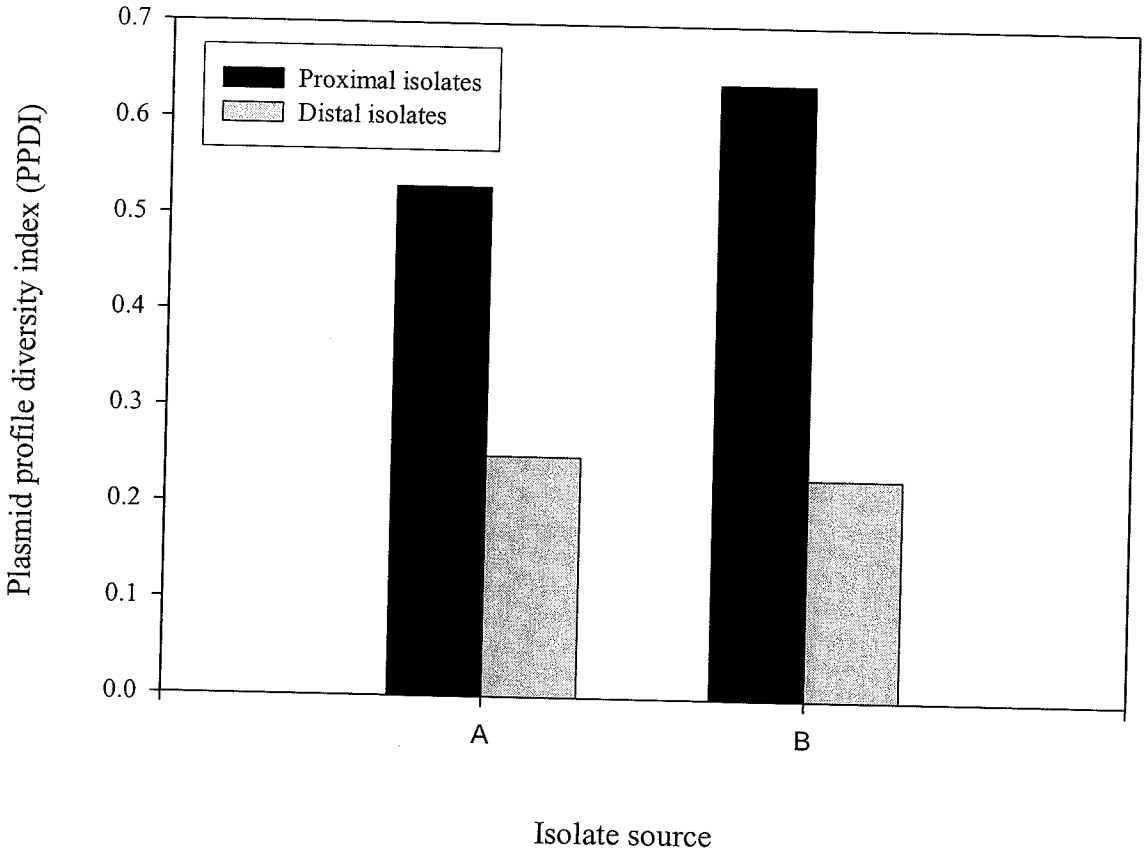

Figure 4.9. Plasmid profile diversity index (PPDI) of proximal and distal isolates obtained from inoculated plants at Morden 2 in 2000. The inoculant strain (PBC108) was included in calculating PPDI values in group A of the graph, but not in group B.

5.0 DISCUSSION

5.1 Presence and Abundance of Pea Rhizobia in Southern Manitoba Soils

The field survey carried out in 1998 demonstrated that soils across southern Manitoba contain resident populations of *Rhizobium leguminasorum* bv. *viciae* able to nodulate field pea. Uninoculated pea plants grown in arable fields were well nodulated, regardless of whether or not the fields had previously been grown to pea, lentil or faba bean. However, nodulation of uninoculated pea plants was very poor or almost absent in the fields with no history of cultivation (Westbourne 2 and Stuartburn), indicating that resident *R. leguminosarum* bv. *viciae* populations were either too low or absent. Previous studies have demonstrated the presence of resident populations of *R. leguminosarum* bv. *viciae* in most western Canadian soils. Kucey and Hynes (1989) showed that wheat fields in southern Alberta that had never been planted to pea contained 1 to 100 *R. leguminosarum* bv. *viciae* per gram of soil. Rice et al. (1993) reported that *R. leguminosarum* bv. *viciae* was present in all but two of 29 soils collected from northwestern Alberta and northeastern British Columbia. In recent studies, Rice et al. (2000a) detected no resident *R. leguminosarum* bv. *viciae* populations in an uncultivated field in Alberta. Resident rhizobia nodulating crops such as common bean and alfalfa have also been recovered from soils in which these crops had never been planted (Kucey and Hynes, 1989; Bottomley and Jenkins, 1983).

The presence of populations of *R. leguminosarum* bv. *viciae* resident in the arable and virgin soils not known to have been grown to their host legumes could be attributed to several sources. The rhizobial populations that nodulated pea could have been native strains that nodulate *Lathyrus* sp. and vetch (*Vicia* sp.) plants which were found growing

close to the experimental "trap" plots. If this was the case, *R. leguminosarum* bv. *viciae* could be considered native to southern Manitoba. Kucey and Hynes (1989) concluded that *R. leguminosarum* bv. *vicia*e was possibly native to southern Alberta when they found these bacteria in soils that had never been sown to pea before. Rhizobia from nearby sites could also be introduced in soils to which their host legumes have never been planted by dispersal agents such as wind, water, man and animals. Large numbers of rhizobia can be found in air-borne dust during harvesting and on clean legume seed. For example, airborne-dust arising from clover plant debris was estimated to contain 10^5 nodulating cells of *R. leguminosarum* bv. *trifolii* per gram, while clean clover seeds contained about 200 nodulating cells per gram (reviewed by Parker et al. al., 1977). Rhizobia may also spread through agricultural implements and in animal forage feed. Some of the aforementioned avenues may have contributed to the presence of rhizobia nodulating pea in the study areas where no deliberate rhizobial inoculation had been carried out. It is conceivable that the little nodulation noticed in pea plants grown at the virgin Westbourne 2 site was caused by rhizobia dispersed from an adjacent arable field situated five metres away.

The arable soils (Carman 4, Fort Garry Campus, Arborg 1, and Teulon 1) that may never have seen pea, lentil or faba bean before appeared to contain much higher levels of resident *R. leguminosarum* bv. *viciae* than the virgin soils (Stuartburn and Westbourne 2). The average nodule number per plant for the arable soils was above 17 times that for the virgin soils (i.e, 50.2 nodules per plant for arable soils compared to 2.9 nodules per plant for virgin soils) (Appendix 8.1). This could be attributed partly to the potential differences in soil fertility between the two groups of soils. Cultivated soils have been

shown to support larger populations of rhizobia than do virgin soils which are normally limited in nutrients, especially phosphorous and nitrogen (Parker et al., 1977; Thies et al., 1992). Arable fields in southern Manitoba are high in organic matter and are routinely fertilized with nitrogen and phosphorous. These fields would thus be expected to promote the growth and survival of pea rhizobia. Superior rhizobial numbers in arable soils could also have resulted from the effects of previous cultivation of legumes not nodulated by *R. leguminosarum* bv. *viciae.* Rhizobial growth has been shown to be preferentially stimulated in the rhizosphere of legumes than that of non-legumes due to the large amounts of exudates released into the rhizosphere, consisting principally of sugars, amino acids, and vitamins such as biotin and pantothenic acid (Rao, 1995). Soil tillage possibly enhanced the dispersal of the root nodule bacteria within and between arable fields, resulting in reduced competition for limited soil resources and better odds of the bacteria encountering and infecting legume host roots. This is unlike in the relatively undisturbed virgin soils in which bacterial cells would be expected to be relatively stationary (Date, 1991; Bottomley, 1992; Streeter, 1994) and the chances of rhizobia encountering and infecting growing legume roots greatly diminished. This may partly explain the poor nodulation of *Lathyrus* sp. and vetch plants observed in this study. It is, however, possible that some of these arable soils may have been previously grown to inoculated or uninoculated host legumes of *R. leguminosarum* bv. *viciae,* even though there were no records to that effect.

Detailed studies and the field survey revealed no differences in nodulation between inoculated (without fertilizer N) and uninoculated pea plants in most sites. Similar and contrasting observations have been made in related studies. Rennie and

Dubetz (1986) reported higher nodule numbers in inoculated than in uninoculated pea plants only in one of two sites in southern Alberta. In contrast, Rice et al. (2000a), using several inoculation techniques in greenhouse experiments, reported significant improvements in nodulation in pea grown in three Alberta soils containing 390, 34 and 0 rhizobial cells per gram of soil, respectively. In other studies, Thies at al. (1991) showed that inoculation levels of eight leguminous crops grown in soils containing 10 to 100 indigenous rhizobial cells per gram of soil increased the number of nodules per plant. Studying two soils with low populations of *R. leguminosarum* bv. *trifolii*, Nasih et al. (1993) demonstrated that a population of 10^2 rhizobia per gram of soil provided little nodulation, but a population of 10^3 rhizobia per gram of soil increased clover nodulation. There was no response of lentil nodulation to inoculation with *R. leguminosarum* bv. *viciae* in Egypt (Moawad et al., 1998). The lack of nodulation response to inoculation in our study indicates that *R. leguminosorum* bv. *viciae* is not only present but also abundant in most southern Manitoba soils. The abundance of pea rhizobia could be attributed to inoculated pea or lentil previously grown in the experimental sites. Legumes tend to stimulate the growth of rhizobia that nodulate them more than those that do not (Bushby, 1993; Thies et al., 1995). In studies in southern Alberta soils, there were 10 to 100 times more *R. leguminosorum* bv. *viciae* in pea fields than in common bean or wheat fields, while common bean-nodulating bacteria were 100 to 1000 times higher in common bean fields than in pea or wheat fields (Kucey and Hynes, 1989). Indigenous *S. meliloti* nodulating alfalfa (Barber, 1982) and *Bradrhizobium* nodulating cowpea and soybean (Thies et al., 1995) have been reported to increase in size following the cropping of the homologous host legume. Selective stimulation of rhizobial growth may occur when host

plants produce substrates that are selectively utilized by rhizobia that nodulate them. For example, host pea plants have been shown to produce homoserine which can be selectively metabolized by *R. leguminosarum* bv. *viciae* (van Egeraat, 1975). In addition, some strains of *R. leguminosarum* bv. *viciae* have demonstrated the capacity to catabolize rhizopines produced in bacteroids within pea plant nodules (Murphy et al.1995; Wexler et al., 1995). Strains capable of utilizing secondary metabolites can, therefore, proliferate in the host legume rhizospheres. However, most of the rhizobial cells found in the soil following homologous legume host plants possibly originate from the nodules. An individual nodule can contain up to 10^6 viable (nodulating) cells of a *Rhizobium* species (reviewed by Hirsch, 1996). These rhizobial cells are released into the soil upon nodule senescence and can colonize the soil and the rhizosphere, thereby becoming persistent and perhaps permanent components of the soil microbial population (Kuykendall, 1989; Thies et al., 1995; Hirsch, 1996).

The uninoculated pea plants were relatively poorly nodulated compared to inoculated (with no N fertilizer) plants at Carman 1 in 2000. In this case, inoculation (with no N fertilizer) doubled the number of nodules per pea plant. In addition, Carman 1 had 1.6 to 2.8 times fewer nodules per plant than the other sites during both years of detailed studies. The soil characteristics and the field history of the Carman site were not unusual relative to the other sites, with the exception that the soil was very mildly acidic (pH 6) and lentil was the previous host legume grown. Nodulation has been shown to be very sensitive to low pH even when rhizobia are abundant. Pea nodulation was reported to be 10-fold more susceptible to acidity than either rhizobial multiplication or plant growth (Evans et al., 1980). The initial stages of nodulation, including rhizobial

attachment and root hair curling, which coincide with nod gene induction, are thought to be the most pH sensitive (Franco and Munns, 1982; Vargas and Graham, 1988). Rice et al. (2000a) reported that inoculation improved nodulation of pea in acid-soils from Alberta. Since the pH at Carman 1 was mildly acidic, other factors such as protozoan predation and microbial antagonism may have played a role in reducing both inoculant and resident rhizobia. Alternatively, pea plants at Carman may not have required more nodules since they fixed more nitrogen than the better nodulated pea plants at the other sites. Host legume plants have been shown to regulate nodule number in a process called autoregulation (Hansen, 1994). According to the soil analysis results (Norwest Labs, Winnipeg, MB), the electrical conductivity of the soil at the Letellier site in 2000 was considered toxic for plant growth. However, pea plants were very well nodulated in this site, suggesting that resident pea rhizobia can survive well in soils prone to salt stress and that pea-*Rhizobium* symbiosis can withstand moderate salt stress levels. In contrast, it appears that the salt stress may have depressed plant growth at this site. Previous studies have demonstrated that rhizobial strains appear to be more tolerant to elevated salinity than their host legumes (Tu, 1981; Zahran and Sprent, 1986).

Application of N fertilizer to inoculated pea depressed nodulation in most cases. This finding is in agreement with the well documented fact that inorganic forms of nitrogen, especially nitrate, interfere with nodulation in leguminous plants. There have been reports of reduced numbers (Rai, 1982), delayed nodulation (Herridge et al., 1984) and even complete cessation of nodulation in the presence of nitrate (da Silva et al., 1993). The mechansim for nitrate inhibition of nodulation is not well understood, although carbohydrate deprivation in the nodules as a result of the energy required for

nitrate reduction is one of the major explanatory hypotheses (Havelka et al., 1982). High levels of nitrate in field soils would, thus, be expected to have an adverse impact on the effectiveness of legume-rhizobial symbiosis under field conditions.

5.2 Symbiotic Effectiveness of Resident Pea Rhizobia

It is often necessary to inoculate legumes in N-deficient soils in which resident rhizobial populations are either too low for adequate nodulation or poor in fixing nitrogen. The relative symbiotic effectiveness of resident rhizobia (i.e. the ability of a nodulated plant to fix nitrogen) can be quantitatively expressed by comparison of dry weights of uninoculated plants and those supplied with N-fertilizer. The differences in dry weights among the uninoculated and inoculated (with and without N-fertilizer) plants reflect the efficiency of N-fixation by the inoculant strain (Graham, 1998). In our study, shoot dry matter produced by plants sampled from inoculated plots was significantly higher (by 17.5 to 40%) than that from uninoculated plants in 1998 and in 2000, with the exception of Westbourne where no treatment effects were detected. In addition, there was no difference in shoot dry matter between N fertilized and non N fertilized inoculated plants during the same period. These results suggest that the locally adapted resident populations of *R. leguminosarum* bv. *viciae* were not as efficient (in most cases) as the inoculant strain in their contribution to dry matter production. The majority of studies have shown that resident rhizobia strains nodulating most-field grown plants, including pea, appear to be ineffective in fixing nitrogen. A two-year field study involving five sites in Saskatchewan and two cultivars each of pea, lentil, and faba bean showed that inoculation increased dry matter accumulation (Bremer et al., 1988). In greenhouse

experiments, inoculation was reported to increase the aboveground biomass of pea plants grown in three acid-soils in Alberta (Rice et al., 2000a). Fesenko et al.(1995) reported that some indigenous isolates of *R. leguminosarum* bv.*viciae* obtained from pea plants were effective nitrogen fixers. However, most rhizobial populations resident in northwestern Alberta and northeastern British Columbia were either partially or completely ineffective (Rice et al., 1993).

