ENDOPHYTIC COLONIZATION AND NITROGEN FIXATION BY *PAENIBACILLUS POLYMYXA* IN ASSOCIATION WITH LODGEPOLE PINE AND WESTERN RED CEDAR

by

Richa Anand

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies
(Soil Science)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

November 2010

Abstract

In this study I provide evidence of biological nitrogen fixation by endophytic, diazotrophic bacteria as a possible source of nitrogen for lodgepole pine (*Pinus contorta* var. latifolia (Dougl. Engelm.) and western red cedar (*Thuja plicata* Donn.); conifers that are known for their ability to grow in the nitrogen-poor forests of western North America. Diazotrophic bacteria were isolated from root, stem and needle tissues of both these tree species, growing on forested sites with contrasting N availability in the interior of British Columbia, Canada. Members of the genera *Bacillus* and *Paenibacillus* genera dominated the culturable, endophytic bacterial community in tissues of both tree species. A *Paenibacillus polymyxa* isolate designated strain P2b-2R from lodgepole pine at the nitrogen deficient site near Williams Lake, BC., demonstrated high (5.1705 µmols C_2H_4/ml), replicable, nitrogenase activity, under laboratory conditions.

P. polymyxa strain P2b-2R inoculated and control lodgepole pine and cedar seedlings were grown in a sand – turface mixture enriched with a 5 atom % excess ^{15}N [$Ca(^{15}NO_3)_2$] solution. Root, shoot and seedling length, fresh weight and dry weight demonstrated that both tree species accumulated significantly higher biomass when inoculated with strain P2b-2R. ^{15}N atom percent excess indicated that P2b-2R inoculated lodgepole pine and western red cedar derived 67.53 and 21.94% of their total foliar nitrogen from the atmosphere, respectively. Using *in situ* confocal laser scanning microscopy, cells of strain P2b-2R tagged with green fluorescent protein were found to colonize the root and stem cortical cells of lodgepole pine, both inter- and intracellularly.

Sequences of *nif* B, H and D genes of strain P2b-2R were obtained using PCR. Phylogenies based on *nif*H and *nif*D genes of *P. polymyxa* strain P2b-2R place these genes in monophyletic groups with those of free-living cyanobacteria and root nodule-forming *Frankia*, respectively. Within the genus *Paenibacillus*, based on *nif*H and *nif*D phylogenies, *P. polymyxa* was most closely related to *P. massiliensis* T7, a bacterium isolated from the rhizosphere of willow trees (*Salix spp*.) in Beijing. These results provide the first evidence of significant endophytic nitrogen fixation in conifer species growing under nitrogen-limited conditions and

support the possibility of a novel and ecologically significant interaction between coniferous trees and diazotrophic bacteria.

Preface

Chapter 1: Introduction

Chapter 1 is an amalgamation of a published book chapter, co-authored by Dr. Leslie Paul and Dr. Chris Chanway and another book chapter authored by Richa Anand (in press). Richa Anand researched and reviewed the literature and wrote all material for the two chapters. Images 1.1 and 1.2 and research related to the images were contributed by Dr. Leslie Paul. Dr Chris Chanway supervised a major part of the scientific research reported in the published chapter and the preparation of both the chapters for publication.

Chapter 2: Isolation and Identification of Potential Endophytic Diazotrophs from *Pinus contorta* and *Thuja plicata.*

This manuscript is co-authored by Amandeep Bal, Dr. Odile Berge and Dr. Chris Chanway. Amandeep Bal sampled plant material and isolated bacteria. Dr. Odile Berge was responsible for identification of the isolated bacteria and providing editorial input to Richa Anand for the writing of this manuscript. Dr Chris Chanway supervised the research reported in the manuscript and provided editorial input to Richa Anand in the writing of this manuscript. Richa Anand interpreted and analyzed the results and wrote this manuscript.

Chapter 3: Conifer Seedlings Derive Nitrogen and Growth Benefits from Naturally Occurring Endophytic Diazotrophic Plant Growth Promoting Rhizobacterium (PGPR) *Paenibacillus polymyxa* Strain P2b-2R.

This manuscript is co- authored by Dr. Sue Grayston and Dr Chris Chanway. Richa Anand was responsible for all the work presented in this manuscript and for writing this manuscript. Dr Chris Chanway supervised the research presented and Dr Sue Grayston supervised the preparation of this manuscript.

Chapter 4: Sequencing of Nitrogen Fixation (*nif)* Genes Using SSP-PCR (Single Specific Primer) and *nif* Phylogeny of *Paenibacillus polymyxa* Strain P2b-2R.

This manuscript is co-authored by Dr Chris Chanway. Richa Anand was responsible for all the work presented in this manuscript and for writing this manuscript. Dr Chris Chaway supervised the research reported in this manuscript.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	= Analysis of Variance
AP	= Ammonia permease
ARA	= Acetylene reduction assay
ATP/ADP	= Adenosine tri-phosphate/Adenosine di-phosphate
BHIG	= Brain heart infusion glycerol
BLAST	= Basic local alignment search tool
BNF	= Biological nitrogen fixation
CCM/CCMA	= Combined carbon medium / Combined carbon medium agar
cfu	= colony forming units
CRD	= completely randomized block design
CSLM	= Confocal Laser scanning microscopy
CWH	= Coastal western hemlock
DABCO	= 1,4-diazabicyclo-[2,2,2]-octane
DIG	= Digoxigenin
GC-FAME	= Gas chromatographic-fatty acid methyl ester
GFP	= Green fluorescent protein
GS	= Glutamine synthetase
LB/LBA	= Luria Broth/ Luria broth agar
MCL	= Maximum composite likelihood
MEGA	= Molecular evolutionary genetic analysis
N	= Nitrogen
Ndfa	= Nitrogen derived from atmosphere
PAR	= Photosynthetically active radiation
PBS/SPB	= Phosphate buffered saline/ Sterile phosphate buffer
PGPR	= Plant growth promoting rhizobacteria
PEB	= Phosphate electroporation buffer
REP-PCR	= interspersed repetitive elements – Polymerase chain reaction

RGR = Relative growth rates

rrs = ribosomal RNA 16S

SBPS = Sub-boreal pine spruce

SSP-PCR = Single specific primer-polymerase chain reaction

TSA/TSB = Tryptic soy agar/ Tryptic soy broth

Acknowledgments

The successful completion of a thesis is a long and ardurous task, requiring more than the inspiration and exertions of one individual. This thesis was no exception, and there are many people to whom I am indebted.I wish to thank Dr Sue Grayston and Dr William Mohn for their invaluable guidance and immense patience as supervisors. I am grateful to Dr. Collette Breuil for bringing her expertise to my supervisory committee and opening up her laboratory facilities for use at all times. I am also thankful to Dr R.E.W. Hancock, Manjeet Bains, Jelena Pistolic and John Hale at the Center for microbial disease and immunity research, UBC, for providing me access to their laboratory facilities.

I am deeply indebted to Dr Chris Chanway for the opportunity to work on this exciting project and his guidance through the thick and thin of it. I also wish to thank Dr Mahesh Upadhyaya, Dr Rob Guy and Dr Cindy Prescott for their unconditional support through the most challenging of professional and personal circumstances.

I extend my sincere gratitude to NSERC, Faculty of Land and Food systems at UBC and the Ministry of Advanced Education of British Columbia for financial support at various stages of my studies.This work would not be complete without the technical support of Kevin Hodgson, Kate Del Bel, Elizabeth Starks, Drs. Brad Sealy and Minako Kaneda. I am also grateful to Amandeep Bal and Ming Luo for their help at the initial stages of this project. I owe special thanks to my dear friend Denise Brooks for inspiration, ideas, encouragement and endless support.

I thank my parents and Raman Anand, who believed in my capabilities and supported me in this undertaking. I am deeply grateful to Devesh Bahuguna and Geoffery Antony who made sure that both, my computer and I, made it to the finish line. I am also indebted to my dear friends Yuexin Li, Dawn Marie Morgan, Shalima Ganesan, Shreya Chandola, Toktam Sajedi, Prabha P., Jason Barker and Li Li who helped me keep my head above the water at all times. Finally, hats off to my little daughter Shereen who waited patiently while mommy was ‘writing her very long book’.

Dedication

To Raman Anand

Hope I have done you proud

1 Introduction[1]

Plants can be considered as complex microecosystems that provide different habitats to a variety of microorganisms. These habitats are represented by the plant external surfaces as well as internal tissues (McInroy and Kloepper, 1994).Whereas the importance of microbial colonisation of plant surfaces in plant growth promotion has been well understood for a long time, interior tissue colonisation was, until recently, largely perceived as being related only to the perpetuation of systemic diseases. It is now well known that tissues of healthy plants are also colonised internally by various microorganisms that establish neutral or, more interestingly, beneficial interactions with their host plants. The term "endophyte" is commonly used to describe such microorganisms.

Although a variety of definitions have been applied to the term "endophyte", it refers mainly to bacteria and fungi that live inside plant tissues without causing disease (Wilson, 1995; Schulz and Boyle, 2006). Whether or not latent pathogens can be considered as endophytes, is a major topic of debate in the general acceptance of this definition (Misaghi and Donndelinger, 1990; James and Olivares, 1998; Schulz and Boyle, 2006).

The best-characterised microbial endophytes are the grass-inhabiting Balansiaceous fungi (ascomycetous genera *Epichloë* and *Balansia* and anamorphs *Neotyphodium* and *Ephelis*), for which the most compelling evidence of plant–microbe mutualism has been provided (Clay, 1988; Schardl *et al.,* 2004). Some of the non-balansiaceous endophytic fungi that typically colonize herbaceous and woody plants, are also mutualistic with their hosts (Carroll, 1988; Schulz, 2006), and produce compounds that render plant tissues less attractive to herbivores, while other strains increase host plant drought resistance (Gange *et al*., 2007) and tolerance to extreme temperatures and salinity (Rodrigues *et al*., 2004). Mutualistic association between such fungi and plants has been projected as a plausible strategy for mitigating the impacts of

[1] This chapter is an updated and amalgamated version of a chapter published in the book, Soil Biology, Volume 9.Anand R., Paul L., Chanway C. (2006) Research on Endophytic Bacteria: Recent Advances with Forest Trees, in: B. J. E. Schulz et al., (Eds.), Microbial Root Endophytes, Springer Berlin Heidelberg. pp. 89-106 and another chapter in the book, Handbook of Soil Science, Second Edition, Anand R.,(in press) Biological nitrogen fixation, in:P.M. Huang et al., (Eds.), Taylor and Francis Group, NY.

global climate change on plants (Rodriguez *et al*., 2004). In return, fungal endophytes are thought to benefit from the comparatively nutrient rich, buffered environment inside plants (Schulz and Boyle, 2006).

Apart from fungi, bacteria belonging to various genera have also been shown to exist inside plants without causing apparent disease symptoms. Some of these bacteria are known to impart benefits to their host plants by the same mechanisms as their soil- or rhizosphere-colonizing counterparts (Ryan *et al.,* 2007). The primary mechanisms thought to lead to beneficial effects for the plant are, nitrogen fixation (Boddey and Döbereiner, 1995) and biocontrol of pathogenic and detrimental microorganisms, either through direct antagonism of pathogens or by inducing systemic resistance to such organisms (Hallman *et al.,* 1997). Other known mechanisms by which beneficial bacteria can have a positive influence on plant performance are the production or stimulation of plant growth hormones, facilitation of nutrient uptake (Kloepper and Ryu, 2006) and as facilitators of mycorrhization (Garbaye, 1994).

Since the first reported isolation of endophytic bacteria from potato plants (Tervet and Hollis, 1948; Hollis, 1951), majority of the information available on these bacteria has been derived from studies on plant species of agricultural and horticultural importance. The endophytic bacteria of rice (Reinhold-Hurek and Hurek, 1998), corn (Triplett, 1996) and sugarcane (Döbereiner *et al.,* 1995) are by far the best studied until now. In contrast to these crop species, much less is known about bacterial endophytes of trees. Some trees survive and grow well in very difficult terrain under extreme conditions, for example lodgepole pine (*Pinus contorta* Dougl. var. *latifolia*) in dry interior regions of British Columbia and western Alberta, Canada, as well as Roheda (*Tecomella undulata,* Bignoniaceae) in the extremely arid deserts of northwestern India (Bhau *et al.*, 2007). It is possible that endophytic bacteria that enhance host survival and growth in exchange for protection in the relatively buffered environment of internal plant tissues may be involved under such extreme environmental conditions (Law and Lewis, 1983).

Although the realization of this possibility has led to occasional reports of endophytic bacteria in asymptomatic angiosperm and gymnosperm tree species, little is known about their diversity and influence on plant growth. The earliest report of bacterial endophytes from trees

was from Gardner *et al.,* (1982), who isolated representatives of 13 genera from xylem fluid of rough lemon rootstock, and found population sizes ranging from 10^2–10^4 colony forming units (cfu) g^{-1} xylem fluid. Only 48 of the 850 isolates turned out to be phytopathogenic, but the role of the other 802 isolates was not determined. Similarly, several strains of *Pseudomonas syringae* were isolated and characterized from inside pear seedlings by Whitesides and Spotts (1991), but their exact role could not be determined.

The procedures of isolation and identification of endophytic bacteria from trees are the same as those used in their isolation from agronomic crops, and suffer from the same limitations, e.g. the difficulty, or perhaps impossibility, of absolute surface-sterilisation of external plant tissues (Hyde and Soytong, 2008), as well as our inability to culture many bacteria we know to exist (Amman, 2000). The impact of these problems can be reduced by the use of standardised sterilization protocols and molecular techniques (James, 2000; Hallmann *et al.*, 2006). The major difficulty, therefore, lies in the evaluation of the effects of these bacteria on their host trees, owing to the long life-cycle of trees and the limited availability of detailed physiological information on trees, particularly forest trees.

1.1 Bacterial Endophytes of Forest Trees

Although limited, the results of research on endophytic bacteria and their role in growth promotion of forest trees so far are very encouraging and will, hopefully, draw more attention to this developing area of study. Brooks *et al.,* (1994) conducted an extensive study in which endophytic bacteria were isolated from surviving live oak (*Quercus fusiformis*) in Texas, (where oak wilt is epidemic) and evaluated as potential biological control agents for the disease. Of the 889 bacterial isolates tested, 183 showed *in-vitro* inhibition of the pathogen, *Ceratocystis fagacearum*. Six isolates were further evaluated for colonization of containerized Spanish oak (*Quercus texana*) and live oak. Interestingly, in containerized live oaks inoculated with the oak wilt pathogen, pre-inoculation with 15 isolates of *Pseudomonas denitrificans* reduced the number of diseased trees by 50% and decreased the percentage of crown loss by 17%. In a subsequent trial, no reduction in numbers of diseased trees was observed, but preinoculation with the same isolates of *P. denitrificans,* or a strain of *P. putida,* significantly

reduced crown loss. These results clearly established the potential of endophytic bacteria as pre-plantation nursery treatments for wilt control.

Several endophytic aerobic heterotrophic bacteria belonging to the genera *Bacillus*, *Curtobacterium*, *Pseudomonas*, *Stenotrophomonas*, *Sphingomonas*, *Enterobacter*, and *Staphylococcus*, have also been isolated from phloem tissue of roots and branches of elm trees (*Ulmus* spp.; Mocali *et al.,* 2003). An attempt was also made to determine the correlation between the seasonal fluctuations in the structure of the endophytic bacterial community and phytoplasma disease infection of these trees; however, no consequential effect of the bacterial community on phytoplasmosis of elm trees could be demonstrated (Mengoni *et al.,* 2003).

There are multiple studies that have reported the isolation and identification of endophytic bacteria in poplar and hybrid poplar (genus *Populus*) trees. Ulrich *et al.*, 2008, found at least 53 taxa of endophytic bacteria that included Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes in hybrid poplars (*Populus sp.*). Alpha-proteobacterial genus *Sphingobacterium* was found to be the most abundant when culture independent methods such as TRFLP and cloning of 16S rDNA were used, whereas the culturable endophytic population was dominated by the genera *Pseudomonas* (gamma-proteobacterium) and *Curtobacterium* (Actinobacterium). Germaine *et al.*, (2004) also isolated three endophytic *Pseudomonas* strains from poplar trees. However, neither study attempted to determine whether these endophytic bacteria had a mutualistic association with the host trees. Moore *et al*., (2006), and Taghavi *et al.*, (2005) have studied the potential of endophytic bacteria of poplar in phytoremdiation of sites infected by toluene and other hydrocarbons.Taghavi *et al.,* (2005) showed that the endophyte *Burkholderia cepacia* VM1468 decreased phytotoxicity in inoculated poplar cuttings grown under conditions of high toluene toxicity (*Populus trichocarpa x deltoides* cv. Hoogvorst), and this bacterium also considerably improved the growth of poplar trees regardless of the presence or absence of toluene.*Enterobacter sp.* strain 638 was also shown to facilitate root development and significantly increase the biomass of inoculated poplar plants (Taghavi *et al.*, 2009). Nitrogen fixing endophytes belonging to the genera Burkholderia, Rahnella, Acinetobacter and Sphingomonas have been isolated from Poplar and Willow (*Salix sitchensis*) growing in a nutrient- poor riparian system in western Washington

state (Doty *et al*., 2005). An endophytic diazotrophic strain similar to *Burkholderia vietnamiensis* isolated from Poplar trees growing in a similar provenanance in western Washington was found to fix nitrogen, produce indole acetic acid and impart biomass and nitrogen gains to test plant, Kentucky Bluegrass, upon inoculation (Xin, G. *et al*., 2009). An endophytic, non-nodule forming strain of *Rhizobium* was also isolated from stems of Poplar growing in western Washington (Doty *et al*., 2009), indicating that Poplar species growing in this area harbor an interesting range of endophytic diazotrophic bacteria.

Apart from these studies on bacterial endophytes of deciduous trees, most other reports of endophytic bacteria and their role in tree growth promotion are from studies on various conifer tree species conducted mostly by our research group at the University of British Columbia led by Dr. Chris Chanway. Our interest in endophytic bacteria of conifers has been largely inspired by the immense commercial, social, and environmental importance of forestry in Canada and the rest of North America and the fact that conifers are the dominant trees in the temperate forests of this region (~ 85% of forests).

1.2 Endophytic Bacteria of Conifers

Conifers are members of the plant division Coniferophyta (3 Domain classification), which are characterized by naked seeds borne in specialized sporophylls or cones. Their vascular tissues differ from angiosperms in not having vessels in the xylem, and companion cells in the phloem. The division is comprised of 550 species spread over seven families, each dating back to the Mesozoic era. Distributed throughout the world with extensive latitudinal and longitudinal ranges, conifers are of great commercial and ecological value.

Traditionally, fungi, particularly mycorrhizae, were considered to be the only microorganisms that could exert a positive influence on the growth and survival of forest trees. The continuity of this trend until now is evident from the results of keyword searches for “endophytic bacteria + conifers” in all well known scientific databases.

Although some confirmed reports of conifer tree growth-promotion by naturally occurring soil and rhizosphere bacteria were available (Pokojska-Burdziej, 1982; Chanway and Holl, 1992, 1993, 1994; O’Neill *et al.,* 1992), the mechanisms employed by these bacteria for

growth promotion were not determined. It was generally believed that the primary mechanism of plant growth promotion by these bacteria was only indirect, i.e. by facilitating the establishment and growth of mycorrhizae (Fitter and Garbaye, 1994). Therefore, the focus of research on endophytic microflora of conifers remained on fungi, even after the importance of endophytic bacteria had been well established in agronomic crop species.

In an initial study of conifer root-associated bacteria, O'Neill *et al.,* (1992) isolated 22 strains from surface-sterilised roots of naturally regenerating white x Engelmann (*Picea glauca* x *P. engelmannii*) hybrid spruce seedlings. A range of effects on seedling growth in a greenhouse-screening assay using spruce were found: three strains were inhibitory, five strains were stimulatory and the remaining strains had no significant effect on seedling growth (O'Neill *et al.,* 1992). Based on the magnitude and consistency of seedling growth effects, the two best plant growth-promoting endophytes were identified and selected for further study: one isolate was *Pseudomonas putida* and the other was a *Staphylococcus spp*. While the positive effect of both of these strains on plant growth was reproducible in the greenhouse, a field trial with two ecotypes of 1-year-old spruce seedlings planted at three different reforestation sites yielded mixed results (Chanway and Holl, 1993). For example, *P. putida* enhanced seedling growth of only one of two spruce ecotypes planted at two of three reforestation sites. In addition, it had inhibitory effects in three of the spruce ecotype x planting site treatment combinations.

Evaluation of gymnosperm bacterial endophytes was only a small part of a larger project designed to characterise gymnosperm root-associated bacterial (i.e. external and internal) colonists (O'Neill *et al.,* 1992; Chanway and Holl, 1992; 1994). Therefore, our group undertook a subsequent bacterial isolation and screening program emphasizing endophytic bacteria as possible tree seedling growth-promoting agents (Chanway *et al.,* 1994, 1997). As seen in the group's earlier work (O'Neill *et al.,* 1992), several bacterial strains isolated from surface-sterilized roots of white x Engelmann hybrid spruce seedlings caused reproducible biomass increases in spruce seedlings, of up to 36%, two months after seeds were sown and inoculated in greenhouse trials (Chanway *et al.,* 1994). Three of these strains belonged to *Paenibacillus spp*. (isolates N3, N4, N5), three were actinomycetes, most likely *Streptomyces*

spp. (isolates A1, N1 and W2), and one was a *Phyllobacterium* (isolate W3). An additional strain that performed well in greenhouse assays could not be identified with certainty.

In addition, the seedling growth promotion efficacy of some of these strains was altered significantly when assays were conducted in the presence of a small amount (2% v/v) of forest soil known to contain seedling growth-inhibiting organisms (i.e. minor pathogens). One of the endophytic actinomycetes (isolate W2) as well as the *Phyllobacterium* isolate (W3) clearly stimulated spruce seedling growth only in the absence of forest soil. In its presence, seedling growth was inhibited, as it was when forest soil alone was used. These results suggested that growth promotion by W2 and W3 occurred via a mechanism unrelated to biocontrol of minor pathogens, and may have involved one of the direct plant growth promotion mechanisms, possibly production of plant growth regulators (Kloepper, 1993; Glick, 1995; Chanway, 1997). Interestingly, actinomycete isolate N1 and *Bacillus* isolate N4 stimulated seedling growth only in the presence of forest soil, which suggested that these strains acted through a biocontrol mechanism, either by direct antagonism or by inducing systemic resistance in the host plant. Elucidation of these possibilities requires further experimentation.

Shishido *et al.,* (1995) isolated several endophytic bacterial strains from lodgepole pine that were screened for effects on seedling growth. Plant-growth-promoting *Bacillus polymyxa* (now *Paenibacillus*, Ash *et al.,* 1993) strain (Pw2) that originated from internal root tissues of a naturally regenerating 2- to 3-year-old pine seedling was found to be most effective. Studies indicate that Pw2 can colonise external and internal pine and spruce root tissues after seed or root inoculation. Colonization of internal root tissues may depend on lateral root development, and results in endophytic bacterial population sizes approaching 10^6 cfu g^{-1} fresh root tissue (Shishido *et al.,* 1995; Chanway, 1997; Shishido, 1997). In addition, using a surface-sterilization, dilution-plating assay, as well as immunofluorescence microscopy, a rifamycin-resistant derivative of this strain, Pw2-R, was shown to be capable of colonizing internal pine and white x Engelmann hybrid spruce stem tissues after soil or root inoculation (Chanway *et al.,* 2000). Five months after root inoculation, internal stem bacterial populations reached 10^5 cfu g^{-1} fresh stem tissue (Shishido, 1997).

In order to examine the effects of endophytes on conifer plant growth and to investigate the host specificity of bacterial endophytes, in terms of the ability to promote growth of inoculated host plants other than the ones from which they were initially isolated, initial field trials with *P. polymyxa* strain Pw2-R and *Pseudomonas chlororaphis* strain Sm3-RN, another bacterial endophyte capable of stimulating white x Engelmann hybrid seedling growth in the greenhouse, were also performed (Chanway *et al.,* 1997). Two years after bacterial inoculation and planting at nine sites, representing a range of forest regions in which spruce naturally occurs in British Columbia and Alberta, Canada, white x Engelmann hybrid spruce treated with strain Pw2-R (initially isolated from pine) showed mean biomass increases up to 33% above controls at seven of the nine sites, but increases were significant at only one site. In contrast, *Pseudomonas* strain Sm3-RN (isolated from white x Engelmann hybrid spruce) caused significant white x Engelmann hybrid spruce biomass increases of up to 57% at three of the nine sites but a significant decrease in spruce biomass at one site. Site productivity was not correlated with plant growth promotion or inhibition.

Contrarily, when seedlings were inoculated with strains Pw2-R and Sm3-RN and grown in the greenhouse for 4 months before planting at four of the reforestation sites described above (Shishido and Chanway, 2000), mean internal root populations reached ca. 10^3–10^4 cfu g^{-1} tissue indicating that pre-inoculation followed by a period of growth in the greenhouse prior to outplanting in the field facilitated internal tissue colonization by these microorganisms. As expected, mean seedling biomass also increased due to bacterial inoculation in the greenhouse. Because seedling growth responses in the field would be inseparable from those that occurred in the greenhouse, simple measurement of biomass accumulation after a period of growth in the field would yield spurious results. therefore plant growth was evaluated using relative growth rates (RGRs), in which plant growth increments over time are expressed as a proportion of the biomass that existed at some previous time in the plant's life (Hunt, 1982). In general, after the first growing season, RGRs of seedlings containing endophytic bacteria were greater than those of control seedlings at all four planting sites (Shishido and Chanway, 2000). In some cases, RGRs of inoculated plants were double the control value. This was particularly interesting in view of results with seedlings that were inoculated and planted immediately at the same

sites. At two of the four sites, seedlings inoculated at the time of planting (i.e., with no greenhouse growth period) did not respond to bacterial treatment, and in one case responded negatively. However, shoot and root RGRs of seedlings pretreated in the greenhouse before planting at the same sites were 23–132% greater than controls, and endophytic populations in root tissues of between 10^2 and 4×10^4 cfu g^{-1} plant tissue were detected in seedlings at three of the four sites.

Similar effects on establishment and functioning of bacterial endophytes were observed by Brooks *et al.,* (1994) in wilt-infested oak trees. These results suggest that a period of growth under a controlled environment to facilitate establishment of endophytic bacterial populations may be an important step in successful application of plant-growth-promoting bacterial endophytes in forestry. It has also been demonstrated that the benefits of pre-outplanting inoculation of seedlings with bacterial endophytes can be maximised by careful matching of the inoculant bacterial strain with outplanting sites to synchronize seedling phenology with regional day length and climate (Chanway *et al.,* 2000). However, much research into site quality and plant growth responses will be required before reliable recommendations can be made. In addition, much more research is warranted to answer the many questions regarding the entry and operation of endophytic bacteria in conifers.

1.3 Modes and Sites of Entry

Endophytic bacteria have been shown to be able to gain entry in plants through wounded, as well as intact, tissues (Sprent and James, 1995; Bloemberg *et al.,* 2006). Izumi *et al.*, (2008), found discrepancies between bacterial genera prevalent in the rhizosphere soil versus those abundant inside the root tissues, leading them to suggest that root endophytic bacteria may be in residence through processes of selection or active colonization, rather than by passive diffusion from the rhizosphere. In an attempt to understand the modes and sites of entry of endophytic bacteria, Timmusk and Wagner (1999), followed the colonization of a green fluorescent protein *gfp*-tagged endophytic strain of *Paenibacillus polymyxa* in *Arabidopsis thaliana*. They observed a slight degradation of the root tips within five hours of inoculation and they found that *P. polymyxa* had two preferred zones of infection. The first is located at the

root tip in the zone of elongation, which sometimes results in the loss of the root cap. The other colonization region was observed in the differentiation zone. Similar colonization zones have been reported for other endophytes, e.g. *Azoarcus* by Hurek *et al.,* (1994), who suggested that plant cells were destroyed after bacteria had penetrated cell walls. Perhaps this is the reason why most endophytic bacteria are limited to the intercellular spaces inside tissues. However, it is not clear how endophytic bacteria are stopped from entering cells and causing necrosis. To determine which microbial characteristic(s) facilitate entrance of bacterial endophytes into plant tissues, Shishido *et al.,* (1995), compared the biochemical capabilities of the endophytic *Paenibacillus polymyxa* strain Pw2 with those of another plant-growth promoting, non-endophytic strain, *P. polymyxa* L6-16R. Interestingly, strain L6-16R is unable to enter plant tissues even when co-inoculated with an endophytic microorganism (Bent and Chanway, 1998).

According to the Biolog physiological profiling system, based on carbon source utilization, both strains possessed similar metabolic capabilities with some potentially important exceptions (Shishido *et al.,* 1995). For example, strain Pw2-R was able to metabolise sorbitol, but strain L6-16R was not. Mavingui *et al.,* (1992), found that, in general, *P. polymyxa* strains isolated from the rhizoplane of wheat (*Triticum aestivum* L.) were capable of metabolising sorbitol, whilst rhizosphere and non-rhizosphere isolates were not. They hypothesized that intense competition for oxygen would occur on the root surface due to root respiration, which would result in selection pressure for bacteria capable of anaerobic growth on highly reduced, scarce substrates, such as sorbitol. In addition, strain Pw2 was able to metabolise D-melezitose, a sugar that has been detected in the sap of conifers (Wherry, 1920). However, the occurrence of sorbitol and d-melezitose in lodgepole pine root tissues and their utilisation by other *Paenibacillus* root endophytes must be demonstrated before a role for these substrates in internal root colonization by *Paenibacillus* can be postulated with greater confidence.

To facilitate root colonization, it is logical to suspect that root endophytic bacteria may also possess the ability to metabolise structural components of plant cells. In particular, the ability to metabolize pectin (polygalacturonic acid), a major component of the middle lamellae

of plant cell walls, has been proposed to, at least partly, explain why bacterial root endophytes are often found in the root cortex intercellularly (Balandreau and Knowles, 1978; Baldani and Döbereiner, 1980). Both strains L6 and Pw2 possessed pectolytic activity *in-vitro*, but only strain Pw2 was able to metabolise D-galacturonic acid (Shishido *et al.,* 1995), the primary monomeric component of pectin (Scheller *et al*., 1999). It is not clear whether the capability of strain Pw2 to metabolise monomeric galacturonic acid, after breakdown of the pectin polymer, was related to its ability to enter root tissues. However, breakdown products of plant cell walls are known to induce systemic disease responses in plants (Brock *et al.,* 1994), which leads to the possibility that Pw2 avoids plant defense mechanisms by metabolizing cell wall components before they elicit a defense response by the host plant. This possibility also requires further investigation. If, in fact, the entry of Pw2 in plant roots is facilitated by its capability to metabolize the primary components of the cell wall, the question as to why it does not cause necrosis of interior tissues, remains.

1.4 Mechanisms of Plant Growth Promotion

Unlike symbiotic rhizobia, mechanisms of plant growth promotion by plant growth-promoting rhizobacteria (PGPR) vary greatly, and have been broadly categorised into two groups, direct and indirect (Kloepper *et al.,* 1989; Kloepper and Ryu, 2006). Direct plant growth promoting mechanisms may involve nitrogen fixation (Cavalcante and Döbereiner, 1988), production of plant growth regulators and antibiotics, or increased availability of plant growth-limiting nutrients. Indirect mechanisms may involve suppression of deleterious microorganisms as well as enhancement of mutualisms between host plants and other symbionts such as mycorrhizae (Kloepper *et al.,* 1989). Similar to other aspects of studies on endophytic bacteria, there is a great deal of information on the mechanisms of plant growth promotion employed by these bacteria in agronomic crops (Lodewyckx *et al.,* 2002). In the case of conifers, it was generally believed that these plants could derive benefits from bacteria only indirectly through their mycorrhizal symbionts (Fitter and Garbaye, 1994). However, growth studies on lodgepole pine seedlings (Chanway and Holl, 1991; Shishido *et al.,* 1996) and hybrid spruce (*Picea glauca* x *P. engelmannii*) (Shishido *et al.,* 1996) co-inoculated with PGPR and mycorrhizal fungi have

clearly shown that growth promotion of these conifers by PGPR is independent of the mycorrhizal status of the seedlings.

Despite many efforts, determination of the exact mechanisms of conifer growth promotion by PGPR has not been possible. *Paenibacillus polymyxa* strain L6-16R was shown to produce cytokinins (Holl *et al.,* 1988), and this property was advanced as a likely explanation of pine growth promotion mediated by this strain.

A detailed study was also conducted to determine the mechanisms of growth promotion of spruce by six *Paenibacillus* and *Pseudomonas* strains, including the endophyte, *B. polymyxa* Pw2 (Shishido, 1997). It could only be concluded that more than one mechanism was responsible for growth promotion by these bacteria. Production of plant growth hormones and enhancement of nutrient uptake were designated as the most likely of these mechanisms.

Paenibacillus polymyxa strain Pw2 isolated from lodgepole pine possessed diazotrophic properties (Shishido, 1997). This led to the intriguing possibility that lodgepole pine harbours a systemically-endophytic nitrogen-fixing bacterial population, similar to that found in sugar cane (Boddey *et al.,* 1995). This would explain the ability of lodgepole pine to grow, and even thrive in arid, nutrient poor, shallow and gravelly soils (Lotan and Critchfield, 1990, Barbour and Minnich, 2000**)** and under nitrogen-deficient conditions, in the absence of significant rhizospheric nitrogen fixation (Binkley, 1995). Indeed, the $^{15}N/^{14}N$ ratio of pine foliage in a central coastal forest in British Columbia, was observed to be low enough to suggest that biological nitrogen fixation supplies plant N (F.B. Holl, personal communication).

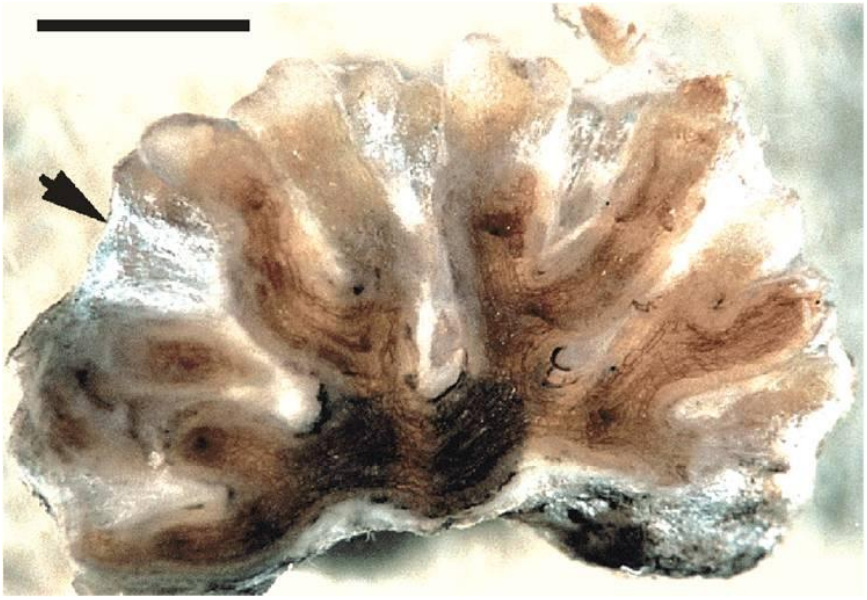
However, nitrogen fixation could not be shown to be the primary mechanism of growth promotion by *P. polymyxa* strain Pw2-R, since seedlings inoculated with this strain failed to support detectable rhizosphere acetylene reduction activity (ARA) even after 48 h of incubation with acetylene (Shishido, 1997). Interestingly, similar limitations were encountered by Rhodes-Roberts (1981) and Achouak *et al.,* (1999) while working with other strains of *P. polymyxa*. However, they were able to measure the nitrogen gains of seedlings by micro-kjeldahl analysis, which led them to suggest that the acetylene reduction assay is not always able to provide positive results for the nitrogen-fixing ability of *P. polymyxa*. Therefore, conclusions on the occurrence of N_2 fixation *in- vivo* should be drawn from a number of lines of

evidence, including a positive nitrogenase activity test (acetylene reduction assay), ^{15}N dilution and detection of conserved *nif* genes in the purported diazotrophic endophyte.

Nitrogen-fixing bacteria have also been observed inside what can only be described as a unique and enigmatic type of mycorrhizae on lodgepole pine (Paul, 2002), first described by Zak (1971), on Douglas-fir (*Pseudotsuga menziesii*) roots. These mycorrhizal structures, often referred to as tuberculate mycorrhizae, look more like leguminous root nodules than mycorrhizae (Fig. 1.1). They are fully enclosed subterranean "nodules" or tubercles attached to the tree root system, with hundreds more typical mycorrhizal root tips crowded inside the outer covering, or peridium (Fig. 1.2). Nitrogen-fixing bacteria have been previously detected on the peridium (Li *et al.,* 1992), but more recently, in our laboratory, a limited number of strains representing three diazotrophic bacterial species *Paenibacillus pabuli*, *Paenibacillus amylolyticus* and *Methylobacterium mesophilicum* have been detected inside the peridium of *Suillus tomentosus*, colonising the fungal hyphae within the tubercle (Paul *et al.*, 2006, 2007). It has yet to be demonstrated that these endophytic diazotrophs fix N_2 *in situ*, let alone transfer it to the host plant, but these intriguing possibilities remain to be evaluated.

Figure 1.1 External morphology of tuberculate ectomycorrhizae on *Pinus contorta* roots. Bar 5mm

Figure 1.2 Cross section through a mature tubercle from *P. contorta* revealing mycorrhizal root tips (brown) and interstitial hyphae (arrow). Note pinnate radiated fan form and dichotomous branching of root tips within the tubercle. Bar 2mm



1.5 Biological Nitrogen Fixation

Nitrogen is abundant in the atmosphere, lithosphere and hydrosphere of the earth. However, 99% of nitrogen is in the form of dinitrogen which is inert and cannot be used by most living organisms. In order for living cells to use it for synthesis of vital bio-organic molecules like proteins, nucleic acids and vitamins, molecular nitrogen (dinitrogen) has to be in its reduced or fixed form. Dinitrogen fixation, the process by which dinitrogen is reduced to ammonia, is therefore a very important processes for the sustenance of life.

Three processes are responsible for most of the dinitrogen fixation in the biosphere. Atmospheric fixation by lightning contributes approximately 5-8% ($5x10^9$ Kg N/yr) of total fixed nitrogen (Myrold and Bottomley, 2007). The enormous energy contained in lightning breaks dinitrogen molecules and enables their atoms to combine with oxygen in the air forming nitrogen oxides that dissolve in rain. These oxides of nitrogen then form nitrates that are carried to the earth in rainfall. Industrial nitrogen fixation occurs through a process called the Haber-Bosch industrial process that was established in 1913. This process uses a catalytic agent (iron with a small amount of aluminium added) at high pressure (as much as 5.06×10^7 Pa) and high temperature (600−800 K) and consumes large amounts of fossil fuel. Ammonia produced

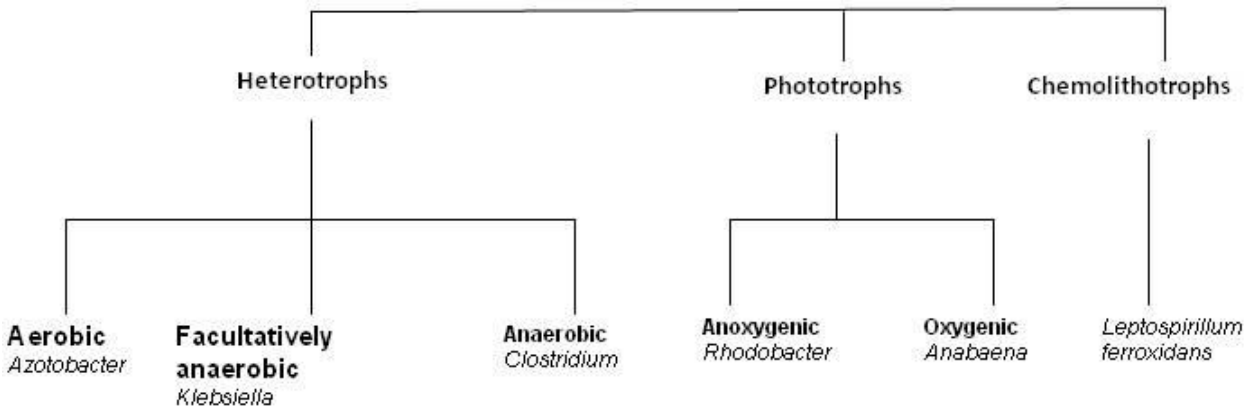
through this highly expensive process is combined with other elements to produce nitrogenous fertilizers like urea and ammonium nitrate. Although the use of these fertilizers is inevitable in meeting rising food demand to sustain the growing global population, their indiscriminate use has set off very negative effects on water resources and the environment. Approximately 1.1×10^{11} kg N (Myrold and Bottomley, 2007) are manufactured by ammonia industry annually. Increasing fossil fuel combustion and burning of forests and grasslands contributes approximately 2.5×10^{10} of anthropogenic reactive nitrogen (Nr)(Galloway and Cowling, 2002) that is proving menacing to the environment. Biological nitrogen fixation**,** a natural process by which certain prokaryotic micro-organisms fix nitrogen by a highly specialized enzyme complex called nitrogenase, is an environmentally benign source of plant usable fixed nitrogen. According to estimates, approximately 1×10^{11}-1.4×10^{11} kg N (Myrold and Bottomley, 2007) are fixed from the atmosphere by biological nitrogen fixation every year. While appreciating the contributions of all sources of fixed nitrogen, this chapter focuses on the process and applications of biological nitrogen fixation.

Farmers since ancient Chinese and Roman civilizations practiced crop rotation with legumes to increase soil fertility and agricultural productivity. However, the science behind such practice was revealed by Boussingault who experimented with leguminous crops fixing N_2 in 1838. In 1886, Hellriegel and Wilfarth showed definitive evidence for N_2 fixation by microbes in legumes.

1.5.1 Nitrogen Fixing Organisms

The ability to fix nitrogen is limited to bacteria and archaea only. Within these groups it is quite widely distributed revealing considerable phylogenetic diversity among diazotrophic organisms. Young (1992), has prepared a comprehensive listing of nitrogen fixing bacteria and archaea, under 12 broad phylogenetic groups based on 16S rDNA phylogeny including green sulphur bacteria (*Chlorobium*), Firmicutes (*Paenibacillus, Clostridium*), Thallobacteria (*Frankia*), cyanobacteria (*Anabaena, Nostoc*) and all subdivisions of the proteo bacteria (α: *Rhizobium*, *Acetobacter*; β: *Azoarcus*, *Herbaspirillum*; γ: *Klebsiella*; δ: *Desulfovibrio*) and Archaea (mostly methanotrophs).Dinitrogen fixing organisms also exhibit quite diverse physiologies (Fig 1.3).

Figure 1.3 Diversity of diazotrophs based on physiology



Diazotrophs are also widely distributed ecologically. They are found free living in soils and water (*Klebsiella*, Cyanobacteria), in root nodule symbiosis with legumes (*Rhizobium*), associative symbiosis with grasses (*Azoarcus, Gluconoacetobacter*), actinorrhizal associations with woody plants (*Frankia*), cyanobacterial symbiosis with various plants and symbiotic associations in termite guts.

