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REGULAR ARTICLE

Evaluating lodgepole pine endophytes for their ability to fix nitrogen and support tree growth under nitrogen-limited conditions

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Abstract
Aims The Sub-Boreal Pine-Spruce xeric-cold (SBPSxc) biogeoclimatic region in British Columbia, Canada is characterized by weakly-developed soils, thin organic forest floor and limited plant-available nitrogen. Yet, lodgepole pine trees are thriving in this region unaffected by these nitrogen-limitations, which led us to hypothesize that endophytic nitrogen-fixing bacteria could be playing a potential role in sustaining pine tree growth.
Methods We evaluated these endophytes in a yearlong greenhouse experiment with their native host (lodgepole pine) for *in planta* nitrogen-fixation (^{15}N isotope dilution assay) and growth-promotion (length and biomass). These endophytes were also evaluated with a foreign host native to the SBPSxc region (hybrid white spruce) in another yearlong greenhouse trial.
Results Each bacterial strain considerably enhanced seedling length and biomass of pine and spruce along with fixing significant amount of nitrogen from atmosphere (15–50%). Notably, *Caballeronia sordidicola* HP-S1r strain fixed 49–50% of the host nitrogen from atmosphere and enhanced seedling length and biomass by up to 1.5-fold and 4-fold, respectively.
Conclusions Endophytic bacteria could be playing a crucial role in the survival of lodgepole pine trees in the SBPSxc region by providing them with significant amounts of fixed nitrogen. Their effectiveness with a foreign host (hybrid white spruce) shows the lack of plant x microbe specificity, indicating their potential role in supporting the growth of multiple boreal forest trees.

Keywords Lodgepole pine · Endophyte · Diazotroph · *Pinus* · *Picea* · Biological nitrogen fixation

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Introduction

Lodgepole pine (*Pinus contorta var. latifolia*) is regarded as a ubiquitous species in Western North America with an extensive ecological amplitude. It has one of the widest ranges of environmental tolerance among any coniferous species in North America since it thrives on an array of soil, moisture and topographical conditions (Lotan and Critchfield 1996). It is unique among conifer species in its ability to thrive on nutrient-poor and fire-affected sites that are severely limited in nitrogen (N) (Weetman et al. 1988). Lodgepole pine stands can be found on rough and rocky terrain as well as steep slopes and ridges, including bare

gravel that contain very minimal amounts of plant-essential nutrients, particularly N (Chapman and Paul 2012).

British Columbia (BC), Canada has some of the most diverse terrestrial ecosystems in North America and is divided into 14 biogeoclimatic zones (Pojar and Meidinger 1991). The Sub-Boreal Pine-Spruce (SBPS) biogeoclimatic zone is a montane region that lies in the central interior of BC. One of the subzones of SPBS – xeric cold (xc) – is an extremely cold and dry region because of its position in the rain-shadow of the Coast Mountains, which has resulted in thin and weakly developed soils comprised of mostly sand or sandy loam texture (Puri et al. 2018a). The organic forest floor is either very thin or completely absent on these soils due to frequent wildfire activity (Steen and Coupé 1997). In addition, analyses of various physico-chemical soil properties such as pH, C:N ratio, cation exchange capacity, base saturation, organic matter, texture and bulk density have revealed that soil health at various sites in this subzone is relatively poor (Puri et al. 2018a). These soils lack several essential macro- and micro-nutrients and, most importantly, they are poor in available as well as mineralizable N. Lodgepole pine are the most common forest stands in this region, which aligns with the ability of this tree species to grow on highly disturbed sites. But the fundamental question is, how can lodgepole pine thrive on such nutrient-poor, N-limited soils of this region?

Since soil N is scarce in the SBPSxc subzone, N-fixing bacteria (diazotrophs) can be viewed as an important pathway by which lodgepole pine trees could be fulfilling their N-requirements. Although associative N-fixation in the rhizosphere is widely recognized for plants, rhizospheric N-fixation in lodgepole pine has been reported to be insignificant (Chanway and Holl 1991). In addition, the harsh environmental and soil conditions in this region may not be suitable for rhizospheric diazotrophs (Chapman and Paul 2012). Interestingly, potential diazotrophs have been observed inside the tubercles of a tuberculate ectomycorrhizae species (*Suillus tomentosus*), but neither their N-fixation marker gene (*nifH*) was successfully identified nor their ecological role was demonstrated *in planta* (Paul et al. 2007, 2013). Endophytic diazotrophs (N-fixing bacteria living inside the plant tissues) could provide a crucial source of N for lodgepole pine trees in this region since they are thought to be better protected from abiotic and biotic stresses inside the tissues (Chanway et al. 2014). In growth chamber experiments, an endophytic diazotrophic strain, *Paenibacillus polymyxa* P2b-2R, isolated from SBPSdc subzone showed significant potential in promoting growth and fulfilling the N-requirements of lodgepole pine seedlings (Anand et al. 2013; Padda et al. 2017b). In addition, endophytic diazotrophs have been speculated to provide significant amounts of fixed N to limber pine (*Pinus flexilis*) and Engelmann spruce (*Picea engelmannii*) trees growing in a subalpine forest in Colorado, USA (Carrell and Frank 2014; Moyes et al. 2016). These studies led us to hypothesize that lodgepole pine trees growing in this SBPSxc subzone harbour endophytic diazotrophs in their internal tissues, which have the potential to provide pine trees with significant amounts of fixed N from the atmosphere.

We previously isolated endophytic bacteria from lodgepole pine trees growing at two different sites in the SBPSxc subzone (52°00′04.2″ N, 124°59′44.7″ W and 52°00′09.1″ N, 124°59′25.2″ W) and tested their ability to fix N by using an acetylene reduction assay (ARA) (Puri et al. 2018a). We isolated 48 endophytic bacteria on N-free culture media, of which 23 bacteria showed positive nitrogenase activity in an ARA (Puri et al. 2018a). In an effort to understand the potential role of these endophytic diazotrophs in fulfilling the N-requirements of lodgepole pine trees and sustaining their growth on N-limited soils, we selected six endophytic diazotrophic strains for this study (Table 1) that showed the best performance in ARA, i.e. they converted highest amounts of acetylene to ethylene (Puri et al. 2018a). In this study, we examined these strains in a yearlong greenhouse experiment by inoculating each strain into lodgepole pine. In an additional greenhouse experiment, we tested each of these strains with another tree species native to the SBPSxc subzone – hybrid white spruce (*Picea glauca x engelmannii*). The motive behind testing these strains with another host was to observe their plant x microbe specificity and ecological functioning in a different plant niche.

This study is part of an ongoing research into exploring the natural ecological association and plant x microbe specificity of endophytes to support tree (lodgepole pine and hybrid white spruce) growth at highly disturbed and nutrient-poor sites. In other studies, endophytic N-fixing bacteria have been isolated from hybrid white spruce trees growing in this SBPSxc region and evaluated for their ability to fix N and promote the growth of spruce and pine trees (Puri et al. 2018a,

2020a). In contrast to previous suggestions regarding how specific the endophyte x tree species association could be (O'Neill et al. 1992; James and Olivares 1998; Shishido et al. 1995; Chanway et al. 2000), no evidence of such plant x microbe specificity was observed in these studies.

In this study, our primary objective was to evaluate pine endophytes for their potential to form beneficial associations with lodgepole pine and hybrid white spruce trees and support their growth under extremely N-poor conditions by supplementing their N-requirements through biological N-fixation. The specific objectives of this study were: (i) to evaluate if each of the selected endophytic diazotrophic strains can colonize the internal tissues of lodgepole pine (native host) and hybrid white spruce (foreign host) following inoculation; (ii) to quantify the proportion of N-requirements of pine and spruce fulfilled by each strain; and (iii) to determine the potential of each strain to enhance pine and spruce seedling growth (biomass and length) upon *in planta* inoculation.

Materials and methods

Antibiotic-resistant derivative strains

Six strains originating from internal root, stem and needle tissues of lodgepole pine trees growing at highly-disturbed, nutrient-poor sites of the West Chilcotin region in BC were used in this study. These strains reduced the highest amounts of acetylene to ethylene in an ARA (Puri et al. 2018a), indicating the possibility of high *in planta* nitrogenase enzyme activity. We derived antibiotic-resistant mutants of these strains so that endophytic colonies formed by each strain after inoculation into lodgepole pine and hybrid white spruce seedlings could be quantified in the greenhouse experiments. The antibiotic-resistant derivative of each strain was raised (Table 1) by streaking multiple times on N-free combined carbon medium (CCM) agar amended with the antibiotic rifamycin (200 mg/L) (Bal and Chanway 2012). The antibiotic-resistant strains were stored in CCM amended with 200 mg/L rifamycin and 20% (*v*/v) glycerol at −80 °C until further evaluation. To confirm the N-fixing ability of the antibiotic-resistant derivative strains, we analyzed their nitrogenase enzyme activity using ARA as outlined by Puri et al. (2018a).