Farmers in southern Manitoba are advised not to apply N-fertilizer if they have inoculated their pea crops with the expectation that the residual soil N and the biologically fixed atmospheric N would meet the crop N-needs (Waterer et al., 1994). The results of the present study strongly support this recommendation as there were no differences in aboveground biomass (with the exception of Souris 2 in 1999) among inoculated (with and without N-fertilizer) plants both in the survey and in the detailed experiments. A similar finding has been reported by several researchers. Waterer et al. (1994) reviewed a number of experiments on yield response to N-fertilizer application and reported positive responses only in 12 of 81 site years of pea. Experiments carried out in Saskatchewan (Cowell et al., 1989) and Alberta (Izzaurralde et al., 1990) showed no yield response by pea to N-fertilizer application. However, in an earlier study in Saskatchewan, Sosulki and Buchan (1978) had shown an increase in pea yield with N-fertilizer application.

N-fertilized pea plants accumulated more biomass than both inoculated (with no fertilizer) and uninoculated pea plants at Souris 2 in 1999. In addition, a heavy dose of N-fertilizer (100 Kg N ha^{-1}) failed to depress pea nodulation at this site. These two observations suggest that residual soil N may have been very low in the early part of the

pea growing period. Since this field site was under wheat stubble and was low in nitrate, incorporation of wheat stubble into the soil at planting could have immobilized some of the soil residual nitrogen (Doran, 1980; Paul and Clark, 1996), making it less available for plant growth. Low soil residual nitrogen is expected to increase nodulation and nitrogen fixation by legumes (Wheatley et al., 1995); however, very low nitrogen is possibly detrimental to these processes. It has been suggested that a moderate dose ("starter") of nitrogen at planting may be necessary to stimulate legume nodulation and nitrogen fixation in N-deficient soils. The "starter" nitrogen could have possibly enhanced growth in the period between root emergence and the onset of active N-fixation (Giller and Cadisch, 1995). However, the poor performance of the inoculated (without N-fertilizer) pea plants relative to N-fertilized inoculated pea plants at Souris 2 could be attributed to the possible failure of the inoculant strain to occupy the majority of nodules in the presence of competitive, but less effective resident rhizobia. Plasmid profile analysis of *R. leguminosarum* bv. *viciae* isolates obtained from inoculated (with no N-fertilizer) pea plants grown at Morden 2 site showed that the inoculant strain occupied only 14% (9/64) of all the nodules isolated. Previous to this finding, plasmid profile analysis of nodule isolates had shown that none of the nodules (12) obtained from inoculated plants grown at Glenlea 1 in 1998 was occupied by the inoculant strain. The observation that inoculated (without N-fertilizer) plants did not accumulate more aboveground biomass than uninoculated plants at Morden 2 and Souris 2 in 2000 suggests that the number of nodules occupied by the inoculant strain may not have been large enough for optimal N-fixation benefits to be realized. These observations are consistent with studies that have shown poor nodule occupancy by inoculant rhizobia in

field soils containing significant populations of resident rhizobia. A study in Egypt by Moawad et al. (1998) demonstrated no significant increases in dry matter yield of lentil with inoculation. They attributed the lack of response to inoculation to the low competitive ability of the *R. leguminosarum* bv. *viciae* inoculant strains which occupied only 12-24% of the lentil nodules. In multi-site experiments in Ontario, Moxley et al. (1986) reported recovery of inoculant strains from common bean nodules in the range of 0 to 31 %.

Preliminary studies were conducted at one site (Morden 2 in 2000) to examine the proportion of nodules (from inoculated pea) occupied by the inoculant strain. The results showed that the inoculant strain occupied 6% (2/32) of the distal root nodules compared to 22% (7/32) of the proximal root nodules. This finding supports other studies that have demonstrated that inoculant rhizobia applied to the seed produce nodules primarily on the upper portion of the tap root, with little nodulation on the more distal portion of the taproot and lateral roots (McDermott and Graham, 1989; Rice et al., 2000a). The disadvantage of this phenomenon is that upper tap root nodules are older than lateral root nodules and may be senescing during the reproductive stage when plant nutrient demand is at its peak (Rice et al., 2000a). Thus, the younger lateral root nodules are especially useful in providing fixed nitrogen during grain-filling. The failure to achieve effective dispersal of bacteria in the bulk soil with seed inoculation may be attributed to the limited mobility of rhizobia in the soil relative to the rate of root exploration of the soil (see review by Streeter, 1994). The movement of rhizobia in soil varies with water movement, soil texture, soil slope, and rhizobial motility (Issa et al., 1993; Parco et al., 1994), but it is generally poor in most soil conditions, especially in the lateral direction (Date, 1991).

Considering that only one single colony per nodule was examined in our study, it is possible that the proportion of the nodules occupied by the inoculant strain may have been underestimated in case individual nodules were inhabited by two or more distinct strains. Corich et al. (2001) examined plasmid profiles of 10 to 15 single colonies per nodule and found double and multiple strains of *R. leguminosarum* bv. *viciae* in only 23% of the single nodules. However, the proportion of the alternative strains sharing the same nodule was numerically dominated by a single strain.

The main aim of inoculation is to maximize survival of the root nodule bacteria in the period between their introduction to the soil and the development of the legume system that they can colonize. Hence, the dominance of the pea nodules by resident rhizobia calls for development of alternative strategies (to complement or replace seed inoculation) that can enhance the proportion of nodules occupied by inoculant strains in both proximal and distal portions of pea roots. Seed application of massive doses of the inoculum can be effective in displacing resident rhizobia (Weaver and Frederick, 1974), but is not economically practical. In contrast to seed inoculation, application of inoculants directly into the seed bed has been shown to achieve good distribution of the inoculant strains throughout the host root system. Rice et al. (2000a) reported that a granular inoculant applied to the soil was more effective in nodule formation in pea than seed-applied peat and liquid inoculants. In addition, whereas seed-applied peat and liquid inoculants produced nodules clustered around the crown root, the granular inoculant produced nodules that were distributed throughout the root system. Liquid inoculants applied to the soil can also provide better distribution of the inoculant strain than seed inoculation (Zablotowicz et al., 1991). Seed inoculation coupled with cover inoculation

with irrigation water results in relatively uniform distribution of inoculant rhizobia in the soil and has been shown to increase infections and nodulation on secondary roots of soybean plants (Ciafardini et al., 1992). Apart from achieving poor soil distribution of inoculant strains, seed inoculation may sometimes be inefficient in the presence of seed coat toxicity, when the soil is too dry, or when the seed is dressed with incompatible pesticides. A lupin inoculation study showed that 95% of the original bacteria present in peat inoculant (with seed adhesives) died during inoculation and sowing and further 83% of the remaining bacteria lost viability during the following day in the soil (Roughley et al., 1993). The failure of the inoculant strain to occupy most of the pea nodules at Morden 2 and any of the nodules at Glenlea 1 could conceivably be attributed to low inoculant bacterial population resulting from mortality on the seed and in the soil. Another strategy that could potentially improve nodule formation by inoculant strains is to use bacteriocidal agents such as fungicides, antibiotics, and bacteriocins with selected or constructed resistant inoculant strains (Ramirez and Alexander, 1980; Li and Alexander, 1990; Triplett, 1990). The proportion of nodules occupied by inoculant strains could also be improved by either selecting legume genotypes that nodulate almost exclusively with a developed superior rhizobia strain (Cregan and Keyser, 1986; Weiser et al., 1990) or developing symbiotically promiscuous lines that nodulate and fix nitrogen vigorously with diverse resident rhizobial populations (Thompson et al., 1991).

Inoculated plants fixed significantly more atmospheric nitrogen in above ground biomass, as well as in seed, than uninoculated plants only at Carman. Based on the N-difference method, the amount of nitrogen fixed in total dry matter varied from 4.6 g N m^{-2} to 15.1 g N m^{-2} in 1999 and 3.2 g N m^{-2} to 12.6 g N m^{-2} in 2000. These figures are lower

than 18.5 g N m^{-2} reported for pea under irrigated conditions in Alberta (Rennie and Dubetz, 1986). The observation that there were no significant differences in shoot dry matter, fixed-N, and seed yield at Westbourne 1, during either of the two years, may suggest the presence of efficient nitrogen-fixing pea rhizobial strains. Although most indigenous strains of *R. leguminosarum* bv. *viciae* appear to be ineffective nitrogen fixers (Rice et al., 1993), there are some that can effectively fix nitrogen (Fesenko et al., 1995). It is also possible that the soil residual nitrogen was not limiting at this site and this may have masked the response to inoculation. In a review, Brockwell et al. (1995) advised against planting a legume in fields with significant amounts of available nitrate.

5.3 Diversity of Resident Pea Rhizobia in Southern Manitoba Soils

5.3.1 Plasmid Profiles

Large plasmids found in most strains of rhizobia provide a convenient method to "fingerprint" or type closely related strains. The plasmid content of strains can be easily visualized by electrophoresis of cell lysates in Eckhardt agarose gels (Hynes et al., 1986; Wheatcroft et al., 1990) and plasmid size can be determined by calibration against the relative mobilities of DNA fragments of known size. In this study, plasmid profile analysis revealed that commercial, field pea and native *Lathyrus* sp. isolates contained one to eight plasmids, with the majority of isolates bearing one to five plasmids. Plasmid size ranged from less than 50 kb to more than 1000 kb, with most isolates harboring at least one plasmid of above 600 kb. In previous studies, isolates from pea, faba bean, and lentil have been shown to vary in plasmid content from about one to seven and in plasmid size from less than 50 kb to 950 kb (Laguerre et al., 1992; van Berkum et al., 1995;

Handley et al., 1998; Wilson,1998). In our study, it appears we had a relatively higher proportion of isolates with single plasmids and megaplasmids (>1000 kb) than previously reported. In a review, Amarger (2001) noted that the presence of megaplasmids was not a general feature of *R. leguminosarum* bv. *viciae* strains, in contrast to *S. meliloti*. It was not possible to isolate plasmids in some field pea and *Lathyrus* sp. isolates, more so in those that produced relatively copious amounts of mucoid material and pigments. Failure to find plasmids by agarose gel electrophoresis of cell lysates has been reported for *R. leguminosarum bv. viciae* and *R. leguminosarum* bv. *trifolii*. Moawad et al. (1998) found no plasmids in some rhizobial isolates obtained from lentil and clover nodules. Using symbiotic probes, Mazurier and Laguerre et al. (1997) did not find plasmids in wild strains of *R. leguminosarum bv. viciae* nodulating vetch. They postulated that either these strains carried symbiotic genes on the chromosome or on a plasmid larger than the already described *pSyms*. The excessive amounts of mucoid material and heavy pigmentation produced by certain strains appeared to have interfered with the isolation of their plasmids in the present study. However, it is also possible that some of the isolates had plasmids that were too small or too large to be resolved on Eckhardt gels.

Out of 230 isolates that included commercial and native *Lathyrus* sp. strains, 77 distinct plasmid profiles were established. The present finding is in agreement with studies that have demonstrated that rhizobial isolates originating from pea, lentil, and faba bean are genetically diverse. Kucey and Hynes (1989) observed a total of 10 plasmid profile types out of 24 pea isolates examined, while van Berkum et al. (1995) found 13 distinct plasmid profiles out of 22 strains from disparate geographical locations. Brockman and Bezdicek (1989) identified slightly fewer distinct plasmid profiles (18)

among 192 isolates of *R. leguminosarum* bv. *viciae* originating from pea root nodules. Wide variations in plasmid profiles have also been observed in isolates of clover, alfalfa, and common bean (Kucey and Hynes, 1989; Hartman and Amarger, 1991; Amarger et al., 1994; Moawad et at., 1998). An examination of all the distinct plasmid profiles of field pea isolates within each site demonstrated a possible case of addition or deletion of plasmid bands at only two sites. Profiles 4a-3 (four bands) and 3a-2 (three bands) both of which were found at Carman 1 and Morden 2 differed by only one plasmid band.

The diversity of *R. leguminosarum* bv. *viciae* strains varied within and between sites and was considerably high in the majority of the sites. Plasmid profile diversity index (PPDI), which we defined as the number of distinct plasmid profiles divided by the total number of isolates profiled, was used as an indicator of the level of diversity at each site. This index was useful in evaluating the relative diversity levels between sites that were different in the number of isolates typed. Among the sites with 10 and more isolates, the PPDIs ranged from 0.2 at Teulon 1 to 0.83 at both Glenlea sites. The PPDIs for all the arable sites, with the exception of Teulon 1, were above 0.5. Variations in diversity among and within sites have been reported previously for nodule isolates from pea and other legumes. On the basis of plasmid profile and RAPD-PCR analyses, Handley et al. (1998) characterised numerous isolates of *R. leguminosarum* from multiple sites in Britain and observed large variations in diversity between the sites. The diversity index (number of RAPD profiles/total number of isolates typed) for the sites varied from less than 0.1 to above 0.65. Aguilar et al. (1998), using DNA fingerprinting and plasmid profiles, found huge diversity of *R. etli* isolates from wild beans within and among sites in northwestern Argentina. Variations among sites in the diversity of *R. leguminosarum*

bv. *trifolii* populations nodulating red clover have also been observed (Hagen and Hamrick, 1996). The high genetic diversity appears to indicate the presence of many distinct microsites or unique environmental niches to which different rhizobial strains are adapted. In the present study, with a few exceptions, it was not common for single strains to dominate all the pea nodules at any given arable site. Handley et al. (1998) made a similar observation about pea isolates in diverse sites in Britain. They hypothesized that arable soils that include host legumes provide a wide range of microhabitats for rhizobia and strains that are competitive for nodulation in the presence of the host legumes and those with superior saprophytic competence in the absence of the host legumes are expected to fluctuate in number. Hence, it is unlikely that a single strain can dominate such soils.

The presence (or absence) of the host plants appears to influence the composition of *R. leguminosarum* bv. *viciae* populations in the soil. Pea isolates from most sites with a history of pea and lentil cultivation were highly diverse, as were native *Lathyrus* sp. isolates. There were three distinct plasmid profiles out of four *Lathyrus* sp. nodule isolates from Teulon 1. In contrast, ninety percent (9/10) of the pea nodule isolates from an arable site (Teulon 1) with no history of pea or lentil cultivation comprised a single strain (1a-4) which also inhabited all the pea nodules (four) from a virgin field (Westbourne 2). In addition, the same strain occupied the only pea nodule from Stuartburn. These observations suggest that the presence of host legumes may increase the diversity of compatible rhizobial populations in the soil. Host legumes release a lot of carbon compounds in their roots, including micromolar concentrations of secondary metabolites such as homoserine in the case of pea (van Egeraat, 1975), which can be

expected to change the composition of bacterial populations during the saprophytic phase. The main impact on the heterogeniety of rhizobial strains by host legumes occurs perhaps during the symbiotic phase in which the nodules provide nutrient-rich environments which are drastically different from the soil environment. Hence, the fluctuations in saprophytic and symbiotic niches for *R. leguminosarum* bv. *viciae* populations that may often occur between the time host plants begin setting seed (when nodules begin to degenerate) and germination the following year (during which there are several months of warm temperature and cold winter conditions) may be responsible for creating the rich diversity in the populations of rhizobia resident in southern Manitoba soils. The dominant strain at Teulon 1 and Westbourne 2 (also found at Stuartburn) was possibly native to these sites that had not seen pea before. It can be assumed that this strain was more efficient in exploiting the limited soil resources than other potential strains in the soil and was more likely present in relatively significant numbers at the Teulon 1 site compared to the Westbourne 2 and Stuartburn sites both of which had exhibited very poor nodulation. It has been demonstrated that *R. leguminosarum* bv. *viciae* can exist in low densities in the soil for many years (Kucey and Hynes, 1989).

