

**Plant Growth Promotion and Nitrogen Fixation by
Paenibacillus polymyxa in Corn and Canola**

by

Akshit Puri

B.Tech., Punjab Agricultural University, 2013

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

MASTER OF SCIENCE

in

The Faculty of Graduate and Postdoctoral Studies

(Soil Science)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

July 2015

© Akshit Puri, 2015

Abstract

Availability of nitrogen is the most yield-limiting mineral factor in crop production. Several *Paenibacillus* bacterial strains that were able to fix nitrogen from atmosphere were isolated from extracts of surface-sterilized lodgepole pine seedling and tree tissues. One strain, *Paenibacillus polymyxa* P2b-2R, was found to derive high amounts of nitrogen from the atmosphere when introduced into gymnosperm species, namely lodgepole pine and western red cedar. I wanted to determine if *Paenibacillus polymyxa* P2b-2R could colonize, fix nitrogen and promote the growth of important agricultural crops such as corn and canola. For this I inoculated corn and canola seeds with *P. polymyxa* P2b-2R and grew them for 30 and 60 days, respectively. Corn seedlings were harvested 10, 20 and 30 days after inoculation and canola seedlings were harvested 20, 40 and 60 days after inoculation for evaluation of rhizospheric and internal tissue (endophytic) colonization (cfu). Seedlings were also evaluated for biological nitrogen fixation (^{15}N dilution) and growth promotion (length and biomass) at these harvest intervals. The entire experiment was repeated to confirm the treatment effects. P2b-2R successfully colonized the corn rhizosphere with 5.52×10^6 cfu/g dry root and the canola rhizosphere with 1.08×10^8 cfu/g dry root. P2b-2R also colonized internal tissues of corn and canola root with population densities of 4.54×10^5 cfu/g fresh weight and 2.36×10^3 cfu/g fresh weight, respectively. Corn seedling growth was promoted significantly by inoculation with P2b-2R with an increase of 35% in length and 31% in biomass, 30 days after inoculation. Similarly, canola seedling length was promoted by 25% and biomass by 30%, 60 days after inoculation. Corn seedlings inoculated with P2b-2R were able to derive about 17% of foliar nitrogen from the atmosphere, 30 days after inoculation. Similarly canola seedlings derived nearly 20% of foliar nitrogen from atmosphere, 60 days after inoculation. These results clearly suggest that *Paenibacillus polymyxa* P2b-2R might have a broad range host capability and is able to fix nitrogen and promote the growth of at least certain important agricultural crops – corn and canola.

Preface

This thesis represents original, unpublished work by the author, Akshit Puri. I was the lead investigator for the projects described in Chapter 2 and 3 where I was responsible for all major areas of research question formation, research work, data collection, data analysis, and thesis composition. Dr. Maja Kržić, Dr. Sue Grayston and Dr. James Kronstad contributed to thesis edits. Dr. Alice Chang from UBC Stable Isotope Facility in the Department of Forest and Conservation Sciences, Faculty of Forestry was involved in analyzing foliar ^{15}N samples of corn and canola seedlings. Dr. Chris Chanway was the supervisory author on this project and was involved throughout the project, from research question formation through to thesis edits.

Table of Contents

Abstract	ii
Preface	iii
Table of Contents	iv
List of Tables	vi
List of Figures	vii
List of Abbreviations	ix
Acknowledgements	x
Dedication.....	xi
Chapter 1 – General Introduction	1
1.1. Plant growth promoting bacteria.....	1
1.1.1. Plant growth promoting rhizobacteria (PGPR).....	2
1.2. Bacterial endophytes.....	3
1.2.1. Diazotrophic endophytes.....	5
1.3. <i>Paenibacillaceae</i> (formerly known as <i>Bacillaceae</i>).....	7
1.3.1. <i>Paenibacillus polymyxa</i>	9
1.3.1.1. <i>Paenibacillus polymyxa</i> P2b-2R: a diazotrophic endophyte..	10
1.4. Corn and canola.....	12
1.5. Research objectives and hypotheses.....	14
1.5.1. Research objectives.....	14
1.5.2. Hypotheses.....	14
Chapter 2 – Evidence of Nitrogen Fixation and Growth Promotion in Corn	
Inoculated with Diazotrophic <i>P. polymyxa</i> P2b-2R.....	15
2.1. Introduction.....	15
2.2. Materials and methods.....	18
2.2.1. Seed and bacteria.....	18
2.2.2. Seed inoculation and plant growth.....	18
2.2.3. Evaluation of endophytic colonization.....	20
2.2.4. Evaluation of rhizospheric colonization.....	20
2.2.5. Nitrogen analysis and seedling biomass.....	21

2.2.6. Statistical analysis.....	22
2.3. Results.....	22
2.3.1. Endophytic and rhizospheric Colonization.....	22
2.3.2. Plant growth promotion.....	23
2.3.3. Nitrogen fixation.....	25
2.4. Discussion.....	28
2.5. Conclusions.....	33
Chapter 3 – Growth Promotion and N ₂ -Fixation in Canola (<i>Brassica napus</i> L.)	
Colonized by an Endophytic Diazotroph <i>Paenibacillus polymyxa</i> P2b-2R.....	35
3.1. Introduction.....	35
3.2. Materials and methods.....	38
3.2.1. Seed and microorganism.....	38
3.2.2. Seedling growth and inoculation.....	38
3.2.3. Quantification of rhizospheric colonization.....	40
3.2.4. Quantification of endophytic colonization.....	40
3.2.5. 15N analysis and seedling growth promotion.....	41
3.2.6. Statistical analysis.....	42
3.3. Results.....	42
3.3.1. Rhizospheric and endophytic colonization by P2b-2R.....	42
3.3.2. Growth promotion and N- fixation.....	43
3.4. Discussion.....	49
3.5. Conclusions.....	54
Chapter 4 – General Summary and Conclusions.....	56
References.....	61
Appendix A – Plant Nutrient Solution.....	74
Appendix B – Steps to Prepare Combined Carbon Medium (CCM).....	75
Appendix C – Recipe to Make Phosphate Buffered Saline.....	76
Appendix D – Additional Figures.....	77

List of Tables

Table 1.1 Studies documenting plant growth promotion by <i>Paenibacillaceae</i> isolates.....	8
Table 2.1 Atom % ¹⁵ N excess in foliage, % foliar N and % N derived from the atmosphere (Ndfa), developed from corn seeds inoculated with <i>P. polymyxa</i> strain P2b-2R and phosphate buffered saline (control) measured over 3 harvests in growth trial 1.....	26
Table 2.2 Atom % ¹⁵ N excess in foliage, % foliar N and % N derived from the atmosphere (Ndfa), developed from corn seeds inoculated with <i>P. polymyxa</i> strain P2b-2R and phosphate buffered saline (control) measured over 3 harvests in growth trial 2.....	27
Table 3.1 Atom % ¹⁵ N excess in foliage, % foliar N and % N derived from the atmosphere (Ndfa), developed from canola seeds inoculated with <i>P. polymyxa</i> P2b-2R and phosphate buffered saline (control) measured 20, 40 & 60 days after inoculation (dai) in growth trial 1.....	47
Table 3.2 Atom % ¹⁵ N excess in foliage, % foliar N and % N derived from the atmosphere (Ndfa), developed from canola seeds inoculated with <i>P. polymyxa</i> P2b-2R and phosphate buffered saline (control) measured 20, 40 & 60 days after inoculation (dai) in growth trial 2.....	48

List of Figures

Figure 2.1 Shoot, root and seedling length of corn seedlings harvested thrice at 10, 20 and 30 days after inoculation (dai) in 1 st growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).....	23
Figure 2.2 Shoot, root and seedling length of corn seedlings harvested thrice at 10, 20 and 30 days after inoculation (dai) in 2 nd growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).....	24
Figure 2.3 Shoot, root and seedling dry weight of corn seedlings harvested 10, 20 and 30 days after inoculation (dai) in 1 st growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).....	24
Figure 2.4 Shoot, root and seedling dry weight of corn seedlings harvested 10, 20 and 30 days after inoculation (dai) in 2 nd growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).....	25
Figure 3.1 Seedling, root and shoot length of canola seedlings harvested 20, 40 and 60 days after inoculation (dai) in 1 st growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).....	44
Figure 3.2 Seedling, root and shoot length of canola seedlings harvested 20, 40 and 60 days after inoculation (dai) in 2 nd growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).....	45
Figure 3.3 Seedling, root and shoot dry weight of canola seedlings harvested 20, 40 and 60 days after inoculation (dai) in 1 st growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).....	45
Figure 3.4 Seedling, root and shoot dry weight of canola seedlings harvested 20, 40 and 60 days after inoculation (dai) in 2 nd growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).....	46
Figure 3.5 Number of canola seedlings having floral buds (reached reproductive stage) of each treatment measured 40 and 60 days after inoculation (dai) in two growth trials (n=14).....	46

Figure D.1 P2b-2R-inoculated (left) and control (right) seedling of corn plant harvested 10 days after inoculation (dai) showing clear difference in length, biomass and plant health.....77

Figure D.2 P2b-2R-inoculated (right) and control (left) seedling of corn plant harvested 20 days after inoculation (dai) showing clear difference in length, biomass and plant health.....77

Figure D.3 P2b-2R-inoculated (left) and control (right) seedling of corn plant harvested 30 days after inoculation (dai) showing clear difference in length, biomass and plant health.....78

Figure D.4 P2b-2R-inoculated (left) and control (right) seedling of canola plant harvested 20 days after inoculation (dai) showing clear difference in length, biomass and plant health.....78

Figure D.5 P2b-2R-inoculated (left) and control (right) seedling of canola plant harvested 40 days after inoculation (dai) showing clear difference in length, biomass and plant health.....79

Figure D.6 P2b-2R-inoculated (right) and control (left) seedling of canola plant harvested 60 days after inoculation (dai) showing clear difference in length, biomass and plant health.....79

List of Abbreviations

ANOVA	Analysis of Variance
BNF	Biological Nitrogen Fixation
C	Carbon
CCM	Combined Carbon Medium
CCMA	Combined Carbon Medium Agar
cfu	colony forming units
dai	days after inoculation
gfp	green fluorescent protein
ISR	Induced Systemic Resistance
N/N ₂	Nitrogen
Ndfa	Nitrogen derived from atmosphere
NF	Nitrogen Fixation
PBS	Phosphate Buffered Saline
PGPB	Plant Growth Promoting Bacteria
PGPR	Plant Growth Promoting Rhizobacteria
Pi	inorganic Phosphorous
rrs	Ribosomal RNA 16S
SAR	Systemic Acquired Resistance
SBPS	Sub-boreal Pine Spruce
TSA	Tryptic Soy Agar
UBC	University of British Columbia

Acknowledgments

I would like to start by acknowledging my research supervisor, Dr. Chris Chanway, for providing financial support and able guidance. I would also like to extend my thanks to my committee members, Dr. Maja Krzic and Dr. Sue Grayston for their constructive feedback on my work. I would like to give special thanks to my former committee member, Dr. Richa Anand, for her suggestions in crop selection and experimental design.

I also owe special thanks to Dr. Alice Chang, for her contribution in analysis of ¹⁵N plant samples and providing great support in the lab work. A huge and sincere thanks to Henry Yang for mentoring me and teaching me microbiological techniques. I am also thankful to Dr. Les Lavkulich and Dr. Sandra Brown for helpful conversations and insights along the way.

I am also grateful to Trent Whiting and SeCan Association for generously providing Canola seeds for this study. I would also like to thank Natasha Thompson at Department of Forest and Conservation Sciences, and Lia Maria Dragan and Shelly Small at Faculty of Land and Food Systems for providing great support in all administrative matters and making me feel comfortable at UBC. I would also like to appreciate the support provided by Dr. Pia Smets in making growth chamber facilities available for this research work. I owe special thanks to my dear friend Kiran Preet Padda for inspiration, ideas, encouragement and endless support.

I would like to thank my parents for believing in my capabilities and supporting me in this undertaking. Last but not the least, I would like to appreciate the contribution provided by my friends and those who kept me in their prayers.

Akshit Puri

To My Father
Late Mr. Darshan K. Puri
(Dec 25, 1956 – Nov 28, 2014)

*From carrying me on your shoulders when I
was a kid to making me capable enough
that I have reached here in my life.*

Hope I have done you proud Papa...

Chapter 1 – General Introduction

1.1. Plant growth promoting bacteria

Soil is replete with microscopic life forms including bacteria, fungi, protozoa, and algae. Of these different microorganisms, bacteria are by far the most common (i.e., 95%) (Glick, 2012). Both the number and the type of bacteria that are found in different soils are influenced by the soil conditions including temperature, moisture, and the presence of salt and other chemicals as well as by the number and types of plants found in those soils (Glick *et al.*, 1999). The interaction between soil bacteria and plants may be (from the perspective of the plant) beneficial, harmful, or neutral (Lynch, 1990). However, the effect that a particular bacterium has on a plant may change as the conditions change. For example, a bacterium that facilitates plant growth by providing either fixed nitrogen or phosphorus compounds that are often present in only limited amounts in many soils, is unlikely to provide any benefit to plants when significant amounts of chemical fertilizer is added to the soil. The bacteria that can promote plant growth, that is plant growth promoting bacteria (PGPB), include those that are free-living, those that form specific symbiotic relationships with plants (e.g., *Rhizobia* spp. and *Frankia* spp.), bacterial endophytes that can colonize some or a portion of a plant's interior tissues, and cyanobacteria (formerly called blue-green algae) (Glick, 2012). The use of microorganisms with the aim of improving nutrient availability for plants is an important practice and necessary for agriculture (Freitas *et al.*, 2007).

1.1.1. Plant growth promoting rhizobacteria (PGPR)

Hiltner (1904) discovered that the rhizosphere, i.e., the layer of soil influenced by the root, is much richer in bacteria than the surrounding bulk soil. These rhizosphere microbes benefit because plant roots secrete metabolites that can be utilized as nutrients. This rhizosphere effect is caused by the fact that a substantial amount of the carbon fixed by the plant, 5–21%, is secreted, mainly as root exudate (Marschner, 1995). The term plant growth promoting rhizobacteria (PGPR) was elaborated by Kloepper & Schroth (1978) and used to designate the rhizobacteria showing significant plant growth promotion, as shown with the substantial increases in fresh matter yield obtained with inoculated radishes. Plant growth promoting rhizobacteria (PGPR) represent a wide variety of soil bacteria which, when grown in association with a host plant, result in stimulation of growth of their host (Vessey, 2003). During the past couple of decades, the use of PGPR for sustainable agriculture has increased tremendously in various parts of the world. Significant increases in growth and yield of agronomically important crops in response to inoculation with PGPR have been repeatedly reported (Figueiredo *et al.*, 2011). There are several PGPR inoculants currently commercialized that seem to promote growth through at least one mechanism: suppression of plant disease (bioprotectants), improved nutrient acquisition (biofertilizers), or phytohormone production (biostimulants). Bacteria in the genera *Bacillus*, *Streptomyces*, *Pseudomonas*, *Burkholderia*, and *Agrobacterium* are the biological control agents predominantly studied and increasingly marketed. They suppress plant disease through at least one

mechanism such as production of antibiotics or induction of systemic resistance (Tenuta, 2003).

The means by which PGPR enhance the nutrient status of host plants can be categorized into five areas: (1) biological N₂ fixation, (2) increasing the availability of nutrients in the rhizosphere, (3) inducing increases in the root surface area, (4) enhancing other beneficial symbioses of the host, and (5) a combination of the previously mentioned modes of action (Vessey, 2003). There is also renewed interest in biological N₂ fixation. The most studied and longest exploited PGPR include the rhizobia, such as *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* for their ability to fix nitrogen in their legume hosts. However, PGPR usually refer to any of number of free-living bacteria, for example *Azospirillum* spp., that are also able to fix nitrogen and provide it to plants whose rhizosphere they have colonized (Bashan & Levanony, 1990).

1.2. Bacterial endophytes

Endophytic bacteria have been defined as 'bacteria that live within living plant tissues without doing substantive harm or gaining benefit other than securing residency'. Endophytic bacteria colonize an ecological niche similar to that of plant pathogens, especially vascular wilt pathogens, which might favor them as potential candidates for biocontrol agents and plant growth promotion agents (Bressan & Borges, 2004). In contrast to free-living, rhizosphere or phyllosphere microorganisms, bacterial endophytes are better protected from

abiotic stresses such as extreme variations in temperature, pH, nutrient, and water availability as well as biotic stresses such as competition. In addition, bacterial endophytes colonize niches that are more conducive to forming mutualistic relationships with plants through NF, for example, as suggested in sugarcane, corn and other crops (Chanway *et al.*, 2014). Many seeds carry a diversity of bacterial endophytes (Coombs & Franco, 2003; Hallmann *et al.*, 1997). By being seedborne, endophytes assure their presence in new plants. Plants that propagate vegetatively (such as potatoes or sugarcane) can transmit their endophytes to the next generation and would not require the infection process (Rosenblueth & Martínez-Romero, 2006). In order to colonize the plant, some bacteria must find their way through cracks formed at the emergence of lateral roots or at the zone of elongation and differentiation of the root. Dong *et al.* (2003) showed that cells of *Klebsiella* sp/ strain Kp342 aggregate at lateral-root junctions of wheat and alfalfa. Similarly, *Gluconacetobacter diazotrophicus* and *Herbaspirillum seropedicae* also colonize lateral-root junctions in high numbers (James & Olivares, 1997).

Endophytic bacteria are able to lessen or prevent the deleterious effects of certain pathogenic organisms. The beneficial effects of bacterial endophytes on their host plant appear to occur through similar mechanisms as described for rhizosphere-associated bacteria. It is believed that certain endophytic bacteria trigger a phenomenon known as induced systemic resistance (ISR), which is phenotypically similar to systemic-acquired resistance (SAR). SAR develops when plants successfully activate their defense mechanisms in response to

primary infection by a pathogen, notably when the latter induces a hypersensitive reaction through which it becomes limited in a local necrotic lesion of brown desiccated tissue. ISR is effective against different types of pathogens but differs from SAR in that the inducing bacterium does not cause visible symptoms on the host plant (van Loon *et al.*, 1998).

1.2.1. *Diazotrophic endophytes*

Diazotrophs are the bacteria and archaea that fix atmospheric nitrogen gas into a biologically usable form such as ammonia. In the 1980's, Brazilian researchers were perplexed by the consistently high yields of field-grown sugarcane, an N-demanding crop, without exogenous N fertilizer application and looked for a microbiological explanation for this apparently anomalous observation. After it was determined that rhizospheric NF did not occur at sufficient rates to facilitate high sugarcane yields, Cavalcante & Döbereiner (1988) looked for microorganisms within sugarcane tissues that might be involved and isolated a diazotrophic bacterium, *Gluconoacetobacter diazotrophicus*, previously known as *Acetobacter diazotrophicus* (Chanway *et al.*, 2014). It is a small, Gram-negative, aerobic rod like bacterium that can grow on very high concentrations of sugar and 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 interior of a sugarcane plant has encouraged researchers to believe that such systems might be in place for other non-legumes, essentially waiting to be discovered (Anand, 2011).