Greenhouse trials

We conducted two separate yearlong greenhouse growth trials – the first involving the inoculation of each selected strain into lodgepole pine (native host) and the second involving the inoculation into hybrid white spruce (foreign host). Therefore, six bacteria-inoculated and one non-inoculated control treatments were evaluated in each growth trial. Acquisition, pre-treatment and inoculation of seeds and greenhouse growth conditions have been described elsewhere (Puri et al. 2020a). Briefly, lodgepole pine and hybrid white spruce seeds were acquired from the BC Ministry of Forests Tree Seed Centre, Surrey, BC. Seeds were surface sterilized and stored at 4 °C for 28 days for stratification. Seed stratification is done to simulate the natural environmental conditions (cold temperature and high moisture) that seeds must experience for several weeks to break dormancy. The combination of cold and moist conditions triggers biochemical changes which transform complex food substances into simpler forms that are utilized by the embryo when it germinates (Willan 1986; Kolotelo et al. 2001). The absence of internal seed contamination by any of the selected bacterial strains prior to inoculation was confirmed after the stratification period as previously described (Puri et al. 2020a). It was assumed that all seeds used in this study harbour similar communities of endophytic bacteria, therefore any effects would be similar in all treatments. Seedlings were grown in Ray Leach Cone-tainers (height: 210 mm; diameter: 38 mm) filled to two-third capacity with an autoclaved soil media (69% *w*/w silica sand; 29% w/w Turface; 2% w/w $CaCO_3$) and fertilized with 20 mL of a nutrient solution (Puri et al. 2015). Three surface-sterilized seeds were sown aseptically into each Cone-tainer. A bacterial suspension (5 mL) of each strain (10^6 cfu/mL) was pipetted directly over the seeds in each Cone-tainer designated for that strain. Non-inoculated control seeds received 5 mL of sterile PBS without any bacteria in each trial. Seventy replicate seedlings were used per treatment per tree species for various analyses. The Cone-tainers were placed in the University of British Columbia Plant Care Services' greenhouse with photosynthetically active radiation at the canopy level of at least 300 μmol/m^2/s during a 16 h photoperiod (6 am to 10 pm). Two weeks after sowing, seedlings

Table 1 Nitrogen-fixing endophytic bacterial strains selected from Puri et al. (2018a) and their antibiotic-resistant derivatives along with the assessment of their nitrogen-fixing ability demonstrated via acetylene reduction activity. Acetylene reduction activity has been expressed as nanomoles of ethylene produced per mL of culture tube headspace (mean ± standard error; $n = 3$)

Bacterial species	Strain	Antibiotic-resistant strain	Acetylene reduction activity (nmol C_2H_4/mL)
Caballeronia sordidicola	HP-S1	HP-S1r	2.7 ± 0.10
Pseudomonas frederiksbergensis	HP-N1	HP-N1r	1.5 ± 0.21
Phyllobacterium myrsinacearum	HP-R1	HP-R1r	1.4 ± 0.14
Pseudomonas mandelii	LP-S1	LP-S1r	1.0 ± 0.09
Paraburkholderia phytofirmans	LP-R1	LP-R1r	2.2 ± 0.25
Caballeronia udeis	LP-R2	LP-R2r	1.9 ± 0.03

were thinned to the largest single germinant per Cone-tainer. Seedlings received 20 mL of the nutrient solution without $Ca(^{15}NO_3)_2$ once per month and were watered as required.

Evaluation of endophytic and rhizospheric colonization by each bacterial strain, seedling length and biomass and foliar ^{15}N analysis was performed for lodgepole pine and hybrid white spruce seedlings according to Puri et al. (2020a). Five seedlings per treatment were harvested each to evaluate internal tissue colonization and rhizospheric colonization 4, 8 and 12 months after sowing. For internal tissue colonization, seedlings were surface sterilized and a sample each of root, stem and needle tissue was triturated separately. Subsequently, serial dilutions were performed and plated onto CCM agar amended with cycloheximide (100 mg/L) to inhibit fungal growth and rifamycin (200 mg/L) to select for inoculum bacteria. The number of colonies formed by each strain (cfu) was evaluated a week after incubation at 30 °C. For rhizospheric colonization, roots were separated from shoots and gently shaken to remove loosely adhering soil media. Roots were then placed in sterile Falcon tubes (50 mL; BD Bioscience, CA) containing 10 mL of sterile PBS and vortexed for 1 min at 1000 rpm. Serial dilutions were performed, and 0.1 mL of each dilution was plated on CCM amended with cycloheximide (100 mg/L) and rifamycin (200 mg/L). Root dry weight was measured after oven-drying at 65 °C for 2 days. The number of colonies formed by each strain was evaluated a week after incubation at 30 °C. Rhizospheric populations were calculated as cfu/g of dry root tissue. Ten lodgepole pine and 10 hybrid white spruce seedlings per treatment were harvested destructively 4, 8 and 12 months after sowing to evaluate seedling growth (length and biomass). For foliar ^{15}N analysis, 10 pine and 10 spruce seedlings were randomly selected from each treatment 12 months after sowing. Needles of each selected seedling were oven-dried and ground to a particle size <2 mm and a 2.5 mg sample of ground foliage of each seedling was sent to the Stable Isotope Facility in the Faculty of Forestry, University of British Columbia, Vancouver, Canada to determine the % foliar N content and atom % ^{15}N excess in foliage. The amount of fixed N accumulated in the foliage of each bacteria-inoculated seedling was estimated (Rennie et al. 1978) by calculating the percent N derived from the atmosphere (%Ndfa) = {atom % ^{15}N excess (control plant) – atom % ^{15}N excess (inoculated plant)} ÷ atom % ^{15}N excess (control plant).

Statistical analyses

Statistical analysis of each greenhouse growth trial was performed separately. To evaluate the treatment effects of each bacterial strain on the growth of seedlings in each growth trial, a completely randomized experimental design with 70 replicates per treatment was used. The statistical package, SAS University Edition (SAS Institute Inc., Cary, NC, USA), was used to perform statistical analyses. Analysis of variance (ANOVA) was done (F-test and Student's t test) to determine significant differences between treatment means for seedling length, seedling biomass, atom % ^{15}N excess in foliage and % foliar N. The confidence level, α, was set to 0.05.

Results

All antibiotic-resistant derivative strains successfully reduced acetylene to ethylene in ARA, producing 1.0 to 2.7 nmol of ethylene per mL capacity of the vial

(Table 1). When each strain was inoculated into their native host (lodgepole pine) and foreign host (hybrid white spruce) in the yearlong greenhouse trial, it was observed that all strains were able to derive significant amounts of N from the atmosphere and provide it to their host. This was evident from the considerably lower atom % ^{15}N excess values for inoculated seedlings as compared to the controls (Tables 2). In particular, seedlings inoculated with strains HP-S1r and LP-R2r had significantly lower atom % ^{15}N excess in foliage than other bacterial treatments. These strains fulfilled 46–50% of the N requirement of both pine and spruce seedlings from the atmosphere (Table 2). The N content (%) in foliar tissues of pine and spruce seedlings inoculated with these strains was also significantly higher than controls and other bacterial treatments (Table 2). Notably, HP-S1r-inoculated pine and spruce seedlings had accumulated 73% and 56% higher N in their foliage, respectively, as compared to the control seedlings.

Apart from fixing N, all six bacterial strains successfully promoted seedling length and biomass of lodgepole pine and hybrid white spruce seedlings (Supplementary Figs. 1 and 2). At the 4-month harvest, pine seedling length of HP-S1r, LP-S1r, LP-R1r and LP-R2r treatments was significantly greater than control, HP-N1r and HP-R1r treatments (Fig. 1a). However, at the 8- and 12-month harvest, all six bacterial treatments were significantly better than the control treatment in terms of pine seedling length (Fig. 1a). For all treatments, the increase in pine seedling length throughout the growth trial, i.e. from 4- to 12- month harvest, was similar (1.2–1.4 fold increase). With regard to pine seedling biomass, strains HP-S1r, LP-S1r and LP-R2r very effectively increased the biomass as compared to the control and all other bacterial treatments at the 4- and 8-month harvests (Fig. 1b). Similar to seedling length, all bacteria-inoculated pine seedlings had accumulated significantly higher biomass (> 100%) than controls at the end of the growth trial (12 months) (Fig. 1b). The seedling biomass of all bacteria-inoculated pine seedlings increased during the growth trial, with more than 2-fold increase observed for HP-S1r, LP-R1r and LP-R2r treatments. Notably, inoculation with strain HP-S1r increased pine seedling length by 53% and biomass by 278% as compared to the control. Additionally, strain LP-R2r also performed considerably well by increasing length and biomass of pine seedlings by 40% and 200%, respectively, in comparison to the control. The ratio of root to shoot length was similar for all treatments (~3:1) at each harvest (Fig. 2a). In addition, no considerable variations were observed between treatments for root to shoot biomass ratio (~1:1) at all harvest intervals (Fig. 2a).

In the case of spruce seedlings, all bacterial treatments except LP-S1r significantly increased the seedling length as compared to the control (> 30%) at the 4-month harvest (Fig. 3a). Later in the growth trial, all bacteria-inoculated spruce seedlings were significantly longer than the controls at the 8- and 12-month harvests (Fig. 3a). Particularly, strain LP-R2r had increased seedling length by nearly 50% in comparison to the control at the end of the growth trial.

