Bacterial seed endophytes of Douglas-fir (*Pseudotsuga menziesii*) and their potential to confer drought tolerance

By

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Abstract

Bacterial endophytes have the potential to confer benefits to Douglas-fir (*Pseudotsuga menziesii*), for instance drought tolerance. Bacterial endophytes originating from seeds are especially important. This is because seed endophytes are more likely to be the first endophytes to get established within the young host plant. The goals of this study were to characterize the bacterial seed endophytes of Douglas-fir from the Pacific Northwest and to test whether some bacterial seed endophytes can help seedlings survive under drought conditions. Using culture-based methods, endophytes were first isolated from 8 different populations of Douglas-fir (50 seeds per population). A total of 61 bacteria were isolated from seeds. Isolates were identified by sequencing the 16S rRNA region. A total of 7 genera were identified. Overall, the composition of bacterial seed endophytes differed among Douglas-fir populations, with *Rahnella* isolated most frequently. To test the hypothesis that bacterial seed endophytes could confer drought tolerance to Douglas-fir, two populations of seedlings (WA1 and CA2) were inoculated with *Paenibacillus* sp., *Bacillus* sp., or sterile water for control, then grown under drought conditions. Survival analysis revealed no significant effect of bacterial seed endophyte inoculation on seedling survival under drought conditions.

Introduction

Douglas-fir trees are ecologically and economically important in Oregon. Their forests provide a unique food source and habitat for wildlife. These trees are a valuable source of timber for people because of how large they can grow, which can be up to 11 feet in diameter and at least 300 feet tall (20). Many nurseries also grow young Douglas-firs because they are sought after as Christmas trees. The total economic value of the forest sector in Oregon was estimated to be $22 billion in 2009, which was 11% of Oregon's total goods and services (19). Many stakeholders depend on this tree specie.

Douglas-fir trees are not drought tolerant plants (17). Their health and growth can be affected by prolonged drought (17). Drought stress can make Douglas-fir trees more prone to disease and insect damage. (17). For Oregon the years 2013-2015 were record drought years and although the cause of this could be peaks in a drought cycle, trends show decreasing average precipitation for summers (17). Climate change could prolong drought in the years to come. In the Pacific Northwest temperatures are projected to increase by 1.5 °C from 2003 to 2020, which will produce dryer summers from the reduced summer precipitation and the reduced winter snowmelt (6). Drought associated with climate change is thus a threat to Douglas-fir trees.

Douglas-fir endophytes could help alleviate drought stress. Endophytes are non-pathogenic microbes from a plant's microbiome (8). Endophytes from other plant species have been known to confer drought tolerance to their host plant (8). Certain species of bacterial endophytes belonging to the genus of *Paenibacillus* and *Bacillus* have been found to confer drought tolerance to their host plants (14 & 15). *Paenibacillus polymyxa* can enhance the drought

tolerance of *Arabidopsis thaliana*, which is done by bacterial release of plant growth hormones that promote root growth and increase root surface area (15). *Bacillus sp.*can also release root growth promoting hormones and in addition to that prevent plant cells from leaking electrolytes, to confer drought tolerance on Maize (14). Bacterial species in the *Paenibacillus* and *Bacillus* genera have the potential to alleviate the drought stress of Douglas-firs.

Only a few studies on the seed endophytes of Douglas-fir have been conducted (2). That makes new research about Douglas-fir seed endophyte interactions valuable. If research shows that some Douglas-fir seed endophytes can confer drought tolerance, then those endophytes can be used commercially and in ecological restoration.

My thesis characterizes the composition of bacterial seed endophyte communities for eight Douglas-fir populations located in the Pacific Northwest. I also conducted an inoculation experiment to test whether some of the isolated endophytes can increase tree survival under drought conditions. The findings of this project can provide significant insight on Douglas-fir bacterial endophyte compositions and the interactions between Douglas-fir bacterial seed endophytes and Douglas-fir facing drought conditions.

Materials and Methods

Bacterial seed endophyte characterization

Douglas-fir seed sourcing. Seeds from eight different sourced populations of Douglas-fir from California to Washington were acquired (Silvaseed, Roy, Washington). We expected that spatial, environmental, and plant genetic variation among these populations would lead to differences in bacterial endophyte frequency and community composition.

