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Communication

Increased Biomass of Nursery-Grown Douglas-Fir Seedlings upon Inoculation with Diazotrophic Endophytic Consortia

Zareen Khan, Shyam L. Kandel, Daniela N. Ramos, Gregory J. Ettl, Soo-Hyung Kim and Sharon L. Doty *

School of Environmental and Forest Sciences, College of the Environment, University of Washington, Seattle, WA 98195, USA; E-Mails: zareen@uw.edu (Z.K.); sk66@uw.edu (S.L.K.); dnavil9@uw.edu (D.R.); ettl@uw.edu (G.J.E.); soohkim@uw.edu (S.-H.K.)

* Author to whom correspondence should be addressed; E-Mail: sldoty@uw.edu; Tel.: +1-206-616-6255.

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Abstract: Douglas-fir (*Pseudotsuga menziesii*) seedlings are periodically challenged by biotic and abiotic stresses. The ability of endophytes to colonize the interior of plants could confer benefits to host plants that may play an important role in plant adaptation to environmental changes. In this greenhouse study, nursery-grown Douglas-fir seedlings were inoculated with diazotrophic endophytes previously isolated from poplar and willow trees and grown for fifteen months in nutrient-poor conditions. Inoculated seedlings had significant increases in biomass (48%), root length (13%) and shoot height (16%) compared to the control seedlings. Characterization of these endophytes for symbiotic traits in addition to nitrogen fixation revealed that they can also solubilize phosphate and produce siderophores. Colonization was observed through fluorescent microscopy in seedlings inoculated with *gfp*- and *mkate*-tagged strains. Inoculation with beneficial endophytes could prove to be valuable for increasing the production of planting stocks in forest nurseries.

Keywords: Douglas-fir; conifers; nurseries; endophytes; diazotrophic; phosphate solubilization; siderophore; biomass; sustainable forestry

1. Introduction

Millions of Douglas-fir (*Pseudotsuga menziesii*) seedlings are grown in nurseries for reforestation purposes in North America, Europe and elsewhere [1]. The majority of seedlingsare grown for more than a year as bare root seedlings, although some are also grown in containers. Environmental disturbances and stresses contribute to seedling failure when transplanted in the field, and in order to maximize production, nurseries apply intensive cultural practices, fertilization being one of the most common [1,2]. This can result in additional costs to nursery operators and could also have negative environmental repercussions if nitrogen leaches from nurseries situated in areas near lakes and rivers and/or where ground water reservoirs are found. An inexpensive and environmentally-benign alternative for enhancing the productivity of newly-established forest plantations involves nursery inoculation of seedlings with plant growth-promoting microorganisms. In forestry, this has traditionally been restricted to inoculation with mycorrhizal fungi [3,4]; however, it is reported that in many cases, mycorrhizal inoculation resulted in low to no measurable benefit in outplanting success. Recently, root-associated bacteria have been shown to stimulate tree seedling growth in addition to improving mycorrhizal colonization and may be valuable in current reforestation efforts [5,6].

Some species of conifers are able to inhabit harsh and nutrient-poor subalpine sites where few other plants grow. A key component of their ecological success may be attributed to the presence of endophytes. Endophytes are microbes (bacteria and fungi) that reside inside plants and do not cause disease [7–10], and there is increasing evidence of their profound impact on plant development, physiology, evolution and adaptation [11]. An important trait of some endophytes is the ability to supply nitrogen to their host plant through biological nitrogen fixation. Nitrogen-fixing endophytes have been isolated from a variety of species, such as sugarcane [12], wild rice [13], corn [14,15], African sweet potato [16], kallar grass [17], coffee [18], cactus [19] and woody plants, including poplar, willow and coniferous trees [20–23]. It has been demonstrated that N fixed by diazotrophic bacteria can be utilized by plants [24]. Endophytic bacteria have been found in different parts of tree tissues, such as roots [25], stems [26], shoots [27], leaves [28] and buds [29]. More recently, diazotrophic endophytes were isolated from conifers growing in nitrogen-poor soils. Inoculation of lodgepole pine and western red cedar seedlings with the isolate *Paenibacillus polymyxa* P2b2R resulted in significant foliar ^{15}N dilution, indicative of biological nitrogen fixation [30,31]. The importance of endophytes to the establishment and persistence of long-lived western conifers is unknown; however, endophytes may allow establishment and growth on nutrient-limited and environmentally-stressed sites; for example, similar endophytes were found in subalpine conifers from distant sites in California and Colorado [32].

Phosphorous is another essential macronutrient promoting plant growth and development. Many woody plants are dependent on ectomycorrhizal fungi for their growth and survival. Mycorrhizae inoculation of Douglas-fir seedlings is a common practice in commercial nurseries [33,34]. However, commercial nursery treatments, including frequent addition of fertilizer and water, may not be favorable for mycorrhizae colonization of container-grown seedlings [35]. The importance of phosphate-solubilizing endophytes in increasing the phosphorous availability by solubilization of inorganic phosphate and mineralization of organic phosphate has recently been demonstrated as one of the important mechanisms of increased plant growth [36]. Siderophores are low molecular weight ferric iron-specific chelating agents produced by bacteria and fungi [37]. Microbial siderophores have been

reported to have a positive correlation with plant growth promotion, and the production of siderophores is being considered as one of the key traits for primary screening of beneficial endophytes [38].