Table 2 Percent foliar N (mean ± standard error), atom percent ^{15}N excess in foliage (mean ± standard error), and percent N derived from the atmosphere (% Ndfa) of lodgepole pine and hybrid white spruce seedlings treated with six endophytic diazotrophic strains and a non-inoculated control, measured 12 months after sowing. Percent foliar N and atom percent ^{15}N excess in foliage values are mean ± standard error (n = 10 seedlings per treatment). Values followed by different letters are significantly different at $P < 0.05$

Treatment	Lodgepole Pine			Hybrid White Spruce		
	% Foliar N	*Atom % ^{15}N excess in foliage*	*% Ndfa*	*% Foliar N*	*Atom % ^{15}N excess in foliage*	*% Ndfa*
C. sordidicola HP-S1r	1.09 ± 0.05^{c}	0.39 ± 0.03^{d}	50	0.98 ± 0.04^{c}	0.35 ± 0.03^{d}	49
P. frederiksbergensis HP-N1r	0.62 ± 0.02^{a}	0.64 ± 0.02^{b}	18	0.60 ± 0.03^{a}	0.58 ± 0.02^{b}	15
P. myrsinacearum HP-R1r	0.65 ± 0.04^{a}	0.52 ± 0.01^{bc}	34	0.61 ± 0.04^{a}	0.53 ± 0.01^{bc}	23
P. mandelii LP-S1r	0.71 ± 0.02^{ab}	0.54 ± 0.01^{b}	31	0.72 ± 0.02^{ab}	0.47 ± 0.02^{c}	32
P. phytofirmans LP-R1r	0.62 ± 0.01^{a}	0.58 ± 0.02^{b}	26	0.72 ± 0.03^{ab}	0.50 ± 0.02^{bc}	27
C. udeis LP-R2r	0.82 ± 0.04^{b}	0.42 ± 0.03^{c}	46	0.80 ± 0.03^{b}	0.36 ± 0.02^{d}	48
Control	0.63 ± 0.03^{a}	0.78 ± 0.03^{a}	–	0.59 ± 0.02^{a}	0.69 ± 0.01^{a}	–

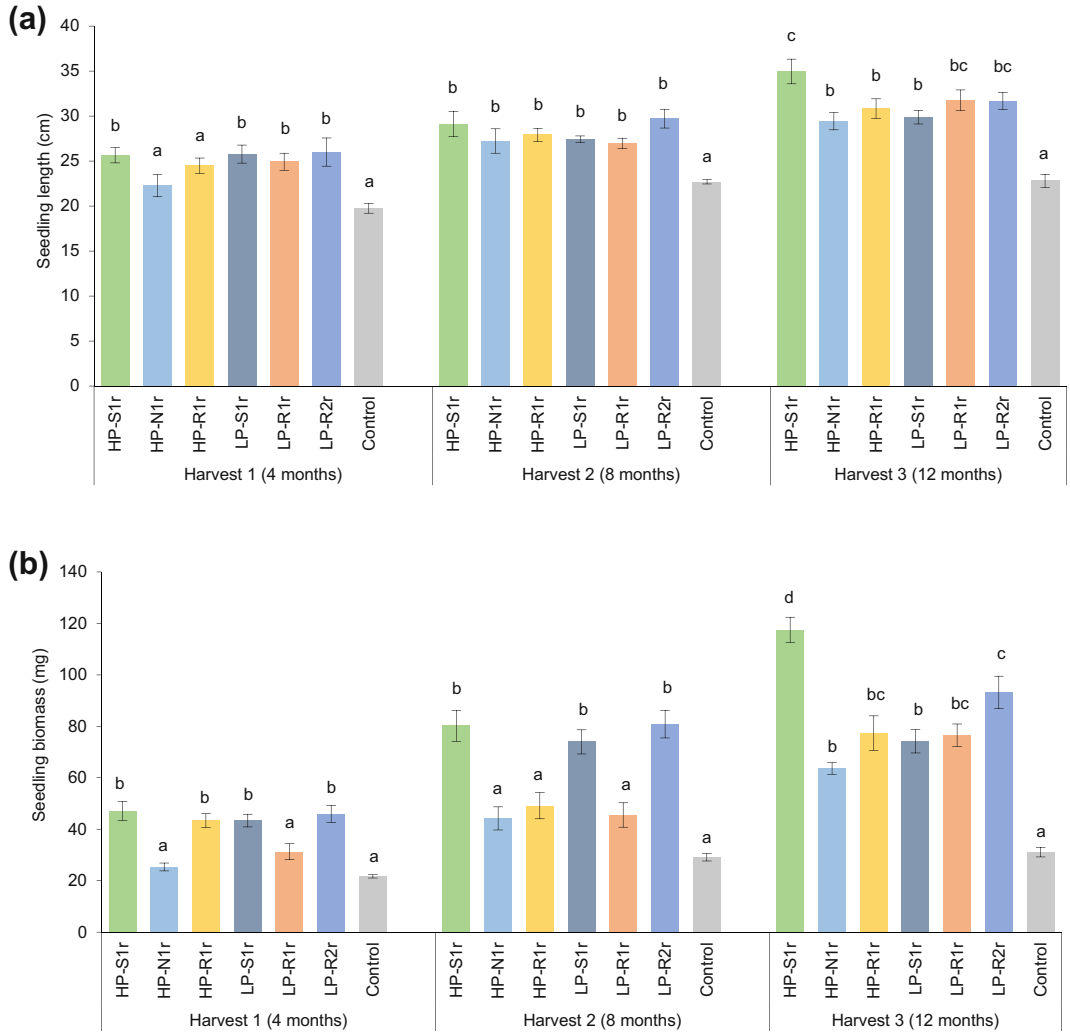

Fig. 1 (a) Length and (b) biomass of **lodgepole pine** seedlings subjected to six bacterial treatments and a non-inoculated control treatment, determined 4, 8 and 12 months after sowing and inoculation (means and standard errors; $n = 10$ seedlings per treatment). Error bars with different letters are significantly different ($P < 0.05$)

Similar to pine, the increase in spruce seedling length was fairly alike for all treatments (1.2–1.4 fold) between 4- and 12-month growth period. All bacterial treatments accumulated significantly greater biomass (nearly 100%) than control in spruce seedlings at the 4-month harvest (Fig. 3b). Whereas at the 8-month harvest, only HP-S1r, LP-S1r and LP-R1r and LP-R2r treatments had significantly higher biomass than control (Fig. 3b), with LP-R2r-inoculated spruce seedlings acquiring 175% more biomass than controls. At the last harvest, all bacteria-inoculated spruce seedlings were significantly greater than controls in terms of biomass (Fig. 3b). Spruce seedling biomass increased by 1.6–2.7 fold during the growth trial (from 4- and 12-month harvest), with largest growth observed for HP-S1r and LP-R2r treated seedlings. Notably, strains HP-S1r, LP-R1r and LP-R2r increased spruce seedling biomass by >200% as compared to the controls. For root to shoot biomass ratio in spruce seedlings, a slight increase was observed between harvest intervals for all treatments, starting from ~0.8:1 and reaching ~1.2:1 by the end (Fig. 2b). However, substantially higher root to shoot length ratio was observed (~4:1) during the 4-month, which further increased to ~6:1 by the end of the growth trial for all treatments (Fig. 2b).

The significant plant-growth-promotion and N-fixation observed for bacteria-inoculated pine and

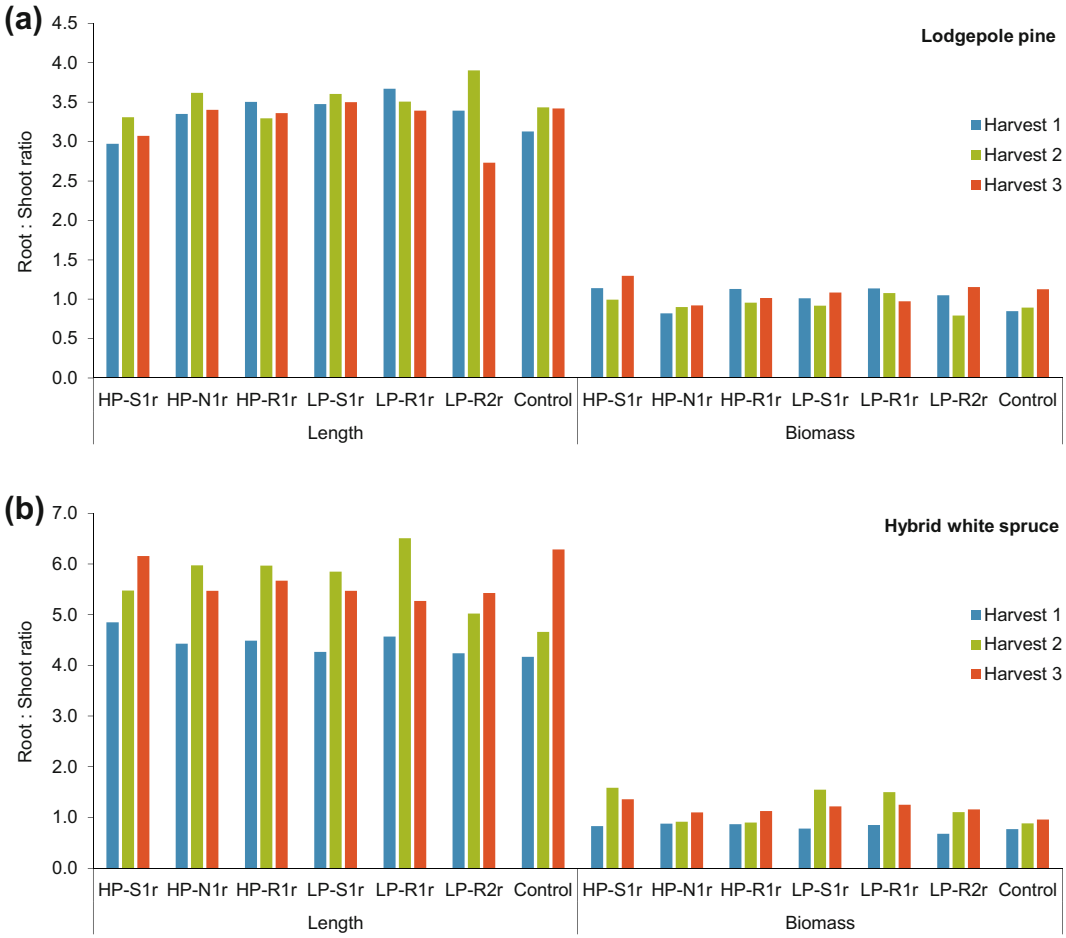

Fig. 2 Root to shoot ratio of length and biomass variables for (a) lodgepole pine and (b) hybrid white spruce seedlings subjected to six bacterial treatments and a non-inoculated control treatment, determined at three harvest intervals (4, 8 and 12 months after sowing)