Döbereiner (1992) introduced the term "endophytic diazotrophic bacteria" in the area of BNF to designate all diazotrophs that survive very poorly in soil but colonize the root interior of graminaceous plants, and fix nitrogen in association with them (Baldani *et al.*, 1998). Since the discovery of diazotrophic endophytes in sugarcane (*Saccharum officinarum* L.) (Ruschel *et al.*, 1975), several other agriculturally important crop species including rice (*Oryza sativa*) (Shrestha & Ladha, 1996), maize (*Zea mays* L.) (Montañez *et al.*, 2009), kallar grass (*Leptochloa fusca* L.) (Malik *et al.*, 1997) and canola (*Brassica napus* L.) (DeFreitas & Germida, 1998) have been postulated to receive significant amounts of fixed N₂ in this way. Inoculation of rice with the diazotrophic endophyte *Azoarcus* sp. strain BH72 significantly promoted plant growth (Hurek *et al.*, 1994). In this particular case, growth promotion also occurred with Nif- mutants, indicating that N₂ fixation by *Azoarcus* sp. was apparently not involved in plant growth promotion. Therefore, the authors speculated that the observed plant growth promotion might have been caused by enhanced plant mineral uptake and improved plant water relationship associated with the colonization by *Azoarcus*.

1.3. Paenibacillaceae (formerly known as Bacillaceae)

The genus *Paenibacillus* was created by Ash *et al.* (1993) to accommodate the former 'group 3' of the genus *Bacillus*. It comprises over 30 species of facultative anaerobes and endospore-forming, neutrophilic, low G+C Gram-positive bacilli (Lal & Tabachonni, 2009). The name Paenibacillaceae (like that of its type genus, *Paenibacillus*) is derived from the Latin *paene* and the Greek *Bacillus*: "almost" or "nearly" a *Bacillus*. The name is meant to indicate a similarity between these organisms and *Bacillus sensu stricto* (Zeigler, 2013). The role of Paenibacillaceae in the environment is an ongoing research focus. Spores are commonly found in soils of all types, as is also the case for the Bacillaceae. Certain species are obligate pathogens of honeybees (Genersch, 2010) or scarab beetles (Pettersson *et al.*, 1999). Others may be adapted to colonize the vertebrate intestinal tract, including that of humans (Hoyles *et al.*, 2012). Perhaps the most frequently reported isolation of Paenibacillaceae, however, is from endophytic and other plant-associated communities. Isolates have been obtained from the interiors of surface-sterilized fruits, seeds, stems, and roots of a variety of wild plants and cultivated crops. Evidence strongly suggests that many of these isolates are actually plant-growth promoting bacteria, and that certain characteristics of these species—including antibiotic production and biofilm formation—play an essential role in this activity (Timmusk *et al.*, 2005). Members of the family Paenibacillaceae (Table 1 and references therein), especially *P. polymyxa*, are well studied and known for their ability to promote plant growth by different mechanisms.

Table 1.1 Studies documenting plant growth promotion by Paenibacillaceae isolates.

Bacterium	Plant	Stress	Proposed Mechanism	References
<i>P. polymyxa</i>		<i>Leptosphaeria maculans</i>	Fusaricidin-like cyclic depsipeptides	(Beatty & Jensen, 2002)
<i>Brevibacillus</i>	Sugarcane	<i>Curvularia</i> and <i>Fusarium</i>		(Raton <i>et al.</i> , 2012)
<i>P. polymyxa</i>		<i>Fusarium oxysporum</i>	Direct interaction	(Dijksterhuis <i>et al.</i> , 1999)
<i>P. polymyxa</i>	Peanut	<i>Aspergillus niger</i>	Biofilm formation, niche exclusion	(Haggag & Timmusk, 2008)
<i>P. polymyxa</i>	Wheat	<i>Fusarium graminearum</i>	Enzymatic or antibiotic activity	(He <i>et al.</i> , 2009)
<i>P. polymyxa</i>	Wheatgrass, Clover		IAA production	(Holl <i>et al.</i> , 1988)
<i>P. polymyxa</i>	Wheat		Cytokinin production	(Timmusk <i>et al.</i> , 1999)
<i>P. polymyxa</i>	Tomato	Root-knot nematode		(Khan <i>et al.</i> , 2008)
<i>P. polymyxa</i>	Tomato	<i>Phytophthora infestans</i>		(Lamsal & Kim, 2013)
<i>P. polymyxa</i>	Arabidopsis	<i>Pseudomonas syringae</i>	Volatile compounds	(Lee <i>et al.</i> , 2012)
<i>P. polymyxa</i>		<i>Xanthomonas campestris</i>	1.3 kDa lipopeptide	(Mageshwaran <i>et al.</i> , 2012)
<i>P. polymyxa</i>	Wheat	<i>Erwinia</i> spp.	Polymyxin P	(Niu <i>et al.</i> , 2013)
<i>P. polymyxa</i>	Pepper	<i>Xanthomonas campestris</i>	Volatile compounds	(Phi <i>et al.</i> , 2010)
<i>Paenibacillus</i> sp.	Soybean	<i>Rhizoctonia bataticola</i>		(Senthilkumar <i>et al.</i> , 2009)
<i>P. polymyxa</i> , <i>P. lentimorbus</i>	Tomato	root-knot nematode and <i>Fusarium</i>		(Son <i>et al.</i> , 2009)
<i>P. polymyxa</i>	Arabidopsis	<i>Erwinia</i> , drought	Induction of plant stress genes	(Timmusk <i>et al.</i> , 2005)
<i>P. polymyxa</i>	Arabidopsis	<i>Phytophthora palmivora</i> , <i>Pythium aphanidermatum</i>	Biofilm formation, niche exclusion	(Timmusk <i>et al.</i> , 2009)
<i>Brevibacillus</i> sp.	White clover	Zn-toxicity	Zn accumulation; IAA stimulation	(Vivas <i>et al.</i> , 2006)
<i>Paenibacillus</i> sp.	Locust	<i>Penicillium expansum</i>	Heat stable 4.5 kDa protein	(Zhou <i>et al.</i> , 2008)
<i>P. polymyxa</i>	Sesame	Nine fungal pathogens		(Ryu <i>et al.</i> , 2006)

1.3.1. *Paenibacillus polymyxa*

Paenibacillus polymyxa (formerly *Bacillus polymyxa*), a non-pathogenic and endospore-forming *Bacillus*, is one of the most industrially significant facultative anaerobic bacterium. It occurs naturally in soil, the rhizosphere and roots of crop plants and in marine sediments. During the last two decades, there has been a growing interest in the ecological and biotechnological importance of this genus, particularly due to their limited genomic information. von der Weid *et al.* (2000) investigated the influence of plant development both at the phenotypic and genotypic levels by *P. polymyxa* populations naturally occurring in the maize rhizosphere. Their investigation(s) suggested that a more homogeneous *P. polymyxa* population was present during the middle stages of maize growth (30 and 60 days after sowing) than in the first stage (10 days) and after 90 days of maize growth. The effect of plant cultivar on the degree of genetic diversity of 67 *P. polymyxa* isolates recovered from the root system of maize planted in a tropical Brazilian soil was evaluated by da Mota *et al.* (2002). Results revealed a high level of genetic polymorphism among isolates recovered from different cultivars, yielding a total of 54 distinct groups. Guemori-Athmani *et al.* (2000) demonstrated the nitrogen fixing ability by *P. polymyxa*. These authors measured nitrogenase activity of some representative isolates of *P. polymyxa* recovered from Algerian soil by acetylene reduction assay (ARA). Results showed that only 14 of the 23 strains tested were able to reduce acetylene. In another study, *P. polymyxa* strain RC05 and RC14 isolated from rhizosphere of wild berries, wheat

and barley exhibited nitrogenase activity, phosphate solubilisation capability and promoted plant growth when inoculated into wheat and spinach plants (Çakmakçı *et al.*, 2007). Antagonistic activity of *P. polymyxa* was demonstrated against the nematode *Meloidogyne javanica*. The inoculation of *P. polymyxa* alone or together with *Rhizobium* increased lentil plant growth both in *M. javanica*-inoculated and non-inoculated plants (Siddiqui *et al.*, 2007).

1.3.1.1 *Paenibacillus polymyxa* P2b-2R: a diazotrophic endophyte

Lodgepole pine (*Pinus contorta* var. *latifolia* (Dougl.) Engelm.) and western red cedar (*Thuja plicata* Donn.) are common, commercially important gymnosperms in western North America with a wide ecological range from Alaska to California. Lodgepole pine grows successfully on nutrient poor sites and often scorched sites that are severely limited in nitrogen (Weetman *et al.*, 1988). As a result, N inputs of lodgepole pine forests are of great interest both from ecological and management perspectives. Similarly, western red cedar forests are known to have limited nitrogen availability due to low rates of nitrogen mineralization in cedar forests (Prescott & Preston, 1994; Prescott *et al.*, 1996). Based on earlier work with lodgepole pine suggesting that rhizospheric BNF contributed only small amounts of N to seedlings (Chanway & Holl, 1991), Bal *et al.* (2012) searched for endophytic diazotrophs in lodgepole pine and western red cedar as a possible explanation for the ability of this species to grow on N-deficient substrates.

Bal *et al.* (2012) isolated several bacterial strains from plant tissues collected from three sampling sites. But only four isolates were able to consistently reduce acetylene and grow on N-free Combined Carbon Medium Agar (CCMA). These diazotrophic strains were refined using 16S rRNA gene analysis. Strains were reintroduced to lodgepole pine and western red cedar to assess their colonization and nitrogen fixing ability in two different growth trials of 27 weeks and 35 weeks respectively. One of the strains, *Paenibacillus polymyxa* strain P2b-2R (GU132543), showed consistent N-fixation with each tree species in each growth trial (Bal & Chanway, 2012a,b). In a long-term experiment (13-month growth trial), P2b-2R showed even better results as the inoculated lodgepole pine and western red cedar seedlings derived 79% and 36% of their foliar N from BNF, respectively and doubled their biomass. In these trials, *P. polymyxa* P2b-2R successfully colonized internal tissues of root, stem and needle of both lodgepole pine and western red cedar with population sizes ranging from $10^{-1} - 10^{-3}$ cfu/g of tissue in needles and $10^{-4} - 10^{-7}$ cfu/g of tissues in stems and roots (Anand *et al.*, 2013; Anand & Chanway, 2013a). Anand & Chanway (2013c) characterized *nif* gene structure of *Paenibacillus polymyxa* strain P2b-2R to determine the arrangement and sequences of genes in the *nif* operon. This strain was found to possess a single copy of the *nifH* gene with *nifB* located directly upstream of *nifH* and *nifD*. Phylogenetic analyses of the full *nifH*, partial *nifB* and *nifD*, and 16s rDNA (*rrs*) gene sequences indicated that P2b-2R was part of a monophyletic cluster with other members of the genus *Paenibacillus*. These findings confirm the ability of *P. polymyxa* P2b-2R to

colonize and fix nitrogen in at least certain gymnosperms, lodgepole pine and western red cedar. But can this bacterial strain fix nitrogen if introduced into agricultural crops? The answer is not known and determining it formed the main objective of my thesis project.

1.4. Corn and canola

In Canada, canola production is very important to the agricultural industry. According to Statistics Canada (n.d.), the total seeded area under canola for the year 2014 was 8,225,074 hectares in Canada, which was the second highest just after wheat. The total production of canola in Canada for the year 2013 was also the second highest with 15,555,271 metric tonnes. A study released in 2013 shows that Canadian-grown canola contributes \$19.3 billion to the Canadian economy each year, and contributes to more than 249,000 Canadian jobs and \$12.5 billion in wages (Canola Council of Canada, n.d. a). Canola is a very popular crop in British Columbia as well. The total seeded area of canola in 2014 was 42,492 ha which was the highest among all crops grown in BC (Statistics Canada, n.d.).

When it comes to cereal crop production around the world, corn is the king with a world production of 990,690,000 metric tonnes in the year 2013-14. Corn is Canada's third most valuable crop with a booming annual production of 14,190,000 metric tonnes in year 2013-14. Canada annually produces approximately 1.2% of the corn produced globally and is ranked 11th in the world (Hamel & Dorff, 2014; United States Department of Agriculture, 2015).

In addition, corn and canola crops differ physiologically as well as botanically. The main physiological difference between corn and canola is that corn belongs to the C4 category of plant species whereas canola belongs to the C3 category. The most important difference between C3 and C4 species is that C3 species continue to increase photosynthesis with rising CO₂ level whereas in C4 species, photosynthesis does not. So, C3 plants respond readily to higher CO₂ levels, and C4 plants make only limited responses. The main botanical difference between corn and canola is that corn is a monocot whereas canola is a dicot. The main reason for choosing corn and canola for this study was that these crops are agronomically important and very different physiologically and botanically.

Several different studies have observed the ability of endophytic diazotrophs to colonize corn and canola plants under field, greenhouse, and aseptic conditions. Studies have shown that a bacterial endophyte found in sugarcane, *G. diazotrophicus* is capable of inhabiting several corn genotypes when different means of inoculation were used: seed coating, applications to the base of stems, and root dipping (Riggs *et al.*, 2001; Cocking *et al.*, 2006; Tian *et al.*, 2009). A study found that a *Paenibacillus polymyxa* strain (PKB1) produces fusaricidin-type antifungal antibiotics which are very active against *Leptosphaeria maculans* (the causative agent of blackleg disease of canola) thus enhancing canola plant growth (Beatty & Jensen, 2002). Lifshitz *et al.* (1987) and Kloepper *et al.* (1988) also found that many PGPR like *Pseudomonas putida* strains are capable of growth promotion of canola.

1.5. Research objectives and hypotheses

1.5.1. Research objectives

The main research objectives of this project were to determine if *Paenibacillus polymyxa* strain P2b-2R is capable of: (i) colonizing rhizosphere and internal tissues of *Zea mays* (corn) and *Brassica napus* L. (canola), (ii) deriving nitrogen from atmosphere in association with these crop plants and, (iii) increasing biomass production and growth rate of *Zea mays* and *Brassica napus* L.

1.5.2. Hypotheses

I developed three main hypotheses:

H1: *Paenibacillus polymyxa* P2b-2R is capable of colonizing rhizosphere and internal tissues (endophytically) of the important agricultural crops, corn and canola.

H2: *P. polymyxa* P2b-2R is capable of supplying a biologically significant amount of fixed nitrogen to colonized plants grown under nitrogen poor conditions.

H3: P2b-2R inoculation enhances biomass production (dry weight) and growth rate of corn and canola seedlings.

Chapter 2 – Evidence of Nitrogen Fixation & Growth Promotion in Corn Inoculated with Diazotrophic *P. polymyxa* P2b-2R¹

2.1. Introduction

For plants, nitrogen is necessary as a primary constituent of nucleotides, proteins, and chlorophyll (Robertson & Vitousek, 2009). Many agricultural scientists see the availability of fixed nitrogen (nitrate or ammonium converted from dinitrogen) as the most yield-limiting factor related to the crop production (Muthukumarasamy *et al.*, 2002). An inexpensive and natural way of providing plants with fixed nitrogen is by biological nitrogen fixation (BNF). Approximately 80% of all BNF is accomplished through the symbiotic interaction between legumes and α -proteobacteria in the order Rhizobiales, family Rhizobiaceae (Garg & Geetanjali, 2007). However, non-specific nitrogen-fixing bacteria also exist and their discovery has opened up the possibility of symbiotic nitrogen fixation in a wide array of agricultural crops like corn.

Endophytic bacteria have been defined as 'bacteria that live within living plant tissues without doing substantive harm or gaining benefit other than securing residency' (Bressan & Borges, 2004). In contrast to free-living, rhizosphere or phyllosphere-dwelling microorganisms, bacterial endophytes are better protected from abiotic stresses such as extreme variations in temperature,

¹ A version of this chapter has been submitted to Soil Biology and Biochemistry Journal for publication under the title 'Can a diazotrophic endophyte originally isolated from lodgepole pine colonize an agricultural crop (corn) and promote its growth?' Authors: Akshit Puri, Kiran Preet Padda and Chris P Chanway and it is currently under review.

pH, nutrient, and water availability as well as biotic stresses such as competition (Chanway *et al.*, 2014). Diazotrophs comprise certain bacteria and archaea that fix atmospheric nitrogen into a biologically usable form such as ammonia. The term "endophytic diazotrophic bacteria" was introduced in the area of BNF by Döbereiner (1992) to designate all diazotrophs able to colonize primarily the root interior of graminaceous plants, survive very poorly in soil and fix nitrogen in association with these plants. Since the discovery of diazotrophic endophytes in sugarcane (*Saccharum officinarum* L.) (Ruschel *et al.*, 1975), several other agriculturally important crop species including rice (*Oryza sativa*) (Shrestha & Ladha, 1996) and maize (*Zea mays* L.) (Montañez *et al.*, 2009) have been postulated to receive significant amounts of fixed N₂ in this way.

Paenibacillus polymyxa is a Gram-positive, rod-shaped, endospore-forming bacterium that is non-pathogenic and found in environments such as plant roots in soil and marine sediments (Timmusk *et al.*, 2005; Ravi *et al.*, 2007; He *et al.*, 2007). The wide range of potential plant growth promoting characteristics this bacterium possess includes the ability to fix nitrogen and produce hormones that promote plant growth as well as hydrolytic enzymes and antibiotics that protect against harmful microorganisms (Lal & Tabacchioni, 2009). Górska *et al.* (2015) isolated two nitrogen-fixing microorganisms from agricultural soil and identified them as *Paenibacillus polymyxa* [laboratory names EG2 and EG14] based on 16S rRNA sequence. The genome of these bacterial strains was found to carry *nif* genes coding the individual components of the

nitrogenase complex. Their nitrogen fixing ability was confirmed by studying nitrogenase activity in cultures of the studied bacteria in N-free medium.

Bal *et al.* (2012) isolated several *Paenibacillus* strains that possessed significant acetylene reduction activity from extracts of surface-sterilized lodgepole pine seedling and tree tissues (Bal & Chanway, 2012a,b). When one of the strains, *Paenibacillus polymyxa* strain P2b-2R, was reintroduced to lodgepole pine and grown in a very N-limited soil for 35 weeks, seedlings were found to derive more than half (66%) of their foliar N from biological nitrogen fixation (BNF) (Bal & Chanway, 2012a). In a long-term experiment (13-month growth trial), P2b-2R showed even better results as the inoculated lodgepole pine seedlings derived 79% of their foliar N from BNF and doubled their biomass (Anand *et al.*, 2013). These results provided confirmation of the ability of *P. polymyxa* strain P2b-2R to colonize gymnosperms (lodgepole pine and western red cedar) and fix nitrogen when associated with them. However, we were interested in determining if this bacterial strain could also fix nitrogen and promote plant growth if introduced into an agricultural crop. I chose corn for our study, as it is an important agricultural crop grown on a large scale worldwide.

In this study, I test the hypothesis that the endophytic diazotroph *P. polymyxa* P2b-2R isolated from lodgepole pine is capable of colonizing rhizosphere and internal tissues of an important agricultural crop, corn (*Zea Mays*), and stimulating biomass production through BNF.