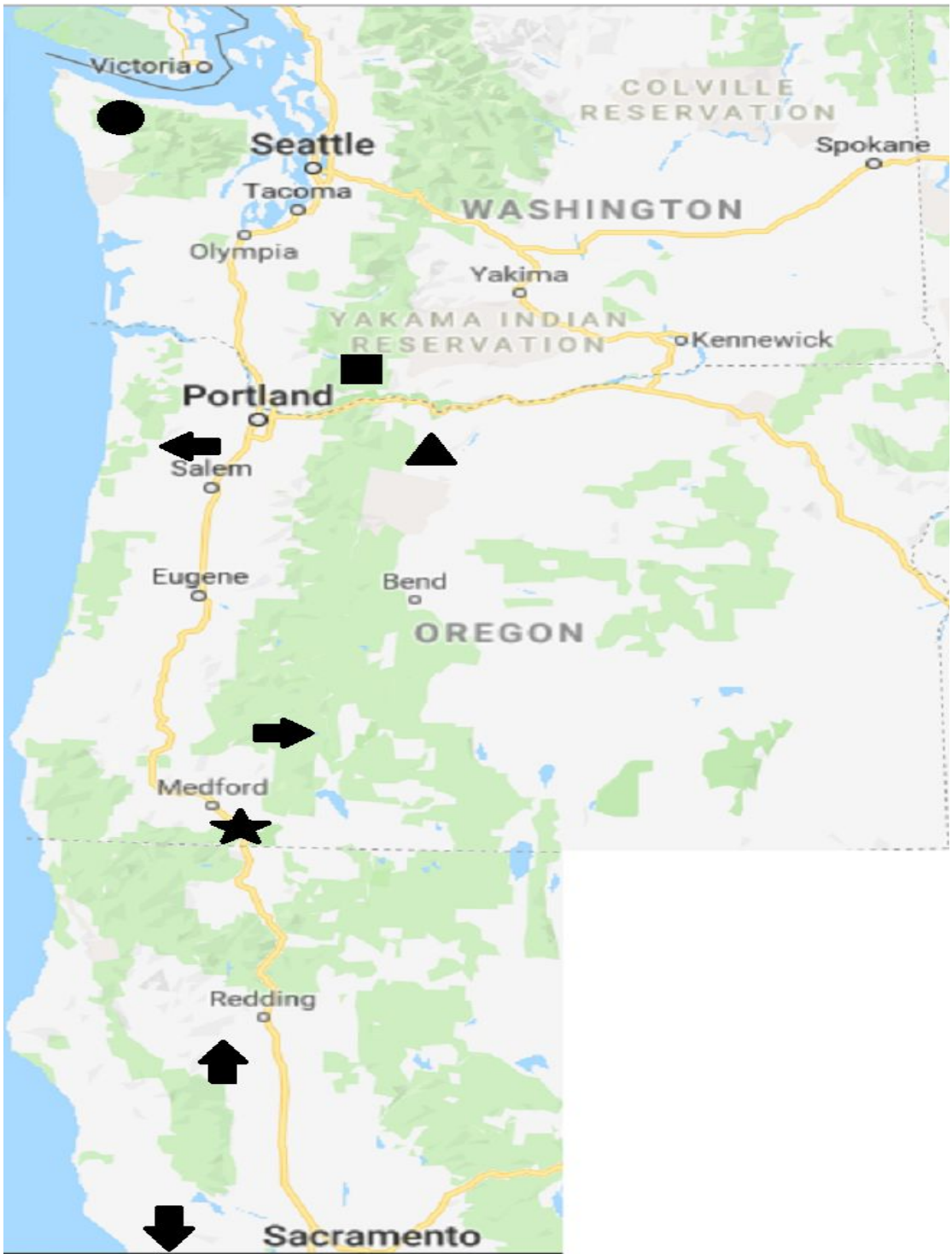

Figure 1. An approximate location of each population of Douglas-fir. WA1 is the circle. WA2 is the square. OR1 is the triangle. OR2 is the left pointing arrow. OR3 is the right pointing arrow. OR4 is the star. CA1 is the up pointing arrow. CA2 is the down pointing arrow.

Douglas-fir stratification. Before any Douglas-fir seeds from the 8 different populations were used, they were stratified. Stratification promotes higher germination rates and more uniform germination of Douglas-fir seeds (11). The Douglas-fir seeds were stratified in cold storage (4 °C) for at least 28 days following the procedure described by (7).

