EFFECTS OF *PAENIBACILLUS POLYMYXA* AND SOIL NITROGEN ON LODGEPOLE PINE (*PINUS CONTORTA* VAR. *LATIFOLIA* (DOUGL.) ENGELM.) SEEDLING GROWTH

by

Henry Yang

B.Sc., The University of Northern British Columbia, 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies

(Forestry)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2015

Abstract

Paenbacillus polymyxa strain P2b-2R has previously been shown to colonize lodgepole pine (*Pinus contorta* var. *latifolia* (Dougl.) Engelm.) internal tissues and stimulate seedling growth possibly through N_2 fixation. I evaluated the biochemical characteristics of *P. polymyxa* strain P2b-2R and the effects of soil nitrogen (N) concentrations on lodgepole pine seedling growth after inoculation with P2b-2R. The bacterium was capable of using major plant cell wall components including carboxymethyl cellulose, xylan and sodium polypectate in addition to 39 of 95 carbon sources from BioLog GP2 microplates. These results suggest that hydrolytic enzymes may facilitate endophytic colonization by P2b-2R.

Bacterial colonization and lodgepole pine seedling growth responses (foliar ^{15}N atom % excess, foliar N concentrations, total foliar N and root and shoot biomass) to inoculation with P2b-2R were assessed at varying soil N concentrations in a one-year greenhouse study. Surface sterilized pine seeds were sown in Ray Leach cone-tainers containing an autoclaved sand-montmorillonite clay mixture and inoculated with log 6 colony forming units (cfu) P2b-2R. Non-inoculated controls received phosphate-buffered saline. Seedlings were then subjected to monthly applications of a nutrient solution containing one of four soil N concentrations as $Ca(NO_3)_2$ (5% ^{15}N label): 0.0029, 0.029, 0.29 and 2.9 mmol L^{-1}, referred to as 'very low', 'low', 'medium' and 'high' soil N treatments, respectively. Rhizospheric and endophytic population sizes of P2b-2R, foliar ^{15}N atom % excess and foliar N concentrations were not significantly affected by P2b-2R during the experiment. Inoculation with P2b-2R resulted in seedling biomass across the four soil N treatments four months after sowing. However, seedling growth inhibition was transient as it was not detectable four months later and by month twelve, inoculated seedlings from the very low soil N treatment had accumulated 56.3 and 46.4% more root and shoot biomass, respectively, than controls. Seedlings from the medium soil N treatment responded similarly to bacterial inoculation, but no biomass enhancement was observed at low and high soil N concentrations. My results suggest that pine biomass stimulation by P2b-2R may depend on soil N concentrations and

that P2b-2R can enhance biomass accumulation of pine seedlings without providing significant amounts of fixed N.

Acknowledgements

I would like to thank my supervisor, Dr. Chris Chanway, for financial support and guidance. I would also like to extend my thanks to my committee members, Dr. Grayston and Dr. Anand, and former committee member, Dr. Breuil, for their constructive feedback on my work. I would like to give much credit to Dr. Guy and Dr. Weetman for their suggestions in my experimental design.

I also owe special thanks to Rolando Descalzo, Norman Hodges, Felix Choi, David Kapland, Kate del Bel, Andrea Chan, Natasha Thompson, Dr. Lee Kalcsits and Dr. Alice Chang for this project would not have been possible without their assistance.

Last but not least, I would like to thank my family, all my friends and those who kept me in their prayers.

Table of Contents

List of Tables

List of Figures

1 Introduction

Plants can be considered as highly complex ecosystems, providing habitats and a rich source of nutrients for soil inhabitants such as fungi and bacteria. Up to 50% of photosynthetically fixed carbon may be exuded at root surfaces (Swinnen *et al*. 1994) in forms that are easily utilized by microorganisms. The rhizosphere, which is the volume of soil surrounding plant roots and under direct influence of root activity, is enriched with organic compounds such as amino acids, peptides, proteins, enzymes, vitamins and phytohormones (Grayston *et al*. 1997; Jones *et al*. 2004; Shi *et al*. 2012). Bacteria inhabiting this nutrient-rich microbabitat are known as rhizobacteria and are thought to benefit from the aforementioned organic compounds as a ready source of nutrients. However, antagonistic interactions such as parasitism (Cacciari *et al*. 1986) and competition for these nutrients (Cocking 2003) may reduce the abundance of certain rhizobacterial species including those known to exert positive effects on plant growth. In addition, a subset of plant-colonizing bacteria and rhizobacteria may acquire these nutrients by colonizing plants internally. The term 'bacterial endophyte' is used to describe a bacterium that colonizes internal plant tissues without inducing disease symptoms (Hallmann *et al.* 1997; Azevedo *et al.* 2000).

Unlike their rhizosphere counterparts, endophytic bacteria are thought to enter plants through pre-existing openings such as emergence sites of lateral roots or wounds to external plant tissues (James *et al.* 1994). Alternatively, it is possible that bacterial endophytes may create openings by hydrolyzing major plant cell wall components such as cellulose, hemicellulose and pectin (Compant *et al*. 2005; Reinhold-Hurek *et al*. 2006). The ability of bacterial endophytes to evade plant defenses is thought to involve production of enzymes that degrade elicitors of plant

immune responses (Fouts *et al*. 2008) and to be essential for successful endophytic colonization (Bulgarelli *et al.* 2013). By occupying the plant interior, bacterial endophytes may have a comparative advantage over rhizosphere-colonizing bacteria due to ready access to continuous supplies of nutrients and an additional degree of protection from competition and predation that likely occur outside plant roots (Hallmann *et al*. 1997). Certain bacterial endophytes may in turn enhance the fitness of their plant hosts by inducing systemic defense (Kavino *et al.* 2006) or providing essential growth-limiting compounds such as nitrogen (N) (Dong *et al.* 1994).

Nitrogen (N) is one of the most plant-growth limiting nutrients in terrestrial ecosystems (Dalton and Kramer 2006). To become bioavailable, atmospheric N must be transformed from its inert gaseous form (N_2) to ammonia (NH_3) through the process of nitrogen fixation (NF). Very large amounts of energy are required to break the triple bond between the two N atoms which comprise N_2. Natural phenomena such as lightning and volcanic activity can provide enough energy to break this bond. Various oxides of N are produced in the atmosphere that then dissolve in rain and descend to the ground as ammonia and ammonium. The amount of NF by lightning is estimated to be 4.0×10^9 kg per year (Schumann and Huntrieser 2007). However, N_2 can also be fixed industrially through the Haber-Bosch process, which accounts for the annual production of 1.1×10^{11} kg NH_3 (Kramer 1999). This industrial process is extremely fossil-fuel-intensive and consumes 3-5% of the world's natural gas annually (Bottomley and Myrold 2007).

Alternatively, N_2 may be fixed through the normal activity of certain microorganisms. This process, known as biological nitrogen fixation (BNF), is also energy-intensive, but differs from the aforementioned abiotic processes in that it usually takes place at ambient temperature and normal atmospheric pressure. It occurs through the action of the oxygen-sensitive enzyme, nitrogenase, which is found only in certain members of the Bacteria and Archaea (Galloway *et*

al. 2008). Each year, these N_2-fixing microorganisms provide approximately 1.1 x 10^{11} and 1.4 x 10^{11} kg of bioavailable N to terrestrial and marine ecosystems, respectively (Galloway *et al.* 2004).

N_2-fixing bacteria are commonly referred to as diazotrophs and may be free-living or occur in symbiotic associations. Free-living diazotrophs are self-sustaining and do not fix N_2 unless the amount of available N limits their growth. However, their capacity to fix N_2 is often limited by a lack of adequate carbon to generate energy (Reed *et al.* 2011) and by nitrogenase-inhibiting concentrations of atmospheric oxygen (Postgate 1998**)**. While the general belief is that free-living diazotrophs do not contribute large quantities of fixed N to most terrestrial ecosystems (Postgate 1982; Newton 2007), they are known to provide a biologically significant proportion of fixed N in some tropical and temperate forest ecosystems (Cleveland et al. 1999; Gehring et al. 2005; Reed et al. 2007).

Nitrogen fixing symbioses are thought to be mutualistic and occur where non-photosynthetic prokaryotes depend on primary producers for photosynthate to fix N (Mylona *et al.* 1995), while photosynthesizers benefit from additional N from NF (James and Olivares 1998; James 1999). Based on the degree of morphological and physiological intimacy with the primary producer, these mutualistic N_2-fixing partnerships range from associative symbioses, comprising loose associations of bacteria colonizing the plant rhizosphere with little or no plant x microbe specificity, to highly specialized symbioses, where specialized plant organs and bacterial cells develop, often with a high degree of plant x microbe specificity. For any symbiotic N_2 fixing system to be effective, the system must involve diazotrophs that can transfer significant amounts of fixed N to the hosts (James and Olivares 1998; James 1999). Rapid transfers of N by

endophytic diazotrophs have been reported to occur in specialized plant organs such as root nodules of legumes (Stacey 2007) or coralloid roots of cycads (Lindblad *et al.* 1991).

Alternatively, significant amounts of N may be provided by endophytic diazotrophs that do not induce macroscopic structural changes on or in the plant hosts (Dobereiner *et al.*1995b; Malik *et al*. 1997). Perhaps the best known example of this symbiosis is sugar cane (*Saccharum officinarum* L.) and one or more possibly endophytic N fixing bacterial species. The discovery of this symbiosis resulted from questioning how such an N-demanding crop could maintain consistently high yields for decades on N-deficient soils without exogenous applications of N fertilizer (Boddey *et al.* 1991; Cavalcante and Dobereiner 1998). Past research had focused primarily on rhizospheric NF as a possible microbiological explanation of this apparently anomalous phenomenon as several N-fixing rhizobacteria belonging to the genera *Beijerinkia, Erwinia, Azotobacter, Derxia*, *Azospirillum* and *Enterobacter* were found to associate with sugar cane and other grasses (Purchase 1980; Graciolli and Ruschel 1981; Graciolli *et al*. 1983). However none of these rhizosphere-colonizing bacteria could account for the observed annual N accumulation in sugar cane (Cavalcante and Dobereiner 1988; Boddey *et al.*1991). The interior of sugar cane was subsequently investigated for microorganisms that might account for the unexplained N input, and a diazotrophic endophyte, *Gluconacetobacter diazotrophicus* (formerly *Acetobacter diazotrophicus*), was isolated and identified as a possible causative agent (Cavalcante and Dobereiner 1988).

G. diazotrophicus was found to possess cellulolytic enzymes (Mateos *et al.* 1992; Adriano-Anaya *et al.* 2005); it could grow on media supplemented with 10% sucrose and tolerate low pH (Boddie *et al.* 1991). These characteristics were thought to facilitate internal colonization of sugar cane tissues by *G. diazotrophicus*, and when re-introduced to sugar cane, *G.*

diazotrophicus reached high population densities ranging from 10^3 to 10^5 colony forming units (cfu) g^{-1} fresh tissue in roots (Dobereiner *et al*. 1988) and 10^5 cfu g^{-1} fresh tissue in shoots (Mufioz-Roja and Caballero-Mellad 2003). It also provided up to 72% of the plant's N requirement (Yoneyama *et al.* 1997). Since this discovery, several other crop plants including rice (Oryza sativa L.) (Malik *et al*. 1997) and maize *(Zea mays* L.) (Montañez *et al.* 2009) have been suggested to receive fixed N from endophytes.