spruce seedlings could be related to the colonization of internal tissues by these bacterial strains. Although needle colonization was not observed by any of the six strains for pine and spruce seedlings initially at the 4-month harvest, colonization was observed for strains HP-S1r and LP-R2r in both pine and spruce needle tissues (pine – 10^3 to 10^5 cfu/g fresh tissue & spruce – 10^2 to 10^3 cfu/g fresh tissue) at the 8- and 12-month harvests (Tables 3 and 4). Colonies of strain LP-R1r were also observed in both pine and spruce needles at the 12-month harvest, whereas strain HP-R1r was only observed in pine needles at this harvest. All strains successfully colonized the stem tissues of pine and spruce seedlings at the 8- and 12-month harvests with population densities ranging from 10^2 to 10^6 cfu/g fresh tissue (Tables 3 and 4). Initially, at the 4-month harvest, only three strains (HP-S1r, LP-S1r and LP-R2r) were able to colonize pine stem tissues and two strains (HP-S1r and LP-R2r) were able to colonize spruce stem tissues. In the case of root tissues, endophytic colonization was observed for each bacterial strain at all harvest intervals in both pine and spruce seedlings with the population densities ranging between 10^3 and 10^6 cfu/g fresh tissue (Tables 3 and 4). Along with colonizing the root tissues, each bacterial strain successfully colonized the rhizosphere of pine and spruce seedlings at all harvests, with population sizes of 10^4–10^6 cfu/g dry root for pine and 10^3–10^5 cfu/g dry root for spruce (Tables 3 and 4). Strains HP-S1r, LP-R1r and LP-R2r had colonized the pine and spruce rhizosphere with highest population densities (10^6 and 10^5 cfu/g dry root, respectively) by the end of the growth trials. No evidence of endophytic and rhizospheric colonization was observed

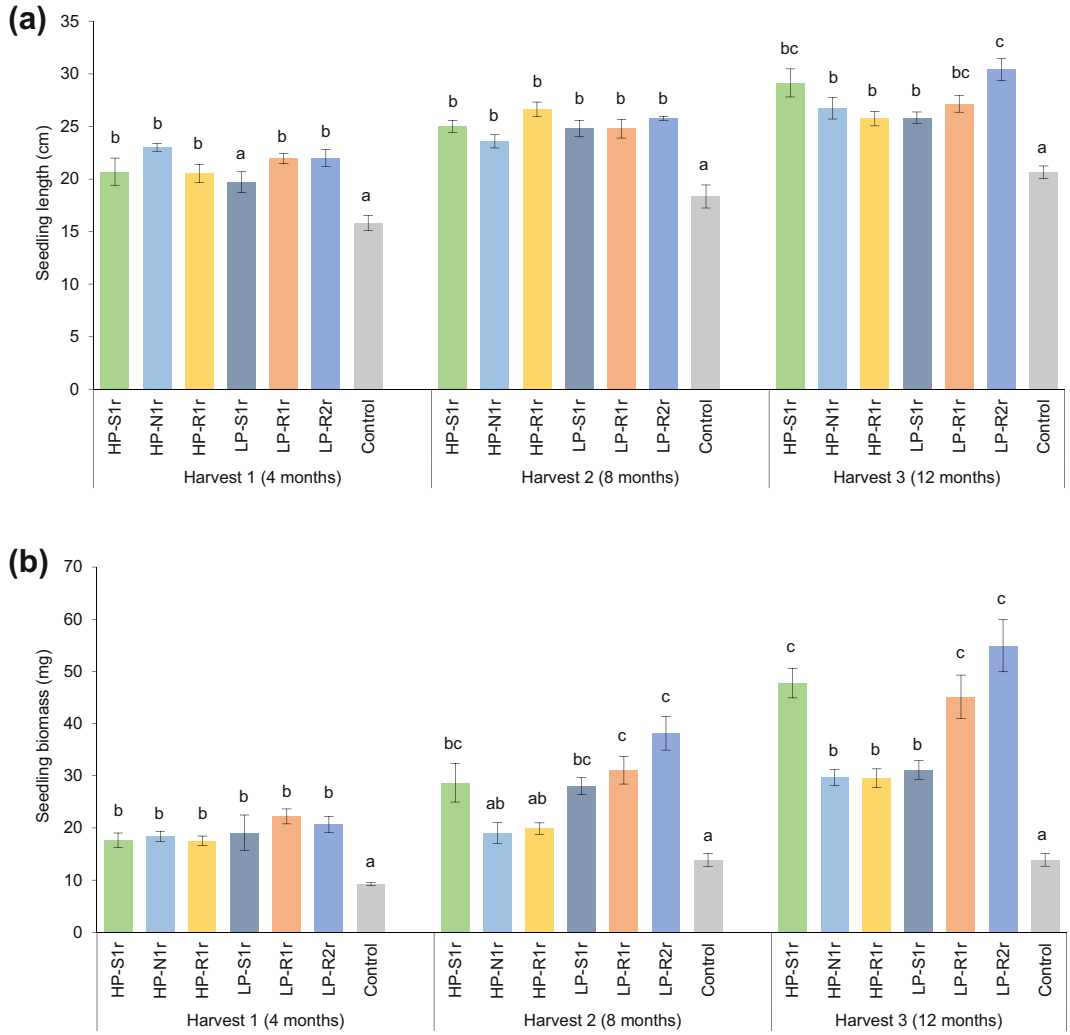

Fig. 3 (a) Length and (b) biomass of **hybrid white spruce** seedlings subjected to six bacterial treatments and a non-inoculated control treatment, determined 4, 8 and 12 months after sowing and inoculation (means and standard errors; n = 10 seedlings per treatment). Error bars with different letters are significantly different ($P < 0.05$)

in control seedlings of pine and spruce at any harvest interval.

The endophytic colonization density (aggregated for all tissue types) in pine seedlings during the growth trial (from 4 to 12 months) increased for all bacterial treatments (Table 3). The most prominent increase was observed for strains HP-S1r and LP-R1r (78 and 40-fold increase, respectively), whereas minimal increase was observed for strains HP-N1r and HP-R1r. Similar trends were observed for rhizospheric colonization increase in pine seedlings (Table 3). For spruce seedlings, endophytic and rhizospheric population densities increased by 73 and 112 fold for strain HP-S1r and by 55 and 80 fold for strain LP-R2r, respectively, between 4- and 12-month harvests (Table 4). Comparatively, slower population growth was observed for strains HP-N1r, HP-R1r and LP-S1r.

Discussion

Notwithstanding the poorly developed, nutrient poor soils that characterize the SBPSxc biogeoclimatic region in BC (Steen and Demarchi 1991; Puri et al. 2018a), lodgepole pine forest stands thrive in this region. We suspected that N-fixation by endophytic diazotrophs could be one of the possible ways through which pine trees are fulfilling their N-requirements (Puri et al.

Table 3 Colonization density (mean) of each of the six bacterial strains in the endophytic tissues (needle, stem and root) and rhizosphere of **lodgepole pine** seedlings at 4, 8 and 12 month harvest intervals, expressed as colony forming unit (cfu) per gram tissue ($n = 5$ seedlings per bacterial treatment for endophytic evaluations; $n = 5$ seedlings per bacterial treatment for rhizospheric evaluations)

	Needle population			Stem population			Root population			Rhizosphere population		
Treatment	Harvest 1 (4 months)	Harvest 2 (8 months)	Harvest 3 (12 months)	Harvest 1 (4 months)	Harvest 2 (8 months)	Harvest 3 (12 months)	Harvest 1 (4 months)	Harvest 2 (8 months)	Harvest 3 (12 months)	Harvest 1 (4 months)	Harvest 2 (8 months)	Harvest 3 (12 months)
HP-S1r	–	6.31×10^3	5.01×10^5	1.58×10^3	3.16×10^4	1.58×10^6	1.26×10^5	1.00×10^6	7.94×10^6	7.94×10^4	6.31×10^5	4.16×10^6
HP-N1r	–	–	–	–	1.26×10^2	1.58×10^3	6.31×10^4	1.26×10^5	1.58×10^5	1.00×10^5	2.00×10^5	2.00×10^5
HP-R1r	–	–	1.58×10^3	–	3.16×10^2	6.31×10^3	1.00×10^5	3.98×10^5	5.01×10^5	5.01×10^4	2.51×10^5	2.51×10^5
LP-S1r	–	–	–	6.31×10^2	6.31×10^3	1.26×10^5	5.01×10^4	6.31×10^5	1.00×10^6	6.31×10^4	5.01×10^5	5.59×10^5
LP-R1r	–	–	7.94×10^3	–	1.26×10^2	5.01×10^3	3.16×10^4	6.31×10^5	1.26×10^6	2.00×10^4	6.31×10^5	9.10×10^5
LP-R2r	–	1.58×10^3	3.16×10^4	1.26×10^2	1.58×10^3	6.31×10^4	1.26×10^5	3.98×10^5	3.16×10^6	7.94×10^4	3.16×10^5	1.38×10^6