Douglas-fir surface sterilization. Before any Douglas-fir seeds were cultured or germinated, they were surface sterilized. The justification for surface sterilization of Douglas-fir seeds was to eliminate the microbes on the seed coat but not kill the microbes deeper within the seeds. Seeds were sterilized in aseptic conditions. First seeds were washed using ethanol (95%) with a drop of TWEEN for around 30 seconds. Then the seeds were washed in hydrogen peroxide (30%) for 2 minutes. After that the seeds were washed in ethanol (70%) for 2 minutes. Last the seeds were washed in autoclaved (1 hour at 121 °C) deionized water for 1 minute.

Douglas-fir bacterial seed endophytes. 50 seeds from each population of Douglas-fir were surface sterilized and then plated in 60 mm diameter petri dishes filled with LB (Luria-Bertani) agar. A maximum of five seeds each were plated per LB agar petri dish. Bacterial endophytes that emerged from the seeds, within three weeks were isolated into pure culture. Additional bacterial seed endophytes were provided by the lab. Those endophytes were isolated from PDA (Potato Dextrose agar).

Bacterial endophyte DNA extractions, PCR and sequencing. DNA was extracted from each bacterial endophyte using Extract-n-Amp kits (Thermo Fisher Scientific, Waltham, MA). Next, we used PCR to amplify the 16S rRNA region of each sample using Bac 797R and Bac 331F primers. Reactions: included: 2 ul of extraction sample with, 1 ul Bac 797R, 1 ul Bac 331F, 12.5

ul of GoTaq Green Mastermix (Promega), and 8.5 ul of molecular grade water. PCR products were cleaned and sequenced at a MCLAB facility (San Francisco, California). Seqtrace (12) was used to clean raw sequence data (e.g., correcting missing or mismatched codons).

Bacterial endophyte identification and characterization. The cleaned sequences were then compared to sequences from the GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to retrieve a genus level match. The criteria for matches was a greater than 93% match for both query cover and identity match. Isolation frequency was calculated for each genus across the eight populations. Qualitative observations were made on potential differences in isolation frequency and community composition among populations.

Drought experiment

Douglas-fir tree germination and growth. Two different populations of Douglas-fir (WA1 and CA2) were selected for the experiment. The populations of WA1 and CA2 were chosen because the locations of those two populations had differences in rainfall. The approximate location (Clallam bay, Washington State) of WA1 had a annual precipitation of 81 inches (16). The approximate location (Mendocino County, California) of CA1 had a annual precipitation of 45 inches (18). Sixty seedlings from each of the two populations were grown. Seeds were first surface sterilized and then plated in 100 mm diameter petri dishes on moistened filter paper (.5 ml water). They were germinated under fluorescent lights in a climate controlled growth chamber (Temperature: 18 °C, Relative humidity: 75%, light/dark hours: 12/12). As the seedlings germinated, they were taken out and planted in pots then placed under fluorescent

lights in the same climate controlled growth chamber . The pot dimensions were 7 cm in height by 7 cm in width by 6 cm in length. They were sterilized by soaking them in 10% bleach for 24 hours. Potting soil (Sun Gro Horticulture, Agawam, Massachusetts) was used as the growing medium. The soil was autoclaved at 121 °C for 2 hours each day, for three consecutive days.

Plants were watered once a week. The seedlings were watered with DI water and the water was applied until soil was saturated. After a month of growth the seedlings were fertilized with 10 ml of diluted 5:1:1 Alaska fish fertilizer (5 ml of 5:1:1 Alaska fish fertilizer to 946 ml of DI water). The diluted Alaska fish fertilizer was autoclaved for 1 hour at 121 °C before use.

Douglas-fir endophyte inoculations. Isolates of *Paenibacillus sp.* and *Bacillus sp.* were grown in LB broth at 30 °C while being shaken at 200 rpm for 2 days (1). After the 2 days both bacterial endophytes solutions were centrifuged at 3000 rpm for 10 minutes. The solutions were then drained leaving only the pellets. The pellets were resuspended with DI water. The pellet solutions were further diluted with the use of a spectrometer to make the inoculums (600 NM .1 A) (5). 10 ml of each of the bacterial inoculums were added to their assigned seedlings. 10 ml of DI water was applied to some seedlings as control. Both populations of Douglas-fir (WA1 and CA2) were inoculated 1 week after the application of the diluted 5:1:1 Alaska fish fertilizer. A week after the inoculations, the seedlings were randomized, and watering was halted. The halting of the watering marked the start of the drought experiment.

Table 2: Experimental design. DI H_2O will be used as a negative control. This figure represents the seeds from only one population of Douglas-fir.