In this preliminary study, we chose a consortia of diazotrophic endophytes, originally isolated from poplar and willow trees [20–22], because they had shown robust growth-promoting activity with agricultural crops, grasses and poplar plants grown under abiotic stress [22,39–41]. Our objective was to determine if these endophytes enhance the growth of Douglas-fir seedlings under nutrient-poor conditions.

2. Experimental Section

2.1. Plant Material and Endophytes

Douglas-fir container (1 + 0) (4 cu. in ; Ray Leach "cone-tainers") seedlings from a local western WA provenance obtained from Silvaseed Company Foresters (Roy, WA, USA) were individually planted into plastic pots containing Sunshine Mix #2 (Steubers, WA, USA). The propagation protocol for seedling production is described elsewhere [42]. Seven bacterial strains and one yeast strain (Table 1) were chosen based on strong growth in nitrogen-free media, the presence of the nitrogen fixation gene (*nifH*) [20,21], production of phytohormones [43] and/or their robust plant growth-promoting abilities.

Table 1. Poplar and willow endophytes used in this study and their phosphate solubilization index (PSI) in the plate assay [20,21].

Endophyte	Closest 16SrDNA Match	PSI
WP1	*Rhodotorula graminis*	1.05
WP5	*Rahnella* sp.	1.64
WP9	*Burkholderia* sp.	1.50
WP19	*Acinetobacter calcoaceticus*	3.10
PTD1	*Rhizobium tropici* bv *populus*	1.40
WW5	*Sphingomonas yanoikuyae*	1.10
WW6	*Pseudomonas putida*	1.64
WW7	*Sphingomonas* sp.	1.82

2.2. Methods

Inoculation suspensions were prepared by first growing the individual endophyte strains on nutrient-rich media from −80 °C glycerol stocks. Bacteria were grown on mannitol glutamate/Luria–Bertani (MG/L) medium [44]. The yeast strain was grown on yeast extract peptone dextrose (YPD) medium. Single colonies were inoculated into liquid broth and grown overnight on a rotary shaker (150 rpm) and agitated for 24 h at 30 °C. Bacteria were harvested by centrifugation at 8000 rpm for 10 min, resuspended in nitrogen-free Murashige and Skoog (NFMS) (Caisson Labs, USA) broth and washed three times in NFMS. The endophyte consortia treatment was prepared by mixing equal concentrations of each inoculation suspension as determined by measuring the optical density at 600 nm (OD_{600}) of the individual strains and adjusting to a final OD_{600} of 0.1. Using a sterile conical tube, a 50-mL sample of the endophyte consortia was then delivered 5–10 cm below the surface and in close proximity to the roots. Mock-inoculated control seedlings received the same volume of sterile

liquid broth. A total of 24 pots, 12 replications of inoculated or mock-inoculated control plants, were arranged in a randomized fashion. Seedlings were kept in an environment with an 18h photoperiod, a 20/14 °C day/night temperature cycle and a relative humidity of 70%–85%. Drip trays were placed under each individual pot to prevent cross-contamination through run-off. Each pot was individually irrigated with tap water. Irrigation frequency and duration were adjusted, such that the pots were not allowed to flood nor dry out between watering. Additionally, each pot was fertigated with received 100 mL of 1/2 strength, 1/8 nitrogen modified Hoagland's medium containing (g L^{-1}): $CaCl_2 \cdot 2H_2O$, 0.22; K_2SO_4, 0.17; $MgSO_4 \cdot 7H_2O$, 0.26; KH_2PO_4, 0.136; NaFeEDTA(10% Fe), 0.015; with 1 mL·L^{-1} micronutrient solution containing (g·L^{-1}): H_3BO_3, 0.773; $MnSO_4$, 0.169; $ZnSO_4 \cdot 7H_2O$, 0.288; $CuSO_4 \cdot 5H_2O$, 0.062; H_2MoO_4 (83% MoO_3), 0.04; weekly for the first two months post-inoculation, then 250 mL twice monthly until the end of the experiment.

Height measurements were recorded after inoculation and every month thereafter. After 15 months, the plants were removed from the soil, and roots and shoots were gently washed to remove dirt and debris. Roots and stems were then blotted dry, cut into root and shoot sections, and the lengths and wet weights were determined. Foliar samples were taken from each plant and oven dried at 65 °C, ground to a fine powder and analyzed for nitrogen content using a PE 2400 series II CHN elemental analyzer (Perkin-Elmer, MA, USA) at the University of Washington's School of Environmental and Forest Sciences Analytical Soils laboratory.

