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Effects of *Paenibacillus polymyxa* inoculation and different soil nitrogen treatments on lodgepole pine seedling growth

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1 **Effects of *Paenibacillus polymyxa* inoculation and different soil nitrogen**
2 **treatments on lodgepole pine seedling growth**

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Abstract

An endophytic diazotroph, *Paenibacillus polymyxa* P2b-2R, has been shown to colonize internal tissues of lodgepole pine and stimulate seedling growth possibly through nitrogen (N) fixation in an N-limited environment. We evaluated the effects of different soil N concentrations on lodgepole pine seedling growth after inoculation with P2b-2R in a year-long greenhouse study. Seedlings were subjected to monthly application of a nutrient solution containing one of four soil N concentrations as $\text{Ca}(\text{NO}_3)_2$ (5% ^{15}N label): 0.0029, 0.029, 0.29 and 2.9 $\mu\text{mol/L}$, referred to as ‘very low’, ‘low’, ‘medium’ and ‘high’ soil N treatments, respectively. Foliar ^{15}N atom% excess and foliar N content values of P2b-2R inoculated seedlings were not significantly different from controls at any N treatment. Similarly, rhizospheric and endophytic population of P2b-2R did not vary significantly across N treatments. Inoculation with P2b-2R resulted in seedling growth inhibition during early stages of the experiment but by month twelve, inoculated seedlings from the very low soil N treatment had accumulated 56.3% and 46.4% more root and shoot biomass than controls, respectively. These results suggest that pine biomass stimulation by P2b-2R may depend on soil N concentrations and that such growth stimulation may occur without providing significant amounts of fixed N.

Keywords: Lodgepole pine; *Paenibacillus polymyxa*; Biological nitrogen fixation; Plant growth promotion; Endophytic diazotroph

25 Introduction

26 Bacteria inhabiting the nutrient-rich microhabitat of rhizosphere, i.e., the soil immediately
27 surrounding plant roots, are known as rhizobacteria and are thought to benefit from the abundant
28 organic compounds released by roots as a ready source of nutrients. However, antagonistic
29 interactions such as parasitism (Cacciari et al. 1986) and competition with plants and other
30 microbes for these nutrients (Cocking 2003) may reduce the abundance of certain rhizobacterial
31 species including those known to exert positive effects on plant growth. In addition, a subset of
32 plant-colonizing bacteria and rhizobacteria may acquire these nutrients by colonizing plants
33 internally and stimulating plant growth in return. The term 'bacterial endophyte' is used to
34 describe a bacterium that colonizes internal plant tissues without inducing disease symptoms
35 (Hallmann et al. 1997; Azevedo et al. 2000). By occupying the plant interior, bacterial
36 endophytes may have a comparative advantage over rhizosphere-colonizing bacteria due to ready
37 access to continuous supplies of nutrients and an additional degree of protection from
38 competition and predation that likely occur outside plant roots (Hallmann et al. 1997).

39 The genus *Paenibacillus* was assigned to accommodate the former 'group 3' of the genus
40 *Bacillus* (Ash et al. 1993). In Latin *paene* means *almost*, and therefore the *Paenibacillus* is
41 almost a *Bacillus*. *Paenibacillus polymyxa* is a facultative anaerobe that is non-pathogenic and
42 form endospores (Lal and Tabacchioni 2009). It is found in environments such as plant roots,
43 soil, and marine sediment (Timmusk et al. 2005; Ravi et al. 2007). The wide range of potential
44 plant growth promoting characteristics of these bacteria includes the ability to fix N and produce
45 plant growth-promoting hormones and solubilize soil phosphorous (Lal and Tabacchioni 2009).