	Drought
Bacterial Endophyte 1	20
Bacterial Endophyte 2	20
DI H_2O	20

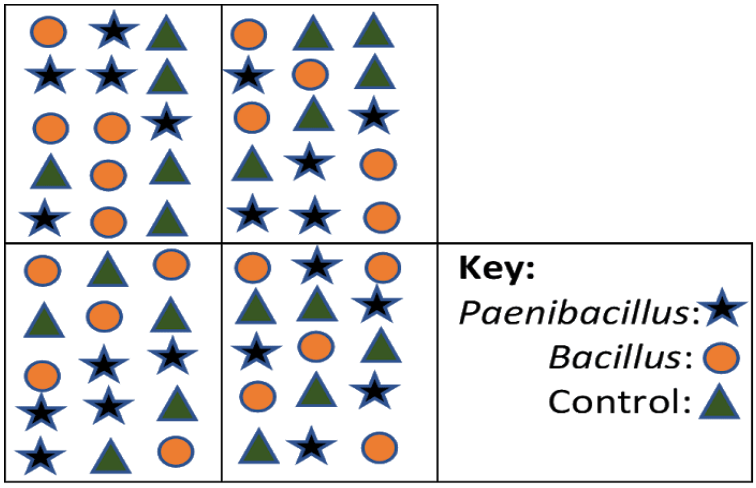

Figure 2: Randomization of the endophyte treatments and control (DI water) for one population of Douglas-fir. There are 4 blocks. Each block has 15 seedlings with their assigned treatments all randomized. There is 5 of each treatment per block.

Douglas-fir Drought experiment data. The height of each of the Douglas-fir seedlings was recorded at around 31 days after watering was halted. The height of seedlings was measured starting from the bottom needles to the apical meristem. The justification for measuring the height of the seedlings was to evaluate whether plant size differed between the different endophyte treatments. If the plant height did differ between endophyte treatments then the size could influence the results of the drought experiment.

Seedling mortality was recorded each day after watering was stopped. Seedling mortality was determined by complete needle death. A needle counted as dead if it was either completely brown/orange or light green and dry or severely wrinkled.

Data analysis. A single factor ANOVA was conducted to test for a statistical relationship between the seedling heights and the endophyte treatments (Excel). The relationship between height and treatments was found to be statistically insignificant for both the WA1 and CA2 Douglas-fir populations (p-values .737 and .407). Douglas-fir seedling size did not differ between the different treatments. So the seedling heights are unlikely to influence the results of the drought experiment, and this data was not included as a covariate in subsequent analyses.

To determine whether the endophyte treatments influenced Douglas-fir seedling survival I conducted a Survival analysis (R, packages: Survivor, Survminer, and ggplot2). For Survival analysis the variables used were endophyte treatments and the days until the Douglas-fir seedling had died.

Results

Douglas-fir bacterial seed endophyte community. A total of 61 bacterial seed endophytes, belonging to 7 different genera, were isolated from the 8 different Douglas-fir populations (figure 4). Those genera of bacteria were *Rahnella*, *Paenibacillus*, *Bacillus*, *Streptomyces*, *Serratia*, *Stenotrophomonas* and *Pseudomonas*. The first, second, and third most isolated genera of bacteria were *Rahnella*, *Paenibacillus*, and *Pseudomonas* (figure 4). *Rahnella* was isolated from 4 different Douglas-fir populations (figure 3), which was a greater number of populations than any of the other genera of bacterial seed endophytes. Each population of Douglas-fir had a different composition of genera of bacterial seed endophytes except for OR4 and WA2 (figure 3). OR4 and WA2 both only had *Rahnella* only (figure 3). OR1 had no bacterial seed endophytes (figure 3). OR4 had the greatest number of isolated bacterial seed endophytes compared to the

rest of the populations (figure 3). OR3 had the most diverse composition of bacterial seed endophytes, consisting of three different genera (*Rahnella*, *Paenibacillus*, and *Pseudomonas*) (figure 3).