Notwithstanding the potential benefits *G. diazotrophicus* may confer to sugar cane, whether this bacterium is the major diazotrophic that enables field-grown sugar cane to thrive on N-limited soils is unclear. Other culturable N_2-fixing bacteria have been shown to enhance the N status of sugar cane. These include *Enterobacter cloacae, Erwinia herbicola, Klebsiella pneumoniae, Azotobacter vinelandii, Paenibacillus polymyxa (formerly Bacillus polymyxa), Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans* (Ruschel 1981; Cavalcante and Dobereiner 1988; Olivares *et al.* 1996), *Azoarcus* spp. (Reinhold *et al*. 1993) and *Azospirillum brasilense* (de Bellone and Bellone 2006). In addition, a consortium of viable, but non-culturable diazotrophs (Fuentes-Ramírez *et al.* 1999; Burbano *et al*. 2011; Taulé *et al*. 2012) may also contribute fixed N to sugar cane, rendering this an ongoing topic of investigation.

In comparison with well characterized NF systems in agricultural crops, studies of diazotrophs within conifer seedlings, particularly those of great commercial importance, have been scarce. In North America, soil N deficiency is widespread and can limit the growth of tree seedlings in boreal forest ecosystems (Mahendrappa and Salonius 1982). However, the growth of certain forest tree species such as lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.) appears to be less affected by low soil N availability (Chapman and Paul 2012).

Lodgepole pine is one of the most important timber species in Canada. In addition to its wide ecological amplitude in western North America (Critchfield and Little 1966), lodgepole is known to not only grow, but thrive on severely N-deficient sites (Weetman *et al.*1988) such as rocky substrates, roadsides and even gravel pits (Chapman and Paul 2012). The ability of this tree species to persist on nutrient-poor soils may relate to its physiological traits and association with soil microorganisms. In comparison with other conifers such as white spruce (*Picea glauca*), lodgepole pine is not considered a nutrient-demanding species (Weetman *et al.* 1984), and may adopt physiological strategies such as efficient internal cycling of N (Bormann *et al.* 1977) and acquisition of N contained in rain and aerosols (Miller *et al.* 1979) to lessen its dependence on soil N. Alternatively, lodgepole pine associated with mycorrhizas may have enhanced access to and uptake of soil organic N (Read 1991; Northup *et al.* 1995). However, in light of its ability to maintain vigorous growth (Chapman and Paul 2012) and adequate foliar N levels (Kranabetter *et al.* 2006) on certain sites with low inputs of organic or atmospherically deposited N, lodgepole pine may also rely on BNF to satisfy its N demand.

Chanway and Holl (1991) determined that rhizospheric BNF contributed only 4% of the total foliar N of lodgepole pine seedlings in one study, indicating that this process likely could not significantly enhance seedling growth in the short term. Bal *et al* (2012) isolated a consortium of diazotrophic bacteria from internal tissues of naturally regenerating lodgepole pine seedlings at an N-poor site near Williams Lake BC and tested the hypothesis that such microorganisms could stimulate pine seedling growth through BNF. One strain of particular interest that was isolated from surface-disinfected pine tissues, *Paenibacillus polymyxa* strain P2b-2R, was subsequently shown to be able to grow on N-free media, to consistently display *in vitro* nitrogenase (acetylene reduction) activity (Bal *et al.* 2012) and was found to harbor the *nifH*

gene (Anand and Chanway 2013b) that encodes nitrogenase. When P2b-2R was re-introduced to pine via seed inoculation, it was able to form persistent rhizospheric (Bal *et al.* 2012) and endophytic (Anand *et al.* 2013) populations. Using a gfp (green fluorescence protein) tagged derivative of the bacterium and confocal laser scanning microscopy, Anand and Chanway (2013a) detected intracellular colonization by P2b-2R and were thus able to confirm its status as a pine endophyte.

Through the use of foliar ^{15}N dilution assays (Rennie *et al.* 1978), pine seedlings inoculated with P2b-2R were observed to have significantly lower ^{15}N enrichment than controls in three growth trials (Bal and Chanway 2012; Anand *et al.* 2013), which suggests that seedlings were obtaining foliar N from a source other than the soil, *i.e.*, NF. When NF was quantified as the percent N derived from the atmosphere (%Ndfa) (Rennie *et al.* 1978), BNF by P2b-2R was shown to account for 30 and 60% of total foliar N in seedlings grown for seven and nine months, respectively (Bal and Chanway 2012). When the growth trial was extended to 13 months, inoculated seedlings derived up to 79% of foliar N from the atmosphere (Anand *et al.* 2013). The amounts of BNF detected by Bal and Chanway (2012) and Anand *et al.* (2013) are comparable to those reported for *G. diazotrophicus* x sugar cane associations and highly suggestive of a long-term mutualism, where the pine host provides the carbon substrates to the endophytic diazotrophs in exchange for fixed N. The possibility that lodgepole pine seedlings inoculated with P2b-2R continue to fix N_2 in the field requires further investigation.

Although P2b-2R was shown to provide substantial amounts of fixed N to its pine hosts, inoculation with this bacterium did not enhance foliar N content and even resulted in a slight growth reduction of seven- and nine-month old pine seedlings (Bal and Chanway 2012). However, when the growth assay was extended to 13 months, inoculated pine seedlings

outperformed the controls and had significantly higher root and shoot biomass (Anand *et al.* 2013). In addition, inoculation with P2b-2R increased seedling foliar N content almost 5-fold (Anand *et al.* 2013), from a severely deficient state to a concentration considered to be adequate for lodgepole pine (Ballard and Carter 1986). These results suggest that: (1) pine seedlings grown in N-deficient soil may acquire most of their foliar N from the atmosphere, (2) the reliance of pine on N fixed by P2b-2R increases with seedling age and (3) temporary seedling growth reduction may characterize early stages in the development of effective BNF, *i.e.* one that is capable of enhancing seedling foliar N content (Anand *et al.* 2013; Chanway *et al.* 2014) and biomass accumulation (Anand *et al.* 2013) in this pine x diazotrophic endophyte association.

The significant contribution of fixed N and concomitant biomass enhancement of pine seedlings by P2b-2R suggest that lodgepole pine may benefit from diazotrophic endophytes in N-deficient soils. However, whether this mutualistic relationship continues to exist at higher soil N concentrations is unclear as high amounts of exogenous N are known to suppress *in vitro* nitrogenase activity (Dixon and Kahn 2010; Wang *et al.* 2013) and can trigger specific metabolic and enzymatic activities in plants (Abellan *et al.* 1994), which may result in reduced populations of endophytic diazotrophs (Fuentes-Ramirez *et al.* 1999; Munoz-Rojas and Caballero-Mellado 2003).

The primary objective of my thesis was to determine if the amount of BNF by P2b-2R-treated pine seedlings decreased in response to increasing soil N concentrations. A secondary objective was to determine if P2b-2R possessed enzyme activities that were consistent with those that characterize known endophytic microorganisms.

To address these objectives, I have tested the following hypotheses:

1. P2b-2R population sizes on and inside pine tissues will decrease with increasing soil N concentrations,
2. The percentage of N derived from the atmosphere (%Ndfa) by pine seedlings inoculated with P2b-2R will decrease as soil N concentrations increase,
3. Pine seedling biomass treated with P2b-2R will be greatest at the low soil N concentrations, and
4. P2b-2R possesses enzymes that hydrolyze the major plant cell wall components: carboxymethyl cellulose, xylan and sodium polypectate.

2 Methods and materials

2.1 Bacteria

Paenibacillus polymyxa strain P2b was isolated from surface-sterilized stem tissue of a lodgepole pine seedling that was naturally regenerating near Williams Lake, British Columbia, Canada (52°05' N lat., 122°54'W long., elevation 1300 m, Sub-Boreal Pine Spruce, SBPSdc Zone) (Bal *et al.* 2012). *P. polymyxa* P2b-2R is a spontaneous mutant that was derived from stain P2b grown on combined carbon medium (CCM; Appendix A) agar (Rennie 1981) amended with 200 mgL^{-1} rifamycin (Bal *et al.* 2012). Strain P2b-2R was stored at -80^{o}C on CCM amended with 20% (v/v) glycerol. Unless otherwise specified, a frozen P2b-2R culture was thawed and subcultured twice on CCM agar amended with 200 mg mL^{-1} rifamycin before use in experiments.

2.2 *in vitro* carbon-source utilization by *Paenibacillus polymyxa* strain P2b-2R in relation to endophytic colonization

To assess the ability of P2b-2R to degrade plant cell wall components, 50 µL of P2b-2R (ca. 10^7 cfu mL^{-1}) were spot-inoculated on Luria Bertani (LB) agar amended with one of the following substrates (5 g L^{-1}): sodium carboxymethylcellulose (CMC) (Sigma-Aldrich, ON, CAN), beechwood xylan (Sigma-Aldrich, ON, CAN) or sodium polypectate (Sigma-Aldrich, ON, CAN) and incubated for two days at 30°C. To visualize substrate utilization, plates with sodium CMC or xylan were flooded with 0.5% (w/v) Congo red for 30 minutes, drained and then rinsed with 1 M NaCl (Cho *et al.* 2007). To visualize pectate lyase activity, sodium polypectate plates were flooded with 10% (w/v) copper acetate for 30 minutes, drained and rinsed with distilled water (Cho *et al.* 2007). Clear zones around bacterial colonies on plates indicated enzyme activity.

BioLog GP2 (BioLog, Hayward, CA) plates were used to evaluate utilization of other carbon sources by P2b-2R. A frozen P2b-2R culture was thawed and subcultured, and a single

colony was used to inoculate tryptic soy broth. After 24 h, bacteria were harvested by centrifugation (5000 x g for 10 min), washed with 0.1M phosphate buffered saline (PBS) and resuspended in the same buffer to achieve 28% transmittance at OD640 (BioLog Inc. 2008). One-hundred and fifty µL aliquots of the P2b-2R-PBS suspension were pipetted into each microwell of a GP2 plate, incubated at 30°C for 24 hours and read at OD595 with a plate reader (Spectra Max 340, Sunnyvale, CA 94089, USA). An OD595 above 0.1 indicated strong substrate utilization and readings below 0.05 were considered negative. Values between 0.05 and 0.1 were considered weakly positive. Assays were repeated to ensure results were reproducible.

2.3 Seedling growth assay

2.3.1 Seed source

Lodgepole pine seed originating from near Williams Lake, British Columbia, Canada (52°05' N lat., 122°54'W long., elevation 1300 m, Sub-Boreal Pine Spruce, SBPSdc Zone) was obtained from the British Columbia Ministry of Forests Tree Seed Centre, Surrey, British Columbia, Canada.