N-fixing bacteria are commonly referred to as diazotrophs and may be free-living or occur in symbiotic associations. Free-living diazotrophs are self-sustaining and do not fix N unless the amount of available N in their intermediate surroundings limit their growth. While the general belief is that free-living diazotrophs do not contribute large quantities of fixed N to most terrestrial ecosystems (Postgate 1998; Newton 2007), they are known to provide a biologically significant proportion of fixed N in some tropical and temperate forest ecosystems (Cleveland et al. 1999; Gehring et al. 2005; Reed and Martiny 2007). Significant amounts of N may be provided by endophytic diazotrophs that do not induce macroscopic structural changes on or in the plant hosts (Dobereiner et al. 1995; Malik et al. 1997).

In comparison with well-characterized N fixing systems in agricultural crops, studies of diazotrophs within conifers, particularly those of commercial importance, have been scarce. In North America, soil N deficiency is widespread and can limit the growth of tree seedlings in the boreal forest and other ecosystems (Mahendrappa and Salonijs 1982). However, the growth of certain forest tree species such as lodgepole pine (*Pinus contorta* var. *latifolia* Engelm. ex S. Watson) appears to be less affected by low soil N availability (Chapman and Paul 2012). The ability of lodgepole pine to persist on N-limited soils may relate to its physiological traits and association with N-fixing microbes living in rhizosphere. But according to Holl and Chanway (1992), rhizospheric biological N fixation (BNF) contribute only 4% to the total foliar N of lodgepole pine, indicating that this process might not be able to enhance seedling growth in the short term. A consortium of endophytic diazotrophic bacteria were isolated from internal tissues of naturally regenerating lodgepole pine seedlings at a N-limited site near Williams Lake, BC, Canada and used to test the hypothesis that such microorganisms could stimulate lodgepole pine seedling growth through BNF (Bal et al. 2012). One strain, *Paenibacillus polymyxa* strain P2b-

2R, was subsequently shown to be able to grow on N-free media, to consistently display *in vitro* nitrogenase (acetylene reduction) activity (Bal et al. 2012) and was found to harbour the *nifH* gene that encodes nitrogenase (Anand and Chanway 2013c). When P2b-2R was re-introduced to lodgepole pine, it was able to form persistent rhizospheric (Bal and Chanway 2012a) and endophytic (Anand *et al.* 2013) populations, derive significant amounts of N from atmosphere (79%) and enhance seedling biomass (Anand et al. 2013). *P. polymyxa* P2b-2R was also tested for its broad range host capability and was found to colonize, fix N and promote growth of another tree species, western red cedar (Bal and Chanway 2012b; Anand and Chanway 2013b), as well as the agricultural crops: corn (Puri et al. 2015; Puri et al. 2016b), canola (Puri et al. 2016a) and tomato (Padma et al. 2016). Using a green fluorescent protein (GFP) tagged derivative of P2b-2R and confocal laser scanning microscopy, intracellular colonization of lodgepole pine was also confirmed (Anand and Chanway 2013a). The significant contribution of fixed N to seedlings and the concomitant biomass enhancement of lodgepole pine seedlings by P2b-2R suggest that lodgepole pine may benefit from diazotrophic endophytes in N-deficient soils. However, whether this mutualistic relationship continues to exist at higher soil N concentrations is unclear as high amounts of exogenous N are known to suppress *in vitro* nitrogenase activity in other symbiotic systems (Dixon and Kahn 2004; Wang et al. 2013) and can trigger specific metabolic and enzymatic activities in plants (Abellan et al. 1994), which may result in reduced populations of endophytic diazotrophs (Fuentes-Ramirez et al. 1999; Munoz-Rojas and Caballero-Mellado 2003).

The primary objective of this study was to determine if the amount of N fixed by *P. polymyxa* P2b-2R treated lodgepole pine seedlings decreased in response to increasing soil N concentrations. To address this objective, we tested the following hypotheses: (i) rhizospheric

and endophytic P2b-2R population sizes will decrease with increasing soil N concentrations when inoculated in lodgepole pine, (ii) the percentage of N derived from the atmosphere (%Nd_{fa}) by lodgepole pine seedlings inoculated with P2b-2R will decrease as soil N concentrations increase, and (iii) biomass of lodgepole pine seedlings treated with P2b-2R will be greatest at the low soil N concentrations.