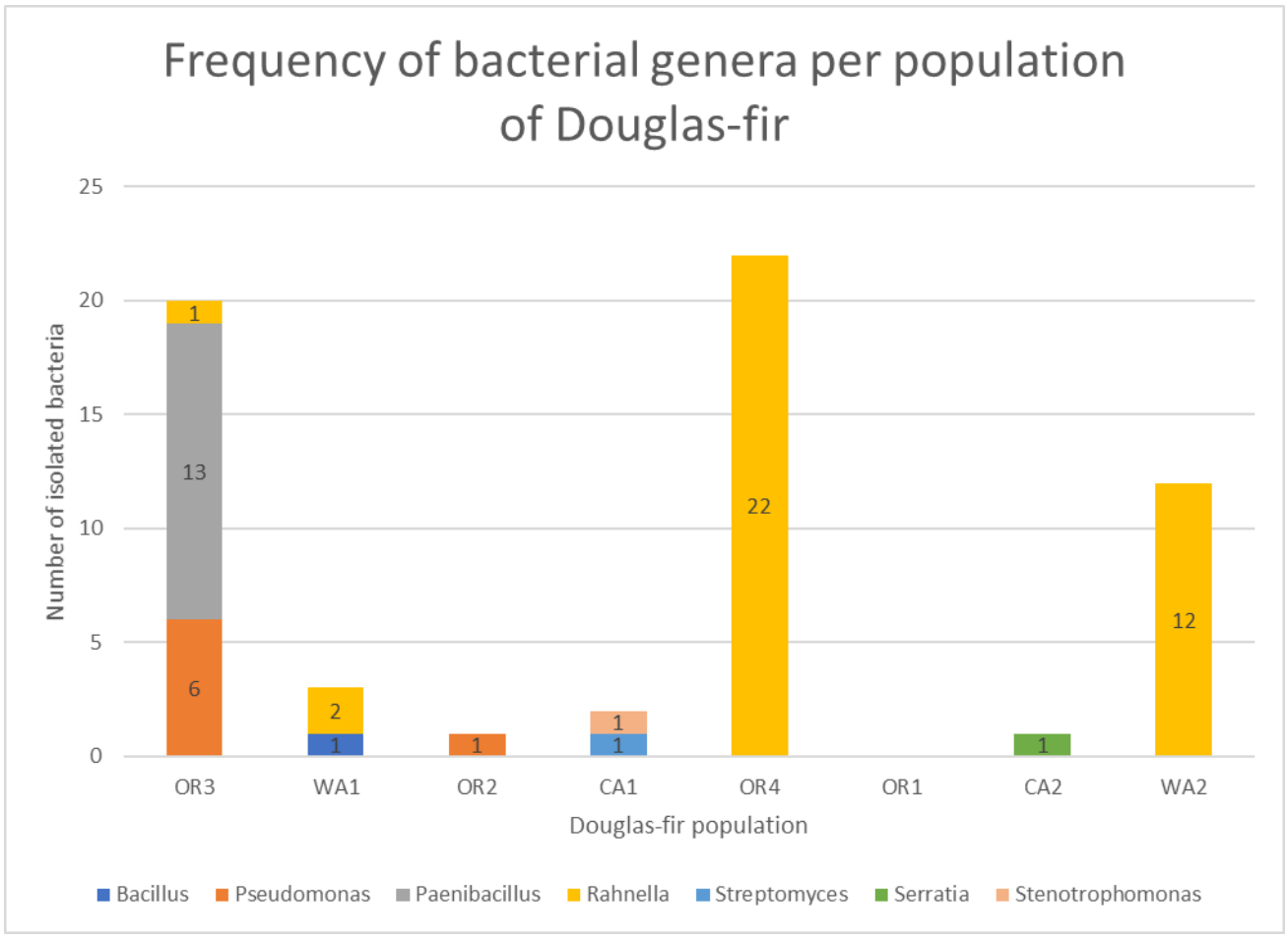

Figure 3. A comparison of the number of bacterial seed endophytes isolated from each of the 8 Douglas-fir populations. Each color represents a genus of bacteria.