2.3.2 Seed inoculation and seedling growth

Seedlings were grown in Ray Leach cone-tainers (210 mm x 38 mm in diameter; Stuewe & Sons, OR, U.S.A.) that were filled to 67% capacity with an autoclaved sand-Turface (montmorillonite clay; Applied Industrial Materials Corporation, Deerfield, IL) mixture (69% w/w silica sand; 29% w/w Turface; 2% (w/w) $CaCO_3$). Two seed inoculation "levels" (*i.e.*, inoculated with (i) live P2b-2R and (ii) sterile PBS) were evaluated at four soil N concentrations (5.9, 5.9 x 10, 5.9 x 10^2 and 5.9 x 10^3 µM, which corresponded to very low, low, medium and high N treatments, respectively), resulting in eight treatment combinations. Each treatment was replicated 60 times in a 12-month experiment. Prior to inoculation, each cone-tainer was

fertilized to saturation with 20 mL of a nutrient solution (Chanway *et al.* 1988), which was modified by replacing KNO_3 and $Ca(NO_3)_2.4H_2O$ with $Ca(NO_3)_2$ (5 atom % ^{15}N; Sigma-Aldrich Co. LLC.) and Sequestrene 330Fe (CIBA-GEIGY, Missisuaga, ON) with $Na_2FeEDTA$ (0.02 g L^{-1}). The modified plant nutrient solution contained one of the four N concentrations described above as well as (g L^{-1}): KH_2PO_4, 0.14; $MgSO_4$, 0.49; H_3BO_3, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001; $CuSO_4 \cdot 5H_2O$, 0.0001; and $NaMoO_4 \cdot 2H_2O$, 0.001.

Pine seeds were surface sterilized by immersion in 30% hydrogen peroxide for 90 seconds followed by three 30-second rinses in sterile distilled water. After surface sterilization, ten randomly selected seeds were imprinted on tryptic soy agar (TSA) (BD Difco) and incubated at 30°C for two days to check for surface contamination. Uncontaminated pine seeds were then placed in sterile cheesecloth bags and stratified on moist, sterile sand for 5 weeks at 4°C. Ten randomly selected stratified seeds were then checked for internal contamination by *P. polymyxa* P2b-2R by aseptically crushing seeds and plating the resulting extracts on TSA amended with 200 mg L^{-1} rifamycin. Plates were examined for contamination two days later.

Three stratified and surface-sterilized seeds were sown aseptically in each cone-tainer and covered with ca. 5 mm of autoclaved silica sand before inoculation. Bacterial inoculum was prepared by thawing a frozen culture of strain P2b-2R, streaking a loopful onto CCM agar amended with 200 mg L^{-1} rifamycin, and incubating at 30°C for two days. A 1-L flask containing 500 mL of CCM broth amended with rifamycin was then inoculated with a loopful of bacterial growth from the agar, secured on a rotary shaker and agitated (150 rpm) at room temperature for two days. Bacterial cells were harvested by centrifugation (3000 x g; 30 min), washed twice in sterile PBS (pH 7.4) and resuspended in the same buffer to a density of ca. 10^6 cfu mL^{-1}. Five mL of the P2b-2R-PBS suspension were then pipetted into each cone-tainer designated for live

P2b-2R treatment. This process was repeated using five mL of sterile PBS without bacteria for non-inoculated (control) cone-tainers.

All cone-tainers were placed in trays (98 cells; RL98; Stuewe & Sons, OR, U.S.A) and transported to the greenhouse at the University of British Columbia. A 16-h photoperiod (6am to 10pm) with an intensity of at least 300·µmol s^{-1}·m^{-2} was achieved with a combination of natural and artificial light. Seedlings were watered as required with tap water and thinned to the largest single germinant per cone-tainer two to three weeks after sowing. Seedlings received 20 mL of modified nutrient solution containing one of the four concentrations of $Ca(NO_3)_2$ (see above) once per month during the 12-month growth trial. Tray position was randomized weekly to reduce positional effects during the experiment.

2.3.3 Rhizospheric and endophytic colonization

For evaluation of rhizosphere colonization, three randomly-selected seedlings from each treatment were harvested destructively four, eight and twelve months after sowing and inoculation. Seedlings were removed from cone-tainers, and loosely adhering soil particles were removed from roots with gentle shaking. Roots were then separated from shoots, placed in sterile polypropylene Falcon tubes (50 mL; BD Biosciences, CA, USA) filled with 10 mL of autoclaved PBS and shaken on a vortex mixer at 1000 rpm for one minute. Tenfold serial dilutions were performed, and 100 µL aliquots were then plated on CCM plates amended with rifamycin (200 mgL^{-1}) and cycloheximide (100 mg L^{-1}). Plates were incubated at room temperature for seven days, after which colonies were counted. Roots were washed and oven-dried at 65°C for two days before weighing. Rhizospheric bacterial populations were then calculated as the number of cfu g^{-1} root (dry weight).

For evaluation of endophytic colonization, three randomly-selected seedlings from each treatment were harvested destructively at the time of rhizosphere colonization sampling. Seedlings were agitated (150 rpm) in a 2-L flask containing 800 mL of PBS for 20 minutes for removal of loosely adhering growth media. Seedlings were then surface-sterilized in 1.3% (w/v) sodium hypochlorite for five minutes, rinsed three times with sterile PBS, imprinted on TSA and left for 24 h to check for surface microbial contamination. Needle, stem and root tissue samples (20 mg fresh weight) were then triturated separately in 1 mL of SPB and tissue homogenates were serially diluted on CCM agar plates supplemented with 100 mgL^{-1} cycloheximide or with cycloheximide (100 mgL^{-1}) and rifamycin (200 mgL^{-1}). This protocol was modified for the second harvest by reducing the concentration of sodium hypochlorite from 1.3% to 1.0%. For the final harvest, seedlings were treated with 0.6% sodium hypochlorite for seven minutes.

2.3.4 Analysis of foliar N and seedling biomass

Nine seedlings from each treatment were harvested destructively four, eight and twelve months after sowing for evaluation of seedling biomass as well as foliar N and ^{15}N content. Roots were separated from shoots and dried at 65°C for two days before weighing. Needles were then removed from shoots, weighed, ground to a particle size of less than 1 mm and mixed thoroughly. To assess the effect of bacterial inoculation on seedling growth parameters, *i.e.* biomass and foliar N content, treatment effects were expressed as the percentage difference from the appropriate controls and were calculated as follows:

$$(Gi-Gc)/Gc \times 100\%,$$

where *Gi* and *Gc* are growth parameter values of the inoculated and control seedlings, respectively.

For foliar N analysis, oven dried foliage was ground to a particle size of less than 1 mm and a 5 mg subsample from each seedling was packaged and sent to the Stable Isotope Facility at

the University of California in Davis. Foliar N content and % ^{15}N excess were determined with an elemental analyzer interfaced with an isotope ratio mass spectrometer (Europa Scientific Integra). The amount of fixed N in foliage was estimated by calculating the percent N derived from the atmosphere (%Ndfa) (Rennie *et al.* 1978) as follows:

$$\%Ndfa = [1 - \text{atom } \%\ ^{15}N \text{ excess (inoculated plant)} / \%\ ^{15}N \text{ excess (non-inoculated plant)}] \times 100\%$$

2.3.5 Statistical analyses

For the in the seedling growth experiment, the eight treatment combinations, *i.e.*, two inoculation "levels" x four soil N concentrations, were arranged according to a completely randomized design. A two-tailed Student's t-test (alpha = 0.05) was performed at each N level to assess inoculation effects on foliar N concentration and ^{15}N atom percent excess amount as well as seedling biomass four, eight and twelve months after inoculation. A two-way analysis of variance (ANOVA) (Steel *et al.* 1996) was performed using SAS 9.3 (Copyright (c) 2002-2010 by SAS Institute Inc., Cary, NC, USA) to evaluate the effects of bacterial inoculation and soil N concentration on the aforementioned growth parameters at the same harvest intervals. Bacterial colony counts were log transformed prior to analysis in order to meet the statistical assumptions of normally distributed residuals and variance homogeneity. Treatment means were separated using Bonferroni's tests (alpha = 0.05).

3 Results

3.1 *in vitro* carbon-source utilization by *Paenibacillus polymyxa* P2b-2R in relation to endophytic colonization

All of the plates containing carboxymethyl cellulose, xylan or pectin showed visible zones of hydrolysis around colonies after incubating for two days. *Paenibacillus polymyxa* P2b-2R was able to use 41% of the carbon sources on BioLog GP2 plates (*i.e.,* 39 of the possible 95 sources; Appendix B). The bacterium readily metabolized arabinose, xylose, mannose, glucose, galactose and pyruvic acid, biochemicals that constitute major components of plant cell walls, but was unable to use other plant cell wall-related compounds such as galacturonic acid, rhamnose, glucuronic acid and mannan. No carbon sources from the amines/amides, amino acids, polysorbates, nucleotides or glycerol derivative group were metabolized by the bacterium.

3.2 Rhizospheric and endophytic colonization

Paenibacillus polymyxa strain P2b-2R was recovered from the rhizosphere of all inoculated seedlings at each harvest. Rhizosphere population densities ranged from 3.1 to 4.4 log cfu g^{-1} root (dry weight) and did not differ significantly with soil N concentration (Table 1). With the exception of those from the high N treatment where P2b-2R densities increased over time, rhizosphere population densities tended to decline between months four and twelve (Table 2). A statistically significant reduction from 4.2 log cfu g^{-1} root at month eight to 3.1 log cfu g^{-1} root at month twelve was detected on inoculated seedlings that received the lowest soil N concentration (*i.e.*, very low – Table 1).

Table 1 Effect of added nitrogen (N treatment) on the P2b-2R rhizosphere population size (log cfu g^{-1} root (dry weight) ± standard error; n = 3) of lodgepole pine seedlings four, eight and twelve months after sowing and inoculation.

	Months since sowing		
Nitrogen treatment	**4**	**8**	**12**
Very Low	3.6 ± 0.0	4.2 ± 0.3	3.1 ± 0.3
Low	3.8 ± 0.1	3.5 ± 0.2	3.1 ± 0.4
Medium	4.0 ± 0.1	3.4 ± 0.5	3.3 ± 0.5
High	3.9 ± 0.1	3.7 ± 0.2	4.4 ± 0.2

Table 2 Temporal variation of P2b-2R rhizosphere population size (log cfu g^{-1} root (dry weight) ± standard error; n = 3) of lodgepole pine seedlings supplied with very low, low, medium or high soil N concentrations.

	Nitrogen treatment			
Months since sowing	**Very Low**	**Low**	**Medium**	**High**
4	3.6[1] ± 0.0ab	3.8 ± 0.1a	4.0 ± 0.1a	3.9 ± 0.1ab
8	4.2 ± 0.3a	3.5 ± 0.2a	3.4 ± 0.5a	3.7 ± 0.2b
12	3.1 ± 0.3b	3.1 ± 0.4a	3.3 ± 0.5a	4.4 ± 0.2a

[1]Means with different letters within columns (nitrogen treatments) are significantly different ($p < 0.05$).

No P2b-2R were recovered from surface-disinfected pine tissues using CCM agar, with or without rifamycin, until the final harvest, twelve months after sowing and inoculation, when the modified surface-sterilization protocol was used. *Paenibacillus polymyxa* strain P2b-2R was detected inside root and stem tissues but not inside needles in twelve-month-old seedlings (Figure 1). Although significant "within treatment" variation prevented soil N effects from being statistically significant, roots from seedlings grown at very low and low N levels harbored larger mean endophytic P2b-2R population sizes than stems (Figure 1).

