Attachment C

Christmas Tree Promotion Board

Final Research Report

CTPB Project Number: 19-02-CAES

Project Title: Investigating Soil Acidification Mechanisms for Inhibiting Phytophthora

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Final Report

Introduction

Phytophthora root rot (PRR) is a serious disease of Christmas trees, causing poor growth and mortality of susceptible trees. Characteristics of these disease organisms include production of long-lived oospores and chlamydospores, and production of zoospores in sporangia when saturated soil conditions occur. The irregular but occasionally intense precipitation patterns we have been experiencing, possibly caused by changes in our climate, exacerbate problems with root rots caused by *Phytophthora* species because periods of soil saturation are becoming more common. Zoospores produced by *Phytophthora* are able to swim from an infected tree to other potential hosts to cause new infections. Zoospores are attracted to root tips or damaged roots, where they encyst, germinate, and enter the root. Once inside the root, mycelium grows within and kills the cortical tissues. Upward growth causes elongated cinnamon-colored lesions in the phloem tissue. Disruption of vascular function may lead to flagging of lower branches and branches arranged in a spiral pattern in the tree. Further enlargement of lesions through growth of the *Phytophthora* may lead to coalescence of the infected area, trunk girdling, and tree death. Trees may continue to appear healthy for a short time after girdling of the cambium and phloem, but then do collapse. Dead trees are recognized by the reddish color of the foliage. Recovery of the disease organism from diseased trees can be made more difficult by the lag between the death of the tree and appearance of symptoms: dead tissues may not yield live cultures of *Phytophthora* as the killed tissue then may support other saprobic microorganisms.

Diverse *Phytophthora* spp. cause root rots in Christmas trees. In Connecticut, Dr. Katie McKeever (at that time a Ph.D. candidate working with Dr. Gary Chastagner) found four named species of *Phytophthora* by sampling diseased tissue from two Christmas tree farms (McKeever and Chastagner 2016). Our examination of the *Phytophthora* organism causing fir mortality at a research site in Brooklyn, CT, revealed that this was a species unknown to science, and which we have named *P. abietivora* (Li et al., 2019). Further surveys are likely to reveal greater biodiversity of *Phytophthora* everywhere Christmas trees are grown.

A complete assessment of methods to prevent root rot occurrence should start with the disease triangle – plant disease occurs only when there is co-occurrence of disease inoculum, a susceptible host, and environmental conditions conducive to infection. Any one of these three sides to the disease triangle could be manipulated to decrease disease. This work focused on the soil environment and the impact of soil acidification, as it relates to suitability for the plant vs. for the disease to flourish. I have discovered that acidification of soil to a pH of 4 through the addition of elemental sulfur provides significant benefits for protecting trees from Phytophthora infection. Infections of trees planted into a field uniformly infested with Phytophthora resulted in 25 and 80% reductions in Fraser and Canaan fir tree losses when soil was acidified, respectively (Cowles 2020).

This project explored via a strong inference paradigm the possible mechanisms for this beneficial effect: many potential mechanisms are possible (which are not necessarily mutually exclusive) each of which have been or are in the process of being systematically tested via experiments.

(1) The growth of *Phytophthora* organisms may be inhibited by low pH.

(2) *Phytophthora* organisms may not survive low pH conditions.

(3) Elemental sulfur could act directly as a biocide.

(4) Soil acidification may liberate cations into soil solution from exchange sites in soil; these cations may interfere with *Phytophthora* biology.

(5) A reduction in pH changes micronutrient (Mn, Cu, and Zn) availability; increased uptake could protect the plants against infection (Duffy 2007; Evans et al. 2007; Thompson and Huber 2007).

(6) Lowered pH may favor microflora antagonistic to *Phytophthora*.

(7) Higher pH may favor organisms that exacerbate *Phytophthora* root rot.

Methods

Low pH and growth of Phytophthora. Growth inhibition directly related to pH was tested by growing cultures of *Phytophthora* obtained from infected Christmas trees in Connecticut, inoculated onto V8 agar plates with pH of 2.5 to 6, and the radial growth of each colony measured over time. Agar would not gel when autoclaved after the addition of acid. Therefore, we determined the amount of acid needed to reach the targeted pH, and that predetermined quantity of acid was mixed into autoclaved agar as it cooled to generate the needed gradient of pH conditions in properly gelled culture plates.

Elemental sulfur or calcium cations and growth of Phytophthora. Powdered elemental sulfur fungicide was incorporated into media to determine if there was an effect on radial

growth of Phytophthora spp. The dose response of calcium chloride added to culture media was observed for radial mycelial growth of *Phytophthora* species.

Influence of soil acidification on fir tissue concentrations of minerals. Foliage samples were collected to compare study trees grown with pH 4 vs. 6 soil conditions, relative to the concentrations of minor and major nutrient levels in the foliage. Foliage was dried, pulverized, and subjected to inductively coupled plasma (ICP) elemental analysis.

Comparison of rhizosphere microorganisms from pH 4 and pH 6 soils.