2.2. Materials and methods

2.2.1. Seed and bacteria

Corn seeds (*var.* Golden Bantam) were obtained from West Coast Seeds (Delta, British Columbia, Canada). *Paenibacillus polymyxa* strain P2b was isolated from surface-sterilized stem tissues of a lodgepole pine seedling 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). *Paenibacillus polymyxa* P2b-2R (GU132543) was a spontaneous antibiotic-resistant derivative of strain P2b, capable of growing on combined carbon medium (CCM) (Rennie, 1981) agar amended with 200mg/L rifamycin (Bal & Chanway, 2012a). Strain P2b-2R was stored at -80°C in CCM amended with 20% (v/v) glycerol.

2.2.2. Seed inoculation and plant growth

Seedling growth assays were performed in small pots (12cmx8cmx4cm) filled to 67% capacity with sterile Sand-Turface (montmorillonite clay, Applied Industrial Materials Corporation, Deerfield, IL, USA) mixture (69% w/w silica sand; 29% w/w Turface; 2% w/w CaCO₃). Each pot was fertilized with 50mL of a nutrient solution (Appendix A) (Chanway *et al.*, 1988), which was modified by replacing KNO₃ and Ca(NO₃)-4H₂O with Ca(¹⁵NO₃)₂ (5% ¹⁵N label) (0.0576g/L) and sequestrene 330 Fe (CIBA-GEIGY, Mississauga, ON, Canada) with Na₂-FeEDTA (0.02g/L).

Corn seeds were surface-sterilized by immersion in 30% hydrogen peroxide for 90 seconds, followed by three 30-second rinses in sterile distilled water. To confirm the effectiveness of surface sterilization, seeds were imprinted on tryptic soy agar (TSA) and checked for microbial contamination 24h later. Two surface sterilized seeds were aseptically sown in each pot.

Two seed inoculation treatments – live P2b-2R and sterile PBS (Control) were evaluated using corn seedlings, replicated 60 times. Bacterial inoculum was prepared by thawing a frozen culture of strain P2b-2R and streaking a loopful onto combined carbon medium (CCM) agar amended with 200mg/L rifamycin, and incubating at 30°C for 2 days. A 1 L flask containing 500mL of CCM broth amended with rifamycin was then inoculated with a loopful of bacterial growth from the agar plate, secured on a rotary shaker and agitated (150 rpm) at room temperature for 2 days. Bacterial cells were harvested by centrifugation (3000x g, 30 min), washed twice in sterile phosphate buffered saline (PBS) (pH 7.4) and resuspended in the same buffer to a density of 10^6 cfu/mL. Immediately after sowing the seeds, 5mL of the P2b-2R – PBS suspension was pipetted directly into each pot designated for live P2b-2R. This process was repeated using 5mL of sterile PBS without bacteria for uninoculated (control) pots. Pots were placed in a growth chamber (Convion CMP3244, Convion Products Company, Winnipeg, MB, Canada) under an 18-h photoperiod with an intensity of at least $300\mu\text{mol s}^{-1} \text{m}^{-2}$ and a 25/18°C day/night temperature cycle. Seedlings were thinned to the largest single germinant per pot 2 days after sowing and were watered as required with sterile distilled water. Seedlings received modified

nutrient solution without $\text{Ca}^{15}\text{NO}_3)_2$, 20 days after inoculation. The entire experiment was repeated to confirm treatment effects.

2.2.3. Evaluation of endophytic colonization

To evaluate endophytic colonization by P2b-2R in corn, 3 randomly chosen seedlings of each treatment were harvested destructively 10, 20 and 30 days after inoculation. Seedlings were rinsed in a 2L flask containing 1L sterile distilled water for removal of loosely adhering growth media. In the first growth trial, seedlings were surface-sterilized in 1.3% (w/v) sodium hypochlorite for 5 minutes, rinsed three times with sterile distilled water and imprinted on TSA plates for 24-h to check for surface contamination. Samples of stem, root and leaf tissues were triturated separately in 1mL of sterile PBS using a mortar and pestle. Triturated tissue suspensions were serially diluted on CCM plates supplemented with 100mg/L cycloheximide and 200mg/L rifamycin. Plates were incubated at room temperature for 7 days and colonies were counted after incubation. Data from seedlings that showed contamination after surface sterilization were excluded from further analysis.

2.2.4. Evaluation of rhizospheric colonization

For evaluation of rhizosphere colonization by P2b-2R, 3 randomly chosen seedlings of each treatment were harvested destructively 10, 20 and 30 days after inoculation. Seedlings were removed from pots and loosely adhering soil particles were removed from roots with gentle shaking. Roots were then

separated from shoots, placed in sterile Falcon tubes (50mL; BD Biosciences, CA, USA) filled with 10mL of sterile PBS and shaken on a vortex mixer at 1000 rpm for 1 minute. Serial dilutions were performed, and aliquots of 100µL were plated on CCM plates amended with rifamycin (200mg/L) and cycloheximide (100mg/L). Plates were incubated at room temperature for 7 days and colonies were counted after incubation. Roots were oven-dried at 65°C for 2 days before weighing. Rhizospheric bacterial populations were then calculated as cfu per gram dry root.

2.2.5. Nitrogen analysis and seedling biomass

Fourteen corn seedlings of each treatment were harvested destructively 10, 20 and 30 days after inoculation for evaluation of seedling biomass as well as foliar N and ¹⁵N content. Roots were separated from shoots, and shoot and root lengths were measured. Shoots and roots of each seedling were then oven dried at 65 °C for 2 days before being weighed. For foliar N analysis, ground and oven dried foliage of each seedling from each treatment was mixed thoroughly and a 1mg sample was sent to the UBC Stable Isotope Facility for determination of foliar N content and %¹⁵N excess 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):

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

2.2.6. Statistical analysis

A completely randomized experimental design with 60 replicates per treatment was used to assess the treatment effects on growth of corn seedlings. The statistical package, SAS v9.4 (Copyright © 2014, SAS Institute Inc., Cary, NC, USA.), was used to perform statistical analyses. A linear generalized model was used to analyze the correlations between the dependent variables viz. plant growth parameters, number of CFUs, and atom percent excess of ^{15}N and independent variable, N. The confidence level, α , was set to 0.05 to determine the significance of the model and treatment effects.

2.3. Results

2.3.1. Endophytic and rhizospheric colonization

Paenibacillus polymyxa strain P2b-2R colonized the corn rhizosphere in both growth trials with 2.18×10^6 cfu/g dry root and 8.85×10^6 cfu/g dry root, respectively. P2b-2R colonies were also observed on both CCM and TSA stem and leaf imprint plates after these tissues were surface sterilized. P2b-2R colonized corn root endophytically with 4.54×10^5 cfu/g fresh weight in the 2nd growth trial but there was no evidence of endophytic colonization in stem and leaf tissues by P2b-2R strain in either growth trial. No evidence of rhizospheric or endophytic colonization was found in control plants.

2.3.2. Plant growth promotion

Seedling growth was promoted significantly by inoculation with P2b-2R. P2b-2R inoculation increased shoot length by 19-40% in the 1st growth trial (Figure 2.1) and 21-45% in the 2nd growth trial (Figure 2.2). Seedling length was promoted by 6-25% in the 1st trial (Figure 2.1) and 10-35% in the 2nd trial due to P2b-2R inoculation (Figure 2.2). P2b-2R inoculation significantly increased shoot dry weight (24-33% in the 1st trial (Figure 2.3) and 27-38% in the 2nd trial (Figure 2.4)) and seedling biomass (18-26% in the 1st trial (Figure 2.3) and 20-30% in the 2nd trial (Figure 2.4)).

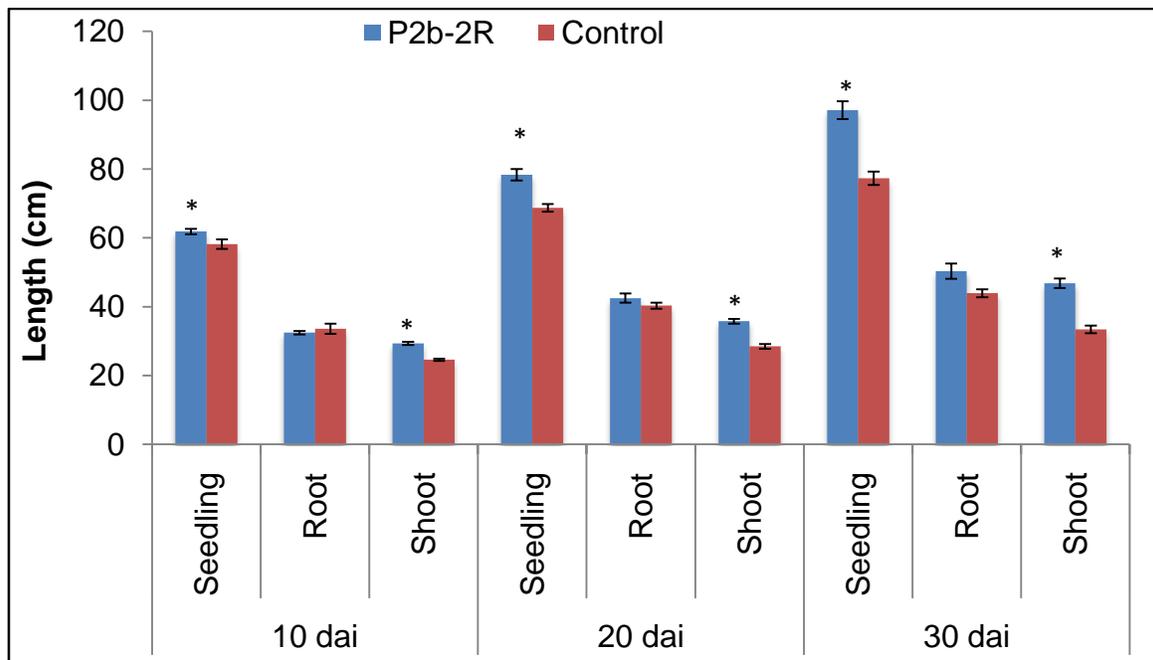


Figure 2.1 Shoot, root and seedling length of corn seedlings harvested thrice at 10, 20 and 30 days after inoculation (dai) in 1st growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).

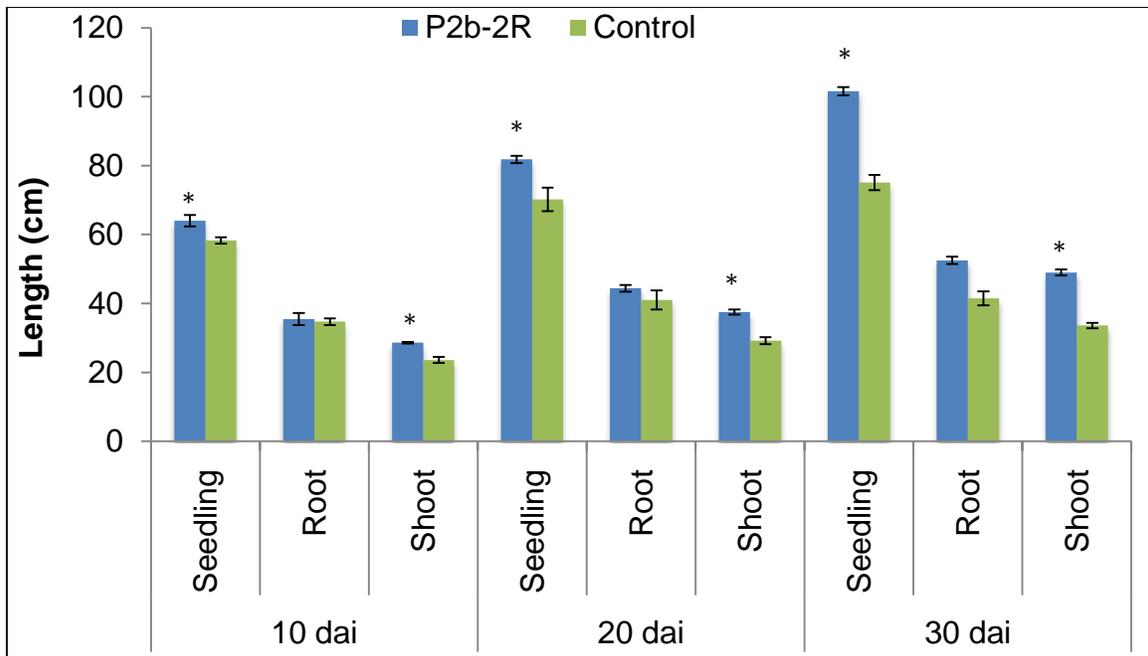


Figure 2.2 Shoot, root and seedling length of corn seedlings harvested thrice at 10, 20 and 30 days after inoculation (dai) in 2nd growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).

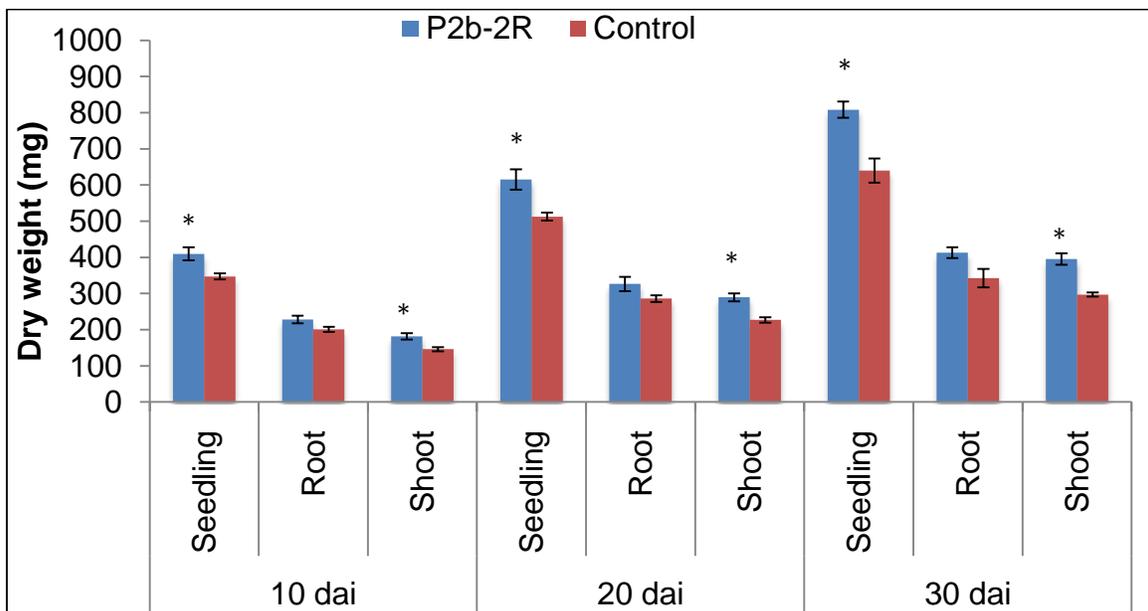


Figure 2.3 Shoot, root and seedling dry weight of corn seedlings harvested 10, 20 and 30 days after inoculation (dai) in 1st growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).

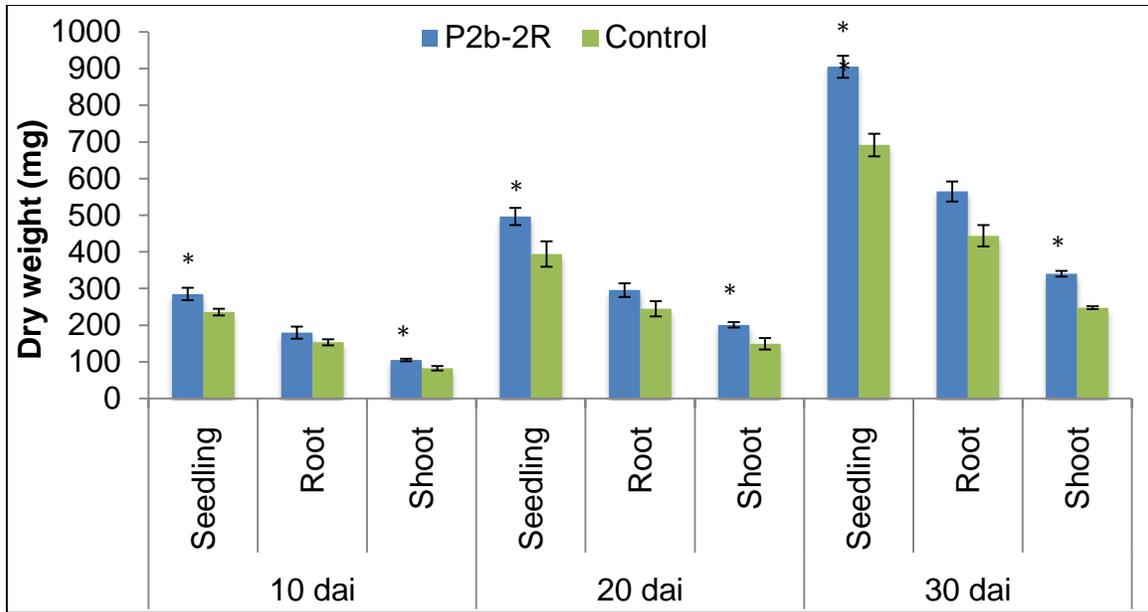


Figure 2.4 Shoot, root and seedling dry weight of corn seedlings harvested 10, 20 and 30 days after inoculation (dai) in 2nd growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).

2.3.3. Nitrogen fixation

In the first growth trial, % foliar N of seedlings inoculated with P2b-2R was 5, 8, and 10% higher than the control seedlings in the 1st, 2nd and 3rd harvests, respectively (Table 2.1). Similarly in the second growth trial, % foliar N of inoculated seedlings was 9, 14, and 19% higher than the control in the 1st, 2nd and 3rd harvest, respectively (Table 2.2). Based on % foliar ¹⁵N atom excess data, it was found that inoculated seedlings derived 5% of the foliar N from atmosphere (Ndfa) in the 1st harvest, which increased to 8% in the 2nd harvest and 15% in the 3rd harvest in first growth trial (Table 2.1). A similar trend was observed in the second growth trial, as inoculated seedlings derived 6% of the foliar N from atmosphere in the 1st harvest, which increased to 10% in the 2nd harvest and 19% in the 3rd harvest (Table 2.2).