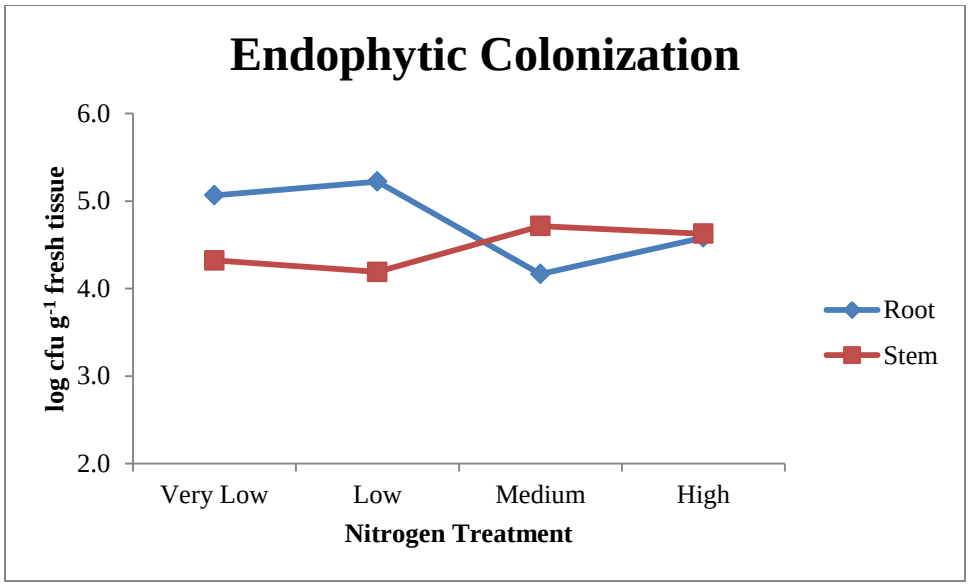

Figure 1 Endophytic population size of P2b-2R (log cfu g^{-1} fresh tissue; n = 1 - 3) in lodgepole pine seedlings twelve months after sowing and inoculation.

3.3 Seedling growth responses to P2b-2R at varying soil N concentrations

Foliar N content (FNC) generally increased between four and eight months after sowing and inoculation and then declined thereafter (Figure 4). Regardless of inoculation, foliar samples from the high soil N treatment had consistently greater FNC than those from the lower soil N treatments, but differences were not significant (Table 3). Inoculation generally had insignificant effects on foliar N content during the experiment. The single exception occurred in the very low soil N treatment, where inoculation increased foliar N concentration by 19.4% (Table 4) four months after inoculation, but decreased this growth parameter by 23.3% by the final harvest.

Table 3 Foliar nitrogen concentration (percentage w/w ± standard error; n = 9) four, eight and twelve months after sowing and inoculation.

		Months after sowing and inoculation		
N treatment	**Inoculation**	**4**	**8**	**12**
Very Low	Inoculated	0.9 ± 0.1	1.2 ± 0.1	0.6 ± 0.1*
	Control	0.7 ± 0.1	1.2 ± 0.1	0.7 ± 0.1
Low	Inoculated	0.9 ± 0.1	1.1 ± 0.1	0.6 ± 0.1
	Control	0.9 ± 0.1	1.2 ± 0.1	0.7 ± 0.0
Medium	Inoculated	1.0 ± 0.1	1.3 ± 0.1	0.7 ± 0.1
	Control	1.0 ± 0.1	1.3 ± 0.1	0.7 ± 0.1
High	Inoculated	1.3 ± 0.1	1.6 ± 0.1	1.1 ± 0.1
	Control	1.3 ± 0.1	1.5 ± 0.1	1.1 ± 0.1
Full Model	I	n.s.	n.s.	n.s.
	N	**	**	**
	I x N	n.s.	n.s.	n.s.

n.s. = not significant
***** $p<0.10$
****** $p<0.05$

Table 4 Differences in mean foliar N concentrations of seedlings inoculated with *Paenibacillus polymyxa* P2b-2R expressed as the percentage of their respective controls four, eight and twelve months after sowing and inoculation.

	Months after sowing and inoculation		
N treatment	**4**	**8**	**12**
Very Low	19.4	2.6	-23.3
Low	1.1	-6.6	-1.5
Medium	-3.0	-0.7	-6.8
High	0.8	1.3	0.9

Seedling total foliar N (TFN) was affected primarily by soil N concentration and not by bacterial inoculation (Table 5). Seedlings from the high soil N treatment generally accumulated more foliar N

than those receiving lower soil N concentrations but inoculation had no clear effect on seedling TFN. Exceptions occurred in the low and medium soil N treatments where inoculated seedlings supplied with low soil N concentrations accumulated 35.3% less foliar N relative to controls four months after sowing and inoculation (Table 6), but inoculation at the medium soil N concentration enhanced TFN of 12-month old pine seedlings by 30.6%.

Table 5 Total foliar N (mg ± standard error; n = 9) of pine seedlings four, eight and twelve months after sowing and inoculation.

		Months after sowing and inoculation		
N treatment	**Inoculation**	**4**	**8**	**12**
Very Low	Inoculated	0.2 ± 0.0	0.3 ± 0.1	0.5 ± 0.1
	Control	0.1 ± 0.0	0.3 ± 0.1	0.5 ± 0.0
Low	Inoculated	0.1 ± 0.0**	0.3 ± 0.0	0.7 ± 0.1
	Control	0.2 ± 0.0	0.3 ± 0.0	0.7 ± 0.1
Medium	Inoculated	0.2 ± 0.0	0.4 ± 0.1	0.9 ± 0.1*
	Control	0.2 ± 0.0	0.4 ± 0.1	0.7 ± 0.1
High	Inoculated	0.3 ± 0.0	0.7 ± 0.1	1.9 ± 0.3
	Control	0.3 ± 0.0	0.7 ± 0.2	2.1 ± 0.3
Full Model	I	n.s.	n.s.	n.s.
	N	**	**	**
	I x N	n.s.	n.s.	n.s.

n.s. = not significant
***** $p<0.10$
****** $p<0.05$

Table 6 Differences in mean total foliar N of seedlings inoculated with *Paenibacillus polymyxa* P2b-2R expressed as the percentage of their respective controls four, eight and twelve months after sowing and inoculation.

	Months after sowing and inoculation		
N treatment	**4**	**8**	**12**
Very Low	15.4	6.9	17.8
Low	-35.3	24.0	6.0
Medium	-15.8	-7.9	30.6
High	3.0	-7.0	-9.6

Foliar ^{15}N atom percent excess increased with increasing soil N concentrations (Table 7), but was unaffected by inoculation. Due to lack of significant inoculation effects on ^{15}N atom percent excess ($p > 0.05$), % N derived from the atmosphere was not calculated.

Table 7 Foliar ^{15}N atom percent excess (‰ ± standard error; n = 9) of lodgepole pine seedlings four, eight and twelve months after sowing and inoculation.

		Months after sowing and inoculation		
N treatment	**Inoculation**	**4**	**8**	**12**
Very Low	Inoculated	6.0 ± 2.0	31.2 ± 5.7	34.0 ± 3.4
	Control	3.9 ± 1.3	22.2 ± 4.2	32.6 ± 4.6
Low	Inoculated	159.2 ± 97.7	237.5 ± 24.7	346.09 ± 40.5
	Control	72.7 ± 18.2	187.9 ± 20.5	326.66 ± 20.1
Medium	Inoculated	1019.5 ± 140.2	2096.0 ± 266.3	3068.96 ± 160.3
	Control	925.1 ± 83.8	2262.5 ± 199.8	3046.39 ± 184.2
High	Inoculated	4971.5 ± 245.3	7247.1 ± 406.4	8337.99 ± 258.7
	Control	5114.2 ± 297.6	6892.4 ± 378.6	8308.93 ± 249.5
Full Model	I	n.s.	n.s.	n.s.
	N	**	**	**
	I x N	n.s.	n.s.	n.s.

n.s. = not significant
** $p<0.05$

Seedlings inoculated with *Paenibacillus polymyxa* P2b-2R generally had less root and shoot biomass compared to their respective controls four months after sowing and inoculation (Table 8). Seedling growth inhibition appeared to be greatest in the low soil N treatment, where inoculated seedlings accumulated 29.7% and 37.0% less root and shoot biomass, respectively (Table 9). However, this tendency was not apparent by month eight as root and shoot biomass of inoculated and non-inoculated seedlings did not differ across four N treatments (Table 8) and by month twelve, inoculated seedlings from the very low N treatment had a 52.3% and 46.4% increase in root and shoot biomass, respectively (Table 9). Seedlings from the medium N treatment responded similarly to inoculation, but no such effects were observed at the low and high soil N concentrations.

Table 8 Seedling biomass (mg ± standard error; n = 9) four, eight and twelve months after sowing and inoculation.

		Months since sowing and inoculation					
		4		**8**		**12**	
Nitrogen treatment	**Inoculation**	**Root**	**Shoot**	**Root**	**Shoot**	**Root**	**Shoot**
Very Low	Inoculated	16.1 ± 2.3	23.1 ± 2.4	53.3 ± 9.6	39.1 ± 9.7	143.3 ± 19.8*	100.7 ± 14.0*
	Control	19.0 ± 1.2	24.0 ± 2.4	46.5 ± 5.1	35.1 ± 2.8	94.1 ± 13.4	68.8 ± 8.6
Low	Inoculated	16.1 ± 2.5	16.9 ± 1.3**	39.8 ± 7.9	38.5 ± 4.5	139.8 ± 18.6	116.0 ± 11.4
	Control	22.8 ± 4.1	26.9 ± 3.5	42.2 ± 11.0	31.4 ± 3.3	148.5 ± 24.4	107.2 ± 13.0
Medium	Inoculated	22.9 ± 2.4	22.2 ± 1.5	48.2 ± 12.8	40.3 ± 6.1	184.1 ± 27.9*	148.9 ± 19.4*
	Control	25.1 ± 3.3	24.8 ± 2.8	57.2 ± 8.2	41.2 ± 6.7	120.9 ± 23.9	102.0 ± 15.3
High	Inoculated	36.4 ± 4.6	35.7 ± 2.1	84.4 ± 12.3	60.8 ± 4.4	195.2 ± 65.2	200.6 ± 46.3
	Control	41.9 ± 6.3	36.8 ± 2.6	67.8 ± 11.9	64.4 ± 11.8	196.8 ± 62.4	216.1 ± 45.60
Full Model	I	*	**	n.s.	n.s.	n.s.	n.s.
	N	**	**	**	**	n.s.	**
	I x N	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

n.s. = not significant
***** $p < 0.10$
****** $p < 0.05$

Table 9 Difference in mean root and shoot biomass of seedlings inoculated with *Paenibacillus polymyxa* P2b-2R expressed as the percentage of their respective controls four, eight and twelve months after sowing and inoculation.