Materials and methods

Seed and bacteria

Lodgepole pine seeds were obtained from the BC Ministry of Forests Tree Seed Centre, Surrey, BC, Canada and originated from a provenance located near Williams Lake, BC, Canada. *Paenibacillus polymyxa* strain P2b was isolated from surface-sterilized stem tissue of a lodgepole pine seedling that was naturally regenerating near Williams Lake, BC, Canada (52°05' N lat., 122°54' W long., elevation 1300m, Sub-Boreal Pine Spruce, SBPSdc Zone) (Bal et al. 2012). *P. polymyxa* P2b-2R is a spontaneous mutant that was derived from strain P2b grown on combined carbon medium (CCM) agar amended with 200mg/L rifamycin (Bal et al. 2012). Strain P2b-2R was stored at -80 °C on CCM amended with 20% (v/v) glycerol.

Seed inoculation and seedling growth

Seedlings were grown in Ray Leach cone-tainers (210mm height x 38mm diameter) that were filled to 67% capacity with an autoclaved sand-Turface mixture (69% w/w silica sand; 29% w/w Turface; 2% w/w CaCO₃). Two seed inoculation “levels” (i.e., inoculated with (i) live P2b-2R and (ii) sterile phosphate buffered saline (PBS)) were evaluated at four soil N concentrations (5.9, 5.9x10¹, 5.9x10² and 5.9x10³ µM, which corresponded to very low, low, medium and high

113 N treatments, respectively), resulting in eight treatment combinations. In total, 45 seedlings were
114 used in each treatment (altogether eight treatments) in this yearlong experiment. Prior to
115 inoculation, each cone-tainer was fertilized to saturation with 20mL of a nutrient solution
116 (Chanway et al. 1988), which was modified by replacing KNO_3 and $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ with
117 $\text{Ca}(^{15}\text{NO}_3)_2$ (5% ^{15}N label) and Sequestrene 330Fe with Na_2FeEDTA (0.02g/L). The modified
118 plant nutrient solution contained one of the four N concentrations described above as well as
119 (g/L): KH_2PO_4 , 0.14; MgSO_4 , 0.49; H_3BO_3 , 0.001; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.001; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001;
120 $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.0001; and $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.001.

121 Lodgepole pine seeds were surface sterilized by immersion in 30% hydrogen peroxide for
122 90 seconds followed by three 30sec rinses in sterile distilled water. After surface sterilization, ten
123 randomly selected seeds were imprinted on tryptic soy agar (TSA) and incubated at 30°C for two
124 days to check for surface contamination. Uncontaminated lodgepole pine seeds were then placed
125 in sterile cheesecloth bags and stratified on moist, sterile sand for five weeks at 4°C. Ten
126 randomly selected stratified seeds were then checked for internal contamination by *P. polymyxa*
127 P2b-2R by aseptically crushing seeds and plating the resulting extracts on TSA amended with
128 200mg/L rifamycin. Plates were examined for contamination two days later. Three surface-
129 sterilized seeds were sown aseptically in each cone-tainer and covered with 5mm of autoclaved
130 silica sand. Bacterial inoculum was prepared by thawing a frozen culture of strain P2b-2R,
131 streaking a loopful onto CCM agar amended with 200mg/L rifamycin, and incubating at 30°C
132 for two days. A 1-L flask containing 500mL of CCM broth amended with rifamycin was then
133 inoculated with a loopful of bacterial growth from the agar, secured on a rotary shaker and
134 agitated (150 rpm) at room temperature for two days. Bacterial cells were harvested by
135 centrifugation (3000 x g; 30 min), washed twice in sterile PBS (pH 7.4) and resuspended in the