Table 4 Colonization density (mean) of each of the six bacterial strains in the endophytic tissues (needle, stem and root) and rhizosphere of **hybrid white spruce** seedlings at 4, 8 and 12 month harvest intervals, expressed as colony forming unit (cfu) per gram tissue (n = 5 seedlings per bacterial treatment for endophytic evaluations; n = 5 seedlings per bacterial treatment for rhizospheric evaluations)

Treatment	Needle population			Stem population			Root population			Rhizosphere population		
	Harvest 1 (4 months)	Harvest 2 (8 months)	Harvest 3 (12 months)	Harvest 1 (4 months)	Harvest 2 (8 months)	Harvest 3 (12 months)	Harvest 1 (4 months)	Harvest 2 (8 months)	Harvest 3 (12 months)	Harvest 1 (4 months)	Harvest 2 (8 months)	Harvest 3 (12 months)
HP-S1r	–	3.16×10^2	1.00×10^3	6.31×10^3	7.94×10^3	2.51×10^4	2.00×10^3	5.01×10^4	5.85×10^5	2.00×10^3	3.16×10^4	2.26×10^5
HP-N1r	–	–	–	–	3.98×10^2	6.31×10^3	3.16×10^3	6.31×10^3	3.16×10^4	1.58×10^3	5.01×10^3	2.00×10^4
HP-R1r	–	–	–	–	1.00×10^3	1.00×10^4	3.98×10^3	3.16×10^4	1.00×10^5	6.31×10^3	3.16×10^4	3.16×10^4
LP-S1r	–	–	–	–	1.58×10^3	7.94×10^3	6.31×10^3	1.58×10^4	6.31×10^4	5.01×10^3	2.00×10^4	6.98×10^4
LP-R1r	–	–	1.26×10^2	–	6.31×10^2	3.16×10^3	1.58×10^4	1.00×10^5	5.01×10^5	1.26×10^4	6.31×10^4	2.66×10^5
LP-R2r	–	6.31×10^2	3.16×10^3	1.58×10^3	6.31×10^3	1.26×10^4	1.00×10^4	1.58×10^5	6.31×10^5	6.31×10^3	1.26×10^5	5.01×10^5

2018a). However, the crucial questions were: (i) how much N can these endophytic diazotrophs provide to lodgepole pine trees via biological N-fixation, and (ii) is there a plant x microbe specificity in this association?

We selected six top-performing endophytic diazotrophs – originally isolated from the internal tissues of lodgepole pine trees growing in the SBPSxc region – based on their nitrogenase enzyme activity (Puri et al. 2018a). Antibiotic-resistant mutants of each strain were derived in order to quantify the population sizes of these endophytes in plant tissues and rhizosphere (Table 1). Although previous reports suggest that the spontaneous antibiotic-resistance mutation to rifamycin has no effect on the nitrogenase activity of endophytic bacteria (Shishido et al. 1995; Bal et al. 2012; Padda et al. 2018), to be sure, we evaluated the antibiotic-resistant strains for nitrogenase enzyme activity using ARA (Table 1). Our results support the previous findings regarding the spontaneous mutation to rifamycin resistance since the amount of ethylene produced in ARA by antibiotic-resistant strains was similar to the wild-type strain reported previously by Puri et al. (2018a).

Looking for further evidence to determine the proportion of the N-requirement of plants fulfilled by these strains via N-fixation, we used the ^{15}N isotope dilution method. The results of this assay revealed that each endophytic diazotrophic strain fixed significant amounts of N from the atmosphere to supplement the N-requirements of pine and spruce seedlings (Table 2). This method works on the principle that bacteria-inoculated seedlings obtain fixed ^{14}N from the atmosphere, thereby resulting in diluted ^{15}N levels in their tissues as compared to the control seedlings (Hardarson and Danso 1993; Puri et al. 2018b). This is clear from the atom % ^{15}N excess values observed for bacteria-inoculated and control seedlings of pine and spruce (Table 2). Irrespective of the plant host, *Caballeronia sordidicola* HP-S1r and *Caballeronia udeis* LP-R2r emerged as potent N-fixing bacteria as they fulfilled 46–50% of the N-requirements of pine and spruce seedlings through biological N-fixation. Certain tree endophytes have shown similar abilities to fulfill such a considerable proportion of N-requirements of their host via N-fixation (Anand et al. 2013; Knoth et al. 2014; Moyes et al. 2016). However, more rigorous testing using ^{15}N incorporation assay and non-nitrogenase mutant strains should be conducted to confirm this observed N-fixation by these strains. In addition, the nif operon of these bacterial strains should be sequenced to further verify their nitrogen-fixation ability. The concentration of N in the foliage of pine and spruce seedlings inoculated with these two strains was also significantly higher than the control seedlings (Table 2). Since the seedling growth media was fertilized with a very limited amount of N at the onset of the experiment, it is reasonable to conclude that higher N concentration in seedlings inoculated with strains HP-S1r and LP-R2r was due to the accumulation of fixed N in their foliage from endophytic diazotrophs. However, it is still unclear how such endophytic diazotrophs provide fixed N to the plant. Two possibilities are that (i) there could be an active transfer of fixed N compounds from the bacteria to the host plant, similar to the rhizobium-legume model, or (ii) bacteria fix N in planta for their own metabolism and provide fixed N indirectly to the plant after they die and decompose in the plant (Doty 2017; Puri et al. 2017a). If N-fixation is calculated on a per year basis, *C. sordidicola* HP-S1r fixed 5.5 g of N per kg pine tissues and 4.8 g of N per kg of spruce tissues while *C. udeis* LP-R2r fixed 3.8 g of N per kg tissue for both pine and spruce. If similar rates of N-fixation occur under field conditions, fixed N from these bacteria could constitute 50–60% of the total N uptake by lodgepole pine trees in a fully stocked forest in the SBPS region of BC (Kimmins et al. 1999; Anand 2010). This estimate is reasonably close to the total N uptake reported for lodgepole pine forests in south-eastern Wyoming, USA (Fahey et al. 1985). Although these N-fixation rates are generally lower than those fixed in the nodules of leguminous plants, this N-accumulation pathway could still be biologically significant for pine trees growing in the SBPSxc region due to the severely limited soil N (Steen and Coupé 1997; Puri et al. 2018a). However, complete accounting of the N cycle in these forest stands should be performed to further explore the importance of this endophytic N-fixation pathway.

The seedling length and biomass of lodgepole pine and hybrid white spruce were evaluated thrice during the greenhouse trials in order to track the growth of inoculated and non-inoculated seedlings. Length and biomass of pine and spruce seedlings increased with time during the growth trial for all bacterial treatments compared to the slower growing control seedlings, thus indicating the growth promoting effects of bacterial inoculation (Figs. 1 and 3). This could be attributed to the limited N-source for control seedlings (i.e. soil N), which likely depleted after the single small N

application at the beginning of the growth trial. However, bacteria-inoculated seedlings had access to an additional N-source (atmospheric N) as evidenced by the results of the ^{15}N isotope dilution assay. The length and biomass of all bacteria-inoculated pine and spruce seedlings was significantly higher than control seedlings at the end of the growth study (Figs. 1a and 3a). It is notable to see that inoculation with each of the six endophytic diazotrophic strains increased biomass accumulation by >2-fold in both pine and spruce seedlings (Figs. 1b and 3b). Such an increase in biomass accumulation could be ascribed to the increased accessibility of inoculated seedlings to fixed N from the atmosphere as postulated in previous studies (Bal and Chanway 2012; Knoth et al. 2014). However, endophytic diazotrophs are also known for their ability to promote plant growth through other mechanisms such as phytohormone modulation, phosphate solubilization, and increased access to certain micronutrients like iron (Kandel et al. 2017; Padda et al. 2017a; Yang et al. 2017). Therefore, it is certainly important to study such mechanisms in the future to further explain the growth promotion observed for bacteria-inoculated pine and spruce seedlings. Regardless of the mechanism(s) at work, our results, including those of a previous study (Puri et al. 2020a), clearly indicate that plant x microbe specificity is unimportant in endophytic diazotroph x tree species interactions.

Depending on the physiology of a plant, the growth of certain tissues could be more pronounced particularly at different life stages. Comparing the growth of aboveground and belowground tissues of pine and spruce seedlings, the ratio of root to shoot length was found to be similar for all treatments (around 3:1) in pine seedlings for the entire growth trial, but for spruce seedlings this ratio increased from 4:1 to 6:1 during the growth trial for all treatments (Fig. 2). Greater elongation of roots in comparison to shoots in the early stages of growth and development is a basic phenotypic trait of plants as they search for nutrients and water supply in the soil media. Spruce is a shade-tolerant species and invests more resources in extending its root network in the early stages of its life cycle so that it can have an adequate supply of nutrients and water for further growth (Valladares and Niinemets 2008; Perry et al. 2008), which is consistent with the increasing trend of root to shoot ratio observed between harvest intervals in our study. In contrast, lodgepole pine is a shade-intolerant species, with the inherent physiology to ascend above the crown cover in order to get adequate light for growth (Lotan and Critchfield 1996; Perry et al. 2008; Axelson et al. 2010), which explains the greater rate of stem elongation in comparison to spruce. In the case of root to shoot biomass ratio, a constant trend (1:1) was observed for all treatments throughout the growth trial for both pine and spruce. This indicates that both tree species accumulated similar biomass in their roots and shoots, regardless of their lengths. Since stem tissues are primarily composed of woody cells and the majority of nutrients are stored in the needle tissues of conifers (Perry et al. 2008), having greater biomass in a shorter shoot is logical. Overall, considering that no differences in root to shoot growth were observed between bacteria-inoculated and non-inoculated control seedlings, it can be suggested that bacterial inoculation might not cause any changes to the fundamental phenotypic characteristics of pine and spruce trees.