	Months since sowing and inoculation					
	4		8		12	
N treatment	Root	Shoot	Root	Shoot	Root	Shoot
Very Low	-15.5	-3.6	14.7	11.5	52.3	46.4
Low	-29.7	-37.0	-5.6	22.7	-5.8	8.2
Medium	-8.5	-10.4	-15.8	-2.2	52.3	45.9
High	-13.0	-3.1	24.6	-5.6	-0.8	-7.2

4 Discussion

The primary objective of this thesis was to evaluate the growth response of lodgepole pine seedlings to inoculation with *P. polymyxa* P2b-2R under different N fertility regimes. A secondary objective was to determine which enzyme activities P2b-2R possessed that could facilitate its previously demonstrated ability to colonize internal tissues of lodgepole pine. I will first discuss endophytic colonization by P2b-2R in this thesis before addressing the response of pine to inoculation with P2b-2R under varying soil N concentrations.

4.1 *in vitro* carbon-source utilization by *Paenibacillus polymyxa* strain P2b-2R in relation to endophytic colonization

Bacteria that are capable of endophytic colonization may enter roots passively via existing cracks such as emergence sites of lateral roots (James *et al.* 1994), or more actively, by using hydrolytic enzymes to break down plant cell wall components and metabolize organic compounds in the apoplast (Hurek *et al.*1994). The latter mode of entry has been reported for plant x microbe interactions ranging from the highly specific symbiosis between *Rhizobium leguminosarum* biovar *trifolii* and white clover (Mateos *et al.*1992) to more casual associations such as those between *Azoarcus* sp. and grasses (Hurek *et al.*1994). I examined the capability of *P. polymyxa* strain P2b-2R to utilize carbon substrates known to comprise typical plant cell walls using *in vitro* plate assays (Appendix B). Of the 39 carbon substrates readily oxidized by P2b-2R, the "carbohydrates" group represents the largest source of utilizable carbon, *i.e.*, 23 of possible 39 substrates or 59 % (Appendix B). These carbon sources are found naturally in plants, as freely available, stored forms such as sucrose, raffinose and maltose while sugars including arabinose, xylose, mannose, glucose and galactose exist in association with major components of plant cell walls. Conclusions regarding the relationship between *in vitro* substrate utilization and the ability to colonize internal tissues of plants cannot be made with certainty, but my data suggest that P2b-2R could

theoretically utilize the aforementioned sugars as well as organic acids such as pyruvic acid for energy generation during endophytic colonization .

It is also interesting to note that P2b-2R was able to hydrolyze sodium polypectate, the main component of pectin, but unable to metabolize its main hydrolysis product, D-galacturonic acid. The bacterium was also incapable of using other pectin-related carbon sources including rhamnose and D-glucuronic acid. It is possible that the partial degradation of pectin, which is known to generate oligogalacturonides (Nothnagel *et al.* 1983; Aziz *et al.* 2004), might have trigged a plant innate immune response (Galletti *et al.* 2009) and eventually limited the endophytic population sizes of P2b-2R. Alternatively, pectinolytic enzymes may provide enough destabilization of plant cell walls to allow endophytic colonization by P2b-2R. By degrading part of the middle lamella, P2b-2R may have better access to organic compounds associated with cellulose and hemicellulose. However, the defense signal pathways in lodgepole pine as well as the actual products of partial pectin degradation by P2b-2R need to be established to evaluate these possibilities.

Because the rhizosphere and plant interiors represent two vastly different environments (Gottel *et al.* 2011), it is tempting to speculate that bacteria adapted to different habitats will exhibit distinctly different metabolic profiles. The link between metabolism of specific carbon substrates and the ability of rhizosphere bacteria to colonize the interior of lodgepole pine seedlings was investigated by Shishido *et al.* (1995) using two strains of *P. polymyxa,* L6-16R and Pw-2R. Strain Pw-2R is a root endophyte of lodgepole pine (Chanway *et al.*1990) and is capable of metabolizing sorbitol and D-melezitose, a carbohydrate commonly found in conifer sap (Lehninger 1975). Strain L6-16R, which was isolated from the rhizosphere of perennial ryegrass (*Lolium perenne* L.) (Chanway *et al.* 1990), could not use these substrates and was unable to enter pine. The ability to metabolize these substrates has also been reported for a spruce endophyte, *Pseudomonas fluorescens* strain Sm3-RN (Shishido *et al.* 1999). Taken together,

these observations suggest that endophytic and non-endophytic bacteria may be differentiated by their ability to utilize specific plant-derived carbon compounds.

Since both P2b-2R and Pw-2R are pine endophytes, I expected that they would have similar carbon source-utilization capabilities that reflect their adaptation to colonizing internal pine tissues. However, based on the 35 Biolog carbon substrates that distinguished Pw-2R and L6-16R (Shishido *et al.* 1999), use of only eight of these carbon compounds was common between Pw-2R and P2b-2R (Table 10). In addition, D-melezitose and sorbitol, which were readily used by other pine endophytes (Shishido *et al.* 1995; Shishido *et al.* 1999), were not metabolized by P2b-2R. In light of the diversity of available nutrients produced by plants, this small overlap in carbon substrate-utilization profiles of P2b-2R and Pw-2R suggests that these two presumably closely related endophytic strains of *P. polymyxa* may occupy two distinct nutritional niches of internal pine tissues.

Table 10 Carbon source-utilization profiles of *P. polymyxa* strains Pw-2R and P2b-2R.

Carbon sources	BIOLOG test reactions[a]	
	Pw-2R[b]	P2b-2R
P-Cyclodextrin	wp	wp
a-Methyl-D-galactoside	+	+
D-Psicose	+	+
L-Malic acid	-	-
Methyl pyruvate	+	+
Pyruvic acid	+	+
Glycerol	+	+
Inosine	-	-

[a] + = positive; wp = weakly positive; - = negative.
[b] adapted from Shishido *et al* (1995)

To summarize my carbon substrate utilization results, I found that P2b-2R was able to degrade cellulose, xylan and sodium polypectate, the main constituents of plant cell walls (Compant *et al*. 2005; Reinhold-Hurek *et al*. 2006), which suggests that hydrolytic enzymes are important facilitators of endophytic colonization. However, it is difficult to interpret *in vitro* substrate hydrolysis without

evidence of *in situ* enzyme activities as plate assays only demonstrate the potential enzyme activities a microbe possesses, which may or may not be expressed in nature. In addition, the role of these enzymes in endophytic colonization is not clear because enzymes known to degrade plant cell wall components have been found in both endophytic and non-endophytic strains of plant-colonizing bacteria (Shishido *et al.* 1995). *P. polymyxa* strains L6-16R and Pw-2R were able to hydrolyze sodium polypectate, the major component of the middle lamella, but only the latter was found to colonize internal tissues of lodgepole pine seedlings. Future experiments involving microscopy and use of bacterial mutants that lack cellulolytic or pectinolytic enzymes are required to ascertain the importance of *in vitro* enzyme activities in endophytic colonization by P2b-2R.

4.2 Rhizospheric and endophytic colonization

The rhizosphere population densities of P2b-2R ranged from 3.1 to 4.4 log cfu g^{-1} root (dry weight) (Table 1). Notwithstanding this variation, the recovery of P2b-2R from the rhizosphere of all harvested pine seedlings 4, 8 and 12 months after sowing and inoculation suggests this strain is a competent rhizosphere colonizer and is in agreement with earlier findings of Bal and Chanway (2012). By the end of the 12-month growth trial P2b-2R densities were comparable to those reported for other strains of *P. polymyxa* on conifers such as lodgepole pine, 5 x 10^7 cfu/g root (Shishido *et al.* 1995), hybrid spruce (*Picea glauca* x *engelmannii*), > 1 x 10^4 (Shishido *et al*. 1996) and western red cedar, 2 x 10^5 (Bal and Chanway 2012).

Despite the large differences in the amount of added N among the three lowest soil N treatments, *i.e.*, 10 – 100-fold, P2b-2R population sizes decreased similarly over time and stabilized to a density of approximately 3.1 log cfu g^{-1} root (dry weight) 12 months after inoculation in each of these treatments. Such a decline of bacterial population densities with seedling age has been reported for *Bacillus* strains colonizing the rhizosphere of lodgepole pine (Holl and Chanway 1992) and spruce (Shishido and

Chanway 2000). Since root exudates are the mostly likely carbon source that sustained rhizosphere-colonizing microorganisms (Bais *et al.* 2006), the population decrease of P2b-2R population size may be attributable to a decrease in the amount of photosynthate translocated to the root as plants age (Keith *et al.* 1986; Domanski *et al.* 2001). A reduction in the density of colonization microsites, such as tips of actively growing roots and emergence sites of lateral roots (Bent *et al*. 2002) where nutrients are released (Charlton 1996), may also be important. In contrast, seedlings from the highest N treatment had larger root systems, which might provide more root exudates and higher densities of nutrient-rich microsites for P2b-2R to colonize.

Endophytic P2b-2R has been isolated from root, stem and root tissues of pine seedlings as early as two months after sowing and inoculation (Anand and Chanway 2012a), however I was not able to isolate P2b-2R from within surface-sterilized tissues of such young seedlings in my experiment. Poor or unsuccessful recovery of endophytes has been observed when sodium hypochlorite was used to sterilize plant samples (Mclnroy *et al.* 1996). At micromolar concentrations, sodium hypochlorite is known to significantly reduce microbial populations (Nakagawara *et al.* 1998) and may render injured bacterial cells non-culturable by predisposing them to oxidative stress (Dukan *et al.* 1999) and DNA damage, which can result in a loss of antibiotic resistance (Heisig 1996; Dukan *et al.* 1999). Surface-sterilization protocols involving sodium hypochlorite as the major sterilant might be improved by using sodium thiosulfate to neutralize remaining hypochlorite in the rinsing step (Miche and Balandreau 2001) as well as by the addition of 'scavengers' of reactive oxygen species such as sodium ascorbate (Ducret *et al.* 2014) and catalase (Kono 1982) to enhance the viability of endophytic bacteria.

When I revised my plant surface sterilization procedure by reducing the concentration of sodium hypochlorite from 1.3% to 0.6%, endophytic P2b-2R was detected in root and stem tissues of one-year-old pine (Figure 1). The average population densities, which ranged from 4.2 to 5.2 log cfu g^{-1} fresh tissue, were similar to those reported by Anand and Chanway (2013a) and were much higher than those

isolated from the rhizosphere 4, 8 or 12 months after inoculation. This observation suggests that the pine interior may be a more protected habitat that allows P2b-2R to reach higher population densities than on the root surface and rhizosphere.