1.5.2 Chemistry of Nitrogen Fixation

The overall chemical reaction of biological dinitrogen fixation by nitrogenase is represented by the equation:

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

Due to the stark disparity of energy and heat requirements between chemical and biological N fixation, the structure and functioning of nitrogenase has always been of immense interest to biochemists (Dance, 2007).

Nitrogenase is a complex enzyme comprised of two metalloproteins, the Mo-Fe protein, also called dinitrogenase or component I and the Fe protein called dinitrogenase reductase or component II.

The dinitrogenase responsible for the actual reduction of dinitrogenase to ammonia, is a heterotetramer composed of 2 alpha and 2 beta subunits with an overall molecular weight of

240 kilo Daltons. The MoFe-protein contains two types of metal centers, the FeMo-cofactor and the P-cluster pair, of which the FeMo- cofactor is the active site where dinitrogen binds whereas the P-cluster mediates electron transfer between the Fe protein and the FeMo-cofacter.

The dinitrogenase reductase or Fe protein is a homodimer of two identical subunits, with an overall molecular mass of ~60kDa. It contains two ATP/ADP molecules and one 4Fe-4S (Kim and Rees, 1994).

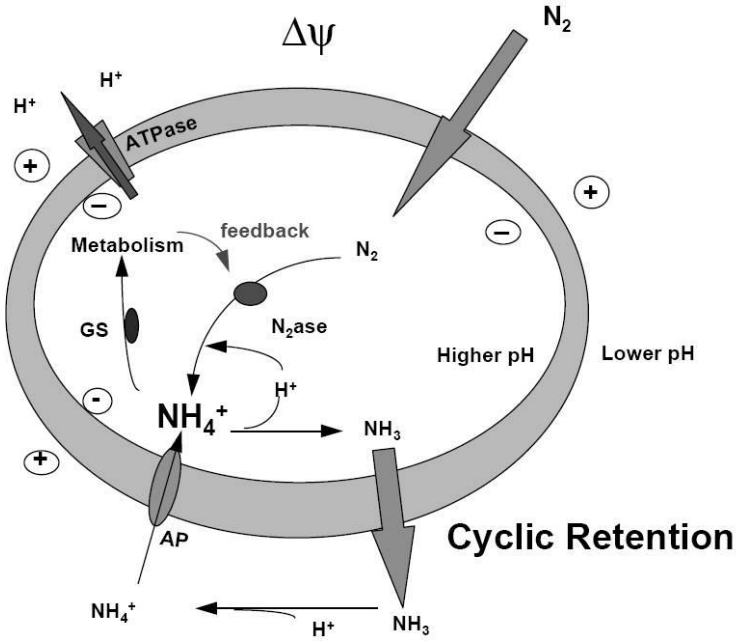
Under conditions of molybdenum depletion, alternative nitrogenase systems may be induced. Molecular genetic studies have shown that Mo independent nitrogenases are quite widely distributed among diazotrophs and vanadium (V)-nitrogenases of *A*. *chroococcum* and *A*. *vinelandii* and the Fe-nitrogenases of *A*.*vinelandii* and *Rhodobacter capsulatus* are the best known of these (Eady, 1996). *Streptomyces thermoautotrophicus* has been recently found to be able to fix dinitrogen, but it harbors a very unusual N_2-fixing system that requires three proteins for nitrogen fixation, a heterotrimeric molybdenum-containing dinitrogenase (St1), a homodimeric manganese-containing superoxide oxidoreductase (St2) and another heterotrimeric molybdenum containing carbon monoxide dehydrogenase (St3 or CODH). These proteins differ entirely from the known nitrogenase protein components and are insensitive to O_2. Compared to conventional or alternative nitrogenases, the St nitrogenase also requires less ATP (Qi, 2008).

The overall functioning of nitrogenase can be summarized as a key biochemical cycle also called Fe protein cycle (Dance, 2007) which involves 5 steps (Dance *et al*., 2007, Kim and Rees,1994) : **a)** the reduction of Fe protein by electron carriers such as flavodoxin or ferredoxin, **b)** Association of the reduced Fe protein (including two MgATP complexes) with the Mo-Fe protein in preparation of electron transfer, **c)** hydrolysis of MgATP, which enables transfer of one electron to the Mo-Fe protein (via Fe_4S_4 and the P cluster), **d)** electron transfer to dinitrogen and thus its reduction, while it is bound to the active site within the Mo-Fe protein and **e)** dissociation of the two protein molecules, exchange of ATP back into the Fe protein, and re-reduction of the Fe protein. Dance (2008), has reviewed chemical catalysis of nitrogenase in

detail, addressing questions such as how N_2 binds the FeMoCo, how exactly electrons travel through the system and finally how NH_3 leaves the complex.

For plants to benefit from biologically fixed nitrogen it is important that the fixed forms of nitrogen (ammonia/ammonium) be released out of the diazotrophic bacterial cells into the soil or surrounding plant tissues. According to the cyclic retention hypothesis of ammonium retention (Kleiner, 1985), bacteria actively retain fixed nitrogen for assimilation by an effective transport mechanism mediated by enzymes like ammonia permease (AP) (Fig 1.4).

Figure 1.4 Model for the active cyclic retention hypothesis of ammonium retention. Fixed nitrogen in ammonia form is released from bacterial cells by passive diffusion but absorbed back immediately after protonation to ammonium ion, with the help of ammonia permease (AP) with the use of ATP and assimilated by the bacterial cell using glutamate synthetase (GS). Mutation in AP or GS causes release of fixed nitrogen for use by plants.



(This figure has been reproduced with the kind permission of Springer science + Business media and Dr Ivan R. Kennedy <Plant and Soil, Biological nitrogen fixation in non-leguminous field crops: Facilitating the evolution of an effective association between *Azospirillum* and wheat. **194:** 65–79, 1997.Ivan R. Kennedy, Lily L. Pereg-Gerk, Craig Wood, Rosalind Deaker, Kate Gilchrist and Sunietha Katupitiya. Fig 3>)

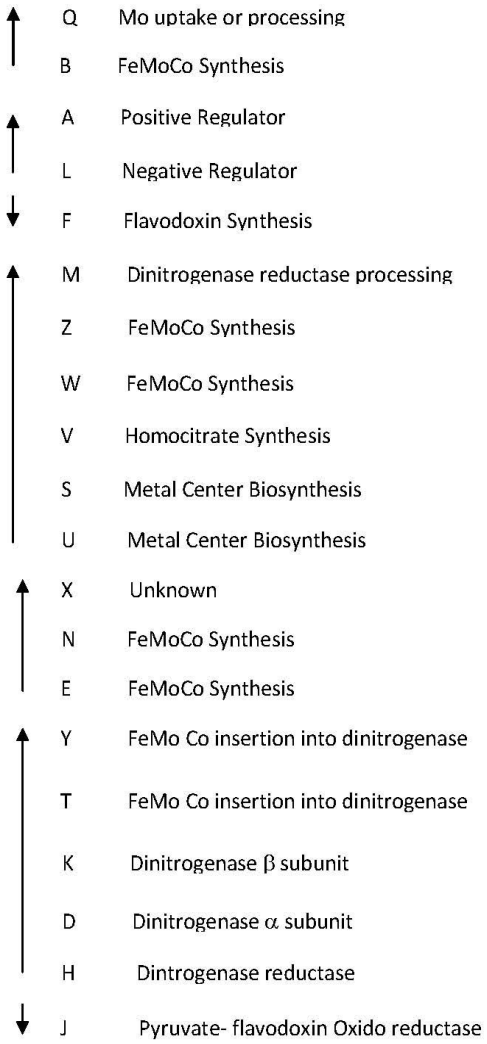
In order for ammonia to be released out of the bacterial cells mutations in ammonia permease, or the ammonium assimilation enzymes such as glutamine synthetase (GS) and/or glutamine synthase are required. Such mutations cause a build up of high concentrations of ammonia within the bacterial cells resulting in ammonia leakage out of the cells. It is possible

that most effective diazotrophic PGPR have such mutations in order for them to benefit their host plants (Colnaghi *et al*., 1997).

1.5.3 Genetics of Nitrogen Fixation

The structure and function of nitrogenase is coded by close to 20 genes (*nif* genes) organized in 7 operons (*nif* cluster) spanning over 24 kb. These genes fall into three categories: structural, regulatory and supplementary and can be housed either in genomic DNA or on plasmids (i.e. sym plasmids in *Sinorhizobium meliloti*). The *nif* cluster of the free living bacterium *Klebsiella pneumoniae* is the most studied of *nif* genes and serves as a model for understanding the regulation, synthesis and assembly of nitrogenase. The function and arrangement of these genes is described in Fig 1.5.

Figure 1.5 Arrangement and functions of various *nif* genes in *Klebsiella pneumonia*. Arrows represent the 7 operons in which *nif* genes are organized and the direction of translation (Modified from Madigan, 2000).



Genes governing alternative and vanadium (V) dependent nitrogenases are called *anf* and *vnf* genes respectively. These genes share extensive homology with *nif* genes with some differences in sequence and arrangement. Eady (1996), has discussed detailed structure, function and genetics of these alternative nitrogenases.

Other plant and bacterial genes involved in establishing plant–diazotroph interactions such as symbiosis are also known. Of these the legume genes called *nod* genes that are required for the early steps in nodule formation (Debelle, 2002). Other genes that are essential for nitrogen fixation by Rhizobia but have no homologue in *K. pneumoniae* are known as '*fix*'

genes. The most commonly known of these are *fix* ABCX which are believed to be involved in electron transport, *fix* NOPQ, which are involved in bacteroid respiration under conditions of low oxygen present in nodules, and *fix* GHIS that are involved in redox (Fischer, 1994).

1.5.4 Methods of Studying Biological Nitrogen Fixation

1.5.4.1 Isolation of Diazotrophs from Plants and Soil

Nitrogen free media with multiple carbon sources are central to the isolation of most culturable diazotrophs. These media used in semi solid state are ideal for simulating the microaerophillic conditions ideal for nitrogen fixation. Examples of some such media are yeast extract mannitol (YEM) medium, Vincent's minimal medium (Vincent, 1970), combined carbon medium (Rennie, 1981). The use of intact pieces (0.5-1.0 cm) of roots and other plant parts has been suggested for isolating diazotrophs adhering to the surface of tissues (Bashan, 1993). For isolation of all diazotrophs from a plant, parts are washed and macerated and serially diluted before incubation on semi solid, nitrogen free medium. Endophytic bacteria are isolated by surface sterilizing tissues before grinding and incubating on medium (Cavalcante and Döbereiner, 1988). Similarly, serial dilutions of rhizospheric and non-rhizospheric soils are used to isolate culturable diazotrophs from soil (Seldin *et al*., 1983).

Complete communities of culturable as well as unculturable diazotrophs associated with soils and plants can also be profiled using various molecular techniques that utilize the polymorphism in the *nif* H gene, which is believed to be quite conserved across diazotrophs (Zehr, 2003), to determine the diversity in the *nif* H gene pool in the target environmental sample. This is done by amplifying the *nifH* gene by the Polymerase chain reaction (PCR) using environmental DNA as a template. The PCR product which is a pool of almost similar amplicons of various *nif*H genes in the sample is subjected to subsequent analyses by cloning and sequencing, restriction fragment length polymorphism (RFLP) (Poly *et al*., 2001), terminal-RFLP (T-RFLP; Deslippe *et al*., 2005), or by denaturing gradient gel electrophoresis (DGGE; Rosado *et al*., 1998). These techniques are limited to detecting the presence of diazotrophs in the community but do not provide any information on the frequency of a particular species. Macro- and microarrays may reveal both, the presence and frequency of different N_2-fixing prokaryotes (Steward *et al*., 2004; Moisander *et al*., 2006).

1.5.4.2 Localization of Endophytic Diazotrophs *in planta*

Endophytic diazotrophs are found in roots, stems, leaves, seeds, fruits, tubers, ovules (Hallmann *et al*., 1997; Verma *et al*., 2001, Muthukumarasamy *et al*., 2002), inside legume nodules (Benhizia *et al*., 2004; Sturz *et al*., 1997) and also inside tubercles of mycorrhizal fungi (Paul, 2002). Localization of these endophytic bacteria within plant tissues requires techniques that facilitate observation of bacteria on a very small spatial scale. Various methods have been used to locate bacteria *in planta* and visualize them at their sites of colonization. Viable staining of vital bacterial cells combined with light microscopy has been used to to detect bacteria in surface-disinfested corn roots (Patriquin and Dobereiner, 1978). This method is based on the ability of bacteria to convert 2, 3, 5-triphenyltetrazolium dichloride to red-colored fromazans (Kuhn and Jerchel, 1941). Another dye, acridine orange in conjunction with epifluorescence microscopy has been used to detect bacteria within xylem tissue of grapevine (*Vitis vinifera* L.) (Bell *et al*., 1995). However, besides being messy and tedious, this technique can lead to false-positive detection of bacteria because reduction of tetrazolium dyes also respond to all chemical compounds that are oxidized in plant tissues through respiratory electron transport (Zimmermann *et al*.,1978), therefore plant particles such as starch grains , that are similar to the bacteria in size, might also get stained (Patriquin and Dobereiner, 1978).Similarly, acridine orange also stains monopolysaccharide particles (Culling, 1974) which makes it very difficult to distinguish bacteria from cell organelles.

Endophytic bacteria can also be detected and identified within plant tissue by *in-situ* hybridization, a technique that detects specific bacterial DNA or RNA sequences in plant tissue. Nucleic acid probes are labeled by the attachment of a hapten (i.e., biotin or digoxygenin), which is then recognized by an antibody coupled to a visual marker, i.e. colloidal gold (McFadden, 1991). Specific RNA or DNA probes labelled with fluorochromes such as FITC (Fluorescein isothiocynate), Cyanine 3 (Cy3) and Cyanine 5 (Cy5) used in conjunction with fluorescent or confocal microscopy for fluorescent *in-situ* hybridization (FISH) have been used to detect endophytic bacteria in plant tissues such as wheat roots (Rothballer *et al*., 2003) and grape leaves (LoPicollo *et al*., 2010). The use of fluorescent probes eliminates the multiple staining and incubation steps required for *in-situ* hybridization. However, the technique is still

tedious due to the complexity involved in preparation of specific probes and hybridization *in-situ*.

Immunological techniques have been used extensively to detect plant-associated microorganisms. Antibodies can be raised against epitopic proteinous components of bacteria such as fimbriae (Korhonen *et al*., 1986), specific enzymes like nitrogenase (Sasakawa *et al*., 1988; Dalton *et al*., 2004; Chelius and Triplett, 2000) or whole bacterial cells by injecting them into rabbits or mice. For visualization, the antibodies themselves or secondary antibodies raised against primary antibodies are coupled with fluorochrome such as fluorescein isothiocyanate (FITC) (Mahaffee *et al*., 1994; Schank *et al*., 1979; Allan and Kelman, 1977), or colloidal gold (Hurek *et al*., 1994; Levanony *et al*., 1989; Quadt-Hallmann and Kloepper, 1996). Problems with immunological technique occur from low antibody specificity and cross reactivity with undesirable epitopes (Quadt-Hallmann and Kloepper, 1996; Hurek *et al*., 1994). The preparation of monoclonal antibodies that are more specific and less cross reactive than polyclonal antibodies is expensive as well as time consuming. In addition, careful sectioning of plant tissue, followed by staining with the primary and secondary antibodies is required prior to microscopic visualization of bacterial cells *in planta* (James and Olivares, 1998).

Molecular techniques are also sensitive tools to detect endophytic bacteria in plant tissues, particularly unculturable bacteria. Polymerase chain reaction (PCR), using primers specific to the 16S rRNA gene has been used to confirm the presence of bacteria in the root and shoot tissues of rice inoculated with *Azoarcus sp*. Hurek *et al*., (1994) and seeds of *Eucalyptus urophylla* (Shen *et al*., 2010). RT-PCR (Reverse transcriptase- PCR) has been used to determine the expression of the dinitrogenase reductase (nifH) gene of Azoarcus sp strain BH72 colonizing rice tissues in an unculturable state (Hurek *et al*., 2002).However, both PCR and RT-PCR are easily inhibited by plant compounds, thus making these techniques highly prone to false negative results.

Chromogenic marker genes coding for metabolic enzymes like β-galactosidase (*lac*Z), β-glucuronidase (*gus*A) have also been used to localize diazotrophic bacteria *in-situ* (Katupitiya *et al*., 1995; Gyaneshwar *et al*., 2001). Both, *gus*A and *lac*Z produce blue color in the presence of appropriate and adequate substrates, which are 5-bromo-4-chloro-3-indolyl-β-D-

galactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside respectively. Expression of *nif* genes of *Azospirillum brasilense* in wheat roots has been shown by fusions of the *lac*Z gene with *nif*H, A and B genes (Arsène *et al*., 1994). However, the use of these chromogenic marker genes is limited by the short- term stability of their enzymatic products and the requirement of substrates or reactives to be expressed or detected (Jansson, 1998).

Electron microscopy has also been used extensively to localize endophytic bacteria in plant tissue. Scanning electron microscopy (SEM) has been successfully used to detect endophytic bacteria in various plants (Turner *et al*., 1993; Gantar *et al*., 1991, Van Doorn *et al*., 1991; Sardi *et al*., 1992). However, during the preparation of internal plant tissues for SEM, the samples need to be sectioned, and bacterial contaminants from tissue surfaces might be introduced and misidentified as endophytes (Hallmann *et al.*, 1997). Embedding of tissue in epoxy resin prior to sectioning for transmission electron microscopy (TEM) reduces these concerns, but the resin itself has been shown to cause leakage of endophytic bacteria from the apoplastic fluid of the cut surface (Dong *et al*., 1994).

All the methods described above require either chemical or physical treatment of plant tissues for detection and visualization of endophytic bacteria *in-situ.* The use of autofluorescent proteins in conjunction with confocal laser scanning microscopy (CLSM) eliminates the need for any chemical treatment of plant tissues and requires minimal physical preparation of plant tissue samples before microscopic visualization. The green fluorescent protein (*gfp*) gene found in the jellyfish *Aequorea aequorea* is the most popular autofluorescent protein system used for localization of endophytic bacteria. GFP is a useful AFP biomarker because it does not require any substrate or cofactor in order to fluoresce. GFP cassettes can be integrated into the bacterial chromosome and expressed through an inducible or constitutive promoter of indigenous or exogenous origin (Tombolini *et al*., 1997; Tombolini & Jansson, 1998; Xi *et al*., 1999; Compant *et al*., 2005). Alternatively, a plasmid borne gfp gene can be introduced into bacterial cells of interest (Timmusk *et al*., 2005; Chelius and Triplett; 2000). Bacterial cells expressing *gfp* can be visualized by epifluorescence microscopy or confocal laser scanning microscopy (CLSM) (Villacieros *et al*., 2003; Germaine *et al*., 2004).

CLSM is a powerful tool for high-resolution visualization of microbial cells labelled by fluorescent markers (Gamalero *et al*., 2009). CLSM has a distinct advantage over electron microscopy because it enables optical sectioning of the sample, thus eliminating the need for ultra thin sectioning and tedious, chemically invasive fixation and embedding procedures. Images of thin optical sections can also be composited to produce a 3- dimensional (3-D) image of the plant tissue sample in which endophytic bacteria are lodged. Another major advantage of CLSM is that the confocal imaging system allows the detection of signals only from the focused plane, limiting background fluorescence arising from materials such as plant tissue, soil particles or organic debris. CLSM also permits the simultaneous detection of different bacterial populations by using different fluorescence channels. The images generated by CLSM are easily subject to digital analysis and processing. Due to these advantages CLSM has become a method of choice to localize endophytic microorganisms tagged with fluorescent markers (Assmus *et al*., 1995; Bloemberg *et al*., 2000; Verma *et al*., 2004). The only limitation to the use of CLSM is the high cost of the instrument.

1.5.4.3 Quantification

The most commonly used methods for measurement of nitrogen fixation are, nitrogen balance (also called the nitrogen accretion or nitrogen difference method), the acetylene reduction assay, xylem solute analysis (ureide production) and stable isotope methods (^{15}N isotope dilution, natural abundance and incorporation). The major considerations for selection are sensitivity, duration, sample type, and whether relative or absolute rates are required (Myrold and Tiedje, 1986). A more detailed review with examples of application has been done by Danso (1995). The nitrogen balance method is the oldest and simplest method that measures biological nitrogen fixation (BNF) as the difference between the total N contents of plants that fix nitrogen versus those that do not. It is highly dependent and thus disadvantaged by its underlying assumption that both nitrogen fixing and non-fixing control plants absorb equal amounts of N from soil. This assumption is hard to justify due to differences in root morphology and other physiological attributes.

The xylem solute method of measuring BNF is based on the determination of the composition of nitrogen compounds in plant tissues or the N flowing through the xylem sap to the shoot. The underlying idea is to differentiate between nitrogen absorbed from soil, which is predominantly nitrate and fixed nitrogen occurring in the form of N solutes, primarily amides and ureides (McClure and Israel, 1979). In the presence of increasing levels of soil nitrate plants get less dependent on nitrogen fixation and the ureide content of xylem solute decreases. Although, the method has sometimes been used to even make quantitative measurements of nitrogen fixation (Herridge, 1984) it is severely hindered by the fact that only a very small proportion of nitrogen fixing plants export fixed nitrogen in the form of ureides (Kessel *et al.*, 1988).

The acetylene reduction assay (ARA) described by Hardy *et al*., (1968) is a very popular technique that is used to indirectly measure BNF by estimating enzyme activity based on electron flux through nitrogenase. It is based on the ability of nitrogenase to reduce acetylene (CH ≡ CH) to ethylene. Samples to be assayed are incubated in a gas tight chamber that is injected with 0.03 to 0.1 (v/v) acetylene. The gas collected from the chamber after the end of incubation is assayed for ethylene production using a gas chromatograph fitted with Poropak N or P column. It is a simple, low cost and sensitive assay that can measure BNF in bacterial cultures, detached nodules, plant parts or even whole plants. The acceptance of ARA as a sole basis of interpretations of BNF has been hindered by several problems including the short-term nature of the assays, the doubtful validity of always using a conversion ratio of 3 and the auto-inhibition of acetylene conversion to ethylene (Danso *et al*., 1995).

The stable isotope methods using ^{15}N are more widely used and accepted over all other methods of BNF measurement. These methods are based on the principle that the soil or medium in which plants are grown has a distinctly different $^{15}N/^{14}N$ ratio than the almost constant 0.3663 % ratio present in the atmosphere. Therefore plants incorporating fixed nitrogen from the atmosphere will have a $^{15}N/^{14}N$ ratio different from the substrate they are growing on. When N fixing plants are grown in air labelled with ^{15}N they are expected to have an enhanced ratio as compared to substrate (^{15}N incorporation method). Where available soil N is labelled with ^{15}N, a reduction in the ratio is expected when plants incorporate fixed nitrogen

from air. This method is also called isotope dilution. Isotope dilution methods rely either on the inherent higher $^{15}N/^{14}N$ ratios of growth substrates than that of the atmosphere (natural abundance), or on ^{15}N enrichment of the substrate via labelled fertilizer. In both cases, a reduced $^{15}N/^{14}N$ ratio indicates nitrogen fixation. However the enrichment method is more common because of the clearer distinction between substrate and atmospheric ratios, which allows easier detection using relatively less costly equipment (Danso *et al*., 1993). Sanborn *et al*., (2002), have shown that whereas ^{15}N natural abundance data failed to provide useful insight into nitrogen fixation by Sitka alder, ^{15}N isotope dilution method was able to provide unambiguous evidence for the same.

1.5.5 BNF: Economy and Environment

The economic and environmental costs of the heavy use of chemical N fertilizers in agriculture are a cause of global concern. The manufacture of nitrogenous fertilizers is heavily dependent on the already dwindling and disputed reserves of fossil fuel. Their excessive application has adverse effects on human health (methemoglobinemia in infants, cancer, respiratory illness) and the environment (nitrate leaching into water sources, eutrophication, plant toxicity, ozone depletion) (Bohlool *et al*., 1992). Biological nitrogen fixation is an economically feasible and environmentally benign process that can be used alone or in conjunction with nitrogenous fertilizers to sustain food production particularly in the poor and developing countries where more than 78% of the world's population resides (Khush, 2001). The Food and Agricultural Organization emphasizes the importance of beans and other legumes in the diets of people in these parts of the world (Jones, web resource, pub date unavailable). The contribution of BNF to the social and economic success of Brazil through bean and sugarcane cultivation (Dobereiner, 1997) is an important model for the potential of this nitrogen input in the developing countries in the tropics and subtropics.

Another important economic and environmental application of biological nitrogen fixation is in the cultivation of energy crops like sugarcane for the production of biofuels (Dobereiner, 1997). The elimination of N-fertilizer for the production of biofuel crops, such as sugarcane, represents the key to high energy balances because N-fertilizer is produced by

reduction of atmospheric N_2 to NH_4, using natural gas as an energy source (Reis *et al*., 2007). Brazil where subsidies on nitrogen fertilizers are traditionally low and sugarcane is grown with the help of natural and inoculants aided biological nitrogen fixation (Boddey *et al*., 1995) has been really successful in producing and utilizing these biofuels. Baldani *et al*., 2002, have reported that as many as three million cars in Brazil run on 95% hydrous ethanol and all gasoline sold in Brazil contains 20-24% ethanol as biofuel.

Nitrogen fixing bacteria have also been found to be useful for bioremediation of industrial waste and hydrocarbon spills. Ikechukwu (1999), found that *Azotobacter* played a role in bioremediation of soil polluted with crude oil by providing other bacteria with nitrogen and through some co-metabolic activities. Some diazotrophs isolated from petrochemical sludge have the ability to degrade organic contaminants like polyethylene glycol, naphthalene and hexadecane (Naumova *et al.*, 2009).

1.5.6 Factors Limiting Biological Nitrogen Fixation

Bohlool et al., (1992), have categorized the constraints to practical application of BNF systems as environmental, biological, methodological, and socio cultural. Major environmental constraints are concerned with conditions affecting the microbe, the host, or their symbiotic interaction. These include soil acidity, aluminum and manganese toxicity, sulphur and phosphorus deficiency (law of minimum), salinity and soil aeration. A thorough understanding of these factors and how they affect different BNF systems is required to overcome these limitations. A major obstacle to successful establishment and effective performance of introduced N_2-fixing systems is also competition from native organisms.

A variety of biological constraints may influence the expression of BNF in all nitrogen-fixing systems. Both plants and bacteria are subject to biological constraints, such as disease and predation which can directly or indirectly affect the amount of N fixed. In general, the amount of nitrogen fixed is directly related to the growth potential of the host in a particular system. When growth is limited, for example by disease, nitrogen fixation will be reduced accordingly.

Identification, preparation and application of diazotrophic inoculants are major methodological constraints to widespread field application of BNF systems. The scale of

production, the availability of suitable carrier material, and shelf-life of the finished product limit the use of inoculants, especially in developing countries. Various cultural, educational, economic and political factors also affect fuller implementation of biological nitrogen fixation. Small and subsistence farmers lack the awareness and means to utilize this technology, especially in developing countries while subsidies on nitrogen fertilizers (Bohlool *et al*., 1992) in both developed and developing countries are a huge disincentive to the use of BNF, for medium and large farms. Giller and Cadish (1995), have discussed these constraints and ideas for overcoming them, with examples from Asia, Africa, Europe and North America.

1.5.7 Nitrogen Fixation in Agriculture and Forestry

Biological N_2-fixation contributes to productivity both directly, where the fixed N_2 is harvested in grain or other food for human or animal consumption, or indirectly, by contributing to the maintenance or enhancement of soil fertility in the agricultural system by adding N to the soil (Giller and Cadisch, 1995). Among symbiotic N_2-fixing systems, nodulated legumes have been used in cropping systems for centuries. They can serve a multitude of purposes in sustainable agriculture. They are used as primary sources of food, fuel, fiber and fertilizer, or, secondarily, to enrich the soil, preserve moisture and prevent soil erosion. The *Rhizobium*-legume symbiosis was the focus of agronomic practices and nitrogen fixation research during the 19^{th} century and for most part of the 20^{th} century. However, since the latter half of the 20^{th} century researchers have started focussing on extending nitrogen fixation to include non-legumes as well. Efforts have been made to induce paranodules on roots and inoculate them with effective rhizobial strains (Cocking *et al*., 1995). The process involves application of auxins, call wall degrading enzymes or genetic modification of bacteria that are not very practical for field application (Bruijn *et al*., 1995). Exploration of naturally occurring diazotrophic associations in non-legumes has proven to be a better approach (James and Olivares, 1998). Sugarcane research in Brazil presents a valuable example of this approach. The observation that sugarcane had always been grown successfully in Brazil with little or no nitrogen inputs, led J. Dobereiner (1961), to suggest that sugarcane might be meeting its nitrogen demands with the help of naturally associated nitrogen fixing bacteria.

Early efforts to explore diazotrophs in sugarcane fields resulted in the isolation of bacteria of the genus *Beijerinckia* in high numbers, with selective enrichment in the rhizosphere and especially on the root surface (Dobereiner, 1961). Diazotrophs of the genera *Erwinia, Azotobacter, Derxia, Azospirillum* and *Enterobacter* (Graciolli *et al*., 1981; Purchase, 1980) were also found from the roots, stems and even leaves of sugar cane. However*, n*one of these bacteria seemed to occur in large enough numbers to account for the 38-77 kg N ha^{-1} of mean annual BNF input reported in various studies (Oliviera *et al*., 1994; Boddey *et al*., 1995). In 1988 Cavalcante and Dobereiner, found an extraordinary diazotroph, *Gluconoacetobacter diazotrophicus*, in large numbers within the roots and stems of sugarcane. It is a small, Gram negative, aerobic rod like bacterium that can grow on very high concentrations of sugar, has the capability to fix N (>100 nmoles C_2H_2 ml^{-1} h^{-1}) at a pH as low as 3.0 (Stephan *et al.*, 1991) and oxygen levels of 4.0 kPa (Reis *et al*., 1990). Its ability to fix nitrogen is also not affected by the presence of high levels of nitrate (Boddey *et al*., 1991). The discovery of a bacterium possessing such unique properties adapted to fixing nitrogen under conditions very specific to the interiors of a sugarcane plant, has encouraged researchers to believe that such systems might be in place for other non-legumes waiting to be discovered. Some other examples of diazotrophic bacteria associated with other non-legumes are *Herbaspirillum*, *Azospirillum* and *Klebsiella* with corn (Chelius and Triplett, 2001), *Herbaspirillum*-rice (Baldani *et al*., 1986), *Azospirillum*-wetland rice (Dobereiner and Pedrosa, 1987), *Azoarcus*- Kallar Grass (Reinhold-Hurek *et al*., 1993). Such associations are also found in horticultural plants eg *Burkholderia* with tomato (Caballero-Mellado, 2007), *Klebsiella variicola* in banana (Martinez *et al*., 2003), *Azotobacter* with pepper (Govedarica *et al*., 1996).

Studies revealing associations between endophytic diazotrophic bacteria and forest trees, particularly Lodgepole pine are underway in our laboratory and have been described in Anand *et al*. (2006). These studies are expected to help answer long-standing questions about the presence of unexplained nitrogen in some forest ecosystems (Fenn *et al*., 1998, Binkley *et al*., 2000).

Despite much advancement in our basic knowledge of associative nitrogen fixation the extent of true benefits from it in the field remains to be established especially in temperate agriculture and forests (Peoples, 2006).

1.5.7.1 Importance of Asymbiotic Nitrogen Fixation in Temperate Forests

Nitrogen deficiency is common in temperate forest ecosystems particularly where input from atmospheric contaminants is low, such as the Pacific northwest of North America where urban centers are less aggregated. Besides mild wet and dry atmospheric deposition of nitrogen in areas downwind of sparse urban centers, the chief sources of nitrogen in these forests are symbiotic nitrogen fixation by nodule forming bacteria like *Rhizobium* with understory plant species such as Cusick's vetch (*Lathyrus nevadensis ssp*. *cusickii* (Watsi) C. L. Hitchcock) and *Frankia* with alder trees (*Alnus spp.*) (which are often the first to colonize forest ecosystems after disturbance), and forest fertilization (Fenn *et al.*, 1998).

Multiple studies have reported inputs of nitrogen that cannot be accounted for, particularly in forests that lack symbiotic nitrogen fixation. Unexplained nitrogen inputs, ranging from 40-285 kg/ha/yr, have been reported in soils under Norway spruce (Son and Grower, 1992), silver fir (Eriksson and Rosen, 1994), Japanese larch (Fisher and Eastburn, 1974) and various species of pine (Fisher and Eastburn, 1974; Dickson and Crocker 1953; Turvey and Smethurst, 1988). Inputs by non-symbiotic N-fixation were suggested as the most likely source of some of these unaccounted N inputs (Dickson & Crocker, 1953; Stevenson, 1959; Day *et al.,* 1975; Bormann *et al.,* 1977; Johnson and Todd, 1998; Wei and Kimmins, 1998). Son (2001) reviewed asymbiotic nitrogen fixation in forest ecosystems and suggested an average asymbiotic N fixation gain of 2-4 kg/ha/yr in temperate forest ecosystems, which translates into substantial total N inputs to the N budget of the forest ecosystems during a rotation period, for example 80-120 years for lodgepole pine.

The possibility that nitrogen inputs by asymbiotic nitrogen fixation could explain the ability of certain coniferous species like lodgepole pine (*Pinus contorta*) to thrive in marginal ecosystems characterized by low nutrient and moisture availability is an exciting hypothesis that is being currently pursued by our research group. The studies reported in this thesis were conducted to explore this possibility.

Two commercially important coniferous tree species, lodgepole pine (*Pinus contorta* (*Dougl.*) Engelm.) and western redcedar (*Thuja plicata*) were used to isolate and identify naturally-occurring, endophytic diazotrophs. Lodgepole pine is a two-needled pine species with wide ecological amplitude. It grows throughout the Rocky mountain and Pacific coast regions, extending North to about latitude 64^{o}N in the Yukon territory and south to about latitude 31^{o}N in Baja, California, and west to east from the pacific ocean to the Black hills of South Dakota. In Canada, forests dominated by lodgepole pine cover some 20 million ha. (Lotan and Critchfield, 1996).

It is a very important commercial tree species in British Columbia where it accounts for approximately 22% of the total inventory and contributes almost 25% to the total annual cut (Ministry of Sustainable Resource Management, Victoria, B.C.). It is also well known for its remarkable ability to grow under adverse climatic conditions like low soil moisture and on nutrient poor mineral soils with little or no organic nitrogen, without any fertilization (Lotan and Critchfield,1996).

Western redcedar is a common tree in Pacific northwest coastal and interior rainforests of British Columbia. Its wood is among the most durable of the native tree species; its reddish-brown color, straight grain, good stability, and excellent paint holding ability make this a preferred siding wood. Practically all shakes and shingles are made of this wood. Other important uses are poles, grape stakes, and paneling. On a standing volume basis BC has been reported to have about 750 million m^3 of western red cedar with about 80% of that found in the coastal region. Western red cedar represents 20% of the total standing volume of mature softwoods on the coast, and 2% of the total BC mature softwood inventory combined (Gonzalez, 2004). Western redcedar forests are also known to have limited nitrogen availability due to low rates of nitrogen mineralization (Prescott and Preston, 1994; Prescott *et al*., 1995). The ability of both lodgepole pine and western redcedar to thrive in conditions of low nitrogen availability makes these two conifer species ideal candidates for examining the possibility of biological nitrogen fixation.

The diazotrophic plant growth promoting bacterium, *Paenibacillus polymyxa* is known to be a predominant nitrogen fixing bacterium in soils (Jurgenson *et al*., 1971), rhizospheres (Deslippe and Egger, 2006) and plant tissues (Shishido *et al*., 1999) in temperate ecosystems, based on both culture dependent isolation (Jurgenson *et al*., 1971; Izumi *et al*., 2009) studies as well as culture independent survey studies (Izumi *et al*., 2009; Deslippe and Egger, 2006; Burke *et al*., 2008). The genus *Paenibacillus* (Ash *et al*., 1993; previously *Bacillus*), comprised of more than thirty species, is facultatively anaerobic and belongs to endospore-forming, low-G+C, Gram-positive bacilli. Species in the genus can fix nitrogen, produce antimicrobial compounds, and secrete diverse hydrolyzing enzymes. *P. polymyxa* is the most common representative of this genus in the soil. It is characterized by ellipsoidal spores, white to translucent, convex and mucoid colonies. It has the ability to grow at pH 5.7, hydrolyze starch, decompose casein and produce acid from glucose, mannitol, arabinose and xylose. (von der Weid *et al*., 2000) Many strains of the species are known to produce antimicrobial compounds (Rosado and Seldin, 1993; Choi *et al*., 2008) exopolysaccharides (Haggag, 2007), chitinase (Mavingui and Heulin, 1994), hydrolytic enzymes (Nielsen and Sorensen, 1997), phytohormones (Gutierrez-Manero *et al*., 2001; Timmusk *et al*., 1999; Lebuhn *et al*., 1997) enhance soil porosity (Gouzou *et al*., 1993), suppress plant diseases (Kloepper *et al*., 2004; Beatty *et al*.,2002) and fix atmospheric nitrogen (Heulin *et al*., 1994; Lindberg *et al*., 1985). These properties make it an effective plant growth promoting bacterium.

Nitrogen fixing strains of *P. polymyxa* are known to be associated with coniferous tree species in British Columbia, for example with roots of hybrid spruce (O' Neill *et al*., 1992), internal root tissues of lodgepole pine (Shishido *et al*., 1995) and tubercles of tuberculate ectomycorrhizae associated with roots of lodgepole pine (Paul, 2002). The ability of these strains to fix and transfer fixed nitrogen has not been studied in depth. Whereas no attempts were made to assess the contribution of nitrogen fixation to the growth promotion effects of the spruce isolates, Shishido et al., (1995), attempted acetylene reduction assays on rhizoshpheres of seedlings inoculated with diazotrophic

Paenibacillus polymyxa strain Pw-2R and found mildly detectable concentration of ethylene. Similar failure to detect sufficient acetylene reduction activity by *P. polymyxa* in soil led Rhodes-Roberts (1981), to suggest that suitable conditions for demonstrating acetylene reduction by this bacterium remain to be found, but they were able to determine nitrogen gains to plants by micro-Kjeldahl analysis. Rhodes–Roberts (1981) and Achouak *et al*., (1999) concluded that acetylene reduction does not always provide positive results for the nitrogen–fixing ability of *P. polymyxa*. Therefore conclusions on occurrence of nitrogen fixation should be drawn from both, a positive nitrogenase activity test (ARA, micro-Kjheldahl analyses or ^{15}N-uptake analysis) and by confirming the presence of a conserved structural *nif* gene such as *nif*H.

Sequencing of *nif* genes of diazotrophic bacteria is a powerful tool to study their nitrogen fixation ability. These sequences are also important to understand the inheritance of nitrogen fixation traits, through phylogenetic analysis. Whether nitrogen fixation genes are inherited laterally or vertically, has been a long standing point of discussion among microbiologists (Zehr *et al*., 2003; Dedysh *et al*., 2004). Despite its recognition as a widely distributed nitrogen fixing organism, sequence information for the *nif* genes of *P. polymyxa* remains limited to a ~361bp internal fragement of the *nif*H gene of various *P.polymyxa* isolates from different habitats ranging from agricultural soils in France (Achouak, *et al*., 1999) to rhizospheres of arctic plants in Alaska (Deslippe and Egger, 2006). A genomic snapshot of *P. polymyxa* strain ATCC 842^{T} provided sequence information for 17.5 % of its genome (Jeong *et al*., 2006), including sequence tags that were identified as *nif*U and *nif*S genes, both of which are involved in metal center biosynthesis. However, sequence tags homologous to the structural nitrogenase genes *nif*HDK were not found even though a partial *nif*H sequence was previously reported for this strain (AJ223997). Since *P. polymyxa* is a promising diazotroph found to be associated with important conifer tree speices in British Columbia (O' Neill *et al*., 1992; Shishido *et al*., 1995, Chapter 2 of this thesis), it is important to obtain more sequence information of the structural genes coding for the nitrogenase enzyme of this bacterial species to

facilitate our understanding of nitrogen fixation by *P. polymyxa* in association with coniferous tree species.

1.6 Thesis Objectives

The overarching question addressed in this thesis is, whether gymnosperm /coniferous tree species can benefit from nitrogen fixation by naturally occurring endophytic diazotrophic bacteria? My studies focussed on the following objectives:

i. To identify potential diazotrophic endophytic bacteria associated with coniferous tree species under natural conditions.
ii. To examine the ability of diazotrophic bacteria to re-colonize conifer tissues internally upon inoculation and to identify sites of colonization.
iii. To study nitrogen fixation and growth promotion effects of endophytic diazotrophic bacteria on inoculated conifers.
iv. To sequence and analyze nitrogen fixation genes of promising endophytic diazotrophic bacteria.

The specific hypotheses I tested were:

i. Lodgepole pine growing in the nitrogen-deficient, Williams Lake site, is likely to harbour more endophytic diazotrophic bacteria than lodgepole pine growing on the nitrogen-rich, Chilliwack Lake site and western red cedar growing in the nitrogen deficient Boston Bar site.
ii. Naturally occuring endophytic, diazotrophic bacteria are capable of entry, re-colonization and survival in biologically-significant numbers, inside conifer tissues following re-inoculation.
iii. Naturally occurring endophytic, diazotrophic bacteria are capable of promoting plant growth of coniferous plants, fixing nitrogen and transferring fixed nitrogen to their host plants, following re-inoculation.
iv. The *nif* genes of endophytic, associative, diazotrophic bacteria of coniferous plants are phylogenetically closer to those of free-living nitrogen-fixing bacteria than to those of nodule-forming, symbiotic, nitrogen-fixing bacteria.

1.7 Thesis Overview

In Chapter 2, I address objective (i), and describe the isolation and identification of potential diazotrophic bacteria from the internal tissues of lodgepole pine from two forest sites contrasting in nitrogen availability and internal tissues of western redcedar from a nitrogen deficient site. Both tree species are known for their ability to grow in N-poor ecosystems. These endophytic diazotrophic bacteria were isolated using culture-based, surface sterilization-grinding and plating methods, using a nitrogen-free culture medium. The preliminary identification of these bacteria was performed using the Gas Chromatographic Fatty Acid Methyl Ester (GC-FAME) method of microbial identification. Preliminary tests of nitrogenase activity were conducted using the acetylene reduction activity (ARA) assay. Isolates showing any acetylene reduction activity were assessed for clonality using REP-PCR (interspersed Repetitive elements -Polymerase chain reaction). Non- clonal isolates that showed consistent ARA were identified to the species level by amplifying and analyzing their 16S rRNA gene. The importance of the presence of bacteria of various genera is discussed. Strain P2b-2R of *Paenibacillus polymyxa* was chosen for further studies due to its high acetylene reduction activity and growth promotion ability in preliminary plant growth trials of short duration (not included in the thesis).

In Chapter 3, I address objectives (ii) and (iii). I marked *P. Polymyxa* strain P2b-2R with constitutively-expressed green fluorescent protein (GFP). I inoculated lodgepole pine seedlings with these GFP-marked bacteria and studied their colonization sites within plant tissues using confocal laser scanning microscopy (CSLM). Colony counts on agar plates spread with extracts of surface-sterilized plants colonized by wild type *P. polymyxa* strain P2b-2R were used to determine population densities of this bacterium in various plant tissues after inoculation.