Potential plant growth-promoting traits, such as phosphate solubilization and siderophore production, were determined for the selected endophytes. The ability of the isolates to solubilize tricalcium orthophosphate (TCP) was tested on the National Botanical Research Institute's growth medium [45] containing g·L^{-1}: glucose, 10; $Ca_3(PO_4)_2$, 5; $MgCl_2 \cdot 6H_2O$, 5; $MgSO_4 \cdot 7H_2O$, 0.25; KCl, 0.2; $(NH_4)_2SO_4$, 0.1. The inoculum was added in quadruplicate on the medium in Petri dishes. The halo and colony diameters were measured after 14 days of incubation of the plates at 25 °C. The ability of the microbes to solubilize the insoluble phosphate is described by the solubilization index (the ratio of the total diameter (halo + colony diameter (mm) to the colony diameter (mm)). Analysis of siderophore production by the endophyte isolates was performed following the chrome azurol S (CAS) method of Alexander and Zuberer [46]. For each isolate, 10 µL of inoculum were placed in quadruplicate on the medium. After incubation at 25 °C for 3 days, the discoloration of the medium (blue to orange) indicated siderophore-producing endophytes.

To evaluate endophytic colonization by some of the isolates, we used strains that were labeled with fluorescent markers with a broad host range plasmid. The *mkate* and *gfp* plasmids were introduced into *Acinetobacter calcoaceticus* strain WP19 and *Rahnella* sp. strain WP5, respectively, via triparental mating using *E. coli* DH5α as the donor and *E. coli* HB101 (pRK 2073) as the helper strain [47]. The inoculations were done as described above, and the colonization of root tissue and needles was verified through fluorescent microscopy using a Zeiss Imager M2 equipped with an AxioCam MRM and recorded with Zeiss AxioVision software (Karl Zeiss, LLC, Thornwood, NY, USA).

2.3. Statistical Analysis

One-way ANOVA (analysis of variance) and Tukey's honestly significant difference (HSD) test were used to identify significant differences between inoculated and mock-inoculated control seedlings.

3. Results and Discussion

Differences in plant heights and the health of inoculated and mock-inoculated control plants were apparent after fifteen months of growth in the greenhouse under N-limited conditions. As shown in Figure 1, the mock-inoculated control plants were stunted, with some being chlorotic and showing signs of nutrient deficiency, whereas the inoculated plants appeared taller, more robust and greener than the controls. The inoculated plants were 16% (p = 0.008) taller, had 13% longer roots (p = 0.024) (Figure 2) and a significant 48% (p = 0.019) increase in biomass (Figures 2 and 3) when compared to their mock-inoculated control plants. The concentration of N in plant tissues was higher in the inoculated plants compared to the controls, although it was not statistically significant (p = 0.156) (Figure 4).

Figure 1. Representative photograph of mock-inoculated (left) and inoculated (right) Douglas-fir seedling after 15 months of growth in nutrient-poor soil.

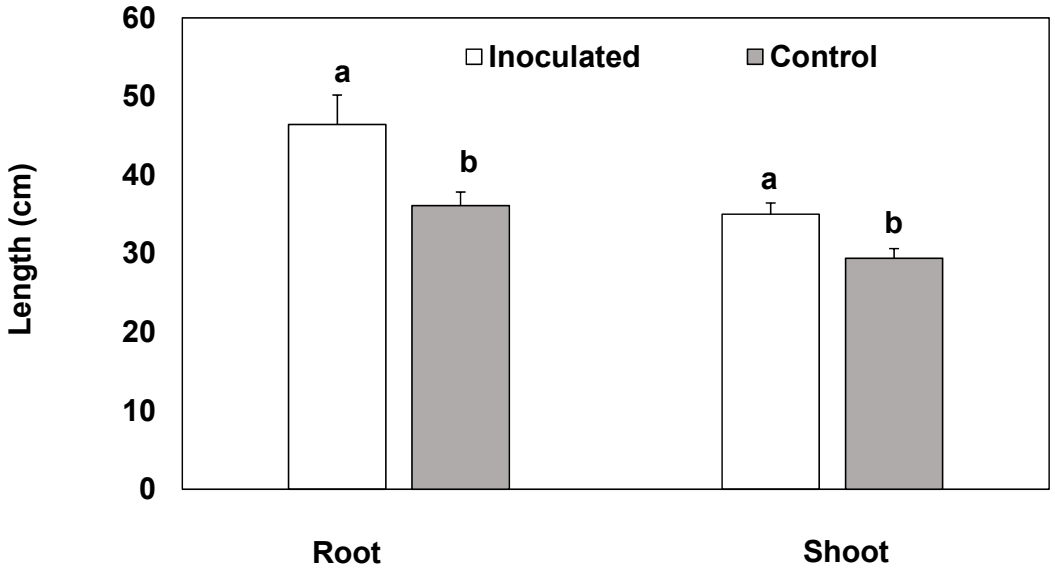


Figure 2. Root and shoot lengths of inoculated and control Douglas-fir seedlings at harvest. Data are the mean of 12 replicates. Error bars show the standard deviation. Means designated with different letters are significantly different.