Some soil ecological conditions have been shown to limit diversity of rhizobial populations. Wilson et al. (1998) reported that the only single strain that dominated one particular site produced bacteriocins to which most of the strains from the other sites were sensitive. Bacteriocins are protein-based antimicrobial compounds that some rhizobial strains produce in order to relieve pressure for identical but limited soil resources with closely related strains (Hirsch, 1979; Hogson et al., 1985; Wilson, 1998). Strain 1a-4 was not tested for bacteriocin production in the present study. Gandee et al. (1999) observed

limited genetic diversity in isolates of alfalfa grown in a sandy soil relative to alfalfa isolates from a relatively fine textured soils. Unlike clay soils, coarse textured soils are prone to water deficits, acidification, and nutrient deficiencies (Mahler and Wollum, 1981; Ozawa, 1988; Bottomley, 1992) and have fewer microniches that can protect rhizobia against protozoan predation. It is tempting to suggest that the limited diversity at Teulon 1 could also possibly be attributed to the relatively coarse texture of the soil at this site compared to most other sites, except that Souris 1 which had a more coarse-textured soil had more diverse pea isolates (PPDI= 0.72). Caballero-Mellado and Martinez-Romero (1999) reported that soil fertilization levels used in agricultural fields in Mexico caused a decline in genetic diversity among isolates of some common bean cultivars. It is possible that the relatively high levels of soil organic matter and soil nitrate may have resulted in the relatively low diversity level at Morden 1 (PPDI=0.33) which had seen pea in recent times. However, no correlation was demonstrated between PPDIs and nitrate level when all the sites were considered. Anyango et al. (1995) reported that 30 out of 35 isolates from *Phaseolus vulgaris* L. grown in a low pH soil in Kenya comprised the acid tolerant *R. tropici* strains but this species of *Rhizobium* was almost absent in a near neutral pH soil where *R. etli* and *R. leguminosarum* bv. *phaseoli* predominated. Labes et al. (1996) observed changes in populations of *R. leguminosarum* bv. *viciae* nodulating pea following the treatment of the soil with polluted or non-polluted slurry. The strains found in non-polluted soils were absent in the polluted soils and new strains appeared in the slurry-polluted soil. Hirsch et al. (1993) reported that soil contamination with heavy metals resulted in drastic decline in the diversity of *R. leguminosarum* bv. *trifolii* isolates recovered from white clover. Based on our results, simple variations in soil

physicochemical characteristics such as organic matter, pH, electrical conductivity, and available nitrate did not appear to have had much influence on the diversity levels of *R. leguminosarum* bv. *viciae* populations. Brockwell and Robinson (1970) observed that environmental factors had no consistent influence on rhizobia occurrence other than through their effects on the components of the vegetation. It is possible that field management may have played a more important role in influencing the level of diversity of soil rhizobial population at any given site. Variations in field management, in terms of crop rotations, tillage, pesticide use, and application of fertilizers and manures would be expected to create diverse microhabitats and microniches for rhizobia.

Each site had identical plasmid profiles with at least one other site, but the number of plasmid profiles common to any two sites was not dependent on the proximity between them. For example, Souris 1 and Souris 2 (1 km apart) had only one plasmid profile in common, while Glenlea and Souris 1, which were about 250 km apart, shared five plasmid profiles. This observation is similar to the one made in related studies in Alberta. Strains with identical plasmid profiles were obtained from pea fields located at 60 and more than 100 km from Lethbridge (Kucey and Hynes, 1989; Hynes and O'Connell, 1990). Isolates of native *Lathyrus* sp. plants from Teulon 1, Morden and Glenlea sites did not have any plasmid profiles in common with field pea isolates, including those from adjacent experimental sites. In addition, the three sites did not share any plasmid profiles (from *Lathyrus* sp. plants).

Plasmid profiles of the field isolates were compared to those of a wide range of commercial inoculants that may have been introduced to southern Manitoba soils in previous years of pea, lentil or faba bean cultivation. Interestingly, only few (about 6%) of

the field pea nodule isolates obtained from sites that had previously been planted to inoculated pea or lentil had plasmid profiles that matched those of the inoculant strains. Similar observations have been made in related studies (Kucey and Hynes, 1989; Hynes and O'Connell, 1990; Moënne-Loccoz et al., 1994; Sullivan et al, 1995). Kucey and Hynes (1989) found that the plasmid profiles of isolates from pea and common bean nodules were different from those of the original inoculants (previously introduced to the soil) as well as from those of the resident rhizobia. The authors speculated that the new strains were possibly inoculant strains whose plasmid profiles had been altered. Moënne-Loccoz et al. (1994) performed detailed examination of the diversity of *R. leguminosarum* bv. *trifolii* in areas where arrowleaf clover (*T. vesiculosum*) and crimson clover (*T. incarnatum)* had previously been grown and found only two of 300 isolates (for three sites examined) had the same plasmid profiles as the inoculant strain. Contrasting reports suggest that inoculant strains can remain genetically stable (in symbiotic characteristics) for long periods under field soil conditions. Populations of *R. leguminosarum* bv. *viciae* inoculants released in Rothamstead soils containing indigenous rhizobia survived at the same level as the indigenous rhizobial population for several years (Hirsh and Spokes, 1994; Hirsch, 1996). A symbiotic plasmid-cured strain of *R. leguminosarum* bv. *viciae* also persisted at the level of the indigenous rhizobia several years after being released into the soil (Hirsch, 1996). However, the behaviour of the inoculant strains appeared to depend on local conditions.

The poor recovery of inoculant strains from the soil several years after inoculation, as observed in our study (6% recovery rate) and in previous studies, could be explained in several ways. First, the success of the inoculant strains in occupying the

legume nodules is highly dependent on the level of resident rhizobia in the soil. Once introduced to the soil, the inoculant strains have to colonize the soil and the rhizospheres of the host legumes and nodulate the roots in the presence of highly competitive, locally adapted resident rhizobial strains. Hence, a small proportion of the nodules is expected to be occupied by inoculant strains if resident rhizobia are abundant in the soil. The inherent competitive potential of the introduced rhizobia may be suppressed under adverse conditions of pH, moisture, temperature, nutrients, and protozoan predation as well as in deleterious interactions with other soil bacteria (Bottomley, 1992) which may reduce inoculant strain numbers. High mortality rate of rhizobia as demonstrated by Roughley et al. (1993) can greatly undermine the success of the inoculant strain in occupying host nodules. Failure of the inoculant strains to occupy a sizable proportion of the nodules during the initial inoculation is expected to make it even more difficult for them to displace the resident strains from the nodules in subsequent years. This is because, apart from the harsh environment to which resident rhizobia are adapted, the numbers of resident rhizobial cells are expected to be augmented more relative to those of inoculant strains upon nodule senescence by virtue of their high capacity to occupy the nodules. An individual nodule can contain up to 10^6 viable cells of *Rhizobium* which are released into the soil upon nodule senescence. Indeed, the recovery of the inoculant strain following initial inoculation has been shown to decline in subsequent years (Vlassak et al., 1996). It is conceivable that most of the inoculant strains previously introduced to southern Manitoba soils survived, but they may be present in such low densities that they can not successfully displace resident rhizobia. Since we did not find the inoculant strain recommended for lentil (RGL4), it is tempting to suggest that the pea plant may have

discriminated against this strain. Pea, lentil and faba bean have been shown to prefer certain strains of *R. leguminosarum* bv. *viciae (*Handley et al., 1998). However, lentil appeared to be less selective for certain rhizobial genotypes than pea and faba bean in studies by Hynes and O'Connell (1990).

One of the major explanatory hypotheses for the poor recovery (or absence) of plasmid profiles among isolates of field-grown pea plants matching known inoculant strains is that there may have been complex recombination in which plasmids were rearranged (Kucey and Hynes, 1989). In *Rhizobium*, *Sinorhizobium* and some *Mesorhizobium* strains, the genes for nodulation and nitrogen fixation are carried on plasmids (Banfalvi et al., 1981; Lamb et al., 1982; Hirsch, 1996; Mazurier and Laguerre, 1997). Transfer of plasmids among rhizobia and between rhizobia and other bacteria has been reported. Conjugal transmission of plasmids among rhizobial strains has been well demonstrated in the laboratory (Kondorosi et al., 1982; Rao et al., 1994) and symbiotic plasmids have been transferred to symbiotic plasmid- cured strains in sterile and non-sterile soils (Kinkle and Schmidt, 1991; Rao et al., 1994). Soil bacteria not related to *Rhizobium* species acquired symbiotic plasmids from *R. leguminosarum* bv. *trifolii* and were able to nodulate clover (Fenton and Jarvis, 1994). Rhizobial strains can lose symbiotic plasmids and hence the ability to nodulate. Segovia et al. (1991) found non-symbiotic bacteria that were identical to *R. etli* in the rhizosphere of *Phaseolus vulgaris* bean. Similarly, Laguerre et al. (1993) were able to isolate nonsymbiotic *R. leguminosarum* from soil. The observations that plasmids can be gained, lost or exchanged in the soil points to the potential importance of inoculant strains (even when they fail to occupy the nodules) and nonsymbiotic rhizobia in contributing to the diversity

of soil rhizobial populations. Inoculant strains introduced to the soil may "lose" their symbiotic plasmids (i.e., be the source of symbiotic genes) to non-symbiotic rhizobia and/or gain new cryptic plasmids. Since the "new" symbiotic strains are likely to be saprophytically adapted to local soil conditions, they are likely to be the dominant occupants of legume nodules. The downside of this phenomenon is that these dominant nodule formers are more likely to be poor-nitrogen fixers compared to the original inoculant strains. The inoculant strains that lose symbiotic plasmids also lose the ability to nodulate legume roots.

Sampling of inoculated plants at Morden 2 site in 2000 showed that the isolates from the proximal portion of the pea root system were more diverse (PPDI= 0.53 or 0.64) than those from the distal portion (PPDI= 0.25 or 0.23) of the root system. In addition, the profiles of the distal root isolates were in most cases different from those of the proximal root isolates. There is no clear explanation for these observations, but they could possibly be attributed to potential differences in the multiplication of rhizobial strains in the period between inoculation and nodule initiation on pea roots. The strains that nodulated soon after inoculation were more likely to reflect the ratio of the strains present in the soil at planting, while those nodulating later possibly reflected changes in the relative proportions of rhizobial strains as a result of potential differences in growth rate (Handley et al., 1998). It is plausible to suggest: (1) that the strains occupying the proximal portion of the pea root system were relatively more saprophytically competent and existed in the soil in relatively larger numbers at planting compared to the strains occupying the distal portion of the pea root system; and (2) that the distal nodule-inhabiting strains might have grown faster than the proximal isolates in the presence of exudates from the host plant,

thereby achieving a competitive advantage in the distal root portions. The alternative explanation for the differences in the proximal and distal isolates is that these strains occupied different positions within the soil profile, the proximal strains occupying mainly the nutrient-rich top few centimetres of the rhizosphere while the distal strains occupied mainly the nutrient-poorer lower portions of the rhizosphere (i.e., the distal isolates were more saprophytically competent). Diversity of microorganisms is normally higher closer to the soil surface than down the soil profile (Paul and Clark, 1996). The variations in the levels of diversity of isolates between proximal and distal portions of the pea root system have important implications for sampling strategies in pea isolate diversity studies. It appears that to reveal as much diversity of *R. leguminosarum* bv. *viciae* strains as possible, more nodules need to be sampled from the proximal portion of the pea root system, where strains are likely to be more diverse, than from the distal portion. However, since the distal isolates tended to be different from the proximal isolates, it is necessary to also sample a few nodules from the distal portions of the root.

Some plasmid profiles that were isolated more than once appeared to be associated more with some pea plants than with others. For example, profile M12 was isolated three times from plant 2 but none in the other plants, while M10 was isolated once in plant 1, three times in plant 3 and none in the other two plants (Table 4.21). A previous study by Handley et al. (1998) demonstrated that plasmid profiles of *R. leguminosarum* bv. *viciae* were not randomly distributed among individual plants of pea. However, these plants were exposed to a mixture of strains in well-mixed soil solution, unlike in our study in which plants were sampled directly from the soil. The finding in the present study may be attributed to non-random distribution of strains in the soil. In fields

that have had the host legumes before, "hot spots" of rhizobial cells would be expected at the points of nodule decay, especially in zero-tillage which involves limited disturbance of the soil. Rhizobia are known to be relatively immobile in the soil and may not possibly be equally distributed within the soil. It would have also been possible for the most competitive strain to prevent other strains from occupying nodules (Dowling and Broughton, 1986), but this may not be the case because no single strain dominated most plants.

The diversity levels observed in the various sites in southern Manitoba is most likely just the tip of the iceberg. This is partly because we examined only the rhizobial populations with the capacity to nodulate pea. Studies have shown that *S. meliloti* strains obtained from alfalfa nodules were not representative of the soil population (Bromfield et al., 1995). The variations in the type of plasmid profiles found among individual pea plants at the Morden 2 site suggests that the diversity of rhizobia had not been fully revealed at this site. If more plants and fewer nodules were sampled, it is more likely that "new" strains of *R. leguminosarum* bv. *viciae* would have been found. In addition, differences in preferences for certain rhizobial strains have been demonstrated among host legumes and their cultivars (Hynes and O'Connell, 1990; Handley et al., 1998). Hence, more strains of *R. leguminosarum* bv. *viciae* could have possibly been revealed in southern Manitoba soils if faba bean, lentil, or even more pea cultivars had been used as "trap" hosts. The fact that *Lathyrus* sp. isolates were diverse and different from pea isolates serves to emphasize the importance of "trap" plants in characterizing diversity of soil rhizobial populations.

5.3.2 PCR-RFLP 16S-23S rDNA IGS

Bacterial ribosomal RNA genes are useful in diversity studies because they contain conserved regions that can be used to define primers and highly variable regions that can be used to distinguish between strains. In the present study, PCR primers derived from conserved sequences of 16S and 23S allowed the specific amplification of the intergenic spacer sequences between 16S and 23S genes coding for rRNA of *Rhizobium leguminosarum* strains. Single PCR bands were generated for all the strains tested, with the exception of one. Estimates showed that the length of the 16S-23S rDNA IGS regions of the strains ranged from 1700 to 2000 bp, confirming that the IGS for rhizobia is much longer than 400 bp which is the average IGS length for most other bacteria (Navarro et al., 1992). The relatively large IGS is favourable for PCR-RFLP analysis because it is more likely to contain more restriction sites. Given a normal bacterial IGS length of about 400 bp, each restriction enzyme would be expected to yield 1-2 fragments per isolate (assuming the theoretical frequency of 1/256 for 4-base enzymes) which may not be enough to discriminate closely related isolates.

The results of this study are consistent with previous studies that have demonstrated that the length of the IGS of 16S-23S rDNA varies within and between bacterial species (Jensen et al., 1993; Laguerre et al., 1996). Laguerre et al. (1996) amplified the 16S-23S rDNA IGS of 43 strains of the *R. leguminosarum* biovars *viciae*, *phaseoli*, and *trifolii* with primers FGPS1490 and FGP132' and demonstrated that it varied in length from 1160 to 1400 bp. This IGS size range is smaller than the one found in our study most likely because we used a different forward primer. In our study, we used the forward primer 16SR11 (corresponds to an oligonucleotide at the 3' part of the

16S rDNA) which permits the specific amplification of *R. leguminosarum* and *R. etli* 16S-23S rDNA IGS when used with the universal FGPL132' (corresponds to the 5' part of the 23S rDNA right next to the IGS) as the reverse primer (Navarro et al., 1992; Laguerre et al., 1996). Out of 28 strains, only one strain of *R. leguminosarum* bv. *viciae,* an inoculant strain RGL4 recommended for lentil inoculation, exhibited an extra PCR band in the same 1700 to 2000 bp range. Laguerre et al. (1996) also found a few strains with one extra band in the normal range of 1160-1400. It has been shown that the rRNA operon is present in multiple copies in bacteria, *Rhizobium* species containing at least three copies (Huber and Selenska-Pobell, 1994). The extra PCR band recorded for the lentil inoculant strain suggests that this strain possibly bears at least two copies of the IGS that are variable in length. Variability in the length of copies of the IGS could be attributed partly to the insertion of various tRNA genes in these regions (Jensen et al.,1993). Alternatively, the extra amplification fragment in RGL4 strain could have resulted from the generation of heteroduplex DNA structures and of single-stranded DNA (Jensen and Straus, 1993)

Digestion of the 16S-23S rDNA IGS regions with restriction enzymes revealed intraspecies variations in *R. leguminosarum* bv. *viciae*. The restriction enzymes differed in their levels of discrimination of isolates, generating 14 to 23 distinct restriction patterns. The least discriminative enzyme was *Alu*I and *Dde*I was the most discriminating. The rest of the enzymes *(Cfo*I, *Msp*I, and *Taq*I) exhibited intermediate levels of discrimination. Each enzyme exhibited two to eight bands per restriction pattern, but the number of the restriction fragments produced by each enzyme did not affect its level of discrimination of isolates. Some strains such as C1, 128C56G and 99AA1 were only

differentiated following digestion with *Dde*I, while strains RP212-19, RGFP, S1B, S20B, S10, RGL4, H441, RP212-37 and S16 showed unique restriction patterns with all the restriction enzymes tested. However, it was not possible to distinguish between strains S5A and S5B which had an identical plasmid profile and were from the same site.