To address objective (iii), I conducted a plant growth trial under controlled conditions. I used the ^{15}N isotope dilution method to assess nitrogen fixation in plants inoculated with *P. polymyxa* strain P2b-2R. Various growth parameters, *i.e.* length, fresh weight and dry weight of plant roots and shoots, and atom percent excess of ^{15}N isotope in harvested foliage were measured to draw conclusions about the abilities of *P. polymyxa* strain P2b-2R to fix nitrogen and promote plant growth. Two conifer species, lodgepole pine and western redcedar, were

used and their growth responses were compared to make inference on whether the plant-microbe interaction between *P. polymyxa* strain P2b-2R and conifers could be a host specific interaction.

In Chapter 4, I address objective (iv), and sequenced unknown nucleotide sequences of three important *nif* genes of *P .polymyxa* strain P2b-2R using a variation of the SSP (single specific primer) - PCR method. I analyzed and discussed the features of the sequences obtained and used these sequences to resolve the phylogenetic relationship of *P. polymyxa* strain P2b-2R with other known diazotrophic bacteria.

In Chapter 5, I summarize the main findings of the research work presented in this thesis. I also discuss the limitations of my research and suggest future research directions.

2 Isolation and Identification of Potential Endophytic Diazotrophs from *Pinus contorta* and *Thuja plicata* [2]

2.1 Introduction

Lodgepole pine (*Pinus contorta*) and western red cedar (*Thuja plicata*) are common gymnosperm trees in western North America ranging from Alaska to California. Lodgepole pine thrives in a wide range of soil, moisture and topographical situations and is unique among conifer species in its ability to thrive on nutrient poor (Weetman *et al*., 1988), fire- affected sites that are severely limited in nitrogen. As a result of its ability to grow on such poor sites, nitrogen (N) inputs in lodgepole pine forests have been a subject of great interest. Similarly, western red cedar forests are known to have limited N availability due to low rates of N mineralization (Prescott and Preston 1994; Prescott *et al*., 1995; Prescott *et al*., 1996). Despite the nutrient poor status of soils that lodgepole pine grows on, it does not show a sustained positive response to the application of N fertilizers (Weetman and Fournier 1982; Weetman *et al*., 1988; Brockley 1989, 1990, 1991, 1996; Yole *et al*., 1991, Marshall *et al.,* 1992). Like lodgepole pine, western redcedar also shows very modest gains in growth following fertilization with N (Prescott *et al*., 1996).

Traditionally, the mineralization of organic N by free-living soil microorganisms has been considered the primary process by which plant available N is released into the soil. In addition, certain mycorrhizal fungi are known to produce enzymes that help release the N tied up in forest floor organic matter (Read 1991; Xiao 1994). However, given the slower mineralization rates in western redcedar forests and relative lack of forest floor organic matter in fire- affected and other nutrient poor sites where lodgepole pine thrives, these processes seem inadequate to explain the release of sufficient plant- available nitrogen for tree growth. Other possilble sources of available nitrogen are wet and dry atmospheric deposition, and biological nitrogen fixation.

[2] A version of this chapter has been submitted for publication. R. Anand, A. Bal, O. Berge and C. Chanway (2010). Isolation and identification of potential endophytic diazotrophs from Pinus contorta and Thuja plicata.

Whereas atmospheric deposition in forests is highly site dependent based on proximity to industrial and agricultural activity (Aber *et al*., 1989), biological nitrogen fixation is very widespread in soils. In western Canada there is little atmospheric deposition and many soils are extremely N deficient. Free- living and root- associated diazotrophic bacteria have been explored as a source of fixed nitrogen in certain conifers including lodgepole pine (Holl and Chanway 1992; Shishido *et* al., 1996; Bormann *et* al., 1993). However, seedlings inoculated with these bacteria have failed to show significant rhizospheric acetylene reduction activity or increase in foliar N content (Chanway and Holl 1991; Shishido 1997). Tubercles of tuberculate mycorrhizal species associated with conifer roots have been found to harbor diazotrophic bacteria that have shown significant acetylene reduction activity (Li *et al.,* 1992; Paul *et al*., 2007). The amount of N gained by trees through this association has yet to be determined.

It has been suggested that endophytic bacteria colonizing plant tissues might interact more closely with the host, with less competition for carbon sources and a more protected environment for N_2 fixation (Quispel, 1991;Reinhold-Hurek and Hurek, 1998), such as that occurring in the relatively efficient N_2-fixing symbioses between rhizobia and legumes (Mylona *et al*., 1995). Such associative endophytic diazotrophic bacteria are well- established as a source of fixed N to crop plants, such as sugarcane (Cavalcante and Doberiener, 1988) and rice (Reinhold Hurek *et al.*, 2000). The presence of such bacteria in conifers and their possible role in N-fixation might explain the abilities of trees like lodgepole pine and western redcedar to thrive in N- limited conditions.

In this study we report the isolation and identification of potential diazotrophic endophytic bacteria from root, stem and needle tissues of naturally- regenerating seedlings of lodgepole pine and western redcedar collected from different sites in the southern interior of British Columbia, Canada.

2.2 Material and Methods

2.2.1 Collection of Plant Samples

Ten entire lodgepole pine seedlings, as well as stem, and needle samples from 10 other randomly chosen mature (stand age class 2, < 40 years old) trees were collected from

stands near Williams Lake (52°05' N lat., 122°54'W long., elevation 1300 m, Sub-Boreal Pine Spruce-very dry cold, SBPS xc zone) and Chilliwack Lake (49° 03'N lat., 121°25' long., elevation 625 m, Coastal Western Hemlock-dry maritime, CWH dm Zone) in British Columbia, Canada. Each seedling and tree sampled was located at least 10 meters apart from any other sampled seedling or tree. Roots were only sampled from seedlings. Stem samples from trees were obtained by taking cores with an increment borer. A scalpel was used to shave off a thin layer of bark from the stem sampling point in order to minimize epiphytic contamination. Both the borer and scalpel were cleaned and disinfected with 6% (w/v) sodium hypochlorite (NaOCl) for 2 minutes, 70% ethanol for 2 minutes followed by three 30 second rinses in sterile distilled water. Before reuse, stem cores were placed in sterile plastic bags, sealed, and transported to the laboratory on ice. Needle samples from trees were obtained by clipping off branches close to the ground. Western redcedar samples were collected from a stand near Boston Bar, British Columbia (49°50'N lat., 121°31'W long., elevation 163 m, Interior Douglas-fir zone-moist warm, IDF mw). Ten randomly-chosen entire seedlings and needles and stems from 10 randomly-chosen trees were sampled exactly as described for pine. Stem samples of cedar were obtained from trees by cutting small wedges from stems using a pruning knife, which was disinfected prior to each sampling.

Mineralizeable N available at the Williams Lake, Chilliwack Lake and Boston Bar sites was 12 ppm, 22 ppm and 13 ppm respectively (see appendix A for other chemical properties and nutrient levels in the soils at the three sites).

2.2.2 Isolation of Endophytic Bacteria

Needle, stem and root samples of seedlings and needle and stem tissue samples of mature trees were surface-sterilized in the laboratory by immersion in 2.5% (w/v) NaOCl for 2 minutes, followed by three 30- second rinses in 10 mM sterile phosphate buffer (SPB) (pH 7).To check for surface contamination, samples were imprinted on triplicate plates of both tryptic soy agar (TSA) (BD Sparks, MD, USA) and nitrogen-free combined-carbon-medium (Appendix B) agar (CCMA) (Rennie 1981).TSA was used to assess endophytic diversity and CCMA was used to select for N-fixing bacteria. Both media were supplemented with 100 mg/L cycloheximide to suppress fungal growth. Tissues free of surface contamination were ground in a small volume

of SPB with a sterile mortar and pestle. Ground tissues were diluted with SPB and plated onto TSA and CCMA. Following aerobic incubation at room temperature for 3 days, representative bacterial colonies were selected, based on colony size, shape, morphology, and color. Selected colonies were purified by streaking onto fresh plates of the same medium used for primary isolations. Purified isolates were grown in the corresponding broths and stored frozen at -80°C in cryovials with 20% (v/v) glycerol.

2.2.3 Identification of Bacteria

Frozen isolates were thawed and streaked onto TSA for identification by gas chromatographic analysis of bacterial fatty acids (as methyl esters) (GC-FAME) using the MIDI (Microbial ID, Inc., NJ, USA) microbial identification system (Kloepper *et al*., 1992) at Auburn University, Auburn, AL, USA.

Isolates that exhibited nitrogenase activity were assessed for clonality using REP-PCR fingerprinting, which is a simple and rapid technique that discriminates between strains at the intraspecies level (Versalovic *et al.*, 1991). Primers REP1R-I and REP2- I, which target conserved sequences known as bacterial repetitive elements (Versalovic *et al*. 1991), were used for the PCR. Amplification was done in 25 µL reaction volumes containing 2.5 µL Goldstar DNA polymerase buffer x 10 (Eurogentec, Seraing, Belgium), 1.25 mM (each) deoxynucleoside triphosphates, 6 mM $MgCl_2$, dimethyl sulfoxide at 10%, 60 µM (each) primers REP1R-I and REP2- I, and 5 µL of bacterial suspension to provide template DNA. The bacterial suspension was prepared by scraping bacterial cells off TSA x 1/10 agar plates that were cultured for 24 to 48 h, then suspending in 0.8 % KCl. The reaction mixture containing bacterial cells was heated at 96 °C for 10 min before the addition of thermo-resistant DNA polymerase. The PCRs were performed in a thermal cycler (Hybaid Omnigene, Thermo scientific, MA,USA) with 30 cycles of denaturation (94°C, 1 min), annealing (40°C, 1 min), and extension (65°C, 8 min), with a final extension (65°C, 16 min) and a final soak at 5°C. Products of PCR amplification were separated by horizontal electrophoresis with a 1.5% agarose gel in 0.5X Tris-borate-EDTA buffer. The 1-kb DNA ladder was used as a molecular weight marker.

Stained gels were digitized as gel images using Enhanced Analysis System software (easy 3.16; Herolab, Wiesloch, Germany). Strains were then assigned to REP genotypes by

comparing their electrophoretic patterns. It was assumed that all the strains sharing the same REP genotype were clonal (Versalovic and Lupski, 2000) and one representative strain per REP genotype was selected for 16s rRNA gene sequencing to make a tentative taxonomic affiliation.

For 16s rRNA gene-sequencing 5 µL of bacterial cell suspension (prepared as described above for REP-PCR) was used for PCR amplification of the 16 rRNA (*rrs*) gene fragments (approx. 1500 bp) using primers fD1 (5´ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG 3´) and rD1 (5´cccgggatccaagcttAAGGAGGTGATCCAGCC3´) (Weisburg *et al*., 1991), corresponding to positions 8-27 and 1524-1540, respectively, on the *Escherichia coli* ***rrs*** sequence. Amplification was performed in 25 µL reaction volumes containing 2.5 µL Goldstar DNA polymerase buffer x 10 (Eurogentec, Seraing, Belgium),1.25 mM (each) deoxynucleoside triphosphates, 6 mM $MgCl_2$, 10 µM (each) primers, 1.0 U Goldstar DNA polymerase (Eurogentec, Seraing, Belgium), and 5 µl of bacterial suspension. The reaction mixture, containing bacterial cells, was heated at 96 °C for 10 min before the addition of thermoresistant DNA polymerase. The PCRs were performed in a thermal cycler (Hybaid, Omnigene) with 30 cycles of denaturation (94°C, 1 min), annealing (53°C, 1 min), and extension (72°C, 2 min), with a final extension (72°C, 10 min). Products of PCR amplification were checked by horizontal electrophoresis with a 1.5% agarose gel in 0.5X Tris-borate-EDTA buffer. A 1-kb DNA ladder was used as a molecular weight marker.

PCR products of strains were purified using the QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed using the ABI PRISM Dye Terminator Ready Reaction kit as specified by the manufacturer (Perkin Elmer, Waltham, MA, USA). Sequences were obtained with an automatic sequencer (ABI PRISM 377 DNA sequencer; Perkin Elmer) using primers corresponded to the following positions in the E. coli ***rrs*** sequence: primer S6 (517-534), S10 (909-925), S12 (1099-1114) and S17 (1492-1509), each producing 400-500 bp of good quality sequence. These approximately 400 bp sequences were used for taxonomic identification of isolates using the Basic local alignment search tool (BLAST, NCBI).

2.2.4 Evaluation of Nitrogenase Activity

Bacterial isolates that grew on CCMA were tested for the presence of *in vitro* N-fixing activity using an acetylene reduction assay (ARA) (Holl *et al*. 1988). Bacterial isolates were grown in quadruplicate, in 5 mL culture vials fitted with a teflon seal. Each vial was filled with 2

mL of CCM broth, inoculated with bacteria and incubated at room temperature with shaking (175 rpm), until the broths reached an OD_{600} of 0.5 (12-14 hrs). Acetylene was then injected into 3 of the 4 vials per isolate, to a final volume of 10% of head space (v/v), after which vials were left to incubate at room temperature. The 4^{th} vial of each culture received no acetylene. After 48 hours a 1 mL sample of gas was removed from each vial. Ethylene content of this gas was measured by flame-ionization gas chromatography on a Hewlett Packard 5830A gas chromatograph using a stainless steel column (0.3 x 180 cm) containing Porapak N (80-100 mesh) at 50°C with N_2 carrier gas at a flow rate of 40 mL/min. Gas samples from the 4^{th} vial of each isolate were used as control for endogenous ethylene production by all isolates. Un-inoculated broth, with and without 10% acetylene, was also used as a control.

2.3 Results

2.3.1 Isolation and Identification

Bacteria isolated on both nitrogen-rich (TSA) and nitrogen-free medium (CCMA), from lodgepole pine tissues collected from two sites and western redcedar tissues from one site are listed in Table 2.1. Ninety-nine different isolates were identified from seedlings and mature tree tissues collected over all three sites. The identities of isolates in Table 2.1 are based on GC-FAME results; therefore some are identified only to the genus level, whilst others were identified to species level.

Twenty of these isolates were selected for their ability to grow on CCMA and for exhibiting any acetylene reduction activity levels above background. Of these, two isolates originated from stems of western redcedar trees, four from western redcedar seedlings both sampled from Boston Bar. The remaining 14 isolates all originated from lodgepole pine samples taken from the Williams Lake site (8 from seedlings, 2 from needle tissue of trees and 4 from tree stems). Of the 20 isolates, 4 were found to be clonal to other isolates based on REP- PCR. However, of the remaining 16 non-clonal isolates, only four, were able to reduce acetylene consistently through 10 replications (Table 2.2). Three of these isolates were from lodgepole pine and the fourth from western redcedar.

Table 2.1 Endophytic bacteria isolated from different tree species collected from different sites

Tree species	Sampling Site	Tryptic soy agar (TSA)	Combined carbon medium (CCM)
Lodgepole pine	Williams Lake	*Bacillus sp* [a,b,c,e] *Bacillus mycoides* *Bacillus pumilus* [b,e] *Brevibacillus sp* *Kocuria rosea* *Paenibacillus sp* [a] *Paenibacillus polymyxa* [a]	*Bacillus sp* *Bacillus longisporus* *Bacillus megaterium* [a,b] *Bacillus mycoides* *Brevundimonas vesicularis* *Cellulomonas biazotea* *Kocuria kristinae* *Dyadobacter sp.*[b] *Paenibacillus sp* [a] *Paenibacillus pabuli* [c] *Paenibacillus peoriae* [a] *Paenibacillus polymyxa* [a,c]
Lodgepole pine	Chilliwack Lake	*Bacillus* sp[a,b,c,e] *Bacillus licheniformis* *Bacillus megaterium* *Bacillus pumilus*[d] *Brevibacillus* [a,b] *Kocuria rosea* *Paenibacillus gordonae* [c] *Paenibacillus polymyxa* [b,d] *Paenibacillus sp* [d]	*Bacillus sp* *Bacillus megaterium* *Bacillus pumilus* *Burkholderia pyrrocinia* *Paenibacillus gordonae* [d] *Paenibacillus polymyxa*
Western redcedar	Boston Bar	*Arthrobacter agilis* *Bacillus sp* [b,c] *Bacillus megaterium* *Bacillus mycoides* *Bacillus pumilus* [a,b] *Bacillus sphaericus* [a,b] *Brevibacillus sp* *Cellulomonas tubata*[d] *Paenibacillus sp* *Paenibacillus gordonae* *Paenibacillus pabuli* *Pseudomonas sp* [a,b]	*Bacillus sp* [b,d] *Bacillus halodenitrificans* [d] *Bacillus megaterium* *Bacillus mycoides* *Bacillus pumilus* [a,b] *Bacillus subtilis* *Brevibacillus sp* *Burkholderia sp* *Paenibacillus sp* *Paenibacillus gordonae* [a,b] *Paenibacillus polymyxa* [a,b] *Pseudomonas sp* [e] *Streptoverticillium reticulum*

[a] Isolated from stem of seedling
[b] Isolated from stem of tree
[c] Isolated from needles of seedling
[d] Isolated from needles of tree
[e] Isolated from roots of seedling
Bacteria not identified by superscripts were present in all tissues

The identities of these four strains that exhibited consistent nitrogenase activity were determined again using 16S rRNA gene analysis. The 16s rRNA genes of strains P2b-2R

(GU132543) and P18b-2R (GU132544) were equally homologous (99%) to *P*. *peoriae* and *P*. *polymyxa*. Also both of these strains are closely related to *P. polymyxa* strains isolated from the rhizospheres of wheat plants CF43 (AJ223989) and PMD230 (AJ223988) (Achouak *et al*., 1999). Therefore, it can be said that these strains belong to the *P. polymyxa* monophyletic group containing, *P. polymyxa*, *P. jamilae*, *P. brasilensis*., *P. peoriae*, *P. kribbensis* and *P. daejonensis* (Akaracharanya *et al*., 2009). Strain C3b (GU132545) belongs to the *P*. *amylolyticus* monophyletic group (99% similarity) (Nelson *et al*., 2009) containing *P. amylolyticus*, *P. pabuli*, *P. tundrae* and *P. xylanexedens* and strain P19a-2R (GU132546) belonged to the Flexibacter group and was closely related to *Dyadobacter fermentans* (99% similarity).

Table 2.2 List of isolates with most consistent nitrogenase activity.

Bacterial strain	Tree species and tissue of origin	Sampling Site	Taxonomic Identity of Bacterial strain [a]	Acetylene reduction activity (µmols C_2H_4/ml [b])
P2b-2R	Lodgepole pine Seedling (stem and needle)	Williams Lake	*Firmicutes*, *Bacillales* *Paenibacillaceae*, *Paenibacillus* ***P.polymyxa/ peoriae***	5.172
P18b-2R	Lodgepole pine Seedling (stem)	Williams Lake	*Firmicutes*, *Bacillales* *Paenibacillaceae*, *Paenibacillus* ***P.polymyxa/peoriae***	4.0638
P19a-2R	Lodgepole pine (Tree stem tissue)	Williams Lake	*Bacteroidetes* *Sphingobacteria* *Sphingobacteriales* *Noctuoidea* *Dyadobacter* ***D. fermentans***	1.0702
C3b	Western red cedar Seedling (root)	Boston Bar	*Firmicutes*, *Bacillales* *Paenibacillaceae*, *Paenibacillus* ***P. amyloliticus***	3.3058

[a] Taxonomic identities based on partial sequence analysis (~400 bp) of 16s rDNA gene.
[b] moles of ethylene per ml of head space in culture tube

2.3.2 Nitrogenase Activity (ARA)

Of all the isolates that grew on combined-carbon, N-free medium, only 4 were found to be capable of reducing acetylene through 10 replications. These were designated as P2b, P18b, P19a, and C3b based on the codes assigned to isolation sites, tissue samples and media. Conversely two strains, P19b and P20b, that were not capable of growing on CCM and were

isolated on nitrogen-rich medium (TSA), exhibited acetylene reduction, although they failed to do so across replications. Many other strains that had grown on CCM and were identified by GC-FAME as being *P*. *polymyxa* could not reduce acetylene. All strains that had acetylene reduction capability were isolated from pine tissue samples taken from the Williams Lake site. The only acetylene reducing strain to have come from western redcedar a different site, Boston Bar, was C3b. All isolates that originated from the Chilliwack Lake site failed to reduce acetylene, regardless of their ability to grow on CCM.

2.4 Discussion

Bacteria belonging to the family Bacillaceae were by far the most common isolates from both tree species and on both isolation media. The GC-FAME identities of bacteria isolated from internal tissues of pine and cedar indicate that many of the bacteria colonizing the tissues were common to both tree species. As many of the isolates identified are common soil bacteria such as *Paenibacillus polymyxa*, *P*. *gordonae*, *P*. *pabuli*, *Bacillus megaterium*, *B*. *mycoides*, *B*. *pumilus*, *B*. *licheniformis*, *B*. *sphaericus*, and *B*. *subtilis* (Slepecky, 1992), the source of many of these bacteria presumably is the soil. Twenty-two of 58 *Bacillus* isolates originated from aerial parts of trees or seedlings that were surface-sterilized, indicating that these isolates are capable of systemic colonization of plant tissues, as suggested for *P. polymyxa* (previously known as *Bacillus*) strain Pw-2R (Shishido *et al*., 1995).

Although a number of strains belonging to the genera *Bacillus* and *Paenibacillus* and some of the genera *Brevundimonas, Cellulomonas, Kocuria, Burkholderia, Brevibacillus, Pseudomonas* and *Streptoverticillum* were able to grow on nitrogen-free medium (CCMA), only 3 strains belonging to the genus *Paenibacillus* and one strain identified as genus *Dyadobacter* exhibited consistently detectable nitrogenase activity. The ability of all other bacteria to grow on CCMA indicated that growth on nitrogen-free media does not necessarily indicate the ability to fix nitrogen under assay conditions. It is possible that these bacteria or strains are highly efficient scavengers of a very small amount fixed nitrogen found in CCMA (100mg/L yeast extract), or ammonia in the atmosphere (Wynn-Williams, 1974; Hill and Postgate, 1969).

It is notable that strains of the genus *Burkholderia,* which is known to be a genus rich in plant- associated nitrogen-fixing species (Estrada-deLos Santos *et al*., 2001), were isolated from both pine and cedar. The strains isolated from pine could be identified as *Burkholderia pyrrocinia* using GC-FAME, whereas strains from cedar could not be identified to the species level. Since none of these strains exhibited any nitrogenase activity, no effort was made to pursue further identification of these strains by 16s rRNA sequencing. Similarly, representatives of the genera *Brevundimonas* and *Pseudomonas* have been reported for their ability to reduce acetylene (Montanez *et al*., 2008), but in our study they did not exhibit any such ability. We suggest that although strains of these genera did not qualify as diazotrophs in our study, their association with gymnosperm trees should not be ignored and that there is a possibility that diazotrophic strains of these genera might be found associated with other trees.

The fact that bacteria of *Cellulomonas spp* were also isolated from both pine and cedar is also very interesting because these bacteria are known to assist nitrogen fixers by breaking down cellulose and hemicelluloses, rendering substrates more available as an energy source for the energy demanding process of nitrogen fixation (Halsall and Gibson, 1986). As long as there are no physical signs of pathogenic cellulase activity, it is a possibility that the presence of *Cellulomonas* might facilitate nitrogen fixation by diazotrophic bacteria *in planta*.

Among the isolates that showed consistent nitrogenase activity, strains P2b-2R and P18b-2R were identified to genus level as *Paenibacillus*, but were equally similar (99%) to both *P*. *polymyxa* and *P. peoriae* at the species level and could not be differentiated.. This was not unexpected, as both species belong to the *P.polymyxa* monophyletic group (Akaracharanya *et al*., 2009). Moreover, Heynderickx *et al*., (1996), have suggested that *P. polymyxa* is phenotypically closely related to *B. peoriae* (now *Paenibacillus*) and can be distinguished only by a very limited number of tests. Similarly, Rodrigues *et al*., (2003), have also reported difficulties in separating these two species based on the 23s rRNA and 16s rRNA-RFLP techniques and have suggested the coupling of these methods with Multi locus enzyme electrophoresis (MLEE) for the conclusive differentiation of these species. However, based on extensive searches of the NCBI database (National Center for Biotechnology Information) we are aware that there are multiple reports of *P. polymyxa* strains having the dinitrogenase

reductase gene (*nif*H), whereas no reports of this gene are available in *P. peoriae* strains. Achouak *et al*., (1999), have also reported the presence of *nif*H gene as well as ARA (0.8-0.7µ mol C_2H_4), in *P. polymyxa* strains, and the lack of both these characteristics in the *P. peoriae* strains tested. Based on this information, we are tentatively identifying both P2b-2R and P18b-2R as *P. polymyxa*, pending further testing by obtaining longer 16s rDNA sequences and sequencing the *nif* genes of these strains.

Two of the isolates capable of acetylene reduction were identified as belonging to species that had not been previously observed to fix nitrogen. P19a-2R, isolated from pine tissues originating from the William's Lake site, was identified as *Dyadobacter fermentans* using 16s rRNA sequencing. While P19a-2R had acetylene reduction activity, the values were lower than other acetylene reducing isolates found in this study. This is contradictory to a previous report (Chelius and Triplett, 2000), where a *D. fermentans* strain isolated from corn tissues failed to show any acetylene reduction or *nif*H amplification. To the best of our knowledge strain P19a-2R is the first reported *D. fermentans* strain to show acetylene reduction activity.

Isolate C3b was placed in the *P. amylolyticus* group by 16s rRNA analysis, which contains *P. pabuli*, *P. tundrae*, *P. xylanexedens*, none of which are reported to be nitrogen-fixing species so far. Similar to isolate P19a-2R, *nif* gene-based testing is required to confirm the diazotrophic potential of this isolate. Isolate C3b is of particular interest as it is the only isolate from cedar tissues and the sole isolate from the Boston bar site that was able to reduce acetylene. All other isolates that had ARA were isolated from pine tissues collected from the Williams Lake site lies in the SBPS xc BEC zone which is characterized by is characterized by cold, dry winters and hot, dry summers. Forest floors are typically thin (<4 cm) and decomposition is slow. The soils are nutrient-deficient with relatively low-productivity (Steen and Demarchi, 1991; Steen and Coupe´, 1997). The prevalence of potential diazotrophic bacteria on such poor sites might be attributed to positive selection for bacteria with diazotrophic characteristics.

Interestingly none of the isolates from the Chilliwack Lake site showed ARA activity. It is notable that the Chilliwack area is known to have high levels of ammonia/ ammonium and nitrate ions in the air and precipitation which are deposited at the average rate of 3,900 µg/m^2/day, with the deposition velocity being higher in forests (0.5-1.77 µm/s) as compared to

grasslands (0.49-0.96 µm/s) (Vingarzan *et al*., 2000). This lack of acetylene reduction among isolates from this nitrogen-rich site indicates that there may be selection against nitrogen fixation ability or simply repression of nitrogenase activity due to presence of fixed nitrogen in the system (Roberts and Brill, 1981).

The presence of endophytic bacteria capable of acetylene reduction in the tissues of lodgepole pine and western red cedar is indicative of potential nitrogen fixation by these bacteria *in planta*. Three of the four acetylene reducing bacterial isolates originated from the tissues of lodgepole pine, a species known for its ability to grow on nitrogen poor sites, and no isolates capable of acetylene reduction were obtained from plants growing on a nitrogen-rich site. *Paenibacillus* dominated the culturable endophytic bacterial community as well as potential diazotrophic community. These findings suggest that endophytic nitrogen fixation might be a source of available nitrogen in forest ecosystems where plant growth or accretion of excess nitrogen (Fenn *et* al., 1998; Binkley *et al*., 1999) cannot be explained by other processes. We are examining further the ability of these potential diazotrophs to re-colonize conifer tissues upon inoculation and their ability to transfer fixed nitrogen to these host plants (Chapter 3, this thesis).

2.5 Acknowledgments

Funding for this project was provided by NSERC and Global Forest (catalogue # GF-18-2000-68) grants to C.P.C. and by the Science Council of British Columbia and Brinkman and Associates Reforestation.

3 Conifer Seedlings Derive Nitrogen and Growth Benefits from Naturally Occurring Endophytic Diazotrophic Plant Growth Promoting Rhizobacterium (PGPR) *Paenibacillus polymyxa* Strain P2b-2R [3]

3.1 Introduction

Endophytic diazotrophs are presumed to have an advantage over free-living and root-associated diazotrophs, because they are better placed to avoid competition and to exploit carbon substrates supplied by the plant (Döbereiner *et al*., 1995a, 1995b; McInroy and Kloepper, 1995; Boddey *et al*., 1995; Sprent and James, 1995; Triplett, 1996). Nitrogen fixation and plant growth promotion by naturally occurring endophytic diazotrophic bacteria has been well studied in both field crops (Kennedy *et* al., 2004) and horticultural plants (Caballero-Mellado, 2007; Martinez *et al*., 2003; Govedarica *et al*., 1996). Endophytic diazotrophs have been applied successfully to crops such as rice (*Oryza sativa*) (Ngyuen *et al*., 2003; Barrett and Marsh, 2002; Malik *et* al., 2002; Hegazi *et* al., 1996) and sugarcane (*Saccharum officinarum*) (Boddey *et* al., 2001) resulting in yield benefits and reduced costs due to decrease in the need for nitrogen fertilizers. Associations between diazotrophs and their hosts have been useful in explaining agro-ecological conundrums, such as the ability to continuously cultivate sugarcane in Brazil with little fertilizer input and yet without apparent depletion of soil nitrogen reserves (Boddey, 2003).

Similar to sugarcane in Brazil, accretion of unexplained nitrogen in temperate forests has also been a subject of curiosity among researchers working on nitrogen budgets in these forests that are primarily vegetated by conifers (Fenn *et* al., 1998; Binkley *et* al., 2000).

[3] A version of this chapter will be submitted for publication. R. Anand, S.J. Grayston and C. Chanway (2010). Conifer seedlings derive nitrogen and growth benefits from naturally occurring endophytic diazotrophic plant growth promoting rhizobacterium (PGPR) Paenibacillus polymyxa strain P2b-2R.

Lodgepole pine (*Pinus contorta* var. latifolia (Dougl.) Engelm.) is unique among conifers for its ability to regenerate and grow on very nutrient poor sites, such as post-fire soils, and dry, sandy and gravelly soils (Lotan and Critchfield, 1996) that are known to lack sufficient organic matter and N to account for tree growth (Neff *et al*., 2005). Well known sources of plant available N in forest soils, such as mineralization of organic N by free-living soil microorganisms and enzymatic release of N sequestered in forest floor organic matter by mycorrhizal fungi (Read, 1991; Xiao, 1994) cannot explain the release of physiologically significant amounts of nitrogen on such sites. Although many diazotrophic free-living and rhizospheric bacteria have been shown to promote the growth of lodgepole pine and other conifers (Holl and Chanway, 1992; Shishido *et al*., 1996; Bormann *et al*., 1993) by alternative mechanisms like phytohormone production, their actual nitrogen contribution to the trees could not be demonstrated (Chanway and Holl, 1991; Shishido, 1997). Despite the demonstration of endophytic nitrogen fixation in other plant systems (Boddey *et al*., 2003, Elbeltagy *et al*., 2001, Malik *et al*., 1997), this phenomenon remains largely unexplored in coniferous forests. Equally, the location of the endophytic diazotrophs in conifers remains elusive (Chanway *et al*., 2000).

A number of potential endophytic diazotrophic bacteria were isolated from surface-sterilized tissues of naturally growing seedlings in a range of nutrient-poor sites in the interior of British Columbia, Canada, close to Williams Lake (52°05’ N lat., 122°54’W long., elevation 1300 m, Sub-Boreal Pine Spruce, SBPSxc Zone), using culture-based techniques (see Chapter 2 of this thesis). Among the isolates, strain P2b-2R identified as *Paenibacillus polymyxa* showed a consistently higher level of acetylene-reduction activity (nitrogen fixation potential) in culture (5.17 µmols C_2H_4/ mL of headspace air, see Chapter 2 of this thesis).

This study investigated the ability of *P. polymyxa* strain P2b-2R to colonize lodgepole pine tissues internally and characterized the location of the bacteria within the tree. In addition, the ability of strain P2b-2R to provide conifers (lodgepole pine and western redcedar) with fixed nitrogen and growth benefits after inoculation was determined. The investigation with western red cedar was to ascertain whether the endophytic colonization, nitrogen fixation and growth promoting abilities of strain P2b-2R were host specific to lodgepole pine. Our objective was to obtain evidence for the two most important criteria of endophytic bacterial nitrogen

fixation in a plant system: a) Confirmed ability of the diazotrophic bacterium to colonize host tissues systemically, and b) Increased accretion of atmospheric nitrogen in plant tissues after inoculation with the diazotrophic bacterium (James and Olivares, 1997; James 2000).

Since, endospore-forming bacteria, like *P. polymyxa*, are known to be susceptible to misidentification as endophytes using culture-based techniques alone (Bent, 2002), here we used green fluorescent protein (GFP) as a bacterial marker coupled with confocal laser scanning microscopy to observe endophytic colonization by P2b-2R. To the best of or knowledge this is the first report of *in situ* microscopic localization of endophytic bacteria in coniferous tissues.

In addition, whereas previous studies in conifer systems have relied on total plant nitrogen and the acetylene reduction assay as indirect evidence of biological nitrogen fixation (Chanway and Holl, 1991; Shishido, 1997), we have used the more robust N^{15} isotope dilution technique (Danso *et al*., 1993) to evaluate the ability of P2b-2R to provide colonized plants with fixed nitrogen *in situ*. The implication of endophytic N-fixation in our understanding of N-cycling in coniferous forests is discussed.

3.2 Materials and Methods

3.2.1 Bacterial Strains

Wild type *P. polymyxa* strain P2b-2R, its derivative P2b-2R*gfp* (Transformant T1, this study) marked with GFP and *Bacillus subtilis* strain BEST 3156 (Itaya *et al*., 2001) were used in this study. Strain P2b-2R is resistant to rifamycin while P2b-2R*gfp* is resistant to rifamycin as well as both tetracyline and chloramphenicol. BEST 3156 is resistant to both tetracycline and chloramphenicol. All bacterial cultures were grown on nitrogen-free, combined carbon medium (CCM) agar (Rennie, 1981) amended with glycerol (20%) for 24-30 hrs at 30 ^{o}C and then kept frozen in cryovials at -80 ^{o} C until use.

3.2.2 Transformation of *P. polymyxa* Strain P2b-2R with GFP

A shuttle plasmid capable of replicating in both *E. coli* and *B. subtilis* was used to introduce the plasmid-borne *gfp* gene into *P. polymya* strain P2b-2R. Plasmid pBSGV104 (Itaya *et* al., 2001), is a low copy number plasmid that carries a 786-bp *gfp* fragment inserted between the *Hin*dIII and *Eco*RI restriction sites of shuttle vector pHY300PLK (Ishiwa and

Shibahara, 1985) and confers resistance to tetracycline and chloramphenicol. The expression of *gfp* is constitutively driven by a *bsr* promoter. Plasmid pBSGV104 was isolated from its carrier strain *Bacillus subtilis* BEST 3156 using the QIAprep spin miniprep kit (Cat # 27104, Qiagen, USA), modified to isolate plasmid from Gram-positive bacteria by adding 1 mg lysozyme mL^{-1} to the P1 solution, and incubating at 37°C for 1 h before adding P2 solution.

Electroporation of *P. polymyxa* P2b-2R was carried out with minor changes to a previously described method (Rosado *et al.,* 1994) using a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA). Strain P2b-2R was grown in Erlenmeyer flasks containing BHIG (BHI + 0.5% Glycerol) without mannitol (BD Sparks, MD,USA) at 30 ^{o}C with shaking (150 rpm) (Orbit shaker 3520, Lab-line instruments, Melrose park, Il, USA) for approximately 24 hours until concentrations of about 10^7 cfu mL^{-1} were reached. Cells were harvested by centrifugation (10,400 x *g*, 15 min), washed twice in double-distilled water and once in phosphate electroporation buffer (PEB) (272 mM sucrose,1 mM $MgCl_2$, 7mM potassium phosphate. pH 7.4) (Luchansky *et al*., 1988), and resuspended in electroporation buffer at 1/100 of the original culture volume to make cells electro-competent. Ten microliters of plasmid DNA was mixed with 0.2 ml of a suspension containing 5 x 10^9 electro-competent cells in a chilled Gene Pulser cuvette (inter electrode gap, 0.2 cm) and kept on ice for approximately 5 min and then subjected to an electric pulse of 2.5kV, at 25uF capacitance and resistance of 5 Ohms. One milliliter of BHI broth was immediately added to the cuvette following application of the electric pulse. The cell suspension was kept on ice for 15 min, then diluted with 10 ml of BHI broth. The cells were incubated at 30°C for 4 h, concentrated to 1 mL by centrifugation and resuspended in Luria Broth (LB, Fisher scientific, NJ, USA). *P. polymyxa* transformants were selected on LBA plates containing 5 µg chloramphenicol mL^{-1} and 200 µg $mL^{-1}$of rifamycin.

Initial confirmation of fluorescence emission by transformants was done by visual inspection under 310 nm UV light. Fluorescence intensity was measured in LB culture using a Cytofluor II multi well plate reader (AB, CA) equipped with 508/20 EM emission filter. Transformant stability was determined by sub- culturing transformants for 10 successive nights in fresh CCM broth without selective pressure (chlormaphenicol) and then plating a dilution series on CCM agar with and without chloramphenicol. The stability figure was computed using

the ratio of the colony number on selective plates to that on the non-selective plates (Lee and Choi, 1987).

3.2.3 Plant Growth Promotion, Nitrogen Fixation Assays and Colonization Studies

For plant growth promotion and nitrogen fixation assays, lodgepole pine and western redcedar germinants were inoculated with either live *P. polymyxa* P2b-2R or heat-killed P2b-2R. Heat-killed P2b-2R served as a control to ascertain any plant growth promotion arising through nutrient or mineralized nitrogen release from dead bacterial cells. Un-inoculated seedlings were used as an overall control. Each tree species, treatment and controls were replicated 70 times and arranged in a completely randomized design (CRD). For microscopic studies lodgepole pine seedlings were inoculated with the transformant P2b-2R*gfp*, whereas un-inoculated plants and those inoculated with wild-type P2b-2R were used as controls. Each treatment had 30 replicates arranged in a completely randomized design.

3.2.3.1 Seeds and Pre-treatment of Seeds

All seeds for this study were supplied by the British Columbia Ministry of Forests & Range tree seed centre, Surrey, BC. Pine seeds originated from provenances of similar location and elevation as the Williams Lake pine site from which strain P2b-2R was originally isolated (52°05' N lat., 122°54'W long., elevation 1300 m, Sub-Boreal Pine Spruce-very dry cold, SBPSxc Zone) and western redcedar seeds originated from a site near Boston bar, BC (49°50'N lat., 121°31'W long., elevation 163 m, Interior Douglas-fir Zone-moist warm, IDFmw). All empty pine seeds were removed by flotation in ethanol (Barnett, 1971). Both cedar and pine seeds were surface sterilized by immersion in 30% hydrogen peroxide (H_2O_2) for 1 minute 30 seconds, followed by three 30 second rinses in sterile distilled water. The effectiveness of the surface sterilization was confirmed by imprinting sterilized seed on tryptic soy agar (TSA) (BD Sparks, MD, USA) and checking for microbial contamination two days later. Seeds found to be free of surface contamination were placed in sterile bags made of cheese cloth, that were placed in sterile (autoclaved), moist sand in a loosely tied autoclavable plastic bag at 4^{o}C for 5 weeks for cold stratification before sowing to optimize and synchronize germination. Stratified seeds were again imprinted on TSA plates for 48 hours before sowing to confirm the absence of

surface contamination. Ten randomly picked surface sterilized seeds were crushed and imprinted on TSA plates supplemented with 200 µg/mL rifamycin, for 48 hours to confirm the absence of internal seed contamination with *P. polymyxa* strain P2b-2R.

3.2.3.2 Seedling Preparation

Pine and cedar seedlings for growth promotion and nitrogen fixation assays and pine seedlings for colonization studies were all grown in glass tubes (25 mm x 150 mm, Sigmaware™, Sigma-Aldrich, Canada) filled two-thirds, with a sand-Turface (montmorillonite clay, Applied Industrial Materials Corporation, Deerfield, IL) mixture (69% w/w silica sand; 29% w/w turface; 2% w/w $CaCO_3$). The sand-turface mixture was washed with 6 % sodium hypochlorite at room temperature with continuous shaking (60 rpm) for 6 hours, in order to remove organic nitrogen (Mikutta *et al.,* 2005) and then washed three times with distilled water prior to drying and filling the glass tubes. Each tube was filled to saturation with 17 mL of a nutrient solution (Appendix C)(Chanway *et al*., 1988) which was modified by replacing KNO_3 and $Ca(NO_3)_2{\cdot}4H_2O$ with $Ca(^{15}NO_3)_2$ (5% ^{15}N label, 0.0576 g/L) (Cambridge isotope laboratories, Inc., USA) (Chanway and Holl, 1991) and Sequestrene 330 Fe (CIBA-GEIGY, Mississauga, Ont.) with $Na_2FeEDTA$ (0.025 g/L). Tubes were then autoclaved for 1 hour. Three surface-sterilized seeds of either pine or cedar were then aseptically sown in each tube and covered with ca. 5 mm of autoclaved silica sand.

3.2.3.3 Seedling Inoculation and Growth

Strain P2b-2R and P2b-2R*gfp* were grown overnight in CCM amended with 200 mg/L rifamycin and 5mg/L chloramphenicol respectively, harvested by centrifugation (5724 x g for 30 minutes) and resuspended in sterile phosphate buffered saline (PBS) to a density of ca. 10^6 cfu/mL for growth promotion and nitrogen fixation assays and 10^5 cfu/mL for colonization studies. Heat-killed P2b-2R inoculum was prepared by autoclaving broth containing strain P2b-2R for 1 hour and streaking on TSA plates to confirm the absence of any live cells in the inoculum. Immediately, after seeds were sown, 5 mL of the appropriate bacterial suspension was pipetted directly into each of the relevant replicate tubes containing either lodegpole pine or western redcedar. The uninoculated control seeds received 5.0 mL of sterile PBS. Tubes

were placed in a growth chamber (Conviron CMP3244, Conviron Products Company, Winnipeg, MB) for a total of 14 weeks for the localization studies and 13 months for the growth promotion and nitrogen fixation assays. Photosynthetically active radiation (PAR) at canopy level was ca. 300 $\mu mol \cdot s^{-1} \cdot m^{-2}$ during an 18-h photoperiod, and 20°C/14°C day/night temperature cycle with 70 % relative humidity.

Pine and cedar seedlings were thinned to the largest single germinant per tube, 2 and 3 weeks after sowing, respectively. Seedlings were watered with nutrient solution without $Ca(NO_3)_2$ once a month and with sterile distilled water daily to constant weight.

3.2.3.4 Seedling Harvest

For the microscopy studies visualizing the colonization and location of the bacteria within pine tissues, 3-4 pine seedlings from P2b-2R*gfp* inoculated, P2b-2R wild-type and uninoculated were harvested destructively every 2 weeks for 14 weeks. For the bacterial enumeration study three seedlings per tree species, treatment and control were harvested destructively every 2-4 months to re-isolate and enumerate bacteria colonizing the internal tissues of host plants at various stages of growth. After 13 months all remaining seedlings were harvested destructively for growth promotion and nitrogen fixation assays. The growth parameters recorded were length, fresh weight and dry weight of complete plant, root and shoot in addition to measuring total nitrogen as well as percentage of nitrogen derived from the atmosphere in pine and cedar foliage.