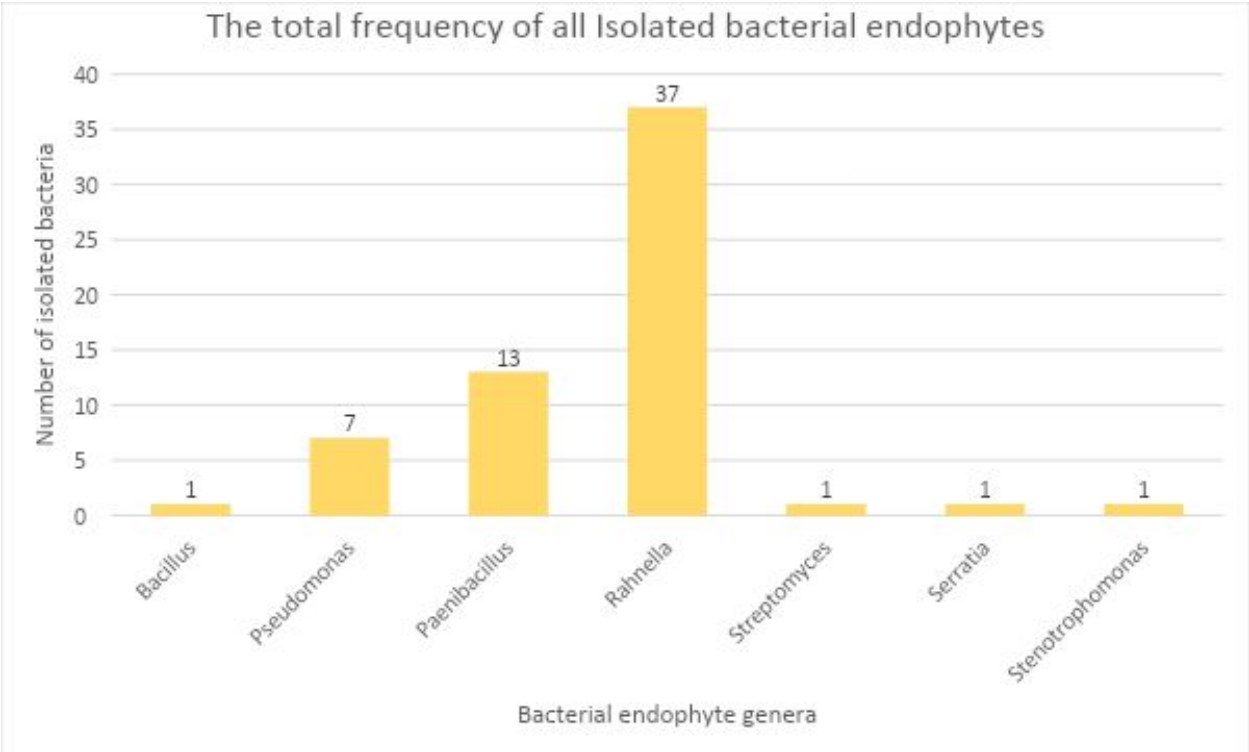

Figure 4. A comparison of the number of bacterial seed endophytes isolated from all the Douglas-fir populations. The bacteria were identified at the genus level.

Drought experiment. I found no evidence that endophyte inoculation with *Paenibacillus* or *Bacillus* influenced seedling survival under drought stress (figures 5 & 6).