4.3 Seedling growth responses to P2b-2R at varying soil N concentrations

The primary objective of my thesis was to determine if the amount of nitrogen fixation by lodgepole pine seedlings colonized by *P. polymyxa* P2b-2R would decrease in response to increasing soil N concentrations. However, even though a decrease in seedling foliar N content was observed across four soil N treatments between months eight and twelve (Table 3), indicating that pine seedlings experienced N deficiency, I could not detect significant foliar ^{15}N dilution and hence, BNF in the assay system I used. In fact, twelve months after sowing and inoculation, the foliar N content of seedlings from the very low, low and medium soil N treatments were at a level that is considered 'very severely deficient' for lodgepole pine (Ballard and Carter 1986). These results are not in agreement with those reported by Bal and Chanway (2012) and Anand *et al.* (2013), who used a glass-tube plant growth and inoculation system. In those studies, P2b-2R provided substantial amounts of fixed N to pine seedlings (Bal and Chanway 2012; Anand *et al.* 2013). Several differences between the two seedling growth trials may have contributed to these discrepant findings. Unlike the glass-tube assay system, where N-fertilizer was applied once, *i.e.*, at the time of sowing and inoculation, monthly application of N-containing fertilizer was used in my experiment to reduce leaching of nitrate from the bottoms of cone-tainers. If my seedlings were fixing N, the increased frequency of N-fertilization may have obscured my ability to detect ^{15}N foliar dilution. In addition, the larger rooting volume provided by cone-tainers might have resulted in enhanced soil N uptake, which is known to suppress BNF (Shrestha and Ladha 1996). Twelve months after sowing and inoculation, the control seedlings from the very low N treatment had accumulated much higher root biomass (94 vs 17 mg) than that reported by Anand *et al.* (2013) although the former received approximately 8.5-fold less mineral N (1.2 vs 9.8 mg $Ca(NO_3)_2$ seedling^{-1}). This

higher seedling biomass accumulation in my assay system may indicate that the amount of soil N from the very low N treatment was not low enough to trigger BNF. It is possible that the higher foliar N content of younger pine seedlings in my experiment which ranged from 0.7% to over 1.0% (Table 3) may have delayed BNF by P2b-2R.

The effects of P2b-2R on seedling foliar N concentrations were most apparent in the very low soil N concentrations during the growth trial compared to the low, medium or high soil N treatments (Table 4). This suggests that the relative significance of P2b-2R to the N status of pine seedlings may be greater in N-poor soils. Even though foliar ^{15}N dilution was not detected, foliar N concentration was enhanced by 19.4% in the very low N treatment four months after sowing and inoculation (Table 4), which is indicative of BNF. Compared with other early successional tree species such as trembling aspen and Douglas-fir, lodgepole pine is poorer at taking up nitrate and does not respond readily when this form of N is present at low concentrations in soils (Min *et al.* 1999). Considering its relatively low ability to take up nitrate from low nitrate soils and the reduced root biomass in response to inoculation (Table 8), inoculated pine seedlings from the very low N treatment were unlikely to accumulate sufficient N from the soil and could have been fixing N.

Previously, Bal and Chanway (2012) reported biomass reduction in N_2-fixing pine seedlings. In my cone-tainer system, the inhibitory effects on seedling biomass were also observed four months after sowing and inoculation (Table 8). Given that diazotrophy is an energetically expensive process, poorer performance of pine seedlings inoculated with P2b-2R has been suggested to result from the maintenance of BNF at the expense of plant-derived carbon substrates (Bal and Chanway 2012). Thus I hypothesized that seedling biomass reduction due to P2b-2R would be more pronounced at the very low soil N level, where pine seedlings were expected to fix N_2. However, due to the insignificant inoculation effects on foliar ^{15}N dilution (Table 7), it is not possible to relate seedling biomass responses to BNF. Nonetheless, considering that inoculated seedlings in all soil N treatments generally had lower root and

shoot biomass than their controls (Table 8), it could be hypothesized that P2b-2R can act as a parasite, at least temporarily, that depends on pine for carbon substrates without providing fixed N in return. Since low soil N availability can limit a plant's ability to produce photosynthate, the greater magnitude of biomass reduction observed at the very low and low N levels at the first harvest (Table 9) might have resulted from the combined effects of reduced photosynthate production and carbon utilization by P2b-2R. Alternatively, P2b-2R could have inhibited pine seedling growth in a similar manner to other root-colonizing bacteria that are known to produce plant-growth-inhibiting amounts of phytohormones or toxic compounds, or to compete with their plant host for nutrients such as iron (Kremer 2006). Regardless of the mechanisms, my results suggest that pine seedling biomass inhibition by P2b-2R can occur at a wide range of soil N concentrations and characterizes early stages of the pine x P2b-2R interaction. Biomass reduction due to bacterial inoculation was not apparent eight months after sowing and inoculation (Table 8) and could have resulted from the uptake of nutrients that were released from dead P2b-2R cells. The exact mechanism by which initial growth inhibition was overcome by older seedlings remains to be evaluated.

The pronounced seedling biomass enhancement by P2b-2R that I observed twelve months after sowing and inoculation (Table 8) is in agreement with previous results (Anand *et al.* 2013), and supports the hypothesis that a mutualism exists between pine and this bacterium. Biomass accumulation due to bacterial inoculation was more pronounced in the very low N treatment than those in the high N treatment: inoculated seedlings from the very low N treatment had a 52.3% and 46.4% increase in root and shoot biomass, respectively, but those receiving high amounts of mineral N showed no such trend (Table 9). Therefore, my data support the hypothesis that seedling growth promotion by P2b-2R is more pronounced at the low soil N concentrations. Anand *et al.* (2013) suggested that P2b-2R enhanced performance of pine seedlings through BNF. However, the same bacterial strain in my experiment was able to stimulate pine biomass without affecting foliar ^{15}N atom % excess (Table 7), suggesting that

P2b-2R might promote the growth of my pine seedlings without providing appreciable amounts of fixed N. It is well known that other *P. polymyxa* strains can enhance plant performance by producing phytohormones (Chanway 2002), but the ability of P2b-2R to synthesize plant growth regulators such as auxins and cytokinins requires further assessment.

5 Conclusions

The following conclusions can be drawn from my study:

(i) Soil mineral N concentrations did not significantly affect the population sizes of *P. polymyxa* P2b-2R on and inside pine tissues.

(ii) BNF by P2b-2R was not detected in my experiment.

(iii) Biomass enhancement of pine seedlings by P2b-2R was greater at low soil N concentrations.

(iv) P2b-2R possesses hydrolytic enzyme activities consistent with those that characterize known bacterial endophytes.

My results indicate a mutualism between P2b-2R and pine seedlings, but several questions regarding the pine x P2b-2R interactions need to be resolved to confirm my results. First, due to great variation in P2b-2R population sizes in my plate assays, it is not possible to evaluate with certainty the effects of soil mineral N concentrations on the population sizes of P2b-2R in association with pine seedlings. Quantification of bacterial cells using more sensitive methods such as indirect enzyme-linked immunosorbent assays may provide more reliable comparisons of bacterial population sizes at different soil N concentrations. Second, considering that P2b-2R did not significantly lower foliar ^{15}N, it may be possible that the bacterium can stimulate pine growth by other mechanisms than BNF. Further evaluation of other plant-growth-promoting characteristics of P2b-2R including production of plant growth regulators may be required to enhance our understanding of the pine x P2b-2R interactions at varying soil N concentrations. Finally, it is not known whether P2b-2R actually used hydrolytic enzymes to destabilize plant cell walls and to acquire nutrients in pine interiors in my study. Molecular techniques such as real-time polymerase chain reactions (PCR) and quantitative PCR combined with microscopy may be used to quantify and localize the activity of plant cell wall-degrading enzymes *in planta*.

Bibliography

Abellan, I.P., Urguiza, R.D.A., Valadier, M.H., Champigny, M.L. 1994. Short-term effect of nitrate on carbon metabolism of two sugarcane cultivars differing in their biomass production. *Phytochemistry*. **36:** 819 – 823.

Adriano-Anaya, M., Salvador-Figueroa, M., Ocampo, J.A., Garcia-Romera, I. 2005. Plant cell-wall degrading hydrolytic enzymes of *Gluconacetobacter diazotrophicus. Symbiosis.* **40:** 151 – 156.

Anand, R., Chanway, C.P. 2013a. Detection of GFP-labeled *Paenibacillus polymyxa* in auto-fluorescing pine seedling tissues. *Biol. Fertil. Soils.* **49:** 111 - 118.

Anand, R., Chanway, C.P. 2013b. *nif* gene sequence and arrangement in the endophytic diazotroph *Paenibacillus polymyxa* strain P2b-2R. *Biol. Fertil. Soils.* **49**: 965 – 970.

Anand, R., Grayston, S., Chanway, C.P. 2013. N_2-fixation and seedling growth promotion of lodgepole pine by endophytic *Paenibacillus polymyxa. Microbial Ecology***. 66:** 369 – 374.

Azevedo, J. L., Maccheroni Jr., W., Pereira, J. O. Araújo, W. L. 2000. Endophytic microorganisms: a review on insect control and recent advances on tropical plants. *EJB: Electronic Journal of Biotechnology*. **3:** 40 – 65.

Aziz, A., Heyraud, A., Lambert, B. 2004. Oligogalacturonide signal transduction, induction of defense-related responses and protection of grapevine against Botrytis cinerea. Planta. 218: 767 – 774.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., Vivanco, J.M. 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* **57:** 233 – 266.

Bal, A., Chanway, C.P. 2012. Evidence of nitrogen fixation in lodgepole pine inoculated with diazotrophic *Paenibacillus polymyxa*. *Botany*. **90:** 891 - 896.

Bal, A.S., Anand, R., Berge, O., Chanway, C.P. 2012. Isolation and identification of diazotrophic bacteria from internal tissues of *Pinus contorta* and *Thuja plicata*. *Can. J. For. Res.* **42:** 807 – 813.

Ballard, T.M., Carter, R.E. 1986. Evaluating forest stand nutrient status. Land Management Report, ISSN 0702–9861; No. 20. British Columbia Ministry of Forests, Victoria, pp 33–34

Beckett, P.H.T., Webster, R. 1971. Soil variability: a review. *Soils and Fertilizers*. **34:** 1-15.

Bent, E., Breuil, C., Enebak, S., Chanway, C.P. 2002. Surface colonization of lodgepole pine (*Pinus contorta* var. *latifolia* [Dougl. Engelm.]) roots by *Pseudomonas fluorescens* and *Paenibacillus polymyxa* under gnotobiotic conditions. *Plant Soil.* **214:** 187 – 196.

Billings, W.D. 1952. The environmental complex in relation to plant growth and distribution. *The Quarterly Review of Biology.* **27:** 251 – 265.

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

Bormann, F.H., Likens, G.E., Melillo, J.M. 1977.Nitrogen budget for an aggrading northern hardwood forest ecosystem. *Science.* **196:** 981 – 983.

Bottomley, P.J., Myrold, D.D. 2007. Biological nitrogen fixation: The return of N to the soil. p. 365-387. In Soil Microbiology, Ecology, and Biochemistry, 3rd Edition (E.A. Paul, ed.). Academic Press, Burlington, MA.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E.V.L., and Schulze-Lefert, P. 2013. Structure and functions of the bacterial microbiota of plants. *Annual Review of Plant Biology.* 64: 807 – 838.

Burbano, C.S., Liu, Y., Rösner, K.L., Reis, V.M., Caballero-Mellado, J., Reinhold-Hurek, B., Hurek, T. 2011. Predominant nifH transcript phylotypes related to *Rhizobium rosettiformans* in field-grown sugarcane plants and in Norway spruce. *Environ Microbiol Rpt*. **3:** 383 – 389.

Cacciari, I., Del Gallo, M., Ippoliti, S., Lippi, D., Pietrosanti, T., Pietrosanti, W. 1986. Growth and survival of *Azospirillum brasilense* and *Arthrobacter giacomelloi* in binary continuous culture. *Plant Soil*. **90:** 107 – 116.