same buffer to a density of ca. 10^6 cfu/mL. P2b-2R-PBS suspension (5mL) was then pipetted into each cone-tainer designated for live P2b-2R treatment. This process was repeated using 5mL of sterile PBS without bacteria for non-inoculated (control) cone-tainers. All cone-tainers were placed in trays (98 cells) and transported to the greenhouse at the University of British Columbia, Vancouver, BC, Canada. A 16-h photoperiod (6am to 10pm) with an intensity of at least $300\mu\text{mol/s/m}^2$ was achieved with a combination of natural and artificial light in the greenhouse. Greenhouse had glass walls and roof to allow the natural light to come in and artificial lighting (incandescent tubes and fluorescent bulbs) was used to supplement the natural light so as to keep the light intensity of at least $300\mu\text{mol/s/m}^2$ inside the greenhouse. Seedlings were watered as required with tap water and thinned to the largest single germinant per cone-tainer two weeks after sowing. Seedlings received 20mL of modified nutrient solution containing one of the four concentrations of $\text{Ca}^{15}\text{NO}_3)_2$ once per month during the twelve-month growth trial. Tray position was randomized weekly to reduce positional effects during the experiment.

Rhizospheric and endophytic colonization

For evaluation of rhizosphere colonization, three randomly selected seedlings from each treatment were harvested destructively four, eight and twelve months after sowing. Seedlings were removed from cone-tainers, and loosely adhering soil particles were removed from roots with gentle shaking. Roots were then separated from shoots, placed in sterile Falcon tubes (50mL) filled with 10mL of autoclaved PBS and shaken on a vortex mixer at 1000 rpm for one minute. Ten-fold serial dilutions were performed, and 100 μL aliquots were then plated on CCM plates amended with rifamycin (200mg/L) and cycloheximide (100mg/L). Plates were incubated at room temperature for seven days, after which colonies were counted. Roots were washed and

oven-dried at 65°C for two days before weighing. Rhizospheric bacterial populations were then calculated as the number of cfu/g dry root.

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

Analysis of foliar N and seedling biomass

Nine seedlings from each treatment were harvested destructively four, eight and twelve months after sowing for evaluation of seedling biomass, foliar N and ¹⁵N content. Root and shoot of seedlings were separated and dried at 65°C for two days before weighing to know the biomass. Needles were then removed from shoot, weighed, ground to a particle size of less than 1mm and mixed thoroughly. For foliar N analysis, a 5mg subsample of ground oven dried foliage from each seedling was sent to the Stable Isotope Facility at the University of California, Davis, CA, USA. Foliar N content and % ¹⁵N excess were determined with an elemental analyzer interfaced

with an isotope ratio mass spectrometer. The amount of fixed N in foliage can be estimated by calculating %Ndfa (Rennie et al. 1978) as follows:

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

Statistical analyses

For the seedling growth experiment, the eight treatment combinations, i.e., two inoculation levels x four soil N concentrations, were arranged according to a completely randomized design. A two-way analysis of variance (ANOVA) was performed using SAS 9.3 (Copyright (c) 2012, SAS Institute Inc., Cary, NC, USA) to evaluate the effects of bacterial inoculation and soil N concentration on growth parameters (Foliar N content, ^{15}N atom percent excess, total foliar N, shoot biomass and root biomass), four, eight and twelve months after inoculation. The confidence level, α , was set to 0.05 to determine the significance of the model and treatment effects. Bacterial colony counts were log transformed prior to analysis in order to meet the statistical assumptions of normally distributed residuals and variance homogeneity.

Results

Rhizospheric and endophytic colonization

P. polymyxa P2b-2R was recovered from the rhizosphere of inoculated seedlings at each harvest. Rhizosphere population densities ranged from 3.1 to 4.4 log cfu/g dry root and did not differ significantly with soil N concentration (Fig. 1). With the exception of those from the high N treatment where P2b-2R densities increased over time, rhizosphere population densities tended to

decline between months four and twelve (Fig. 1). P2b-2R was not recovered from surface-disinfected lodgepole pine tissues until the final harvest when the modified surface-sterilization protocol was used. *Paenibacillus polymyxa* strain P2b-2R was detected inside root and stem tissues but not inside needles in twelve month-old seedlings (Fig. 2).