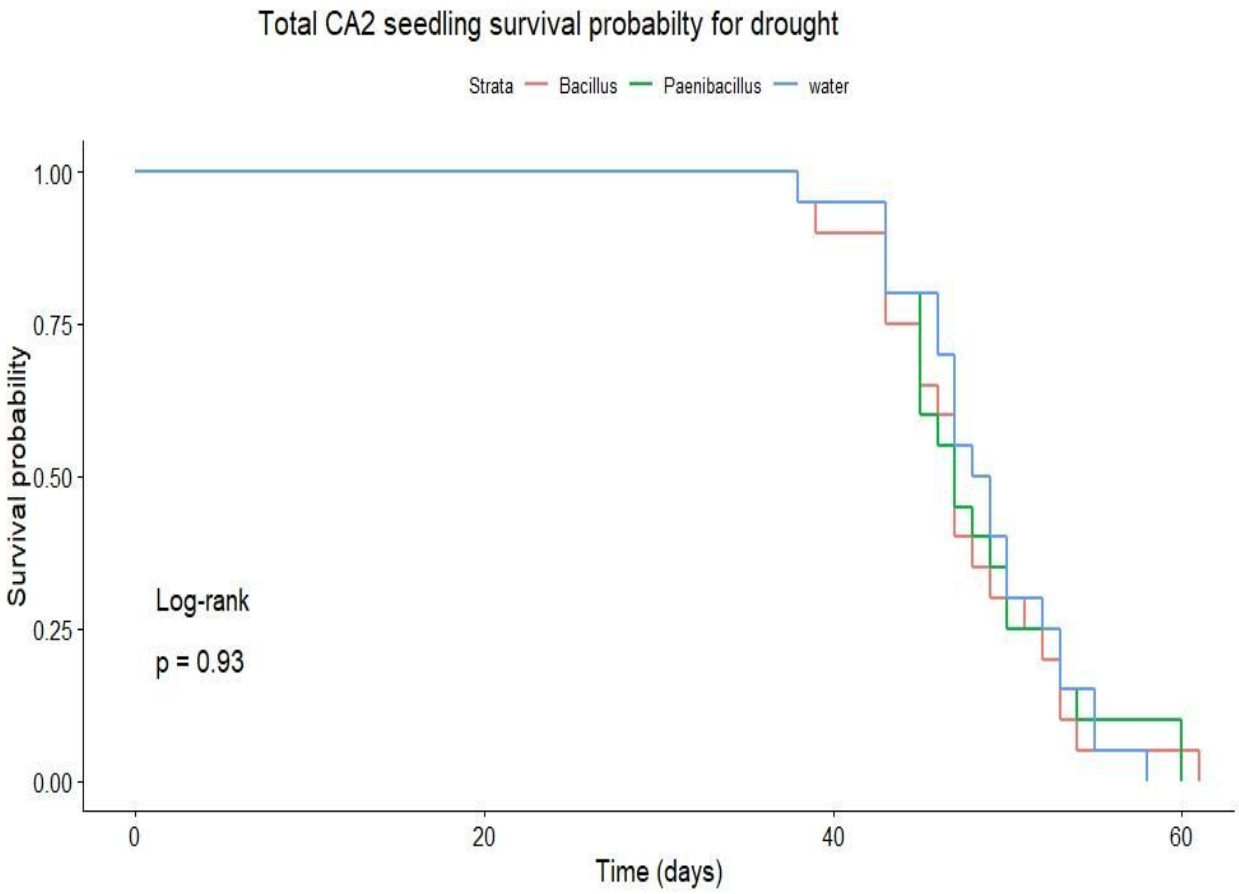

Figure 5. The comparison of the drought survival curves of CA2 seedlings, that were inoculated with their respective treatment. The different colors represent the different drought treatments, water is the control.