Table 2.1 Atom % ¹⁵N excess in foliage, % foliar N and % N derived from the atmosphere (Ndfa), developed from corn seeds inoculated with *P. polymyxa* strain P2b-2R and phosphate buffered saline (control) measured over 3 harvests in growth trial 1

	Harvest 1			Harvest 2			Harvest 3		
	Atom % ¹⁵ N			Atom % ¹⁵ N			Atom % ¹⁵ N		
	Excess in Foliage	%Foliar N	%Ndfa	Excess in Foliage	%Foliar N	%Ndfa	Excess in Foliage	%Foliar N	%Ndfa
P2b-2R	0.41*±0.006 ^a	1.60*±0.05	5.18	0.39*±0.003	1.78*±0.02	8.25	0.38*±0.006	1.97*±0.01	15.35
Control	0.43±0.005	1.52±0.06	-	0.42±0.009	1.65±0.05	-	0.45±0.007	1.80±0.05	-

^a Mean ± standard error; n=14 for atom %¹⁵N excess & %foliar N

*P<0.05 (significantly different from control)

Table 2.2 Atom % ¹⁵N excess in foliage, % foliar N and % N derived from the atmosphere (Ndfa), developed from corn seeds inoculated with *P. polymyxa* strain P2b-2R and phosphate buffered saline (control) measured over 3 harvests in growth trial 2

	Harvest 1			Harvest 2			Harvest 3		
	Atom % ¹⁵ N			Atom % ¹⁵ N			Atom % ¹⁵ N		
	Excess in Foliage	%Foliar N	%Ndfa	Excess in Foliage	%Foliar N	%Ndfa	Excess in Foliage	%Foliar N	%Ndfa
P2b-2R	0.40*±0.005 ^a	1.75*±0.05	6.65	0.39*±0.014	1.84*±0.04	10.78	0.33*±0.005	2.06*±0.02	19.62
Control	0.43±0.006	1.66±0.05	-	0.43±0.006	1.70±0.05	-	0.41±0.006	1.87±0.01	-

^a Mean ± standard error; n=14 for atom %¹⁵N excess & %foliar N

*P<0.05 (significantly different from control)

2.4. Discussion

The objective of this study was to determine if an endophytic diazotroph *P. polymyxa* P2b-2R isolated from internal tissues of lodgepole pine is capable of colonizing rhizosphere and internal tissues of corn. We were also interested to see if it is capable of promoting growth and fixing nitrogen in association with this crop plant. P2b-2R colonized the corn rhizosphere with population sizes similar to those observed previously in gymnosperms (Holl & Chanway, 1992; Bal & Chanway, 2012a,b). This shows that P2b-2R possess the necessary adaptations for rhizosphere colonization of this agricultural crop despite being isolated from internal tissues of a tree species. Internal tissue (endophytic) colonization of corn roots was observed in the second growth trial with population densities similar to those observed in other published reports of this bacterial strain (Anand *et al.*, 2013; Anand & Chanway, 2013a). This strain was also present on CCM and TSA, stem and leaf imprint plates and thus we feel it would be premature to rule out endophytic colonization of above ground parts of corn by this strain. The native environment of *P. polymyxa* P2b-2R is the plant interior (Bal *et al.*, 2012) and it was previously reported to colonize stem and leaf tissues of the plant with higher population densities than the root tissues (Anand *et al.*, 2013; Anand & Chanway, 2013a). Thus, we expected this strain to colonize extracts of surface sterilized stem and leaf tissues of corn with high population densities, but this was not the case. The comparatively harsh protocol that was required to ensure complete surface sterilization of seedling samples may have disinfected internal

cortical tissues and precluded the detection of this bacterial strain inside stem and leaf tissues. Future studies utilizing a direct detection technique such as *gfp*-labeled bacteria (Gage *et al.*, 1996) with confocal scanning laser microscopy will facilitate more sensitive studies of endophytic colonization by this strain (Anand & Chanway, 2013b).

Paenibacillus polymyxa P2b-2R showed consistent and significant effect on corn foliar ¹⁵N abundance. Based on atom % ¹⁵N excess in foliage data, seedlings treated with live P2b-2R derived 15% of foliar N at the end of the 1st growth trial (Table 2.1) and 19% at the end of the 2nd growth trial (Table 2.2) from a source other than soil N, likely the atmosphere. The proportion of fixed N assimilated in inoculated corn seedlings was lower than that observed in sugar cane (*Saccharum officinarum* L.) (Lima *et al.*, 1987; Boddey *et al.*, 1991), kallar grass (*Leptochloa fusca*) (Malik *et al.*, 1987), and rice (*Oryza sativa* L.) (Malik *et al.*, 1997), but were consistent with estimates from wheat (*Triticum aestivum*) (Rennie & Larson, 1979; Rennie *et al.*, 1983) and western red cedar (*Thuja plicata*) (Bal & Chanway, 2012b). The amount of nitrogen fixed in P2b-2R inoculated plants was significant considering the fact that this was a short growth trial. The percent nitrogen derived from atmosphere increased at each harvest in both the growth trials (Table 2.1 and 2.2) suggesting that BNF is an important component of N nutrition of corn growing in a nitrogen limited soil medium. But the source of this BNF is still unknown and it could be speculated that it is induced by either rhizospheric P2b-2R or endophytic P2b-2R found in root tissues. Significant foliar ¹⁵N dilution with no concomitant increase in foliar N

concentration during nitrogen fixation has been observed previously and is thought to be due to a compensatory mechanism where less N is taken up from soil when fixed N is assimilated by plants (Rennie *et al.*, 1983; Rennie & Thomas, 1987; Kucey, 1988). Uriquaga *et al.* (1992) previously observed an increasing reliance on BNF with seedling age and concomitant decreasing dependence on soil N in sugarcane, as %Ndfa rose from 6 to 55 % during the interval of 100-250 days after emergence.

Werner *et al.* (2014) recently reported that *Zea mays* (Corn) does not belong to a phylogenetic clade of angiosperms with an N-fixing precursor. They also reported that species that are in the precursor state are roughly 100 times more likely to evolve a functioning N₂-fixation state than a non-precursor is to evolve a precursor. But interestingly, *Paenibacillus polymyxa* and other diazotrophs have been reported to form a symbiotic relation with corn in many reports. Crops like corn and sugarcane have high sucrose content and sucrose is postulated as an important factor to the colonization success of many diazotrophic species (Muthukumarasamy *et al.*, 2002). High sucrose concentrations (10%) are the best source of carbon for the bacterium's growth (Cavalcante & Dobereiner, 1988). Additionally, when looking specifically at the process of nitrogen fixation, sucrose levels play an invaluable role, by providing the bacterium with a sufficient source of energy for the process of nitrogen fixation (Galar & Boiardi, 1995). It was recently reported that *P. polymyxa* P2b-2R could readily oxidize about 39 different carbon substrates, out of which 'carbohydrates' group represent the largest source of utilizable carbon. These

carbon sources are found naturally in plants as freely available forms such as sucrose, raffinose and maltose (Yang, 2015). Thus, it is possible that *P. polymyxa* strain P2b-2R interacts mutualistically with corn by supplying biologically significant amounts of fixed N to its plant host in return for nutrient rich colonization sites on or in the plant.

P2b-2R inoculation of corn seedlings increased shoot length by 40% in the 1st growth trial and 45% in the 2nd growth trial, 30 days after inoculation. Seedling length was also increased significantly (25% in the 1st trial and 35% in the 2nd trial), 30 days after inoculation. This indicates that P2b-2R inoculation enhances the vertical growth of corn seedlings especially above ground, which is consistent with a previous report of this bacterial strain (Anand *et al.*, 2013). P2b-2R also enhanced biomass production in corn seedlings, particularly the above ground biomass. Above ground biomass of P2b-2R inoculated seedlings was 33-38% greater than the control seedlings. The significant difference in shoot length and shoot dry weight between P2b-2R inoculated and control seedlings suggests that P2b-2R promotes plant growth both vertically (length) as well as horizontally (biomass), unlike some previous reports about this bacterial strain using lodgepole pine and western red cedar seedlings (Bal & Chanway, 2012a,b). While *P. polymyxa* possess several characteristics that can result in plant growth promotion (Chanway, 2008), foliar N data (Table 2.1 and 2.2) suggests that the enhanced growth promotion was caused primarily by an increase in the amount of N derived from atmosphere. The concentration of foliar N increased

consistently during both growth trials (Table 2.1 and 2.2) leading to a consistent increase in biomass and length of seedlings.

There are many ways in which *Paenibacillus polymyxa* P2b-2R might have promoted plant growth in corn. One of the most commonly reported plant growth promoting mechanism of *Paenibacillus* is the production of substances like auxins, cytokinins and/or gibberellins by this bacterium. *Bacillus amyloliquefaciens* FZB42, a model strain for scientific research was reported to be involved in auxin biosynthesis, leading to the re-modeling of Pi transporter expression as well as elevating organic C exudation in wheat (*Triticum aestivum*). This shows the importance of rhizobacterial-derived auxin following colonization of root surfaces by a PGPR (Talboys *et al.*, 2014). Another way by which PGPR bacteria can stimulate plant growth is by suppressing endogenous ethylene production in the host plant's rhizosphere and root system. Various *Paenibacillus* species are also responsible for increased nutrient availability in the rhizosphere. For example, phosphorous is often abundant in soils, but in forms mostly unavailable to plants, as part of insoluble or poorly soluble inorganic or organic phosphate pools. Studies have shown that *Paenibacillus* sp. is capable of phosphate mineralization by producing enzymes, which cleave phosphate groups (Chanway, 2008). *Paenibacillus polymyxa* is also capable of synthesizing many antibacterial and antifungal secondary metabolites (Borriss, 2015). A study was conducted with *Paenibacillus polymyxa* HT16 bacterial strain to evaluate its ability to reduce white rot disease caused by the fungus *Coniella diplodiella* on table grapes. HT16 strain showed significant inhibitory effects on *C.*

diplodiella in vitro, reducing white rot disease in 'Kyoho' and 'Thompson Seedless' table grape varieties by more than 40% after inoculation (Han, 2015). In another study, *P. polymyxa* strain Sb3-1 (syn. *Paenibacillus kribbensis* Sb3-1) isolated from agricultural, organically managed soil in Egypt showed antifungal and antibacterial characteristics *in vitro* (Köbrel *et al.*, 2013). The genome of this strain revealed several genes that potentially contribute to its antagonistic and plant growth promotion activity (Rybakova *et al.*, 2015). Lei *et al.* (2015) reported the complete genome sequence of *P. polymyxa* CF05 strain isolated from the interior of an ancient tree, *Cryptomeria fortunei*, in China. This bacterial strain displayed potent biocontrol effects against certain soil-borne diseases and the elicitation of induced systemic resistance in tomatoes.

2.5. Conclusions

Paenibacillus polymyxa P2b-2R, a bacterial strain originally isolated from a lodgepole pine tree (gymnosperm) is capable of colonizing rhizosphere and internal tissues of root (endophytically) of an important cereal crop corn (angiosperm). I think that this is an important finding in terms of this bacterial strain's broad range host capability. This bacterial strain might possess necessary adaptations for root colonization of corn plant but the mechanisms involved in these adaptations are still unknown. P2b-2R was not successful in endophytically colonizing above ground parts of the corn in this study for unknown reasons. Two possibilities are: either the surface sterilization protocol used was too harsh or the bacterium was not able to reach the above ground

parts of the plant in such short duration. Corn seedlings inoculated with P2b-2R were successful in deriving nitrogen from atmospheric N pool. The amount of nitrogen fixed was significant considering the fact that this was a short growth trial. But the question is, does this BNF originate from endophytic or rhizospheric P2b-2R? Our results also suggest that P2b-2R inoculation promotes growth (length and biomass) of corn seedlings. A consistent increase in foliar N of seedlings inoculated with P2b-2R is a major contributing reason for growth promotion. Other possibilities include production of plant growth promoting substances, nutrient mineralization and assistance in nutrient uptake by this bacterial strain. Future studies might involve an in-depth investigation of the abilities of *P. polymyxa* to endophytically colonize above ground parts of corn, especially in a long-term growth trial and to look for possible role of this bacterial strain in nutrient mineralization and uptake.

Chapter 3 – Growth Promotion and N₂-Fixation in Canola (*Brassica napus* L.) Colonized by an Endophytic Diazotroph *Paenibacillus polymyxa* P2b-2R²

3.1. Introduction

Nitrogen is one of the most common nutrients required for plant growth and productivity as it forms an integral part of proteins, nucleic acids and other essential biomolecules (Bøckman, 1997). Almost 80% of our atmosphere is nitrogen, but plants can't use it. It needs to be converted into ammonia, a form available to plants. Atmospheric nitrogen is converted into forms utilized by plants by three different processes- i) conversion of atmospheric nitrogen into oxides of nitrogen in the atmosphere by lightning, ii) industrial nitrogen fixation using catalysts and high temperature (300-500°C) to convert nitrogen to ammonia, and iii) biological nitrogen fixation involving the conversion of nitrogen to ammonia by microorganisms using a complex enzyme system identified as nitrogenase (Kim & Rees, 1994). Biological nitrogen fixation represents an economically beneficial and environmentally sound alternative to chemical fertilizers (Ladha *et al.*, 1997). The rhizobia-legume symbiosis is perhaps the most common N₂ fixing agent in agricultural systems (Herridge *et al.*, 2008). But unlike nodule-forming bacteria there is another group of bacteria that fixes nitrogen even in non-leguminous plant species. Plant growth-promoting

² A version of this chapter has been submitted to Biology and Fertility of Soils Journal for publication under the title 'Plant Growth Promotion and N₂-Fixation in Canola (*Brassica napus* L.) by an Endophytic Diazotroph *Paenibacillus polymyxa* P2b-2R' Authors: Akshit Puri, Kiran Preet Padda and Christopher P Chanway and it is currently under review.

rhizobacteria that fix nitrogen in non-leguminous plants are known as diazotrophs. They form a non-obligate interaction with the host (Glick *et al.*, 1999).

Endophytic bacteria have been known for more than 120 years (Hardoim *et al.*, 2008). In 1926, Perotti was the first to use the term endophytes to describe non-pathogenic bacteria that had been isolated from within plants, other than rhizobia (Hallmann *et al.*, 1997; Hardoim *et al.*, 2008). In comparison with rhizosphere and rhizoplane bacterial communities, endophytic bacteria are likely to interact more closely with their host plant as they are provided with a readily available source of nutrients and a secure residence inside intercellular spaces of plants (Rosenblueth & Martinez-Romero, 2006; Weyens *et al.*, 2009). In return, the endophytic bacteria could enhance host plant growth and health via various direct and indirect mechanisms. The most important direct growth promotion mechanism is nitrogen fixation.

Döbereiner (1992) introduced the term "endophytic diazotrophic bacteria" in the area of BNF to designate all diazotrophs that survive very poorly in soil but colonize the interior tissues of roots of graminaceous plants, and fix nitrogen in association with them (Baldani *et al.*, 1998). Some examples of endophytic diazotrophs of rice, maize and sugarcane are *Azotobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Azoarcus spp.* and *Enterobacter asburiae*, which serve as nitrogen fixers when other available sources of nitrogen are absent or at low levels (Reinhold *et al.*, 1986, Döbereiner *et al.*, 1993; Kirchhof *et al.*, 1997).

Canola was developed in the early 1970s using traditional plant breeding techniques by Canadian plant breeders to remove the anti-nutritional components (erucic acid and glucosinolates) from rapeseed to assure its safety for human and animal consumption (Soyatech, n.d.). Global canola production has grown rapidly over the past 40 years, rising from the sixth largest oil crop to the second largest. Canola oil, obtained from crushing canola seed, was the third-most-produced vegetable oil globally in 2008/09 (United States Department of Agriculture, n.d.). Canola seeds are 44% oil, more than double the oil content of soybeans. High-protein meal is produced from the other 56% of the canola seed, which is an excellent animal feed for cattle, poultry, swine and fish. When fed to dairy cows, it can increase milk production by one litre per day. Canola meal is the second largest feed meal after soybean meal (Canola Council of Canada, n.d. b).

P. polymyxa P2b-2R was reported to colonize and fix nitrogen in gymnosperms (lodgepole pine and western red cedar) in both short duration and long duration growth trials (Bal & Chanway, 2012a,b; Anand & Chanway, 2013a; Anand *et al.*, 2013). But can this bacterial strain associate with Canola (*Brassica napus* L.), an important oil crop? The main objectives of this study were to determine if *Paenibacillus polymyxa* strain P2b-2R is capable of: (i) colonizing rhizosphere and internal tissues of Canola (*Brassica napus* L.), (ii) fixing N and stimulating growth rate in association with Canola.

3.2. Materials and methods

3.2.1. Seed and microorganism

Canola (*Brassica Napus* L.) seeds (*var.* Rugby Roundup ready) were obtained from SeCan Association's Alberta branch (Lamont, Alberta, Canada). *Paenibacillus polymyxa* strain P2b was isolated from internal tissues of a lodgepole pine seedling stem and needles 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). *Paenibacillus polymyxa* P2b-2R is a spontaneous antibiotic-resistant mutant that was derived from strain P2b (Bal *et al.*, 2012). *P. polymyxa* P2b-2R is resistant to 200 mg/L rifamycin and was stored at -80 °C on combined carbon medium (CCM) amended with 20% glycerol (Bal & Chanway, 2012a).

3.2.2. Seedling growth and inoculation

Seedling growth assays were performed in small pots (12cmx8cmx4cm) filled to 67% capacity with sterile Sand-Turface (montmorillonite clay, Applied Industrial Materials Corporation, Deerfield, Ill., USA) mixture (69% w/w industrial sand; 29% w/w Turface; 2% w/w CaCO₃). Each pot was fertilized with 50 mL of a sterile nutrient solution (Appendix A) (Chanway *et al.*, 1988), which was modified by replacing KNO₃ and Ca(NO₃)₂·4H₂O with Ca(¹⁵NO₃)₂ (5% ¹⁵N label) (0.0576 g/L) and sequestrene 330 Fe (CIBA-GEIGY, Mississauga, Ont., Canada) with Na₂-FeEDTA (0.02 g·L⁻¹).

Canola seeds were surface-sterilized by immersion in 30% hydrogen peroxide (H₂O₂) for 1.5 min, followed by three 30-second rinses in sterile distilled water. To confirm the effectiveness of surface sterilization, seeds were imprinted on tryptic soy agar (TSA) and checked for microbial contamination after 2 days. Three surface-sterilized seeds were then aseptically sown in each pot.

Two seed inoculation treatments – live P2b-2R and control (phosphate buffered saline) were evaluated using canola seedlings, with 60 replications per treatment. Bacterial inoculum was prepared by thawing a frozen culture of strain P2b-2R and streaking onto combined carbon medium (CCM) plates amended with 200 mg/L rifamycin. After colonies grew, a loopful of the strain was inoculated into a 1-L flask containing 500 mL of fresh CCM broth amended with rifamycin. Flasks were then secured on a rotary shaker (150 rpm) and agitated for 24 h at room temperature. Bacteria were harvested by centrifugation (3000x g, 30 min) and re-suspended in phosphate buffered saline (PBS) to a density of 10⁶ cfu/mL. For inoculation, 5 mL of the P2b-2R-PBS suspension was pipetted directly into each of 60 replicate pots immediately after sowing canola seeds. This process was repeated using 5 mL of sterile phosphate buffered saline (PBS) without bacteria for control (noninoculated) pots. The pots were placed in a growth chamber (Convion CMP3244, Convion Products Company, Winnipeg, MB, Canada) with photosynthetically active radiation at canopy level of 300 μmol s⁻¹ m⁻², an 18-h photoperiod and a 25/18 °C day/night temperature cycle. Seedlings were thinned to the largest single germinant per pot 5 days after sowing and were watered as required with sterile distilled water. A modified

nutrient solution was provided to each pot (without $\text{Ca}^{15}\text{NO}_3)_2$), 20 and 40 days after inoculation. The entire experiment was repeated again to confirm the treatment effects.