3.2.3.5 Preparation of Bacterial and Plant Samples for Colonization Studies

To confirm observable *gfp* production by strain P2b-2R*gfp*, cells were grown overnight in CCM broth, centrifuged at 805 x g in a minicentrifuge (Hettich Mikro20, GMI, MN), washed twice in 0.1 M PBS pH 7.2 and resuspended to 50X concentration in 4% formaldehyde (w/v freshly prepared from paraformaldehyde) in 0.1 M PBS pH 7.2 for 3 hours. Fixed cells were centrifuged again at 805 x g, cells from the pellet were lifted with a wire inoculation loop, spread onto a glass microslide and immediately mounted with a drop of 2.5 % 1, 4-diazabicyclo-[2,2,2]-octane (DABCO) antifade mounting medium prepared with 50 % glycerol in 20mM PBS (pH 8.5) (Ono *et al*., 2001) and covered with a 1.5mm glass coverslip which was then sealed on all 4 sides with a thin layer of paraffin wax.

For confocal laser scanning microscopy of plant tissues 2-14 week old lodgepole pine seedlings were harvested destructively, surface disinfested by washing in 10 % NaOCl for 3 minutes with mild shaking followed by three 30 second rinses in sterile distilled water. Roots and stems of seedlings were detached, cut into 3 mm segments and immediately fixed in 4% formaldehyde solution as described above. Lower, middle and upper segments of stem were fixed separately for distinction. Fixed segments were embedded in low melting point agar (Sigma- Aldrich Canada Ltd, Ontario) and cut into 6-8 µm thick longitudinal sections using a vibratome (Leica VT1000S). Sections were mounted on glass microslides as described above.

3.2.3.6 Microscopy

For detection of GFP, bacterial cells and plant tissues were observed under a Ziess 510 Meta laser scanning head mounted on an Axiovert 200M inverted microscope. GFP was excited at 488 nm with an argon laser and fluorescence was detected at 500-540 nm. Cells were visualized using Plan-Apochromat 20X/0.8, C-Apochromat 40X/ 1.1 W and EC Plan-Neofluar 100x/1.3 oil objectives. Images obtained were viewed with the help of the LSM image browser and spectral analysis of images was performed using the LSM 510 Meta AIM software.

3.2.3.7 Re-isolation and Enumeration of Inoculum

Seedlings were harvested destructively at 2, 4, 8, and 12 months after sowing, washed thoroughly under running water for 3 hours, surface sterilized in 1.3 % sodium hypochlorite for 5 minutes than washed three times with sterile distilled water. Seedlings were imprinted on TSA plates for 24 hours after which roots, stems and needles were crushed separately in sterile PBS with sterile pestles and mortars. The extract was serially diluted and plated on TSA plates with and without rifamycin, and incubated at 30^0C for 72 hours before counting the number of colony forming units (cfu). CFU data was converted to base 10 log for statistical manipulation and graphing.

3.2.3.8 Determination of Plant Growth Promotion and Nitrogen Fixation

After 13 months, 50 seedlings of pine and cedar that were initially inoculated with either P2b-2R, heat-killed P2b-2R or uninoculated were assessed for growth promotion. Total plant height, (root and shoot length) and fresh and dry weights of the whole plant as well as root, stem and needles were recorded. Plants were dried for 48 hours at 65 ^{o}C before recording

dry weight. For assessment of nitrogen fixation, foliage of 7 seedlings per treatment was randomly chosen and then ground to a particle size <2 mm. Each sample was measured to ~ 5.0 µg and sent to the Stable Isotope Facility at the University of California at Davis for determination of percent N^{15} levels using an elemental analyzer interfaced with an isotope ratio mass spectrometer (Europa Scientific Integra). The N content of foliage (dry weight basis) was also assessed. The amount of fixed N in foliage was calculated using the formula provided by Rennie et al. (1978) which involves determining the percent N derived from the atmosphere (% Ndfa) as follows:

$$\%\text{Ndfa} = 1 - \frac{\text{atom \% } ^{15}\text{N excess (inoculated plant)}}{\text{atom \% } ^{15}\text{N excess(control plant)}^{*}} \text{ x } 100$$

* Dual calculations were performed for % Ndfa using (i) Uninoculated plants in the first instance and then (ii) those inoculated with heat killed P2b-2R as control plants.

3.2.4 Statistical Analyses

Analysis of variance (ANOVA) $\alpha < 0.05$ was performed using the general linear model procedure (GLM, SAS Institute Inc., Carey, NC) to determine whether the plant growth parameters (plant, root and shoot lengths, fresh weights, and dry weights), log of colony forming units of bacteria in plants, total foliar nitrogen and atom percent excess of N^{15} varied significantly among inoculant treatments (live P2b-2R cells, heat killed P2b-2R cells and uninoculated control). The general model of ANOVA with source, degrees of freedom, mean square formula and error term is given in Table 3.1. Least significant difference (SAS Institute Inc, Carey, NC) tests were used to determine statistical significance of differences between any two pairs of treatment means.

Table 3.1 General Model for ANOVA design

Source of variance	df formula	MS	F –Ratio
T	t-1	MST	MST/MSE
r(t)	t(r-1)	MSE	
Total	r(t)-1		

Where t is treatment (P2b-2R, heat killed P2b-2R and un inoculated control), r is the number of replications.

Differences in seedling mortality were tested for significance using the two sided chi square (χ^2) contingency test using the JMP 8.0.2 statistical discovery software (SAS Institute Inc. Carey, NC). The observed frequencies of dead and surviving seedlings of inoculated treatments were tested against the frequencies of dead and surviving seedlings of the uninoculated control (expected frequencies) in order to test the hypothesis (H_0) that seedling mortality was lower in the inoculated treatments as compared to the uninoculated control. Both inoculated treatments (heat killed and live P2b-2R) were tested against uninoculated control separately.

Table 3.2 Layout of the χ^2 contingency table for seedling mortality

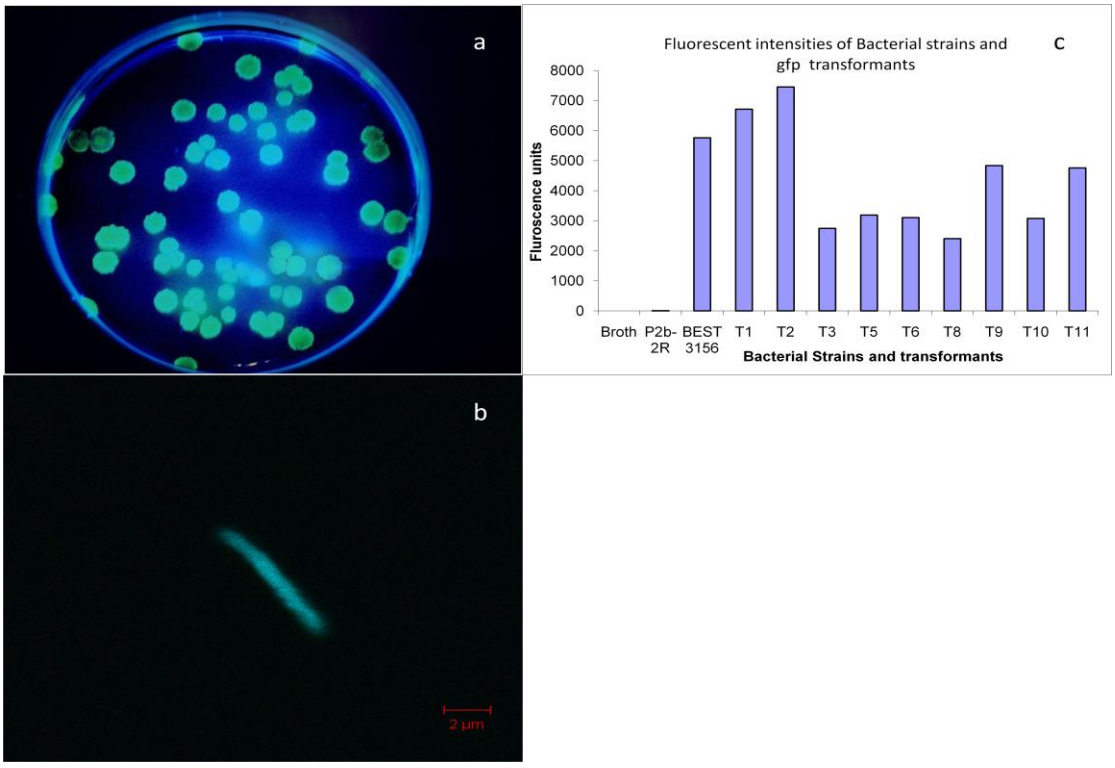
Category	Observed Frequency	Expected Frequency
Dead seedlings	Frequency of dead seedlings of heat killed or live P2b-2R inoculated treatment	Frequency of dead seedlings of uninoculated control
Surviving seedlings	Frequency of surviving seedlings of heat killed or live P2b-2R inoculated treatment	Frequency of surviving seedlings of uninoculated control

3.3 Results

3.3.1 GFP Tagging of *P.polymyxa* P2b-2R

Electroporation of strain P2b-2R with *gfp* plasmid pBSVG104 resulted in 9 transformant colonies selected on LB agar amended with 5µg/ml chloramphanicol. All transformants clearly emitted green fluorescence under the 310 nm UV light. A representative transformant T1 (P2b-2R *gfp)* is shown in Fig 3.1a. Two transformants (numbers T1 and T2) were found to have higher fluorescent intensity than the carrier strain BEST 3156 (Fig 3.1c). Although transformant T2 had higher fluorescent intensity than T1, T1 was chosen for endophytic localization experiments since its growth rate was similar to the parent strain P2b-2R, whereas the growth rate of transformant T2 was only 54 % of the parent strain. Plasmid stability was also higher in T1 (88.2%) as compared to T2 (66.4%) after 10 passages through non-selective medium. Transformant T1 was named strain P2b-2R*gfp* for name conformity. Green fluorescent protein was clearly detectable microscopically, in cells of P2b-2R*gfp*, that emitted light consistently between 506-540 nm when excited with a 488 nm argon laser (Fig 3.1b).

Figure 3.1 Fluorescence images of GFP-transformed P. polymyxa strain P2b-2R. a) P2b-2R gfp colonies formed on LB-Agar plates as seen under 310 nm UV light. b) Fig 1b: Single cell of P2b-2R gfp under a 100X/1.3 oil objective under a Ziess 510 Meta confocal microscope upon excitation with 488 nm laser. c) Comparison of fluorescence signal intensities (units) of controls and gfp transformants. (Standard errors of the mean have not been shown due to very small values (0.88-18.0), as compared to the 1000X scale of fluorescence intensity units).



3.3.2 Visualization of P.polymyxa in Lodgepole Pine Tissues

Bacteria were detected in both pine seedling roots and stems. The best visualization of the association was demonstrated in longitudinal sections of stem between 4-6 weeks of growth. Visualization in roots and stems of seedlings younger than 4 weeks and older than 6 weeks was problematic due to excessive plant auto-fluorescence, which is characteristic of conifers (Timonen, 1995). However, wherever green fluorescent bacterial cells could be observed without much interference from the background, the peak emission frequency of GFP (517-528nm) could be clearly distinguished from background auto-fluorescence of plant material (560-570nm) (Fig. 3.2 b).

Bacteria typically colonized the stem cortical tissues inter- (Fig. 3.2a) and intra-cellularly (Fig. 3.3, 3.5a, 3.5b). A large number of bacteria were detected within one or more

cortical cells of stems (see appendix D, Fig D-1 for a labeled diagram of pine stem histology) from at least 6, randomly chosen, seedlings. In some cells a few bacteria were found in the vicinity of, or possibly adherent to, the chloroplasts, while others were distributed throughout the cell (Fig. 3.5). To the best of our knowledge this is the first report of intracellular colonization of plant tissues by a growth promoting bacterium in association with a non-leguminous host. Colonization was also evident in longitudinal sections of root (Fig. 3.2b), but endophytic localization was problematic due to the difficulty in surface sterilizing the root surface completely before sectioning.

Figure 3.2 a) Longitudinal section of lodgepole pine stem colonized by GFP-labelled *P.polymyxa* strain P2b-2Rgfp (Excitation 488nm, 40X/1.1W C-apochromat objective). b) Longitudinal section of pine root showing P2b-2Rgfp and background fluorescence (488 nm, EC Plan Neofluar 100 X/ 1.3 oil objective). The arrows indicate GFP tagged cells

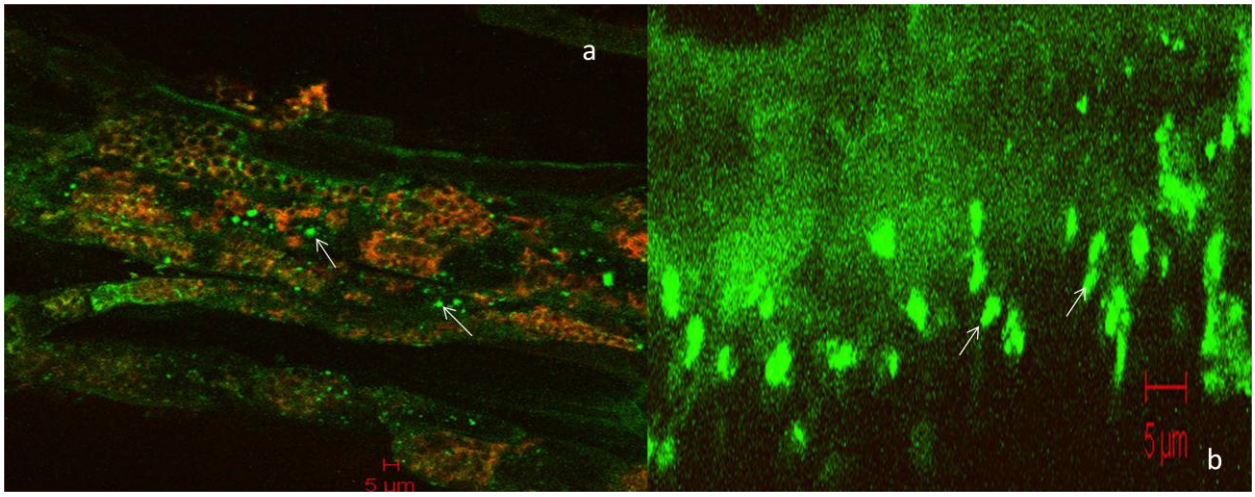


Figure 3.3 Colonization of a cortical cell of lodgepole pine with P2b-2Rgfp seen in a longitudinal section (Excitation 488nm, 40X/1.1W C-apochromat objective). The stem epidermis is marked by a white triangle. Arrow points to gfp tagged cells.

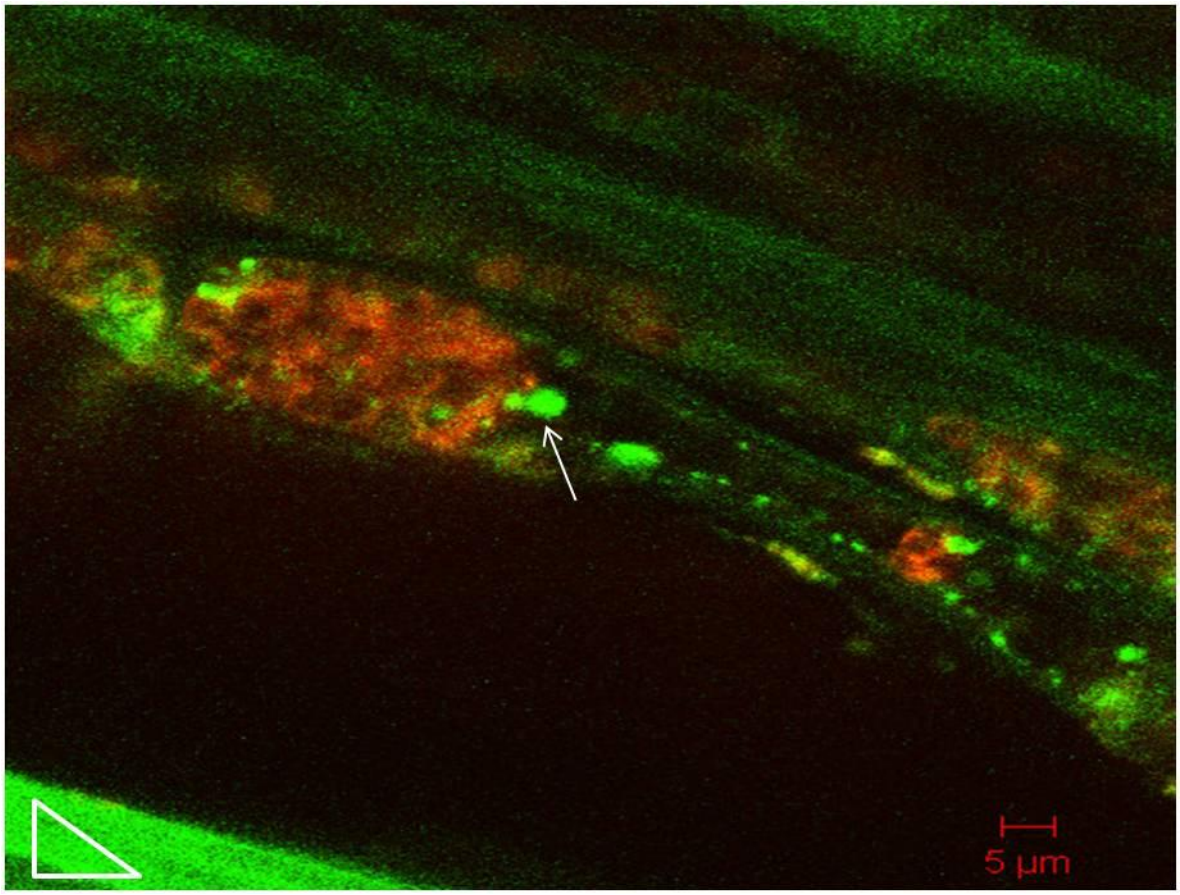
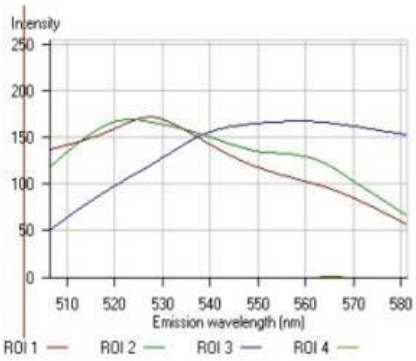
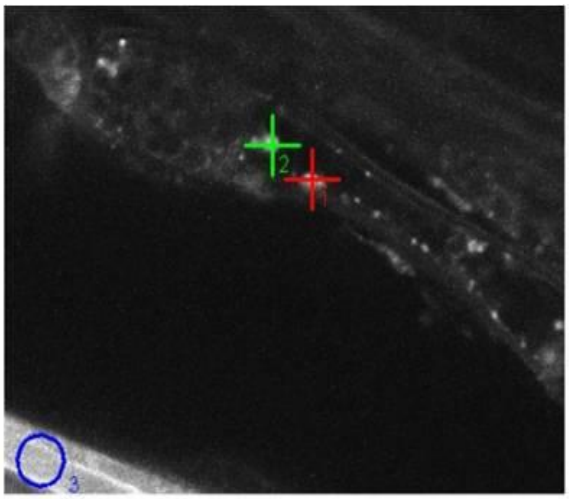


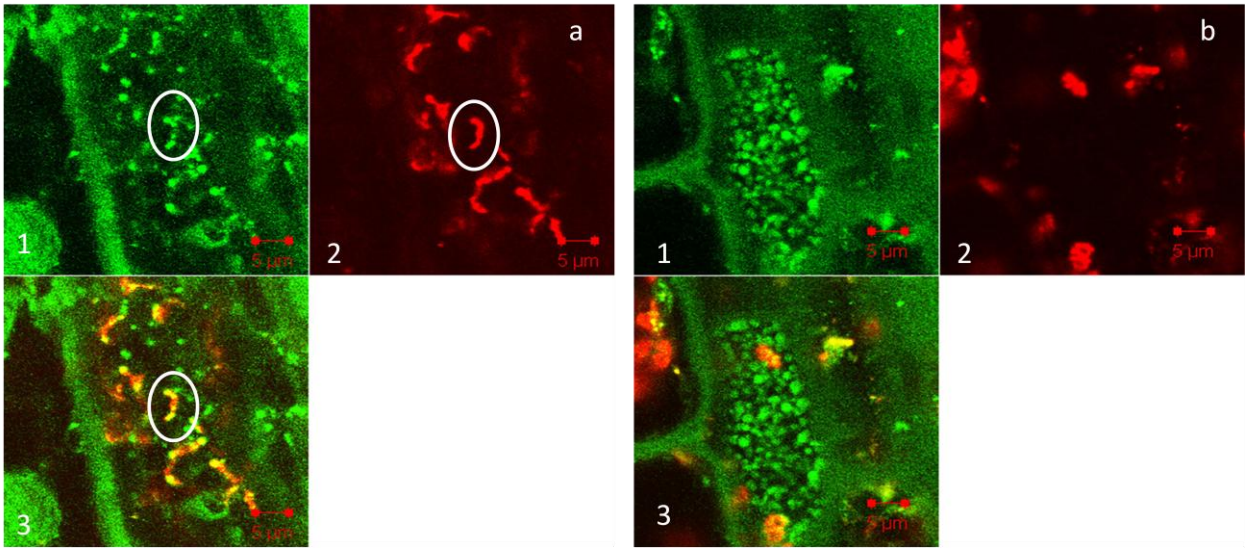
Figure 3.4 Spectral emission profiles of points of fluorescence emissions shown in Fig 3a. Blue circle and blue line graph represent auto-fluorescence of plant material (maximum emission intensity at 560 nm). P2b-2R*gfp* cells and their emission profiles are represented by green and red crosses and associated line graphs, respectively (maximum emission intensity at 528 nm).





Emission wavelength [nm]	IntensityROI 1	IntensityROI 2	IntensityROI 3	IntensityROI 4
517	153.0	160.4	88.6	0.0
528	171.6	166.4	120.5	0.0
538	147.2	153.4	152.6	0.0
549	119.6	136.0	164.9	0.0
560	102.7	130.1	167.7	0.0
570	84.6	102.4	161.8	0.0
581	57.0	66.8	152.7	0.0

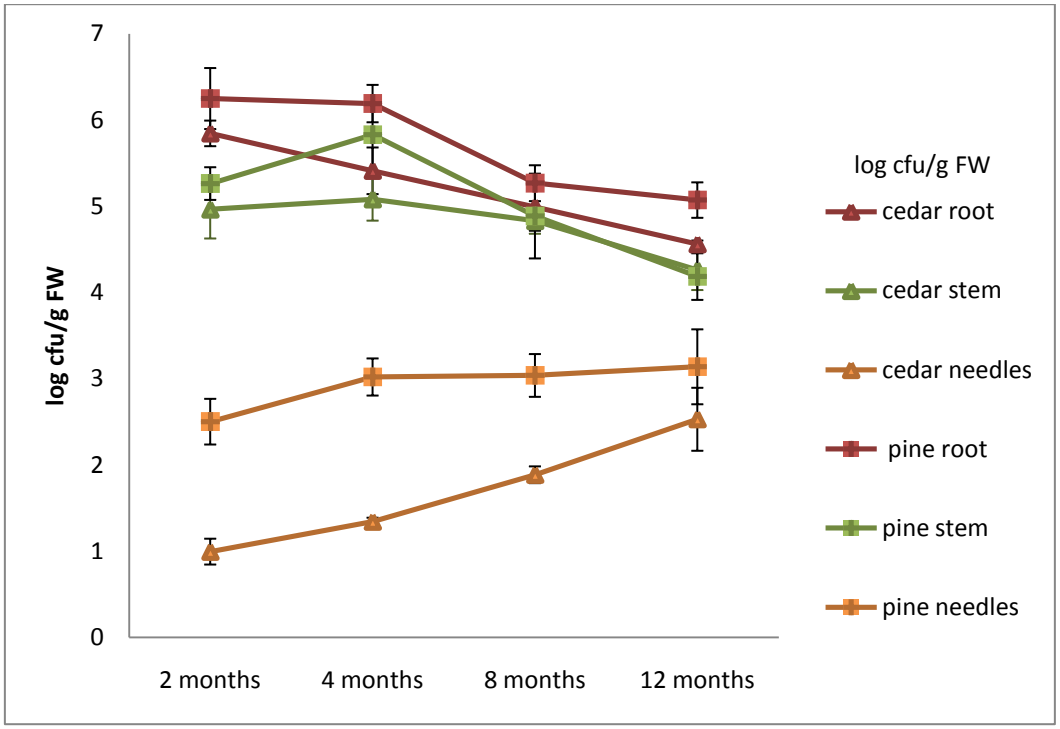
Figure 3.5 Colonization of cortical cells of pine by *P. polymyxa* P2b-2Rgfp (a and b). Series of images display GFP and plant material in green (a 1 and b 1), chloroplasts in red (a 2 and b 2) and a combination of both green and red channels (a 3 and b 3). White circles (a 1, 2, 3) mark close arrangement of P2b-2Rgfp cells in close proximity to chloroplasts.



3.3.3 Endophytic Population Density

Using surface sterilization, grinding and selective plating techniques we were able to re-isolate strain P2b-2R from root, stem and needle tissues of lodgepole pine and western red cedar at 2, 4, 8 and 12 months after inoculation. Root, stem and needle tissues of cedar and pine harboured between (log cfu/ g fresh weight) 4.56-6.24, 4.18-5.8 and 0.9-3.19 bacteria, respectively, over the 12 month growth period (Fig 3.6). For all plant tissues across all sampling dates pine contained higher population densities of *P. polymyxa* P2b-2R than cedar, and this was significant ($p<0.05$) for needle tissues over the entire study period and for root tissues upto the 8 month sampling point. In both tree species, bacterial population densities in roots declined over the 12 month period, whereas the population in needles showed an increase (Fig. 3.6). The bacterial populations in stem tissues increased from months 2 to 4, but then declined.

Figure 3.6 Population densities of *P. polymyxa* strain P2b-2R in surface sterilized tissues of lodgepole pine and western red cedar at 2, 4, 8 and 12 months after inoculation. Error bars represent standard errors of mean.



3.3.4 Growth Promotion and Nitrogen Fixation

After a 13-month growth period both pine and cedar seedlings inoculated with live cells of wild type P2b-2R accumulated significantly higher biomass (plant fresh and dry weights, $P < 0.05$), than both un-inoculated control seedlings and those inoculated with heat-killed bacteria (Table 3.2)(Fig.3.7). Pine seedlings inoculated with live bacterial cells were also significantly longer (total stem plus root length), than uninoculated seedlings, however, there was no effect of P2b-2R on the total length of cedar seedlings (Table 3.2) (Fig.3.7). There was no significant difference between plant length of un-inoculated control seedlings and those treated with heat-killed bacteria for both plant species. It was also observed that seedling mortality of both pine and cedar was highest in un-inoculated controls (24%), followed by the heat-killed bacterial treatment (19%) compared to the seedings inoculated with P2b-2R (12%) (Table 3.4). Based on the two sided chi square contingency test of goodness of fit, seedling mortality of the P2b-2R inoculated treatment was significantly lower than that of uninoculated

pine control (P=0.0205). Seedling mortality of pine inoculated with heat killed P2b-2R was not significantly different from uninoculated controls or the live P2b-2R inoculated treatment (P=0.3025). There was no significant differences in the seedling mortalities of heat killed (P=0.5078) and live P2b-2R (P=0.0978) inoculated treatments and uninoculated control for western redcedar.

Figure 3.7 Comparison of effect of P2b-2R inoculation on plant growth. (Error bars represent standard errors of mean). Significant differences between treatments for each parameter for each plant species are represented by different letters).

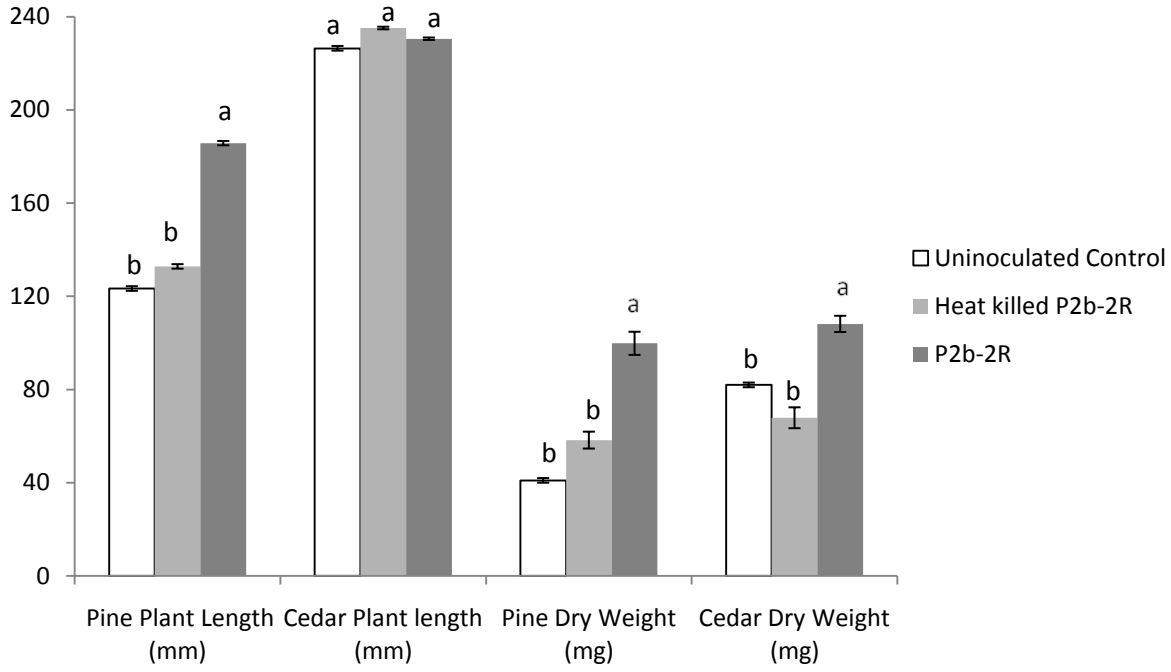


Table 3.3 Analysis of Variance of seedling growth and nitrogen fixation data. Bold letters indicate significant results for α=0.05.

Variable	*p*- value	
	Lodgepole pine	**Western red cedar**
Plant length	**<.0001**	0.392
Plant dry weight	**<.0001**	**<.0001**
N^{15} atom percent excess	**0.01**	**0.0075**
Total Foliar Nitrogen	**<.0001**	0.828

Table 3.4 Seedling mortality over 13 month growth period

Treatment	Seedling mortality (percent)	
	Lodgepole pine	Western red cedar
Uninoculated	24	24
Heat-Killed P2b-2R	18	20
P2b-2R	10	14

Total foliar nitrogen was significantly higher in pine seedlings inoculated with live P2b-2R, whereas cedar showed no significant difference in total foliar nitrogen between treatments (Table 3.3, Fig.3.8). N^{15} atom percent excess (atom % N^{15}-0.3663) was used as a measure of nitrogen derived from sources other than the enriched planting medium. Both pine and cedar inoculated with live cells of strain P2b-2R had significantly lower N^{15} atom percent excess as compared to un-inoculated controls, indicating a greater proportion of N not derived from the planting medium in the inoculated seedlings (Table 3.3, Fig. 3.9).

Figure 3.8 Total foliar nitrogen in microgram/mg of foliar dry weight. (Error bars represent standard errors of mean).Significant differences between treatments for each plant species are marked by different letters.

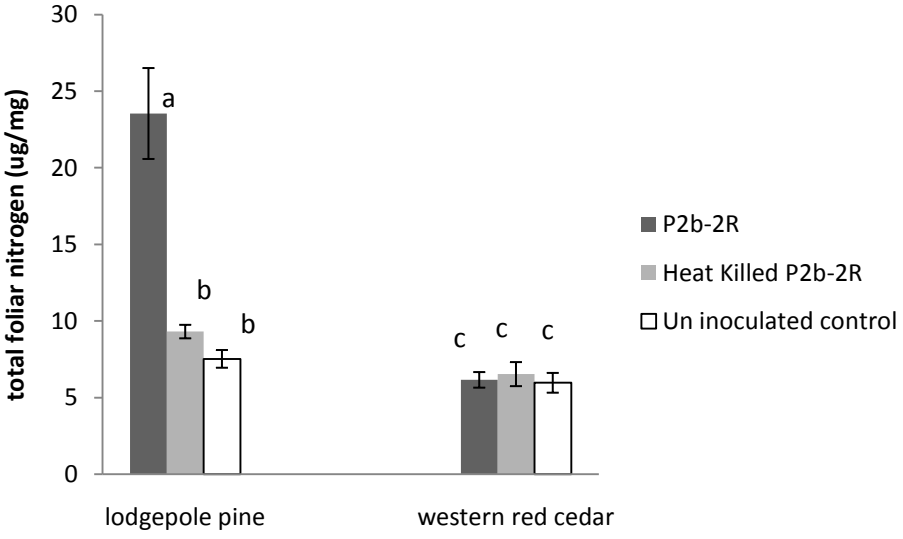
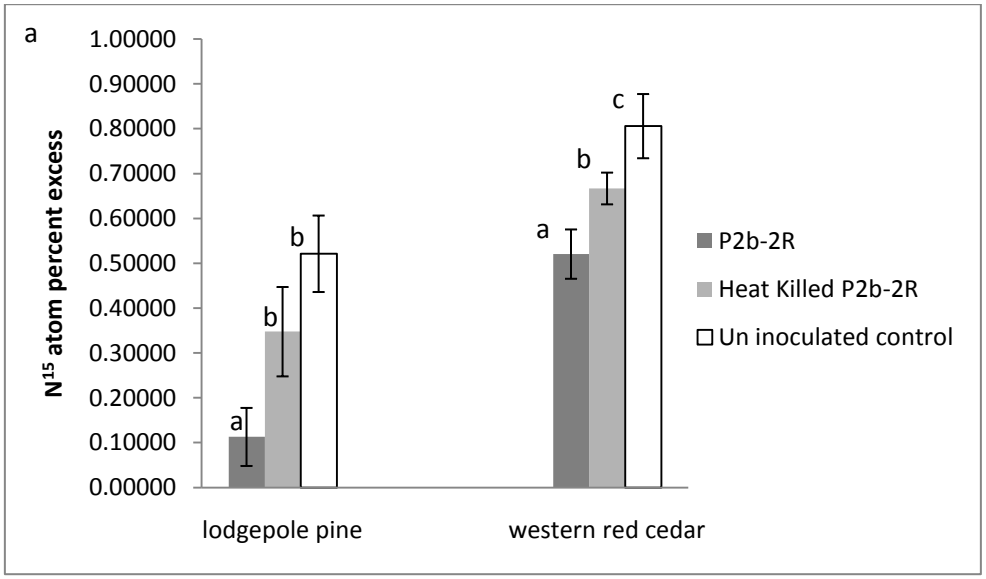


Figure 3.9 N^{15} atom percent excess in lodgepole pine and cedar foliage after a 13 month growth period. (Error bars represent standard error of means). Significant differences between treatments for each plant species are marked by different letters



Lodgepole pine seedlings inoculated with live P2b-2R and heat-killed cells derived 78 % and 33% of their nitrogen from the atmosphere or sources other than the N^{15} enriched planting medium, respectively (%Ndfa). Western redcedar seedlings treated with live cells and heat-killed cells derived 35.4 % and 17.2 % of their nitrogen from the atmosphere or sources other than the N^{15} enriched planting medium, respectively. When the N^{15} atom percent excess of the heat-killed treatment was used as the non-nitrogen fixing control, pine derived 67.53 % and cedar derived 21.94 % of its nitrogen from the atmosphere

3.4 Discussion

3.4.1 Colonization of Pine by *P. polymyxa*

This study clearly demonstrated that *P. polymyxa* strain P2b-2R can form sustaining endophytic populations in roots, stems and needles of lodgepole pine and western red cedar seedlings. Re-isolation of strain P2b-2R in culture, from inoculated seedlings satisfies a basic requirement of the Koch's postulates that an effect causing bacterium can be re-isolated from the affected individual after infection. However, since direct plating of surface-sterilized tissue extract alone has previously led to population overestimation and outright misidentification of

spore-forming *P. polymyxa* strain PW-2R (Shishido, 1995; Bent and Chanway, 2002) as a pine endophyte, we used GFP-tagged microscopic detection of bacteria *in situ* to obtain additional evidence that strain P2b-2R is capable of colonizing pine tissue internally.

The recovery of a consistent population of P2b-2R from stem and needle tissues over a 13 month growth period suggests that this bacterium is able to move up the plant into aerial parts after inoculation. Although bacterial population levels were generally higher in roots and stems as compared to the needles, it is interesting that populations decreased in root and stem over the growth period, whereas they increased in the needles. Compant *et al*., 2005, made a similar observation, after inoculation of grape plantlets with *Burkholderia sp*. Strain PsJN and suggested that bacterial cells eventually accumulate in the leaf which can be considered as sink, whereas the stem serves only for transition. The population densities of bacteria in our study are comparable to densities reported for other effective endophytic diazotrophs in other field and horticulture crops, which have ranged from $2.1x\ 10^4$ to $3.2x10^5$ cfu/g fresh weight in rice (Elbeltagy *et al*., 2001), $1.67x10^5$ to $5.5x10^7$ in sugarcane (Sevilla*et al*., 2001), $7.1x10^5$ to $1.4x10^9$ in grape (Compant *et al*., 2005). Although the present study was only conducted for a period of 13 months, there is evidence (Izumi *et al*., 2008) that bacterial endophytes of the genera *Bacillus* and *Paenibacillus* are able to colonize internal tissues of roots, stems and needles of coniferous trees aged up to 150 years. Sustained colonization over time would be required for ecologically significant nitrogen fixation in a forest stand, though availability of nitrogen during seedling establishment is also likely very important (Jose *et al*., 2003).

Green fluorescent protein has been effectively used to visualize endophytic bacteria *in situ* (Chelius and Triplett, 2000; Egener *et al*., 1998; Tanaka *et al.*, 2006) due to ease of detection without an exogenous substrate or cofactor. Since the chromophore requires both oxygen and water for the development of visible absorbance and fluorescence (Zimmer, 2002) the potential for misidentifying endospores as live bacteria is also avoided. Despite these advantages, the use of GFP for studying plant colonization by *P. polymyxa* is limited to only one report (Timmusk *et al*., 2005). In our efforts to tag strain P2b-2R, we found that successful tagging of this bacterium with constitutively expressed GFP was limited by the relatively few plasmid choices available for Gram-positive bacteria (Dunn and Handelsman, 1999; Carniol *et*

al., 2005; Itaya *et al*., 2001) and difficulties in delivery (Rosado *et al.*, 1994, observations in our lab) and maintenance (Timmusk *et al.*, 2005) of the plasmid in the bacterial cells. We found that electroporation protocols developed for *Bacillus* species worked well in general, with slight variations like growing bacterial cells in BHI medium (Macaluso and Mettus, 1991) without mannitol, as opposed to Glucose Broth (Rosado *et al*., 1994) or Tryptic soy broth (TSB) (Turgeon *et al*., 2006) and extra washing steps with the electroporation buffer to overcome the high exo-polysaccharide production that is characteristic of *P. polymyxa* (Yegorenkova *et al.*, 2008).

A big deterrent to using GFP with conifers is the autofluorescence generated from tissues (mostly lignin) and secondary metabolites (eg. phenolic compounds). In this work we observed very high auto-fluorescence in tissues of young roots, which may be due to the presence of large quantities of polysaccharide grains that emit a wide spectrum of auto-fluorescence with 340-560 nm excitation wavelength (Timonen, 1995). Although we were able to distinguish background fluorescence from fluorescence generated by GFP, based on the emission spectral profile it was difficult to derive any specific localization information from root sections (Fig.3.2 b). We also found that young needle tissues mostly lost their turgidity during sample preparation for microscopy due to loss of sap. Therefore, studying needle colonization might require freezing and embedding techniques followed by immune- localization of GFP or electron microscopy. Longitudinal sections of 4-6 week old stems were found to be ideal candidates for GFP-aided colonization studies of pine, if GFP expression can be maintained at adequately high levels to be visually distinguishable from the auto-fluorescence.

Although endophytic bacteria are generally found to colonize intercellular spaces of cortical cells and xylem vessels (Chelius and Triplett, 2000; Lodewyck, 2002), there are some reports of intracellular colonization by non-symbiotic endophytic bacteria (Compant *et al*., 2005; Hurek *et al*., 1994; James and Olivares, 1998). In our study we found that *P. polymyxa* strain P2b-2R colonizes inter- as well as intra-cellular spaces of cortical cells of pine stem. Since we were unable to detect bacteria in vascular tissues we believe that after gaining entry through the root (Timmusk *et al*., 2005), these bacteria might spread systemically through apoplastic transport (Reinhold-Hurek *et al*., 2007) which is defined as the movement of water and solutes through a continuum of cell walls of adjacent cells as well as extracellular spaces. We observed

at least three intact cells or cell-like structures, in different stem sections, that were internally colonized with GFP-marked bacteria. We hypothesize that these cells or cell-like structures could be specialized seats of nitrogen fixation, similar to, but not as highly specialized as, *Rhizobium* or *Frankia* nodules. Such specialized structures have also been reported by You *et al*. (1983 and 1991) in wetland rice tissues enclosing *Alcaligenes faecalis*. The fact that all of the observed structures had chloroplasts in them, leads us to believe that these structures were plant cells initially.

The apparent association of bacteria with chloroplasts (fig 3.5a) and in the vicinity of chloroplasts (3.5a, 3.5b) is of interest because chloroplasts are oxygen-rich microenvironments within the cell, due to photosynthetic oxygen evolution (Dixon *et al*., 1997) and the Fe protein of the nitrogenase enzyme (NifH) is known to be oxygen sensitive. To the best of our knowledge this is the first observation of such an arrangement of endophytic bacteria in higher plants, although some algae have been found to harbour virus like particles and endophytic bacteria near their chloroplasts (Colombo, 1978, Preisig and Hibberd, 1984). Cheng *et al*., (2005), successfully demonstrated expression of the *Klebsiella pneumoniae* NifH protein in chloroplasts of *Chlamydomonas reinhardtii*. By analogy, it is possible that the *nif*H of *P. polymyxa* retains function at high oxygen concentrations, while bacterial cells derive photosynthetic carbon from chloroplasts, which would be a distinct advantage due to the high carbon demand of the diazotrophic process.

Another possibility is that *P. polymyxa* P2b-2R employs specialized oxygen protection systems similar to those employed by non-heterocystous cyanobacteria like *Oscillatoria sp*., that are known to fix nitrogen aerobically without the use of structural mechanisms like leghaemoglobin in rhizobial nodules, actinorhizal vesicles or cyanobacterial heterocysts. Some members of the genus *Oscillatoria* are known to synthesize nitrogenase at a high rate to counteract the loss of nitrogenase due to photosynthetic oxygen while others employ a switch off mechanism to respond to short term exposure to oxygen, in addition to a temporal separation of nitrogen fixation and the oxygenic process of photosynthesis by chloroplasts (Carpenter and Price, 1976).

It is also possible that the bacterial cells located close to the chloroplasts sacrifice their nitrogenase activity by absorbing oxygen evolving from the chloroplasts, creating a relatively anoxic environment for other bacterial cells located elsewhere in the plant cells, thus protecting their nitrogenase enzyme. Such a micro-spatial protection mechanism is also know in the planktonic cyanobacterium *Oscillatoria erythraea* that protects its nitrogenase enzyme in oxygen-deficient microsites within macroscopic aggregates of trichomes (Bryceson & Fay, 1981).