The genetic relationships among the isolates were determined by analyzing the combined data from all the seven restriction enzymes. Each isolate was scored for the presence (coded as 1) and absence (coded as 0) of a restriction band, producing148 polymorphic bands (for all the enzymes) that were used to generate simple matching coefficients (SMC). The SMC values were used to develop a phenogram based on the unweighted pair group method with an arithmetic mean (UPGMA) and the neighbour-joining method, showing similarity in the rDNA IGS regions of *Rhizobium* strains. In addition, the simple matching coefficient matrix was reduced to two dimensions and displayed as a multi-dimensional scaling plot depicting genetic relationships among the isolates. Analysis by UPGMA placed the isolates in several clusters and subclusters consistent with the similarity in restriction patterns demonstrated in Table 4.19. The NJ method and MDS techniques clustered the strains in a similar manner as the UPGMA, but they displayed a few glaring inconsistencies. For example, the NJ method separated strains 5A and S5B yet they had identical restriction patterns with all the enzymes while the MDS plot could not distinguish strain S20 from strain RP212-37 which had no common restriction patterns. Only the results based on the UPGMA technique will be discussed.

The UPGMA analysis placed strains H441 (a *R. leguminosarum* bv. *phaseoli* strain obtained from France), RGFP (an inoculant strain for faba bean), S10, and RP212-

37 in unique individual groups. It is interesting to note that although H441 was clearly differentiated from all the pea nodulating strains, it was not possible to tell whether it was *R. leguminosarum* biovar *viciae* or *phaseoli*. This finding supports previous observations that the strains of the three biovars of *R. leguminosarum* (biovars *viciae, phaseoli*, and *trifolii*) are not easily distinguishable using techniques that assess chromosomal variation. These observations were based on multi-locus enzyme electrophoresis (Segovia et al., 1991), RFLP analysis with chromosome gene probes (Laguerre et al., 1993), and randomly amplified polymorphic DNA profiles (Dooley et al., 1993).

Four major groupings could be discerned from the UPGMA analysis when field pea isolates were considered separately. The strains within the first grouping (S15A, S15B, S17, S15C, and S1A) had an average SMC of close to 0.73 as did the strains within the second grouping (S18, S5A, S5B, S12, S20A, S20B, and S1B). The strains in the third grouping (S13 and S16) had an average SMC of close to 0.8, while strain S10 was in a unique fourth individual grouping with an average SMC of about 0.7 relative to the other groupings. The average SMC (about 0.7) among the field pea isolate groupings was similar to the average SMC between H441 (*R. leguminosarum* bv. *phaseoli)* and the field pea isolate groupings. Although the 16S-23S rDNA IGS regions of only a limited number of isolates (15 isolates) were examined, these observations suggest a high chromosomal diversity in field pea isolates across southern Manitoba. In addition, there was evidence for both low and high chromosomal diversity of isolates within sites. Strains S5A and S5B (Fort Garry Campus) were identical (average SMC=1), while strains S15A and S15C (Morris 1) had a high level of chromosomal similarity (average SMC of above 0.9). In contrast, the average SMC of S1A and S1B (based on UPGMA analysis)

was close to 0.7 as was the average SMC between S15B and its sister isolates S15A and S15B.

Strains with identical plasmid profiles did not necessarily have close chromosomal backgrounds. Strains S5A and S5B from the same site had an identical plasmid profile and were not differentiated by UPGMA clustering, suggesting that these isolates were of the same strain. In contrast, strain S1B and S15B from different sites had the same plasmid profile but were diverse in their 16S-23S rDNA IGS regions (average SMC close to 0.7). In related studies, Corich et al. (2001) found that some strains of *R. leguminosarum* bv. *viciae* with identical plasmid profiles could be further distinguished by pulse-field gel electrophoresis of genomic large fragment digests. This finding points to the fact that plasmid profile analysis is limited as a fingerprinting technique for rhizobia in cases where the same plasmids (or different plasmids of equal molecular weights) are harboured by strains of dissimilar chromosomal backgrounds. Hence, it may be necessary to combine plasmid profiling with molecular techniques that evaluate chromosomal variations when characterizing rhizobial strains.

PCR-RFLP analysis demonstrated that field isolates with different plasmid profiles (from the same or different sites) can be similar or dissimilar chromosomally. Strains S15A and S15C (from the same site) had different plasmid profiles, but were very similar in their 16S-23S rDNA IGS regions (average SMC of about 0.94). In contrast, strains S20A and S20 B (from the same site) were dissimilar in both plasmid profiles and in the 16S-23S IGS regions as were strains S1A and S1B (from the same site). Field strain S5A/S5B from Fort Garry Campus had exhibited a different plasmid profile from strain S12 (Morden 2), but they were very similar in the IGS (average SMC of above

0.95). Strains 15A and S15C (from the same site) had different plasmid profiles from S17, but were similar chromosomally (average SMC of about 0.94). Hence, strains of very similar chromosomal backgrounds can harbour different plasmid profiles. Similar observations have been made in previous studies (Laguerre et al., 1993; Schofield et al., 1987).

The genetic similarity of the isolates was not apparently dependent on the proximity of the sites from which they were obtained. One of two strains from Carman 1 was chromosomally closer to a commercial inoculant while the other was closer to strains from Morden and Fort Garry Campus. Similarly, two isolates from Morris 1 were chromosomally closer to an isolate from Souris 1 than to a sister Morris 1 isolate. In addition, it was found that an isolate from Morris 2 was much more similar to an isolate from Westbourne 1 than it was to the three isolates from the nearby Morris 1 site. The same genotypes have been found in populations of *R. leguminosarum* bv. *viciae* in Britain, France and the United States of America (Laguerre et al., 1992; Strain et al., 1995). These observations suggest that the rate of migration of these genotypes was high and genetic exchange was limited. However, in the present study, it is plausible that differences in the chromosomal elements were due to differences in microsital niches rather than migration. Locations in southern Manitoba could, perhaps, be considered homogenous such that the similarity levels in the genotypes reflected differences in microsites.

The present studies have demonstrated that resident populations of *R. leguminosarum* bv. *viciae* in southern Manitoba soils are chromosomally highly diverse. All the strains tested, with exception of two, were differentiated by PCR-RFLP analysis

of their 16S-23S rDNA IGS regions. In addition, chromosomal diversity was also high within isolates from the same site. Similarly, the diversity of plasmid profiles was huge in most sites. These observations emphasize the need to look at the diversity of strains from both chromosomal and plasmidic perspectives. This huge diversity may arise through various ways. It appears that random genetic mutation occurs among rhizobial populations (Streeter and Smith, 1998), creating strains with the ability to survive well in the diverse microsites that occur in the soil. Another previously discussed mechanism is through the transfer of symbiotic plasmids to non-symbiotic rhizobia, and possibly to other bacteria, that eventually become competent nodulators. Cryptic plasmids could also be lost or exchanged with other rhizobia. New nodulating strains could also arise through the transfer of chromosomal symbiotic genes as previously demonstrated (Sullivan et al., 1995).

6.0 CONCLUSIONS

The present study has shown that resident populations of *Rhizobium leguminasorum* bv. *viciae* able to nodulate field pea are abundant in cultivated soils of southern Manitoba (including soils with no history of host legume cultivation), but not in virgin soils. Previous cultivation of inoculated legumes and higher fertility levels in the cultivated fields are the possible reasons for this observation.

In most cases, aboveground biomass of inoculated plants was higher than that of uninoculated plants, suggesting that locally adapted resident populations of *R. leguminosarum* bv. *viciae* may be less effective at fixing nitrogen than the inoculant strain (PBC108) used in this study. Inoculated plants produced higher seed yield than uninoculated plants only at Carman. Based on these observations, it may be advisable for farmers in southern Manitoba to routinely inoculate their pea crops with commercial inoculants, especially on an insurance basis. In addition, the absence of differences between the inoculated (with and without N fertilizer) treatments in aboveground biomass and seed yield support the official recommendation that farmers in southern Manitoba need not apply N fertilizer if they have inoculated their pea crops with a good quality inoculant.

The inoculant strain was more prevalent in the proximal nodules than in the distal nodules, indicating that seed inoculation may not permit good dispersal of rhizobial cells in the soil. These findings call for improvement in the systems used to deliver rhizobia to pea plants in the field if the capacity of the inoculant strain to overcome the dominance of the pea nodules by resident rhizobia is to be enhanced.

Our study has revealed that *R. leguminosarum* bv. *viciae* populations in southern

Manitoba soils are highly diverse both chromosomally and in terms of plasmid profiles. Strains with the same plasmid profiles did not necessarily have similar chromosomal backgrounds, while strains of very similar chromosomal backgrounds could harbour different plasmid profiles. These observations emphasize the need to look at the diversity of strains from both chromosomal and plasmidic perspectives.

Diversity levels varied within and among sites. Plasmid profiles of pea isolates from fields with a history of pea and lentil cultivation appeared to be more diverse than plasmid profiles of pea isolates from fields that had not seen pea in at least 15 years. Simple variations in soil physicochemical characteristics among the sites did not appear to have had much influence on the diversity levels of *R. leguminosarum* bv. *viciae* populations. Field management may have played a more important role in influencing the level of diversity of soil rhizobial populations at any given site. Hence, to understand the cause(s) of the variations in diversity among the sites, detailed field history of the sites (e.g., tillage index, crop rotations, and fertilizer and pesticide use) need to be taken into account. Many sites had common plasmid genotypes, but the number shared by any two sites was not dependent on their proximity. It is plausible to suggest that the trial sites were possibly ecologically homogenous such that the genetic similarity of isolates among the sites was mainly a function of microsital effects within each location.

Only few field pea nodule isolates from sites that had seen inoculated field pea or lentil had plasmid profiles that matched those of the inoculant strains. This could be attributed to complex plasmid rearrangements in the soil, death of the inoculant strain upon delivery into the soil, or the failure of the inoculant strain to occupy the nodules in the presence of abundant and highly competitive resident strains.

The proximal root isolates were more diverse than the distal root isolates and some plasmid profiles appeared to be associated more with some individual pea plants than with others. Thus, potentially more diversity of *R. leguminosarum* bv. *viciae* can be revealed if more nodules are sampled from the proximal portion of the pea root system than from the distal portion, and if more plants and fewer nodules per plant are sampled. Given that variations exist within cultivars of the same species and among species in preferences for certain rhizobial, only a small portion of the diversity of *R. leguminosarum* bv. *viciae* populations in southern Manitoba soils was possibly captured in the present study.

The tremendous diversity among nodule-forming rhizobial strains in southern Manitoba soils could be attributed to random genetic mutations which enable rhizobia populations to adapt to diverse microniches. There may also be exchanges of symbiotic and cryptic plasmids (or even genes borne on chromosomes) among bacterial populations in these soils, resulting in new strains.

Some of the following investigations might be useful in addressing the questions arising out of the present study: (1) tagged inoculant strains could be introduced in native sites with no effective resident rhizobia and monitored over several years in terms of survival, competitiveness for nodulation, symbiotic effectiveness, and genetic stability. Plasmid profile and PCR-RFLP (of 16S-23S rDNA IGS) analyses could be used to monitor any changes in diversity of rhizobia; (2) detailed field studies comparing nodule occupancy and performance of several, locally available commercial inoculants across southern Manitoba. Attention could be paid to possible differences in the levels of diversity between proximal and distal root portions of pea. In addition, different cultivars

of pea (or even lentil and faba bean) could be included in this proposed investigation; (3) Rigorous testing of the diverse field isolates for symbiotic effectiveness with a view to selecting the most competitive and superior nitrogen fixers; and (4) Evaluate various inoculation techniques across southern Manitoba.

7.0 REFERENCES

Aguilar, O.M., Lopez, M.V., Ricillo, P.M., Gonzalez, R.A., Pagano, M., Grasso, D.H., Pühler, A., and Favelukes, G. 1998. Prevalence of the *Rhizobium etli* like allele in genes coding for 16S rRNA among the indigenous rhizobial populations found associated with wild beans from the southern Andes in Argentina. Appl. Environ. Microbiol. **64**: 3520-3524.

Amarger, N. 2001. Rhizobia in the field. Adv. Agron. **73**: 110-148.

Amarger, N., Bours, M., Revoy, F., Allard, M.R., and Laguerre, G. 1994. *Rhizobium tropici* nodulates field-grown *phaseolus vulgaris* in France. Plant Soil **161**: 147-156.

Andrés, J.A., Corea, N.S., Rosas, S.B. 1998. Survival and symbiotic properties of *Bradyrhizobium japonicum* in the presence of thiram: isolation of fungicide resistant strains. Biol. Fertil. Soils 1998. **26**: 141-145.

Anyango, B., Wilson, K.J., Beynon, J.L., and Giller, K.E. 1995. Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. Appl. Environ. Microbiol. **61**: 4016-4021.

Baldani, J.I., Weaver, R.W., Hynes, M.F., and Eardly, B.D. 1992. Utilization of carbon substrates, electrophoretic enzyme patterns, and symbiotic performance of plasmid-cured clover rhizobia. Appl. Environ. Microbiol. **58**: 2308-2314.

Banfalvi, Z., Sakanyan, V., Koncz, C., Kiss, A., Dusha, I., and Kondorosi, A. 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *R. meliloti*. Mol. Gen. Genetic. **184**: 318.

Barber, L.E. 1982. *Rhizobium meliloti* distribution in the soil following alfalfa inoculation. Plant Soil **64**: 363-368.

Barea, J.M., and Azcón-Aguilar, C. 1983. Mycorrhizas and their significance in nodulating nitrogen-fixing plants. Adv. Agron. **36**: 1-54.

Beattie, G.A. and Handelsman, J. 1989. A rapid method for the isolation and identification of *Rhizobium* from root nodules. J. Microbiol. Methods **9**: 29-33.

Bell, D.K. and Crawford, J.L. 1967. A Bortran amended medium for isolating *Aspergillus flavus* from peanuts and soil. Phytopath. **57**: 939-941.

Beringer, J.E. 1974. R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. **84**: 188-198.

Boivin, C., Barran, L.R., Malpica, C.A., Truchet, G., and Rosenberg, C. 1991. Genetic analysis of a region of the *Rhizobium meliloti pSym* plasmid specifying catabolism of trigonelline, a secondary metabolite present in legumes. J. Bacteriol. **173**: 2809-2817.

Booth, I.R. 1985. Regulation of cytoplasmic pH in bacteria. Microbiol. Rev. **49**: 359-378.

Bordeleau, L.M. and Prévost, D. 1994. Nodulation and nitrogen fixation in extreme environments. Plant Soil **161**: 115-125.