3.2.3. Quantification of rhizospheric colonization

To evaluate rhizosphere colonization, 3 randomly selected seedlings from each treatment were harvested destructively. Seedlings were removed from pots and gently shaken to remove loosely adhering soil. Roots were then separated from shoots, placed in falcon tubes (BD Biosciences, CA, USA) containing 10 mL of sterile PBS and shaken on a vortex mixer at 1000 rpm for 1 min. Serial dilutions were made in 9 mL of PBS using 1 mL pipetted from the original suspension. Aliquots of the serial dilutions (0.1 mL) were plated onto CCM amended with 200 mg/L rifamycin plates and incubated for 7 days at 30°C. Colonies were counted after 7 days of incubation. Roots were oven-dried at 65°C for 2 days before weighing to determine the dry root weight. Rhizospheric bacterial populations were then calculated as cfu per gram dry root.

3.2.4. Quantification of endophytic colonization

Three randomly selected seedlings from each treatment were harvested destructively 20, 40 and 60 days after sowing to evaluate endophytic colonization. At each harvest, seedlings were surface-sterilized with 1.3% sodium hypochlorite for 5 min, and washed thrice with sterile distilled water. Seedlings were imprinted on TSA plates for 24 h to check for surface contamination. Root,

stem, and leaf tissue samples were then triturated separately in 1 mL PBS using a mortar and pestle. Triturated tissue suspensions were then diluted serially, and 0.1 mL of each dilution was plated onto CCM supplemented with cycloheximide (100 mg/L) to suppress fungal growth and rifamycin (200 mg/L) to suppress bacterial growth. The number of colony forming units (cfu) was evaluated 7 days after incubation at 30°C.

3.2.5. ¹⁵N analysis and seedling growth promotion

Fourteen canola seedlings were harvested destructively 20, 40 and 60 days after sowing for evaluation of seedling growth promotion as well as foliar N and ¹⁵N content. Seedling growth promotion was evaluated in three ways—lengths, dry weight and no. of seedlings with floral buds. Roots were separated from shoots, and shoot and root lengths were measured. Shoots and roots of each seedling were then oven dried at 65 °C for 2 days before being weighed to determine the dry weight. For foliar N analysis, oven dried and ground foliage of each seedling from each treatment was mixed thoroughly and a 1 mg sample was sent to the Stable Isotope Facility (Department of Forest and Conservation Sciences, Faculty of Forestry, The University of British Columbia, Vancouver, Canada) for determination of foliar N content and %¹⁵N excess with an elemental analyzer interfaced with an isotope ratio mass spectrometer (Isoprime, GV Instruments). The amount of fixed N in foliage was estimated by calculating the percent nitrogen derived from the atmosphere (%Ndfa) (Rennie *et al.*, 1978):

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

3.2.6. *Statistical analysis*

A completely randomized experimental design with 60 replicates per treatment was used to assess the treatment effects on growth of canola seedlings. The statistical package, SAS v9.4 (Copyright © 2014, SAS Institute Inc., Cary, NC, USA.), was used to perform statistical analyses. Analysis of variance (ANOVA), $P < 0.05$, was performed to determine significant differences between treatment means for atom percent ^{15}N excess, foliar N content and all plant growth parameters. Since residuals were well distributed, data was left untransformed. Treatment means were separated using Fisher's protected least significant difference (LSD).

3.3. Results

3.3.1. *Rhizospheric and endophytic colonization by P2b-2R*

Paenibacillus polymyxa strain P2b-2R colonized canola rhizosphere with 1.41×10^8 cfu/g dry root in 1st growth trial and 7.49×10^7 cfu/g dry root in 2nd growth trial. P2b-2R colonized internal tissues of canola root (endophytically) with 3.48×10^5 cfu/g fresh weight in the 1st growth trial and 2.36×10^3 cfu/g fresh weight in the 2nd growth trial. But there was no evidence of endophytic colonization in stem and leaf tissues in either growth trials. Although, P2b-2R colonies were observed on TSA stem and leaf imprint plates after these tissues

were surface sterilized. No evidence of rhizospheric or endophytic colonization was found in control plants.

3.3.2. *Growth promotion and N- fixation*

Seedling growth was enhanced significantly by inoculation with *P. polymyxa* P2b-2R. P2b-2R inoculation increased shoot length by 9%, 19%, and 31% in 1st, 2nd and 3rd harvest respectively in the 1st growth trial (Figure 3.1). Similarly in the 2nd growth trial, P2b-2R inoculation increased shoot length by 17%, 20%, and 30% at each harvest interval respectively (Figure 3.2). Seedling length of P2b-2R inoculated seedlings was promoted by 8%, 18%, and 25% at each harvest interval respectively in the 1st trial (Figure 3.1). Similarly in the 2nd growth trial, P2b-2R inoculation promoted seedling length by 14%, 15%, and 21% at each harvest interval respectively (Figure 3.2). P2b-2R inoculation significantly increased shoot dry weight (22%, 32%, and 38% at each harvest interval respectively in the 1st trial (Figure 3.3) and 18, 42 and 31% at each harvest interval respectively in the 2nd trial (Figure 3.4)) and seedling biomass (17, 26, 30% at each harvest interval respectively in the 1st trial (Figure 3.3) and 16, 32, 24% at each harvest interval respectively in the 2nd trial (Figure 3.4)). Canola plants reached reproductive stage after 40 days of inoculation, as the floral buds started blooming. It was observed that a greater number of inoculated canola seedlings reached reproductive stage than the control seedlings in both growth trials (Figure 3.5).

Based on percent foliar ^{15}N atom excess of P2b-2R inoculated and control seedlings, 7% of the foliar N of inoculated seedlings was derived from the atmosphere (Ndfa) at the time of 1st harvest, which increased to 11% at the time of 2nd harvest and 22% at the time of 3rd harvest in the first growth trial (Table 3.1). A similar trend was observed in the second growth trial, as inoculated seedlings derived 5% N from atmosphere in the 1st harvest, which increased to 8% in the 2nd harvest and 19% in the 3rd harvest (Table 3.2). In the 1st growth trial, percent foliar N of seedlings inoculated with P2b-2R was 14, 23, and 41% higher than the control seedlings at the time of 1st, 2nd and 3rd harvests respectively (Table 3.1). Similarly in the second growth trial, percent foliar N of inoculated seedlings was 23, 29, and 38% higher than the control at the time of 1st, 2nd and 3rd harvest respectively (Table 3.2).

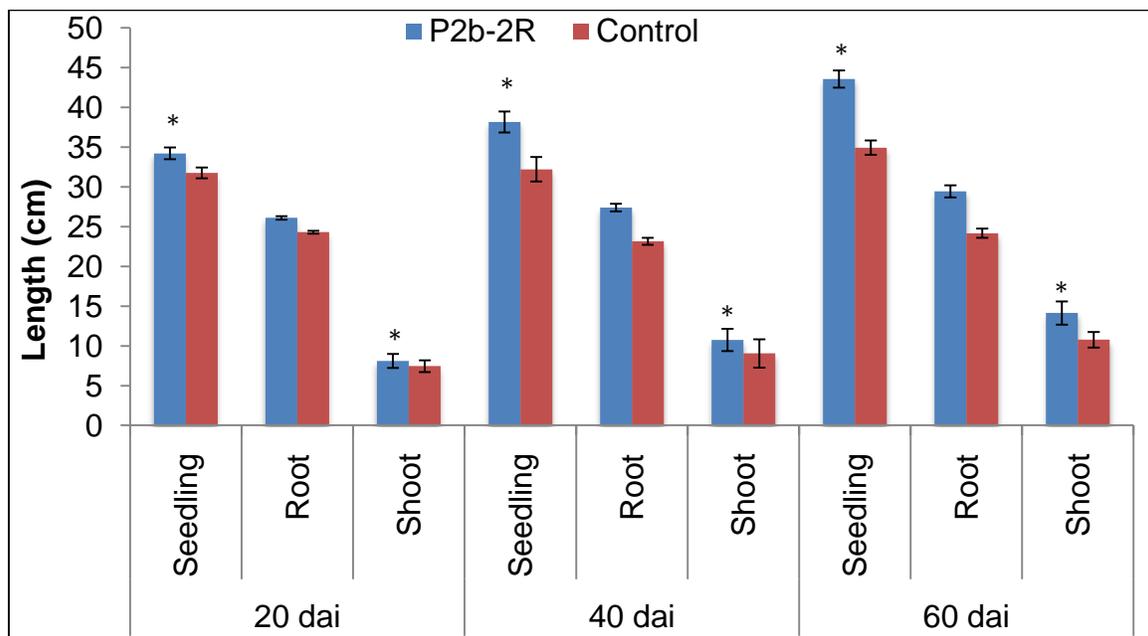


Figure 3.1 Seedling, root and shoot length of canola seedlings harvested 20, 40 and 60 days after inoculation (dai) in 1st growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).

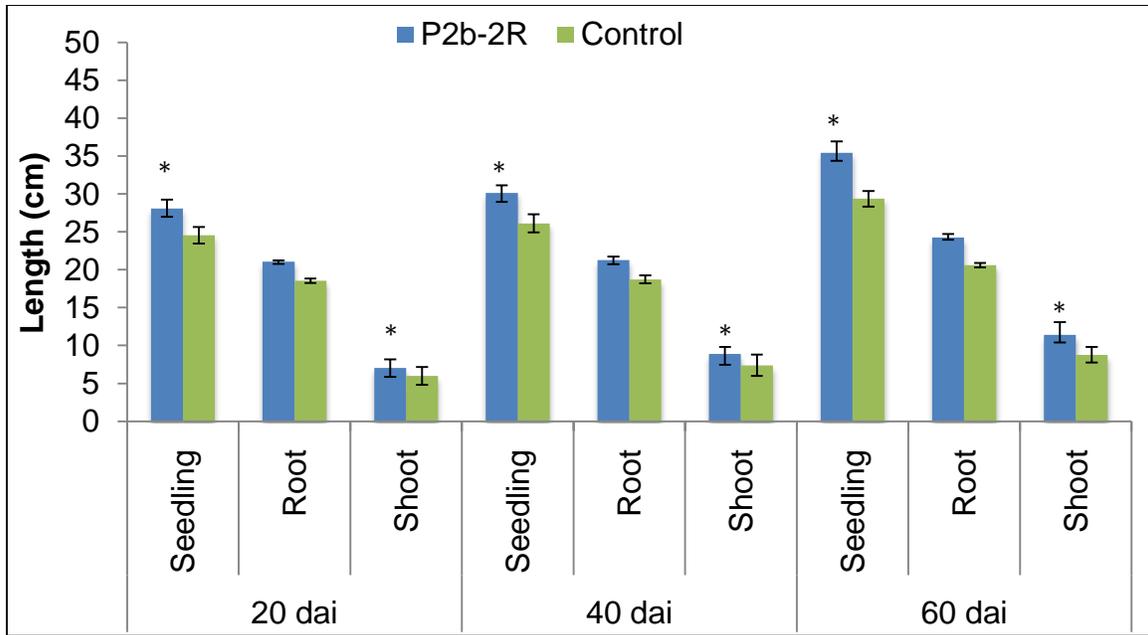


Figure 3.2 Seedling, root and shoot length of canola seedlings harvested 20, 40 and 60 days after inoculation (dai) in 2nd growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).

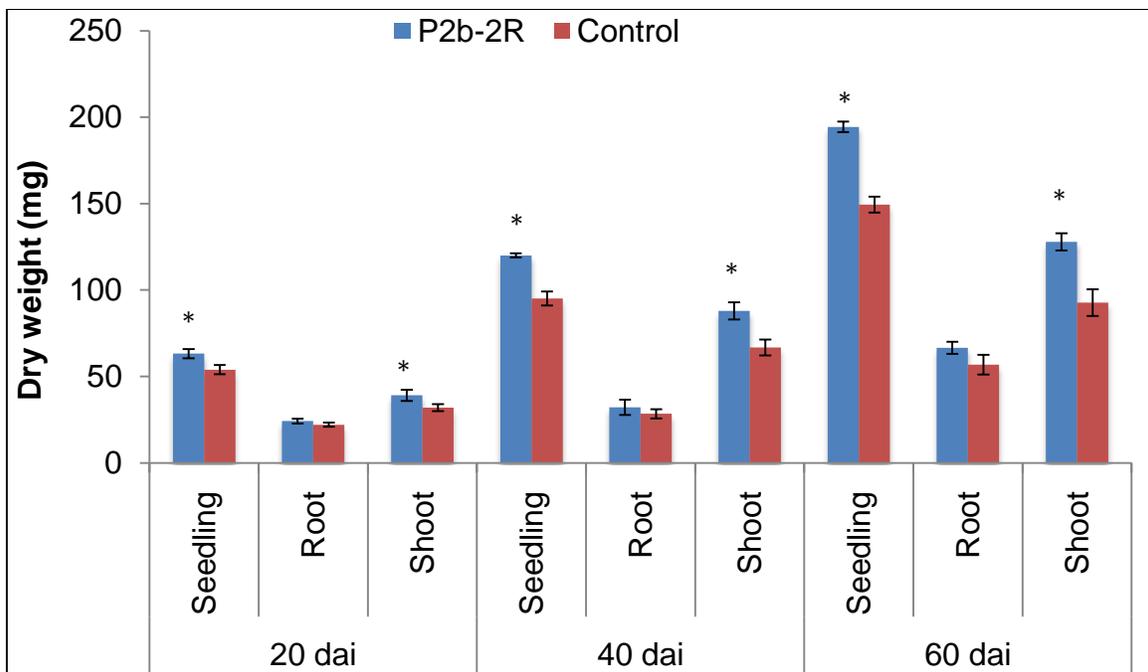


Figure 3.3 Seedling, root and shoot dry weight of canola seedlings harvested 20, 40 and 60 days after inoculation (dai) in 1st growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).

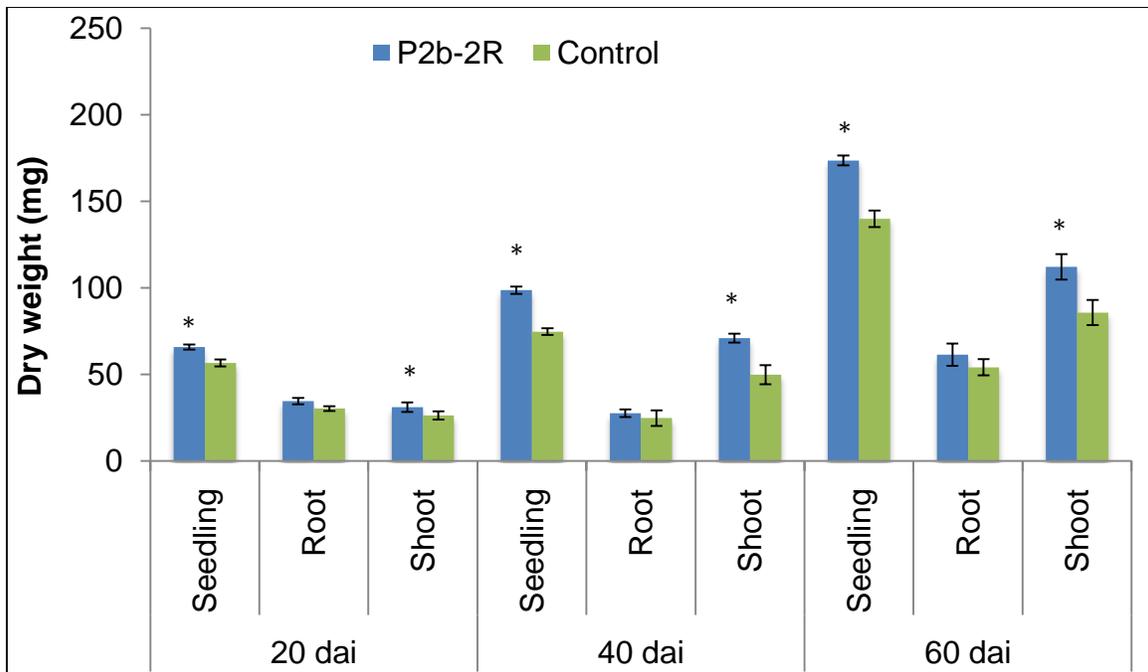


Figure 3.4 Seedling, root and shoot dry weight of canola seedlings harvested 20, 40 and 60 days after inoculation (dai) in 2nd growth trial. Error bars represent standard error of the mean (n=14). *P<0.05 (significantly different from control).

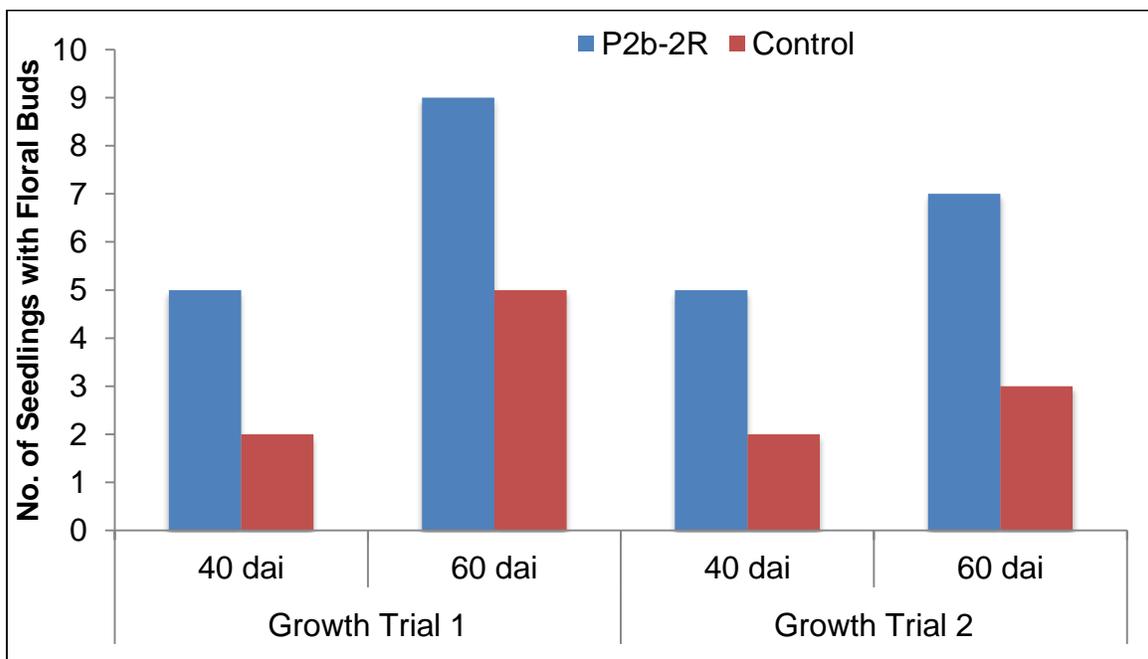


Figure 3.5 Number of canola seedlings having floral buds (reached reproductive stage) of each treatment measured 40 and 60 days after inoculation (dai) in two growth trials (n=14).