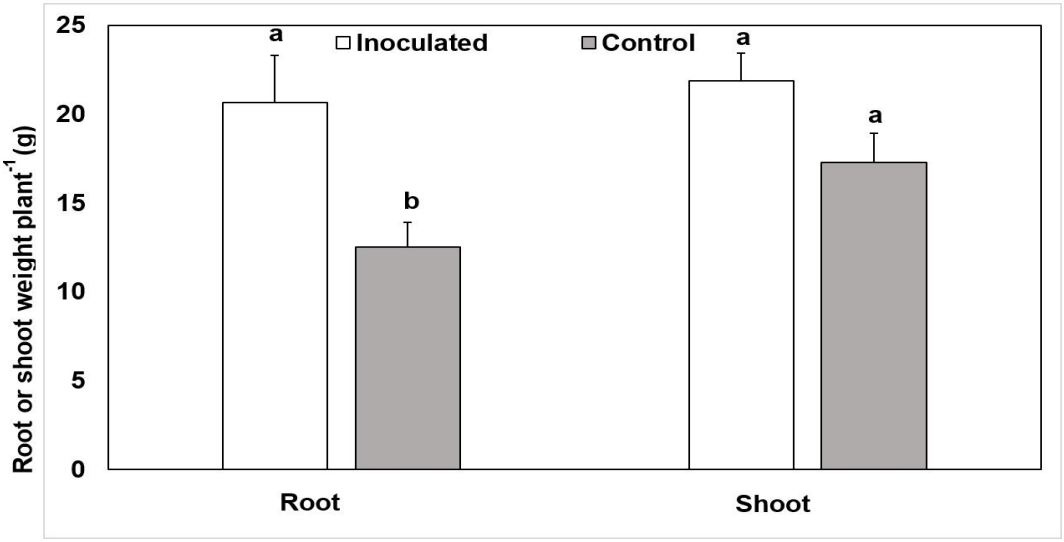


Figure 3. Root and shoot weights (g) of inoculated and control Douglas-fir seedlings at harvest. Data are the mean of 12 replicates. Error bars show the standard deviation. Means designated with different letters are significantly different.

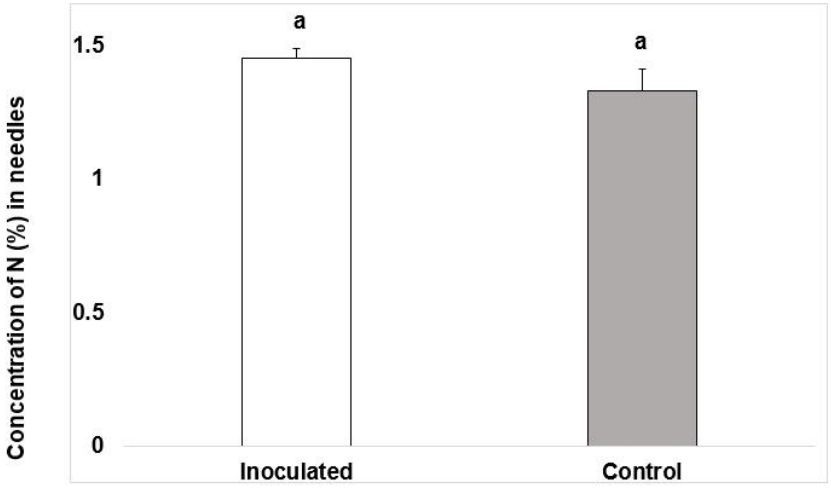


Figure 4. Total N concentration in the inoculated and control Douglas-fir seedlings needles at harvest. Data are the mean of 12 replicates. Error bars show the standard deviation. ($p = 0.156$).

Our results demonstrate that inoculation with the poplar and willow endophyte consortia stimulated growth of Douglas-fir seedlings under low nutrient conditions. It was previously concluded that these endophytes are not host-specific colonizers [39]. Poplar and willow plants are angiosperms, which diverged from gymnosperms more than 100 million years ago [48]. Remarkably, the endophytes from poplar and willow plants colonized Douglas-fir, a gymnosperm. It is possible that the required plant-microbe communication may be ancient, pre-dating the divergence of angiosperms and gymnosperms. Interestingly, the response of Douglas-fir to inoculation with the endophytes took more than a year to develop, which suggests that this plant species may require a period of growth under controlled conditions to facilitate the establishment of the symbiotic relationship with these endophytes. Parker and Dangerfield [49] also reported a delayed response to microbial inoculation in Douglas-fir. In

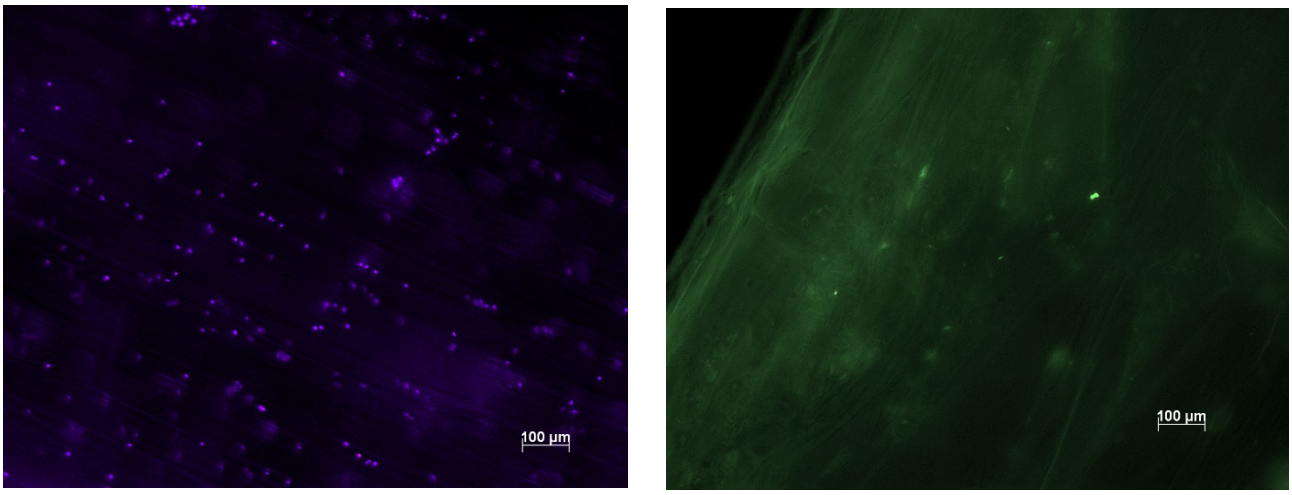
studies done by Chanway *et al.* [50], rhizospheric bacteria isolated from perennial rye grass and white clover stimulated the growth of container Douglas-fir seedlings after 12 weeks of inoculation. More recently, Anand *et al.* [31] showed that seedling growth enhancement in other conifers, lodgepole pine and western red cedar occurred only after 13 months.