Bottomley, P.J. 1992. Ecology of *Bradyrhizobium* and *Rhizobium*. *In* Biological nitrogen fixation. Edited by G. Stacey, R.H. Burris and H.J. Evans. Chapman and Hall, London. pp. 293-348.

Bottomley, P.J., and Jenkins, M.B. 1983. Some characteritsics of *Rhiozbium meliloti* isolates from alfalfa fields in Oregon. Soil Sci. Soc. Am. J. **47**: 1153-1157.

Bremer, E. 1988. Dinitrogen fixation of lentil, field pea and faba bean under dryland conditions. Can. J. Soil Sci. **68**: 553-562.

Brockman, F.J., and Bezdicek, D.F. 1989. Diversity within serogroups of *Rhizobium leguminosarum* bv. *viciae* in the Palouse region of eastern Washington as indicated by plasmid profiles, intrinsic antibiotic resistance and topography. Appl. Environ. Microbiol. **55**: 109-115.

Brockwell, J., Bottomley, P.J.and Thies, J.E. 1995. Manipulation of rhizobia microflora for improving productivity and soil fertility: a critical assessment. Plant Soil **174**: 143-180.

Brockwell, J., Pilka, A., and Holliday, R.A. 1991. Soil pH is a major determinant of the numbers of naturally-occurring *Rhizobium meliloti* in non-cultivated soils of New South Wales. Aust. J. Exp. Agric. **31**: 211-219.

Brockwell, J., and Robinson, A.C. 1970. Observations on the natural distribution of *Rhizobium* spp. relative to the physical features of the landscape. *In* Proc. 11th Int. Grassl. Congr. Univ. Queensland Press, Brisbane, Australia. pp. 438-441.

Bromfield, E.S.P., Barran, L.R., and Wheatcroft, R. 1995. Relative genetic structure of a population *Rhizobium meliloti* isolated directly from soil and from nodules of alfalfa (*Medicago sativa*) and sweet clover (*Melilotus alba*). Mol. Ecol. **4**: 183-188.

Bushby, H.V.A. 1993. Colonization of rhizospheres by *Bradyrhizobium* sp. in relation to strain persistence and nodulation of some pasture legumes. Soil Biol. Biochem. **25**: 597-605.

Buss, T.J. 1998. Effects of co-inoculation with *Bacillus cereus* UW85 and (brady)rhizobia on the nodulation, nitrogen fixation and dry matter accumulation of grain legumes. M.Sc. Thesis, University of Manitoba, Winnipeg, MB.

Caballero-Mellado, J., and Martinez-Romero, E. 1999. Soil fertilization limits the genetic diversity of *Rhizobium* in bean nodules. Symbiosis **26**: 111-121.

Caetano-Anollés, and Greshoff, P. M. 1991. Efficiency of nodule initiation and autoregulatory responses in a supernodulating soybean mutant. Appl. Environ. Microbiol. **57**: 2205-2210.

Carrol, B. J., McNeil, D. L., and Gresshoff, P. M. 1985. A supernodulation and nitrate-tolerant symbiont (nts) soybean mutant. Plant Physiol. **78**:34-40.

Castro, S., Vinocur, M., Permigiani, M., Halle, C., Taurian, T., and Fabra, A. 1997. Interaction of the fungicide in culture and under field conditions. Biol. Fertil. Soils **25**: 147-151.

Catroux, G., Hartmann, A., and Revelin, C. 2001. Trends in rhizobial inoculant production and use. Plant Soil **230**: 21-30.

Chakrabarti, S., Lee, M.S., and Gibson, A.H. 1981. Diversity in the nutritional requirements of strains of various *Rhizobium* species. Soil Biol. Biochem. **13**: 349-354.

Chao, W.L., and Alexander, M. 1981. Interaction between protozoa and *Rhizobium* in chemicallly amended soil. Soil Sci. Soc. Am. J. **45**: 48-50.

Chatel, D.L., and Parker, C.A. 1973. Survival of field-grown rhizobia over the dry summer period in western Australia. Soil Biol. Biochem. **5**: 415-423.

Chen, H., Richardson, A.E., Gartner, E., Djordjevic, M.A., Roughley, R.J., and Rolfe, B.G. 1991. Construction of an acid-tolerant *Rhizobium leguminosarum* biovar *trifolii* strain with enhanced capacity for nitrogen fixation. Appl. Environ. Microbiol. **57**: 2005-2011.

Chhongai, P.K.and Subba-Rao, N.S. 1966. Fungi associated with legume root nodules and their effect on rhizobia. Can. J. Microbiol. **12**: 1253-1261.

Ciarfardini, G., Marinelli, G., and Missich, R. 1992. Soil biomass of *Bradyrhizobium japonicum* inoculated via irrigation water. Can. J. Microbiol. **38**:584-587

Cooper, J.E. 1982. Acid production, acid tolerance and growth of *Lotus* rhizobia in laboratory media. Soil Biol. Biochem. **14**: 127-131.

Corich, V., Giacomini, A., Carlot, M., Simon, R., Tichy, H., Squartini, A., and Nuti, M. 2001. Comparative strain typing of *Rhizobium leguminosarum* bv. *viciae* natural populations. Can. J. Microbiol. **47**: 580-584.

Cowell, L.E., Bremer, E. and van Kessel, C. 1989. Yield and N_2 fixation of pea and lentil as affected by intercropping and N application. Can. J. Soil Sci. **69**: 243-251.

Cregan, P.B., and Keyser, H.H. 1986. Host restriction of nodulation by *Bradyrhizobium japonicum* strain USDA 123 in soybean. Crop Sci. **26**: 911-916.

Crozat, Y., Cleyet-Marel, J.C., Giraud, J.J., and Obaton, M. 1982. Survival rates of *Rhizobium japonicum* populations introduced into different soils. Soil Biol. Biochem.**14**: 401-405.

Danso, S.K.A. and Alexander, M. 1975. Regulation of predation by prey density: the protozoan-*Rhizobium* relationship. Appl. Microbiol. **29**: 515-521.

da Silva, P.M., Tsai, S.M., and Bonneti, R., 1993. Reponse to inoculation and N fertilization for increased yield and biological nitrogen fixation of common bean (*Phaseolus vulgaris* L.). Plant Soil **152**: 123-130.

Date, R.A. 1991. Lateral movement of strains of *Bradyrhizobium* from inoculated seed of *macroptilium atropurpureum* and *Desmodium intortum* sown in the field. Soil Biol. Biochem. **23**: 543-549.

de Bruijn, F. J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. **58**: 2180-2187.

de Lajudie, P., Fulele, E., Willems, A., Tork, U., Coopman, R., Collins, M.D., Kersters, K., Dreyfus, B., Dreyfus, B., and Gillis, M.1998. *Allorhizobium undicola* gen. nov., sp.nov., nitrogen fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. Int. J. Syst. Bacteriol. **48**: 1277-1290.

de Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M., Collins, M.D., Dreyfus, B., Kersters, K., and Gillis, M. 1994. Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. Int. J. Syst. Bacteriol. **44**: 715-733.

Del Papa, M.F., Balague, L.J., Sowinski, S.C., Wegener, C., Segundo, E., Abarca, F.M., Toro, N., Niehaus, K., Pühler, A., Aguilar, O.M., Martinez-Drets, G., and Lagares, A.

1999. Isolation and characterization of alfalfa nodulating rhizobia present in acidic soils of central Argentina and Uruguay. Appl. Environ. Microbiol. **65**: 1420-1427.

Demezas, D.H., and Bottomley, P.J. 1986. Autecology in rhizospheres and nodulating behaviour of indigenous *Rhizobium trifolii*. Appl. Environ. Microbiol. **52**: 1014-1019.

Demezas, D.H., Reardon, T.B., Watson, J.M., and Gibson, A. H. 1991. Genetic diversity among *Rhizobium leguminosarum* bv. *trifolii* strains revealed by allozyme and restriction fragment length polymorphism analyses. Appl. Environ. Microbiol. **57**: 3489-3495.

Deng, W., Gordon, M.P., and Nester, E.W. 1995. Sequence and distribution of *IS1312*: evidence for horizontal DNA transfer from *Rhizobium meliloti* to *Agrobacterium tumasaciens*. J. Bacteriol. **177**: 2554-2559.

Dooley, J.J., Harrison, S.P., Mytton, L.R., Dye, M., Cresswell, A., Skot,, L., and Beeching, J.R. 1993. Phylogenetic grouping and identification of *Rhizobium* isolates on the basis of random amplified polymorphic DNA profiles. Can. J. Microbiol. **39**: 665-673.

Doran, J.W. 1980. Soil microbial and biochemical changes associated with reduced tillage. Soil Sci. Soc. Am. J. **44**: 765-771.

Dowling, D. N., and Broughton, W.J. 1986. Competition for nodulation of legumes. Annu. Rev. Microbiol. **40**: 131-157.

Dreyfus, B.L., Garcia, J.L., and Gillis, M. 1988. Characterization of *Azorhizobium caulinodans* gen. nov., sp. Nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. Int. J. Syst. Bacteriol. **38**: 89-98.

Drouin, P., Prévost, D., and Antoun, H. 1996. Classification of bacteria nodulating *Lathyrus japonicus* and *Lathyrus pratensis* in northern Quebec as strains of *Rhizobium leguminosarum* bv. *viciae*. Int. J. Syst. Bacteriol. **46**: 1016-1024.

Drouin, P., Prévost, D., and H. Antoun. 1998. Cold shock proteins, fatty acids composition and symbiotic effectiveness of cold adapted rhizobia associated with *Lathyrus* sp. *In* Biological nitrogen fixation for the 21st century. Edited by C. Elemerich, A. Kondorosi and W.E. Newton. Academic Publishers, London. p. 527.

Eckhardt, T. 1978. A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. Plasmid **1**:584-588.

Elkan, G.H., and Kwik, I. E.M. 1968. Nitrogen, energy and vitamin nutrition of *Rhizobium japonicum*. J. Appl. Bacteriol. **31**: 399-404.

England, L.S., Lee, H., and Trevors, J.T. 1993. Bacterial survival in soil: effect of clays and protozoa. Soil Biol. Biochem. **25**: 525-531.

Engvild, K.C., Jensen, E.S., and Skot, L. 1990. Parallel variation in isozyme and nitrogen fixation markers in a *Rhizobium* population. Plant Soil **128**: 283-286.

Eskew, D. L., Kapuya, J., and Danso, S.K.A. 1989. Nitrate inhibition of nodulation and nitrogen fixation by supernodulating nitrate tolerant symbiotic mutants of soybean. Crop Sci. **29**:1491-1496.

Evans, J., Barnet, Y.M., and Vincent, J.M. 1979. Effect of bacteriophage on the colonization and nodulation of clover roots by a strain of *Rhizobium trifolii*. Can. J. Microbiol. **25**: 968-973.

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

Fenton, M., and Jarvis, B.D.W. 1994. Expression of the symbiotic plasmid from *Rhizobium leguminosarum* biovar *trifolii* in *Sphingobacterium multivorum*. Can. J. Microbiol. **40**: 873.

Fesenko, A.N., Provorov, N.A., Orlova, I.F., Orlov, V.P., and Simarov, B.V. 1995. Selection of *Rhizobium leguminosarum* bv. *viciae* strains for inoculation of *Pisum sativum* L. cultivars: Analysis of symbiotic efficiency and nodulation competitiveness. Plant Soil **172**: 189-198.

Finan, T.M., Wood, J.M., and Jordan, D.C. 1983. Symbiotic properties of C_4-dicarboxylic acid transport mutants of *Rhizobium leguminosarum*. J. Bacteriol. **154**: 1403-1413.

Flis, S.E., Glenn, A.R., and Dilworth, M. J. 1993. The interaction between aluminium and root nodule bacteria. Soil Biol. Biochem. **25**: 403-417.

Flores, M., Gonzalez, V., Pardo, M.A., Leija, A., Martinez, E., Romero, D., Pinero, D., Davilla, G., and Palacios, R. 1988. Genomic instability in *Rhizobium phaseoli*. J. Bacteriol. **170**: 1191.

Fobert, P.R., Roy, N., Nash, J.H.E., and Iyer, V.N. 1991. Procedure for obtaining efficient root nodulation of a pea cultivar by a desired *Rhizobium* strain and preempting nodulation by other strains. Appl. Environ. Microbiol. **57**: 1590-1594.

Franco, A.A., and Munns, D.N. 1982. Acidity and aluminium restraints on nodulation,

nitrogen fixation, and growth of *Phaseolus vulgaris* in solution culture. Soil Sci. Soc. Am. J. **46**: 296-301.

Fuhrmann, J., and Wollum, A.G., II. 1989. Nodulation competition among *Bradyrhizobium japonicum* strains as influenced by rhizosphere bacteria and iron availability. Biol. Fertil. Soils **7**: 108-112.

Gandee, C.M, Harrison, S.P., and Davies, W.P. 1999. Genetic characterization of naturally occurring *Rhizobium meliloti* populations and their potential to form effective symbiosis with lucerne. Lett. Appl. Microbiol. **28**: 169-174.

Gibson, A.H., Curnow, B.C., Bergersen, F.J., Brockwell, J. and Robinson, A.C. 1975. Studies of field populations of *Rhizobium*: Effectiveness of strains of *Rhizobium trifolii* associated with *Trifolium subterraneum* L. pastures in southeastern Australia. Soil Biol. Biochem. **7**: 95-102.

Giller, K.E., and Cadisch, G. 1995. Future benefits from biological nitrogen fixation: An ecological approach to agriculture. Plant Soil **174**: 255-277.

Gleddie, S.C. 1993. Response of pea and lentil inoculation with the phosphate-solubilizing fungus *Penicillium bilaji* (Provide). *In* Proc. Soils and Crops Workshop, Saskatoon, S.K. pp. 47-52.

Glick, B. 1995. The enhancement of plant growth by free-living bacteria. Can. J. Microbiolol. **41**: 109-117.

Graham, P.H. 1992. Stress tolerance in *Rhizobium* and *Bradyrhizobium*, and nodulation under adverse conditions. Can. J. Microbiol. **38**:475-484.

Graham, P.H. 1998. Biological dinitrogen fixation. *In* Principles and applications of soil microbiology. Edited by D.M. Sylvia, J. Furhmann, P.G. Hartel and D.A. Zuberer. Prentice Hall, NJ. pp.322-345

Graham, P.H., Viteri, S.E., Markie, F., Vargas, A.T., and Palacios, A. 1982. Variation in acid tolerance among strains of *Rhizobium phaseoli.* Field Crops Res. **5**: 121-128

Gross, D.C., and Vidaver, A.K. 1978. Bacteriocin-like substances produced by *Rhizobium japonicum* and other slow-growing rhizobia. Appl. Environ. Microbiol. **36**: 936-943.

Gulden, R.H., and Vessey, J.K. 2000. *Penicillium bilaii* inoculation increases root-hair production in field pea. Can. J. Plant Sci. **80**: 801-804.

Hagedorn, C. 1978. Effectiveness of *Rhizobium trifolii* populations associated with

Trifolium subterraneum L. in southwest Oregon soils. Soil Sci. Soc. Am. J. **42**: 447-451.

Hagen, M.J., and Hamrick, J.L. 1996. A hierarchical analysis of population genetic structure in *Rhizobium leguminosarum* bv. *trifolii*. Mol. Ecol. **5**: 177-186.

Handley, B.A., Hedges, A.J., and Beringer, J.E. 1998. Importance of host plants for detecting the population diversity of *Rhizobium leguminosarum* bv. *viciae* in soil. Soil Biol. Biochem. **30**: 241-249.

Hansen, A.P. 1994. Symbiotic N_2 fixation of crop legumes: Achievements and perspectives. Centre for Agriculture in the Tropics and Subtropics, University of Hohenheim, Germany.