Table 3.1 Atom % ¹⁵N excess in foliage, % foliar N and % N derived from the atmosphere (Ndfa), developed from canola seeds inoculated with *P. polymyxa* P2b-2R and phosphate buffered saline (control) measured 20, 40 & 60 days after inoculation (dai) in growth trial 1

	20 dai			40 dai			60 dai		
	Atom % ¹⁵ N			Atom % ¹⁵ N			Atom % ¹⁵ N		
	Excess in	% Foliar N	%Ndfa	Excess in	% Foliar N	%Ndfa	Excess in	% Foliar N	%Ndfa
	Foliage			Foliage			Foliage		
P2b-2R	0.72*±0.014 ^a	1.48*±0.06	7.24	0.63*±0.011	1.16*±0.05	11.46	0.54*±0.006	0.94*±0.09	21.76
Control	0.77±0.016	1.21±0.06	-	0.71±0.017	0.94±0.05	-	0.69±0.010	0.67±0.03	-

^a Mean ± standard error; n=14 for atom % ¹⁵N excess & % foliar N

*P<0.05 (significantly different from control)

Table 3.2 Atom % ¹⁵N excess in foliage, % foliar N and % N derived from the atmosphere (Ndfa), developed from canola seeds inoculated with *P. polymyxa* P2b-2R and phosphate buffered saline (control) measured 20, 40 & 60 days after inoculation (dai) in growth trial 2

	20 dai			40 dai			60 dai		
	Atom % ¹⁵ N			Atom % ¹⁵ N			Atom % ¹⁵ N		
	Excess in Foliage	% Foliar N	%Ndfa	Excess in Foliage	% Foliar N	%Ndfa	Excess in Foliage	% Foliar N	%Ndfa
P2b-2R	0.70*±0.011 ^a	1.21*±0.06	5.38	0.59*±0.012	1.13*±0.05	8.48	0.52*±0.010	0.90*±0.08	18.63
Control	0.74±0.010	0.94±0.08	-	0.64±0.019	0.92±0.04	-	0.63±0.015	0.65±0.03	-

^a Mean ± standard error; n=14 for atom % ¹⁵N excess & % foliar N

*P<0.05 (significantly different from control)

3.4. Discussion

The primary objective of my study was to determine if *Paenibacillus polymyxa* strain P2b-2R is capable of colonizing rhizosphere and internal tissues of canola (*Brassica napus* L.), fixing N and stimulating plant growth. *P. polymyxa* P2b-2R was able to colonize rhizosphere of canola seedlings with population sizes greater than those previously reported for this bacterial strain (Bal & Chanway, 2012a,b; Yang, 2015). The population size was also greater than other reported *P. polymyxa* strains (Holl & Chanway, 1992; Heulin *et al.*, 1994; Defreitas & Germida, 1998). This shows that P2b-2R possesses necessary adaptations for rhizosphere colonization of this agricultural crop despite being isolated from internal tissues of a tree species. Timmusk *et al.* (2005) previously reported that colonization of *P. polymyxa* is initiated by bacterial accumulation at the root tip and subsequent formation of biofilms around the root tip. One of the most important factors that determine bacterial colonization on the plant root is root exudation (Walker *et al.*, 2003; Weller & Thomashow, 1994). *Paenibacillus polymyxa* has been found to colonize rhizosphere of canola plant in some previous reports as well. Defreitas & Germida (1998) isolated more than 800 bacterial strains from rhizosphere of field-grown canola and found that about 5% of the isolates were diazotrophs. The most promising diazotrophic bacteria of the collection were two *Bacillus polymyxa* strains (ES600A and RSN17).

Endophytic colonization of canola roots by P2b-2R was observed in both growth trials with population sizes comparable to those observed in other

published reports of this bacterial strain (Anand & Chanway, 2013a; Anand *et al.*, 2013). However, there was no evidence of endophytic colonization in stem and leaf tissues by P2b-2R strain in either growth trial. This was unexpected as P2b-2R previously colonized above ground parts of the plant in lodgepole pine and western red cedar (Anand & Chanway, 2013a; Anand *et al.*, 2013). Possible reasons could be that, either the bacteria was not able to reach above ground parts of the plant in such short time duration or the comparatively harsh protocol that was required to ensure complete surface sterilization of tissue samples disinfected internal cortical tissues thereby precluding detection of this bacterial strain. Additional studies involving direct detection using confocal laser microscopy need to be done to confirm colonization in stem and leaf tissues as was done with lodgepole pine seedlings (Anand & Chanway, 2013b). According to our results, the preferred endophytic colonization region of canola was root tissue, which is consistent with reports about some other endophytic bacteria (Timmusk *et al.*, 2005; Hurek *et al.*, 1994). Quadt-Hallmann & Kloepper (1997) observed that hydrolytic enzymes synthesized by *P. polymyxa* aid entry into intact root tissue.

Paenibacillus polymyxa P2b-2R had consistent and significant effects on canola foliar ¹⁵N content. The magnitude of ¹⁵N foliar dilution suggests that canola seedlings derived 19-22% of foliar N from the activity of P2b-2R (Table 3.1 and 3.2). Similar amounts of N derived from the atmospheric pool have been observed in other plant species including sugar cane (*Saccharum officinarum* L.) (Lima *et al.*, 1987; Boddey *et al.*, 1991), kallar grass (*Leptochloa fusca*) (Malik *et*

al., 1987), rice (*Oryza sativa* L.) (Malik *et al.*, 1997), wheat (*Triticum aestivum*) (Rennie & Larson, 1979; Rennie *et al.*, 1983), western red cedar (*Thuja plicata*) (Bal & Chanway, 2012b; Anand & Chanway, 2013a), lodgepole pine (*Pinus contorta*) (Bal & Chanway, 2012a), and corn (*Zea mays* L.) (Araújo *et al.*, 2015). The percent nitrogen derived by inoculated seedlings from atmosphere increased at each harvest in both growth trials (Table 3.1 and 3.2) suggesting that BNF is an important component of N nutrition of canola growing in a nitrogen limited soil medium. But the source of this BNF is still unknown and it could be speculated that it is either induced by the rhizospheric P2b-2R or endophytic P2b-2R present in root tissues. Nitrogen was added to the soil mix only once at the onset of this experiment. In the absence of further additions, N would be expected to deplete with continued seedling growth and ultimately restrict their growth rate. But it was observed that nitrogen fixation by P2b-2R contributed positively to plant's performance as the inoculated seedlings were having higher foliar N content than the control seedlings (nearly 40%). These results are much better than what was found with corn (presented in Chapter 2), where this bacterial strain increased the foliar N content of corn seedlings by 20%.

Growth promotion of canola seedlings by *P. polymyxa* P2b-2R was inferred on the basis of two parameters- length and biomass. Inoculated seedlings were 25% taller than the control seedlings after 60 days of inoculation. This was primarily due to a significant difference of shoot length (about 30%) between inoculated and control seedlings (Figure 3.1 and 3.2). These results are consistent with previous reports about this bacterial strain, where P2b-2R

inoculation significantly enhanced shoot and seedling length in lodgepole pine and western red cedar (Anand & Chanway, 2013a; Anand *et al.*, 2013). In another report, two strains of *Bacillus polymyxa* species, previously isolated from field-grown canola, were found to enhance plant growth when reintroduced into canola in growth chamber and field trials (Defreitas & Germida, 1998). Seedling biomass of P2b-2R inoculated seedlings was nearly 30% greater than control seedlings after 60 days of inoculation and I think that a significant difference in shoot dry weight (35%) between inoculated and control seedlings was the primary reason for this (Figure 3.3 and 3.4). The substantial differences of shoot length and shoot dry weight between inoculated and control seedlings suggest that P2b-2R promotes plant growth both vertically (length) and horizontally (biomass). The reason for this enhanced growth of canola seedlings is likely related to the nitrogen fixing ability of this bacterium, leading to a higher content of N especially in the above ground parts of the plant. Canola seedlings started blooming after 40 days of inoculation and it was observed that P2b-2R inoculation enhanced the ability of seedlings to reach reproductive stage by 2-2.5 times as compared to the control (Figure 3.5). This clearly indicates that P2b-2R inoculation boosts the ability of canola seedlings to self-pollinate and form floral buds. Thus, P2b-2R might play a significant role in increasing the yield of canola in a field trial. In a previous study, a plant growth promoting bacterium showed the ability to increase yield of canola crop by 57% in field trials (Kloepper *et al.*, 1988).

Plant growth promotion can be achieved by the direct interaction between beneficial microbes and their host plant and also indirectly due to their antagonistic activity against plant pathogens (Prasad *et al.*, 2015). One of the most commonly reported direct plant growth promoting mechanism by *Bacillus* and relatives is the production of plant growth substances such as auxins, cytokinins and giberellins (Chanway, 2008). The production of plant growth promoting compounds by *P. polymyxa* similar in activity to indole-3-acetic acid has been suggested to stimulate growth in crested wheatgrass (Holl *et al.*, 1988). It also releases iso-pentenyladenine and one unknown cytokinin-like compound during its stationary phase of growth which promotes seed germination, de novo bud formation, release of buds from apical dominance, stimulation of leaf expansion and reproductive development and retardation of senescence (Mok, 1994). Timmusk & Wagner (1999) reported that natural isolates of *P. polymyxa* induce drought tolerance and antagonize pathogens in *Arabidopsis thaliana*. Studies have shown that bacteria belonging to *Paenibacillus* genus are capable of mineralizing phosphorous by producing enzymes, which cleave phosphate groups (Chanway, 2008). *Paenibacillus polymyxa* is also capable of synthesizing many antibacterial and antifungal secondary metabolites (Borriss, 2015). Beatty & Jensen (2002) isolated a *P. polymyxa* strain PKB1 capable of inhibiting the growth of *Leptosphaeria maculans*, the causative agent of blackleg disease of canola (*Brassica napus* L. and *Brassica rapa* L.). They found that *P. polymyxa* produces fusaricidin-type antifungal antibiotics, which are quite active against *Leptosphaeria maculans*.

3.5. Conclusions

In conclusion, my results suggest that canola seedlings are able to access an N pool other than the soil, likely the atmospheric pool, when colonized by *P. polymyxa* P2b-2R and demonstrate that N₂-fixing seedlings develop higher foliar N level and grow much better than non-fixing seedlings in N-limited soil. *Paenibacillus polymyxa* P2b-2R bacterial strain was originally isolated from internal stem tissues of lodgepole pine, a gymnosperm species. Successful association of this bacterial strain with canola, an important agricultural crop, indicates that it might be able to colonize broad range of hosts. No bacterial population was observed in stem and leaf tissues of canola, which was unexpected as this strain was originally isolated from stem tissues of lodgepole pine (Bal *et al.*, 2012) and was found to colonize stem and leaf tissues of western red cedar and lodgepole pine (Anand & Chanway, 2013a,b; Anand *et al.*, 2013). Either the surface sterilization protocol was too harsh for stem and leaf tissues or the bacterium was not able to form detectable population sizes. Direct detection techniques such as using confocal laser scanning microscopy with *gfp*-labelled bacteria might be more useful to detect endophytic colonization of stem and leaf tissues of canola. P2b-2R inoculated canola seedlings successfully derived significant amounts of N from atmosphere. But we don't know if this BNF originated from rhizospheric or endophytic P2b-2R populations. P2b-2R inoculation promoted seedling growth by increasing length and biomass. Inoculated seedlings were directly accessing atmospheric N pool, thereby increasing foliar N content, which could be the reason for enhanced seedling

growth. Other possibilities could be that this bacterial strain was involved in nutrient mineralization and uptake, or pathogen suppression. P2b-2R inoculation also boosted the ability of canola seedlings to reach reproductive stage as greater numbers of inoculated seedlings were having floral buds than the control during 2nd and 3rd harvest. This finding reflects the ability of P2b-2R to increase yield of canola plant.

Chapter 4 – General Summary and Conclusions

Nitrogen is recognized as the most frequently limiting nutrient for plant growth in ecosystems (Vitousek & Howarth, 1991). Despite its abundance, nitrogen (N) is one of the most growth-limiting nutrients in terrestrial and aquatic ecosystems (Dalton & Krammer, 2006) because its gaseous form is inert and unusable by most living organisms except for nitrogen fixing microorganisms. For it to become biologically available, atmospheric nitrogen must be transformed or “fixed” from its inert gaseous form (N_2) to ammonia (NH_3).

Nitrogen is fixed naturally through energy-releasing abiotic processes such as lightening, forest fires and volcanic activity. Manufacturing of artificial nitrogen fertilizers became very common in the post-industrial revolution era. Fertilizer production using high temperatures and pressures in the Haber-Bosch process occurs widely and accounts for approximately 20% of annual global NF (Bezdicek & Kennedy, 1998). However, the process is fossil fuel intensive and consumes 3-5% of the world's natural gas annually (Myrold & Bottomley, 2007). Alternatively, NF occurs through the normal metabolic activity of many diazotrophs, through a process commonly referred to as biological nitrogen fixation (BNF) (Chanway *et al.*, 2014). 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 focused on the *Rhizobium*–legume symbiosis and the organisms involved. But since the discovery of

endophytic diazotroph *Gluconoacetobacter diazotrophicus* from internal tissues of sugarcane plant in Brazil, many researchers have shifted their focus on isolation and identification of bacterial endophytes capable of deriving nitrogen from atmosphere.

One such strain, *Paenibacillus polymyxa* P2b-2R, was isolated from internal tissues of lodgepole pine naturally regenerating at a site near Williams Lake, British Columbia, Canada (Bal *et al.*, 2012). When this bacterial strain was reintroduced into lodgepole pine and grown in nitrogen limited environment, it was found to derive 79% of nitrogen from atmosphere (Anand *et al.*, 2013). Inoculation of another gymnosperm species, western red cedar, with this bacterial strain showed significant results as the inoculated seedlings derived 56% of nitrogen from atmosphere (Bal & Chanway, 2012b). Endophytic and rhizospheric colonization of lodgepole pine and western red cedar by P2b-2R was also reported (Anand *et al.*, 2013; Anand & Chanway, 2013a). Thus, P2b-2R possesses the ability to colonize rhizosphere and internal tissues of lodgepole pine and western red cedar (gymnosperm species) and fix nitrogen. But I was interested in investigating this bacterial strain's ability to colonize a broad range of plant hosts, specifically agricultural crops. I chose two important agricultural crops, corn and canola, for my study. Corn is a principal cereal crop grown on a large scale worldwide and canola is an important oil crop generally grown in Canada and exported globally generating very significant revenues. Also, corn and canola crop species differ physiologically as well as botanically.

The ability of P2b-2R to colonize, fix nitrogen and promote plant growth in Corn is presented in Chapter 2. P2b-2R colonized the corn rhizosphere with population sizes consistent with previous reports about this bacterial strain (Bal & Chanway, 2012a,b). P2b-2R was also successful in colonizing internal tissues of corn root with population sizes similar to those observed in reports about lodgepole pine and western red cedar (Anand *et al.*, 2013; Anand & Chanway, 2013a). Endophytic colonization of stem and leaf tissues was not observed, which was unexpected. However, it would be premature to completely rule out the ability of this bacterial strain to colonize aboveground parts of corn, as this strain was present on tissue imprint plates. A direct detection technique such as *gfp*-labeling with the use of confocal laser microscopy will provide more insight in this matter. Successful association of P2b-2R with corn seedlings was observed in terms of N-fixation and growth promotion. P2b-2R showed consistent and significant effect on corn foliar ¹⁵N abundance. According to atom % ¹⁵N excess in foliage data, P2b-2R inoculated corn seedling derived 20% of the foliar N from atmosphere and their foliar N content was also higher than the control. P2b-2R significantly enhanced plant growth by increasing length and biomass (dry matter content) of corn seedlings. Plant length was increased by 35% and biomass by 30% due to the inoculation of this bacterial strain. A consistent increase in foliar N content of inoculated seedlings could be a major contributing reason for growth promotion.

Growth promotion and N-fixation ability of P2b-2R in association with an important oil crop, canola, is reported in chapter 3. Growth promotion of canola

seedlings by P2b-2R was inferred on the basis of length and biomass. P2b-2R inoculated canola seedlings were taller and possessed greater biomass than the control. These results were consistent with previous reports about this bacterial strain (Anand & Chanway, 2013a; Anand *et al.*, 2013). Two strains of *Bacillus polymyxa* species isolated from field-grown canola were also reported to enhance seedling growth in growth chamber and field trials (Defreitas & Germida, 1998). P2b-2R was also involved in increasing the reproductive ability of canola seedlings as greater number of inoculated seedlings were having flower buds as compared to the control, which indicates that this bacterial strain might play a significant role in increasing the yield of canola. A long-term growth trial could be designed to see the effects of this bacterial strain on yield of canola plant. P2b-2R inoculation showed consistent and significant effect on canola foliar ¹⁵N content as the seedlings were found to derive more than 20% of foliar N from atmosphere. Similar amounts of N derived from atmospheric pool have been observed in other plant species including sugar cane (*Saccharum officinarum* L.) (Lima *et al.*, 1987; Boddey *et al.*, 1991), rice (*Oryza sativa* L.) (Malik *et al.*, 1997), wheat (*Triticum aestivum*) (Rennie & Larson, 1979; Rennie *et al.*, 1983), western red cedar (*Thuja plicata*) (Bal & Chanway, 2012b; Anand & Chanway, 2013a), lodgepole pine (*Pinus contorta*) (Bal & Chanway, 2012a), and corn (*Zea mays* L.) (Araújo *et al.*, 2015). Nitrogen fixation by P2b-2R contributed positively to plant's performance by increasing the foliar N content of inoculated seedlings. Rhizospheric and endophytic colonization experiments revealed that P2b-2R successfully colonized canola rhizosphere and internal tissues of roots

with population sizes comparable to those observed in other published reports about this bacterial strain (Anand & Chanway, 2013a; Anand *et al.*, 2013).

In summary, my thesis provides insight into the ability of a PGPB isolated from a gymnosperm species to colonize diverse plant hosts (agricultural crops). The ability of *Paenibacillus polymyxa* P2b-2R to colonize, fix N and promote growth of two agronomically important crops, corn and canola, is an effective finding in terms of sustainable agriculture. With an increasing interest of researchers worldwide towards diazotrophic endophytes as means of increasing plant growth, this PGPB promises to be an endophyte that could be commercialized in the future.