To assess other potential mechanisms in addition to N fixation for the improved growth of the inoculated plants, we investigated the potential of phosphate solubilization by the endophytes. The majority of the isolates were able to produce a clear zone in minimal medium containing insoluble phosphorous. As shown in Table 1, the PSI of isolate WP19 was the highest, indicating the high solubilization capacity of this endophyte compared to the rest of the endophytes. There have been a number of reports on plant growth promotion by bacteria that have the ability to convert the insoluble inorganic forms of P into soluble forms through acidification, secretion of organic acids or protons and exchange reactions [36]. Many symbiotic phosphate-solubilizing microorganisms belonging to species of the genera *Bacillus, Pseudomonas*, *Rhizobium*, *Enterobacter* and *Burkholderia* and certain fungi produce phytase to mineralize organic phosphate [51]. In our study, phosphate solubilization may be one of the important mechanism through which these endophytes promoted growth of Douglas-fir seedlings; however, additional studies including phosphorous quantification and mutant analysis will be necessary to determine what symbiotic factors contribute the most to growth enhancement. The role of mycorrhizal symbiosis in increasing phosphorous uptake has been studied in a number of conifer species, with recent studies showing how mycorrhizal effects on seedling establishment change with soil conditions [52]. In dry Douglas-fir forests, reduced mycorrhizal diversity and abundance have been associated with reduced survival and growth of newly-planted conifer seedlings [53,54]. Inoculating nursery seedlings with beneficial endophytes may help offset some of these disadvantages. Furthermore, endophytes may increase mycorrhizal development by affecting root colonization, as well as by enhancing N and P uptake.

Rungin *et al.* [55] recently demonstrated plant growth-enhancing effects by a siderophore-producing endophytic streptomycete isolated from Thai jasmine rice plants. Significant increases in total biomass of rice and mung bean plants were observed in the inoculated controls compared to the untreated controls and siderophore-deficient mutant treatment. Most of the endophytes in our study produced siderophores, which may have been beneficial in plant growth promotion. However, this is largely speculative and warrants further research. Siderophore-producing microbes may also have significant activity against plant pathogens [56,57]. Interestingly, we found that Douglas-fir seeds inoculated with these endophytes were significantly less affected by Fusarium, a common Douglas-fir pathogen [58].Therefore, these endophytes merit further investigation, as reducing mortality by inoculations could prove valuable to tree nurseries.

To evaluate endophytic colonization, Douglas-fir seedlings were inoculated with fluorescent-labelled strains of WP5 and WP19. As seen from the fluorescent microscope images, *mkate*-tagged cells of *Acinetobacter* strain WP19 were localized in the intercellular spaces of root tissue of the inoculated seedlings (Figure 5A). In addition, GFP-tagged cells of *Rahnella* sp. strain WP5 colonized the needles (Figure 5B), suggesting that this endophyte is able to move up the plant into aerial parts after inoculation. Similar colonization zones have been reported for other endophytes [30].

The enhanced growth in inoculated seedlings seen in this preliminary study merits further research in larger greenhouse trials and field trials to assess the long-term benefits of endophyte inoculations.



(**A**) (**B**)

Figure 5. Fluorescence micrographs showing intercellular colonization of *mkate*-tagged strain WP19 in the root tissue of the inoculated seedling of Douglas-fir (**A**) and GFP-tagged strain WP5 in the needle of the inoculated Douglas-fir (**B**). Samples were surface-sterilized and visualized (1000×) three weeks after inoculation.

4. Conclusions

The results of the present study indicate that inoculation with a diazotrophic endophytic consortia significantly increased Douglas-fir seedling growth under nutrient-limited conditions. Most commercial nursery treatments add high rates of fertilizers to maximize seedling shoot and total growth to increase establishment and success in the field [59] which is not only expensive, but also suppresses mycorrhiza development of container seedlings [60]. Therefore, endophyte inoculation of seedlings may be considered as a cost-effective technique to increase growth in the early stage for the nursery stock, which can lead to later stage successes in transplanting and establishment after transplanting.