Among all bacterial treatments, *C. sordidicola* HP-S1r, *C. udeis* LP-R2r and *Paraburkholderia phytofirmans* LP-R1r enhanced the seedling length of pine and spruce the most (35–53%) by the end of the growth trials. Pine seedlings inoculated with *C. sordidicola* HP-S1r accumulated 278% more biomass than control at the end of the growth trial, which was significantly higher than all other bacterial treatments. This strain performed equally well in spruce by accumulating 243% more biomass than the control by the end of the growth trial. This strain increased the biomass of both pine and spruce seedlings by more than 2.5-fold between 4- and 12-month harvests. Such considerable increase in biomass accumulation could be crucial to sustain tree growth, especially under adverse conditions such as those found in the SBPSxc region. Previous studies have suggested that *C. sordidicola* strains possess multifarious plant-growth-promoting traits such as phosphate solubilization, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, siderophore production and N-fixation (Palaniappan et al. 2010; Lladó et al. 2014; Puri et al. 2020a, 2020b). Similarly, *C. udeis* LP-R2r increased the biomass of pine and spruce seedlings by double or more between the first and last harvest, leading to the accumulation of 200% and 295% more biomass than control in pine and spruce seedlings, respectively, by end of the growth trial. Another noteworthy strain was *P. phytofirmans* LP-R1r which increased the biomass of pine and spruce by nearly 2-fold during the growth study. *P. phytofirmans* species is widely-known, mainly due to the endophytic strain PsJn, which was initially

isolated from onion roots and later reported in numerous studies to endophytically colonize and promote the growth of diverse host species ranging from monocots to dicots (reviewed by Puri et al. 2017b). It is important to mention that both *Caballeronia* and *Paraburkholderia* genera were previously part of the plant-beneficial-environmental group of the *Burkholderia* genus (Dobritsa and Samadpour 2016; Sawana et al. 2014), which is rich in symbiotic and associative N-fixing as well as plant-growth-promoting bacteria (Estrada-De Los Santos et al. 2001).

Each of the six endophytic strains colonized one or more internal tissues and the rhizosphere of pine and spruce seedlings during the growth trial (Tables 3 and 4), indicating that survival and multiplication in biologically significant numbers is likely necessary for these bacteria to perform N-fixation and provide growth-promoting benefits to the host (Chanway et al. 2000; Germaine et al. 2004; Padda et al. 2016a, 2016b). The rhizospheric and endophytic bacterial populations observed in pine and spruce for our strains are comparable to previous studies where coniferous trees were artificially inoculated with endophytic bacteria (Shishido et al. 1999; Tang et al. 2017; Yang et al. 2016). Though all strains were able to colonize the internal root and stem tissues of pine and spruce seedlings, needle colonization was observed for certain strains only. This may be explained by the following postulations – either the plant drives the selection process regarding where certain endophytic bacteria would be harboured, or the endophytic bacteria have a preference for certain plant tissues types. However, neither of these hypotheses has been proven, so it is not possible to conclude how and why endophytic bacteria harbour certain plant tissues. The endophytic and rhizospheric populations of all strains in pine and spruce seedlings increased with time during the greenhouse trials, with the largest increase observed for *C. sordidicola* HP-S1r in both tree species (~75-fold increase in endophytic and 52 to 112-fold increase in rhizospheric), followed by *P. phytofirmans* LP-R1r and *C. udeis* LP-R2r (Tables 3 and 4). The increase in endophytic colonization was substantially greater than the increase in rhizospheric colonization for *C. sordidicola* HP-S1r and *C. udeis* LP-R2r in pine seedlings, however, the opposite trend was observed in spruce seedlings. This could be explained by the fact that certain endophytic bacteria are niche-specific, proliferating in niches more favourable to their growth. Since these bacteria live naturally in association with pine trees, their internal tissues may be a more conducive niche for these bacteria to re-colonize (Shishido and Chanway 2000; Chanway et al. 2000; Bal and Chanway 2012; Anand and Chanway 2013). Whether the strains were slow or fast to colonize the internal tissues, the total endophytic population of all strains in both pine and spruce seedlings was greater than rhizospheric population at the end of the growth trial, clearly highlighting their inherent endophytic nature. In addition, the N-fixation and plant-growth-promotion effects of our strains were similar in both tree species, indicating their non-specific nature when it comes to providing benefits to their host. Although it is tempting to speculate that endophytic populations of our strains might be primarily responsible for providing fixed N to pine and spruce along with enhancing their overall growth in this study, the role played by the rhizospheric populations cannot be underestimated, particularly because of the fact that rhizospheric N-fixation and plant-growth-promotion have been reported more widely in the literature. Therefore, future studies must be designed to determine whether the plant-beneficial effect of these strains is due to: (i) the rhizospheric population, (ii) the endophytic population, or (iii) a synergistic effect of both populations. In addition, endophytic and rhizospheric colonization should also be ascertained through molecular means including real-time qRT-PCR and 16S rRNA gene sequencing to further confirm that the inoculated strains were able to colonize pine and spruce seedlings.

Comparing the total population size (endophytic + rhizospheric) of each bacterial strain with N-fixation and plant-growth variables of pine and spruce at the end of the greenhouse study, a very strong correlation ($R^2 = 0.92$–0.99) was observed with seedling biomass, a strong correlation ($R^2 = 0.82$–0.88) was observed with seedling length and a moderately-strong correlation ($R^2 = 0.63$–0.65) was observed with %Ndfa (Fig. 4). These observations suggest that plant colonization size of these bacterial strains may have a direct role in the N-fixation and growth enhancement of pine and spruce seedlings. Similar observations have been reported in inoculation studies with interior spruce, lodgepole pine and poplar (Shishido et al. 1995; Germaine et al. 2004; Yang et al. 2016; Padda et al. 2019). It should also be noted that the most aggressive plant colonizers of pine and spruce – *C. sordidicola* HP-S1r, *P. phytofirmans* LP-R1r and *C. udeis* LP-R2r – were also the best N-fixers and plant-growth-promoters in the greenhouse study (Fig. 4).

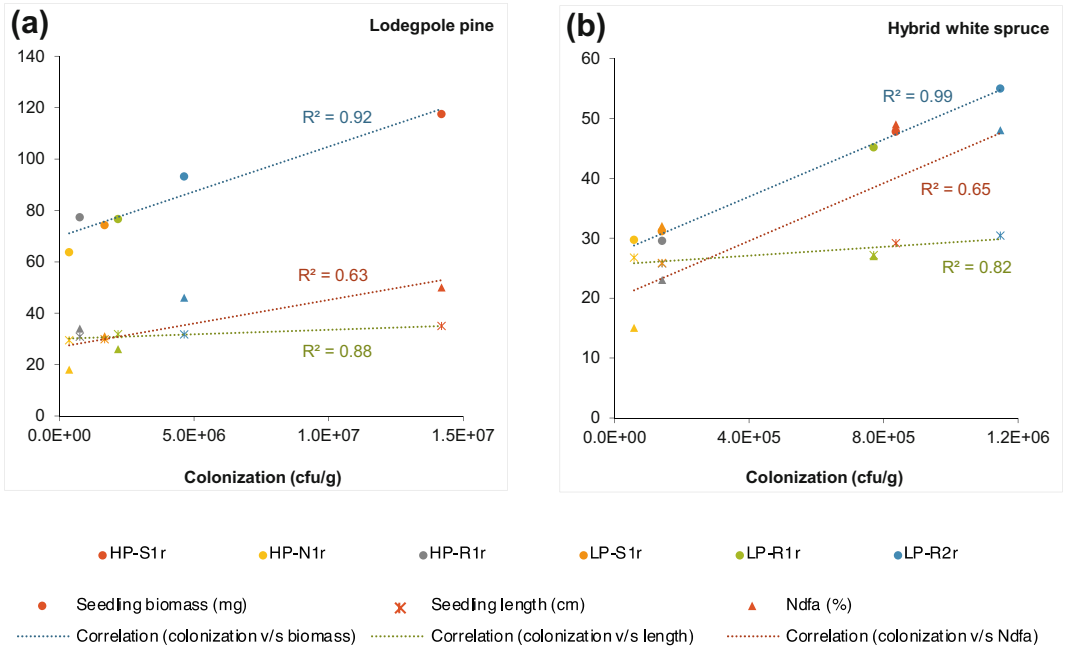

Fig. 4 Correlation of total colonization (endophytic + rhizospheric) with length, biomass and nitrogen derived from atmosphere (%Ndfa) for lodgepole pine and hybrid white spruce seedlings inoculated with six bacterial strains, one year after inoculation