References

- Anand, R. (2011). Endophytic colonization and nitrogen fixation by *Paenibacillus polymyxa* in association with lodgepole pine and western red cedar (Doctoral Dissertation). Available from: https://circle.ubc.ca/bitstream/handle/2429/30254/ubc_2011_spring_anand_richa.pdf?sequence=6 (accessed on: July 21, 2015).
- Anand, R., Grayston, S., & Chanway, C. (2013). N₂-fixation and seedling growth promotion of lodgepole pine by endophytic *Paenibacillus polymyxa*. *Microbial Ecol* 66(2): 369-374.
- Anand, R., & Chanway, C. (2013a). N₂-fixation and growth promotion in cedar colonized by an endophytic strain of *Paenibacillus polymyxa*. *Biol Fert Soils* 49(2): 235-239.
- Anand, R., & Chanway, C. (2013b). Detection of GFP-labelled *Paenibacillus polymyxa* in autofluorescing pine seedling tissues. *Biol Fert Soils* 49(1): 111-118.
- Anand, R., & Chanway, C. P. (2013c). nif gene sequence and arrangement in the endophytic diazotroph *Paenibacillus polymyxa* strain P2b-2R. *Biol Fert Soils* 49(7): 965-970.
- Araújo, É. de O., Martins, M. R., Vitorino, A. C. T., Mercante, F. M., & Urquiaga, S. S. (2015). Effect of nitrogen fertilization associated with diazotrophic bacteria inoculation on nitrogen use efficiency and its biological fixation by corn determined using ¹⁵N. *Afr J Microbiol Res* 9(9): 643-650.
- 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(3-4): 253-260.
- Bal, A., 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 Forest Res* 42(4): 807-813.
- Bal, A., & Chanway, C. P. (2012a). Evidence of nitrogen fixation in lodgepole pine inoculated with diazotrophic *Paenibacillus polymyxa*. *Botany* 90(9): 891-896.
- Bal, A., & Chanway, C. P. (2012b). ¹⁵N foliar dilution of western red cedar in response to seed inoculation with diazotrophic *Paenibacillus polymyxa*. *Biol Fert Soils* 48(8): 967-971.

- Baldani, J. I., Olivares, F. L., Hemerly, A. S., Reis Jr, F. B., Oliveira, A. L. M., Baldani, V. L. D., ... & Dobereiner, J. (1998). Nitrogen-fixing endophytes: recent advances in the association with graminaceous plants grown in the tropics. In *Biological Nitrogen Fixation for the 21st century* (pp. 203-206). Springer Netherlands.
- Bashan, Y., & Levanony, H. (1990). Current status of Azospirillum inoculation technology: Azospirillum as a challenge for agriculture. *Can J Microbiol* 36(9): 591-608.
- 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(2): 159-169.
- Bezdicsek, D. F., & Kennedy, A. C. (1998). In Lynch, J. M., Hobbie, J. E. (eds), *Microorganisms in Action*. Blackwell, Oxford, UK.
- Bøckman, O.C. (1997). Fertilizers and biological nitrogen fixation as sources of plant nutrients: perspectives for future agriculture. *Plant Soil* 194(1-2): 11-14.
- Boddey, R. M., Urquiaga, S., Reis, V., & Döbereiner, J. (1991). Biological nitrogen fixation associated with sugar cane. In *Nitrogen Fixation* (pp. 105-111). Springer Netherlands.
- Borriss, R. (2015). Bacillus, a plant-beneficial bacterium. In *Principles of Plant-Microbe Interactions* (pp. 379-391). Springer International Publishing, Switzerland.
- Bressan, W., & Borges, M. T. (2004). Delivery methods for introducing endophytic bacteria into maize. *BioControl* 49(3): 315-322.
- Çakmakçı, R., Erat, M., Erdoğan, Ü., & Dönmez, M. F. (2007). The influence of plant growth-promoting rhizobacteria on growth and enzyme activities in wheat and spinach plants. *J Plant Nut Soil Sci* 170(2): 288-295.
- Canola Council of Canada (n.d. a). What is Canola? Available at: <http://www.canolacouncil.org/oil-and-meal/what-is-canola/> (accessed on: July 21, 2015).
- Canola Council of Canada (n.d. b). Industry Overview. Available from: <http://www.canolacouncil.org/markets-stats/industry-overview> (accessed on July 21, 2015).

- Cavalcante, V. A., & Dobereiner, J. (1988). A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. *Plant soil*, 108(1): 23-31.
- Chanway, C. P., Nelson, L. M., & Holl, F. B. (1988). Cultivar-specific growth promotion of spring wheat (*Triticum aestivum* L.) by coexistent *Bacillus* species. *Can J Microbiol* 34(7): 925-929.
- 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(3): 507-511.
- Chanway, C.P. (2008). Plant growth promotion by *Bacillus* and relatives. In *Applications and Systematics of Bacillus and Relatives* (eds. Berkeley, R., Heyndrickx, M., Logan, N. & De Vos, P.) (pp. 219-235). Blackwell Science Ltd, Oxford, UK.
- Chanway, C. P., Anand, R., & Yang, H. (2014). Nitrogen Fixation Outside and Inside Plant Tissues. In *Advances in Biology and Ecology of Nitrogen Fixation* (eds Takuji Ohyama) (pp. 3-23). Available from: <http://cdn.intechopen.com/pdfs-wm/46070.pdf> (accessed on: July 21, 2015).
- Cocking, E. C., Stone, P. J., & Davey, M. R. (2006). Intracellular colonization of roots of *Arabidopsis* and crop plants by *Gluconacetobacter diazotrophicus*. *In Vitro Cell Dev-Pl* 42(1): 74-82.
- Coombs, J. T., & Franco, C. M. (2003). Visualization of an endophytic *Streptomyces* species in wheat seed. *Appl Environ Microb* 69(7): 4260-4262.
- da Mota, F. F., Nóbrega, A., Marriel, I. E., Paiva, E., & Seldin, L. (2002). Genetic diversity of *Paenibacillus polymyxa* populations isolated from the rhizosphere of four cultivars of maize (*Zea mays*) planted in Cerrado soil. *Appl Soil Ecol* 20(2): 119-132.
- Dalton, D. A., & Kramer, S. (2006). Nitrogen-fixing bacteria in non-legumes. In *Plant-Associated Bacteria* (pp. 105-130). Springer Netherlands.
- Defreitas, J. R., & Germida, J. J. (1998). Nitrogen Fixing Rhizobacteria as Biofertilizers for Canola. *First Report submitted to the Canola Council of Canada (Period: April 1, 1995 – March 1, 1998), Project no. CARP AG #97-95-13*. Available at: <http://www.saskcanola.com/quadrant/System/research/reports/report-Germida-nitrogenfixing-long.pdf> (accessed on: July 21, 2015).

- Dijksterhuis, J., Sanders, M., Gorris, L. G. M., & Smid, E. J. (1999). Antibiosis plays a role in the context of direct interaction during antagonism of *Paenibacillus polymyxa* towards *Fusarium oxysporum*. *J Appl Microbiol* 86(1): 13-21.
- Döbereiner, J. (1992). Recent changes in concepts of plant bacteria interactions: endophytic N₂ fixing bacteria. *Ciência e Cultura* 44(5): 310-313.
- Döbereiner, J., Reis, V. M., Paula, M. A., & Olivares, F. D. (1993). Endophytic diazotrophs in sugar cane, cereals and tuber plants. In *New horizons in nitrogen fixation* (eds. Palacios, R., Mora, J., and Newton, W.E) (pp. 671-676). Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Dong, Y., Iniguez, A. L., & Triplett, E. W. (2003). Quantitative assessments of the host range and strain specificity of endophytic colonization by *Klebsiella pneumoniae* 342. *Plant Soil* 257(1): 49-59.
- Figueiredo, M. D. V. B., Seldin, L., de Araujo, F. F., & Mariano, R. D. L. R. (2011). Plant growth promoting rhizobacteria: fundamentals and applications. In *Plant growth and health promoting bacteria* (pp. 21-43). Springer Berlin, Heidelberg.
- Freitas, A. D. S., Vieira, C. L., Santos, C. E. R. S., Stamford, N. P., Lyra, M. C. C. P. (2007). Caracterização de rizo'bios isolados de Jacatupé cultivado em solo salino no Estado de Pernambuco, Brasil. *Bragantia* 66: 497–504.
- Gage, D. J., Bobo, T., & Long, S. R. (1996). Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). *J Bacteriology* 178(24): 7159-7166.
- Galar M.L., and Boiardi J.L., (1995). Evidence for a membrane-bound pyrroloquinoline quinone-linked glucose dehydrogenase in *Acetobacter diazotrophicus*. *Appl Microbiol Biotechnol* 43(4): 713-716
- Garg, N. & Geetanjali (2007). Symbiotic nitrogen fixation in legume nodules: process and signaling. A review. *Agron Sustainable Dev* 27(1): 59-68.
- Genersch, E. (2010). American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *J Invertebr Pathol* 103 S1:S 10-19.
- Glick, B.R., Patten, C.L., Holguin, G. & Penrose, D.M. (1999). Biochemical and genetic mechanisms used by plant growth promoting bacteria (pp. 267). Imperial College Press, London, United Kingdom.
- Glick, B. R. (2012). Plant growth-promoting bacteria: mechanisms and applications. *Scientifica*, 2012. doi:10.6064/2012/963401

- Górska, E. B., Jankiewicz, U., Dobrzyński, J., Russel, S., & Pietkiewicz, S. (2015). Degradation and Colonization of Cellulose by Diazotrophic Strains of *Paenibacillus polymyxa* Isolated from Soil. *J Bioremed Biodeg* 6(2): 271.
- Guemouri-Athmani, S., Berge, O., Bourrain, M., Mavingui, P., Thiéry, J. M., Bhatnagar, T., & Heulin, T. (2000). Diversity of *Paenibacillus polymyxa* populations in the rhizosphere of wheat (*Triticum durum*) in Algerian soils. *Eur J Soil Biol* 36(3): 149-159.
- Haggag, W. M., & Timmusk, S. (2008). Colonization of peanut roots by biofilm-forming *Paenibacillus polymyxa* initiates biocontrol against crown rot disease. *J Appl Microbiol* 104(4): 961-969.
- Hamel M-A, and Dorff E. (2014). Corn: Canada's third most valuable crop. Available from: <http://www.statcan.gc.ca/pub/96-325-x/2014001/article/11913-eng.htm> (accessed on July 21, 2015).
- Han, J., Chen, D., Huang, J., Li, X., Zhou, W. W., Gao, W., & Jia, Y. (2015). Antifungal activity and biocontrol potential of *Paenibacillus polymyxa* HT16 against white rot pathogen (*Coniella diplodiella* Speq.) in table grapes. *Biocontrol Sci Techn* 25(10): 1120-1132.
- Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., & Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Can J Microbiol* 43(10): 895-914.
- Hardoim, P. R., van Overbeek, L. S., & van Elsas, J. D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol* 16(10): 463-471.
- He, Z., Kisla, D., Zhang, L., Yuan, C., Green-Church, K. B., & Yousef, A. E. (2007). Isolation and identification of a *Paenibacillus polymyxa* strain that coproduces a novel lantibiotic and polymyxin. *Appl Environ Microb* 73(1): 168-178.
- He, J., Boland, G. J., & Zhou, T. (2009). Concurrent selection for microbial suppression of *Fusarium graminearum*, *Fusarium* head blight and deoxynivalenol in wheat. *J Appl Microbiol* 106(6): 1805-1817.
- Herridge, D. F., Peoples, M. B., & Boddey, R. M. (2008). Global inputs of biological nitrogen fixation in agricultural systems. *Plant Soil* 311(1-2): 1-18.
- Heulin T., Berge, O., Mavingui, P., Gouzou, L., Hebbar, K. P., Balandreau, J. (1994). *Bacillus polymyxa* and *Rahnella aquatilis*, the dominant N₂-fixing

- bacteria associated with wheat rhizosphere in french soils. *Eur J Soil Biol* 30(1): 35-42.
- Hiltner, L. (1904). Über neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie unter besonderer Berücksichtigung der Grundung und Brache. *Arb Dtsch Landwirtsch Ges Berl* 98: 59–78.
- Holl, F. B., Chanway, C. P., Turkington, R., & Radley, R. A. (1988). Response of crested wheatgrass (*Agropyron cristatum* L.), perennial ryegrass (*Lolium perenne* and white clover (*Trifolium repens* L.) to inoculation with *Bacillus polymyxa*. *Soil Biol Biochem* 20(1): 19-24.
- Holl, F. B., & Chanway, C. P. (1992). Rhizosphere colonization and seedling growth promotion of lodgepole pine by *Bacillus polymyxa*. *Can J Microbiol* 38(4): 303-308.
- Hoyles, L., Honda, H., Logan, N. A., Halket, G., La Ragione, R. M., & McCartney, A. L. (2012). Recognition of greater diversity of *Bacillus* species and related bacteria in human faeces. *Res Microbiol* 163(1): 3-13.
- 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(7): 1913-1923.
- 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(1): 77-119.
- Khan, Z., Kim, S. G., Jeon, Y. H., Khan, H. U., Son, S. H., & Kim, Y. H. (2008). A plant growth promoting rhizobacterium, *Paenibacillus polymyxa* strain GBR-1, suppresses root-knot nematode. *Bioresour Technol* 99(8): 3016-3023.
- Kim, J., & Rees, D.C. (1994). Nitrogenase and biological nitrogen fixation. *Biochemistry* 33(2): 389-397.
- Kirchhof, G., Reis, V. M., Baldani, J. I., Eckert, B., Döbereiner, J., & Hartmann, A. (1997). Occurrence, physiological and molecular analysis of endophytic diazotrophic bacteria in graminaceous energy plants. *Plant Soil* 194: 45-55.
- Kloepper, J. W., & Schroth, M. N. (1978). Plant growth-promoting rhizobacteria on radishes. In *Proc. IVth Int. Conf. Plant Pathogenic Bacteria* (pp. 879-882). Vol:2. Angers, France.

- Kloepper, J. W., Hume, D. J., Scher, F. M., Singleton, C., Tipping, B., Laliberte, M., ... & Lee, L. (1988). Plant growth-promoting rhizobacteria on canola (rapeseed). *Plant Dis* 72(1): 42-46.
- Köberl, M., Ramadan, E. M., Adam, M., Cardinale, M., Hallmann, J., Heuer, H., ... & Berg, G. (2013). Bacillus and Streptomyces were selected as broad-spectrum antagonists against soilborne pathogens from arid areas in Egypt. *FEMS Microbiol Lett* 342(2): 168-178.
- Kucey, R. M. N. (1988). Alteration of size of wheat root systems and nitrogen fixation by associative nitrogen-fixing bacteria measured under field conditions. *Can J Microbiol* 34(6): 735-739.
- Ladha, J.K., de Bruijn, F.J., & Malik, K.A. (1997). Introduction: Assessing opportunities for nitrogen fixation in rice – a frontier project. *Plant Soil* 194(1-2): 1-10.
- Lal, S., & Tabacchioni, S. (2009). Ecology and biotechnological potential of *Paenibacillus polymyxa*: a minireview. *Indian J Microbiol* 49(1): 2-10.
- Lamsal, K., & Kim, Y. S. (2013). Biocontrol of late blight and plant growth promotion in tomato using rhizobacterial isolates. *J Microbiol Biotechnol* 23(7): 897-904.
- Lee, B., Farag, M. A., Park, H. B., Kloepper, J. W., Lee, S. H., & Ryu, C. M. (2012). Induced resistance by a long-chain bacterial volatile: elicitation of plant systemic defense by a C13 volatile produced by *Paenibacillus polymyxa*. *PLoS One* 7(11): e48744.
- Lei, M., Lu, P., Jin, L., Wang, Y., Qin, J., Xu, X., ... & Wang, Y. (2015). Complete genome sequence of *Paenibacillus polymyxa* CF05, a strain of plant growth-promoting rhizobacterium with elicitation of induced systemic resistance. *Genome announcements* 3(2): e00198-15.
- Lifshitz, R., Kloepper, J. W., Scher, F. M., Tipping, E. M., & Laliberté, M. (1986). Nitrogen-fixing pseudomonads isolated from roots of plants grown in the Canadian High Arctic. *Appl Environ Microb* 51(2): 251-255.
- Lima, E., Boddey, R. M., & Döbereiner, J. (1987). Quantification of biological nitrogen fixation associated with sugar cane using a ¹⁵N aided nitrogen balance. *Soil Biol Biochem* 19(2): 165-170.
- Lynch, J. M. (1990). The rhizosphere. *Wiley Series in Ecological & Applied Microbiology* (pp. 40056575-6). John Wiley: somerset, New Jersey & Chichester, England.

- Mageshwaran, V., Walia, S., & Annapurna, K. (2012). Isolation and partial characterization of antibacterial lipopeptide produced by *Paenibacillus polymyxa* HKA-15 against phytopathogen *Xanthomonas campestris* pv. phaseoli M-5. *World J Microbiol Biotechnol* 28(3): 909-917.
- Malik, K. A., Zafar, Y., Bilal, R., & Azam, E. (1987). Use of ¹⁵N isotope dilution for quantification of N₂ fixation associated with roots of kallar grass (*Leptochloa fusca* (L.)). *Biol Fert Soils* 4(1-2): 103-108.
- 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* 194(1-2): 37-44.
- Marschner, H. (1995). *Mineral Nutrition of Higher Plants*. London: Academic. 2nd ed.
- Mok, M. C. (1994). Cytokinins and plant development: an overview. In *Cytokinins: Chemistry, Activity, and Function* (eds. Mok, D.W.S., Mok, M.C.) (pp. 155-166). CRC Press, Boca Raton, FL.
- Montañez, A., Abreu, C., Gill, P. R., Hardarson, G., & Sicardi, M. (2009). Biological nitrogen fixation in maize (*Zea mays* L.) by ¹⁵N isotope-dilution and identification of associated culturable diazotrophs. *Biol Fert Soils* 45(3): 253-263.
- Muthukumarasamy, R., Revathi, G., Seshadri, S., & Lakshminarasimhan, C. (2002). *Gluconacetobacter diazotrophicus* (syn. *Acetobacter diazotrophicus*), a promising diazotrophic endophyte in tropics. *Curr Sci India* 83(2): 137-145.
- Myrold, D. D. & Bottomley, P. J. (2007). Biological N inputs. In *Soil Microbiology, Ecology and Biochemistry* (pp. 365-388). Elsevier, Burlington, MA.
- Niu, B., Vater, J., Rueckert, C., Blom, J., Lehmann, M., Ru, J. J., ... & Borriss, R. (2013). Polymyxin P is the active principle in suppressing phytopathogenic *Erwinia* spp. by the biocontrol rhizobacterium *Paenibacillus polymyxa* M-1. *BMC Microbiol* 13(1): 137.
- Pettersson, B., Rippere, K. E., Yousten, A. A. & Priest, F. G. (1999). Transfer of *Bacillus lentimorbus* and *Bacillus popilliae* to the genus *Paenibacillus* with emended descriptions of *Paenibacillus lentimorbus* comb. nov. and *Paenibacillus popilliae* comb. nov. *Int J Syst Bacteriol* 49 Pt 2: 531-540.
- Phi, Q. T., Park, Y. M., Seul, K. J., Ryu, C. M., Park, S. H., Kim, J. G., & Ghim, S. Y. (2010). Assessment of root-associated *Paenibacillus polymyxa* groups

- on growth promotion and induced systemic resistance in pepper. *J Microbiol Biotechnol* 20(12): 1605-1613.
- Prasad, R., Kumar, M., & Varma, A. (2015). Role of PGPR in Soil Fertility and Plant Health. In *Plant-Growth-Promoting Rhizobacteria (PGPR) and Medicinal Plants* (eds. Egamberdieva, D., Shrivastava, S., Varma, A.) (pp. 247-260). Springer International Publishing, Switzerland.
- 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. *Can J For Res* 24(12): 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. *For Chron* 72(3): 293-302.
- Quadt-Hallmann, A., Kloepper, J. W., & Benhamou, N. (1997). Bacterial endophytes in cotton: mechanisms of entering the plant. *Can J Microbiol* 43(6): 577-582.
- Ratón, T. D. L. M. O., Yano, R., Gámez, O. R., Floh, E. I. S., Díaz, M. D. J. S., & Barbosa, H. R. (2012). Isolation and characterisation of aerobic endospore forming Bacilli from sugarcane rhizosphere for the selection of strains with agriculture potentialities. *World J Microb Biot* 28(4): 1593-1603.
- Ravi, A. V., Musthafa, K. S., Jegathambal, G., Kathiresan, K., & Pandian, S. K. (2007). Screening and evaluation of probiotics as a biocontrol agent against pathogenic Vibrios in marine aquaculture. *Lett Appl Microbiol* 45(2): 219-223.
- Reinhold, B., Hurek, T., Niemann, E. G., & Fendrik, I. (1986). Close association of *Azospirillum* and diazotrophic rods with different root zones of Kallar grass. *Appl Environ Microb* 52(3): 520-526.
- 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*. Knoxville, Ten. USA.
- Rennie, R. J., Fried, M., & Rennie, D. A. (1978). Concepts of ¹⁵N usage in dinitrogen fixation studies. In *Isotopes in Biological Dinitrogen Fixation* (pp. 107-131). International Atomic Energy Agency, Vienna (Austria).
- Rennie, R. J., & Larson, R. I. (1979). Dinitrogen fixation associated with disomic chromosome substitution lines of spring wheat. *Can J Bot* 57(24): 2771-2775.