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

Foliar N content (FNC) generally increased between four and eight months after sowing and inoculation and then declined thereafter (Table S1). Inoculation generally had insignificant effects on foliar N content during the experiment. Seedling total foliar N (TFN) was affected primarily by soil N concentration and not by bacterial inoculation (Table S2). Seedlings from the high soil N treatment generally accumulated more foliar N than those receiving lower soil N concentrations but inoculation had no clear effect on seedling TFN. Foliar ^{15}N atom percent excess increased with increasing soil N concentrations (Table S3), but was unaffected by inoculation. Due to lack of significant inoculation effects on ^{15}N atom percent excess ($p > 0.10$), %Ndfa was not calculated.

Seedlings inoculated with *Paenibacillus polymyxa* P2b-2R generally had less root and shoot biomass compared to their respective controls four months after sowing and inoculation (Table 1). Seedling growth inhibition appeared to be greatest in the low soil N treatment after four months of inoculation, where inoculated seedlings accumulated 29.7% and 37.0% less root and shoot biomass, respectively. However, this tendency was not apparent by month eight as root and shoot biomass of inoculated and non-inoculated seedlings did not differ across four N treatments (Table 1) and by month twelve, inoculated seedlings from the very low N treatment had a 52.3% and 46.4% increase in root and shoot biomass, respectively. Seedlings from the

medium N treatment responded similarly to inoculation, but no such effects were observed at the low and high soil N concentrations.

Discussion

Recovery of *P. polymyxa* P2b-2R from the rhizosphere of lodgepole pine seedlings harvested four, eight and twelve months after inoculation suggests that this strain is a competent rhizosphere colonizer. By the end of the twelve-month growth trial P2b-2R densities were comparable to other reported studies about this bacterial strain (Anand et al. 2013; Bal and Chanway 2012a). Despite the large differences in the amount of added N among the three lowest soil N treatments, *i.e.*, 10-100 fold, P2b-2R population sizes decreased over time and stabilized to a density of approximately 3.1 log cfu/g dry root, twelve months after inoculation. Such a decline of bacterial population densities with seedling age has been reported for *Bacillus* strains colonizing the rhizosphere of lodgepole pine (Holl and Chanway 1992) and spruce (*Picea glauca* (Moench) Voss x *Picea engelmannii* Parry ex Engelm.) (Shishido and Chanway 2000). Since root exudates are the most probable carbon source that could sustain rhizosphere-colonizing microorganisms (Bais et al. 2006), the population decrease of P2b-2R may be attributed to a decrease in the amount of photosynthate translocated to the root as plants age (Keith and Oades 1986; Domanski et al. 2001).

Endophytic P2b-2R has been isolated from stem and root tissues of lodgepole pine seedlings as early as two weeks after inoculation (Anand and Chanway 2013a), however we were not able to isolate P2b-2R from surface-sterilized tissues of lodgepole pine until twelve months after inoculation. Unsuccessful recovery of endophytes has been observed when sodium hypochlorite was used to sterilize plant samples (McInroy et al. 1996). At micro molar

concentrations, sodium hypochlorite is known to significantly reduce microbial populations (Nakagawara et al. 1998) and may render injured bacterial cells non-culturable by predisposing them to oxidative stress (Dukan et al. 1999) and DNA damage, which can result in loss of antibiotic resistance (Dukan et al. 1999; Heisiq 1996). When the surface sterilization protocol was revised by reducing the concentration of sodium hypochlorite from 1.3% to 0.6%, endophytic P2b-2R was detected in root and stem tissues of twelve-month-old lodgepole pine (Fig. 2). This reduction in concentration of sodium hypochlorite was efficient enough to kill all microbes except the endophytic P2b-2R. The average endophytic population densities were similar to those reported by Anand et al. (2013) and were much higher than those isolated from the rhizosphere in this study, four, eight or twelve months after inoculation. This indicates that the lodgepole pine interior may be a more protected habitat that allows P2b-2R to reach higher population densities than in the rhizosphere.