In addition to lodgepole pine, *Caballeronia* strains with potential N-fixing ability were also isolated from stem and root tissues of hybrid white spruce trees growing in the SBPSxc region (Puri et al. 2018a), which suggests the possibility of the widespread presence of the genus *Caballeronia* in this region. In a similar study, a *C. udeis* strain provided 36–39% of host nitrogen and a *C. sordidicola* strain provided 52–56% of host nitrogen from atmosphere in spruce and pine while significantly enhancing their length and biomass (Puri et al. 2020a). The N-fixation and plant-growth-promotion observed for *C. sordidicola* HP-S1r and *C. udeis* LP-R2r inoculated pine and spruce seedlings in this study provides further evidence that the genus *Caballeronia* may play a significant role in supporting the growth of lodgepole pine and hybrid white spruce trees in the SBPSxc region. Interestingly, the non-specific nature of *C. sordidicola* and *C. udeis* strains observed in this study and Puri et al. (2020a) has also been observed in other endophytic diazotrophic strains isolated from poplar, willow, lodgepole pine and western red cedar trees (Doty et al. 2009; Bal et al. 2012), as those strains have shown N-fixing capabilities not only in deciduous and coniferous trees but also in agricultural crops (Knoth et al. 2014; Khan et al. 2015; Kandel et al. 2015; Puri et al. 2016a, 2016b). This study as well as that of Puri et al. (2020a) describe a series of experiments conducted by our lab group to explore the ecological associations of pine and spruce endophytes with their natural hosts in the extremely N-poor SBPSxc region. From these studies, we can conclude that N-fixation and plant-growth-promotion by bacterial endophytes may play a crucial role in sustaining the growth of trees in this region. The lack of plant x microbe specificity observed in this study and by Puri et al. (2020a) suggests that plant growth promoting endophytic diazotrophs could be generalists, which are able to enhance the N content and growth of gymnosperms naturally regenerating on nutrient poor soils in the boreal forests of BC.

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References

Anand R (2010) Endophytic colonization and nitrogen fixation by *Paenibacillus polymyxa* in association with lodgepole pine and western red cedar. Doctoral dissertation, University of British Columbia, Vancouver. https://doi.org/10.14288/1.0071481

Anand R, Chanway C (2013) N_2-fixation and growth promotion in cedar colonized by an endophytic strain of *Paenibacillus polymyxa*. Biol Fertil Soils 49:235–239. https://doi.org/10.1007/s00374-012-0735-9

Anand R, Grayston S, Chanway CP (2013) N_2-fixation and seedling growth promotion of lodgepole pine by endophytic *Paenibacillus polymyxa*. Microb Ecol 66:369–374. https://doi.org/10.1007/s00248-013-0196-1

Axelson JN, Alfaro RI, Hawkes BC (2010) Changes in stand structure in uneven-aged lodgepole pine stands impacted by mountain pine beetle epidemics and fires in Central British Columbia. For Chron 86:87–99. https://doi.org/10.5558/tfc86087-1

Bal A, Chanway CP (2012) Evidence of nitrogen fixation in lodgepole pine inoculated with diazotrophic *Paenibacillus polymyxa*. Botany 90:891–896. https://doi.org/10.1139/b2012-044

Bal A, Anand R, Berge O, Chanway C (2012) Isolation and identification of diazotrophic bacteria from internal tissues of *Pinus contorta* and *Thuja plicata*. Can J For Res 42:807–813. https://doi.org/10.1139/x2012-023

Carrell AA, Frank AC (2014) *Pinus flexilis* and *Picea engelmannii* share a simple and consistent needle endophyte microbiota with a potential role in nitrogen fixation. Front Microbiol 5:333. https://doi.org/10.3389/fmicb.2014.00333

Chanway CP, Holl FB (1991) Biomass increase and associative nitrogen fixation of mycorrhizal *Pinus contorta* seedlings inoculated with a plant growth promoting *Bacillus* strain. Can J Bot 69:507–511. https://doi.org/10.1139/b91-069

Chanway CP, Shishido M, Nairn J, Jungwirth S, Markham J, Xiao G, Holl F (2000) Endophytic colonization and field responses of hybrid spruce seedlings after inoculation with plant growth-promoting rhizobacteria. For Ecol Manag 133:81–88. https://doi.org/10.1016/S0378-1127(99)00300-X

Chanway CP, Anand R, Yang H (2014) Nitrogen fixation outside and inside plant tissues. In: Ohyama T (ed) Advances in biology and ecology of nitrogen fixation. InTechOpen, London, pp 3–23. https://doi.org/10.5772/57532

Chapman WK, Paul L (2012) Evidence that northern pioneering pines with tuberculate mycorrhizae are unaffected by varying soil nitrogen levels. Microb Ecol 64:964–972. https://doi.org/10.1007/s00248-012-0076-0

Dobritsa AP, Samadpour M (2016) Transfer of eleven *Burkholderia* species to the genus *Paraburkholderia* and proposal of *Caballeronia* gen. Nov., a new genus to accommodate twelve species of *Burkholderia* and *Paraburkholderia*. Int J Syst Evol Microbiol 66:2836–2846. https://doi.org/10.1099/ijsem.0.001065

Doty SL (2017) Endophytic N-fixation: controversy and a path forward. In: Doty SL (ed) Functional importance of the plant microbiome. Springer, Cham, pp 7–20. https://doi.org/10.1007/978-3-319-65897-1_2

Doty SL, Oakley B, Xin G, Kang JW, Singleton G, Khan Z, Vajzovic A, Staley JT (2009) Diazotrophic endophytes of native black cottonwood and willow. Symbiosis 47:23–33. https://doi.org/10.1007/BF03179967

Estrada-De Los Santos P, Bustillos-Cristales R, Caballero-Mellado J (2001) *Burkholderia*, a genus rich in plant-associated nitrogen fixers with wide environmental and geographic distribution. Appl Environ Microbiol 67:2790–2798. https://doi.org/10.1128/AEM.67.6.2790-2798.2001

Fahey TJ, Yavitt JB, Pearson JA, Knight DH (1985) The nitrogen cycle in lodgepole pine forests, southeastern Wyoming. Biogeochemistry 1:257–275. https://doi.org/10.1007/BF02187202

Germaine K, Keogh E, Garcia-Cabellos G, Borremans B, Van Der Lelie D, Barac T, Oeyen L, Vangronsveld J, Moore FP, Moore ER, Campbell CD (2004) Colonisation of poplar trees by *gfp* expressing bacterial endophytes. FEMS Microbiol Ecol 48:109–118. https://doi.org/10.1016/j.femsec.2003.12.009

Hardarson G, Danso SKA (1993) Methods for measuring biological nitrogen fixation in grain legumes. Plant Soil 152:19–23. https://doi.org/10.1007/BF00016330

James EK, Olivares F (1998) Infection and colonization of sugar cane and other graminaceous plants by endophytic diazotrophs. Crit Rev Plant Sci 17:77–119. https://doi.org/10.1080/07352689891304195

Kandel SL, Herschberger N, Kim SH, Doty SL (2015) Diazotrophic endophytes of poplar and willow for growth promotion of rice plants in nitrogen-limited conditions. Crop Sci 55:1765–1772. https://doi.org/10.2135/cropsci2014.08.0570

Kandel SL, Firrincieli A, Joubert PM, Okubara PA, Leston ND, McGeorge KM, Mugnozza GS, Harfouche A, Kim SH, Doty SL (2017) An *in vitro* study of bio-control and plant growth promotion potential of *Salicaceae* endophytes. Front Microbiol 8:386. https://doi.org/10.3389/fmicb.2017.00386

Khan Z, Kandel S, Ramos D, Ettl G, Kim SH, Doty S (2015) Increased biomass of nursery-grown Douglas-fir seedlings upon inoculation with diazotrophic endophytic consortia. Forests 6:3582–3593. https://doi.org/10.3390/f6103582

Kimmins JP, Mailly D, Seely B (1999) Modelling forest ecosystem net primary production: the hybrid simulation approach used in FORECAST. Ecol Model 122:195–224. https://doi.org/10.1016/S0304-3800(99)00138-6

Knoth JL, Kim SH, Ettl GJ, Doty SL (2014) Biological nitrogen fixation and biomass accumulation within poplar clones as a result of inoculations with diazotrophic endophyte consortia. New Phytol 201:599–609. https://doi.org/10.1111/nph.12536

Kolotelo D, van Steenis E, Peterson M, Bennett R, Trotter D, Dennis J (2001) Seed handling guidebook. British Columbia Ministry of Forests, Tree Seed Centre. https://www2.gov.bc.ca/assets/gov/farming-natural-resources-and-industry/forestry/tree-seed/tree-seed-publications/seed_handling_guidebook_hi.pdf. Accessed 16 May 2020

Lladó S, Xu Z, Sørensen SJ, Baldrian P (2014) Draft genome sequence of *Burkholderia sordidicola* S170, a potential plant growth promoter isolated from coniferous forest soil in the Czech Republic. Genome Announc 2:e00810–e00814. https://doi.org/10.1128/genomeA.00810-14

Lotan JE, Critchfield WB (1996) Lodgepole pine. In: burns RM, Honkala BH (tech Coords) Silvics of North America – volume 1: conifers. Agriculture handbook 654. US Department of Agriculture, Forest Service, Washington DC. https://www.srs.fs.usda.gov/pubs/misc/ag_654/volume_1/silvics_vol1.pdf. Accessed 16 May 2020

Moyes AB, Kueppers LM, Pett-Ridge J, Carper DL, Vandehey N, O'Neil J, Frank AC (2016) Evidence for foliar endophytic nitrogen fixation in a widely distributed subalpine conifer. New Phytol 210:657–668. https://doi.org/10.1111/nph.13850

O'Neill GA, Chanway CP, Axelrood PE, Radley RA, Holl FB (1992) Growth response specificity of spruce inoculated with coexistent rhizosphere bacteria. Can J Bot 70:2347–2353. https://doi.org/10.1139/b92-294