Çakmakçı, Ramazan, Donmez, M.F., Erturk, Y., Erat, M., Haznedar, A., Sekban, R. 2010. Diversity and metabolic potential of culturable bacteria from the rhizosphere of Turkish tea grown in acidic soils. *Plant Soil.* **332:** 299 - 318.

Carrim, A.J.I., Barbosa, E.C., and Vieira, J.D.G. 2006. Enzymatic activity of endophytic bacterial isolates of *Jacaranda decurrens* Cham. (Carobinha-do-campo). *Braz. Arch. Biol. Technol.* **49:** 353 – 359.

Carrim, A.J.I., Barbosa, E.C., Vieira, J.D.G. 2006. Enzymatic activity of endophytic bacterial isolates of *Jacaranda decurrens* Cham. (Carobinha-do-campo). *Braz. Arch. Biol. Technol.* **49:** 353 – 359.

Cavalcante, V.A., Dobereiner, J. 1988. A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. *Plant Soil.* **108:** 23 – 31.

Chanway, C. P. 1996 Endophytes: they're not just fungi! *Can J Bot.* **74:** 321 – 322.

Chanway, C.P. 2002. Plant growth promotion by Bacillus and relatives. In: Berkeley R, Heyndrickx M, Logan N, De Vos P (eds) Applications and systematics of Bacillus and relatives. Blackwell, Oxford, pp 219 – 235.

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, 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. *Can J Bot*., **69:** 507 – 511.

Chapman, W.K., Paul, L. 2012. Evidence that northern pioneering pines with tuberculate mycorrhizae are unaffected by varying soil nitrogen levels. *Microb Ecol.* **64:** 964 – 972.

Charlton, W.A. 1996. Lateral root initiation. *In* Plant Roots: The Hidden Half. Eds. Y Waisel, A Eshel and U Kafkafi. Pp 149-173. Marcel Dekker, New York.

Compant, S., Reiter, B., Sessitsch, A., Nowak, J., Clément, 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.

Critchfield, W. B., Little, Jr. E. L. 1966. Geographic distribution of the pines of the world. *Misc Publ U.S. Dep Agric*. No. 991. Washington, DC.

Dalton, D.A. and Kramer, S. 2006. Nitrogen-fixing bacteria in non-legumes. In Springer Netherlands, Dordrecht pp. 105-130

de Bellone, S.C., Bellone, C.H. 2006. Presence of endophytic diazotrophs in sugarcane juice. *World J Microb Biot*. **22:** 1065 – 1068.

Dixon. R., Kahn, D. 2004. Genetic regulation of biological nitrogen fixation. *Nat Rev Microbiol.* **2:** 621 – 631.

Dobereiner, J., Baldani, V. L. D., Reis, V.M. 1995b. Endophytic occurrence of diazotrophic bacteria in non-leguminous crops. In *Azospirillum* VI and related microorganisms. Eds. I Fendrik, M del Gallo, J Vanderleyden and M de Zamarocy. pp 3–14. Springer Verlag, Berlin, Germany.

Dobereiner, J., Reis, V., Lazarine, A.C. 1988 A new N_2-fixing bacteria in association with cereals and sugar cane. In Nitrogen Fixation: Hundred Years After. Eds. H Bothe, F J de Bruijn and W E Newton. pp. 717-722. Gustav Fischer, Stuttgart.

Dobereiner, J., Ruschel, A.P. 1958. Uma nova espdcie de *Beijerinckia*. *R Biol*. **1:** 261 – 272.

Domanski, G., Kuzyakov, Y., Siniakina, S.V., Stahr, K. 2001. Carbon flows in the rhizosphere of ryegrass (*Lolium perenne*). *J. Soil Sci. Plant Nutr*. **164:** 381 – 387.

Dong, Y., Iniquez, A.L., Ahmer, B.M., and Triplett, E.W. 2003. Kinetics and strain specificity of rhizosphere and endophytic colonization by enteric bacteria on seedlings of *Medicago sativa* and *Medicago truncatula. Appl. Environ. Microbiol.* **69:** 1783 – 1790.

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.

Dukan, S., Belkin, S., Touati, D. 1999. Reactive oxygen species are partially involved in the bacteriocidal action of hypochlorous acid. *Arch Biochem Biophys.* **367:** 311 – 316.

Fouts, D.E., Tyler, H.L., DeBoy, R.T., Daugherty, S., Ren, Q., Badger, J.H., Durkin, A.S., Huot, H., Shrivastava, S., Kothari, S., Dodson, R.J., Mohamoud, Y., Khouri, H., Roesch, L.F., Krogfelt, K.A., Struve, C., Triplett, E.W., Methe, B.A. 2008. Complete genome sequence of the N_2-fixing broad host range endophyte *Klebsiella pneumoniae* 342 and virulence predictions verified in mice. *PLoS Biol*. **4:** 1 – 18.

Fuentes-Ramirez, L.E., Caballero-Mellado, J., Supulveda, J., Martinez-Romero, E. 1999. Colonization of sugarcane by *Acetobacter diazotrophicus* is inhibited by high N-fertilization. *FEMS Microbiology Ecology*. **29:** 117 – 128.

Galloway, J.N., Dentener, F.J., Capone, D.G., Boyer, E.W., Howarth, R.W., Seitzinger, S.P., Asner G.P., Cleveland, C.C., Green, P.A., Holland, E.A., Karl, D.M., Michaels, A.F., Porter, J.H., Townsend, A.R., Voosmarty, C. 2004. Nitrogen cycles: Past, present and future. *Biogeochemistry.* **70:** 153 – 226.

Gottel, N.R., Gasto, H.F., Kerley, M., Yang, Z., Pelletier, D.A., Podar, M., Karpinets, T., Uberbacher, E., Tuskan, G.A., Vilgalys, R., Doktycz, M.J., Schadt, C.W. 2011. Distinct microbial communities within the endosphere and rhizosphere of *Populus deltoides* roots across contrasting soil types. *Appl Environ Microbiol.* **77:** 5934 – 5944.

Graciolli, L.A., de Freitas, J.R., Ruschel, A.P. 1983. Bactdrias fixadoras de nitrog~nio nas rafzes, caules e folhas de cana-de-agficar (*Saccharum* sp.). *R Microbiol*. **14:** 191 – 196.

Graciolli, L.A., Ruschel, A.P. 1981. Microorganisms in the phyllosphere and rhizosphere of sugar cane. In Associative N_2 Fixation. Eds. P B Vose and A P Ruschel. Vol. 2. pp 91-101. CRC Press, Boca Raton, FL.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F., and Kloepper, J.W. 1997. Bacterial endophytes in agricultural crops. *Can. J. Microbiol*., **43:** 895 – 914.

Heisiq, P. 1996. Genetic evidence for a role of *parC* mutations in development of high-level fluoroquinolone resistance in *Escherichia coli*. *Antimicrob Agents Chemother.* **40:** 879 – 885.

Holl, F.B., Chanway, C.P. 1992. Rhizosphere colonization and seedling growth promotion of lodgepole pine by *Bacillus polymyxa. Can J Microbiol.* **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 Biol Biochem*. **20:** 19 - 24.

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

James, E. K., Reis, V. M., Olivares, F. L., Baldani, J. I., Döbereiner J. 1994. Infection of sugar cane by the nitrogen-fixing bacterium *Acetobacter diazotrophicus*. *J Exp Bot.* **45:** 757 - 766.

James, E.K. 2000. Nitrogen fixation in endophytic and associative symbiosis. *Field Crop Res.* **65:** 197 – 209.

James, E.K., Olivares, F.L., 1998. Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. *Crit Rev Plant Sci*. **17:** 77 – 119.

Kavino, M., Harish, S., Kumar, N., Saravanakumar, D., Damodaran, T., Sooianathasundaram, K., Samiyappan, R. 2007. Rhizosphere and endophytic bacteria for induction of systemic resistance of banana plantlets against bunchy top virus. *Soil Biol Biochem.* **39:** 1087 – 1098.

Keith, H., Oades, J.M. 1986. Input of carbon to soil from wheat plants. *Soil Biol Biochem.* **18:** 445 – 449.

Kolb, W., Martin, P. 1988. Influence of nitrogen on the number of N_2-fixing and total bacteria in the rhizosphere. *Soil Biol. Biochem.* **20:** 221 – 225.

Kono, Y. 1982. Oxygen enhancement of bactericidal activity of rifamycin SV on Escherichia coli and aerobic oxidation of rifamycin SV to rifamycin S catalyzed by manganous ions: the role of superoxide. *J. Biochem.* **91:** 381 – 395.

Kramer, D.A. 1999. Minerals Yearbook. Nitrogen. U.S. Geological Survey Minerals Information. http://minerals.usgs.gov/minerals/pubs/commodity/nitrogen/

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 subboreal forests. *Soil Sci Soc Am J.* **70:** 1591 – 1599.

Kremer, R.J. 2006. Deleterious rhizobacteria. In Plant-associated bacteria. Edited by S.S. Gnanamanickam. Springer, Dordrecht, The Netherlands. pp. 335 – 358.

Lee, E.J., Matsumura, Y., Soga, K., Hoson, T., Koizumi, N. Glycosyl hydrolases of cell wall are induced by sugar starvation in *Arabidopsis. Plant Cell Physiol.* **48:** 405 – 413.

Lehninger, A.L. 1975. Biochemistry: the molecular basis of cell structure and function. Worth Publishers, Inc., New York.

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

Lindblad, P., Atkins, C.A., Pate, J.S. 1991. N_2-fixation by freshly isolated *Nostoc* from coralloid roots of the cycad *Macrozamia riedlei* (Fisch. ex Gaud.) Gardn. *American Society of Plant Biologists.* **95:** 753 – 759.

Mahendrappa, M.K., Salonius, P.O. 1982. Nutrient dynamics and growth response in a fertilized black spruce stand. *Soil Sci. Soc. Am. J.* **46:** 127–133.

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 Soil*. **199:** 37 – 44.

McInroy, J.A., Musson, G., Wei, G., Kloepper, J.W. 1996. Masking of antibiotic-resistance upon recovery of endophytic bacteria. **186:** 213 – 218.

Miché, L., Balandreau, J. 2001. Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis. American Society for Microbiology.* **67:** 3046 – 3052.

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

Min, X., Siddiqi, M.Y., Guy, .D., Glass, A.D.M., Kronzucker, H.J. 1999. A comparative study of fluxes and compartmentation of nitrate and ammonium in early-successional tree species. *Plant, Cell & Environment*. **22:** 821 – 830.

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

Munoz-Rojas, J., Caballero-Mellado, J. 2003. Population dynamics of *Gluconacetobacter diazotrophicus* in sugarcane cultivars and its effect on plant growth. *Microb Ecol.* **46:** 454 – 464.

Mylona, P., Pawlowski, K., Bisseling, T. 1995. Symbiotic nitrogen fixation. *Plant Cell*. **7:** 869 – 885.

Nakagawara, S., Goto, T., Nara, M, Ozawa, Y., Hotta, K., Arata, Y. 1998. Spectroscopic characterization and the pH dependence of bactericidal activity of the aqueous chlorine solution. The Japan Society for Analytic Chemistry. 14: 691 – 698.

Northup, R.R., Yu, Z., Dahlgren, R.A., Vogt, K.A. 1995. Polyphenol control of nitrogen release from pine litter. *Nature*. **377:** 227 – 229.