Part 1. Detection of culturable bacteria antagonistic to Phytophthora. Root fragments with adhering soil were collected from the field site and washed with distilled water to provide a suspension containing rhizosphere bacteria. This suspension of bacteria was plated directly onto non-selective V8 medium, after it had already been inoculated with a homogenized culture of *Phytophthora*, which contained mycelial fragments and oospores. The plates were observed at 48 hours after inoculation of the soil bacteria to observe formation of zones of growth inhibition. Candidate bacteria were isolated via conventional plate-streaking methods to start cultures from single colonies. Antagonism of these bacteria to *Phytophthora* was verified by inoculating spots onto non-selective V8 agar media in a dose-response array, and then introducing agar with Phytophthora into the center of where bacteria had been inoculated. Growth inhibition was detected by comparing radial growth of the Phytophthora relative to the number of bacterial colonies.

Part 2. A microbiome comparison. Rhizosphere soil was shaken with phosphate buffered saline in the field, the DNA extracted and analyzed to identify the number of species present.

Results

Low pH and growth of Phytophthora. Growth of mycelium from CT Christmas tree *Phytophthora* isolates is proportional to pH of their culture media, through the range of pH from 2.5 to 4.1, and species differ in their response to pH (Fig. 1). The growth of each species was suppressed, with a linear response over most of the pH range tested. The pH at which growth was inhibited by 50% ranged from a value of 2.9 for *P. kelmania*, which was the most acid-tolerant species, to a value of 4.3 for *P. cactorum*, which was the most acid-sensitive species.

Influence of elemental sulfur and calcium cations on Phytophthora. There was no evidence of direct suppression in mycelial growth with elemental sulfur or with calcium.

Influence of soil acidification on fir tissue concentrations of minerals. The only mineral component that showed dramatic differences in foliar tissue concentrations, relative to soil pH, was manganese (Fig. 2).

Comparison of rhizosphere microorganisms from pH 4 and pH 6 soils.

Part 1. Detection of culturable bacteria antagonistic to Phytophthora. The bioassay method detected many intriguing interactions between bacterial colonies and *Phytophthora* isolates, including antagonism for some and "aphrodisiac" properties for others. For those displaying aphrodisiac properties, certain Phytophthora were strongly stimulated to produce oospores in the presence of bacteria. The most notable characteristic of antagonistic bacteria isolated from the acidified soil was a commonly cultured species producing an aquamarine pigment (Fig. 3); this bacterial type was not cultured from the higher pH rhizosphere soil. However, interestingly there was no statistically significant difference in the proportion of Phytophthora-inhibiting bacterial colonies from the acidified soil vs. the non-acidified conditions. Furthermore, we were surprised to discover that a remarkably high proportion (20 – 30%) of culturable bacteria could inhibit *Phytophthora* growth. Follow-up comparisons of these bacteria with the entire range of our collection of Phytophthora spp. demonstrated clear bacterial-*Phytophthora* species interactions, both for antagonism and for the aphrodisiacal species (Table 2).

Part 2. A microbiome comparison. Soil from treatment plots that had been acidified in 2014 with the addition of elemental sulfur still differed in pH, and demonstrated differences in mineral nutrient availability (Table 1). Analyses of the DNA extracted from the soil microbiome associated with fir roots in these plots revealed an extraordinary situation in which there was a stable community of species found under the higher pH conditions, and communities of bacteria unique to each tree found under the lower pH conditions (Fig. 4)! The populations of rhizosphere bacteria shared relatively few species in common under these two pH conditions (Fig. 5).

Discussion

Our studies have led to a better understanding of basic principles involved with the improved establishment and growth of Christmas trees, when planted into soils recently acidified through the addition of elemental sulfur. The better initial color and growth of these trees is partly due to direct mineral nutrition differences for these trees. In particular, the generation of acid through the oxidation of elemental sulfur into sulfuric acid causes greater soil mobility of other cations held on exchange sites in the soil. This is a short-term benefit, with respect to availability of calcium, magnesium, iron, and manganese, because in the long run, their displacement from cation exchange sites can lead to their loss through leaching in the root zone within the soil profile – evident from the soil mineral analyses (especially divalent cations: calcium and magnesium, see Table 1). Five years after planting, the only mineral compositional difference between trees growing in these two pH soil conditions was a 20-fold greater tissue concentration of manganese for trees grown in the lower pH soil (Fig. 2). The clear difference in mineral nutrient status of newly planted bare-root trees, when planted into recently acidified soil may explain some of the improvement in initial growth (Cowles 2020). However, the nutrient hypothesis cannot fully explain the doubling of growth in the

second year of these plants, as direct supplementation of mineral nutrition has at most generated ~30% improvement in growth (see Final Research Report CTPB Project Number: 18-02-CAES). Therefore, there remains an unaccounted for difference in growth performance related to soil acidification, at least twice the magnitude in effect accounted for by mineral nutrition, that is likely due to improvement of root health and prevention of root losses from various root rot organisms.