- Rennie, R. J. (1981). A single medium for the isolation of acetylene reducing (dinitrogen-fixing) bacteria from soils. *Can J Microbiol* 27(1): 8-14.
- Rennie, R. J., DeFreitas, J. R., Ruschel, A. P., & Vose, P. V. (1983). ¹⁵N isotope dilution to quantify dinitrogen (N₂) fixation associated with Canadian and Brazilian wheat. *Can J Bot* 61(6): 1667-1671.
- Rennie, R. J., & Thomas, J. B. (1987). ¹⁵N-Determined effect of inoculation with N₂ fixing bacteria on nitrogen assimilation in Western Canadian wheats. In *Plant and Soil Interfaces and Interactions* (pp. 213-223). Springer Netherlands.
- Riggs, P. J., Chelius, M. K., Iniguez, A. L., Kaeppler, S. M., & Triplett, E. W. (2001). Enhanced maize productivity by inoculation with diazotrophic bacteria. *Funct Plant Biol* 28(9): 829-836.
- Robertson, G. P., & Vitousek, P. M. (2009). Nitrogen in agriculture: balancing the cost of an essential resource. *Ann Rev Environ Resour* 34: 97-125.
- Rosenblueth, M., & Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Mol Plant Microbe In* 19(8): 827-837.
- Ruschel, A.; Henis, Y. & Salati, E. (1975). Nitrogen-15 tracing of N₂-fixation with soil-grown sugarcane seedlings. *Soil Biol Biochem* 7(2): 181-182.
- Rybakova, D., Wetzlinger, U., Müller, H., & Berg, G. (2015). Complete genome sequence of *Paenibacillus polymyxa* strain Sb3-1, a soilborne bacterium with antagonistic activity toward plant pathogens. *Genome Announcements* 3(2): e00052-15.
- Ryu, C. M., Kim, J., Choi, O., Kim, S. H., & Park, C. S. (2006). Improvement of biological control capacity of *Paenibacillus polymyxa* E681 by seed pelleting on sesame. *Biol Control* 39(3): 282-289.
- Senthilkumar, M., Swarnalakshmi, K., Govindasamy, V., Lee, Y. K., & Annapurna, K. (2009). Biocontrol potential of soybean bacterial endophytes against charcoal rot fungus, *Rhizoctonia bataticola*. *Curr Microbiol* 58(4): 288-293.
- Shrestha, R. K., & Ladha, J. K. (1996). Genotypic variation in promotion of rice dinitrogen fixation as determined by nitrogen-15 dilution. *Soil Sci Soc Am J* 60(6): 1815-1821.
- Siddiqui, Z. A., Baghel, G., & Akhtar, M. S. (2007). Biocontrol of *Meloidogyne javanica* by *Rhizobium* and plant growth-promoting rhizobacteria on lentil. *World J Microbiol Biotechnol* 23(3): 435-441.

- Son, S. H., Khan, Z., Kim, S. G., & Kim, Y. H. (2009). Plant growth- promoting rhizobacteria, *Paenibacillus polymyxa* and *Paenibacillus lentimorbus* suppress disease complex caused by root- knot nematode and fusarium wilt fungus. *J Appl Microbiol* 107(2): 524-532.
- Soyatech (n.d.). Canola Facts and Information. Available at: http://www.soyatech.com/canola_facts.htm (accessed on: July 21, 2015).
- Statistics Canada (n.d.). *Table 001-0010 - Estimated areas, yield, production and average farm price of principal field crops, in metric units, annual*, CANSIM (database). Available from: <http://www5.statcan.gc.ca/subject-sujet/resultat/resultat?pid=920&id=2024&lang=eng&type=ARRAY&sortType=1&pageNum=2> (accessed on July 21, 2015).
- Stephan, M. P., Oliveira, M., Teixeira, K. R. S., Martinez-Drets, G., & Döbereiner, J. (1991). Physiology and dinitrogen fixation of *Acetobacter diazotrophicus*. *FEMS Microbiol Lett* 77(1): 67-72.
- Talboys, P. J., Owen, D. W., Healey, J. R., Withers, P. J., & Jones, D. L. (2014). Auxin secretion by *Bacillus amyloliquefaciens* FZB42 both stimulates root exudation and limits phosphorus uptake in *Triticum aestivum*. *BMC Plant Biol* 14(1): 51.
- Tenuta, M. (2003). Plant Growth Promoting Rhizobacteria: Prospects for Increasing Nutrient Acquisition and Disease Control. In *Proc. 4th Annual Manitoba Agronomists Conference* (pp. 72-77). Winnipeg, Canada. Available from: http://www.umanitoba.ca/faculties/afs/MAC_proceedings/2003/pdf/tenuta_rhizobacteria.pdf (accessed on: July 21, 2015).
- Tian, G., Pauls, P., Dong, Z., Reid, L. M., & Tian, L. (2009). Colonization of the nitrogen-fixing bacterium *Gluconacetobacter diazotrophicus* in a large number of Canadian corn plants. *Can J Plant Sci* 89(6): 1009-1016.
- 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. *Mol Plant Microbe In* 12(11): 951-959.
- Timmusk, S., Nicander, B., Granhall, U., & Tillberg, E. (1999). Cytokinin production by *Paenibacillus polymyxa*. *Soil Biol Biochem* 31(13): 1847-1852.

- Timmusk, S., Grantcharova, N., & Wagner, E. G. H. (2005). *Paenibacillus polymyxa* invades plant roots and forms biofilms. *Appl Environ Microb* 71(11): 7292-7300.
- Timmusk, S., Van West, P., Gow, N. A. R., & Paul Huffstutler, R. (2009). *Paenibacillus polymyxa* antagonizes oomycete plant pathogens *Phytophthora palmivora* and *Pythium aphanidermatum*. *J Appl Microbiol* 106(5): 1473-1481.
- United States Department of Agriculture, Economic Research Service (n.d.). Soyabeans and Oil Crops: Canola. Available at: <http://www.ers.usda.gov/topics/crops/soybeans-oil-crops/canola.aspx> (accessed on: July 21, 2015).
- United States Department of Agricultural, Foreign Agricultural Services (2015). *World Agricultural Production, Circular Series WAP 7-15*, July 2015. Available at: <http://apps.fas.usda.gov/psdonline/circulars/production.pdf> (accessed on: July 21, 2015).
- Van Loon, L. C., Bakker, P. A. H. M., & Pieterse, C. M. J. (1998). Systemic resistance induced by rhizosphere bacteria. *Ann Rev Phyto* 36(1): 453-483.
- Vessey, J. K. (2003). Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil* 255(2): 571-586.
- Vitousek, P. M., & Howarth, R. W. (1991). Nitrogen limitation on land and in the sea: how can it occur? *Biogeochemistry* 13(2): 87-115.
- Vivas, A., Biro, B., Ruiz-Lozano, J. M., Barea, J. M., & Azcon, R. (2006). Two bacterial strains isolated from a Zn-polluted soil enhance plant growth and mycorrhizal efficiency under Zn-toxicity. *Chemosphere* 62(9): 1523-1533.
- von der Weid, I., Paiva, E., Nóbrega, 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(5): 369-381.
- Walker, T. S., Bais, H. P., Grotewold, E., & Vivanco, J. M. (2003). Root exudation and rhizosphere biology. *Plant Physiol* 132(1): 44-51.
- 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(3): 833-839.
- Weller, D. M., & Thomashow, L. S. (1994). Current challenges in introducing beneficial microorganisms into the rhizosphere. In *Molecular ecology of*

- rhizosphere microorganisms: biotechnology and the release of GMOs* (eds F. O'Gara, D. N. Dowling and B. Boesten) (pp. 1-18), Wiley-VCH Verlag GmbH, Weinheim, Germany.
- Werner, G. D. A., Cornwell, W. K., Sprent, J. I., Kattge, J., & Kiers, E. T. (2014). A single evolutionary innovation drives the deep evolution of symbiotic N₂-fixation in angiosperms. *Nat Commun* 5: 4087.
- Weyens, N., van der Lelie, D., Taghavi, S., & Vangronsveld, J. (2009). Phytoremediation: plant–endophyte partnerships take the challenge. *Current Opin Biotech* 20(2): 248-254.
- Yang, H. (2015). Effects of *paenibacillus polymyxa* and soil nitrogen on lodgepole pine (*Pinus contorta* var. *Latifolia* (dougl.) Engelm.) seedling growth (Master's Thesis). Available at: https://circle.ubc.ca/bitstream/handle/2429/53004/ubc_2015_september_yang_henry.pdf?sequence=11 (accessed on: July 21, 2015).
- Zeigler, D. R. (2013). The Family Paenibacillaceae, Bacillus Genetic Stock Center Catalog of Strains Part 5. *Bacillus Genetic Stock Center*. Columbus, OH, USA. Available from: http://bgsc.org/_catalogs/Catpart5.pdf (accessed on: July 21, 2015).
- Zhou, W. W., Huang, J. X., & Niu, T. G. (2008). Isolation of an antifungal Paenibacillus strain HT16 from locusts and purification of its medium-dependent antagonistic component. *J Appl Microbiol* 105(3): 912-919.

Appendix A – Plant Nutrient Solution³

KH_2PO_4	0.14g/L
H_3BO_3	0.001g/L
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.001g/L
$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	0.001g/L
$\text{Na}_2\text{Fe EDTA}$	0.025g/L
MgSO_4	0.49g/L
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.001g/l
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.0001g/L
$\text{Ca}^{15}(\text{NO}_3)_2$	0.0576g/L

³ Modified from Chanway *et al.* (1988)

Appendix B – Steps to Prepare Combined Carbon Medium (CCM)

Solution 1:

Sucrose	5 g/L
Mannitol	5 g/L
Sodium Lactate (ml, 60%, v/v)	0.5 ml/L
K ₂ HPO ₄	0.80 g/L
KH ₂ PO ₄	0.20 g/L
NaCl	0.10 g/L
Na ₂ MoO ₄ .2H ₂ O	0.025 g/L
Na ₂ FeEDTA	0.028 g/L
Yeast Extract	0.1 g/L
Distilled Water	900ml

Solution 2:

MgSO ₄ .7H ₂ O	0.20 g/L
CaCl ₂	0.06 g/L
Distilled water	100 ml

Autoclave solution 1 and 2 separately, cool and mix.

Add filter sterilized Biotin: 5µg/L and Para Amino Benzoic Acid (PABA): 10µg/L

Appendix C – Recipe to Make Phosphate Buffered Saline

NaCl	8 g/L
KH ₂ PO ₄	0.24 g/L
KCl	0.2 g/L
Na ₂ HPO ₄	1.44 g/L
Distilled Water	1 L

Appendix D – Additional Figures

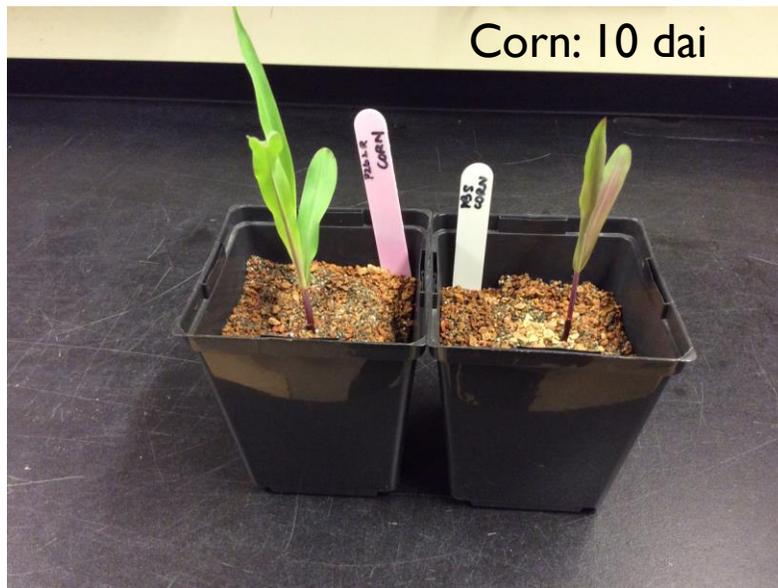


Figure D.1 P2b-2R-inoculated (left) and control (right) seedling of corn plant harvested 10 days after inoculation (dai) showing clear difference in length, biomass and plant health

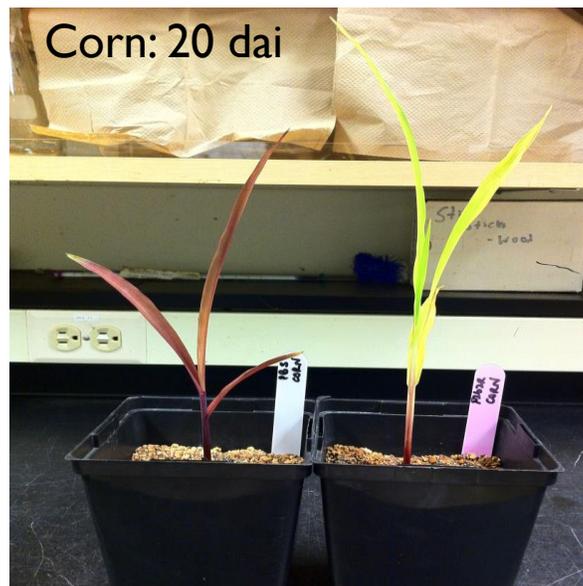


Figure D.2 P2b-2R-inoculated (right) and control (left) seedling of corn plant harvested 20 days after inoculation (dai) showing clear difference in length, biomass and plant health



Figure D.3 P2b-2R-inoculated (left) and control (right) seedling of corn plant harvested 30 days after inoculation (dai) showing clear difference in length, biomass and plant health

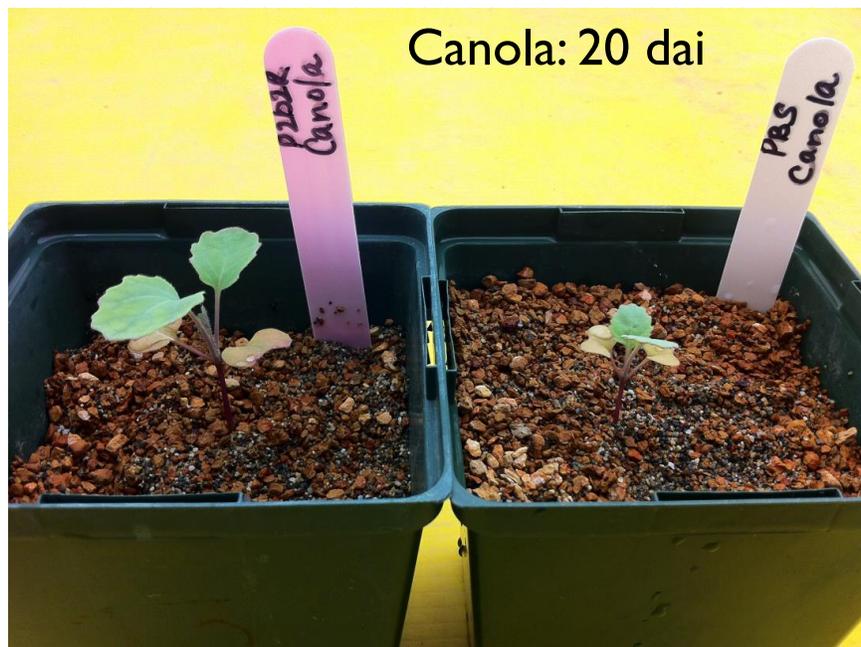


Figure D.4 P2b-2R-inoculated (left) and control (right) seedling of canola plant harvested 20 days after inoculation (dai) showing clear difference in length, biomass and plant health

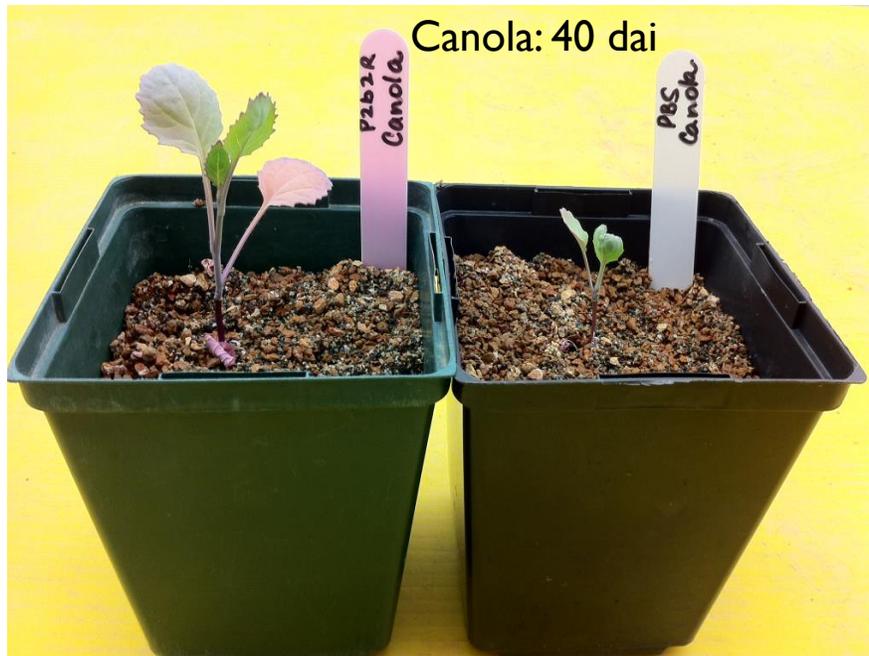


Figure D.5 P2b-2R-inoculated (left) and control (right) seedling of canola plant harvested 40 days after inoculation (dai) showing clear difference in length, biomass and plant health



Figure D.6 P2b-2R-inoculated (right) and control (left) seedling of canola plant harvested 60 days after inoculation (dai) showing clear difference in length, biomass and plant health*