Padda KP, Puri A, Chanway CP (2016a) Effect of GFP tagging of *Paenibacillus polymyxa* P2b-2R on its ability to promote growth of canola and tomato seedlings. Biol Fertil Soils 52:377–387. https://doi.org/10.1007/s00374-015-1083-3

Padda KP, Puri A, Chanway CP (2016b) Plant growth promotion and nitrogen fixation in canola by an endophytic strain of *Paenibacillus polymyxa* and its GFP-tagged derivative in a long-term study. Botany 94:1209–1217. https://doi.org/10.1139/cjb-2016-0075

Padda KP, Puri A, Zeng Q, Chanway CP, Wu X (2017a) Effect of GFP-tagging on nitrogen fixation and plant growth promotion of an endophytic diazotrophic strain of *Paenibacillus polymyxa*. Botany 95:933–942. https://doi.org/10.1139/cjb-2017-0056

Padda KP, Puri A, Chanway CP (2017b) *Paenibacillus polymyxa* – a prominent biofertilizer and biocontrol agent for sustainable agriculture. In: Meena VS, Mishra P, Thakuria D, Bisht J, Pattanayak A (eds) Agriculturally important microbes for sustainable agriculture. Springer, Singapore, pp 165–191. https://doi.org/10.1007/978-981-10-5343-6_6

Padda KP, Puri A, Chanway CP (2018) Isolation and identification of endophytic diazotrophs from lodgepole pine trees growing at unreclaimed gravel mining pits in central interior British Columbia, Canada. Can J For Res 48:1601–1606. https://doi.org/10.1139/cjfr-2018-0347

Padda KP, Puri A, Chanway CP (2019) Endophytic nitrogen fixation–a possible 'hidden'source of nitrogen for lodgepole pine trees growing at unreclaimed gravel mining sites. FEMS Microbiol Ecol 95:fiz172. https://doi.org/10.1093/femsec/fiz172

Palaniappan P, Chauhan PS, Saravanan VS, Anandham R, Sa T (2010) Isolation and characterization of plant growth promoting endophytic bacterial isolates from root nodule of *Lespedeza* sp. Biol Fertil Soils 46:807–816. https://doi.org/10.1007/s00374-010-0485-5

Paul LR, Chapman BK, Chanway CP (2007) Nitrogen fixation associated with *Suillus tomentosus* tuberculate ectomycorrhizae on *Pinus contorta* var. *latifolia*. Ann Bot 99:1101–1109. https://doi.org/10.1093/aob/mcm061

Paul LR, Chapman WK, Chanway CP (2013) Diazotrophic bacteria reside inside *Suillus tomentosus*/*Pinus contorta* tuberculate ectomycorrhizae. Botany 91:48–52. https://doi.org/10.1139/cjb-2012-0191

Perry DA, Oren R, Hart SC (2008) Forest ecosystems, 2nd edn. The John Hopkins University Press, Baltimore

Pojar J, Meidinger DV (1991) Introduction. In: Meidinger DV, Pojar J (eds) Ecosystems of British Columbia. British Columbia Ministry of Forests, Lands, Natural Resource Operations and Rural Development, Victoria, Canada, pp. 1–8. https://www.for.gov.bc.ca/hfd/pubs/docs/Srs/Srs06/chap1.pdf. Accessed 16 May 2020

Puri A, Padda KP, Chanway CP (2015) Can a diazotrophic endophyte originally isolated from lodgepole pine colonize an agricultural crop (corn) and promote its growth? Soil Biol Biochem 89:210–216. https://doi.org/10.1016/j.soilbio.2015.07.012

Puri A, Padda KP, Chanway CP (2016a) Evidence of nitrogen fixation and growth promotion in canola (*Brassica napus* L.) by an endophytic diazotroph *Paenibacillus polymyxa* P2b-2R. Biol Fertil Soils 52:119–125. https://doi.org/10.1007/s00374-015-1051-y

Puri A, Padda KP, Chanway CP (2016b) Seedling growth promotion and nitrogen fixation by a bacterial endophyte *Paenibacillus polymyxa* P2b–2R and its GFP derivative in corn in a long-term trial. Symbiosis 69:123–129. https://doi.org/10.1007/s13199-016-0385-z

Puri A, Padda KP, Chanway CP (2017a) Beneficial effects of bacterial endophytes on forest tree species. In: Maheshwari DK, Annapurna K (eds) Endophytes: crop productivity and protection. Springer, Cham, pp 111–132. https://doi.org/10.1007/978-3-319-66544-3_6

Puri A, Padda KP, Chanway CP (2017b) Plant growth promotion by endophytic bacteria in nonnative crop hosts. In: Maheshwari DK, Annapurna K (eds) Endophytes: crop productivity and protection. Springer, Cham, pp 11–45. https://doi.org/10.1007/978-3-319-66544-3_2

Puri A, Padda KP, Chanway CP (2018a) Evidence of endophytic diazotrophic bacteria in lodgepole pine and hybrid white spruce trees growing in soils with different nutrient statuses in the west Chilcotin region of British Columbia, Canada. For Ecol Manag 430:558–565. https://doi.org/10.1016/j.foreco.2018.08.049

Puri A, Padda KP, Chanway CP (2018b) Nitrogen-fixation by endophytic bacteria in agricultural crops: recent advances. In: Khan A, Fahad S (eds) Nitrogen in agriculture – updates. IntechOpen, London, pp 73–94. https://doi.org/10.5772/intechopen.71988

Puri A, Padda KP, Chanway CP (2020a) Can naturally-occurring endophytic nitrogen-fixing bacteria of hybrid white spruce sustain boreal forest tree growth on extremely nutrient-poor soils? Soil Biol Biochem 140:107642. https://doi.org/10.1016/j.soilbio.2019.107642

Puri A, Padda KP, Chanway CP (2020b) In vitro and in vivo analyses of plant-growth-promoting potential of bacteria naturally associated with spruce trees growing on nutrient-poor

soils. Appl soil Ecol 806 149:103538. https://doi.org/10.1016/j.apsoil.2020.103538

Rennie RJ, Fried M, Rennie DA (1978) Concepts of ^{15}N usage in dinitrogen fixation studies. In: Isotopes in Biological Dinitrogen Fixation, pp. 107–131

Sawana A, Adeolu M, Gupta RS (2014) Molecular signatures and phylogenomic analysis of the genus *Burkholderia*: proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. Nov. harboring environmental species. Front genet 5:429. https://doi.org/10.3389/fgene.2014.00429

Shishido M, Chanway CP (2000) Colonization and growth of outplanted spruce seedlings pre-inoculated with plant growth-promoting rhizobacteria in the greenhouse. Can J For Res 30:848–854. https://doi.org/10.1139/x00-010

Shishido M, Loeb BM, Chanway CP (1995) External and internal root colonization of lodgepole pine seedlings by two growth-promoting *Bacillus* strains originated from different root microsites. Can J Microbiol 41:707–713. https://doi.org/10.1139/m95-097

Shishido M, Brevil C, Chanway CP (1999) Endophyic colonization of spruce by plant growth promoting rhizobacteria. FEMS Microbiol Ecol 29:191–196. https://doi.org/10.1111/j.1574-6941.1999.tb00610.x

Steen OA, Demarchi DA (1991) Sub-boreal pine-spruce zone. In: Meidinger DV, Pojar J (eds) Ecosystems of British Columbia. British Columbia Ministry of Forests, Lands, Natural Resource Operations and Rural Development, Victoria, Canada, pp. 195–207. https://www.for.gov.bc.ca/hfd/pubs/docs/Srs/Srs06/chap13.pdf. Accessed 16 May 2020

Steen OA, Coupé RA (1997) A field guide to forest site identification and interpretation for the Cariboo forest region. British Columbia ministry of forests, Lands, Natural Resource Operations and Rural Development, Victoria, Canada. https://www.for.gov.bc.ca/hfd/pubs/docs/lmh/lmh39.htm. Accessed 16 May 2020

Tang Q, Puri A, Padda KP, Chanway CP (2017) Biological nitrogen fixation and plant growth promotion of lodgepole pine by an endophytic diazotroph *Paenibacillus polymyxa* and its GFP-tagged derivative. Botany 95:611–619. https://doi.org/10.1139/cjb-2016-0300

Valladares F, Niinemets Ü (2008) Shade tolerance, a key plant feature of complex nature and consequences. Annu Rev Ecol Evol Syst 39:237–257. https://doi.org/10.1146/annurev.ecolsys.39.110707.173506

Weetman GF, Fournier RM, Schnorbus E (1988) Lodgepole pine fertilization screening trials: four-year growth response following initial predictions. Soil Sci Soc Am J 52:833–839. https://doi.org/10.2136/sssaj1988.03615995005200030042x

Willan RL (1986) A guide to forest seed handling. Food and Agricultural Organization of United Nations, Rome. http://www.fao.org/3/ad232e/ad232e00.htm. Accessed 16 May 2020

Yang H, Puri A, Padda KP, Chanway CP (2016) Effects of *Paenibacillus polymyxa* inoculation and different soil nitrogen treatments on lodgepole pine seedling growth. Can J For Res 46:816–821. https://doi.org/10.1139/cjfr-2015-0456

Yang H, Puri A, Padda KP, Chanway CP (2017) Substrate utilization by endophytic *Paenibacillus polymyxa* that may facilitate bacterial entrance and survival inside various host plants. FACETS 2:120–130. https://doi.org/10.1139/facets-2016-0031