Other possible mechanisms of nitrogenase protection could be metabolic consumption of oxygen by higher bacterial respiration rates, enhanced superoxide dismutase activity and cell aggregation, similar to those employed by *Azotobacter vinelandii* (Gallon, 1992).

However, these are speculations and further research is required to determine the mechanism by which *P.polymyxa* nitrogenase is protected in, what is likely an oxygen-rich micro-environment.

3.4.2 Growth Promotion and Nitrogen Fixation

Lodgepole pine and western redcedar accumulated significantly higher biomass when inoculated with strain *P. polymyxa*. P2b-2R. Strain P2b-2R is a naturally occurring diazotroph isolated from 2 year-old seedlings of lodgepole pine growing naturally in a forest near Williams Lake, BC. We included western redcedar in our study to test whether growth promotion and nitrogen fixation by P2b-2R is host specific. Growth parameters and total nitrogen between the two species are not comparable due to variable growth and soil N uptake patterns between plant species (vonWiren *et al*., 1997). However, it is evident that the effects of inoculation on growth promotion and nitrogen fixation were larger in lodgepole pine. Upon inoculation with strain P2b-2R, lodgepole pine seedlings (grown from seeds obtained from the same provenances as the sites of isolation of P2b-2R) accumulated more foliar total nitrogen/mg than western redcedar seedlings, when compared to their respective controls. Lodgepole pine seedlings also derived a higher percentage of their nitrogen from the atmosphere as compared to cedar seedlings. This finding is in agreement with the approach suggested by James and Olivares (1998),that use of specific, naturally-occurring; PGPR for growth promotion would be more effective than introducing non-specific inoculants. It also upholds the recommendation of

Chanway *et al*., 2000, that PGPR inoculants should be matched with host plant species and sites of origin in order for the inoculants to impart optimum plant growth benefits.

Seedlings of both tree species inoculated with live cells of *P. polymyxa* P2b-2R also showed higher survival rates than the uninoculated controls and those inoculated with heat killed bacteria, although statistically significant differences in seedling survival were only observed between pine seedlings inoculated with strain P2b-2R and uninoculated pine seedlings. This observation suggests that inoculation with *P. polymyxa* strain P2b-2R facilitates early establishment and survival of seedlings. This effect may be due to improved nitrogen nutrition of seedlings or other growth promoting properties of *P. polymyxa* such as phytohormone production (Gutierrez-Manero *et al*., 2001) improved phosphate uptake (Singh and Singh, 1993) and antibiotic production (Roasado *et al*., 1993; Choi *et al*., 2008). Postive effects of PGPR inoculation on seedling survival and establishment have been reported (Chanway and Holl, 1994; Enebak *et al*., 1998).

Although, a number of strains of *P. polymyxa* (previously *Bacillus polymyxa*) have been found to promote conifer growth (Holl and Chanway, 1992; Shishido *et al*., 1996), the observed effects were attributed mainly to phytohormone production (Timmusk *et* al., 1999) whereas nitrogen fixation, estimated through acetylene reduction could not be correlated to plant growth promotion (Shishido, 1997; Rhodes-Roberts, 1981; Lindberg *et al*., 1985). We used an N^{15} isotope dilution assay and compared the N^{15} atom percent excesses in plants treated with live P2b-2R and heat-killed P2b-2R to the N^{15} atom percent excess of un-inoculated controls. We demonstrated that lodgepole pine seedlings inoculated with live P2b-2R derive up to 78.35 % of their foliar nitrogen from the atmosphere. Our results are in agreement with preliminary results obtained by our research group, in which pine derived 27% (unpublished data), 30 % and 66 % (Bal, 2000) of its foliar nitrogen from the atmosphere 8, 9 and 11 months, respectively, after inoculation with *P. polymyxa* P2b-2R. This shows that nitrogen fixation by P2b-2R in association with pine, under controlled conditions, is in fact repeatable and not a one-time experimental artefact. It is noteworthy that the percentage of foliar nitrogen derived from the atmosphere increased with time over the duration of the growth trial. A similar trend

was reported in the sugarcane cultivar, Krakatau, which obtained an increasing percentage (6.2 to 54.8 %) of its nitrogen from the atmosphere over 4 samplings between 100 and 250 days after emergence (Uriquaga *et al*., 1992). This trend may be due to the decline in available nitrogen in the planting medium, as well as due to the establishment of the nitrogen fixation process and increasing populations of the bacteria in the foliage (Fig. 3.6).

Although we also found that pine and cedar seedlings inoculated with heat-killed bacteria derived 33.33 % and 17.25% of their nitrogen from sources other than the enriched planting medium, we suggest that this nitrogen might have been released from dead bacterial cells after inoculation. Considering that consistent populations of live P2b-2R were maintained in plant tissues over the growth period, it is evident that bacterial cell division, as well as death, would be occurring within these tissues, therefore releasing unlabelled nitrogen into the plant system resulting in overestimation of nitrogen truly derived from the atmosphere. To discount for unlabelled nitrogen gained from dead bacterial cells we also calculated a corrected % Ndfa of the live P2b-2R treated seedlings using the N^{15} atom percent excess of the heat-killed treatment as control instead of that of un-inoculated controls.

Although we cannot partition the growth promotion effects into those induced by other possible mechanisms such as phytohormone production from those caused by nitrogen fixation, our results show that *P. polymyxa* strain P2b-2R is an endophytic diazotrophic bacterium capable of promoting growth, fixing nitrogen and contributing fixed nitrogen to two important conifer species under nitrogen limitation that is characteristic of most temperate forests in western North America. It is possible that the endophytic colonization ability of this strain contributes to its plant growth promotion and nitrogen fixing ability. Pending further experimentation in the field, this and other such naturally occurring diazotrophs associated with conifer species can provide very important information regarding the nitrogen economy of coniferous forests. Field and greenhouse applications of such associations may parallel those of endophytic bacteria in agricultural crops such as sugarcane and rice (Boddey *et al*., 1995).

3.5 Acknowledgements

Funding for this study was provided by NSERC and a UBC Faculty of Forestry IMAJO Award to Chris Chanway and by NSERC (Canada Graduate Scholarship D 3) and The Ministry of Advanced Education of British Columbia (Pacific Leaders Graduate Student Fellowship) to Richa Anand. We are deeply indebted to Dr. M Itaya for gifting us plasmid pBSGV104 for this study.

4 Sequencing of Nitrogen Fixation (*nif*) Genes Using SSP (Single Specific Primer) and *nif* Phylogeny of *Paenibacillus polymyxa* Strain P2b-2R [4]

4.1 Introduction

Nitrogen fixation (*nif*) genes are widely distributed and highly conserved among eubacteria and archaea (Birgle *et al*., 1985, Dean and Jacobson, 1992, Kessler *et al*., 1998; Young, 1992). The nitrogenase enzyme complex is encoded for by three structural genes *nif* H, D and K which are arranged contiguously in a single operon in most diazotrophs. There are at least 17 other *nif* genes that have regulatory or supportive functions in nitrogen fixation (Madigan *et al*., 2000, functions and arrangement of all *nif* genes is described in Chapter 1 of this thesis). Variations in sequences and arrangement of *nif* genes provide important information on the inheritance of nitrogen fixation and its mechanism across diverse groups of bacteria and archaea (Chen *et al*., 1996; Potrich *et al*., 2001; Arnold *et al*., 1988; Kallas *et al*., 1985; Corbin *et al*., 1982; Haselkom, 1992; Sevilla, 1997).

The availability of reliable and adequate *nif* sequence information is useful in the study of gene function, regulation and *in-vivo* nitrogen fixation (Sevilla *et al*., 2001), where lack of function mutants are helpful tools. Direct methods of mutant development like gene disruption through double homology based recombination (Sato *et al*., 2003) or targeted-PCR (Taroncher-Oldenburg and Stephanopoulos, 2000) are dependent on the availability of sequence information and are faster and more reliable than chemical or radiation-based methods. Therefore, the study of known and newly identified diazotrophic bacteria can be expedited by the availability of gene sequence information.

Sequence information is also important for the purposes of understanding the evolutionary pathway of diazotrophy through lateral gene transfer (Zehr *et al.*, 2003). The

[4] A version of this chapter will be submitted for publication. R. Anand and C. Chanway (2010). Sequencing of nitrogen fixation (*nif)* genes using SSP-PCR (single specific primer) and *nif* phylogeny of *Paenibacillus polymyxa* Strain P2b-2R.

evolutionary history of nitrogen fixation is a much debated subject due to the random distribution of *nif* genes in distantly related lineages of prokaryotes, with remarkable conservation (Henson *et al*., 2004; Ruvkun and Ausubel, 1980). Some scientists have proposed that *nif* genes have been transferred laterally to various lineages from one ancestor in evolutionary history (Normand and Bousquet, 1989) while others believe that nitrogen fixation was vertically inherited and ubiquitous at one time but was lost by many lineages through evolution and retained by a few distantly related lineages that are represented by the present day diazotrophs (Kleiner *et al*., 1995).

Despite the use of advanced molecular phylogenetic techniques, examination of nif H, D and K genes continues to produce conflicting results supporting both lateral transfer and vertical descent when different methods of analysis are used (Henson *et al*., 2004; Hirsch *et al*., 1995). Henson *et al*. (2004) have found that phylogenetic trees based on the *nif*D gene support vertical descent when the NifD amino acid sequences were analyzed by parsimony analysis and when *nif*D nucleotide sequences were analyzed using the maximum likelihood method however distance analysis of nucleotide sequences provided evidence for lateral transfer. Similarly, Hirsch *et al*. (1995) found that parsimony analysis of the *nif* K gene of Frankia strain HFPCc13 provided support for vertical descent whereas bootstrapped distance analyses provided stronger evidence for horizontal gene transfer. Although, there is higher evidence of lateral transfer of the *nif*H gene based on both distance based and parsimony methods (Normand and Bousquet, 1989; Normand *et al*., 1992; Hirsch *et al*., 1995) there are a few reports that support vertical descent of *nif*H as well. Mathur and Tuli (1990), have shown that the divergence of *nif*D is intermediate between that of *nif*H and *nif*K making it a better marker to resolve *nif* phylogeny and the debate on lateral versus vertical inheritance (Hirsch *et al*., 1995; Normand *et al*., 1992) of nitrogen fixation. It is interesting that all phylogenetic studies cited above have found discrepancies among the placement of actinobacteria (*Frankia*), cyanobacteria and the Gram-positive bacteria (*Clostridium, Paenibacillus*) making these groups pivotal to the resolution of the debate about *nif* gene inheritance.

P. polymyxa strain P2b-2R is a recently identified Gram-positive endophyte of lodgepole pine that has been shown to fix nitrogen with high efficiency under controlled

conditions (Chapter 3 of this dissertation), making it a good candidate for studying the genetics and phylogeny of nitrogen fixation of this bacterial species. Although, many other nitrogen-fixing strains of *P. polymyxa* have been isolated from soil and the rhizospheres and internal tissues of various crop plants (von der Weid *et al*., 2002; Ding *et al*., 2005; Beneduzi *et al.*, 2008; Coelho *et al*, 2009) and naturally occurring forest trees (Shishido *et al.*,1995), the genetics of nitrogen fixation in this species is not well studied and has been limited to phylogenetic analyses of small internal fragments of the *nif*H gene (Achouak *et al*., 1999; Rosado *et al*., 1998; Zehr *et al*., 2003),

Recently, full sequences of some nitrogen fixation (*nif*) genes particularly, *nif* B, H, D and K have been reported for a few other species of *Paenibacillus* like *P. azotofixans* (Choo *et al*., 2003), *P. massiliensis* (Zhao *et al*., 2006), *P. graminis*, *P*. *fujiensis* and *P*. *abekawaensis* (sequences published in GenBank only). Despite a large gene snapshot survey of *P. polymyxa* (Jeong *et al*., 2007) sequence information for *nif* genes of the species remains limited to approximately 361 bp of a partial internal *nif*H fragment from various cultured and uncultured strains.

The aim of this study was to obtain increased *nif* sequence information for *P. polymyxa* in the region flanking the frequently sequenced internal *nif*H fragment and to study the arrangement of *nif* genes in the operon. Here I report the full *nif*H and partial *nif*B and *nif*D sequences of *P. polymyxa*. To the best of my knowledge, this is the first full sequence of *nif*H and first partial sequences of *nif*B and D genes reported for *P. polymyxa*. I also provide the first comparison of *Paenibacillus nif* phylogenies based on both *nif* H and D gene sequences to determine the taxonomic position of *P. polymyxa* strain P2b-2R relative to representatives of other groups of diazotrophic bacteria and archaea.

4.2 Materials and Methods

4.2.1 Extraction of Genomic DNA

P. polymyxa strain P2b-2R that was isolated from the internal tissues of lodgepole pine growing in the northern interior of British Columbia, Canada, using a nitrogen-free culture medium (Chapter 2, this thesis), was grown overnight in a 250 ml Erlenmeyer flask filled with

100 mL of full strength tryptic soy broth (BD, Sparks, USA) amended with 200ug/mL rifamycin (Sigma-Aldrich, ON, Ca) at 30^{o}C with shaking (150 rpm)(orbit shaker 3520, Lab-line instruments, Melrose park, Il, USA). One milliliter of the overnight grown culture was aliquoted into an eppendorf tube and centrifuged at 805 x g in a table top micro centrifuge (Hettich Mikro 20, Diamed, ON, Ca) to harvest cells of *P.polymyxa* P2b-2R. Total genomic DNA was extracted from harvested cells using the Qiagen DNAeasy kit (Cat no. 69504, Qiagen, Alamaeda, CA, USA) with lysozyme (37^{o}C for 30 min) and proteinase K (56^{o}C for 30 min) pre-treatment for optimum DNA yield from Gram-positive bacteria (page 45 of the Qiagen DNAeasy blood and tissue kit protocol).

4.2.2 Oligonucleotide Synthesis and DNA Sequencing

All oligonucleotide primers used in this study were custom synthesized at Integrated DNA technologies (San Diego, CA, USA). All PCR products and DNA fragments were sequenced using an Applied Biosystems 3730S 48-capillary DNA Analyzer (x2), with 50cm array and POP-7 BigDye® Terminator v3.1 Sequencing Chemistry at the Nucleic Acid Protein Service Unit (NAPS) at the University of British Columbia, Vancouver, Canada.

4.2.3 Preparation of *nif*H Probe

A 388-bp internal fragment (gb HM 185813) of the *nif*H gene of *P. polymyxa* P2b-2R was amplified using degenerate primers, 5´-TAY GGN AAR GGN GGN ATN GGN AA-3´ and 5´-GCR AAN CCN CCR CAN ACN ACR TC-3´(Choo *et. al*., 2003) corresponding to positions 19 to 42 and 383 to 405 of the *nif* H gene of *P. massiliensis* T7 (gb AY912109.3 region 3096-3962). The PCR reaction was performed using purified total genomic DNA of strain P2b-2R as template with *Taq* polymerase premixed in a 2X PCR master mix (Cat. No. K 0171, Fermentas, ON, Ca) and a Bio-Rad MJ mini personal thermal cycler (Cat. N0. PTC 1148 C, Bio-Rad, ON, Ca). The amplification conditions were as follows: initial denaturation 94^{o}C for 5 min, followed by 30 cycles of 30 sec at 94^{o}C, 30 sec at 48^{o}C and 30 sec at 72^{o}C and then a final extension step of 5 min at 72^{o}C.

The amplified fragment was sequenced and the sequence was compared to nucleotide sequences available in GenBank using the Basic local alignment search tool (BLAST,http:// www. ncbi.nlm.nih.gov/) to confirm its identity.

The 388 bp *nif*H fragment was labelled with DIG using the DIG high prime DNA labelling and detection starter kit II (Cat. No.11 585 614 910, Roche, Germany) for use as a probe in southern blot analysis.

4.2.4 Southern Blot Analysis

To identify enzymes that do not digest within the known region of the *nif*H gene, the 388 bp *nif*H sequence was analyzed using the NEB cutter tool (New England Biolabs, Ipswich, MA, USA). Two enzymes *Pst*I and *Hin*dIII (New England Biolabs, Ipswich, MA, USA) were used to digest purified, total genomic DNA of *P. polymyxa* P2b-2R in triplicate, for 10 hrs at 37^{o}C, after which the restriction enzymes were deactivated by heating the reaction mixture at 65^{o}C for 20 minutes. The digested DNA was cleaned up using the MinElute reaction clean up kit (Cat. No. 28204, Qiagen, Alameda, CA, USA). The digested genomic DNA was electrophoretically run on a 1% agarose gel at 30 volts over night and then transferred on to a Amersham Hybond^{TM}-N+ (132 mm) nylon membrane (GE healthcare, USA) with baking at 120^{o}C for 30 min. Southern blotting was performed using the DIG high prime DNA labelling and detection starter kit II (Cat. No.11 585 614 910, Roche,Germany) with chemiluminiscent detection with CSPD (Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl)phenyl phosphate), following the manufacturer's instructions.

4.2.5 SSP-PCR

To identify genomic sequences that lie adjacent to the known 388 bp *nif*H sequence of *P.polymyxa* P2b-2R we used a modification of the single specific primer–polymerase chain reaction (SSP-PCR) (Shyamala and Ames, 1989). The overall scheme of modified SSP-PCR is depicted in Fig 4.1. Genomic DNA of *P.polymyxa* P2b-2R and plasmid pSK were digested using restriction enzyme *Xba*I (Fig 4.1, step A) (New England Biolabs, Ipswich, MA,USA) at 37^{o}C for 10 hrs followed by deactivation of *Xba*I by a 20 min incubation at 65^{o}C. Both digestion reactions were cleaned up using the MinElute reaction clean up kit (Cat. No. 28204, Qiagen, Alamaeda, CA, USA) before the two were ligated (Fig 4.1, step B) by incubation for 16 hrs at 16^{o}C, using T4 DNA ligase (Cat. No. M1804, Promega corp., Madison, WI, USA). The ligation mixture was used as template DNA for PCR in a total volume of 25 µl using Taq polymerase premixed in a 2X PCR master mix (Cat. No. K 0171, Fermentas, ON, Ca) and a Bio-Rad MJ mini personal thermal cycler

(Cat. N0. PTC 1148 C, Bio-Rad, ON,Ca). The primers used for PCR are listed in Table 4.1. Primers T3 and T7 are widely used primer sequences, flanking the multiple cloning site of pSK whilst primes FH5 and RH5 were designed corresponding to the 5' and 3' ends of the 388bp known *nif*H gene sequence respectively, using a web based primer design tool, Primer 3 (Rosen and Skaletsky, 2000) available at http://primer3.sourceforge.net/releases.php.

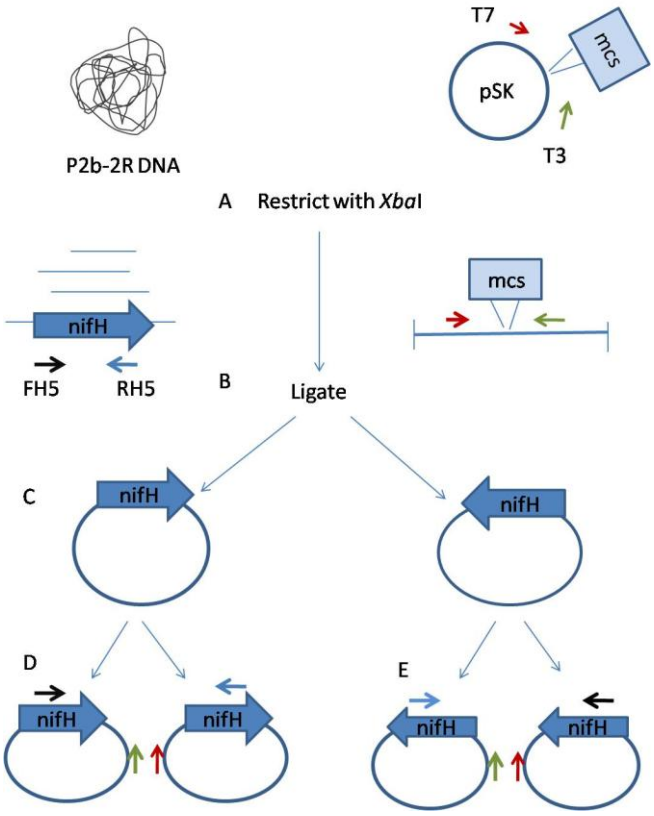
Because the restricted *P. polymyxa* P2b-2R DNA containing the known sequence could ligate into pSK in either direction (fig. 4.1, step c), all 4 possible combinations of T3, T7 and FH5, RH5 primers (Fig 4.1, steps D&E) were used for amplifying DNA regions adjacent to the known *nif*H sequence. The amplification conditions were as follows: initial denaturation 94^{o}C for 5 min, followed by 30 cycles of 30 sec at 94^{o}C, 30 sec at 48^{o}C and 60 sec at 72^{o}C and then a final extension step of 5 min at 72^{o}C. The PCR product was run on a 1% agarose gel and extracted from the gel using a MinElute gel extraction kit (Cat. No. 28604, Qiagen, Alamaeda, CA, USA) before being sequenced. Each PCR product was sequenced twice, separately using both primers corresponding to the initial PCR reaction that yielded the product being sequenced.

Table 4.1 Summary of primers used for SSP-PCR for amplification of unknown DNA sequences flanking the known 388bp *nif*H gene sequence of *P.polymyxa* P2b-2R

Primer name	Source	Primer sequence
T3	pSK (Stratagene,USA)	ATT AAC CCT CAC TAA AGG GA
T7	pSK (Stratagene, USA)	TAA TAC GAC TCA CTA TAG GG
FH5	This study	GGGGATGGGGAAATCGACAACC
RH5	This study	TGCAGACCACGTCCCCCAGTACA

Primer abbreviations: F, forward; R, reverse; H, nifH. All primer sequences are presented in 5' to 3' orientation.

Figure 4.1 Outline of the technique used to identify unknown nucleotide sequences flanking the 388 bp long known *nif*H sequence. *P. polymyxa* P2b-2R genomic DNA and Plasmid pSK (BlueScript, Agilent technolgies, Stratagene division, USA) were restricted with XbaI (A) and then ligated together (B). The restricted *P. polymyxa* P2b-2R DNA can ligate into pSK in either orientation (C). PCR was performed using different combinations of the *nif*H primers (FH5 and RH5) located in the known region of the *nif*H gene and the T7 and T3 primers located on the plasmid pSK.



The sequences recovered by this method were analyzed using the BLAST tool, ORF (open reading frame) finder (National center for biotechnology information) and manual examination. Overlapping (contigs) sequences of interest were joined together using ContigExpress® (Invitrogen, ON, Ca).

4.2.6 Phylogenetic Analysis

The *nif*H (this Chapter), *nif*D (this chapter) and 16s rDNA (*rrs*) (GU132543, Chapter 2 this thesis) were used for phylogenetic analyses. The *nif*H, *nif*D and *rrs* sequences of other organisms included in the phylogentic analyses were obtained by conducting GenBank searches through NCBI (National center for biotechnology information). Organisms representing same

genera and species were included in all three trees to maintain consistency and facilitate comparison. Seaparate, detailed trees based on *nif*H and D phylogenies were constructed for better comparison within the genus *Paenibacillus*.

Sequences were aligned using *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. (Tamura *et al*., 2007). MEGA4 uses clustal W (Thompson *et al*., 1994) to align sequences, the Tamura-Nei (1993) model to correct matrix pairwise comparisons for multiple base substitutions and the Maximum Composite Likelihood (MCL) method for estimating evolutionary distances (*dij*) between DNA sequences. Phylogentic trees were also constructed and drawn with *MEGA4* by the neighbour- joining method (Saitou & Nei, 1987). A bootstrap analysis was performed on 1000 replicates to determine the reliability of the distance tree. The neighbor-joining (N-J) method was used to infer trees to facilitate direct comparison with previous studies (Achouak *et al*., 1999; Zehr *et al*., 2003., Hirsch *et al*., 1995; Henson *et al*., 2004) that also used this method. Another rationale for using the N-J method is that it generates a tree quickly for data sets containing many sequences and the difference between N-J trees and those produced by other time-consuming methods are localized to parts of the trees that were statistically weakly supported, thus making it a popular and reliable method, cited in more than 16,000 publications until 2008 (Kumar *et al*., 2008).

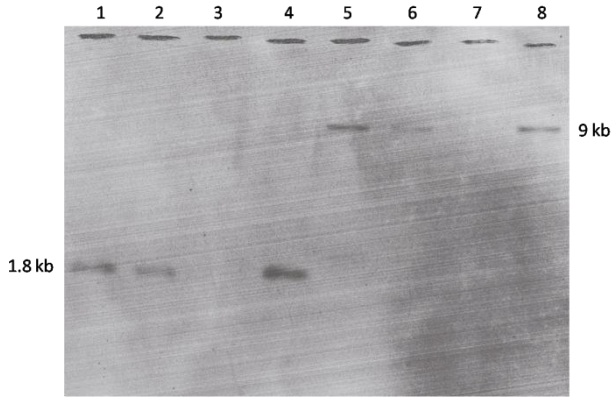
4.3 Results

4.3.1 PCR Amplification of nifH Fragment and Southern Blot Analysis

A 388-bp fragment (HM 185813) was PCR-amplified using degenerate primers to target the *nif*H gene of *P. polymyxa*. BLAST analysis of this fragment confirmed its identity as a partial coding sequence (cds) for the dinitrogenase reductase (NifH) protein, corresponding to positions 97-399 and positions 30-393 of the approximately 874-bp long complete cds of the *nif*H genes of *Klebsiella pneumoniae* 342 (AY242355) (84 % similarity) and *P. massiliensis* (AY912109.3, region 3096-3962)(98% similarity) respectively. After confirmation of its identity this 388-bp *nif* H fragment was used as a DIG-labelled probe for southern blot analysis of the *Pst* I/*Hin*dIII digested genomic DNA of *P.polymyxa* P2b-2R. The southern blot profile showed

only one positive signal band at 1.8 kb and 9 kb of the *Pst*I and *Hin*dIII digests respectively (Fig.4.2), in all three replicates, suggesting that there is only one copy of *nif*H in *P. polymyxa* P2b-2R.

Figure 4.2 Southern blot profile of Pst I (Lanes 1,2 and 4)/*Hin*dIII (Lanes 5,6 and 8)- digested *P.polymyxa* P2b-2R total DNA probed with *nif* H fragment



4.3.2 SSP-PCR Amplification of *nif*BHD from *P.polymyxa* P2b-2R

SSP-PCR amplification was performed in order to obtain sequence information of the unknown DNA regions flanking the known 388-bp *nif*H gene. PCR amplification with primer combination T7 and RH5 resulted in a 1070-bp product, while a 1400 bp product was recovered from primer combination T3 and FH5. The other two primer combinations, namely T3-RH5 and T7-FH5 did not yield a PCR product, suggesting that the *Xba*I digested fragments of *P.polymyxa* P2b-2R genomic DNA containing the known 388-bp *nif*H sequence were ligated into the pSK vector in the forward orientation.

Upon sequencing with both primers T7 and RH5 separately, the 1070 bp product yielded a 1042-bp readable nucleotide sequence. The 1400-bp product was sequenced using both primers T3 and FH5 separately, resulting in a 1390-bp readable nucleotide sequence.

4.3.3 Sequence Analysis of SSP-PCR Products

According to BLAST analysis nucleotides 1-582 of the 1042 bp fragment were identified as partial sequence of the *nif*B gene including its termination codon at the 3' end. The *nif*B gene codes for the NifB cofactor which acts as an iron and sulphur donor to the FeMo-co site of substrate reduction that is buried in the α subunit of dinitrogenase encoded by *nif*D (Fischer,

1994). Nucleotides 693-1042 of this fragment were identified as a partial sequence of the *nif*H gene including its start codon ATG at the 5'end. Nucleotides 724-1042 of this sequence are 99% similar to the known 388 bp *nif*H sequence of *P.polymyxa* strain P2b-2R, that was used as a probe for southern blot analysis in this study. The two genes are separated by an intervening 111 bp nucleotide sequence which does not code for a protein. This intervening sequence was examined for the presence of a sigma 54 type promoter sequence, characteristic of *nif* genes (Barrios *et al.*, 1999) and ribosome binding sites, but no promoter-like sequence or ribosomal binding sites were detected.

Nucleotides 1-771 of the 1400-bp T3-FH5 fragment were identified as a partial sequence of the *nif*H gene including the termination codon TAG. The first 280-bp of this 770-bp region was found to overlap the last 280-bp of the 1042 bp T7-RH5 sequence. Nucleotides 1-304 of this sequence also had 99% similarity to the known 388-bp *nif*H sequence of *P.polymyxa* strain P2b-2R used as probe for the southern blot analysis in this study. Nucleotides 986-1390 of the 1400-bp T3-FH5 fragment were identified as a partial sequence of the *nif*D gene, including its start codon. The *nif*H and *nif*D gene sequences are separated by a 215-bp non-coding nucleotide sequence.

The 280-bp overlap between the two SSP-PCR fragments suggests that they are contiguous sequences, which on joining with ContigExpress® resulted in a 2208-bp sequence (GenBank HM146187), containing a full sequence of the *nif*H gene flanked by a partial *nif*B gene sequence at its 5´end and a partial *nif*D gene sequence at its 3´ end. To the best of our knowledge this is the first full gene sequence of a *P.polymyxa nif*H gene, whereas the partial *nif*B and *nif*D sequences are the only reported sequences of these genes of this species of *Paenibacillus*. Important features of this 2208-bp nucleotide sequence are depicted in Fig 4.3.

Figure 4.3 Characteristics of the 2208-bp *nif*BHD sequence of *P. polymyxa* P2b-2R. The start codon ATG of *nif*H and *nif*D genes are indicated by the red and blue ovals respectively. The termination codons of *nif*B and *nif*H are indicated by the blue and red rectangles respectively. Primer FH5 is underlined in red whereas the reverse complement of primer RH5 is underlined in blue. The area including and enclosed by these two primer sequences corresponds to the known 388-bp *nif*H sequence of *P.polymyxa* strain P2b-2R.

4.3.4 Phylogenetic Analysis

Phylogenetic trees were constructed for the full *nif*H polypeptide sequences (Fig. 4.4), partial n*if*D polypeptides (Fig. 4.5) and partial sequence of the *rrs* genes (Fig. 4.6) of selected diazotrophic bacteria. For ease of comparison we attempted to include similar bacterial genera and species in all trees depending upon availability of reliable sequences. *P. polymyxa* P2b-2R is part of a monophyletic cluster with other paenibacilli based on all three gene sequences. The genus *Paenibacillus* also forms a distinct group in all phylogenetic trees except the *nif*D tree, where it clusters with actinobacterium *Frankia* within a clade. Although the Gram-positive actinobacterium *Frankia* forms a clade with the paenibacilli on the basis of the *rrs* gene and *nif*D polypeptide phylogenies, it does not cluster with the paenibacilli on the basis of *nif*H phylogeny. Similarly, Gram-positive clostridia cluster with the paenibacilli in the *rrs*

phylogenetic tree but are very distant from the paenibacilli based on the *nif*H and *nif*D phylogenies where they form monophyletic groups with the δ proteobacteria and archaea respectively. The archaea represented by *Methanosarcina mazei* and *Methanothermobacter thermoautotrophicus* are clear outgroups in all 3 phylogenies. The paenibacilli are clustered the closest with cyanobacteria based only on the *nif*H amino acid sequence whereas they form a distant and separate cluster based on the *nif*D phylogeny that places them in a larger clade with the β and γ proteobacteria. The cyanobacteria form an exclusive clade based on the *rrs* phylogeny. The paenibacilli do not cluster closely with α, β andγ proteobacteria based on either *nif* D or *rrs* phylogenies. However, based on *nif*H phylogeny they form a separate cluster in a deeply bifurcated clade, including β and γ proteobacteria like *Azoarcus sp.*, *Klebsiella pneumoniae* , *Azotobacter vinelandii* and *Pseudomonas stutzeri*.

Within the paenibacilli (Fig.4.7), *P. polymyxa* P2b-2R forms a monophyletic group with *P. massiliensis* based on the *nif*H and D phylogenies, but fails to cluster with other species of the genus, except *P. graminis* based on *nif*H phylogeny.

Figure 4.4 Phylogeny of *nif*H polypeptide sequences analyzed by the neighbour-joining method. The percentage of 1000 bootstrap replications that support each topological element by more than 50% is indicated near nodes. The scale bar represents 0.05 substitutions per site.

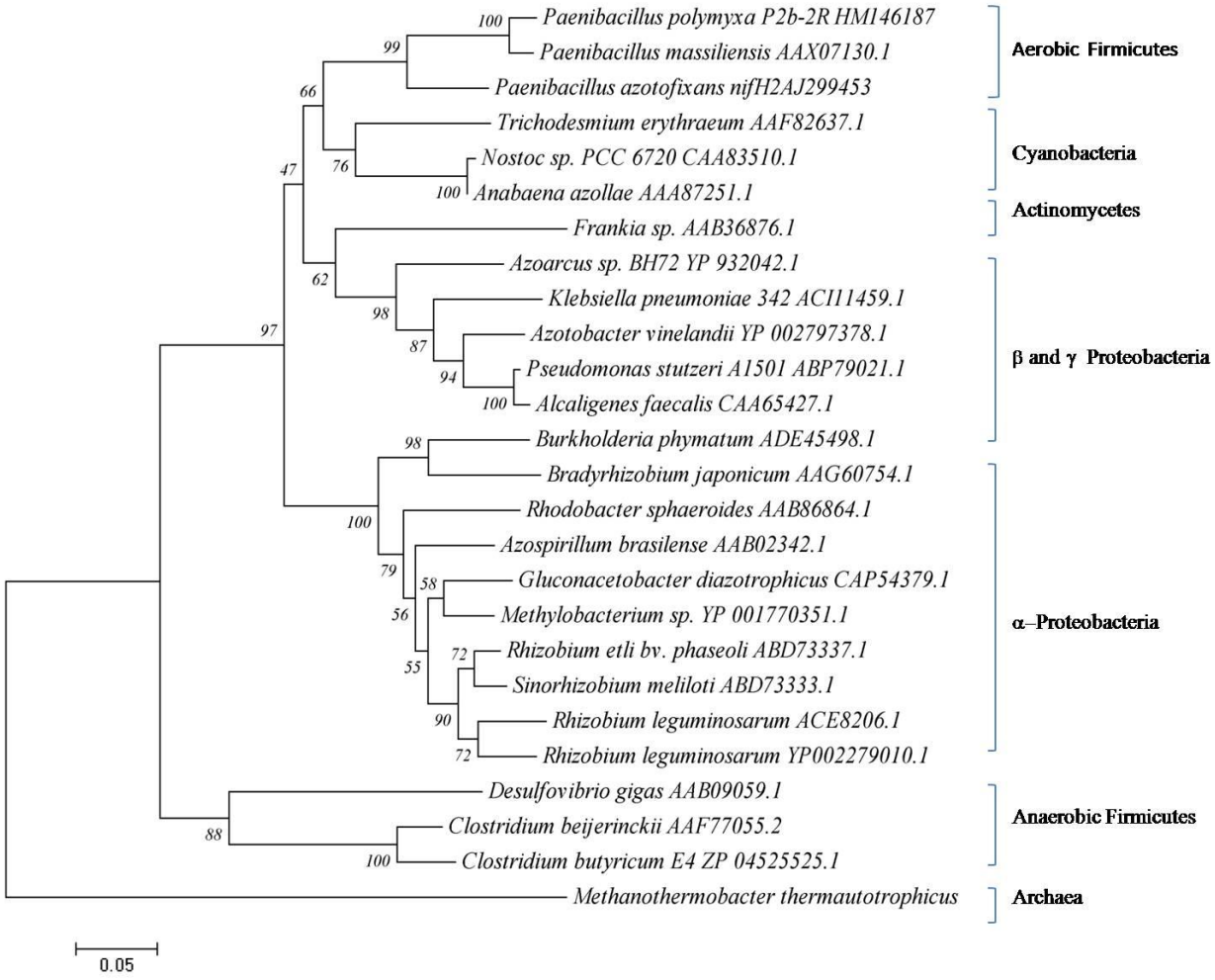


Figure 4.5 Phylogeny of partial *nif*D polypeptide sequences analyzed by the neighbour-joining method. The percentage of 1000 bootstrap replications that support each topological element are indicated near nodes. The scale bar represents 0.05 substitutions per site.

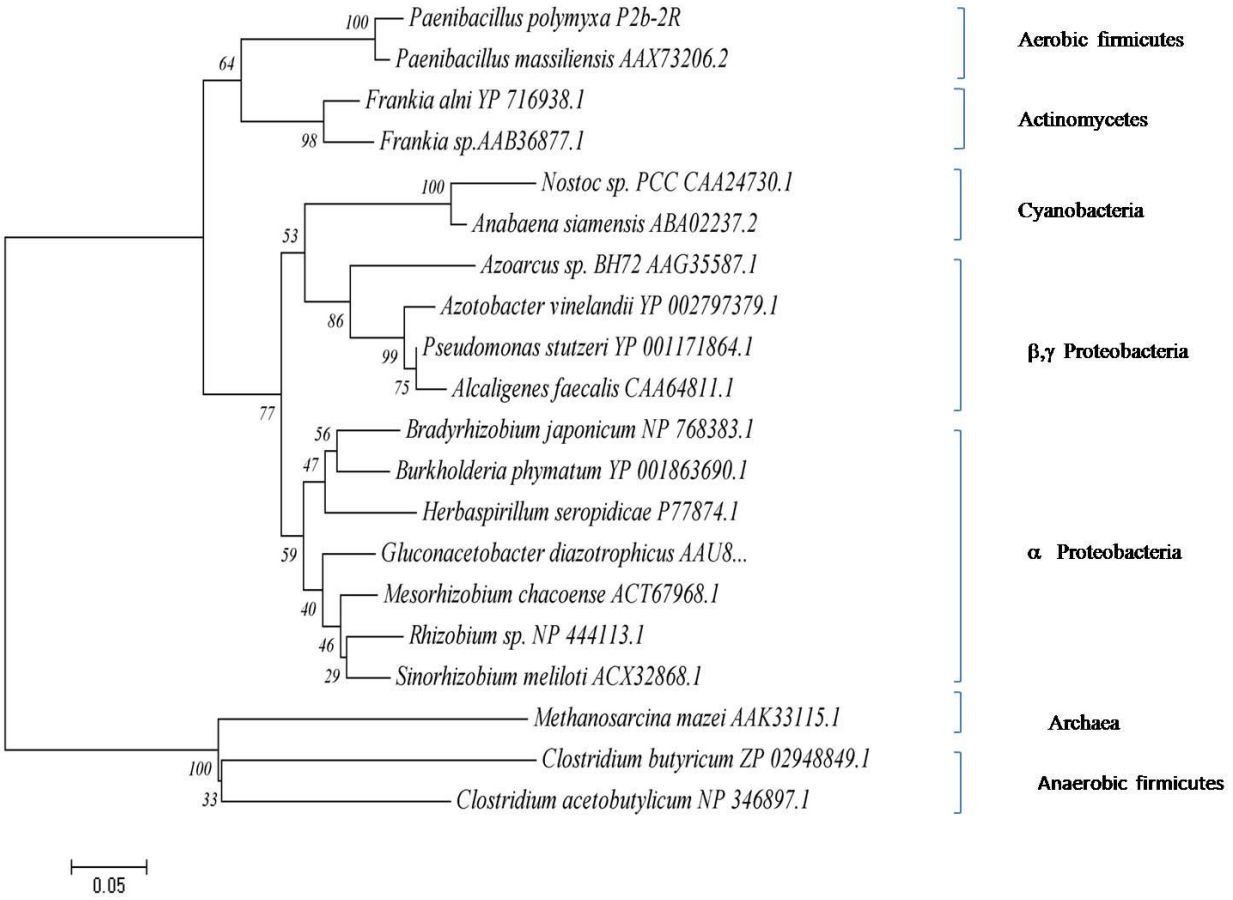


Figure 4.6 Phylogeny of *rrs* gene of selected diazotrophic bacteria analyzed by neighbour-joining method. The percentage of 1000 bootstrap replications that support each topological element by more than 50% is indicated near nodes. The scale bar represents 0.05 substitutions per site.