Nothnagel, E. A., McNeil, M., Albersheim, P., and Dell, A. 1983. Host–pathogen interactions. XXII. A galacturonic acid oligosaccharide fromplant cell walls elicits phytoalexins. Plant Physiol. **71:** 916 – 926.

Olivares, F.L., Baldani, V.L.D., Reis, V.M., Baldani, J.I., Döbereiner, J. 1996. Occurrence of the endophytic diazotrophs *Herbaspirillum* spp. in roots, stems, and leaves, predominantly of *Gramineae. Biol Fertil Soils*. **21:** 197 – 200.

Postgate, J.R. 1998. Nitrogen fixation. Cambridge University Press, Cambridge, UK.

Purchase, B. S. 1980. Nitrogen fixation associated with sugar-cane. *Proc. S. Aft. Sugar Technol. Assoc*., June, 173 – 176.

Quispel, A. 1992. A search for signals in endophytic microorganisms. In Molecular signals in plant - microbe communications. Edited by D.P.S. Verma. CRC Press, Boca Raton, Fla. pp. 471 -490.

Ray, T.B., Peters, G.A., Toia, Jr. R.E., Mayne, B.C. 1978. *Azolla-Anabaena* relationship. *Plant Physiol.* **62:** 463 – 467.

Read, D.J. 1991. Mycorrhizas in ecosystems. *Experientia*. **4:** 376 – 391.

Reed., S.C.; Cleveland, C.C., Townsend, A.R. 2011. Functional ecology of free-living nitrogen fixation: a contemporary perspective. *Annu Rev Ecol Evol Syst*. **42:** 489 – 512.

Reinhold, 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 Evol Microbiol.* **43:** 574 – 584.

Reinhold-Hurek, B., Maes, T., Gemmer, S., Van Montagu, M., Hurek, T. 2006. An endoglucanase is involved in infection of rice roots by the not-cellulose-metabolizing endophyte *Azoarcus* sp. strain BH72. *Mol Plant-Microbe Interact*. **19**: 181-188.

Ridley, B.L., O'Neill, M.A., Mohnen, D. 2001. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry.* **56:** 929 - 967.

Ruschel, A.P. 1981. Associative N_2-fixation by sugar cane. In: Associative N_2-Fixation, Vol. 2 (Vose, P.B. and Ruschel, A.P., Eds.), pp. 81–90. CRC Press, Baton Rouge.

Sasaki, K., Ikeda, S., Eda, S., Mitsui, H., Hanzawa, E., Kisara, C., Kazama, Y., Kushida, A., Shinano, T., Minamisawa, K., and Sato, T. 2010. Impact of plant genotype and nitrogen level on rice growth response to inoculation with *Azospirillum* sp. strain B510 under paddy field conditions. *Soil Science and Plant Nutrition.* **56:** 636 – 644.

Schumann, U., Huntrieser, H. 2007 The global lightning-induced nitrogen oxides source. *Atmos. Chem. Phys. Discuss.* **7:** 2623– 2818.

Shishido, M., and 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., Breuil, C., Chanway, C.P. 1999. Endophytic colonization of spruce by plant growth-promoting rhizobacteria. *FEMS Microbiology Ecology.* **29:** 191 – 196.

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. *Can J Microbiol*. **45:** 707 – 713.

Shishido, M., Massicotte, H.B., and Chanway, C.P. 1996. Effect of plant growth promoting *Bacillus* strains on pine and spruce seedling growth and mycorrhizal infection. *Annals of Botany*. **77:** 433 - 441.

Shrestha, R.K., Ladha, J.K. 1996. Genotypic variation in promotion of rice nitrogen fixation as determined by nitrogen ^{15}N dilution. *Soil Sci. Soc. Am. J.* **60:** 1815 – 1821.

Silvester, W.B., Parsons, R., Watt, P.W. 1996. Direct measurement of release and assimilation of ammonia in the *Gunnera-Nostoc* symbiosis. *New Phytol*. **132:** 617 – 625.

Sindhu, S.S., Dadarwal, K.R. 2001. Chitinolytic and cellulolytic *Pseudomonas* sp. antagonistic to fungal pathogens enhances nodulation by *Mesorhizobium* sp. Cicer in chickpea. *Microbiol Res.* **156:** 353 – 358.

Sturz, A. V., Nowak, J. 2000. Endophytic communities of rhizobacteria and the strategies required to create yield enhancing associations with crops. *Appl Soil Ecol.* **15:** 183 – 190.

Swinnen, J., Van Veen, J.A., Merckx, R. 1994. ^{14}C pulse labeling of field-grown spring wheat: an evaluation of its use in rhizosphere carbon budget estimations. *Soil Biol Biochem*., **26:** 161-170.

Taulé, C., Mareque, C., Barlocco, C., Hackembruch, F., Reis, V.M., Sicardi, M., Battistoni, F. 2012. The contribution of nitrogen fixation to sugarcane (*Saccharum officinarum* L.), and the identification and characterization of part of the associated diazotrophic bacterial community. *Plant Soil*. **36:** 35 – 49.

Timmusk, S., Grantcharova, N., Wagner, E.G.H. 2005. *Paenibacillus polymyxa* invades plant roots and forms biofilms. *Appl Environ Microbiol.* **71:** 7292 – 7300.

Urquiaga, S., Cruz, K.H.S., Boddey, R.M. 1992. Contribution of nitrogen fixation to sugar cane: nitrogen-15 and nitrogen-balance estimates. *Soil Sci Soc Am J.* **56:** 105 – 114.

von der Weid, I., Paiva, E., Nobrega, A., van Elsas, J.D., Seldin, L. 2000. Diversity of *Paenibacillus polymyxa* strains isolated from the rhizosphere of maize planted in Cerrado soil. *Res Microbiol.* **151:** 369 - 381.

Wang, L., Zhang, L., Liu, Z., Zhao, D., Liu, X., Zhang, B., Xie, J., Hong, Y., Li, P., Chen, S., Dixon, R., Li, J. 2013. A minimal nitrogen fixation gene cluster from *Paenibacillus* sp. wly78 enables

expression of active nitrogenase in *Escherichia coli. PLoS Genet.* DOI: 10.1371/journal.pgen.1003865.

Weetman, G.F., Fournier, R.M., Schnorbus, E. 1988 . Lodgepole pine fertilization screening trials: four-year growth response following initial predictions. *Soil Sci Soc Am J*. **52:** 833 – 839.

Weetman, G.F., Yang, R.C., Bella, I.E. 1985. Nutrition and fertilization of lodgepole pine. In Lodgepole Pine the Species and its Management, May 8-10, 1984, Spokane, Wash. and May 14-16, 1984, Vancouver, B.C. Edited by D.M. Baumgartner, R.G. Krebill, J.T. Arnott, and G.F. Weetman. Office of Conferences and Institutes, Cooperative Extension, Washington State University, Pullman. pp. 225-232.

Yoneyama, T., Muraoka, T., Kim, T.H., Dacanay, E.V., Nakanishi, Y. 1997. The natural ^{15}N abundance of sugarcane and neighbouring plants in Brazil, the Philippines and Miyako (Japan). *Plant and Soil*. **189:** 239 – 244.

Appendices

Appendix A Combined Carbon Medium

Solution 1:

a.	Sucrose	5.00 g/L
b.	Mannitol	5.00 g/L
c.	Sodium Lactate (ml, 60%, v/v)	0.50 ml/L
d.	K_2HPO_2	0.80 g/L
e.	KH_2PO_2	0.20 g/L
f.	NaCl	0.10 g/L
g.	$Na_2MoO_2.2H_2O$	25.00 mg/L
h.	$Na_2FeEDTA$	28.00 mg/L
i.	Yeast Extract	100.00 mg/L
j.	Distilled Water	900.00 ml

Solution 2:

a.	$MgSO_2.7H_2O$	0.20 g/L
b.	$CaCl_2$	0.06 g/L
c.	Distilled Water	100.00 ml

Autoclave solutions 1 and 2 separately, cool and mix. Add filter-sterilized biotin (5 µg/L) and Para Amino Benzoic Acid (10 µg/L).

Appendix B Carbon substrate-utilization by *Paenibacillus polymyxa* strain P2b-2R in BioLog GP2 plates (n = 5).

	Test Reaction[1]		
Substrate Group	**Positive**	**Weakly Positive**	**Negative**
Carbohydrates	L-Arabinose	Palatinose	D-Tagatose
	α-D-Lactose	Turanose	L-Rhamnose
	Lactulose	D-Melezitose	Mannan
	D-Trehalose		Gentiobiose
	Maltose		Sedoheptulosan
	D-Cellobiose		N-Acetyl-D-Glucosamine
	Maltotriose		N-Acetyl-D-Mannosamine
	D-Psicose		m-Inositol
	D-Fructose		α-cyclodextrin
	D-Raffinose		Glycogen
	D-Xylose		
	D-Mannose		
	D-Galactose		
	D-Ribose		
	D-Melibiose		
	α-D-Glucose		
	Stachyose		
	Sucrose		
	β-Cyclodextrin		
	Dextrin		
Carbohydrate derivatives	β-Methyl D-Glucoside	D-Mannitol	L-Fucose
	Arbutin	3-Methyl Glucose	α-Methyl D-Glucoside
	Salicin		α-Methyl D-Mannoside
	α-Methyl D-Galactoside		Inulin
	β-Methyl-D-Galactoside		Amygdalin
			D-Arabitol
			Xylitol
			D-Sorbitol
			D-Fructose-6-Phosphate
			α-Glucose-1-Phosphate
			D-Glucose-6-Phosphate
Carboxylic Acids	Pyruvic Acid Methyl Ester	L-Malic Acid	L-Lactic Acid
	Pyruvic Acid	Succinic Acid Mono-methyl Ester	D-Malic Acid
			Acetic Acid
			α-Hydroxybutyric Acid

	Test Reaction[1]		
Substrate Group	**Positive**	**Weakly Positive**	**Negative**
			D-Galacturonic acid
			β-Hydroxybutyric Acid
			Propionic Acid
			γ-Hydroxybutyric Acid
			D-Gluconic Acid
			p-Hydroxyphenylacetic Acid
			α-Ketoglutaric Acid
			Succinic Acid
			α-Ketovaleric Acid
			N-Acetyl L-Glutamic Acid
			D-Lactic Acid Methyl Ester
Amines/Amides			Lactamide
			L-Alaninamide
			Succinamic Acid
			Putrescine
Amino Acids			D-Alanine
			L-Alanine
			L-Alanyl-glycine
			L-Asparagine
			L-Glutamic Acid
			Glycyl- L-Glutamic Acid
			L-Pyroglutamic Acid
			L-Serine
Polysorbates			Tween 40
			Tween 80
Nucleosides	Thymidine	Inosine	Adenosine
		Uridine	2'-Deoxyadenosine
Nucleotides			Adenosine-5'- Monophosphate
			Thymidine-5'-Monophosphate
			Uridine-5'-Monophosphate
Alcohols	2,3-Butanediol		
	Glycerol		
Glycerol derivative			D-L-α-Glycerol Phosphate

[1] OD595 > 0.1 = positive; OD595 < 0.05 = negative; OD595 between 0.05 and 0.1 = weakly positive.