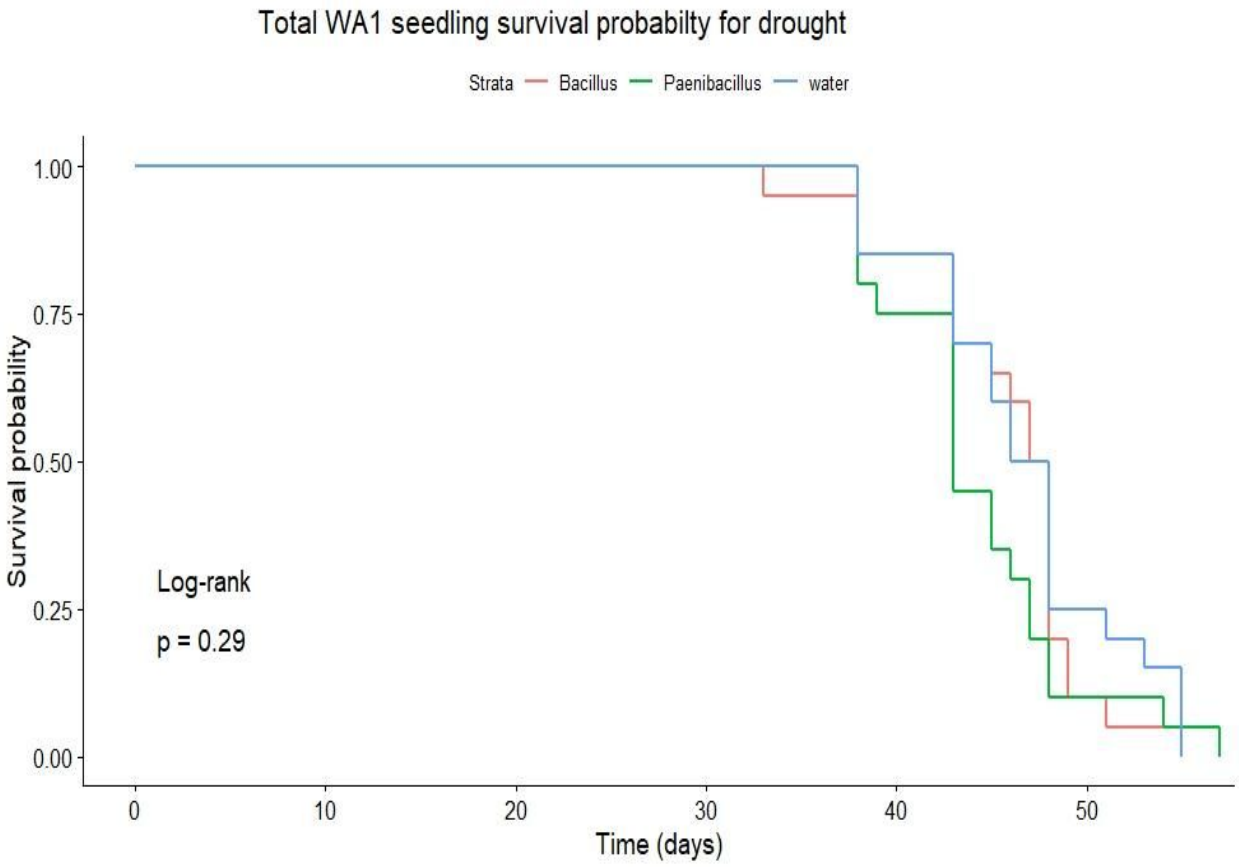

Figure . The comparison of the drought survival curves of WA1 seedlings, that were inoculated with their respective treatment. The different colors represent the different drought treatments, water is the control.

Discussion

I found a total of 61 bacteria from 7 different genera in the seeds of Douglas fir from eight populations located in the Pacific Northwest. The 8 different populations of Douglas-fir except for OR4 and WA2, had different compositions of bacterial seed endophyte genera, as we expected. These differences may be driven by the spatial, environmental, and/or plant genetic differences among the eight populations. The three most frequently isolated genera of bacteria, going by descending order, were *Rahnella*, *Paenibacillus*, and *Pseudomonas*. Not only was *Rahnella* the most frequently isolated bacteria, it was also found in more populations of Douglas-fir than any other genera. Since the bacterial endophytes were isolated by culturing methods, there could be genera of nonculturable bacteria that inhabit the seeds of the 8 different populations of Douglas-fir.

Certain species of bacteria within the 7 genera isolated from Douglas fir seed can have unique effects on plants. *Stenotrophomonas rhizophila* can help combat fungal diseases in cotton and peppers (10). *Rahnella aquatilis* can alleviate Crown Gall disease in grape (3). *Serratia Marcescens* is a pathogen for humans (4). It is possible that *Serratia* was isolated due to contamination. *Streptomyces olivaceus* can confer drought tolerance to wheat (14). *Pseudomonas viridiflava* can be a plant pathogen (10). *Paenibacillus polymyxa* can enhance the drought tolerance of *Arabidopsis thaliana* (15). *Bacillus sp.* can confer drought tolerance to Maize (13).

Species from most of 7 genera of bacteria have the potential to convey beneficial effects on plants.

One change that would improve the characterization of bacterial seed endophytes in this study would be to sequence the entire 16S region. With a longer DNA sequence there is a better chance to get a unique species match, when comparing sequence data to databases. A greater variety of bacterial seed endophytes could be shown when they are identified at the species level.

I found that there was no effect from the bacterial endophyte treatments on the survival of the Douglas-fir seedlings experiencing drought conditions. Thus *Paenibacillus* and *Bacillus* bacteria may not confer drought tolerance in Douglas-fir. However, it is possible that other isolates within these genera, and/or in combination with different tree populations, may result in drought tolerance. There are also several methodological factors that may have led to the negative result that I observed. First, there could have been other microbes that were introduced from the soil that prevented the treatment endophytes from forming a relationship with the seedlings. Second, letting the endophytes stay on the Douglas-fir seedlings longer before halting watering could provide different results. Third, increasing the sample size of Douglas-fir seedlings would provide more statistical power. And finally, introducing the bacterial endophytes to the Douglas-fir seedlings while they have just started to sprout could improve the chance of the endophytes conferring drought tolerance. This increases the chance that the treatment bacterial endophytes are the first endophytes to meet the Douglas-fir seedlings.

Conclusion

This project provides insight into the community composition and potential function of bacterial endophytes of Douglas-fir seeds. In addition, it opens the doors for more in-depth, future studies. In this project, a diverse group of bacterial endophytes were discovered in the seeds of several geographically varied populations of Douglas-firs. Bacterial seed endophytes belonging to the genus of *Rahnella* were the most widespread and frequently isolated. The endophytes used in the drought experiment, *Bacillus* sp. and *Paenibacillus* sp., did not confer drought tolerance to Douglas-fir trees. However, further study is needed to fully explore the functional roles of seed endophytes of Douglas fir, and to evaluate their potential applications in commercial forestry and restoration.

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