Harrison, S.P., Mytton, L.R., Skøt, L., Dye, M., and Cresswell, A. 1992. Characterization of *Rhizobium* isolates by amplification of DNA polymorphisms using random primers. Can. J. Microbiol. **38**: 1009-1015.

Harrison, S.P., Young, J.P., and Jones, D.G. 1989. *Rhizobium* population genetics: host preference and strain competition on the range of *Rhizobium leguminosarum* bv. *trifolii* genotypes isolated from natural populations. Soil Biol. Biochem. **21**: 981-986.

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

Hartman, A., and Amarger, N. 1991. Genotypic diversity of an indigenous *Rhizobium meliloti* field population assessed by plasmid profiles, DNA fingerprinting, and insertion sequence typing. Can. J. Microbiol. **37**: 600-608.

Hartwig, U.A., Joseph, C.M., and Phillips, D.A. 1991. Flavonoids released naturally from alfalfa seeds enhance growth rate of *Rhizobium meliloti.* Plant Physiol. **95**: 797-803.

Hashem, F.M., and Angle, J.S. 1988. Rhizobiophage effects on nodulation, nitrogen fixation, and yield of field-grown soybeans (*Glycine max* L. Merr). Soil Biol. Biochem. **20**: 69-73.

Haukka, K., Lindström, K., and Young, J.PW. 1998. Three phylogenetic groups of n*od*A and *nif*H genes in *Sinorhizobium* and *Mesorhizobium* isolates from leguminous trees growing in Africa and Latin America. Appl. Environ. Microbiol. **64**: 419-426.

Havelka, U.D., Boyle, M.G., and Hardy, R.W.F. 1982. Biological nitrogen fixation. *In* Nitrogen in agricultural soils. Edited by F. J. Stevenson. Agron. Monogr. No. 22. ASA, CSSA, and SSSA, Madison, W1. pp. 365-422.

Heijnen, C.E., Hok-A-Hin, C.H., and van Veen, J.A. 1991. Protection of *Rhizobium* by bentonite clay against predation by flagellates in liquid cultures. FEMS Microbiol. Ecol. **85**: 65-72.

Herridge, D.F., and Betts, J.H. 1988. Field evaluation of soybean selected for enhanced capacity to nodulate and fix nitrogen in the presence of nitrate. Plant Soil **110**:129-135.

Herridge, D.F., Roughley, R.J., and Brockwell, J. 1984. Effect of rhizobia and soil nitrate on the establishment and functioning of the soybean symbiosis in the field. Aust. J. Agric. Res. **35**: 149-161.

Hirsch, P.R. 1979. Plasmid-determined bacteriocin production by *Rhizobium leguminosarum*. J. Gen. Microbiol. **113**: 219-228.

Hirsch, P.R. 1996. Population dynamics of indigenous and genetically modified rhizobia in the field. New Phytol. **133**: 159-171.

Hirsch, P. R., Jones, M.J., McGrath, S.P., and Giller, K.E. 1993. Heavy metals from past applications of sewage sludge decrease the genetic diversity of *Rhizobium leguminosarum* biovar *trifolii*. Soil Biol. Biochem. **25**: 1485-1490.

Hirsch, P.R., and Spokes, J.D. 1994. Survival and dispersion of genetically modified *Rhizobia* in the field and genetic interactions with native rhizobia. FEMS Mircobiol. Ecol. **15**: 147-159.

Hirsch, P.R, van Montagu, M., Johnston, A.W.B., Brewin, N.J., and Schell, J. 1980. Physical identification of bacteriocinogenic, nodulation and other plasmids in strains of *Rhizobium leguminosarum*. J. Gen. Microbiol.**120**: 403-412.

Hogson, A.L.M., Roberts, W.P., and Waid, J.S. 1985. Regulated nodulation of *Trifolium subterraneum* inoculated with bacteriocin-producing strains of *Rhizobium trifolii*. Soil Biol. Biochem. **17**: 475-478.

Huba, I., and Selenska-Pobell, S.1994. Pulse-field electrophoresis-fingerprinting, genome size estimation and *rrn* loci number of *Rhizobium galegae*. J. Appl. Bacteriol. **77**: 528-533.

Hungria, M., And Franco, A.A. 1993. Effects of high temperature on nodulation and nitrogen fixation by *Phaseolus vulgaris* L. Plant Soil **149**:95-102.

Hungria, M., Franco, A.A., and Sprent, J. I. 1993. New sources of high temperature tolerant rhizobia for *Phaseolus vulgaris* L. Plant Soil **149**: 103-109

Hynes, M.F., and McGregor, N.F. 1990. Two plasmids other than the nodulation plasmid are necessary for formation of nitrogen-fixing nodules by *Rhizobium leguminosarum*. Mol. Microbiol. **4**: 567-574.

Hynes, M.F., and O'Connell, M.P. 1990. Host plant effect on competition among strains of *Rhizobium leguminosarum*. Can. J. Microbiol. **36**: 864-869.

Hynes, M.F., Simon, R., Müller, P., Niehaus, K., Labes, M., and Pühler, A. 1986. The two megaplasmids of *Rhizobium meliloti* are involved in the effective nodulation of alfalfa. Mol. Gen. Genet. **202**: 356-362

Ikeda, J. 1994. The effect of short term withdrawal of NaCl stress on nodulation of white clover. Plant Soil **158**: 23-27.

Issa, S., Wood, M., and Simmonds, L.P. 1993. Active movement of chickpea and bean rhizobia in dry soil. Soil Biol. Biochem. **25**: 951-958.

Izaurralde, R.C., Juma, N.G., and McGill, W.B. 1990. Plant and nitrogen yield of barley-field pea intercrop in cryoboreal-subhumid central Alberta. Agron. J. **82**: 295-301.

Jenkinson, D.S., and Ladd, J.N. 1981. Microbial biomass in soil: measurement and turnover. Soil Biochem. **6**: 415-471.

Jensen, M.A., and Straus, N. 1993. Effects of PCR conditions on the formation of heteroduplex and single-stranded DNA products in the amplification of bacterial ribosomal DNA spacer regions. PCR methods Appl. **3**: 186-194.

Jensen, M.A., Webster, J.A., and Straus, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction -amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol. **59**: 945-952.

Johnston, A.W.B., Beynon, J.L., Buchanan-Wollaston, A.V., Setchell, S.M., Hirsch, P.R. and Beringer, J.E. 1978. High-frequency transfer of nodulating ability between strains and species of *Rhizobium*. Nature **276**: 634-636.

Joseph, M.V., Desai, J.D. and Desai, A.J. 1983. Production of antimicrobial and bacteriocin-like substances by *Rhizobium trifolii*. Appl. Environ. Microbiol. **45**: 532-535.

Judd, A. K., Schneider, M., Sadowsky, M.J., and de Bruijn, F.J. 1993. Use of repetitive sequences and the polymerase reaction technique to classify genetically related *Bradyrhizobium japonicum* serocluster 123 strains. Appl. Environ. Microbiol. **59**: 1702-1708.

Kankila, J., and Lindstöm, K. 1994. Host range, morphology and DNA restriction patterns of bacteriophage isolates infecting *Rhizobium leguminosarum* bv. *trifolii*. Soil Biol. Biochem. **26**: 429-437.

Karanja, N.K., and Wood, M. 1988. Selecting *Rhizobium phaseoli* strains for use with beans (*Phaseolus vulgaris* L.) in Kenya. Tolerance of high temperatures and antibiotic resistance. Plant and Soil. **112**: 15-22.

Kapulnik, Y. 1991. Plant-growth-promoting rhizobacteria. *In* Plant roots the hidden half. Edited by Y. Waisel, A. Eshel and U. Kafkafi. Marcel Dekker Inc. New York. pp. 771-729.

Keyes, D.O. 1990. *Penicillium bilaji*: interactions with barley or canola, growth in rhizosphere soil, and overwinter survival. M.Sc. Thesis, University of Alberta, Edmonton.

Keyser, H.H., and Munns, D.N. 1979. Tolerance of rhizobia to acidity, aluminium and phosphate. Soil Sci. Soc.Am. J. **43**: 519-523.

Kinkle, B.K., and Schmidt, E.L. 1991. Transfer of the pea symbiotic plasmid pJB5JI in nonsterile soil. Appl. Environ. Microbiol. **57**: 3264-3269.

Kondorosi, A., Kondorosi, E., Pankhurst, C.E., Broughton, W.J., and Banfalvi, Z. 1982. Mobilization of *Rhizobium meliloti* megaplasmid carrying nodulation and nitrogen fixation genes in other rhizobia and *Agrobacterium*. Mol. Gen. Genetic. **188**: 433-439.

Kremer, R.J., Polo, J., and Peterson, H.L. 1982. Effect of inoculant carrier on survival of *Rhizobium* on inoculated seed. Soil Sci. **134**: 117-125.

Kucey, R.M.N. 1983. Phosphate-solubilizing bacteria and fungi in various cultivated and virgin Alberta soils. Can. J. Soil Sci. **63**: 671-678.

Kucey, R.M.N., and Hynes, M.F. 1989. Populations of *Rhizobium leguminosarum* biovars *phaseoli* and *viciae* in fields after bean or pea in rotation with nonlegumes. Can. J. Microbiol. **35**: 661-667.

Kucey, R.M.N., Janzen, H.H., and Legget, M.E. 1989. Microbially mediated increases in plant-vailable phosphorous. Adv. Agron. **42**: 199-227.

Kündig, C., Henneke, H., and Göttfert, M. 1993. Correlated physical and genetic map of *Bradyrhizobium japonicum* strain 110 genome. *In* New horizons in nitrogen fixation: Proc. of the 9th Intern. Congr. on nitrogen fixation. Edited by R. Palacios, J. Mora and W.E. Newton. pp. 623.

Kuykendall, L.D. 1989. Influence of *Glycine max* nodulation on the persistence in soil of a genetically marked *Bradyrhizobium japonicum* strain. Plant Soil. **116**: 275-277.

Kuykendall, L.D., Swellim, D.M., Hashem, F.M., Abdelwahab, S.M., and Hegazi, N.I. 1996. Symbiotic competence, genetic diversity and plasmid profiles of Egyptian isolates of a *Rhizobium* species from *Leaucaena leucocephala* (Lam) Dewit. Lett. Appl. Microbiol. **22**: 347-352.

Labes, G., Ulrich, A., and Lentzsch, P. 1996. Influence of bovine slurry deposition on the structure of nodulating *Rhizobium leguminosarum* bv. *viciae* soil populations in a natural habitat. Appl. Environ. Microbiol. **62**: 1717-1722.

La Favre, A.K. and Eaglesham, A.R.J. 1986. The effect of high temperatures on soybean nodulation and growth with different strains of bradyrhizobia. Can. J. Microbio. **32**: 22-27.

Laguerre, G., Allard, M.R., Revoy, F. and Amarger, N. 1994. Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ.Microbiol. **60**:56-63

Laguerre, G., Geniaux, S.I., Mazurier, S.I., Rodriguez-Casartelli., R., and Amarger, N. 1993. Conformity and diversity among field isolates of *R. leguminosarum* bv. *viciae*, bv. *trifolii,* and bv. *phasoli* revealed using chromosome and plasmid probes. Can. J. Microbiol. **39**: 412-419.

Laguerre, G., Mavingui, P., Allard, M.R., Charnay, M.P., Louvrier, P., Mazurier, S.I., Gois, L. R. and Amarger, N. 1996. Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application of *Rhizobium leguminosarum* and its different biovars. Appl. Environ.Microbiol. **62**:2029-2036.

Laguerre, G., Mazurier, S.I., and Amarger, N. 1992. Plasmid profiles and restriction fragment length polymorphism of *Rhizobium leguminosarum* bv. *viciae* in field populations. FEMS microbiol. Ecol.**101**:17-2

Lamb, J.W., Hombrecher, G., and Johnston, A.W.B. 1982. Plasmid determined nodulation and nitrogen fixation abilities in *Rhizobium phaseoli*. Mol. Gen. Genetic. **186**: 449.

Lawson, K.A., Barnet, Y.M., and McGilchrist, C.A. 1987. Environmental factors influencing numbers of *Rhizobium leguminosarum* bv. *trifolii* and its bacteriophages in field soils. Appl. Environ. Microbiol. **53**: 1125-1131.

Lennox, L.B., and Alexander, M. 1981. Fungicide enhancement of nitrogen fixation and colonization of *Phaseolus vulgaris* by *Rhizobium phaseoli*. Appl. Environ. Microbiol. **41**: 404-411.

Leung, K., Yap, K., Dashti, N., and Bottomley, P.J. 1994. Serological and ecological characteristics of a nodule-dominant serotype from an indigenous soil population of *Rhizobium leguminosarum* bv. *trifolii*. Appl. Environ. Microbiol. **60**: 408-415.

Li, D., and Alexander, M. 1988. Co-inoculation with antibiotic-producing bacteria to increase colonization and nodulation by rhizobia. Plant Soil **108**: 211-219.

Li, D., and Alexander, M 1990. Factors affecting coinoculation with antibiotic-producing bacteria to enhance colonization and nodulation. Plant Soil **129**: 195-201.

Lowendorf, H.S. 1980. Factors affecting survival of *Rhizobium* in soil. Adv. Microbiol. Ecol. **4**: 87-124.

Lowendorf, H.S., and Alexander, M. 1993. The identification of *Rhizobium phaseoli* strains that are tolerant or sensitive to soil acidity. Appl. Environ. Microbiol. 45: 737-742.
Lynch, D.H., and Smith, D.L. 1993. Soybean (*Glycine max*) nodulation and N_2 fixation as affected by exposure to a low root-zone temperature. Physiol. Plant. **88**: 212-220.

Madrzak, C.J., Golinska, B., Kroliczak, J., Pudelko, K., Lazewka, D., Lampka, B., and Sadowsky, M.J. 1995. Diversity among field populations of *Bradyrhizobium japonicum* in Poland. Appl. Environ. Microbiol. **61**: 1194-1200.

Maier, R. J. and Triplett, E.W. 1996. Toward more productive, efficient, and competitive nitrogen-fixing symbiotic bacteria. Crit. Rev. Plant Sci. **15**: 191-234.

Mahler, R. L., and Wollum, A.G. 1981. The influence of water potential and soil texture on the survival of *Rhizobium japonicum* and *Rhizobium leguminosarum* isolates in the soil. Soil Sci. Soc. Am. J. **44**: 988-992.

Mahmoud, A.L.E. and Abd-Alla, M.H. 1994. Natural occurrence of mycotoxins in broad bean (*Vicia faba* L.) seeds and their effect on *Rhizobium*-legume symbiosis. Soil Biol. Biochem. **26**: 1081-1085.

Manly, B.F.J. 1994. Multivariate Statistical Methods. Chapman and Hall.

Martínez-Romero, E., and Caballero-Mellado, J. 1996. *Rhizobium* phylogenies and bacterial genetic diversity. Crit. Rev. Plant Sci. **15**: 113-140.

Martínez, E., Romero, D., and Palacios, R. 1990. The *Rhizobium* genome. Crit. Rev.

Plant Sci. **9**: 59-93.

Martinez, E., Segovia, L., Mercante, F.M., Franco, A.A., Graham, P., and Pardo, M.A. 1991. A novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. **41**: 1125-1132.

Mary, P., Dupuy, N., Dolhem-Biremon, C., Defives, C., and Tailliez, R. 1994. Differences among *Rhizobium meliloti* and *Bradyrhizobium japonicum* strains in tolerance to dessication and storage at different relative humidities. Soil Biol. Biochem. **26**: 1125-1132.

Materon, L.A., and Weaver, R.W. 1984. Toxicity of arrowleaf seed to *Rhizobium trifolii*. Agron. J. **76**: 471-473.

May, S.W., and Bohlool, B.B. 1983. Competition among *Rhizobium leguminosarum* strains for nodulation of lentils (*Lens esculenta*). Appl. Environ. Microbiol. **45**: 960-965.