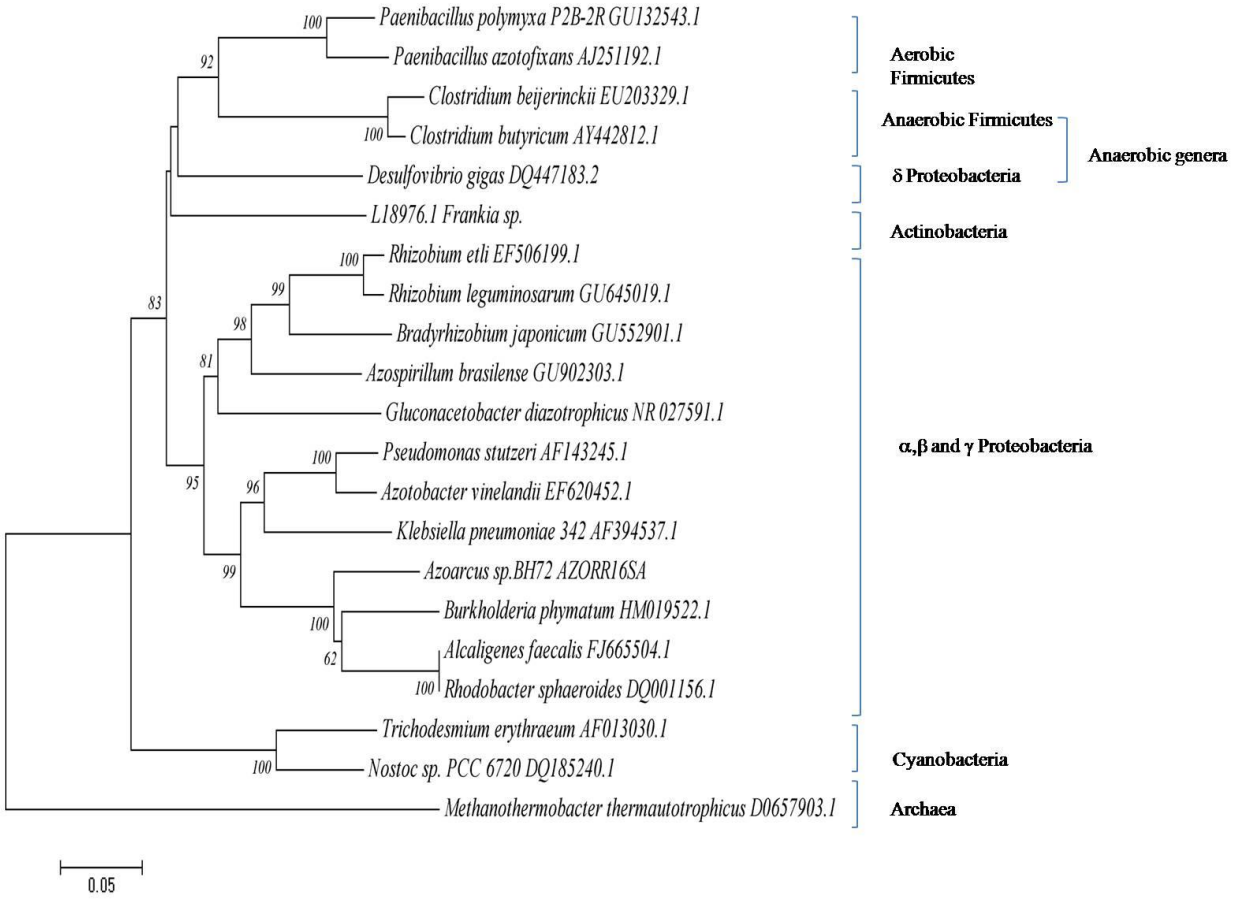
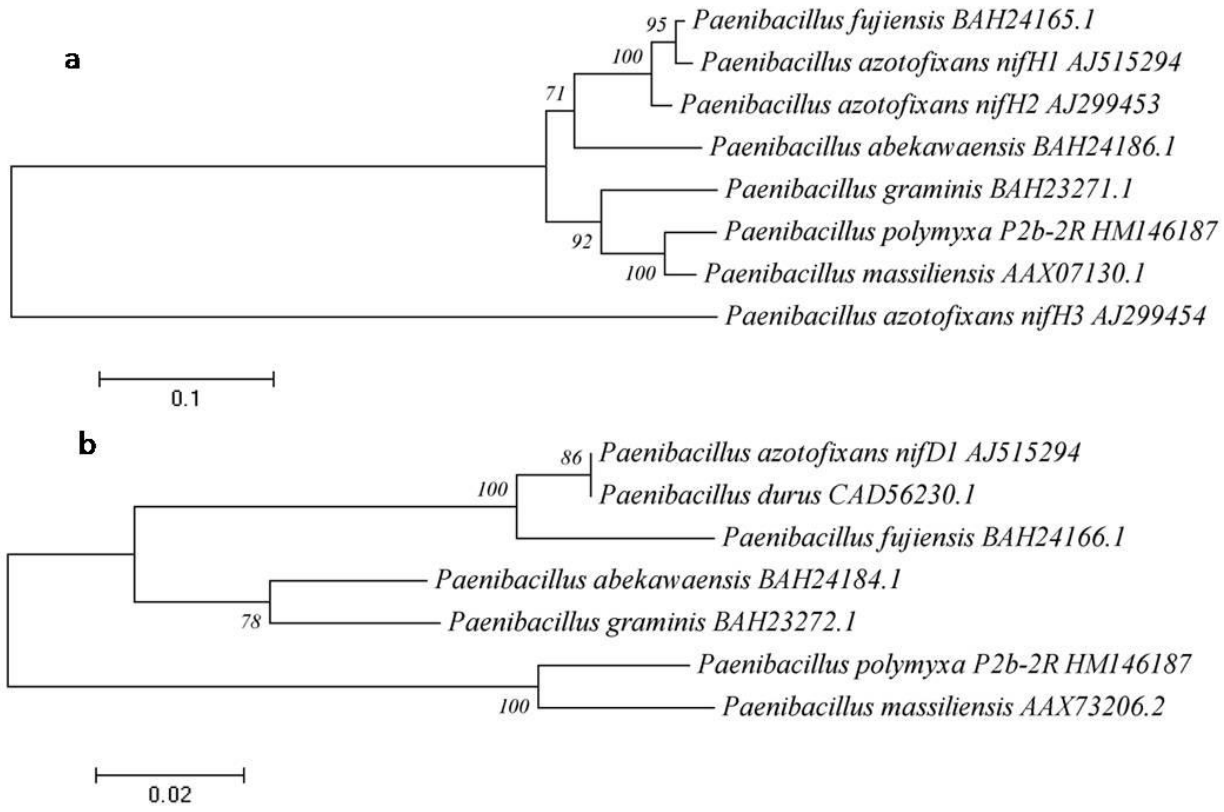


Figure 4.7 Phylogeny of *nif*H (a) and *nif*D (b) polypeptide sequences of diazotrophic bacteria of the genus *Paenibacillus* analyzed by neighbour-joining method. The percentage of 1000 bootstrap replications that support each topological element by more than 50% is indicated near nodes. The scale bar represents substitutions per site



4.4 Discussion

This study was undertaken to sequence and analyze the structural *nif* genes in the region including and flanking the *nif*H fragment, of *P. polymyxa* P2b-2R. Only one positive signal for *nif*H was detected upon southern blotting of total genomic DNA digested by *Pst*I and *Hind*III, suggesting the presence of only one copy of the *nif*H gene in *P. polymyxa* P2b-2R, as opposed to the 2 and 3 copies in *P. massiliensis* (Zhao *et al*., 2006) and *P. azotofixans* (Choo *et al*., 2003), respectively. This finding is in agreement with a previous study by Oliveira *et al*., (1993), who also found only one positive signal upon hybridizing the DNA of *P. polymyxa* (Previously *Bacillus polymyxa*) with the *nif*H gene of *Klebsiella pneumoniae*. Considerable sequence homology (80% and 74% respectively between *P.polymyxa nif*H gene and those of *P. massiliensis* and *P.*

azotofixans) as well as close phylogenetic clustering (Fig 4.4; Fig 4.7 a) within the genus *Paenibacillus* suggest common ancestry and simultaneous evolution of the *nif* genes among species. The disparity in copy number of these genes among species can be attributed to possibilities of reiteration or rearrangement of the *nif*H gene in some species during evolution (Roasdo *et al*., 1998) and that multiple copies of this gene are not a uniform feature within the genus.

The 2208-bp sequence of the *nif* genes of *P. polymyxa* P2b-2R obtained by SSP-PCR showed that a *nif*B gene was present immediately upstream of the *nif*H and D genes. This is a deviation from the most common *nif* gene arrangement in well-studied bacteria like *Rhizobium leguminosarum* (Long, 1989), *Klebsiella pneumonieae* (Arnold *et al*., 1988), *Gluconoacetobacter diazotrophicus* (Lee *et al*., 2000) and *Azotobacter vinelandii* (Jacobson *et al*., 1989). In these other diazotrophs, *nif*HDK genes are arranged in one co-transcribed operon and regulated by a promoter directly upstream of *nif*H, whereas *nif*B is located elsewhere in the *nif* cluster (Martinez-Romero, 2006). No promoter sequence was found in the interspacing region between *nif*B and *nif*H, suggesting that *nif*B, H and D might be co-transcribed through a promoter located upstream of *nif*B (Zhao *et al*., 2006). More sequence information is required to identify the promoter that regulates the structural *nif* genes of *P. polymyxa*. Similar arrangements of the *nif*B gene upstream of *nif*H, D and K have been reported in other paenibacilli like *P. massiliensis* (Zhao *et al*., 2006), *P. azotofixans* (Choo *et al*., 2003) and *P. graminis* (Database only, AB485747), *P. fujiensis* (Database only, AB 489070) and *P. abekawaensis* (Database only, AB 489138), suggesting that this arrangement of *nif*B gene relative to the structural *nif* genes is a consistent, and so far, exclusive, feature of the paenibacilli.

The use of phylogenetic analysis to resolve the conflict between lateral transfer and vertical descent of the *nif* genes relies upon the detection of discrepancies between the phylogenies of *nif* genes and a reference gene such as 16s rRNA gene which is known to be vertically inherited. The *nif*H phylogenetic tree based on the translated amino acid sequence of the full *nif*H gene of *P. polymyxa* P2b-2R is in agreement with previously reported *nif*H phylogenies derived using the partial *nif*H sequences (Achouak *et al*., 1999; Zehr *et al*., 2003)

and places the endophytic *P. polymyxa* strain P2b-2R in a monophyletic group with other paenibacilli and closely within a clade with cyanobacteria that are free-living diazotrophs. However, phylogenies based on 16s rRNA (*rrs*) (this study; Zehr *et al*., 2003) clearly place the cyanobacteria in a separate group evolutionarily distant from the paenibacilli. Interestingly, the *nif*D phylogeny presented in our study also does not show clustering between the paenibacilli and cyanobacteria. Therefore, in terms of the relationship between cyanobacteria and paenibacilli the *nif*D phylogeny is congruent with the *rrs* phylogeny, but is incongruent with the *nif*H phylogeny. Similar incongruence between the *nif*H and D phylogenies was also found with reference to the placement of the high G+C, Gram-positive, aerobic, nodule forming, actinobacterium *Frankia*. Whereas *nif*H phylogenies (Achouak *et al*., 1999; this study) place *Frankia* and paenibacilli in separate clades, both *rrs* and *nif*D phylogenies place *Frankia* makes a sister clade with the paenibacilli. Within the context, Achouak *et al*. (1999) and Normand and Bousquot (1989), placed *Frankia* in a clade within the α proteobacteria, whereas our *nif*H phylogeny places it in a clade with the β and γ proteobacteria. This discrepancy is likely because we were able to use full or larger *nif*H sequences due to considerable enrichment of the public-domain databases since the earlier studies.

The discrepancy among *nif*H and *nif*D phylogenies in terms of the placement of two major groups of bacteria, namely cyanobacteria and actinobacteria suggests the possibility of differential inheritance of the *nif*H and D genes. Because, in both cases the *nif*D phylogeny is in congruence with that of the *rrs* gene, it is likely that in certain bacteria *nif*D is inherited vertically like the *rrs* gene as suggested by Henson *et al*.,(2004). Notwithstanding these disparities, both *nif*H and *nif*D phylogenies place the Gram-positive, anaerobic clostridia in deeply divergent groups away from the Gram-positive, aerobic paenibacilli despite their close phylogenetic clustering in the *rrs* phylogeny (this study; Zehr *et al*., 2003). The *nif*H phylogeny places the clostridia in a monophyletic group with the anaerobic, sulphate-reducing, δ-proteobacterium *Desulfovibrio gigas*, supporting the assumption of Chien and Zinder (1994, 1996), that based on the *nif*HDKE sequences, diazotrophic bacteria could be distributed into four clusters (aerobes, anaerobes, alternative nitrogenases and archaea). However, both our study, as well as Henson *et al*.,(2004), place the clostridia in a monophyletic outgroup with the

archaeon *Methanosarcina mazei* on the basis on *nif*D phylogeny. Leigh (2000) has reported similar unexpected clustering based on *nif*D and *nif*E genes and described it as an exception to the normal phylogenetic separation of clostridia and archaea on the basis of other *nif* genes.

The *nif*H and *nif*D phylogenies fail to provide evidence of clustering of diazotrophs based on their free-living, non-nodule forming endophytic or nodule forming habit. For example, based on *nif*H phylogeny the nodule-forming, actinobacterium *Frankia sp.* clusters with the free-living β and γ proteobacteria and the non-nodule forming, grass endophytic, β proteobacterium *Azoarcus sp.* Similarly, the well known non-nodule forming, sugarcane endophyte, α proteobacterium *Gluconoacetobacter diazotrophicus* clusters with nodule-forming, α proteobacteria of the *Rhizobiaceae* family, based on both *nif*H and *nif*D phylogenies. The free living (*P. massiliensis*, *P. azotofixans*) and endophytic paenibacilli (*P. polymyxa* strain P2b-2R) cluster with nodule-forming *Frankia* based on *nif*D phylogeny, whereas they cluster with free-living cyanobacteria based on *nif*H phylogeny.

The *nif*H and *nif*D phylogenies within the genus *Paenibacillus* show that *P. polymyxa* P2b-2R was most closely related to *P. massiliensis*, a species isolated from the roots of willow trees growing near Beijing (Ding *et al.*, 2005) and most distant from *P. azotofixans*, which is mostly isolated from roots of grasses in Brazil (Rosado *et al*., 1998; Seldin *et al*., 1984). Incidentally, other paenibacilli included in our study, for which sufficient sequence information was available, were also all isolated from roots of various grasses, *P. abekawaensis* from *Miscanthus sinensis* in Japan, *P. fujiensis* from rice in Japan, (both published in GenBank only, Uozomi,T.) and *P.graminis* from wheat roots in France (Berge *et al*., 2002). The divergence between the grass-isolated species and the tree isolates (*P.polymyxa* P2b-2R and *P. massiliensis*) is deeper, based on *nif*D phylogeny. Although, this observation is biased by the limited availability of full *nif*H and at least partial *nif*D sequences of various paenibacilli in the public-domain databases, it leads us to suggest the possibility of a pattern of inheritance of *nif* genes similar to the phylogeographic pattern shown in the genus *Bradyrhizobium* (Parker *et al*., 2002), based on host plant association in this case. We recommend future studies to explore the possibility of such a pattern in diazotrophs of the genus *Paenibacillus*.

In conclusion, our study provides *nif* sequence information of *P. polymyxa* which is useful for experimental and phylogenetic purposes. Based on our data and other available sequences of *nif* operons of paenibacilli, we propose that the presence of *nif*B gene directly upstream of *nif*H and D is a consistent and peculiar feature of diazotrophs of the genus *Paenibacillus*. Comparisons between *nif*H, *nif*D and 16s rRNA phylogenies provide mixed evidence of possible differential inheritance of the *nif*H (horizontal) and *nif*D (vertical) genes. In future studies it would be important to include more *nif* genes and more representatives of each gene, belonging to other organisms, to resolve the inheritance of nitrogen fixation.

4.5 Acknowledgements

We are grateful to Sean Graham and Denise Brooks for advice and assistance with phylogentic analysis. Funding for this study was provided by an NSERC discovery grant to CPC and an NSERC CGSD3 scholarship to Richa Anand. We also thank the Pacific leaders graduate student program of the Ministry of Advanced Education of British Columbia for providing financial support to Richa Anand during manuscript preparation.

5 Conclusions and Future Perspectives

Nitrogen is recognized as the most frequently limiting nutrient for plant growth in ecosystems (Vitousek and Howarth, 1991). On the other hand, in the post industrial revolution world, anthropogenic inputs of nitrogen have led to excesses of this nutrient in certain ecosystems (Aber, 1992, Fenn *et al*., 1998). Whether limited or excess, nitrogen is a major nutritional factor affecting the productivity of our ecosystems. Biological nitrogen fixation is an important component of the nitrogen cycle and has been proven to play a pivotal role in the productivity of agricultural and forest ecosystems dominated by leguminous plants. As a result, most previous research has been focussed on the *Rhizobium*–legume symbiosis and the organisms involved. However, after the 1950s the focus of this research shifted because of a curious observation made about sugarcane cultivation in Brazil. It was noted that sugarcane cultivated in Brazil neither depletes soil nitrogen reserves nor suffers a decline in yield after many decades, or even centuries, of sugarcane cropping. This suggests that sugarcane may benefit significantly from inputs from biological nitrogen fixation (Boddey *et al*., 1995). Since then, many researchers focussed on isolating potential diazotrophs from roots and rhizoshperes of sugarcane and other graminaceous crops (Döbereiner *et al*., 1972; Yoshida and Ancajas, 1971).

The focus of research moved to endophytic diazotrophs following the isolation and identification of endophytic bacterium *Gluconoacetobacter diazotrophicus* within sugarcane tissues (Cavalcante and Döbereiner 1988; Baldani *et al*. 1997). Subsequently, *G. Diazotrophicus* has been shown to fix nitrogen *in planta* (Sevilla *et al*., 2001). Other bacteria have been shown to be endophytic diazotrophs, such as *Azoarcus* sp. in Kallar Grass (Reinhold-Hurek and Hurek, 1998), *Herbaspirillum sp.* in rice (Elbeltagy *et al*., 2001) and species of *Alcaligenes*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Herbaspirillum*, *Klebsiella* and *Pseudomonas*, in rice and maize (*Zea mays*) (Boddey *et al*., 1995; Triplett, 1996; Malik *et al*., 1997; Stoltzfus *et al*., 1997; James *et al*., 1999). It is now widely believed that endophytic diazotrophs may have a distinct advantage over their free-living and rhizospheric counterparts by means of having direct access to fixed carbon and other nutrients from the host plants and by means of having a relatively protected habitat, free

of predators and without much competition from other microorganisms (James, 2000). At the same time it is also possible that transfer of fixed nitrogen to host plants from diazotrophic bacteria lodged within the plant tissue is more efficient than that from free-living and rhizospheric bacteria.

In recent years, the study of endophytic diazotrophic bacteria has since been extrapolated to plants of horticultural importance, for example banana (*Musa paradisiaca*, Martinez *et al*., 2003), grape (*Vitis vinifera*, Compant *et al*., 2005), pineapple (*Ananas comosus* [L.] Merr., Tapia-Hernández,2000) and forest trees, for example oak and poplar. In conifers, reports of endophytic diazotrophs have remained limited to broad surveys of endophytic bacteria and their *nif* H genes (Widmer *et al*., 1999; Izumi *et al*., 2008)

In this thesis, I have investigated the ability of a naturally occurring endophytic, diazotrophic bacterium to colonize coniferous host plants internally, promote their growth, fix nitrogen and transfer fixed nitrogen to its host plants. In Chapter 2, I have presented a survey of endophytic bacteria that were recovered from surface-sterilized tissues of lodgepole pine and western redcedar trees and seedlings. Based on its consistent and higher nitrogenase activity, one isolate, *Paenibacillus polymyxa* P2b-2R was chosen for further studies reported in Chapters 3 and 4. In Chapter 3, I determined population density of this bacterium within lodgepole pine and wester redcedar tissues to confirm the endophytic nature of this bacterium. I then visualized GFP-marked cells of *P*. *polymyxa* P2b-2R within lodgepole pine tissues. Various growth parameters like lengths, fresh and dry weights of roots, shoots and seedlings, were measured to assess its ability to promote the growth of inoculated lodgepole pine and western redcedar and ^{15}N-dilution (in enriched plant foliage) was used to determine the percentage of nitrogen that lodgepole pine and western redcedar, inoculated with *P. polymyxa* P2b-2R derived from the atmosphere. In Chapter 4, I sequenced and analyzed some nitrogen fixation genes of *P. polymyxa* P2b-2R, and used this sequence information to resolve the phylogenetic relationship of these genes with homologs from other known diazotrophs. Chapter 1, of this thesis is an updated version of a published book chapter about the importance of endophytic plant growth promoting bacteria in trees and research conducted so far on this subject, with emphasis on nitrogen fixation.

5.1 Main Findings

The survey of potential diazotrophic endophytic bacteria presented in Chapter 2 revealed that *Bacillus spp.* and *Paenibacillus spp*. comprised the predominant fraction of the cultivable endophytic bacteria from the lodgepole pine and western redcedar tissues. This is in agreement with previous reports describing *Bacillus* spp. that have been isolated frequently from tissues of other tree species like Scots pine (*Pinus sylvestris* L.), silver birch (*Betula pendula* Roth), and rowan (*Sorbus aucuparia* L.) (Izumi *et al*., 2008), chestnut (*Castanea sativ*a, Willhelm *et al*., 1998), Citrus rootstocks (Araujo *et al*., 2001) and tissues of saffron (*Crocus albiflorus*) (Reiter and Sessitsch, 2006). *Bacillus* spp. and *Paenibacillus spp*. may be as widespread as endophytes among different plant hosts in agricultural, as well as in natural ecosystems, as they are in soils (Berge *et al*., 2002; Bezzate *et al*., 2001), humus (Elo, *et al.*, 2001) and rhizospheres (von der Weid *et al*., 2000; Daane *et al*., 2002) of various plants.

Three of four isolates that demonstrated consistent acetylene reduction activity over background (0.187 µmol C_2H_4/ml of headspace (Table 1.2) belonged to the genus *Paenibacillus*. Two of these, P2b-2R and P18b-2R, were identified to species level as being *Paenibacillus polymyxa.* Under the assay conditions used, *P. polymyxa s*trains P2b-2R and P18b-2R also had the highest acetylene reduction activities among the four nitrogen-fixing isolates (5.172 µmol and 4.0938 µmol C_2H_4/ml respectively). These acetylene reduction assay values are in agreement with those reported for rhizospheric bacteria like *Azotobacter armeniacus* (5.487 µmol C_2H_4/ml), *Bacillus azotoformans* (3.57 µmol C_2H_4/ml) (Piao *et al*., 2005) *Klebsiella pneumonia* (6.61 µmol C_2H_4/ml) (Haahtela *et al.*, 1983) in pure culture.

P. polymyxa is known to possess many plant-growth-promoting characteristics like soil phosphorus solubilisation (Singh and Singh, 1993), production of antibiotics (Rosado and Seldin, 1993; Choi *et al*., 2008), exopolysaccharides (Haggag, 2007), chitinase (Mavingui and Heulin, 1994), hydrolytic enzymes (Nielsen and Sorensen, 1997), enhancement of soil porosity (Gouzou *et al*., 1993), phytohormone production (Gutierrez-Manero *et al.*, 2001; Timmusk *et al.*, 1999; Lebuhn *et al*., 1997), disease suppression (Kloepper *et al*., 2004; Beatty *et al*.,2002) and nitrogen fixation (Heulin *et al*., 1994; Lindberg *et al*., 1985). Some of these properties, especially antibiotic production, chitinase production and disease suppression, together with its

endospore forming potential, may enable it to resist a wide range of environmental stresses (Lal and Tabacchioni, 2009) in the soil and plant system where competitiveness of a PGPR strain is a necessary requirement for colonization and to demonstrate the biological effect (Barriuoso *et al*., 2008). This may contribute to the prevalence of these bacteria in various plants across ecosystems, for example in rhizospheres of wheat in Nigeria (Guemouri-Athmani *et al*., 2000), corn in Brazil (von der Weid *et al*., 2000) and lodgepole pine and spruce in western Canada (Holl and Chanway, 1992; Shishido *et al*., 1996).

I found that none of the isolates recovered from lodgepole pine growing at the Chilliwack Lake site, showed acetylene reduction activity, even though they were known diazotrophs like *P. polymyxa* and *Burkholderia* (Minerdi *et al.*, 2001). This finding is of ecological significance because the Chilliwack Lake site is close to the industrial and agricultural town of Chilliwack and is characterized by high atmospheric deposition of nitrogen (Vingarzan *et al*., 2000) and high availability of mineralizable N (Appendix A, this thesis). It is possible that the lack of nitrogenase activity in isolates from this site is either due to evolutionary selection against nitrogen fixation ability or a simple repression of nitrogenase activity due to presence of fixed nitrogen in the ecosystem (Roberts and Brill, 1981). A study of the *nif* genes of these isolates in the future, may provide information on this matter.

Growth promoting effects of *P. polymyxa* strain P2b-2R on lodgepole pine and western redcedar were evident in my study (Chapter 3). Seedling survival, dry weight accumulation, as well as percent nitrogen derived from the atmosphere, were higher in both plant species when inoculated with live cells of strain P2b-2R. Along with uninoculated seedlings, I used seedlings inoculated with heat-killed cells of strain P2b-2R as an additional control to account for growth benefits and nitrogen gains derived from the release of nitrogen and other nutrients from dead bacterial cells. The overall growth effect of the heat-killed bacterial treatment was found to be intermediate between the un-inoculated control and the live P2b-2R treatments for both plant species (Table 3.3, Figs 3.7, 3.8, 3.9) indicating that this treatment was effective in controlling the overestimation of growth promotion effects of the live P2b-2R treatment (inevitable if the growth and nitrogen fixation parameters were compared only to the uninoculated control treatment). Plants inoculated with heat-killed cells of P2b-2R had a significantly lower survival

rate, dry weight accumulation and nitrogen-fixation than those inoculated with live cells of the strain. This indicates that the sustained growth promoting effects of *P. polymyxa* strain P2b-2R are enhanced by the activity of live bacterial cells *in planta* and are not mere artefacts of sudden nutrient release by dead bacterial cells.

So far evidence for the diazotrophic abilities of different species of the genus *Paenibacillus* and isolates of the species *P. polymyxa* is limited to their isolation on nitrogen-free media and the presence of *nif* genes (von der Weid *et al*., 2002, Rosado *et al*., 1998, Choo *et al*., 2003). In this thesis I have provided evidence of nitrogen fixation by *P. polymyxa* in association with plants by using the ^{15}N isotope dilution technique (Fig 3.9). The ^{15}N atom percent excess values of plant tissues inoculated with *P. polymyxa* will be a useful reference for other researchers attempting to estimate nitrogen-fixation by Paenibacilli in plant growth experiments.

Lodgepole pine seedlings colonized by live cells of strain P2b-2R derived as high as 67.53 % of their nitrogen from the atmosphere (% Ndfa), when the seedlings treated with heat killed bacteria were used as the non nitrogen-fixing controls. Western redcedar seedlings under similar conditions derived 21.94 % of its nitrogen from the atmosphere. This is the first report of such high levels of associative nitrogen-fixation in non-leguminous trees. These results indicate that *P. polymyxa* strain P2b-2R is able to fix nitrogen *in-planta* and transfer fixed nitrogen to the host plants, which is also a very important requirement for this plant- microbe interaction to qualify as an associative diazotrophic relationship (James and Olivares, 1998; Kennedy *et al*., 1997). High values of % Ndfa similar to the values reported in this thesis, have also been found in the highly successful, sugarcane – *Gluconoacetobacter diazotrophicus* (65%) interaction (Uriquaga *et al*., 1992). This parallel between the two systems gives rise to an exciting possibility that (if proven to be as effective in the forests as it was in this controlled condition study), the lodgepole pine and *P. polymyxa* association might be as consequential to the nitrogen budgets of lodgepole pine forests as the sugarcane-*G. diazotrophicus* association is to the agriculture and economy of Brazil (Baldani *et al*., 2002). I have discussed the importance of nitrogen in temperate forest ecosystems, especially in western North America in Chapters 1, 2 and 3.

Another important requirement for an endophytic plant-growth-promoting bacterium to be effective is its ability to establish within plant tissues and maintain sufficient population over time. In Chapter 3, I found that the naturally occurring endophyte, *P. polymyxa* P2b-2R, upon re-inoculation under controlled conditions, is able to colonize both lodgepole pine, the plant it was originally isolated from and western redcedar,. In doing so *P. polymyxa* P2b-2R fulfills the basic requirement of an effective endophytic association as well as the tenet of Koch's postulates that the microorganism must be re-isolated from the inoculated experimental host, with the difference that in this case the underlying idea of the postulates applies to the relationship between a causative microbe and the beneficiary host plant, rather than a diseased plant.

Population levels of *P. polymyxa* P2b-2R in both lodgepole pine and western redcedar were found to be closer to, or over the upper limit of the range of population sizes of non-pathogenic endophytic bacteria (1x 10^3 - 1x 10^5 cfu/g plant fresh weight) reported in most plants (Lodewyckx *et al*., 2002; Dong *et al*., 1994; Frommel *et al*., 1991; Quadt-Hallmann and Kloepper, 1996; Lamb *et al*., 1991), especially in the roots and stems of both conifers (1.7x10^6 cfu/g root tissue and 6.7x10^5 cfu/g stem tissue for lodgepole pine; 7.0x10^5 cfu/g root tissue and 1.2 x 10^5 cfu/g stem tissue for western redcedar. Population levels over the range suggested by Lodewyckx *et al*., (2002) (above) have also been reported in a few other plants like sugarcane (Sevilla *et al*., 2001) and grape (Compant *et al*., 2005), where endophytic bacteria have reached population levels of 10^7 -10^9 cfu/g plant tissue. These high population levels are expected for pathogenic bacteria in diseased plants (Tsiantos and Stevens, 1986; Grimault and Prior, 1994). However, neither the sugarcane nor grape plants showed any symptoms of disease or stress. Similarly in my study, neither lodgepole pine nor western redcedar inoculated with *P. polymyxa* P2b-2R showed any disease symptoms. This suggests that population levels of beneficial endophytic bacteria that can be sustained within healthy plant tissues is higher than previously believed (Lodewyckx *et al*., 2002).

I also observed that the population density of *P.polymyxa* strain P2b-2R was highest in the roots of both conifer species up to 2 months after inoculation, after which the population declined steadily over the rest of the sampling period. *P. polymyxa* population density, although

lower in stems, as compared to roots, also followed the same pattern of decline through time, whereas it increased in the needles, where populations were highest at the end of the 12 month sampling period. The important implications of this trend are that the root is the primary site of infection where non-seed-borne endophytes gain entry into the plant (Lodewycx *et al*., 2002) and that bacterial populations tend to accumulate in the foliage during plant growth (Compant *et al*., 2005).

In situ microscopic observation of GFP-marked *P. polymyxa* strain P2b-2R in lodgepole pine stems revealed that this bacterium colonizes stem cortical cells inter and intracellularly. Most endophytic bacteria observed have been found to colonize intercellular spaces of cortical cells (Germaine *et al*., 2004; Chelius and Triplett, 2000, Dalton *et al*., 2006) using confocal or electron microscopy to visualize bacteria marked with GFP. There are very few reports of intracellular colonization (Compant *et al*., 2005; Hurek *et al*., 1994). The ability of these bacteria to gain entry into plant cells without causing necrosis is very intriguing. However, it is possible that these cells of lodgepole pine are specialized structures enveloping the bacteria, as in the well known symbiotic bacteria like Rhizobia and *Frankia* as well as non- symbiotic bacterium *Alcaligenes faecalis* that was reported to be encased in specialized structures within wet land rice plants (You *et al*., 1983 and 1991).

I also found that some GFP-marked bacterial cells of *P*. *polymyxa* strain P2b-2R were located in the vicinity of, or adherent to, chloroplasts within the plant cells. This is of much micro-ecological and evolutionary importance because chloroplasts are oxygen-rich microenvironments within the cell and the Fe protein of the nitrogenase enzyme (NifH) is known to be oxygen sensitive. Although such reports are missing in higher plants, some algae have been found to harbour endophytic bacteria near their chloroplasts (Colombo, 1978, Preisig and Hibberd, 1984) and the NifH protein of *Klebsiella pneumoniae* has been shown to retain its expression in chloroplasts of *Chlamydomonas reinhardtii* (Cheng *et al*., 2005). By analogy, it is tempting to speculate that the NifH of *P. polymyxa* retains function at high oxygen concentrations, while the bacterial cells derive photosynthetic carbon from chloroplasts. This carbon gain would be a distinct advantage due to the high carbon demand of the diazotrophic process.

Another possibility is that the bacterial cells that are in the vicinity of chloroplasts absorb the oxygen evolving from chloroplasts, thus creating a relatively anoxic environment protecting the nitrogenase enzyme of other bacterial cells located elsewhere within the plant cells. Highly evolved and specialized oxygen protection systems in effective diazotrophic associations are not uncommon. Some examples of such systems are leghaemoglobin in rhizobial nodules, passive lipid gas diffusion barriers in Actinobacterial (*Frankia*) vesicles (Murry *et al*., 1984; Parsons *et al*., 1987; Berry *et al*., 1993), heterocysts in cyanobacteria (Fay *et al*., 1968; Haselkorn, 1978; Murry *et al*., 1984). However, the example that is likely most similar to nitrogen fixation by *Paenibacillus* might be that of non-heterocystous cyanobacteria like *Oscillatoria sp.* that are known to fix nitrogen aerobically by non-structural mechanisms. *Oscillatoria spp*. synthesize nitrogenase at a high rate to counteract losses of irreversibly inactivated enzyme and a switch off mechanism to respond to short-term exposures to oxygen, in addition to an essential temporal separation of nitrogen fixation and oxygenic photosynthesis. It has been suggested that nitrogenase activity in the planktonic species *O. erythraea* may result from protection of the enzyme system in oxygen-deficient microsites within macroscopic aggregates or bundles of trichomes (Carpenter & Price, 1976; Bryceson & Fay, 1981). Interestingly, the Fe protein (NifH) of *P. polymyxa* falls in a single monophyletic group with the cyanobacteria based on phylogenetic analysis presented in Chapter 4 of my thesis (Figure 4.3) as well as other published studies (Achouak *et al*., 1999; Zehr *et al*., 2003).

Results reported in chapter 3 also suggest that even though *P. polymyxa* strain P2b-2R was able to colonize western redcedar, promote its growth and fix nitrogen, the levels at which it did so were lower than that for lodgepole pine from which the strain was originally isolated, thus strengthening the idea that use of naturally-occurring, plant species and provenance-specific PGPR for growth promotion would be more effective than introducing non-specific inoculants (James and Olivares, 1998).

Reliable and adequate information of *nif* gene sequences is important for studies using contemporary methods for gaining full understanding of the mechanism of nitrogen fixation by a bacterium at the functional, biochemical or physiological level. Prior to my study, the sequence information available for the *P. polymyxa nif* operon was limited to a small 361 bp

internal fragment of the *nif*H gene for various strains. As a result, I encountered difficulties in my initial attempts to make chromosomal integrations of antibiotic and fluorescent marker genes by single or double homology-based recombination. In Chapter 4, I report the first full *nif*H sequence of *P. polymyxa* and partial sequences of *nif*B and *nif*D genes. This sequence information will be helpful for further studies on this strain as well as other strains of *P. polymyxa*. In this study I have utilized the full *nif*H sequence to understand whether the phylogenetic relationship of *P. polymyxa* with other diazotrophs varies with the use of the full *nif*H sequence versus the partial *nif*H sequence used by previous studies (Achouak *et al*., 1999; Zehr *et al*., 2003), as suggested by Choo *et al*., (2003). However, with the exception of minor discrepancies that are discussed in Chapter 4, I did not find much difference in the phylogenies based on full and partial *nif*H sequences. I have also presented a comparison of *nif*H, and 16s rDNA phlogenies with *nif*D phylogeny of selected diazotrophic organisms for the first time. I found that the the *nif*D phylogeny was more congruent with the 16s rDNA phylogeny than with *nif*H phylogeny when it comes to the relationships between Paenibacilli and other important groups of diazotrophs especially, cyanobacteria and actinobacteria (*Frankia*), indicating that the *nif*H (lateral inheritance) and *nif*D (vertical inheritance) genes might be inherited in different ways as suggested by Henson *et al*., (2004). However, overall comparison of the *nif*H and *nif*D phylogenies does not provide sufficient evidence for such disparity in inheritance.

In summary, my thesis provides evidence of a novel and effective association between an endophytic diazotrophic bacterium and a conifer plant species, which might be of high ecological importance to understand the nitrogen economy of lodgepole pine forests in particular but can also be extrapolated to other bacterial and conifer species in different forest ecosystems.

5.2 Significance and Management Implications

The ability of lodgepole pine to thrive in the nutrient poor soils of interior British Columbia has been a long-standing conundrum. Ecosystem disturbances like wildfires, timber harvest and mountain pine beetle (*Dendroctonus ponderosae*) attacks are common in these forest and affect long term-site productivity by causing gradual loss of nitrogen and other

nutrients (Wei *et al*., 1997). Lodgepole pine has been shown to perform better than other conifer species at such sites (Kranabetter *et al*., 2006; Vyse *et al*., 2009). Productivity and foliar N content of lodgepole pine were significantly higher when compared to hybrid white spruce across a range of soil disturbance and organic matter removal treatments in the Sub-boreal spruce BEC zone in interior British Columbia (Kranabetter *et al*., 2006). Enhanced soil N accumulation has been shown in young pine stands in some ecosystems (Williams *et al*., 1979; Williams, 1992; Krause, 1998) leading some scientists to hypothesize that lodgepole pine might have access to intractable sources of nitrogen (Miller *et al*., 1979). Studies have attributed these unexplained nitrogen inputs to asymbiotic nitrogen fixation in woody debris (Wei and Kimmins, 1998) as well as added nitrogen deposits through symbiotic nitrogen fixation by alder in young lodgepole pine stems (Sanborn *et al*., 2002).

Wei and Kimmins (1998) found that the contribution of fixed nitrogen by coarse woody debris diminishes with stand age but replenishment of lost nitrogen in the ecosystem continues. This led them to suggest that these forests may have other sources of nitrogen such as symbiotic nitrogen fixation by understory plants like *Shepherdia canandensis* (L). My study shows that nitrogen fixation by endophytic diazotrophic bacteria within lodgepole pine trees could explain the long- term, sustained nitrogen inputs in these forests after the effect of asymbiotic nitrogen fixation in woody debris has declined. Nitrogen fixation by bacteria lodged with in the tissues of growing trees would be more plausible than understory fixation after canopy closure because nitrogen fixation increases with the rate of photosynthesis (Hungate *et al*., 1999; Norby, 2006; Kirizii *et al*., 2007).

Actinorhizal nitrogen fixation by Sitka alder has also been shown to contribute 20-150 kg N/ha/yr (Binkley, 1986), but it has been suggested that Sitka alder would have to cover up to 50 % of a young lodgepole pine stands in order to contribute such amounts of nitrogen to the ecosystem, leading to competitive effects on the lodgepole pine (Sanborn *et al*., 2002). In forests where lodgepole pine is harvested for timber value, this would be a major setback to timber productivity. Haeussler and Coates (1986), have suggested that in such situations it is important to determine whether the nitrogen contributions by Sitka alder in these forests are a worthy trade-off for timber value. In addition lodgepole pine dominated stands that were

attacked or threatened by mountain pine beetle, in south central BC, were able to sustain sufficient regeneration of non-pine species such as Douglas fir, interior spruce and sub-alpine fir, even though alder was not found to be a major presence in these stands (Vyse *et al*., 2009).

Based on evidence of successful endophytic associative nitrogen fixation, presented in this thesis, it is tempting to speculate that lodgepole pine forests are able to provide for their own nitrogen requirements thus offsetting the need to sacrifice timber value in favor of alder in these forests. This possibility is valuable from a forest management stand point and needs to be explored further through well-laid out field experiments. As a rough estimate, I used the total nitrogen content of P2b-2R inoculated lodgepole pine seedlings (fig 3.8) to estimate the total uptake of nitrogen per hectare per year, using the stand-level ecosystem simulation model FORECAST (Kimmins *et al*., 1999). It was found that a fully stocked lodgepolepine forest, growing in a forest with a site index of 17m, in the Williams Lake area of British Columbia could uptake upto 24.9 Kg N/ha/yr over a 200 year growth cycle, of which at least 16.81 Kg/ha/yr (67.53%) could be derived by endophytic associative nitrogen fixation such as the one reported in this thesis. This estimated uptake is reasonably close to the 12.5 Kg/ha/yr nitrogen uptake reported by lodgepole pine forests in south-eastern Wyoming over 80-110 years of growth cycle (Fahey *et al*., 1985)

Although, such an extrapolation is not scientifically accurate because the seedlings in my study were grown under extremely controlled conditions and very little input of nitrogen at the onset of the experiment and in the absence of other factors that complicate nitrogen availability and uptake in real forest conditions. In addition, this experiment was done for a very short –term, as compared to a full rotation period of a lodgepole pine forest described above, and thus cannot account for environmental changes over time. Extrapolation is also limited by the assumption that endophytic diazotrophic nitrogen fixation will continue to contribute the same percentage of the total nitrogen over the years. The results of such extrapolation should therefore be treated with extreme caution.

With that caution in mind, it is safe to say that endophytic associative nitrogen fixation is an environmentally benign source of nitrogen that could offset or at least reduce the need for the currently used periodic application of 200 kg of N as urea fertilizer, in lodgepole pine

forests in BC (Brockley and Sanborn, 2009), that is not only expensive but also has adverse effects on soil, water and the environment. It has also been shown that application of nitrogen in urea and ammonium form has adverse effects on the germination of grass seeds (Bremner and Krogmeier, 1989) that could affect understory vegetation. Also, the cost of fertilizer and application can be diverted towards application of other elements like sulphur and boron that are known to become limiting for tree growth when nitrogen is adequately available (Brockley, 2000; Brockley, 2003).

Upon further testing in greenhouse and field settings, inoculation with *P. polymyxa* P2b-2R can prove to be an environmentally benign nursery treatment for lodgepole pine before outplanting in the field, in order to facilitate seedling survival and provide fixed nitrogen. Other plant growth promoting bacteria have been shown to be effective as nursery treatments for coniferous tree species like lodgepole pine (Chanway *et al*., 1991) and spruce (Shishido and Chanway, 2000). Inoculation with an effective plant growth promoting diazotroph such as *P. polymyxa* strain P2b-2R, can prove very beneficial for efficient re-plantation of lodgepole pine after harvest or after a mountain pine beetle attack, which is high priority in BC at this time.

5.3 Future Directions

The key elements of finding and understanding an endophytic diazotrophic association are as follows: a) recognizing potential diazotrophic bacteria within the plant tissue by culture or non-culture based methods, b) confirming their ability to re-colonize plant tissues upon inoculation, c) determining their modes of entry and sites of colonization, d) determining the mechanisms of their beneficial effect on plants, e) determining the importance of their activity for the host plants in question and ultimately their influence at the ecosystem level.

The findings of my studies have addressed many of these important questions regarding the endophytic diazotrophic interaction between *P. polymyxa* stain P2b-2R and its coniferous host of origin, lodgepole pine, as well as an in additional coniferous species western redcedar. However, many aspects of this interaction remain to be explored.

Although, I have provided evidence for the ability of strain P2b-2R to recolonize plant tissues inter- and intracellularly upon inoculation, the sites and mode by which it gains entry

into plant tissues remains to be determined. I found that due to the high autofluorescence of conifer roots, it is very difficult to follow the infection process by microscopic observation of GFP marked cells of strain P2b-2R, therefore I recommend the use of scanning electron microscopy (SEM, Goldstein *et al*., 1977) or transmission electron microscopy (TEM, Hayat *et al*., 1986) to observe the specific sites on the plant surface where bacteria enter the plant system and to follow the bacteria within the plant system after entry. This information is important because *P. polymyxa* is generally considered to be a free living or rhizospheric bacteria (McSpadden Gardener, 2004). Timmusk *et al*., (2005) were unable to show systemic spread of *P. polymyxa* strain B2 (isolated from wheat rhizosphere, Lindberg and Granhall, 1984), in *Arabidopsis thaliana*. However, I found more than one representative strain of *P. polymyxa* to be endophytic. Also, Izumi *et al*., (2008) found other species of the genus *Paenibacillus* to be endophytic in coniferous and deciduous tree species.

Physiological characterization of strain P2b-2R might also be helpful in providing information on whether this strain of *P. polymyxa* possesses certain distinct physiological characteristics that confer on it endophytic colonization ability. Physiological characterization of strain P2b-2R also needs to be conducted in order to determine the other mechanisms of plant growth promotion, if any, by *P. polymyxa* P2b-2R besides nitrogen fixation, that lead to plant growth promotion for example phytohormone or antibiotic production. Similarly, genetic characterization of the bacterium will also be useful in answering questions of endophytic lifestyle, mechanisms of growth promotion and nitrogen fixation, as in the case of the mutualistic, nitrogen-fixing grass endophyte, *Azoarcus sp* strain BH72, for which the full genomic sequence (Krause *et al*., 2006) has provided useful insight into the aforesaid questions.

My finding that strain P2b-2R is able to colonize cells of the stem cortex internally is very important from a micro-ecological standpoint and therefore merits further scrutiny. I suggest the use of 3D confocal imaging, also known as z-stack imaging (Agard, 1984), for its ability to optically section a specimen in the z- axis, for better resolution of the microsites of intracellular colonization. The arrangement of bacterial cells close to chloroplasts within plant cells also merits further examination not only for a better understanding of this particular plant microbe interaction, but also to further our understanding of nitrogen fixation by aerobic

bacteria in the absence of structural oxygen barriers. Extensive physiological and biochemical studies are required to dissect this mechanism. It will also be useful to tag the *nif*H gene of *P. polymyxa* P2b-2R, with a reporter gene like *gfp*, transcribed by the *nif*H promoter. This can be used to understand whether and under what conditions the transcription of the *nif*H genes is initiated *in planta*.

In my study inoculation of conifer seedlings by *P. polymyxa* strain P2b-2R resulted in significant plant growth promotion and nitrogen fixation under controlled conditions. However, it remains to be seen whether the same holds true in field conditions where microbial competition and environmental factors can influence a plant-microbe interaction. In addition, it is important to partition the growth benefits to plants, into those resulting from nitrogen fixation versus those resulting from other mechanisms. For this purpose I suggest the use of non- nitrogen fixing mutants of the strain P2b-2R in plant growth studies and compare their effect with that of the wild-type strain. I have provided important *nif* gene sequence information that can be helpful in the generation of such mutants.