The primary objective of this study was to determine if the amount of N fixation by lodgepole pine seedlings colonized by *P. polymyxa* P2b-2R would decrease in response to increasing soil N concentrations. However, even though a decrease in seedling foliar N content was observed across four soil N treatments between months eight and twelve (Table S1) indicating that lodgepole pine seedlings experienced N deficiency, we were unable to detect significant foliar ^{15}N dilution (hence BNF) in the assay system. In fact, twelve months after inoculation, the foliar N content of seedlings from very low, low and medium soil N treatments was at a level that is considered 'very severely deficient' for lodgepole pine (Ballard and Carter 1986). These results are not in agreement with those reported in previous studies using a glass-tube plant growth and inoculation system (Bal and Chanway 2012a; Anand et al. 2013). Several differences between the two seedling growth trials may have contributed to these discrepant

findings. Unlike the glass-tube assay system, where N-fertilizer was applied once, *i.e.*, at the time of sowing and inoculation, monthly application of N-containing fertilizer was used in our experiment to reduce leaching of nitrate from the bottom of cone-tainers. If our inoculated seedlings were deriving N from the atmosphere, the frequency of N-fertilization may have obscured our ability to detect ^{15}N foliar dilution. In addition, the larger rooting volume provided by cone-tainers might have resulted in enhanced soil N uptake, which is known to suppress BNF (Shrestha and Ladha 1996). There is a possibility that this bacterial strain might have infected the rhizosphere and internal tissues but was not able to form a mutualistic relationship with the plant and fix N. A possible reason for this could be that, since the N-fertilizer was applied every month, conditions were not optimum (N-limited) for bacteria to trigger N-fixation mechanism (Bal and Chanway 2012a, b).

Seedling biomass reduction by P2b-2R inoculation in lodgepole pine seedlings was previously reported in a study (Bal and Chanway 2012a). In our cone-tainer system, the inhibitory effects on seedling biomass were also observed four months after inoculation (Table 1). Given that N-fixation is an energetically expensive process, poorer performance of lodgepole pine seedlings inoculated with P2b-2R has been suggested to result from the maintenance of BNF at the expense of plant-derived carbon substrates (Bal and Chanway 2012a). However, due to the insignificant inoculation effects on foliar ^{15}N dilution (Table S3), it is not possible to relate seedling biomass responses to BNF. Nonetheless, considering that inoculated seedlings in all soil N treatments had lower root and shoot biomass than their controls after four and eight months of inoculation (Table 1), it could be hypothesized that P2b-2R can act as a parasite (at least temporarily), depending on lodgepole pine for carbon substrates without providing fixed N in return. Since low soil N availability can limit a plant's ability to produce photosynthate, the