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Author Contributions

Zareen Khan carried out the experiments and wrote the manuscript. Shyam L Kandel and Daniela N Ramos contributed to the experiments and data analysis. Doty served as the Principal Investigator (Mc Intire-Stennis and NIFA grants) and directed the project. Ettl and Kim served as co-PI's for the project. Doty, Ettl and Kim edited the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Van den Driessche, R. Growth, survival and physiology of Douglas-fir seedlings following root wrenching and fertilization. *Can. J. For. Res.* **1983**, *13*, 270–278.
2. Aldhous, I. Nursery Practice. *For. Comm. Bull*. **1972**, *43*, 184.
3. Cram, M.M.; Dumroese, K.R. Mycorhizae in Forest Tree nurseries. In *Forest Nursery Pests. Agriculture Handbook*; Cram, M.M.F., Mallams, K.M., Eds.; Forest Service: Washington, DC, USA, 2012; pp. 20–25.
4. Kropp, B.R.; Langlois, C.G. Ectomycorrhizae in reforestation. *Can. J. For. Res*. **1990**, *20*, 438–451.
5. Tien, T.M.; Gaskins, M.H.; Hubbell, D.H. Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of Pearl Millet. *Appl. Environ. Microbiol.* **1979**, *37*, 1016–1024.
6. Chanway, C.P.; Holl, F.B.; Rurkington, R. Genotypic coadaptation in growth promotion of forage species by *Bacillus polymyxa*. *Plant Soil* **1988**, *106*, 281–284.
7. Ryan, R.P.; Germaine, K.; Franks, A.; Ryan, D.J.; Dowling, D.N. Bacterial endophytes: Recent developments and applications. *FEMS. Microbiol. Lett.* **2008**, *278*, 1–9.
8. Hardoim, P.R.; van Overbeek, L.S.; van Elsas, J.D. Properties of bacterial endophytes and their proposed role in plant growth. *Trends Microbiol.* **2008**, *16*, 463–471.
9. Gaiero, J.R.; McCall, C.A.; Thompson, K.; Day, N.J.; Best, A.S.; Dunfield, K.E. Inside the root microbiome: Bacterial root endophytes and plant growth promotion. *Am. J. Bot*. **2013**, *100*, 1738–1750.
10. *Symbiotic Endophytes*; Springer-Verlag Berlin Heidelberg: New York, NY, USA, 2014.
11. Compant, S.; van der Heijden, M.G.A.; Sessitch, A. Climate change on beneficial plant microorganism interactions. *FEMS Microbiol. Ecol.* **2010**, *73*, 197–214.
12. Welbaum, G.E.; Sturz, A.V.; Dong, Z.; Nowak, J. Endophytic nitrogen fixation in sugarcane: Present knowledge and future applications. *Plant Soil* **2003**, *252*, 139–149.
13. Peng, G.; Zhang, W.; Luo, H.; Xie, H.; Lai, W.; Tan, Z. Enterobacter oryzae sp. nov., a nitrogen-fixing bacterium isolated from the wild rice species *Oryza latifolia*. *Int. J. Syst. Evol. Microbiol.* **2009**, *59*, 1650–1655.
14. Caballero-Mellado, J.; Martinez-Aguilar, L.; Paredes-Valdez, G.; Estrada-de Los Santos, P. *Burkholderia unamae* sp. nov., an N_2-fixing rhizospheric and endophytic species. *Int. J. Syst. Evol. Microbiol.* **2004**, *54*, 1165–1172.
15. Montanez, A.; Abreu, C.; Gill, P.R.; Hardarson, G.; Sicardi, M. Biological nitrogen fixation in maize (*Zea mays* L.) by N-15 isotope-dilution and identification of associated culturable diazotrophs. *Biol. Fert. Soils* **2009**, *45*, 253–263.
16. Reiter, B.; Burgmann, H.; Burg, K.; Sessitsch, A. Endophytic nifH gene diversity in African sweet potato. *Can. J. Microbiol.* **2003**, *49*, 549–555.
17. Reinhold-Hurek, B.; Hurek, T. Life in grasses: Diazotrophic endophytes. *Trends Microbiol.* **1998**, *6*, 139–144.