Furthermore, the findings of this study also indicate that there might be other beneficial endophytic diazotrophic interactions between aerobic and anaerobic diazotrophic bacteria and coniferous plant species that remain to be explored, which together with the interaction presented here, may have a significant contribution to the nitrogen budgets of our forest ecosystems.

Bibliography

Aber J.D. (1992) Nitrogen cycling and nitrogen saturation in temperate forest ecosystems. Trends in Ecology & Evolution 7:220-224.

Achouak W., Normand P., Heulin T. (1999) Comparative phylogeny of rrs and nifH genes in the Bacillaceae. Int J Syst Bacteriol 49:961-967.

Agard D.A. (1984) Optical Sectioning Microscopy: Cellular Architecture in Three Dimensions. Annual Review of Biophysics and Bioengineering 13:191-219.

Allan E. & Kelman A. (1977). Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var. *atroseptica. Phytopathlogy 67,* 1305-13 12.

Amann R. (2000) Who is out there? Microbial aspects of diversity. Syst. Appl. Microbiol. 23 1-8.

Anand R., Paul L., Chanway C. (2006) Research on Endophytic Bacteria: Recent Advances with Forest Trees, in: B. J. E. Schulz, et al. (Eds.), Microbial Root Endophytes, Springer Berlin Heidelberg. pp. 89-106.

Araújo W.L., Maccheroni W., Jr., Aguilar-Vildoso C.I., Barroso P.A., Saridakis H.O., Azevedo J.L. (2001) Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. Canadian Journal Of Microbiology 47:229-236.

Arnold W., Rump A., Klipp W., Priefer U.B., Pühler A. (1988) Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation gene cluster of Klebsiella pneumoniae. Journal of Molecular Biology 203:715-738.

Arsène F., Katupitiya S., Kennedy I.R., Elmerich C., (1994). Use of *lac*Z fusions to study the expression of *nif* genes of *Azospirillum brasilense* in association with plants. Molecular plant-microbe interactions. 7 (6):748-757

Ash C., Priest F.G., Collins M.D. (1993) Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Antonie van Leeuwenhoek 64:253-260.

Assmus B., Hutzler P., Kirchhof G., Amann R., Lawrence J.R., Hartmann A. (1995) *In situ* localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. Appl Environ Microbiol 61 : 1013-1019

Bal A. (2002) Isolation of endophytic bacteria from lodgepole pine (Pinus contorta var. latifolia (Dougl.) Engelm.), Forest Sciences, MSc Thesis. The University of British Columbia, Vancouver.

Balandreau J., Knowles R. (1978) The rhizosphere, in: Y. R. Dommergues and S. V. Krupa (Eds.), Interactions between non-pathogenic soil microorganisms and plants, Elsevier, Amsterdam. pp. 243–268.

Baldani J., Caruso L., Baldani V.L.D., Goi S.R., Döbereiner J. (1997) Recent advances in BNF with non-legume plants. Soil Biology and Biochemistry 29:911-922.

Baldani J.I., Baldani V.L.D., Seldin L., Döbereiner J. (1986) Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a Root-Associated Nitrogen-Fixing Bacterium. Int J Syst Bacteriol 36:86-93.

Baldani J. I., Reis V. M., Baldani V. L. D., and Döbereiner J. 2002. A brief story of nitrogen fixation in sugarcane -reasons for success in Brazil. *Functional plant biology* 29:417-423.

Bashan Y., Holguin G., and Lifshitz R. 1993. Isolation and characterization of plant growth-promoting rhizobacteria. p 331-345. *In* B. R. Glick and J. E. Thompson(ed.) Methods in plant molecular biology and biotechnology. CRC Press, Boca Raton, Fla.

Ballard R. (1984) Fertilization of plantations., in: Bowen G. D. and Nambiar E. K. S. (Eds.), Nutrition of Plantation Forests. , Academic Press, London. pp. 327-360.

Barbour M.G., Minnich R.A. (2000) California upland forests and woodlands, in: M. G. Barbour and W. D. Billings (Eds.), North American Terrestrial Vegetation. 2nd edition., Cambridge University Press, Cambridge, UK. pp. 161-202.

Barnett J.P. (1971) Flotation in ethanol reduces storability of southern pine seeds. Forest Sciences 17:50-51.

Barrett G., Marsh S. (2002) An economic analysis of inoculant biofertiliser production and use in Vietnam, in: I. R. a. C. Kennedy, A.T.M.A. (Ed.), Biofertilisers in Action, Rural Industries Research and Development Corporation,, Canberra. pp. 102-111

Barriuso J., Solano B.R., Lucas J.A., Lobo A.P., Garcia V.A., Guiterrez Manero F.J. (2008) Ecology, genetic diversity and screening strategies of plant growth promoting rhizobacteria (PGPR). in: I. Ahmad, et al. (Eds.), Plant-Bacteria Interactions: Strategies and techniques to promote plant growth, Wiley-VCH, Germany. pp. 1-18.

Beatty P.H., Jensen S.E. (2002) *Paenibacillus polymyxa* produces fusaricidin-type antifungal antibiotics active against Leptosphaeria maculans, the causative agent of blackleg disease of canola. Can. J. Microbiol. : 48:159-169.

Bell C.R., Dickie G.A., Chan J.W.Y.F. 1995. Variable Response of Bacteria Isolated From Grapevine Xylem to Control Grape Crown Gall Disease in planta. Am. J. Enol. Vitic. 46: 499-508.

Beneduzi A., Peres D., Vargas L.K., Bodanese-Zanettini M.H., Passaglia L.M.P. (2008) Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing bacilli isolated from rice fields in South Brazil. Applied Soil Ecology 39:311-320.

Benhizia, Y., Benhizia, H., Benguedouar, A., Muresu, R., Giacomini, A., Squartini, A. 2004. Gamma proteobacteria can nodulate legumes of the genus *Hedysarum*. Syst. Appl. Microbiol. 27:462-468.

Bent E., Chanway C.P. (1998) The growth-promoting effects of a bacterial endophyte on lodgepole pine are partially inhibited by the presence of other rhizobacteria. Canadian Journal of Microbiology 44:980-988.

Bent E., Chanway C.P. (2002) Potential for Misidentification of a Spore-Forming *Paenibacillus polymyxa* Isolate as an Endophyte by Using Culture-Based Methods. Appl. Environ. Microbiol. 68:4650-4652.

Berge O., Guinebretiere M.H., Achouak W., Normand P., Heulin T. (2002) *Paenibacillus graminis* sp. nov. and *Paenibacillus odorifer* sp. nov., isolated from plant roots, soil and food. Int J Syst Evol Microbiol 52:607-616.

Berry A.M., Harriott O.T., Moreau R.A., Osman S.F., Benson D.R., Jones A.D. (1993) Hopanoid lipids compose the *Frankia* vesicle envelope, presumptive barrier of oxygen diffusion to

nitrogenase. Proceedings of the National Academy of Sciences of the United States of America 90:6091-6094.

Bezzate S., Aymerich S., Chambert R., Czarnes S., Berge O., Heulin T. (2000) Disruption of the *Paenibacillus polymyxa* levansucrase gene impairs its ability to aggregate soil in the wheat rhizosphere. Environmental Microbiology 2:333-342.

Bhau B.S., Negi M.S., Jindal S.K., Singh M., Lakshmikumaran M. (2007) Assessing genetic diversity of *Tecomella undulata* (Sm.) – An endangered tree species using amplified fragment length polymorphisms-based molecular markers. Current Science Vol. 93:67-72.

Binkley D. (1982) Nitrogen fixation and net primary production in a young Sitka alder stand. Can.J. Bot. 60,281-284.

Binkley D. (1995) The influence of tree species on forest soils: processes and patterns., in: D. J. Mead and I. S. Cornforth (Eds.), Proceedings of the trees and soil workshop, Lincoln University Press, Christchurch, New Zealand. pp. 1–33.

Binkley D., Son Y., Valentine D.W. (2000) Do Forests Receive Occult Inputs of Nitrogen? Ecosystems 3:321-331.

Bloemberg GV, O'Toole GA, Lugtenberg BJJ, Kolter R (1997) Green fluorescent protein as a marker for *Pseudomonas* spp. *Appl. Environ. Microbiol.* 63: 4543–4551.

Bloemberg G.V., Camcho Carvajal M.M. (2006) Microbial interactions with plants: A hidden world?, in: B. Schulz, et al. (Eds.), Soil biology: Microbial Endophytes, Springer-Verlag, Berlin. pp. 320-336.

Boddey R., Urquiaga S., Reis V., Döbereiner J. (1991) Biological nitrogen fixation associated with sugar cane. Plant and Soil 137:111-117.

Boddey R.M., Dobereiner J. (1995) Nitrogen fixation associated with grasses and cereals: Recent progress and perspectives for the future. Nutrient Cycling in Agroecosystems 42:241-250.

Boddey R.M., Urquiaga S., Alves B.J.R., Reis V. (2003) Endophytic nitrogen fixation in sugarcane: present knowledge and future applications. Plant and Soil 252:139-149.

Boddey R.M., Polidoro J.C., Resende A.S., Alves B.J.R., Urquiaga S. (2001) Use of the 15N natural abundance technique for the quantification of the contribution of N2 fixation to sugar cane and other grasses. Australian Journal of Plant Physiology 28:889-895.

Boddey R.M., Oliveira O.C., Urquiaga S., Reis V.M., Olivares F.L., Baldani V.L.D., Döbereiner J. (1995) Biological nitrogen fixation associated with sugar cane and rice: Contributions and prospects for improvement. Plant and Soil 174:195-209.

Bohlool B.B., Ladha J.K., Garrity D.P., George T. (1992) Biological nitrogen fixation for sustainable agriculture: A perspective. Plant and Soil 141:1-11.

Bormann B.T., Bormann F.H., Bowden W.B., Piece R.S., Hamburg S.P., Wang D., Snyder M.C., Li C.Y., Ingersoll R.C., . (1993) Rapid N_2 Fixation in Pines, Alder, and Locust: Evidence From the Sandbox Ecosystems Study. Ecology 74:583-598.

Bormann F.H., Likens G.E., Melillo J.M. (1977) Nitrogen budget for an aggrading northern hardwood forest ecosystem. Sci. 196:981-983.

Bremner J.M., Krogmeier M. J. 1989. Evidence that the adverse effect of urea fertilizer on seed germination in soil is due to ammonia formed through hydrolysis of urea by soil urease.

Proceedings of the National Academy of Sciences of the United States of America 86: 8185-8188.

Brock P.M., Inwood J.R.B., Deverall B.J. (1994) Systemic induced resistance to Alternaria macrospora in cotton (*Gossypium hirsutum*). Australasian Plant Pathology 23:81-85.

Brockley R.P. (1989) Response of thinned, immature lodgepole pine to nitrogen fertilization: three-year growth response. FRDA Report No. 036.

Brockley R.P. (1990) Response of thinned, immature lodgepole pine to nitrogen and boron fertilization. Canadian Journal of Forest Research 20:579-585.

Brockley R.P. (1991) Response of thinned, immature lodgepole pine to nitrogen fertilization: six-year growth response. FRDA Report No. 184.

Brockley R.P. (1996) Lodgepole nutrition and fertilization: a summary of B.C. Ministry of Forests research results. B.C. Ministry of Forests, Victoria, B.C.

Brockley R.P. 2003. Effects of nitrogen and boron fertilization on foliar boron nutrition and growth in two different lodgepole pine ecosystems. Can. J. For. Res. 33: 988-996.

Brockley R.P. and Sanborn, P.T. 2009. Effects of repeated fertilization on forest floor and mineral soil properties in young lodgepole pine and spruce forests in central British Columbia.

Brooks D.S., Gonzalez C.F., Appel D.N., Filer T.H. (1994) Evaluation of endophytic bacteria as potential biological-control agents for Oak Wilt. Biological Control 4:373-381.

Bruijn F.J., Jing Y., Dazzo F.B. (1995) Potential and pitfalls of trying to extend symbiotic interactions of nitrogen-fixing organisms to presently non-nodulated plants, such as rice. Plant and Soil 174:225-240.

Bryceson I., Fay P. (1981) Nitrogen fixation in Oscillatoria (Trichodesmium) erythraea in relation to bundle formation and trichome differentiation. Marine Biology 61:159-166.

Burke DJ, Dunham SM, Kretzer AM. 2008. Molecular analysis of bacterial communities associated with the roots of Douglas fir (*Pseudotsuga menziesii*) colonized by different ectomycorrhizal fungi. FEMS Microbiology Ecology 65: 299-309.

Caballero-Mellado J., Onofre-Lemus J., Estrada-de los Santos P., Martinez-Aguilar L. (2007) The Tomato Rhizosphere, an Environment Rich in Nitrogen-Fixing Burkholderia Species with Capabilities of Interest for Agriculture and Bioremediation. Appl. Environ. Microbiol. 73:5308-5319.

Carniol K., Ben-Yehuda S., King N., Losick R. (2005) Genetic Dissection of the Sporulation Protein SpoIIE and Its Role in Asymmetric Division in *Bacillus subtilis.* J. Bacteriol. 187:3511-3520.

Carpenter E., Price C. (1976) Marine *oscillatoria* (*Trichodesmium*): explanation for aerobic nitrogen fixation without heterocysts. Science 191:1278-1280.

Carroll G. (1988) Fungal Endophytes in Stems and Leaves: From Latent Pathogen to Mutualistic Symbiont. Ecology 69:2-9.

Cavalcante V., Döbereiner J. (1988) A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. Plant and Soil 108:23-31.

Chanway C., Holl F., Turkington R. (1988) Genotypic coadaptation in plant growth promotion of forage species by *Bacillus polymyxa*. Plant and Soil 106:281-284.

Chanway C.P. (1997) Inoculation of tree roots with plant growth promoting soil bacteria: an emerging technology for reforestation. For Sci 43:99-112.

Chanway C.P., Holl F.B. (1991) Biomass increase and associative nitrogen fixation of mycorrhizal *Pinus contorta* seedlings inoculated with a plant growth promoting *Bacillus* strain. Canadian Journal of Botany. 69:507-511.

Chanway C.P., Holl F.B. (1992) Influence of soil biota on Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedling growth the role of rhizosphere bacteria. Can. J. Bot. 70:1025-1031.

Chanway C.P., Holl F.B. (1993) First year field performance of spruce seedlings after inoculation with plant growth promoting rhizobacteria. Can. J. Microbiol. 39:1084-1088.

Chanway C.P., Holl F.B. (1994) Growth of Outplanted Lodgepole Pine Seedlings One Year After Inoculation with Plant Growth Promoting Rhizobacteria. Forest Science 40:238-246.

Chanway CP, Radley RA, Holl FB. 1991. Inoculation of conifer seed with plant growth promoting *Bacillus* strains causes increased seedling emergence and biomass. Soil Biology and Biochemistry 23: 575-580

Chanway C.P., Shishido M., Holl F.B. (1994) Root-endophytic and rhizosphere plant growth promoting rhizobacteria for conifer seedlings, in: M. H. Ryder, et al. (Eds.), Improving plant productivity with rhizosphere bacteria, CSIRO Division of Soils, Blackmountain, Australia. pp. 72-74.

Chanway C.P., Shishido M., Jungwirth S., Nairn J., Markham J., Xiao G., Holl F.B. (1997) Second year growth responses of outplanted conifer seedlings inoculated with PGPR, in: A. Ogoshi, et al. (Eds.), 4th International Workshop on Plant Growth-Promoting Rhizobacteria: Plant Growth-Promoting Rhizobacteria—Present Status and Future Prospects, Sapporo, Japan. pp. 172–176.

Chanway C.P., Shishido M., Nairn J., Jungwirth S., Markham J., Xiao G., Holl F.B. (2000) Endophytic colonization and field responses of hybrid spruce seedlings after inoculation with plant growth-promoting rhizobacteria. Forest Ecology and Management 133:81-88.

Chelius M.K., Triplett E.W. (2000) *Dyadobacter fermentans* gen. nov., sp. nov., a novel Gram-negative bacterium isolated from surface-sterilized *Zea mays* stems. International Journal of Systematic and Evolutionary Microbiology 50:751-758.

Chelius M.K., Triplett E.W. (2001) The Diversity of Archaea and Bacteria in Association with the Roots of *Zea mays* L. Microbial Ecology 41:252-263.

Chen Y.-B., Zehr J.P., Mellon M.T. (1996) Growth and nitrogen fixation of the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium* sp. IMS 101 in defined media: evidence for a circadian rhythm. Journal of Phycology 32:916–923.

Cheng Q., Day A., Dowson-Day M., Shen G.-F., Dixon R. (2005) The *Klebsiella pneumoniae* nitrogenase Fe protein gene (*nif*H) functionally substitutes for the *chl*L gene in *Chlamydomonas reinhardtii*. Biochemical and Biophysical Research Communications 329:966-975.

Choi O., Kim J., Ryu C.-M., Park C.S. (2004) Colonization and Population Changes of a Biocontrol Agent, *Paenibacillus polymyxa* E681, in Seeds and Roots. Journal of Plan Pathology 20:97-102.

Choi S.-K., Park S.-Y., Kim R., Lee C.-H., Kim J.F., Park S.-H. (2008) Identification and functional analysis of the fusaricidin biosynthetic gene of *Paenibacillus polymyxa* E681. Biochemical and Biophysical Research Communications 365:89-95.

Choo Q.-C., Samian M.-R., Najimudin N. (2003) Phylogeny and Characterization of Three nifH-Homologous Genes from *Paenibacillus azotofixans*. Appl. Environ. Microbiol. 69:3658-3662.

Clay K. (1988) Fungal Endophytes of Grasses: A Defensive Mutualism between Plants and Fungi. Ecology 69:10-16.

Cocking E.C., Kothari S.I., Batchelor C.A., Jain S., Webster G., Jones J., Jotham J., Davey M.R. (1995) Interaction of rhizobia with non-legume crops for symbiotic nitrogen fixation nodulation. , in: I. Fendrik, et al. (Eds.), *Azospirillum* VI and related Microorganisms: Genetics, Physiology,Ecology., Springer-Verlag, New York. pp. 197-205.

Coelho M.R.R., Marriel I.E., Jenkins S.N., Lanyon C.V., Seldin L., O'Donnell A.G. (2009) Molecular detection and quantification of nifH gene sequences in the rhizosphere of sorghum (Sorghum bicolor) sown with two levels of nitrogen fertilizer. Applied Soil Ecology 42:48-53.

Colnaghi R, Green A, He L, Rudnick P, Kennedy C. 1997. Strategies for increased ammonium production in free-living or plant associated nitrogen fixing bacteria. Plant and Soil 194: 145-154.

Colombo P.M. (1978) Occurrence of Endophytic Bacteria in Siphonous Algae. Phycologia Vol. 17:148-151.

Compant S., Reiter B., Sessitsch A., Nowak J., Clement C., Ait Barka E. (2005) Endophytic Colonization of Vitis vinifera L. by Plant Growth-Promoting Bacterium *Burkholderia* sp. Strain PsJN. Appl. Environ. Microbiol. 71:1685-1693.

Corbin D., Ditta G., Helinski D.R. (1982) Clustering of nitrogen fixation (nif) genes in *Rhizobium meliloti*. J. Bacteriol. 149:221-228.

Culling, C.F.A. (1974) Modern microscopy: Elementary theory and practice. Butterworth and company. London. p. 148

Daane L.L., Harjono I., Barns S.M., Launen L.A., Palleroni N.J., Haggblom M.M. (2002) PAH-degradation by *Paenibacillus spp*. and description of *Paenibacillus naphthalenovorans* sp. nov., a naphthalene-degrading bacterium from the rhizosphere of salt marsh plants. Int J Syst Evol Microbiol 52:131-139.

Dalton D.A., Kramer S., Azios N., Fusaro S., Cahill E., Kennedy C. (2004) Endophytic nitrogen fixation in dune grasses (*Ammophila arenaria* and *Elymus mollis*) from Oregon. FEMS Microbiology Ecology 49:469-479.

Dance I. (2007) Elucidating the Coordination Chemistry and Mechanism of Biological Nitrogen Fixation. Chemistry - An Asian Journal 2:936-946.

Dance, I. 2008. The chemical mechanism of nitrogenase: calculated details of the intramolecular mechanism for hydrogenation of 2-N_2 on FeMo-co to NH_3. *Dalton Transactions*: 5977 - 5991.

Danso S.K.A. (1995) Assessment of biological nitrogen fixation. Nutrient Cycling in Agroecosystems 42:33-41.

Danso S.K.A., Hardarson G., Zapata F. (1993) Misconceptions and practical problems in the use of^{15}N soil enrichment techniques for estimating N_2 fixation. Plant and Soil 152:25-52.

Day J.M., Neves M.C.P., Döbereiner J. (1975) Nitrogenase activity on the roots of tropical forage grasses. Soil Biology and Biochemistry 7:107-112.

Dean D.R., Jacobson M.R. (1992) Biochemical genetics of nitrogenase, in: G. Stacy, et al. (Eds.), Biological nitrogen fixation, Chapman and Hall, New York. pp. 763–834.

Debellé F., Moulin L., Mangin B., Dénarié J., Boivin C. (2002) Nod genes and Nod signals and the evolution of the Rhizobium legume symbiosis. Acta Biochim Polon 48:359-365.

Deslippe J.R., Egger K.N., Henry G.H.R. (2005) Impacts of warming and fertilization on nitrogen-fixing microbial communities in the Canadian High Arctic. FEMS Microbiology Ecology 53:41-50.

Deslippe J, Egger K. 2006. Molecular diversity of *nif*H genes from bacteria associated with high Arctic dwarf shrubs. Microbial Ecology 51: 516-525.

Dedysh SN, Ricke P, Liesack W. 2004. NifH and NifD phylogenies: an evolutionary basis for understanding nitrogen fixation capabilities of methanotrophic bacteria. Microbiology 150: 1301-1313.

Dickson B.A., Crocker R.L. (1953) A chronosequence of soils and vegetation near Mt. Shasta, California. . J. Soil Sci. 4:123-141.

Ding Y., Wang J., Liu Y., Chen S. (2005) Isolation and identification of nitrogen-fixing bacilli from plant rhizospheres in Beijing region. Journal of Applied Microbiology 99:1271-1281.

Dixon R., Cheng Q., Shen G.-F., Day A., Dowson-Day M. (1997) *Nif* gene transfer and expression in chloroplasts: Prospects and problems. Plant and Soil 194:193-203.

Döbereiner, J. 1997. Biological nitrogen fixation in the tropics: Social and economic contributions. *Soil Biology and Biochemistry* 29:771-774.

Döbereiner J. (1961) Nitrogen-fixing bacteria of the genus *Beijerinckia* Derx in the rhizosphere of sugar cane. Plant and Soil 15:211-216.

Döbereiner J., Day J.M., Dart P.J. (1972) Nitrogenase Activity and Oxygen Sensitivity of the *Paspalum notatum-Azotobacter paspali* Association. J Gen Microbiol 71:103-116.

Döbereiner J., Urquiaga S., Boddey R.M. (1995) Alternatives for nitrogen nutrition of crops in tropical agriculture. Nutrient Cycling in Agroecosystems 42:339-346.

Döbereiner J., Pedrosa F.O. (1987) Nitrogen-Fixing Bacteria in Nonleguminous Crop Plants Brock/Springer Series in Contemporary Biosciences, Science Tech Publishers, Madison. pp. 155.

Dong Z., Canny M.J., McCully M.E., Roboredo M.R., Cabadilla C.F., Ortega E., Rodes R. (1994) A Nitrogen-Fixing Endophyte of Sugarcane Stems (A New Role for the Apoplast). Plant Physiol. 105:1139-1147.

Doty S.L., Oakley B., Xin G., Kang J.W., Singleton G.L., Khan Z., Vajzovic A., Staley J.T. (2009) Diazotrophic endophytes of native black cottonwood and willow. Symbiosis 47:23–33

Doty, S. L., Dosher, M. R., Singleton, G. L., Moore, A. L., Aken, B. van, Stettler, R. F., Strand, S. E., Gordon, M. P. (20050 Identification of an endophytic *Rhizobium* in stems of *Populus*. Symbiosis (Rehovot), , 39, 1, pp 27-35.

Dunn A.K., Handelsman J. (1999) A vector for promoter trapping in *Bacillus cereus*. Gene 226:297-305.

Eady R.R. (1996) Structure-Function Relationships of Alternative Nitrogenases. Chemical Reviews 96:3013-3030.

Egener T., Hurek T., Reinhold-Hurek B. (1998) Use of Green Fluorescent Protein to Detect Expression of *nif* Genes of *Azoarcus sp*. BH72, a Grass-Associated Diazotroph, on Rice Roots. Molecular Plant-Microbe Interactions 11:71-75.

Elbeltagy A., Nishioka K., Sato T., Suzuki H., Ye B., Hamada T., Isawa T., Mitsui H., Minamisawa K. (2001) Endophytic Colonization and *In Planta* Nitrogen Fixation by a *Herbaspirillum sp*. Isolated from Wild Rice Species. Appl. Environ. Microbiol. 67:5285-5293.

Elo S., Suominen I., Kampfer P., Juhanoja J., Salkinoja-Salonen M., Haahtela K. (2001) *Paenibacillus borealis sp.* nov., a nitrogen-fixing species isolated from spruce forest humus in Finland. Int J Syst Evol Microbiol 51:535-545.

Enebak SA, Wei G, Kloepper JW. 1998. Effects of Plant Growth-Promoting Rhizobacteria on Loblolly and Slash Pine Seedlings. Forest Science 44: 139-144.

Eriksson H., Rosen K. (1994) Nutrient distribution in a Swedish tree species experiment. Plant and Soil 164:51-59.

Estrada-De Los Santos P., Bustillos-Cristales R., Caballero-Mellado J. (2001) *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl. Environ. Microbiol. 67:2790-2798.

Fahey T, Yavitt J, Pearson J, Knight D. 1985. The nitrogen cycle in lodgepole pine forests, southeastern Wyoming. Biogeochemistry 1: 257-275.

Fay P., Stewart W.D.P., Walsby A.E., Fogg G.E. (1968) Is the heterocyst the site of nitrogen fixation in blue-green algae. Nature 220:810-812.

Fenn M.E., Poth M.A., Aber J.D., Baron J.S., Bormann B.T., Johnson D.W., Lemly A.D., McNulty S.G., Ryan D.F., Stottlemyer R. (1998) NIitrogen excess in North American ecosystems: Predisposing factors, ecosystem responses, and management strategies. Ecological Applications 8:706-733.

Fischer H.M. (1994) Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Mol. Biol. Rev. 58:352-386.

Fisher R.F., Eastburn R.P. (1974) Afforestation alters Prairie soil nitrogen status. Soil Sci Soc Am J 38:366-368.

Fitter A., Garbaye J. (1994) Interactions between mycorrhizal fungi and other soil organisms. Plant and Soil 159:123-132.

Frommel M.I., Nowak J., Lazarovits G. (1991) Growth Enhancement and Developmental Modifications of *in Vitro* Grown Potato (*Solanum tuberosum spp. tuberosum*) as affected by a nonfluorescent Pseudomonas sp. Plant Physiol. 96:928-936.

Gallon J.R. (1992). Reconciling the incompatible: N_2 fixation and O_2.New Phytologist 122: 571-609.

Galloway J.N., Cowling E.B. (2002) Reactive nitrogen and the world: 200 Years of change. AMBIO: A journal of the human environment 31:64-71.

Gamalero E., Lingua G., Berta G., Lemanceau P. 2009. Methods for studying root colonization by introduced beneficial bacteria. Sustainable Agriculture. 5: 601-615.

Gange A.C., Dey S., Currie A.F., Sutton B.C. (2007) Site- and species-specific differences in endophyte occurrence in two herbaceous plants. Journal of Ecology 95:614-622.

Gantar M., Kerby N.W., Rowell P. 1991. Colonization of wheat (*Triticum vulgare* L.) by N_2-fixing cyanobacteria: II. An ultrastructural study. New Phytologist 118: 485-492.

Garbaye, J. (1994) Helper Bacteria: A New Dimension to the Mycorrhizal Symbiosis. New phytologist 128 (2) pp. 197-210.

Gardner J.M., Feldman A.W., Zablotowicz R.M. (1982) Identity and Behavior of Xylem-Residing Bacteria in Rough Lemon Roots of Florida Citrus Trees. Appl. Environ. Microbiol. 43:1335-1342.

Germaine K., Keogh E., Garcia-Cabellos G., Borremans B., Lelie D., Barac T., Oeyen L., Vangronsveld J., Moore F.P., Moore E.R.B., Campbell C.D., Ryan D., Dowling D.N. (2004) Colonisation of poplar trees by *gfp* expressing bacterial endophytes. FEMS Microbiology Ecology 48:109-118.

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

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

Goldstein J.I., Costley J.L., Lorimer G.W., Reed S.J.B. (1977) Quantitative X-ray Analysis in the Electron Microscope, in: O. Johari (Ed.), SEM, IIT Research Institute, Chicago. pp. 315-324.

Gonzalez, J.S. (2004). Growth properties and uses of western redcedar (Special publication). Forintek Canada corporation, Vancouver.BC pp:4.

Gouzou L., Burtin G., Philippy R., Bartoli F., Heulin T. (1993) Effect of inoculation with *Bacillus polymyxa* on soil aggregation in the wheat rhizosphere: preliminary examination. Geoderma 56:479-491.

Govedarica M., Nada M., Mirjana J., Milošev D., Simonida D. (1997) Diazotrophs and their activity in pepper. Acta Hort. (ISHS) 462:725-732.

Gracioli L.A., Ruschel A.P. (1981) Microorganisms in the phyllosphere and rhizosphere of sugarcane, in: P. B. Vose and A. P. Ruschel (Eds.), Associative Nitrogen Fixation, CRC Press, Florida. pp. 87-101.

Grimault V., Prior P. (1994) Invasiveness of *Pseudomonas solanacearum* in tomato, eggplant and pepper: a comparative study. European Journal of Plant Pathology 100:259-267.

Guemouri-Athmani S., Berge O., Bourrain M., Mavingui P., Thiery J.M., Bhatnagar T., Heulin T. (2000) Diversity of *Paenibacillus polymyxa* populations in the rhizosphere of wheat (*Triticum durum*) in Algerian soils. European Journal of Soil Biology 36:149-159.

Gutierrez-Manero F.J., Ramos-Solano B., Probanza A., Mehouachi J., Tadeo F R., Talon M. (2001) The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. Physiologia Plantarum 111:206-211.

Gyaneshwar P, James EK, Mathan N, Reddy PM, Reinhold-Hurek B, Ladha JK. 2001. Endophytic Colonization of Rice by a diazotrophic strain of *Serratia marcescens*. J. Bacteriol. 183: 2634-2645

Haahtela K., Laakso T., Nurmiaho-Lassila E.-L., Korhonen T. (1988) Effects of inoculation of *Poa pratensis* and *Triticum aestivum* with root-associated, N_2-fixing *Klebsiella, Enterobacter and Azospirillum*. Plant and Soil 106:239-248.

Haggag W.M., Timmusk S. (2008) Colonization of peanut roots by biofilm-forming *Paenibacillus polymyxa* initiates biocontrol against crown rot disease. Journal of Applied Microbiology 104:961-969.

Hallmann J., Berg G., Schulz B. (2006) Isolation procedures for endophytic microorganisms. , in: B. Schulz, et al. (Eds.), Microbial root endophytes, Springer Verlag, Berlin. pp. 299-319.

Hallmann J., Quadt-Hallmann A., Mahafee W.F., Kloepper J.W. (1997) Bacterial endophytes in agricultural crops. Can. J. Microbiol. 43:895-914.

Halsall D.M., Gibson A.H., (1986) Comparison of two *Cellulomonas* strains and their interaction with *Azospirillum brasilense* in degradation of wheat straw and associated nitrogen fixation. Appl Environ Microbiol. 51:855-861.

Hardy R.W.F., Holsten R.D., Jackson E.K., Burns R.C. (1968) The Acetylene-Ethylene assay for N_2 fixation: Laboratory and field evaluation. Plant Physiol. 43:1185-1207.

Haselkorn R. (1992) Developmentally regulated gene rearrangements in prokaryotes. Annual Review of Genetics 26:113-130.

Haselkorn R. (2003) Heterocysts. Annual Review of Plant Physiology 29:319-344.

Hayat M.A. (1986) Basic techniques for transmission electron microscopy. Academic Press, Inc., N. Y.

Hegazi N.A., Fayez M., Amin G., Hamza M.A., Abbas M., Youssef, H., Monib M. (1998) Diazotrophs associated with non-legumes grown in sandy soils, in: K. A. Malik, Mirza, M.S. and Ladha, J.K. (Ed.), Nitrogen Fixation with Non-Legumes, Kluwer Academic Publishers, Dordrecht. pp. 209-222.

Haeussler, S., Coates, D., 1986. Autecological characteristics of selected species that compete with conifers in British Columbia: a literature review. FRDA Report No. 001. BC Ministry of Forests, Canadian Forestry Service, Victoria, 180 pp

Henson B., Watson L., Barnum S. (2004) The evolutionary history of nitrogen fixation, as assessed by NifD. Journal of Molecular Evolution 58:390-399.

Herridge D.F. (1984) Effects of nitrate and plant development on the abundance of nitrogenous solutes in root-bleeding and vacuum-extracted exudates of soybean. Crop Sci 24:173-179.

Heulin T., Berge O., Mavingui P., Gouzou L., Hebbar K.P., Balandreau J. (1994) *Bacillus polymyxa* and *Rahnella aquatilis*, the dominant N_2-fixing bacteria associated with wheat rhizosphere in French soils. European Journal of Soil Biology 30:35-42.

Heyndrickx M., Vandemeulebroecke K., Scheldeman P., Kersters K., De Vos P., Logan N.A., Aziz A.M., Ali N., Berkeley R.C.W. (1996) A Polyphasic reassessment of the genus *Paenibacillus*, reclassification of *Bacillus lautus* (Nakamura 1984) as *Paenibacillus lautus* comb. nov. and of *Bacillus peoriae* (Montefusco *et al*. 1993) as *Paenibacillus peoriae* comb. nov., and emended descriptions of *P. lautus* and of *P. peoriae*. Int J Syst Bacteriol 46:988-1003.

Hill S., Postgate J.R. (1969) Failure of putative nitrogen-fixing bacteria to fix nitrogen. J . gen. Microbiol. 58:277.

Hirsch A.M., McKhann H.I., Reddy A., Liao J., Fang Y., Marshall C.R. (1995) Assessing horizontal transfer of *nif*HDK genes in eubacteria: nucleotide sequence of *nif*K from *Frankia* strain HFPCcI3. Mol Biol Evol 12:16-27.

Holl F.B., Chanway C.P. (1992) Rhizosphere colonization and seedling growth promotion of lodgepole pine by *Bacillus polymyxa*. Canadian Journal of Microbiology. 38:303-308.

Holl F.B., Chanway C.P., Turkington R., Radley R.A. (1988) Response of crested wheatgrass (*Agropyron cristatum* L.), perennial ryegrass (*Lolium perenne* L.), and white clover (*Trifolium repens* L.) to inoculation with *Bacillus polymyxa*. . Soil Biology and Biochemistry. 20:19-24.

Hollis J.P. (1951) Bacteria in healthy potato tissue. Phytopathology 41:320-366.

Hunt R. (1982) Plant growth curves. University Park Press, Baltimore.

Hungate BA, Dijkstra P, Johnson DW, Hinkle CR, Drake BG. 1999. Elevated CO2 increases nitrogen fixation and decreases soil nitrogen mineralization in Florida scrub oak. Global Change Biology 5: 781-789.

Hurek T, Handley LL, Reinhold-Hurek B, Piché Y. 2007. Azoarcus grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Molecular Plant-Microbe Interactions 15: 233-242.

Hurek T., Reinhold-Hurek B., Van Montagu M., Kellenberger E. (1994) Root colonization and systemic spreading of *Azoarcus sp*. strain BH72 in grasses. J. Bacteriol. 176:1913-1923.

Hyde K.D., Soytong K. (2008) The fungal endophyte dilemma. Fungal Diversity 33:163-173.

Onwurah I. N. E. (1999) Role of diazotrophic bacteria in the bioremediation of crude oil-polluted soil. Journal of Chemical Technology & Biotechnology 74:957-964.

Ishiwa H., Shibahara H. (1985) New shuttle vectors for *Escherichia coli* and *Bacillus subtilis.* Japanese Journal of Genetics 60:485-498.

Itaya M., Shaheduzzaman S.M., Matsui K., Omori A., Tsuji T. (2001) Green marker for colonies of *Bacillus subtilis*. Bioscience, Biotechnology, and Biochemistry 65:579-583.

Izumi H., Anderson I.C., Killham K., Moore E.R.B. (2008) Diversity of predominant endophytic bacteria in European deciduous and coniferous trees. Canadian Journal Of Microbiology 54:173-179.

James E.K. (2000) Nitrogen fixation in endophytic and associative symbiosis. Field Crops Res. 65:197-209.

James E.K., Olivares F. (1998) Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. Critical Reviews in Plant Sciences 17:77-119.

James E.K., Gyaneshwar G., Barraquio W.L., Ladha J.K. (1999) Endophytic diazotrophs associated with rice, in: J. K. Ladha and P. N. Reddy (Eds.), The quest for nitrogen Fixation in Rice, International Rice Research Institute, Manila.

Jansson J. K. (1998) Marker genes as tags for monitoring microorganisms in nature. An opinion. MAREP (Marker/reporter genes in microbial ecology): A concerted action; european commision biotechnology programme, DGXII, Borås, Sweden.

Jeong H-Y, Kim J-H, Park Y-K, Kim S-B, Kim C-H, Park S-H. 2006. Genome snapshot of *Paenibacillus polymyxa* ATCC 842^{T}. J. Microbiol. Biotechnol., 16:10, 1650–1655

Johnson D.W., Todd D.E., Jr. (1998) Harvesting effects on long-term changes in nutrient pools of mixed oak forest. Soil Sci Soc Am J 62:1725-1735.

Jones, A.L. *PHASEOLUS BEAN: Post-harvest Operations*. FAO [cited. Available from http://www.fao.org/inpho/content/compend/text/ch04.htm.

Jose S., Merritt S., Ramsey C.L. (2003) Growth, nutrition, photosynthesis and transpiration responses of longleaf pine seedlings to light, water and nitrogen. Forest Ecology and Management 180:335-344.

Jurgensen M, Davey C. 1971. Nonsymbiotic nitrogen-fixing micro-organisms in forest and tundra soils. Plant and Soil 34: 341-356.

Kallas T., Coursin T., Rippka R. (1985) Different organization of *nif* genes in nonheterocystous and heterocystous cyanobacteria. Plant Molecular Biology 5:321-329.

Katupitiya S, New PB, Elmerich C, Kennedy IR. Improved N_2 fixation in 2,4-D treated wheat roots associated with *Azospirillum lipoferum*: Studies of colonization using reporter genes. Soil Biology and Biochemistry 27: 447-452.

Kennedy I.R., Choudhury A.T.M.A., Kecskés M.L. (2004) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biology and Biochemistry 36:1229-1244.

Kennedy I.R., Pereg-Gerk L.L., Wood C., Deaker R., Gilchrist K., Katupitiya S. (1997) Biological nitrogen fixation in non-leguminous field crops: Facilitating the evolution of an effective association between Azospirillum and wheat. Plant and Soil 194:65-79.

Kessel C.V., Roskoski J.P., Keane K. (1988) Ureide production by N_2-fixing and non-N_2-fixing leguminous trees. Soil Biol. Biochem. 20:891-.897.

Khush, G. 2001. Challenges for meeting the global food and nutrient needs in the new millennium. *Proceedings of the Nutrition Society* 60:15-26.

Kessler P.S., Blank C., Leigh J.A. (1998) The *nif* Gene Operon of the Methanogenic Archaeon *Methanococcus maripaludis*. J. Bacteriol. 180:1504-1511.

Kim J., Rees D.C. (1994) Nitrogenase and biological nitrogen fixation. Biochemistry 33:389-397.

Kimmins J.P., Mailly D., Seely B. 1999. Modelling forest ecosystem net primary production: the hybrid simulation approach used in. Ecological Modelling 122: 195-224.

Kirizii D., Vorobei N., Kots' S. 2007. Relationships between nitrogen fixation and photosynthesis as the main components of the productivity in alfalfa. Russian Journal of Plant Physiology 54: 589-594.

Kishchuk, B.E., and Brockley, R.P. 2002. Sulfur availability on lodgepole pine sites in British Columbia. Soil Sci. Soc. Am. J. **66**: 1325–1333.

Kleiner D. 1985. Bacterial ammonium transport. FEMS Microbiology Letters 32: 87-100.

Kloepper J.W. (1993) Plant growth-promoting rhizobacteria as biological control agents, in: M. FB (Ed.), Soil microbial ecology applications in agricultural and environmental management., Dekker, New York. pp. 255–274.

Kloepper J.W., Ryu C.M. (2006) Bacterial endophytes as elicitors of induced systemic resistance. pp. 33-52.

Kloepper J.W., Lifshitz R., Zablotowicz R.M. (1989) Free-living bacterial inocula for enhancing crop productivity. Trends in Biotechnology 7:39-44.

Kloepper J.W., McInroy J.A., Bowen K.L. (1992) Comparative identification by fatty acid analysis of soil, rhizosphere, and geocarposphere bacteria of peanut (*Arachis hypogaea* L.). Plant and Soil 139:85-90.

Kloepper J.W., Ryu C.-M., Zhang S. (2007) Induced systemic Resistance and Promotion of Plant Growth by *Bacillus* spp. Phytopathology 94:1259-1266.

Korhonen T.K., Virkola R., Holthofer H. 1986. Localization of binding sites for purified *Escherichia coli, P. fimbriae* in the human kidney. Infect. Immun. 54: 328-332.

Krause A., Ramakumar A., Bartels D., Battistoni F., Bekel T., Boch J., Bohm M., Friedrich F., Hurek T., Krause L., Linke B., McHardy A.C., Sarkar A., Schneiker S., Syed A.A., Thauer R., Vorholter F.-J., Weidner S., Puhler A., Reinhold-Hurek B., Kaiser O., Goesmann A. (2006) Complete genome of the mutualistic, N_2-fixing grass endophyte *Azoarcus sp*. strain BH72. Nat Biotech 24:1384-1390.

Kranabetter J.M., Sanborn P., Chapman B.K., Dube S. 2006. The contrasting response to soil disturbance between lodgepole pine and hybrid white spruce in sub-boreal forests. Soil Sci. Soc. Am. J. 70: 1591-1599

Krause, H.H. 1998. Forest floor mass and nutrients in two chronosequences of plantations: Jack pine vs. black spruce. Can. J. Soil Sci. 78:77–83.

Kuhn, R., Jerchel, D. (1941) Über Invertseifen VIII. Reduktion von Tetrazoliumsalzendurch Bakterien, gãrende Hefe und keimende Samen. Ber. deut. chem. Ges.,74, pt. 1, 949-952.

Lal S., Tabacchioni S. (2009) Ecology and biotechnological potential of *Paenibacillus polymyxa*: a minireview. Indian Journal of Microbiology 49:2-10.

Lamb T.G., Tonkyn D.W., Kluepfel D.A. (1996) Movement of *Pseudomonas aureofaciens* from the rhizosphere to aerial plant tissue. Can. J. Microbiol. 42:1112-1120.