greater magnitude of biomass reduction was observed at the very low and low N levels at the first and second harvest (Table 1). Our results suggest that lodgepole pine seedling growth inhibition by P2b-2R can occur at a wide range of soil N concentrations and characterizes early stages of the lodgepole pine x P2b-2R interaction. The pronounced seedling biomass enhancement by P2b-2R that was observed twelve months after inoculation for very low N treatment (Table 1) is in agreement with previous results (Anand et al. 2013), and supports the hypothesis that a mutualism exists between lodgepole pine and this bacterium. Biomass accumulation due to bacterial inoculation was more pronounced in the very low N treatment, since inoculated seedlings from the very low N treatment had a 52.3% and 46.4% increase in root and shoot biomass, respectively. This is in agreement with the claim reported in previous studies about this bacterial strain (Bal and Chanway 2012a, b; Anand and Chanway 2013b), that inoculated seedlings trigger N fixation process when cultured in N-limited conditions. Biologically fixed N is stored in the plant thus increasing the biomass. Therefore, our data support the hypothesis that seedling growth promotion by P2b-2R is more pronounced at the low soil N concentrations. Seedlings growth inhibition was observed at high soil N treatment level during this whole year-long growth trail, supporting the hypothesis (Anand et al. 2013) that plant growth promotion by P2b-2R occurs in N-limited conditions. Anand et al. (2013) suggested that P2b-2R enhanced performance of lodgepole pine seedlings through BNF. However, the same bacterial strain in our experiment was able to stimulate lodgepole pine biomass without affecting foliar ^{15}N atom % excess (Table S3), suggesting that P2b-2R might promote the growth of our lodgepole pine seedlings without providing appreciable amounts of fixed N. It is well known that other *P. polymyxa* strains can enhance plant performance by producing phytohormones

(Chanway 2008), but the ability of P2b-2R to synthesize plant growth regulators such as auxins and cytokinins requires further assessment.

To conclude, we observed that P2b-2R acts as a parasite by suppressing the growth of lodgepole pine during the initial stages of plant growth. But once established inside the plant, it forms a mutualistic relation by taking carbon from the plant and promoting plant growth in return at very low and medium N concentrations. It was also observed that this bacterial strain wasn't able to fix N from the atmosphere but was still able to stimulate plant growth, likely through other means.

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Table 1 Seedling biomass (mg ± standard error; n = 9) of lodgepole pine seedlings inoculated with *P. polymyxa* P2b-2R and phosphate-buffered saline (control) measured four, eight and twelve months after inoculation at different soil N treatments

N treatment	Inoculation	Months after sowing					
		4		8		12	
		Root	Shoot	Root	Shoot	Root	Shoot
Very Low	P2b-2R	16.1 ± 2.34	23.1 ± 2.43	53.3 ± 9.60	39.1 ± 9.71	143 ± 19.8*	100 ± 14.0*
	Control	19.0 ± 1.23	24.0 ± 2.41	46.5 ± 5.12	35.1 ± 2.81	94.1 ± 13.4	68.8 ± 8.62
Low	P2b-2R	16.1 ± 2.55	16.9 ± 1.31*	39.8 ± 7.93	38.5 ± 4.54	139 ± 18.6	116 ± 11.4
	Control	22.8 ± 4.13	26.9 ± 3.50	42.2 ± 11.0	31.4 ± 3.33	148 ± 24.4	107 ± 13.0
Medium	P2b-2R	22.9 ± 2.42	22.2 ± 1.52	48.2 ± 12.8	40.3 ± 6.14	184 ± 27.9*	148 ± 19.4*
	Control	25.1 ± 3.33	24.8 ± 2.84	57.2 ± 8.23	41.2 ± 6.72	120 ± 23.9	102 ± 15.3
High	P2b-2R	36.4 ± 4.63	35.7 ± 2.12	84.4 ± 12.3	60.8 ± 4.41	195 ± 65.2	200 ± 46.3
	Control	41.9 ± 6.34	36.8 ± 2.61	67.8 ± 11.9	64.4 ± 11.8	196 ± 62.4	216 ± 45.6

*P<0.05 (significantly different from control). Student’s t-test was used to determine the significant difference between two inoculation treatments.

450 **Figure Legends**

451 **Fig. 1** Rhizospheric population size of *P. polymyxa* P2b-2R in lodgepole pine seedlings
452 evaluated four, eight and twelve months after inoculation at different soil N treatments (very low,
453 low, medium and high). Error bars represent standard error of the mean (n = 3).