Mazurier, S.I., and Laguerre, G. 1997. Unusual localization of *nod* and *nif* genes in *Rhizobium leguminosarum* bv. *viciae*. Can. J. Microbiol. **43**: 399-402.

McAndrew, D. 1997. Research Scientist, Agriculture and AgriFood, Canada, Morden Research Station.

McDermott, T.R., and Graham, P.H. 1989. *Bradyrhizobium japonicum* inoculant mobility, nodule occupancy, and acetylene reduction in the soybean root system. Appl. Environ. Microbiol. **55**: 2493-2498.

McKay, I.A., and Djordjevic, M.A. 1993. Production and extension of Nod metabolites by *Rhizobium leguminosarum* bv. *trifolii* are disrupted by the same environmental factors that reduce nodulation in the field. Appl. Environ. Microbiol. 59: 3385-3392.

McLoughlin, T.J., Alt, S.G., and Merlo, P.A. 1990. Persistence of introduced *Bradyrhizobium japonicum* strains in forming nodules in subsequent years after inoculation in Wisconsin soils. Can. J. Microbiol. **36**: 794-800.

Moawad, H., ElDin, S.M.S.B., and AbdelAziz, R.A. 1998. Improvement of biological nitrogen fixation in Egyptian winter legumes through better management of *Rhizobium*. Plant Soil **204**: 95-106.

Moënne-Loccoz, Y., Sen, D., Krause, E.S., and Weaver, R. W. 1994. Plasmid profiles of rhizobia used in inoculants and isolated from clover fields. Agron. J. **86**: 117-121.

Moxley, J.C., Hume, D.J., and Smith, D.L. 1986. N_2 fixation and competitiveness of

Rhizobium phaseoli strains isolated from Ontario soils. Can. J. Plant Sci. **66**: 825-836.

Murphy, P.J., Heycke, N., Banfalvi, Z., Tate, M.E., de Bruijn, F., Kondorosi, A., Tempe, J., and Schell, J. 1987.Genes for the catabolism and synthesis of an opine-like compound are closely linked and on the symbiotic plasmid. Proc. Nat. Acad. Sci., USA. **84**: 493-497.

Murphy, P. J., Wexler, W., Grzeneski, W., Rao, J.P., and Gordon, D. 1995. Rhizopines-their role in symbiosis and competition. Soil Biol. Biochem. **27**: 525-529.

Navarro, E., Simmonet, P., Normand, P., and Bardin, R. 1992. Characterization of natural populations of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer. Arch. Microbiol. **157**: 107-115.

Nazih, N., Sen, D., and Weaver, R.W. 1993. Population densities of clover rhizobia in Texas pastures and response to liming. Biol. Fertil. Soils **15**: 45-49.

Niemann, S., Pühler, A., Tichy, H.V., Simon, R., and Selbitschka, W. 1997. Evaluation of the resolving power of three different DNA fingerprinting methods to discriminate among isolates of a natural *Rhizobium meliloti* population. J. Appl. Microbiol. **82**: 477-484.

Nienhuis, J. Tivang, J. and Skroch, P. 1994. Analysis of genetic relationships among genotypes based on molecular marker data. *In* Analysis of molecular marker data. ASHS/CSSA, USA.

Nour, S.M., Cleyet-Marel, J.C., Beck, D., Effosse, A., and Fernandez, M.P. 1994. Genotypic and phenotypic diversity of *Rhizobium* isolated from chickpea (*Cicer arietinum* L.). Can J. Microbiol. **40**: 345-354

Oresnik, I.J., Twelker, S., and Hynes, M.F. 1999. Cloning and characterization of a *Rhizobium leguminosarum* gene encoding a bacteriocin with similarities to RTX toxins. Appl. Environ. Microbiol. **65**: 2833-2340.

Ozawa, T., Shima, S., and Yamaguchi, M. 1988. Soil aggregate as a favourable habitat for *Bradyrhizobium japonicum*. Soil Sci. Plant Nutr. **34**: 605-608.

Parco, S.Z., Dilworth, M.J., and Glenn, A.R. 1994. Motility and the distribution of introduced root nodule bacteria on the root system of legumes. Soil Biol. Biochem. **26**: 297-300.

Parker, C.A., Trinik, M.J., and Chatel, D.L. 1977. Rhizobia as soil rhizosphere inhabitants. *In* A treatise on dinitrogen fixation. Edited by R.W.F. Hardy and A.H. Gibson. John and Willey, NY. pp. 311-353.:

Paul, E.A. and Clark, F.E. 1996. Soil Microbiology and Biochemistry. Academic Press, San Diego.

Peña-Cabriales, J.J. and Castellanos, J.Z. 1993. Effects of water stress on N_2 fixation and grain yield of *Phaseolus vulgaris* L. Plant Soil. **152**: 151-155.

Pepper. I.L., and Josephson, K.L. 1998. Molecular genetic analysis in soil ecology. *In* Principles and applications of soil microbiology. Edited by D.M. Sylvia, J. Furhmann, P.G. Hartel and D.A. Zuberer. Prentice Hall, NJ. pp.168-185.

Postma, J., van Veen, J.A., and Walter, S. 1989. Influence of different initial moisture contents on the distribution and population dynamics of introduced *Rhizobium leguminosarum bv. trifolii.* Soil Biol. Biochem. **21**: 437-442.

Prakash, R.K., and Atherly, A.G. 1986. Plasmids of *Rhizobium* and their role in symbiotic nitrogen fixation. Int. Rev. Cytol. **104**: 1-24.

Prevost, D., and Bromfield, E.S.P. 1991. Effect of low temperature on symbiotic nitrogen fixation and competitive nodulation of *Onobrychis viciifolia* (sainfoin) by strains of arctic and temperate rhizobia. Biol. Fertil. Soils **12**:161-164.

Rai, R. 1992. Effect of nitrogen levels and *Rhizobium* strains on symbiotic nitrogen fixation and grain yield of *Phaseolus vulgaris* L. genotypes in normal and saline-sodic soils. Biol. Fertil. Soils **14**: 293-299.

Ramirez, C., and Alexander, M. 1980. Evidence suggesting protozoan predation on *Rhizobium* associated with germinating seeds and in the rhizosphere of beans (*Phaseolus vulgaris* L.). Appl. Environ. Microbiol. **40**: 492-499.

Ramos, M.L.G. and Ribeiro, W. 1993. Effect of fungicides on survival of *Rhizobium* on seeds and the nodulation of bean (*Phaseolus vulgaris* L.). Plant and Soil **152**:145-150.

Rao, N.S. 1995. Soil microorganisms and plant growth. Science Publishers, NH, USA.

Rao, J.R., Fenton, M., and Jarvis, B.D.W. 1994. Symbiotic plasmid transfer in *Rhizobium leguminosarum* bv. *trifolii* and competition between the inoculant strain ICMP2163 and transconjugant soil bacteria. Soil Biol. Biochem. **26**: 339-351.

Rennie, R.J., and Dubetz, S. 1984. Effects of fungicides and herbicides on nodulation and nitrogen fixation in soybean fields lacking indigenous *Rhizobium japonicum.* Agron. J. **76**: 451-454.

Rennie, R.J., and Dubetz, S. 1986. Nitrogen-15-determined nitrogen fixation in field-

grown chickpea, lentil, faba bean, and field pea. Agron. J. **78**: 654-660.

Revellin, C., Leterme, P., and Catroux, G. 1993. Effect of some fungicide treatments on the survival of *Bradyrhizobium japonicum* and on nodulation and yield of soybean (*Glycine max* (L).). Biol. Fertil. Soils **6**: 137-140.

Rice, W.A., Clayton, G.W., Olsen, P.E., Collins, M.M., Park, B., and Coy, G. 1993. Requirement for inoculation of peas in northern and central Alberta. *In* 30th Annual Alberta Soil Science Workshop proceedings. Alberta Soil Science Workshop. Edmonton pp.164-166

Rice, W.A., Clayton, P.E., Olsen, P.E., and Lupwayi, N.Z. 2000a. Rhizobial inoculation formulations and soil pH influence field pea nodulation and nitrogen fixation. Can. J. Soil Sci. **80**: 395-400.

Rice, W.A., Lupwayi, N.Z., Olsen, P.E., Schletchte, D., and Gleddie, S.C. 2000b. Field evaluation of dual inoculation of alfalfa with *Sinorhizobium meliloti* and *Penicillium bilaii*. Can. J. Plant Sci. **80**: 303-308.

Rice, W.A., and P.E. Olsen. 1993. Root nodule bacteria and nitrogen fixation. *In* Soil sampling and methods of analysis. Edited by M.R. Carter. CSSS. pp.303-317

Rice, W.A., Olsen, P.E., and Legget, E. 1994. Co-culture of *Rhizobium meliloti* and a phosphorous-solubilizing fungus (*Penicillium bilaii)* in sterile peat. Soil Biol. Biochem. **27**: 703-705.

Richardson, A.E.,and Simpson, R.J. 1989. Acid-tolerance and symbiotic effectiveness of *Rhizobium trifolii* associated with a *Trifolium subterraneum* L. based pasture growing in acid soil. Soil Biol. Biochem. **21**: 87-95.

Richardson, A.E., Viccars, L.A., Watson, J.M., and Gibson, A.H. 1995. Differentiation of *Rhizobium* strains using the polymerase reaction with random and directed primers. Soil Biol. Biochem. **27**: 515-524.

Rohlf, F.L. 1997. NTSYSpc: Numerical taxonomy and multivariate analysis system. Exter Publishers, Setauket, NY.

Romero, D., Martínez-Salazar, J., Girard, L., Palacios, R., Flores, M., and Rodriguez, C. 1995. Discrete amplifiable regions (amplicons) in symbiotic plasmid of *Rhizobium etli* CFN42. J. Bacteriol. **177**: 973-980.

Roughley, R.J., Gemell, L.G., Thompson, J.A., and Brockwell, J. 1993. The number of *Bradyrhizobium* sp (*Lupinus*) applied to seed and its effect on the rhizosphere

colonization, nodulation and yield of lupin. Soil Biol. Biochem. **25**:1453-1458.

Rusanganwa, E., and Gupta, R.S. 1993. Cloning and characterization of multiple *groEL* chaperon-encoding genes in *Rhizobium meliloti.* Gene **126**: 67-75.

Saber, S.M. 1992. Fungal contamination, natural occurrence of mycotoxins and resistance of aflatoxin accumulation of some broadbean (*Vicia faba* L.) cultivars. J. Basic Microbiol. **32**: 249-258.

Saint, C.P., Wexler, M.,Tempe, J., Tate, M.E and Murphy, P.J. 1993. Characterization of genes for synthesis and catabolism of a new rhizopine introduced in nodules by *Rhizobium meliloti Rm220-3*: extension of the rhizopine concept. J. Bacteriol. **175**: 5205-5215.

Salema, M.P., Parker, C.A., Kidby, D.K., and Chatel, D.L. 1982. Death of rhizobia on inoculated seed. Soil Biol. Biochem. **14**: 13-14.

Sande, E., Joseph, C.M., de Bruijn, F.J., and Philips, D.A. 1995. Stachydrine utilization confers a competitive advantage for alfalfa root colonization by *Rhizobium meliloti.* Abstr. 15th North American Conference on Symbiotic Nitrogen Fixation. North Carolina State University, N. C.

Santamaria, M., Agius, F., Monza, J., Gutierreznavarro, A.M., and Corzo, J. 1999. Comparative performance of enterobacterial repetitive intragenic consensus polymerase chain reaction and lipopolysaccharide electrophoresis for the identification of *Bradyrhizobium* sp. (*Lotus*) strains. FEMS Microbiol. Ecol. **28**: 163-168.

Schofield, P.R., Gibson, A.H., Dudman, W.F. and Watson, J.M. 1987. Evidence for genetic exchange and recombination of *Rhizobium* symbiotic plasmids in a soil population. Appl. Environ. Microbiol. **53**: 2942-2947.

Segovia, L., Pinero, D., Palacios, R., and Martínez-Romero, E. 1991. Genetic structure of a soil population of nonsymbiotic *Rhizobium leguminosarum*. Appl. Environ. Microbiol. **57**: 426-433.

Sethi, R.P., and Subba-Rao, N.S. 1968. Inhibitory or stimulatory effects of soil fungi on rhizobia. J. Appl. Microbiol. **14**: 325-327.

Shelp, B.J., Kaiser, B.N., and Deschesne, A.M. 1998. Registration of five near-isogenic stocks of 'Juneau' pea with altered nodulation and nitrate reductase deficiency: A3171, A317nod31, E1351, and R251. Crop Sci. **38**: 554.

Smith, R. S. 1992. Legume inoculant formulation and application. Can. J. Microbiol. **38**:

485-492.

Sneath, P.H.A., and Sokal, R.R. 1973. Numerical Taxonomy. W.H. Freeman and Co., San Francisco.

Sobral, B.W.S., Honeycutt, R.J., and Atherly, A.G. 1991. The genomes of the family Rhizobiacea: Size, stability, and rarely cutting restriction endonucleases. J. Bacteriol. **173**: 704-709.

Sokal, R.R., and Michener, C.D. 1958. A statistical method for evaluating systematic relationships. Univ. Kans. Sci. Bull. **38**: 1409-1438.

Sosulski, F., and Buchan, J.A. 1978. Effects of *Rhizobium* and nitrogen fertilizer on nitrogen fixation and growth of field pea. Can. J. Plant Sci. **58**:553-556.

Sreenivasa, M.N., and Bagyaraj D.J. 1989. Use of pesticides for mass production of vesicular-arbuscular mycorrhizal inoculum. Plant Soil **119**: 127-132.

Strain, S.R., Whittam, T.S., and Bottomley, P.J. 1995. Analysis of genetic structure in soil populations of *Rhizobium leguminosarum* recovered from the USA and the UK. Mol. Ecol. **4**: 105-114.

Streeter, J.G. 1994. Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. Can. J. Microbiol. **40**: 513-522.

Streeter, J.G., and Smith, R.S. 1998. Introduction of rhizobia into soil- problems, achievements, and prospects for the future. *In* Microbial Interactions in Agriculture and Forestry. Vol. 1. Edited by N.S. Subba Rao and Y.R. Dommergues. pp. 45-64.

Sullivan, J.T., Patrick, H.N., Lowther, W.L., Scott, D.B., and Ronson, C.W. 1995. Nodulating strains of *Rhizobium loti* arise through chromosomal symbiotic gene transfer in the environment. Proc. Natl. Acad. Sci. USA. **92**: 8985-8989.

Sutton, W.D. 1974. Some features of the DNA of *Rhizobium* bacteroids and bacteria. Biochem. Biophys. Acta. **306**: 1-10.

Sylvia, D.M. 1998. Mycrobial symbioses. *In* Principles and applications of soil microbiology. Edited by D.M. Sylvia, J. Furhmann, P.G. Hartel and D.A. Zuberer. Prentice Hall, NJ. pp. 408-426.

Thiagarajan, T.R., and Ahmad, M.H. 1993. Influence of a vesicular-aburscular mycorrzal fungus on the competitive ability of *Bradyrhizobium* spp. for nodulation of cowpea *Vigna unguiculata* (L.) Walp in non-sterilized soill. Biol. Fertil. Soils **15**: 294-296.

Thies, J.E., Bohlool, B.B., and Singleton, P.W. 1992. Environmental effects on the competition for nodule occupancy between introduced and indigenous rhizobia and among introduced strains. Can. J. Microbiol. **38**: 493-500.

Thies, J.E., Singleton, P.W., and Bohlool, B.B. 1991. Influence of the size of indigenous rhizobial populations on the establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Appl. Environ. Microbiol. **57**: 19-28.

Thies, J.E., Woomer, P.L., and Singleton, P.W. 1995. The enrichment of *Bradyrhizobium* spp populations in soil due to cropping of the homologous host legume. Soil Biol. Biochem. **27**: 633-636.