18. Jimenez-Salgado, T.; Fuentes-Ramirez, L.F.; Tapia-Hernandez, A.; Mascarua-Esparza, M.A.; Martinez-Romero, E.; Caballero-Mellado, J. *Coffea arabica* L., A new host plant for *Acetobacter diazotrophicus*, and isolation of other nitrogen-fixing acetobacteria. *Appl. Environ. Microbiol.* **1997**, *63*, 3676–3683.
19. Lopez, B.R.; Bashan, Y.; Bacilio, M. Endophytic bacteria of Mammillaria fraileana, an endemic rock-colonizing cactus of the southern Sonoran Desert. *Arch. Microbiol*. **2011**, *193*, 527–541.
20. Doty, S.L.; Dosher, M.R.; Singleton, G.L.; Moore, A.L.; Aken, B.; van Stettler, R.F.; Gordon, M.P. Identification of an endophytic Rhizobium in stems of Populus. *Symbiosis* **2005**, *39*, 27–35.
21. Doty, S.L.; Oakley, B.; Xin, G.; Kang, J.W.; Singleton, G.; Khan, Z.; Vajzovic, A.; Staley, J.T. Diazotrophic endophytes of native black cottonwood and willow. *Symbiosis* **2009**, *47*, 23–33.
22. Xin, G.; Zhang, G.; Kang, J.W.; Staley, J.T.; Doty, S.L. A diazotrophic, indole-3-acetic acid-producing endophyte from wild cottonwood. *Biol. Fert. Soils* **2009**, *45*, 669–674.
23. Bormann, B.T.; Bormann, W.B.; Bowden, R.S.; Piece, S.P.; Hamburg, D.; Wang, M.; Snyder, C.; Ingersoll, R.C. Rapid N_2 Fixation in Pines, Alder, and Locust: Evidence From the Sandbox Ecosystems Study. *Ecosystems* **1993**, *74*, 583–598.
24. Pankievicz, V.C.S.; do Amaral, F.P.; Santos, K.F.D.N.; Agtuca, B.; Xu, Y.; Schueller, M.J.; Arisi, A.C.M.; Steffens, M.B.R.; de Souza, E.M.; Pedrosa, F.O.; *et al*. Robust biological nitrogen fixation in a model grass-bacterial association. *Plant J.* **2015**, *81*, 907–919.
25. Mocali, S.; Bertelli, E.; Dicello, F.; Mengoni, A.; Falanga, A.; Villani, F.; Caciotti, A.; Tegli, S.; Surico, G.; Fani, R. Fluctuation of bacteria isolated from elm tissues during different seasons and from different plant organs. *Res. Micribiol.* **2003**, *154*, 105–114.
26. Bal, A.S.; Anand, R.; Berge, O.; Chanway, C.P. Isolation and identification of diazotrophic bacteria from internal tissues of *Pinus contorta* and *Thuja plicata*. *Can. J. For. Res.* **2012**, *42*, 807–813.
27. Izumi, H.; Anderson, I.C.; Killham, K.; Moore, E.R. Diversity of predominant endophytic bacteria in European deciduous and coniferous trees. *Can. J. Microbiol.* **2008**, *54*, 173–179.
28. Ulrich, K.; Ulrich, A.; Ewald, D. Diversity of endophytic bacterial communities in poplar grown under field conditions. *FEMS Microbiol.* **2008**, *63*, 169–180.
29. Pirttila, A.M.; Laukkanen, H.; Pospiech, H.; Myllyla, R.; Hohtola, A. Detection of intracellular bacteria in the buds of scotch pine (*Pinus sylvestris* L.) by insitu hybridization. *Appl. Environ. Microbiol.* **2000**, *66*, 3073–3077.
30. Bal, A.S.; Chanway, C.P. Evidence of nitrogen fixation in lodgepole pine inoculated with diazotrophic *Paenibacillus polymyxa*. *Botany* **2012**, *90*, 891–896.
31. Anand, R.; Grayston, S.; Chanway, C.P. N_2-fixation and seedling growth promotion of lodgepole pine by endophytic *Paenibacillus polymyxa. Microb. Ecol.* **2013**, *66*, 369–374.
32. Carrell, A.A.; Frank, A.C. *Pinus flexilis* and *Picea engelmannii* share a simple and consistent needle endophyte microbiota with a potential role in nitrogen fixation. *Front. Microbiol*. **2014**, doi:10.3389/fmicb.2014.00333.
33. Stack, R.W.; Sinclair, W.A. Protection of Douglas-fir seedlings against *Fusarium* root rot by a mycorrhizal fungus in the absence of mycorrhiza formation. *Phytopathology* **1975**, *65*, 468–472.
34. Horton, T.R.; Bruns, T.D.; Parker, V.T. Ectomycorrhizal fungi associated with *Arctostaphylos* contribute to *Pseudotsuga menziesii* establishment. *Can. J. Bot.* **1999**, *77*, 93–102.