454 **Fig. 2** Endophytic population size of *P. polymyxa* P2b-2R in lodgepole pine seedlings evaluated
455 twelve months after inoculation at different soil N treatments (very low, low, medium and high).
456 Error bars represent standard error of the mean (n = 3).

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457 **Fig. 1**

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459 **Fig. 2**

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Supplementary material: Effects of *Paenibacillus polymyxa* inoculation and different soil nitrogen treatments on lodgepole pine seedling growth

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Contents: Supplementary Tables S1, S2, S3

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Table S1 Foliar N content (percentage w/w $\pm$ standard error; n = 9) of lodgepole pine seedlings inoculated with *P. polymyxa* P2b-2R and phosphate-buffered saline (control) measured four, eight and twelve months after inoculation

N treatment	Inoculation	Months after sowing		
		4	8	12
Very Low	P2b-2R	0.87 $\pm$ 0.96	1.23 $\pm$ 0.78	0.58 $\pm$ 0.74
	Control	0.72 $\pm$ 0.99	1.23 $\pm$ 0.13	0.71 $\pm$ 0.13
Low	P2b-2R	0.89 $\pm$ 0.96	1.09 $\pm$ 0.89	0.57 $\pm$ 0.87
	Control	0.90 $\pm$ 0.12	1.19 $\pm$ 0.95	0.68 $\pm$ 0.01
Medium	P2b-2R	1.02 $\pm$ 0.97	1.31 $\pm$ 0.12	0.73 $\pm$ 0.12
	Control	1.00 $\pm$ 0.14	1.29 $\pm$ 0.95	0.67 $\pm$ 0.97
High	P2b-2R	1.30 $\pm$ 0.96	1.63 $\pm$ 0.14	1.12 $\pm$ 0.12
	Control	1.32 $\pm$ 0.13	1.53 $\pm$ 0.83	1.14 $\pm$ 0.93

Table S2 Total foliar N (mg± standard error; n = 9) of lodgepole pine seedlings inoculated with *P. polymyxa* P2b-2R and phosphate-buffered saline (control) measured four, eight and twelve months after inoculation

N treatment	Inoculation	Months after sowing		
		4	8	12
Very Low	P2b-2R	0.21 ± 0.01	0.31 ± 0.11	0.49 ± 0.11
	Control	0.11 ± 0.01	0.30 ± 0.13	0.53 ± 0.00
Low	P2b-2R	0.75 ± 0.02	0.33 ± 0.01	0.70 ± 0.07
	Control	0.19 ± 0.01	0.34 ± 0.00	0.72 ± 0.11
Medium	P2b-2R	0.22 ± 0.01	0.44 ± 0.09	0.90 ± 0.13
	Control	0.24 ± 0.04	0.39 ± 0.12	0.73 ± 0.98
High	P2b-2R	0.32 ± 0.00	0.73 ± 0.08	1.90 ± 0.32
	Control	0.28 ± 0.03	0.74 ± 0.17	2.11 ± 0.33

Table S3 Foliar ^{15}N atom percent excess ($\% \pm$ standard error; $n = 9$) of lodgepole pine seedlings inoculated with *P. polymyxa* P2b-2R and phosphate-buffered saline (control) measured four, eight and twelve months after inoculation

N treatment	Inoculation	Months after sowing		
		4	8	12
Very Low	P2b-2R	6.02 ± 2.00	31.2 ± 5.70	34.0 ± 3.44
	Control	3.87 ± 1.29	22.2 ± 4.22	32.6 ± 4.62
Low	P2b-2R	159 ± 97.7	237 ± 24.7	346 ± 40.5
	Control	72.2 ± 18.2	187 ± 20.5	326 ± 20.1
Medium	P2b-2R	1019 ± 140	2096 ± 266	3068 ± 160
	Control	925 ± 83.8	2262 ± 199	3046 ± 184
High	P2b-2R	4971 ± 245	7247 ± 406	8337 ± 258
	Control	5114 ± 297	6892 ± 378	8308 ± 249