Thompson, J.A., Bhromsiri, A., Shutsrirung, A., and Lillakan, S. 1991. Native root-nodule bacteria of traditional soybean-growing areas of northern Thailand. Plant Soil **135**: 53-65.

Thurman, N.P., and Bromfield, E.S.P. 1988. Effect of variation within and between *Medicago* and *Melilotus* species on the composition and dynamics of indigenous populations of *Rhizobium meliloti* Soil Biol. Biochem. **20**: 31-38.

Toro, N., and Olivares, J. 1986. Analysis of *Rhizobium meliloti* sym mutants obtained by heat treatment. Appl. Environ. Microbiol. **51**: 1148-1150.

Triplett, E.W. 1990. Construction of a symbiotically effective strain of *Rhizobium leguminosarum* bv. *trifolii* with increased nodulation competitiveness. Appl. Environ. Microbiol. **56**: 98-103.

Tsien, H.C., Cain, P.S., and Schmidt, E.L. 1977. Viability of *Rhizobium* bacteroids. Appl. Environ. Microbiol. **34**: 854-856.

Tu, J.C. 1981. Effect of salinity on *Rhizobium* root hair interaction, nodulation and growth of soybean. Can. J. Plant Sci. **61**: 231-239.

van Berkum, P., Beyene, D., Vera, F.T., and Keyser, H.H. 1995. Variability among *Rhizobium* strains originating from nodules of Vi*cia faba*. Appl. Environ. Microbiol. **61**: 2649-2653.

van Egeraat, A.W.S.M. 1975. The possible role of homoserine in the development of *Rhizobium leguminosarum* in the rhizosphere of pea seedlings. Plant Soil **42**: 381-386.

Vargas, A.A.T., and Graham, P.H. 1988. *Phaseolus vulgaris* cultivar and *Rhizobium* strain variation in acid-tolerance and nodulation under acidic conditions. Field Crops Res.

19: 91-101.

Venter, A.P., Twelker, S., Oresnik, I.J., and Hynes, M.F. 2001. Analysis of the genetic region encoding a novel rhizobiocin from *Rhizobium leguminosarum* bv. *viciae* strain 306. Can. J. Microbiol. **47**: 495-502.

Vessey, J.K., and Heisinger, K.G. 2001. Effect of *Penicillium bilaii* inoculation and phosphorous fertilization on root and shoot parameters of field-grown pea. Can. J. Plant Sci. **81**: 361-366.

Vincent, J. M. 1970. A Manual for the practical study of root nodule bacteria. Blackwell, Oxford, Edinburg, U. K.

Vlassak, K.M. and Vanderleyden, J. 1997. Factors influencing nodule occupancy by inoculant rhizobia. Crit. Rev. Plant Sci. **16**:163-229.

Vlassak, K.M., Vanderleyden, J., and Franco, A. 1996. Competition and persistence of *Rhizobium tropici* and *Rhizobium etli* in tropical soil during successive bean (*Phaseolus vulgaris* L.) cultures. Biol. Fertil. Soils **21**: 61-68.

Waterer, J.G., Vessey, J.K., Stobbe, E.H., and Soper, R.J. 1994. Yield and symbiotic nitrogen fixation in a pea-mustard intercrop as influenced by fertilizer addition. Soil Biol. Biochem. **26**: 447-453.

Weaver, R.W., and Frederick, L.R. 1974. Effect of inoculum rate on competitive nodulation of *Glycine max L.* Merrill. II. Field studies. Agron. J. **66**: 233-236.

Weiser, G.C., Skipper, H.D., and Wollum, A.G., II. 1990. Exclusion of inefficient *Bradyrhizobium japonicum* serogroups by soybean genotypes. Plant Soil **121**: 99-105.

Wexler, M., Gordon, D., Murphy, P.J. 1995. The distribution of inositol rhizopine genes in *Rhizobium* populations. Soil Biol. Biochem. **27**: 531-537.

Wheatcroft, R., McRae, D.G., and Miller, R.W. 1990. Changes in the *Rhizobium meliloti* genome and the ability to detect supercoiled plasmids during bacteroid development. Mol. Plant-Microbe Interact. **3**: 9-17.

Wheatly, D.M., Macleod, D.A., and Jessop, R.S. 1995. Influence of tillage treatments on N_2 fixation of soybean. Soil Biol. Biochem. **27**: 571-574.

Wilson, R.A., Handley, B.A., and Beringer, J.E. 1998. Bacteriocin production and resistance in a field population of *Rhizobium leguminosarum* bv. *viciae*. Soil Biol. Biochem. **30**: 413-417.

Woese, C.R. 1987. Bacterial evolution. Microbiol. Rev. **51**: 221-271.

Woomer, P., Singleton, P.W., and Bohlool, B.B. 1988. Ecological indicators of native rhizobia in tropical soils. Appl. Environ. Microbiol. **54**: 1112-1116.

Worall, V.S., and Roughley, R.J. 1976. The effect of moisture stress on infection of *Trifolium subterraneum* L. by *Rhizobium trifolii* Dang. J. Exp. Bot. **27**: 1233-1241.

Yahalom, E., Okon, Y., and Dorvat, A. 1990. Possible mode of action of *Azospirillum brasilense* strain Cd on the root morphology and nodule formation in burr medic (*Medicago polymorpha*). Can. J. Microbiol. **33**: 510-514.

Young, J.P.W. 1996. Phylogeny and taxonomy of rhizobia. Plant Soil **186**: 45-52.

Young, C.C., and Cheng, K.T. 1998. Genetic diversity of fast-and slow-growing soybean rhizobia determined by random amplified polymorphic DNA analysis. Biol. Fertil. Soils **26**: 254-256.

Young, J.P. W., and Hauka, K.E. 1996. Diversity and phylogeny of rhizobia. New Phytol. **133**: 87-94

Zablotowicz, R.M., Tipping, E.M., Scher, F.M., Ijzerman, M., and Koepper, J.W. 1991. In-furrow spray as a delivery system for plant growth-promoting rhizobacteria and other rhizosphere-competent bacteria. Can. J. Microbiol. **37**: 632-636.

Zahran, H.H. and Sprent, J.J. 1986. Effects of sodium chloride and polyethylene glycol on root-hair infection and nodulation of *Vicia faba* L. plants by *Rhizobium leguminosarum*. Planta **167**: 303-309.

8.0 APPENDICES

Appendix 8.1 Number of pea nodules plant^{-1} as affected by *Rhizobium* inoculation with and without N-fertilization in 17 sites in southern Manitoba in 1998.

Site	Treatment		
	Uninoculated	Inoculated	Inoculated +N
Carman1	30	26.4	12.1
Carman 2	36.7	45.7	36.3
Carman 3	28.3	59.5	67.8
Carman 4	59.7	49.3	54.8
Fort Garry Campus	52.3	74.3	44
Letellier	50.5	40.1	40.5
Morden 1	25.8	39	39.8
Arborg 1	15.5	7.3	4.3
Arborg 2	13.8	32.9	2.8
Teulon 1	73.2	82.1	34.2
Morden 2	59.3	78.4	63.1
Morris 1	11.3	14.7	6.8
Morris 2	11.8	9.5	4.7
Stuartburn	0	30.8	32.6
Glenlea 1	22.7	28.5	31.3
Glenlea 2	44.2	43.1	35.7
Westbourne 1	28.3	26.6	27.9
Mean (nodules plant^{-1})	33.1	40.5	31.7

Note: Only uninoculated seed sown at Westbourne 2 (recorded 5.8 nodules plant^{-1}). Nodule counts not taken for Souris 1, Souris 2, and Teulon 2, but the pea plants were well nodulated.

Appendix 8.2 Pea nodule dry weight (mg plant^{-1}) as affected by *Rhizobium* inoculation with and without N-fertilization in 17 sites in southern Manitoba in 1998.

Site	Treatment		
	Uninoculated	Inoculated	Inoculated +N
Carman1	135	127	61
Carman 2	267	174	138
Carman 3	54	150	286
Carman 4	663	620	298
Fort Garry Campus	167	235	94
Letellier	298	143	75
Morden 1	43	129	73
Arborg 1	6	28	25
Arborg 2	6	14	0
Teulon	66	59	5
Morden 2	71	12	69
Morris 1	4	8	2
Morris 2	6	8	4
Stuartburn	0	29	14
Glenlea 1	12	13	17
Glenlea 2	92	75	34
Westbourne 1	3	25	1
Mean (mg plant^{-1})	111.4	108.8	70.4

Note: Nodule dry weights not recorded for Westbourne 2, Souris 1 and Souris 2.

Appendix 8.3 Shoot dry matter (g plant^{-1}) of pea plants as affected by *Rhizobium* inoculation with and without N-fertilization in 19 sites in southern Manitoba in 1998.

Site	Treatment		
	Uninoculated	Inoculated	Inoculated +N
Carman1	9.6	8.1	11.1
Carman 2	9.6	9.3	20.1
Carman 3	12.8	15.6	12.4
Carman 4	6.1	8.9	7.3
Fort Garry Campus	3.4	9.3	9.7
Letellier	10	11.9	10.9
Morden 1	7.8	10.3	10.4
Arborg 1	8.1	9.4	11.8
Arborg 2	5.8	6.2	6.5
Teulon	3.7	5	6.7
Morden 2	10.9	9.9	10
Morris 1	4.3	6.3	4.9
Morris 2	4.9	5.1	4.1
Souris 1	3.4	4.8	5.6
Souris 2	7.2	8.5	9.2
Stuartburn	0.46	3.06	3.6
Glenlea 1	3.4	2.5	5.1
Glenlea 2	5.3	5.8	6
Westbourne 1	1.4	1.4	2.8
Mean (g plant^{-1})	6.2	7.4	8.3

Appendix 8.4 Distinct plasmid profiles of *Rhizobium* isolates (1998 survey).

Plasmid size (kb)	Isolate									
	1a-1	1a-2	1a-3	1a-4	1a-5	1a-6	1a-7	1a-8	1a-9	1a-10
<50	–									
50										
85		–								
140										
175			–							
225										
270										
310										
350				–						
400										
450					–					
500										
550						–				
600										
650							–			
700										
750								–		
800										
850									–	
900										
950										
1000										
>1000										–
Sites	21	12,4,17	1,7,8,13, 15, 16, 18	10,14,19, 21, 12	6,15,9,12,	13,9	13,2,12	13,18	13	21
# of Isolates	1	4	16	16	12	4	6	2	2	1

Appendix 8.4 (cont'd)

Plasmid size (kb)	Isolate								
	2a-1	2a-2	2a-3	2a-4	2a-5	2a-6	2a-7	2a-8	2a-9
<50									
50									
85									
140	–	–	–						
175								–	
225									–
270									–
310						–			
350	–								
400									
450		–							
500							–		
550			–			–			
600									
650								–	
700									
750				–					
800									
850									
900									
950									
1000				–	–		–		
>1000					–				
Sites	21,8	13	13	16,21,18	8	16	6,20	16	Glenlea *Lathyrus*
# of Isolates	2	1	1	3	1	1	3	1	1

Appendix 8.4 (cont'd)

Plasmid size (kb)	Isolate									
	3a-1	3a-2	3a-3	3a-4	3a-5	3a-6	3a-7	3a-8	3a-9	3a-1
<50										
50										–
85	–									
140			–		–		–			
175										
225		–	–							
270	–									
310			–			–				
350		–		–	–					–
400								–	–	
450										
500										
550		–								–
600						–				
650					–					
700				–				–		
750										
800						–				
850										
900							–			
950				–				–		
1000							–		–	
>1000	–								–	
Sites	RGAA1	12,15,18,41,2,3,13,20	3,12,8	13,16,21,5	15,16	21	8	20	12	20
# of Isolates	1	15	4	5	2	1	1	2	1	1

Appendix 8.4 (cont'd)

Plasmid size (kb)	Isolate									
	4a-1	4a-2	4a-3	4a-4	4a-5	4a-6	4a-7	4a-8	4a-9	4a-10
<50										
50				–						
85										
140										–
175		–								–
225										
275			–						–	
310	–			–	–	–				
350							–			
410	–		–		–			–	–	–
460		–				–	–			
510				–						
550		–					–	–		
600	–		–		–	–				
650										–
700										
750								–		
800										
850	–	–					–		–	
900										
950										
1000										
>1000				–	–	–			–	
			–					–		
Sites	16, 13,15, 17, PBC	RGP4	1,17,12, RGP2	RGL4	RP212-37 16,15	RP212-2 99AA1,12, 21	PEA082	RP212-19, 13,12	175G10B	12
# of Isolates	6	1	5	1	4	5	1	4	1	2

Appendix 8.4 (cont'd)

Plasmid size (kb)	Isolate										
	4a-11	4a-12	4a-13	4a-14	4a-15	4a-16	4a-17	4a-18	4a-19	4a-20	4a-21
<50											
50											
85											
140											
175						–		–	–	–	–
225											
275										–	
310	–	–	–			–	–				–
350				–		–		–			
410	–		–	–			–		–	–	
460						–		–			
510					–						
550		–									
600					–						
650			–		–		–				–
700	–										
750				–					–	–	
800								–			
850											–
900				–							
950	–				–						
1000		–									
>1000							–				
		–	–						–		
Sites	16,21,20 17,18	7,13,17	12,17	17, 21	21	21	21	2,20	15	18	20
# of Isolates	8	4	4	8	1	1	1	2	1	1	2

Appendix 8.4 (cont'd)

Plasmid size (kb)	Isolate								
	5a-1	5a-2	5a-3	5a-4	5a-5	5a-6	5a-7	5a-8	5a-9
<50									
50									
85									
140									
175				–	–	–			–
225		–			–	–	–	–	
275	–			–				–	–
310		–	–						
350	–		–		–	–	–	–	–
410		–		–					
460				–			–		
510		–		–				–	
550	–		–						–
600									
650	–				–	–	–		
700		–	–						
750									
800									
850						–		–	
900					–				
950	–						–		
1000									
>1000			–						–
Sites	RP213-5,C1 128C56G	RGFP	RP212-13	12,16	12,15,16 21,7,17	12,16	15,21	16,17	3
# of Isolates	3	1	1	2	11	2	2	3	1

Appendix 8.4 (cont'd)

Plasmid size (kb)	Isolate									
	5a-10	5a-11	5a-12	5a-13	5a-14	5a-15	5a-16	5a-17	5a-18	5a-19
<50		–								
50										
85		–							–	
140									–	
175				–	–					–
225	–	–				–		–		
275							–			
310			–	–		–			–	
350				–	–		–	–		
410	–		–		–			–	–	–
460	–					–	–			
510				–		–				
550			–							
600										–
650						–		–		–
700		–		–	–					
750	–									
800							–			
850										
900	–		–							
950					–			–		
1000									–	
>1000							–			
		–	–							–
Sites	17,21	6	13	15,16,17, 20	17	17	18	20	Teulon *Lathyrus*	Morden *Lathyrus*
# of Isolates	2	2	1	5	2	1	1	1	1	2

Appendix 8.4 (cont'd)

Plasmid size (kb)	Isolate							
	6a-1	6a-2	6a-3	6a-4	7a-1	7a-2	7a-3	8a-1
<50								
50		–						–
85				–				
140		–	–	–		–	–	–
175	–				–			–
225			–				–	–
275				–		–	–	
310			–		–			
350	–	–		–	–			–
410	–	–	–		–	–		
460				–			–	–
510						–		
550	–						–	
600						–		
650		–	–		–		–	
700						–		
750								
800				–		–		
850	–				–			
900			–					
950		–				–	–	
1000	–							
>1000					–			–
Sites	16	21	(21,12,20)	Teulon *Lathyrus*	15,10	21	Teulon *Lathyrus*	12
# of Isolates	1	1	3	1	2	1	2	1