Law R., Lewis D.H. (1983) Biotic environments and the maintenance of sex-some evidence from mutualistic symbioses. Biological Journal of the Linnean Society 20:249-276.

Lebuhn M., Heulin T., Hartmann A. (1997) Production of auxin and other indolic and phenolic compounds by *Paenibacillus polymyxa* strains isolated from different proximity to plant roots. FEMS Microbiology Ecology 22:325-334.

Lee K., Choi C. (1987) Growth and plasmid stability of recombinant *E. coli* cells producing hepatitis B surface antigen. Korean Journal of Chemical Engineering 4:182-186.

Leigh J.A. (2000) Nitrogen fixation In methanogens: The archaeal perspective. current issues Molecular Biology 2:125-131.

Levanony H, Bashan Y, Romano B, Klein E. 1989. Ultrastructural localization and identification of *Azospirillum brasilense* Cd on and within wheat root by immuno-gold labeling. Plant and Soil 117: 207-218.

Li C.Y., Massicote H.B., Moore L.V.H. (1992) Nitrogen-fixing *Bacillus* sp. associated with Douglas-fir tuberculate ectomycorrhizae. Plant and Soil 140:35-40.

Lindberg T., Granhall U. (1984) Isolation and characterization of dinitrogen-fixing bacteria from the rhizosphere of temperate cereals and forage grasses. Appl. Environ. Microbiol. 48:683-689.

Lindberg T., Granhall U., Tomenius K. (1985) Infectivity and acetylene reduction of diazotrophic rhizosphere bacteria in wheat (*Triticum aestivum*) seedlings under gnotobiotic conditions. Biology and Fertility of Soils 1:123-129.

Lo Piccolo S, Ferraro V, Alfonzo A, Settanni L, Ercolini D, Burruano S, Moschetti G. 2010. Presence of endophytic bacteria in Vitis vinifera leaves as detected by fluorescence *in situ* hybridization. Annals of Microbiology 60: 161-167.

Lodewyckx C., Vangronsveld J., Porteous F., Moore E.R.B., Taghavi S., Mezgeay M., LelieD. v.d. (2002) Endophytic bacteria and their potential applications. . Critical Reviews in Plant Sciences 21:583-606.

Long SR. 1989. Rhizobium-legume nodulation: Life together in the underground. Cell 56: 203-214.

Lotan J.E., Critchfield W.B. (1990) *Pinus contorta spp*. murrayana - lodgepole pine forest, in: R. M. Burns and B. H. Honkala (Eds.), Silvics of North America,Conifers. Agriculture Handbook 654, USDA, Washington, D.C. pp. 302-313.

Lotan J.E., Critchfield W.B. (1996) Lodgepole Pine in North America, in: P. Koch (Ed.), Forest Products Society, Madison, Wisconson. pp. 343.

Luchansky J.B., Muriana P.M., Klaenhammer T.R. (1988) Application of electroporation for transfer of plasmid DNA to *Lactobacillus, Lactococcus, Leuconostoc, Listeria, Pediococcus, Bacillus, Staphylococcus, Enterococcus* and *Propionibacterium*. Molecular Microbiology 2:637-646.

Macaluso A., Mettus A.M. (1991) Efficient transformation of *Bacillus thuringiensis* requires nonmethylated plasmid DNA. J. Bacteriol. 173:1353-1356.

Madigan M.T., Martinko J.M., Parker J. (2000) Metabolic Diversity, Brock Biology of Microorgansms, Prentice Hall, New Jersey. pp. 586-590.

Mahaffee, W.F., Kloepper, J.W., Van Vuurde, J.W.L., Van der Wolf, J.M., and Van den Brink, M. 1997. Endophytic colonization of *Phaseolus vulgaris* by *Pseudomonas fluorescens* strain 89B-27 and *Enterobacter asburiae* strain JM22. **In:** *Improving Plant Productivity in Rhizosphere Bacteria*. Ryder M.H., Stephens P.M., and Bowen G.D., Eds., CSIRO, Melbourne, Australia.

Malik K.A., Bilal R., Mehnaz S., Rasul G., Mirza M.S., Ali S. (1997) Association of nitrogen-fixing, plant-growth-promoting rhizobacteria (PGPR) with kallar grass and rice. Plant and Soil 194:37-44.

Malik K.A., Mirza M.S., Hassan U., Mehnaz S., Rasul G., Haurat J., Bally R., Normand P. (2002) The role of plant-associated beneficial bacteria in rice-wheat cropping system., in: I. R. a. C. Kennedy, A.T.M.A. (Ed.), Biofertilisers in Action, Rural Industries Research and Development Corporation, Canberra. pp. 73-83.

Mathur M, Tuli R. 1991. Analysis of codon usage in genes for nitrogen fixation from phylogenetically diverse diazotrophs. Journal of Molecular Evolution 32: 364-373.

Marshall V.G., Barclay H.J., Hetherington E.D. (1992) Lodgepole pine response to nitrogenous fertilizers applied on and off snow, and to associated damage by small mammals. . Forest Ecology and Management. 54 225-238.

Martinez-Romero E. (2006) Dinitrogen fixing prokaryotes, in: M. Dworkin, et al. (Eds.), The prokaryotes: ecophysiology and biochemistry, Springer.

Martínez L., Caballero-Mellado J., Orozco J., Martínez-Romero E. (2003) Diazotrophic bacteria associated with banana (*Musa* spp.). Plant and Soil 257:35-47.

Mavingui P., Heulin T. (1994) In vitro chitinase and antifungal activity of a soil, rhizosphere and rhizoplane population of *Bacillus polymyxa*. Soil Biology and Biochemistry 26:801-803.

Mavingui P., Laguerre P.G., Berge O., Heulin T. (1992) Genotypic and phenotypic variability of *Paenibacillus polymyxa* in soil and in the rhizosphere of wheat. Appl. Environ. Microbiol. 58:1894-1903.

McClure P.R., Israel D.W. (1979) Transport of nitrogen in the xylem of soybean plants. Plant Physiol. 64:411-416.

McFadden G.I. (1991) *In-situ* hybridisation techniques:Molecular cytology goes ultrastructural. In *Electron Microscopy of Plant Cells* (Hall, J.M. and Hawes, C., eds). London: Academic Press, pp. 219-255.

McInroy J.A., Kloepper J.W. (1994) Novel bacterial taxa inhabiting internal tissue of sweet corn and cotton., in: M. H. Ryder, et al. (Eds.), Improving plant productivity with rhizosphere bacteria, CSIRO, , Melbourne, Australia. pp. 19-27.

McSpadden Gardener B.B. (2004) Ecology of *Bacillus* and *Paenibacillus spp*. in agricultural systems. Phytopathology 94:1252-1258.

Mead D.J., Preston C.M. (1992) Nitrogen fxation in Sitka alder by ^{15}N isotope dilution after eight growing seasons in a lodgepole pine site. Can. J. For. Res., 22:1192-1194.

Mengoni A., Mocali S., Surico G., Tegli S., Fani R. (2003) Fluctuation of endophytic bacteria and phytoplasmosis in elm trees. Microbiological Research 158:363-369.

Mergel A., Schmitz O., Mallmann T., Bothe H. (2001) Relative abundance of denitrifying and dinitrogen-fixing bacteria in layers of a forest soil. FEMS Microbiology Ecology 36:33-42.

Metting, F.B. 1993. Structure and physiological ecology of soil microbial communities. P. 3-25. *In* F.B. Metting (ed.) Soil microbial ecology: Applications in agricultural and environmental management. M. Dekker, New York.

Mikutta R., Kleber M., Kaiser K., Jahn R. (2005) Review: Organic Matter Removal from Soils using Hydrogen Peroxide, Sodium Hypochlorite, and Disodium Peroxodisulfate. Soil Sci Soc Am J 69:120-135.

Miller, H.G., J.M. Cooper, J.D. Miller, and O.J.L. Pauline. 1979. Nutrient cycles in pine and their adaptations to poor soils. Can. J.For. Res. 9:19–26.

Minerdi, D., Fani, R., Gallo, R., Boarino, A., Bonfante, P. (2001) Nitrogen fixation genes in an endosymbiotic *Burkholderia* strain**.** Appl. Environ. Microbiol. 67: 725-732

Misaghi I.J., Donndelinger C.R. (1990) Endophytic bacteria in symptom-free cotton plants. Phytopathology 80:808-811.

Mocali S., Bertelli E., Di Cello F., Mengoni A., Sfalanga A., Viliani F., Caciotti A., Tegli S., Surico G., Fani R. (2003) Fluctuation of bacteria isolated from elm tissues during different seasons and from different plant organs. Research in Microbiology 154:105-114.

Moisander P.H., Shiue L., Steward G.F., Jenkins B.D., Bebout B.M., Zehr J.P. (2006) Application of a *nif*H oligonucleotide microarray for profiling diversity of N_2-fixing microorganisms in marine microbial mats. Environmental Microbiology 8:1721-1735.

Montañez A., Abreu C., Gill P., Hardarson G., Sicardi M. (2009) Biological nitrogen fixation in maize (*Zea mays* L.) by ^{15}N isotope-dilution and identification of associated culturable diazotrophs. Biology and Fertility of Soils 45:253-263.

Moore F.P., Barac T., Borremans B., Oeyen L., Vangronsveld J., van der Lelie D., Campbell C.D., Moore E.R.B. (2006) Endophytic bacterial diversity in poplar trees growing on a BTEX-

contaminated site: The characterisation of isolates with potential to enhance phytoremediation. Systematic and Applied Microbiology 29:539-556.

Murry M.A., Fontaine M.S., Tjepkema J.D. (1984) Oxygen protection of nitrogenase in *Frankia* sp. HFPArI3. Archives of Microbiology 139:162-166.

Musson G., McInroy J.A., Kloepper J.W. (1995) Development of delivery systems for introducing endophytic bacteria into cotton. Biocontrol. Sci. Technol. 5:407-416.

Muthukumarasamy, R., Revathi, G., Seshadri, S., Lakshminarasimhan, C., 2002. Gluconacetobacter diazotrophicus (Syn. Acetobacter diazotrophicus), a promising diazotrophic endophyte in tropics. Current Science 83,137–145.

Mylona P., Pawlowski K., Bisseling T. (1995) Symbiotic Nitrogen Fixation. Plant Cell 7:869-885.

Myrold, D. D., and P. J. Bottomley. 2007. Biological N inputs. p365-388. *In* E. A. Paul (ed.) Soil microbiology, ecology and biochemistry. Elsivier Academic Press Burlington, MA, USA.

Myrold D.D., Tiedje J.M. (1986) Simultaneous estimation of several nitrogen cycling rates using ^{I5}N: theory and application. Soil Biology and Biochemistry 18:559- 568.

Naumova R.P., Grigoryeva T.V., Rizvanov A.A., Gogolev J.V., Kudrjashova N.V., Laikov A.V. (2009) Diazotrophs originated from petrochemical sludge as a potential resource of waste remediation. World Applied Sciences Journal 6 154-157.

Nguyen T.H., Deaker R., Kennedy I.R.a., Roughley R.J. (2003) The positive yield response of field-grown rice to inoculation with a multi-strain biofertiliser in the Hanoi area, Vietnam. . Symbiosis 35:231-245.

Nielsen P., Sørensen J. (1997) Multi-target and medium-independent fungal antagonism by hydrolytic enzymes in *Paenibacillus polymyxa* and *Bacillus pumilus* strains from barley rhizosphere. FEMS Microbiology Ecology 22:183-192.

Norby RJ. 1987. Nodulation and nitrogenase activity in nitrogen-fixing woody plants stimulated by CO_2 enrichment of the atmosphere. Physiologia Plantarum 71: 77-82

Normand P, Bouquet J. 1989. Phylogeny of nitrogenase sequences in *Frankia* and other nitrogen-fixing microorganisms. Journal of Molecular Evolution 29: 436-447.

Normand P, Gouy M, Cournoyer B, Simonet P. 1992. Nucleotide sequence of nifD from *Frankia alni* strain ArI3: phylogenetic inferences. Mol Biol Evol 9: 495-506.

O'Neill G.A., Chanway C.P., Axelrood P.E., Radley R.A., Holl F.B. (1992) An assesment of growth response specificity of spruce inoculated with coexistent rhizosphere bacteria. Can. J. Bot. 70: 2347-2353.

Oliveira O.C., Urquiaga S., Boddey R.M. (1994) Burning cane: the long term effects. Int. Sugar J 96:272-275.

Ono M., Murakami T., Kudo A., Isshiki M., Sawada H., Segawa A. (2001) Quantitative comparison of anti-Fading mounting media for Confocal Laser Scanning Microscopy. J. Histochem. Cytochem. 49:305-312.

Parker MA, Lafay B, Burdon JJ, van Berkum P. 2002. Conflicting phylogeographic patterns in rRNA and nifD indicate regionally restricted gene transfer in *Bradyrhizobium*. Microbiology 148: 2557-2565.

Parsons R., Silvester W.B., Harris S., Gruijters W.T.M., Bullivant S. (1987) Frankia vesicles provide inducible and absolute oxygen protection for Nitrogenase. Plant Physiol. 83:728-731.

Patriquin, D.G., and Dobereiner, J. *1978.* Light microscopy observations of tetrazolium-reducing bacteria in the endorhizosphere of maize and other grasses in Brazil. Can. J. Microbiol. **24:**734 - 742.

Paul L.R. (2002) Nitrogen fixation associated with tuberculate ectomycorrhiza on lodgepole pine (*Pinus contorta*), Faculty of Forestry, University of British Columbia, B.C., Canada, Vancouver.

Paul L.R., Chapman B.K., Chanway C.P. (2006) *Suillus tomentosus* tuberculate ectomycorrhizal abundance and distribution in *Pinus contorta* woody debris. Canadian Journal of Forest Research 36:460-466.

Paul L.R., Chapman B.K., Chanway C.P. (2007) Nitrogen Fixation Associated with *Suillus tomentosus* Tuberculate Ectomycorrhizae on *Pinus contorta* var. latifolia. Ann Bot 99:1101-1109.

Pedrosa F.O., Teixeira K.R.S., Machado I.M.P., Steffens M.B.R., Klassen G., Benelli E.M., Machado H.B., Funayama S., Rigo L.U., Ishida M.L., Yates M.G., Souza E.M. Structural organization and regulation of the *nif* genes of *Herbaspirillum seropedicae*. Soil Biology and Biochemistry 29:843-846.

Peoples, M.B. 2006. Biological Nitrogen Fixation: contributions to agriculture. p 162-165. *In* R. Lal (ed.) *Encyclopedia of Soil Science*. CRC Press.

Piao Z., Cui Z., Yin B., Hu J., Zhou C., Xie G., Su B., Yin S. (2005) Changes in acetylene reduction activities and effects of inoculated rhizosphere nitrogen-fixing bacteria on rice. Biology and Fertility of Soils 41:371-378.

Pokojska-Burdziej A. (1982) The effect of microorganisms, microbial metabolites and plant growth regulators on the growth of pine seedlings (*Pinus sylvestris* L.). Pol J Soil Sci 15:137-143.

Poly F., Monrozier L.J., Bally R. (2001) Improvement in the RFLP procedure for studying the diversity of *nif*H genes in communities of nitrogen fixers in soil. Research in Microbiology 152:95-103.

Potrich D.P., Passaglia L.M.P., Schrank I.S. (2001) Partial characterization of *nif* genes from the bacterium *Azospirillum amazonense*. Brazilian Journal of Medical and Biological Research 34:1105-1113.

Preisig H.R., Hibberd D.J. (1984) Virus-like particles and endophytic bacteria in *Paraphysomonas* and *Chromophysomonas* (*Chrysophyceae*). Nordic Journal of Botany 4:279-285.

Prescott C.E., Preston C.M. (1994.) Nitrogen mineralization and decomposition in forest floors in adjacent plantations of western red cedar, western hemlock and Douglas-fir. . Canadian Journal of Forest Research. 24:2424-2431.

Prescott C.E., Weetman G.F., Barker J.E. (1996) Causes and amelioration of nutrient deficiencies in cutovers of cedar-hemlock forests in coastal British Columbia. . Forestry Chronicle. 72:293-302.

Prescott C.E., deMontigny L.E., Preston C.E., Keenan R.J., Weetman G.F. (1995) Carbon chemistry and nutrient supply in cedar-hemlock and hemlock-anabilis fir forest floors.,

in: J. M. Kelly and W. W. McFee (Eds.), Carbon forms and functions in forest soils., Soil Science Society of America, Madison, Wisconsin. pp. 377-396.

Purchase B.S. (1980) Nitrogen fixation associated with sugarcane, in: D. Collingwood (Ed.), South African Sugar Technologists Association, Natal, South Africa. pp. 173-176.

Qi C. (2008) Perspectives in Biological Nitrogen Fixation Research. Journal of Integrative Plant Biology 50:786-798.

Quadt-Hallmann A., Kloepper J.W. (1996) Immunological detection and localization of the cotton endophyte *Enterobacter asburiae* JM22 in different plant species. Can. J. Microbiol. 42:1144-1154.

Quispel A. (1991) A critical evaluation of the prospects for nitrogen fixation with non-legumes. Plant and Soil 137:1-11.

Read D.J. (1991) Mycorrhizas in ecosystems. Experentia 47:376-391.

Reinhold-Hurek B., Hurek T. (1998a) Life in grasses: diazotrophic endophytes. Trends in Microbiology 6:139-144.

Reinhold-Hurek B., Hurek T. (1998b) Interactions of Gramineous plants with *Azoarcus* spp. and other diazotrophs: identification, localization, and perspectives to study their function. Crit Rev Plant Sci 17:29-54.

Reinhold-Hurek B., Dörr J., Egener T., Martin D., Hurek T. (2000) Interactions of Diazotrophic *Azoarcus spp*. With Rice, Nitrogen Fixation: From Molecules to Crop Productivity, Springer, Netherlands. pp. 405-408.

Reinhold-Hurek B., Krause A., Leyser B., Miché L., Hurek T. (2007) The Rice Apoplast as a Habitat for Endophytic N_2-Fixing Bacteria, The Apoplast of Higher Plants: Compartment of Storage, Transport and Reactions. pp. 427-443.

Reinhold-Hurek B., Hurek T., Gillis M., Hoste B., Vancanneyt M., Kersters K., De Ley J. (1993) *Azoarcus* gen. nov., Nitrogen-Fixing Proteobacteria Associated with Roots of Kallar Grass (*Leptochloa fusca* (L.) Kunth), and Description of Two Species, *Azoarcus* indigens sp. nov. and *Azoarcus communis* sp. nov. Int J Syst Bacteriol 43:574-584.

Reis V., Lee S., and Kennedy C. 2007. Biological Nitrogen Fixation in Sugarcane. p 213-232. *In* C. Elmerich and W.E. Newton (ed.) Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations.Springer, Netherlands.

Reis V.M., Urquiaga S., Paula M.A., Döbereiner J. (1990, 20-26 May) Infection of sugar cane by *Acetobacter diazotrophicus* and other diazotrophs, 8th International Congress on Nitrogen Fixation, Knoxvill,Ten. USA.

Reiter B., Sessitsch A. (2006) Bacterial endophytes of the wildflower *Crocus albiflorus* analyzed by characterization of isolates and by a cultivation-independent approach. Canadian Journal Of Microbiology 52:140-149.

Rennie R.J. (1981) A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils. Canadian Journal of Microbiology. 27:8-14.

Rennie R.J., Rennie D.A., Fried M. (1978) Concepts of ^{15}N usage in dinitrogen fixation studies, Isotopes in Biological Dinitrogen Fixation, International Atomic Energy Agency,, Vienna. pp. 107-130.

Rhodes-Roberts M., London P. (1981) The taxonomy of some nitrogen fixing *Paenibacillus* species with special reference to nitrogen fixation, in: R. C. W. Berkeley and G. M. (Eds.),

The aerobic-endosperm forming bacteria classification and identification, London Press, London.

Roberts G.P., Brill W.J. (1981) Genetics and regulation of nitrogen fixation. Annu. Rev. Microbiol. 35:207-235.

Rodrigues Coelho M.R., Weid I., Zahner V., Seldin L. (2003) Characterization of nitrogen-fixing *Paenibacillus* species by polymerase chain reaction-restriction fragment length polymorphism analysis of part of genes encoding 16S rRNA and 23S rRNA and by multilocus enzyme electrophoresis. FEMS Microbiology Letters 222:243-250.

Rodrigues R.J., Redman R.S., Henson J.M. (2004) The Role of Fungal Symbioses in the Adaptation of Plants to High Stress Environments. Mitigation and Adaptation Strategies for Global Change 9 (3): 261-272.

Rosado A., Duarte G.F., Seldin L. (1994) Optimization of electroporation procedure to transform *B. polymyxa* SCE2 and other nitrogen-fixing *Bacillus*. Journal of Microbiological Methods 19:1-11.

Rosado A.S., Seldin L. (1993) Production of a potentially novel anti-microbial substance by *Bacillus polymyxa*. World Journal of Microbiology and Biotechnology 9:521-528.

Rosado A.S., Duarte G.F., Seldin L., Van Elsas J.D. (1998) Genetic diversity of *nif*H gene sequences in *Paenibacillus azotofixans* strains and soil samples analyzed by denaturing gradient gel electrophoresis of PCR-amplified gene fragments. Appl. Environ. Microbiol. 64:2770-2779.

Rosch C., Mergel A., Bothe H. (2002) Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Appl. Environ. Microbiol. 68:3818-3829.

Rothballer, M., Schmid,M., Hartmann, A. 2003. Symbiosis 34 261-279. *In-situ* localization and PGPR effect of *Azospirilum brasiliense* strains colonizing roots of different wheat varieties.

Rozen S., Skaletsky H. (2000) Primer3 on the WWW for general users and for biologist programmers. Methods in molecular biology (Clifton, N.J.) 132:365-386.

Rózycki H., Dahm H., Strzelczyk E., Li C.Y. (1999) Diazotrophic bacteria in root-free soil and in the root zone of pine (*Pinus sylvestris* L.) and oak (*Quercus robur* L.). Applied Soil Ecology 12:239-250.

Ruvkun G.B., Ausubel F.M. 1980. Interspecies homology of nitrogenase genes. Proceedings of the National Academy of Sciences of the United States of America 77: 191-195.

Ryan R.P., Germaine K., Franks A., Ryan D.J., Dowling D.N. (2008) Bacterial endophytes: recent developments and applications. FEMS Microbiology Letters 278:1-9.

Saitou N., Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425.

Sanborn P., Preston C., Brockley R. 2002. N_2-fixation by Sitka alder in a young lodgepole pine stand in central interior British Columbia, Canada. Forest Ecology and Management 167: 223-231.

Sardi P., Saracchi M., Quaroni S., Petrolini B., Borgonovi G.E., Merli S. 1992. Isolation of Endophytic Streptomyces Strains from Surface-Sterilized Roots. Appl. Environ. Microbiol. 58: 2691-2693.

Sasakawa,H., Hiyoshi, T., Sugiyama T. (1988). Immuno-Gold Localization of Nitrogenase in Root Nodules of *Elaeagnus pungens* Thunb. Plant and Cell Physiology, Vol. 29 (7): 1147-1152.

Sato T., Fukui T., Atomi H., Imanaka T. (2003) Targeted Gene Disruption by Homologous Recombination in the Hyperthermophilic Archaeon *Thermococcus kodakaraensis* KOD1. J. Bacteriol. 185:210-220. Schardl C.L., Leuchtmann A., Spiering M.J. (2004) Symbioses Of Grasses With Seedborne Fungal Endophytes. Annual Review of Plant Biology 55:315-340.

Schank S.C., Smith R.L., Weiser G.C., Zuberer D.A., Bouton J.H., Quesenberry K.H., Tyler M.E., Milam J.R., Littell R.C. (1979). Fluorescent antibody technique to identify *Azospirillum brasilense* associated with roots of grasses. Soil Biology and Biochemistry 11: 287-295.

Scheller H.V., Doong R.L., Ridley B.L., Mohnen D. (1999) Pectin biosynthesis: a solubilized α1,4-galacturonosyltransferase from tobacco catalyzes the transfer of galacturonic acid from UDP-galacturonic acid onto the non-reducing end of homogalacturonan. Planta 207:512-517.

Schulz B., Boyle C. (2005) The endophytic continuum. Mycological Research 109:661-686.

Schulz B., Boyle C. (2006) What are Endophytes? in: B. J. E. Schulz, et al. (Eds.), Microbial Root Endophytes, Springer Berlin Heidelberg. pp. 89-106.pp. 1-13.

Seldin L., Elsas J.D.V., Penido E.G.C. (1983) *Bacillus* nitrogen fixers from Brazilian soils. Plant and Soil 70:243-255.

Seldin L., Van Elsas J.D., Penido E.G.C. 1984. *Bacillus azotofixans* sp. nov., a Nitrogen-Fixing Species from Brazilian Soils and Grass Roots. Int J Syst Bacteriol 34: 451-456.

Sevilla M., Burris R.H., Gunapala N., Kennedy C. (2001) Comparison of Benefit to Sugarcane Plant Growth and $^{15}N_2$ Incorporation Following Inoculation of Sterile Plants with *Acetobacter diazotrophicus* Wild-Type and NifÂ¯ Mutant Strains. Molecular Plant-Microbe Interactions 14:358-366.

Sevilla M., Meletzus D., Teixeira K., Lee S., Nutakki A., Baldani I., Kennedy C. (1997) Analysis of *nif* and regulatory genes in *Acetobacter diazotrophicus*. Soil Biology and Biochemistry 29:871-874.

Shishido M. (1997) PGPR for interior spruce seedlings. Department of Forest Sciences, PhD thesis. University of British Columbia, Vancouver, BC. pp. 324

Shen H, Li Z, Han D, Yang F, Huang Q, Ran L. 2010. Detection of indigenous endophytic bacteria in Eucalyptus urophylla in vitro conditions. Frontiers of Agriculture in China 4: 37-41.

Shishido M., Chanway C.P. (2000) Colonization and growth promotion of outplanted spruce seedlings pre-inoculated with plant growth-promoting rhizobacteria in the greenhouse. Canadian Journal of Forest Research 30:845-854.

Shishido M., Loeb B.M., Chanway C.P. (1995) External and internal root colonization of lodgepole pine seedlings by two growth-promoting *Bacillus* strains originated from different root microsites. . Canadian Journal of Microbiology. 41:707-713.

Shishido M., Massicotte H.B., Chanway C.P. (1996) Effect of Plant Growth Promoting *Bacillus* Strains on Pine and Spruce Seedling Growth and Mycorrhizal Infection. Ann Bot 77:433-442.

Singh H.P., Singh T.A. (1993) The interaction of rockphosphate, *Bradyrhizobium*, vesicular-arbuscular mycorrhizae and phosphate-solubilizing microbes on soybean grown in a sub-Himalayan mollisol. Mycorrhiza 4:37-43.

Slepecky R.A. (1992) What is a *Bacillus*?, in: R. H. Doi and M. McGloughlin (Eds.), Biology of Bacilli: Applications to Industry., Butterworth-Heinemann, Stoneham, MA, USA. pp. 1-21.

Son Y. (2001) Non-symbiotic nitrogen fixation in forest ecosystems. Ecological Research 16:183-196.

Son. Y., Gower S.T. (1992) Nitrogen and phosphorus distribution for five plantation species in southwestern Wisconsin. For. Ecol. Manag. 53:175-193.

Sprent J.I., James E.K. (1995) N_2-Fixation by Endophytic Bacteria: Questions of entry and operation. In: Fendrik I, del Gallo M, Vanderleyden J, de Zamaroczy M, eds. *Azospirillum VI and related microorganisms.* Berlin, Heidelberg: Springer-Verlag,15-30.

Steen O., Demarchi D.A. (1991) Sub-boreal pine - spruce zone, in: D. Meidinger and J. Pojar (Eds.), Ecosystems in British Columbia., Ministry of Forests, Victoria: B.C., Canada. pp. 195–207.

Steen O., Coupe. R.A. (1997) A field guide to forest site identification and interpretation for the Cariboo Forest Region., Ministry of Forests. Victoria: B.C., Canada.

Stephan M.P., Oliveria M., Teixeira K.R.S., Martinez-Drets G., Döbereiner J. (1991) Physiology and dinitrogen fixation of *Acetobacter diazotrophicus*. FEMS Microbiology Letters 77:67-72.

Steveson G. (1959) Fixation of Nitrogen by Non-nodulated Seed Plants. Ann Bot 23:622-635.

Steward G.F., Jenkins B.D., Ward B.B., Zehr J.P. (2004) Development and testing of a DNA macroarray to assess nitrogenase (*nif*H) gene diversity. Appl. Environ. Microbiol. 70:1455-1465.

Stoltzfus J.R., So R., Malarvithi P.P., Ladha J.K., de Bruijn F.J. (1997) Isolation of endophytic bacteria from rice and assessment of their potential for supplying rice with biologically fixed nitrogen. Plant and Soil 194:25-36.

Sturz A.V., Christie B.R., Matheson B.G., Nowak J. 1997. Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. Biology and Fertility of Soils 25: 13-19.

Taghavi S., Barac T., Greenberg B., Borremans B., Vangronsveld J., van der Lelie D. (2005) Horizontal gene transfer to endogenous endophytic bacteria from Poplar improves phytoremediation of toluene. Appl. Environ. Microbiol. 71:8500-8505.

Taghavi S., Garafola C., Monchy S., Newman L., Hoffman A., Weyens N., Barac T., Vangronsveld J., van der Lelie D. (2009) Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of Poplar trees. Appl. Environ. Microbiol. 75:748-757.

Tanaka K., Shimizu T., Zakria M., Njoloma J., Saeki Y., Sakai M., Yamakawa T., Minamisawa K., Akao S. (2006) Incorporation of a DNA sequence encoding Green Fluorescent Protein (GFP) into endophytic diazotroph from sugarcane and sweet potato and the colonizing ability of these bacteria in *Brassica oleracea*. Microbes and Environments 21:122-128.

Tapia-Hernández A., Bustillos-Cristales M.R., Jiménez-Salgado T., Caballero-Mellado J., Fuentes-Ramírez L.E. (2000) Natural Endophytic Occurrence of *Acetobacter diazotrophicus* in Pineapple Plants. Microbial Ecology 39:49-55.

Taroncher-Oldenburg G., Stephanopoulos G. (2000) Targeted, PCR-based gene disruption in cyanobacteria: inactivation of the polyhydroxyalkanoic acid synthase genes in *Synechocystis sp.* PCC6803. Applied Microbiology and Biotechnology 54:677-680.

Tarone R.E. 1979. Testing the goodness of fit of the binomial distribution. Biometrika 66: 585-590.

Tervet I.W., Hollis J.P. (1948) Bacteria in the storage organs of healthy plants. Phytopathology 38:960-967.

Timmusk S., Wagner E.G.H. (1999) The Plant-Growth-Promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: A possible connection between Biotic and Abiotic stress responses. Molecular Plant-Microbe Interactions 12:951-959.

Timmusk S., Grantcharova N., Wagner E.G.H. (2005) *Paenibacillus polymyxa* Invades Plant Roots and Forms Biofilms. Appl. Environ. Microbiol. 71:7292-7300.

Timmusk S., Nicander B., Granhall U., Tillberg E. (1999) Cytokinin production by *Paenibacillus polymyxa*. Soil Biology and Biochemistry 31:1847-1852.

Timonen S. (1995) Avoiding autofluorescence problems: time-resolved fluorescence microscopy with plant and fungal cells in ectomycorrhiza. Mycorrhiza 5:455-458.

Tombolini, R. and Jansson, J.K. 1998. Monitoring of GFP tagged bacterial cells. In LaRossa, R. (Ed.). Methods in Molecular Biology, Vol. 102: Bioluminescence Methods and Protocols. Humana Press, Totowa, NJ. pp. 285–298.

Tombolini R, Unge A, Davey ME, Bruijn FJ, Jansson JK. 1997. Flow cytometric and microscopic analysis of GFP-tagged *Pseudomonas fluorescens* bacteria. FEMS Microbiology Ecology 22: 17-28.

Triplett E.W. (1996) Diazotrophic endophytes: progress and prospects for nitrogen fixation in monocots. Plant and Soil 186:29-38.

Tsiantos J., Stevens W.A. (1986) The population dynamics of *Corynebacterium michiganense* pv. michiganensis and other selected bacteria in tomato leaves. Phytopathol Mediterr 25:160-162.

Turgeon N., Laflamme C., Ho J., Duchaine C. (2006) Elaboration of an electroporation protocol for *Bacillus cereus* ATCC 14579. Journal of Microbiological Methods 67:543-548.

Turner, J.T., Jeffrey, L.K., and Carlson, P.S. 1993. Endophytes: an alternative genome for crop improvement. *In* International crop science. *Eds* D.R. Buxton, R. Shibles, R.A. Forsberg, B.L. Blad, K.H. Asay, G.M. Paulsen, and R.F. Wilson. Crop Science Society of America, Madison, Wis. pp. 555-560.

Turvey N.D., Smethurst P.J. (1988.) Apparent accumulation of nitrogen in soil under radiata pine: misleading results from a chronosequence. , in: W. J. Dyck and C. A. Mees (Eds.), Re- search strategies for long-term site productivity., New Zealand Forest Research Institute, Rotorua, New Zealand. pp. 39-43.

Ulrich K., Ulrich A., Ewald D. (2008) Diversity of endophytic bacterial communities in poplar grown under field conditions. FEMS Microbiology Ecology 63:169-180.

Urquiaga S., Cruz K.H.S., Boddey R.M. (1992) Contribution of nitrogen fixation to sugar cane: Nitrogen-15 and Nitrogen-Balance Estimates. Soil Science Society of America Journal 56:105-114.

Van Doorn, W.G., Clerkx, A,, and Boekestein, A. 1991. Bacteria as a cause of vascular occlusion in cut fronds of *Adiantuym raddianum:* a scanning electron microscope study. Sci. Hortic. 48: 299-309.

Verma S.C., Ladha J.K., Tripathi A.K. (2001) Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. *Journal of Biotechnology*, 91 (2-3), pp. 127-141.

Verma S.C., Singh A., Chowdhury S.P., Tripathi A.K. 2004. Endophytic colonization ability of two deep-water rice endophytes, *Pantoea sp*. and *Ochrobactrum sp*. using green fluorescent protein reporter. Biotechnology Letters 26: 425-429.

Versalovic J., Lupski J.R. (2002) Molecular detection and genotyping of pathogens: more accurate and rapid answers. Trends in Microbiology 10:15-21.

Versalovic J., Koeuth T., Lupski R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to finerpriting of bacterial enomes. Nucl. Acids Res. 19:6823-6831.

Villacieros, M., Power, B., Sanchez-Contreras, M., Lloret, J., Oruezabal, R.I., Martin, M., Fernandez-Pinas, F., Bonilla, I. (2003) Colonization behaviour of *Pseudomonas fluorescens* and *Sinorhizobium meliloti* in the alfalfa (*Medicago sativa*) rhizosphere. *Plant Soil* **251**, 47–54.

Vincent J.M. (1970) A Manual for the practical study of root-nodule bacteria. (IBP Handbook 15.) Oxford : Blackwell Scientific Publications.

Vingarzan R., Belzer W., Thomson B. (2000) Nutrient levels in the atmosphere of the Elk Creek Watershed, Chilliwack, B.C. EC/GB-02-038. Environment Canada,Pacific and Yukon Region.Georgia Basin Ecosystem Initiative (Canada) Available from :Roxanne.Vingarzan@ec.gc.ca.

Vitousek P.M., Howarth R.W. (1991) Nitrogen limitation on land and in the sea: How can it occur? Biogeochemistry 13:87-115.

von der Weid I., Frois Duarte G., van Elsas J.D., Seldin L. (2002) *Paenibacillus brasilensis* sp. nov., a novel nitrogen-fixing species isolated from the maize rhizosphere in Brazil. Int J Syst Evol Microbiol 52:2147-2153.

von Wirén N., Gazzarrini S., Frommer W.B. (1997) Regulation of mineral nitrogen uptake in plants. Plant and Soil 196:191-199.

Vyse A., Ferguson C., Huggard D.J., Roach J., Zimonick B. 2009. Regeneration beneath lodgepole pine dominated stands attacked or threatened by the mountain pine beetle in the south central Interior, British Columbia. Forest Ecology and Management 258: S36-S43.

Weetman G.F., Fournier R.M. (1982) Graphical diagnoses of lodgepole pine response to fertilization. Soil Science Society of America Journal 46:1280-1289.

Weetman G.F., Fournier R.M., Schnorbus E. (1988) Lodgepole pine fertilization screening trials: four-year growth response following initial predictions. Soil Science Society of America Journal. 52:833-839.

Wei X., Kimmins J.P. (1998) Asymbiotic nitrogen fixation in harvested and wildfire-killed lodgepole pine forests in the central interior of British Columbia. Forest Ecology and Management 109:343-353.

Weisburg W.G., Barns S.M., Pelletier D.A., Lane D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173:697-703.

Wherry E.T. (1920) The Crystallography Of Melezitose. Journal of the American Chemical Society 42:125-128.

Whitesides S.K., Spotts R.A. (1991) Frequency, distribution, and characteristics of endophytic *Pseudomonas syringae* in pear trees. Phytopathology 81:453-457.

Widmer F., Shaffer B.T., Porteous L.A., Seidler R.J. (1999) Analysis of *nif*H Gene Pool Complexity in Soil and Litter at a Douglas Fir Forest Site in the Oregon Cascade Mountain Range. Appl. Environ. Microbiol. 65:374-380.

Wilhelm E., Arthofer W., Schafleitner R., Krebs B. (1998) *Bacillus subtilis* an endophyte of chestnut (*Castanea sativa*) as antagonist against chestnut blight (*Cryphonectria parasitica*). Plant Cell, Tissue and Organ Culture 52:105-108.

Williams B.L., Cooper J.M., Pyatt D.G. 1979. Some effects of afforestation with lodgepole pine on rates of nitrogen mineralization in peat. Forestry 52: 151-160.

Williams, B.L. 1992. Nitrogen dynamics in humus and soil beneath Sitka spruce (*Picea sitchensis* (Bong.) Carr.) planted in pure stands and in mixture with Scots pine (*Pinus sylvestris* L.). Plant Soil 144:77–84.

Wilson D. (1995) Endophyte: The Evolution of a term, and clarification of Its use and definition. Oikos 73:274-276.

Wynn-Williams D.D., Rhodes M.E. (1974) Nitrogen fixation in seawater. Journal of Applied Microbiology 37:203-216.

Xi C., Lambrecht M., Vanderleyden J., Michiels J. 1999. Bi-functional *gfp*-and *gus*A-containing mini-*Tn*5 transposon derivatives for combined gene expression and bacterial localization studies. Journal of Microbiological Methods 35: 85-92.

Xiao G. (1994) The role of root-associated fungi in the dominance of *Gaultheria shallon*., Department of Soil Science, PhD thesis. University of British Columbia, Vancouver, BC. pp. 137.

Xin G, Zhang GY, Kang JW, Staley JT, Doty SL (2009) A diazotrophic, indole-3-acetic acid-producing endophyte from wild cottonwood. Biol Fert Soils 45:669–674.

Yegorenkova I., Tregubova K., Matora L., Burygin G., Ignatov V. (2008) Composition and immunochemical characteristics of exopolysaccharides from the rhizobacterium *Paenibacillus polymyxa* 1465. Microbiology 77:553-558.

Yoshida T., Ancajas R.R. (1971) Nitrogen fixation by bacteria in the root zone of rice. Soil Sci Soc Am J 35:156-158.

You C., Song W., Wang H., Li J., Lin M., Hai W. (1991) Association of *Alcaligenes faecalis* with wetland rice. Plant and Soil 137:81-85.

You C. B., Li X., Wang Y. W., Qiu Y. S., Mo X .Z., Y. Z. (1983) Associative N_2 fixation of *Alcaligenes faecalis* with rice plant. Biological N_2 Fixation Newsletter, Sydney. 11.

Young J.P.W. (1992) Phylogenetic classification of nitrogen-fixing organisms, in: B. R. Stacey G, Evans HJ (Ed.), Biological Nitrogen Fixation, Chapman and Hall, New York. pp. 43-86.

Zak B. (1971) Characterization and classification of mycorrhizae of Douglas fir. II. *Pseudotsuga menziesii* + *Rhizopogon vinicolor*. Can. J. Bot. 49:1079-1084.

Zehr J.P., Jenkins B.D., Short S.M., Steward G.F. (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environmental Microbiology 5:539-554.

Zhao H., Xie B., Chen S. (2006) Cloning and sequencing of *nif*BHDKENX genes of *Paenibacillus massiliensis* T7 and its nif promoter analysis. Science in China Series C: Life Sciences 49:115-122.

Zimmer M. (2002) Green Fluorescent Protein (GFP): Applications, Structure, and Related Photophysical Behavior. Chemical Reviews 102:759-782.

Zimmermann, R., Iturriaga, R., and Becker-Birck, J. 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl. Environ.
Microbiol. ***36:*** 926-935.

Appendices

Appendix A Chemical Properties of Soils Collected from Williams Lake Pine, Chilliwack Lake Pine, and Boston Bar Cedar Stands[1]

	Stand Type		
Property	**Williams Lake (lodgepole pine)**	**Chilliwack Lake (lodgepole pine)**	**Boston Bar (western redcedar)**
pH	6.0	5.2	5.2
Organic matter (%)	1.8	5.5	2.2
Total N (%)	0.045	0.07	0.05
Mineralizable N (ppm)[3]	12	22	13
P (ppm)	15	33	9
K (ppm)	116[2]	30	18
Ca (ppm)	1055[2]	200	500
Mg (ppm)	273[2]	15	60
Cu (ppm)		1.7	1.2
Zn (ppm)		1.6	0.6
Fe (ppm)	7[2]	50	19
Mn (ppm)	55[2]	20	20

[1]All soil analysis shown here was done by the Pacific Soil Analysis Inc. , Richmond, B.C.

[2] Exchangeable nutrients. All other values are available nutrients. Cu and Zn contents were not determined for Williams Lake soil.

[3]The mineralizable N content was done using a 2 week anaerobic incubation followed by KCl extraction.

Appendix B Combined Carbon Medium

Combined Carbon Medium

1. Solution 1:

a. Sucrose	5 g/L
b. Mannitol	5 g/L
c. Sodium Lactate (ml, 60%, v/v)	0.5 ml/L
d. K_2HPO_4	0.80 g/L
e. KH_2PO_4	0.20 g/L
f. NaCl	0.10 g/L
g. $Na_2Mo0_4.2H_20$	25.0 mg/L
h. Na2FeEDTA	28.0 mg/L
i. Yeast Extract	100mg/L
j. Distilled Water	900ml

2. Solution 2:

a. $MgSO_4.7\ H_20$	0.20 g/L
b. $CaCl_2$	0.06 g/L
c. Distilled water	100 ml

Autoclave separately, cool and mix.

Add filter sterilized

Biotin: 5ug/L and Para Amino Benzoic Acid (PABA): 10ug/L

Appendix C Plant Nutrient Solution

Nutrient Solution for Growth Media

KH_2PO_4	0.14g/L
$MgSO_4$	0.49g/L
H_3BO_3	0.001g/L
$MnCl_2.4H_2O$	0.001g/l
$ZnSO_4.7H_2O$	0.001g/L
$CuSO_4.5H_2O$	0.0001g/L
$NaMoO_4.2H_2O$	0.001g/L
$Ca(NO_3)_2$	0.0576g/L
Na_2Fe EDTA	0.025g/L

Appendix D Cross section of pine stem

Figure D-1 Labelled cross ection of a young pine stem