35. Nara, K. Ectomycorhizal networks and seedling establishment during early primary succession. *New Phytol.* **2006**, *169*, 169–178.
36. Rodríguez, H.; Fraga, R. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol. Adv.* **1999**, *17*, 319–339.
37. Neilands, J.B. Siderophores: Structure and function of microbial iron transport compounds. *J. Biol. Chem.* **1995**, *270*, 26723–26726.
38. Glick, B.R. Plant Growth-Promoting Bacteria: Mechanisms and Applications. *Scientifica* **2012**, *2012*, doi:10.6064/2012/963401.
39. Khan, Z.; Guelich, G.; Phan, H.; Redman, R.; Doty, S. Bacterial and Yeast Endophytes from Poplar and Willow Promote Growth in Crop Plants and Grasses. *ISRN Agric*. **2012**, *2012*, 890280:1–890280:11.
40. Knoth, J.L.; Kim, S.H.; Ettl, G.J.; Doty, S.L. Effects of cross host species inoculation of nitrogen-fixing endophytes on growth and leaf physiology of maize. *GCB Bioenergy* **2013**, *5*, 408–418.
41. Knoth, J.L.; Kim, S.H.; Ettl, G.J.; Doty, S.L. Biological nitrogen fixation and biomass accumulation within poplar clones as a result of inoculations with diazotrophic endophyte consortia. *New Phytol.* **2014**, *201*, 599–609.
42. Dumroese, K.R.; Wenny, L.D. Propagation protocol for production of Container (plug) *Pseudotsuga menziesii* Franco plants 66 mL (4cu.in) Ray Leach "Cone-tainers". 2009. Available online: http://www.NativePlantNetwork.org (accessed on 28 September 2015).
43. Khan, Z.; Rho, H.; Firrincieli, A.; Hung, S.H.; Luna, V.; Masciarelli, O.; Kim, S.H.; Doty, S.L. Growth improvement and drought tolerance of hybrid poplar by inoculation with endophyte consoria. **2015**, Submitted.
44. Cangelosi, G.A.; Best, E.A.; Martinetti, G.; Nester, E.W. Genetic analysis of *Agrobacterium*. *Meth. Enzymol.* **1991**, *204*, 384–397.
45. Nautiyal, C.S. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett.* **1999**, *170*, 265–270.
46. Alexander, D.B.; Zuberer, D.A. Use of chrome azurol S reagent to evaluate siderohore production by rhizosphere bacteria. *Biol. Fert. Soils* **1991**, *12*, 39–45.
47. Doty, S.; Chang, M.; Nester, E.W. The chromosomal virulence gene. ChvE of *Agrobacterium tumafaciens* is regulated by a LysR family member. *J. Bacteriol.* **1993**, *175*, 7880–7886.
48. Martin, W.; Lydiate, D.; Brinkmann, H.; Forkmann, G.; Saedle, R.H.; Cerff, R. Molecular phylogenies in angiosperm evolution. *Mol. Biol. Evol.* **1993**, *10*, 140–162.
49. Parker, A.K.; Dangerfield, J.A. Influence of bacterial inoculations on growth of containerized Douglas-fir seedlings. *Can. J. For. Res.* **1975**, *31*, 14–15.
50. Chanway, C.P.; Radley, R.A.; Holl, F.B. Bacterial Inoculation of Lodgepole Pine, White Spruce. In Proceedings of the Intermountain Forest Nursery Association Annual Meeting, Bismarck, ND, USA, 14–18 August 1989; Landis, T.D., Ed.; USDA Forest Service: Fort Collins, CO, USA, 1989.
51. Singh, P.; Kumar, V.; Agrawal, S. Evaluation of phytase producing bacteria for their plant growth promoting activities. *Int. J. Microbiol.* **2014**, *2014*, 7.

52. Horton, T.R.; Cazaras, E.; Bruns, T.D. Ectomycorrhizal, vesicular-arbuscular and dark septate fungal colonization of bishop pine (*Pinus muricata*) seedlings in the first 5 months of growth after wildfire. *Mycorrhiza* **1998**, *7*, 11–18.
53. Kazantseva, O.; Bingham, M.; Simard, S.W.; Berch, S.M. Effects of growth medium, nutrients, water, and aeration on mycorhization and biomass allocation of greenhouse-grown Douglas-fir seedlings. *Mycorrhiza* **2009**, *20*, 51–66.
54. Teste, F.P.; Schmidt, M.G.; Berch, S.M.; Bulmer, C.; Egger, K.N. Effects of ectomycorrhizal inoculants on survival and growth of interior Douglas-fir seedlings on reforestation sites and partially rehabilitated landings. *Can. J. For. Res.* **2004**, *4*, 2074–2088.
55. Rungin, S.; Indananda, C.; Suttiviriya, P.; Kruasuwan, W.; Jaemsaeng, R.; Thamchaipenet, A. Plant growth enhancing effects by a siderophore producing endophytic streptomycete isolated from a Thai jasmine rice plant (*Oryza sativa* L. KDML105). *Antonie Leeuwenhoek* **2012**, *102*, 463–472.
56. Thomashow, L.S. Biological control of plant root pathogens. *Curr. Opin. Biotech.* **1996**, *7*, 343–347.
57. Duijiff, B.J.; Alabouvette, C.; Lemanceau, P. Involvement of pseudobactin 358 in the inhibition of *Fusarium oxysporum* f.sp.lini in flax rhizosphere by the combination of *Pseudomas putida* WCS358 and nonpathogenic *Fusarium oxysporum* F047 and *Pseudomas putida* WCS358. *Phytopathology* **1997**, *89*, 1073–1079.
58. Khan, Z.; Ramos, D.; Matthew, A.; Ettl, J.G.; Doty, S.L. Impact of endophyte inoculation on health of Douglas-fir seedlings. Unpublished work, 2015.
59. Hunt, G.A. Effects of Mycorrhizal Fungi on Quality of Nursery Stock and Plantation Performance in the Southern Interior of British Columbia; FRDA Report ISSN 0835-0752; Natural Resources Canada: Victoria, BC, Canada, 1992; p. 185.
60. Marx, D.H.; Hatch, A.B.; Mendicino, J.F. High soil fertility decreases sucrose content and susceptibility of loblolly pine to ectomycorrhizal infection by *Pisolithis tinctorius*. *Can. J. Bot.* **1977**, *55*, 1569–1574.