We have generated direct evidence that low pH conditions can slow the growth of Phytophthora organisms, but the growth of mycelium in a Petri dish may not represent the real-world biology of the stages of Phytophthora, especially zoospores, that would be most exposed to these low pH conditions. We will continue to investigate the direct influence of pH and mineral composition on the survival and growth of all stages of Phytophthora to help fill this data gap.

Studies on the microbiology and microbiome of fir roots revealed just how great a shock to the microbial community is experienced when there is a two-unit shift in pH. The higher pH rhizosphere community was stable: all trees growing under those conditions had similar species of bacteria colonizing their roots, whereas there were unique rhizosphere communities growing in the lower pH conditions for each individual tree. This can be likened to a combination of Oklahoma land rush and island biogeography dynamics. It appears that the acidification of soil creates such drastically changed conditions that many microbial niches suddenly become available. The rare species of bacteria that can withstand low pH and happen to be present near the roots have a wide-open opportunity to exploit the opening, and consequently multiply, take over the niches, and become important parts of the newly forming community. There is a strong element of chance involved, and so different species are found to be members of communities that form on each tree.

The fact that culturable rhizosphere bacteria can be recovered from fir roots that have antagonistic interactions with Phytophthora was a new discovery. The presence of these antagonistic bacteria suggests opportunities to identify, culture, and use these bacteria as inoculants to help protect roots from phytophthora root rot. This was counterbalanced by finding rhizosphere bacteria that had aphrodisiac properties for *Phytophthora*, also a new discovery. It is likely that bacteria with each property can be found near roots in both lower and higher pH conditions. As a practical matter, it will be important, before attempting to commercialize *Phytophthora*-antagonistic bacteria, to determine whether soil acidification will be required to permit full colonization of roots by these protective bacteria.

Summary of Research

Firs grown as Christmas trees are susceptible to a root disease called phytophthora root rot. Experiments started in 2016 have demonstrated that the addition of elemental sulfur to acidify the soil to a pH of 4 has a dramatic effect in improving the health of susceptible trees when grown in a soil harboring this disease, and reduces losses of Canaan fir by two-thirds compared to soil with a pH of 6. The current best non-mutually exclusive explanations for the improved growth and survival of trees under low pH conditions are (1) enhanced mineral nutrient availability to the tree, resulting in better tree color and growth, (2) direct inhibition at low pH of Phytophthora spp growth, and (3) a dramatically changed microbial community associated with the roots of these fir trees, which might favor better root health. There are remarkably diverse bacteria found associated with fir roots that are antagonistic to the growth of Phytophthora spp. These need to be further studied to determine whether they can become a practical tool as a root dip at the time of planting to protect the roots from infection.

References

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Thompson, I.A., and Huber, D.M., 2007. Manganese and plant disease. In: Datnoff, L.E., Elmer, W.H., Huber, D.M. (Eds.), Mineral Nutrition and Plant Disease. St. Paul, MN: American Phytopathological Society, pp. 139–154.

Tables

Table 1. Soil Chemistry

Sample	pH	Ca (ppm/m^2)	Mg (ppm/m^2)	P (ppm/m^2)	K (ppm/m^2)	Organic Matter (%)	Cation Exchange Capacity (meq/100 g)
Row1 acidified	5.1	641	54	560	98	3.6	11.4
Row2 acidified	4.9	401	40	470	78	3.8	12.8
Row3 acidified	5.2	816	54	567	88	3.9	12.0
Acidified mean	**5.1*** **	**619.3** **	**49.3** **	**532.3**	**88.0**	**3.8***	**12.1**
Row4 control	6.4	1725	87	444	103	4.7	9.7
Row5 control	6.5	2039	85	517	76	4.1	10.8
Row6 control	6.6	2228	91	575	103	4.6	11.6
Control mean	**6.5*** **	**1997.3** **	**87.7** **	**512.0**	**94.0**	**4.5***	**10.7**

Asterisks denote means that were determined to be significantly different by Student's t-test; level of significance*>0.05, **>0.01 , ***>0.001.

Table 2. Species-species interactions between those bacteria showing some antagonism towards *Phytophthora*, and the level of inhibition displayed. The greater the number, the greater the degree of inhibition. A rating of 5 indicates inhibition across the entire Petri dish, possibly signifying the presence of a volatile inhibitor. Note that the interactions are somewhat species-specific, and that, based upon the interactions, the unidentified Phytophthora "CAES2" could be an isolate of P. cactorum. Also note that the isolate CAES4 is more weakly inhibited by these bacteria than are the other species of *Phytophthora*.

Accession	P. abietivora	P. plurivora	P_CAES2	P_CAES4	P. cactorum
2_G11	5	0	4	3	4
6_U40	4	3	0	0	0
5_S27	4	3	4	0	4
5_S34	4	3	4	0	4
2_G10	4	4	4	0	4
2_G13	4	4	4	0	4
5_S31	4	4	4	0	4
2_G8	3	0	0	0	0
6_U35	3	0	0	0	3
2_G7	2	4	2	0	2
4_P23	2	4	2	3	2
3_I18	0	0	4	0	4
5_S32	0	0	4	0	4
3_I17	0	4	2	3	2
4_P22	0	4	2	0	2

Figures

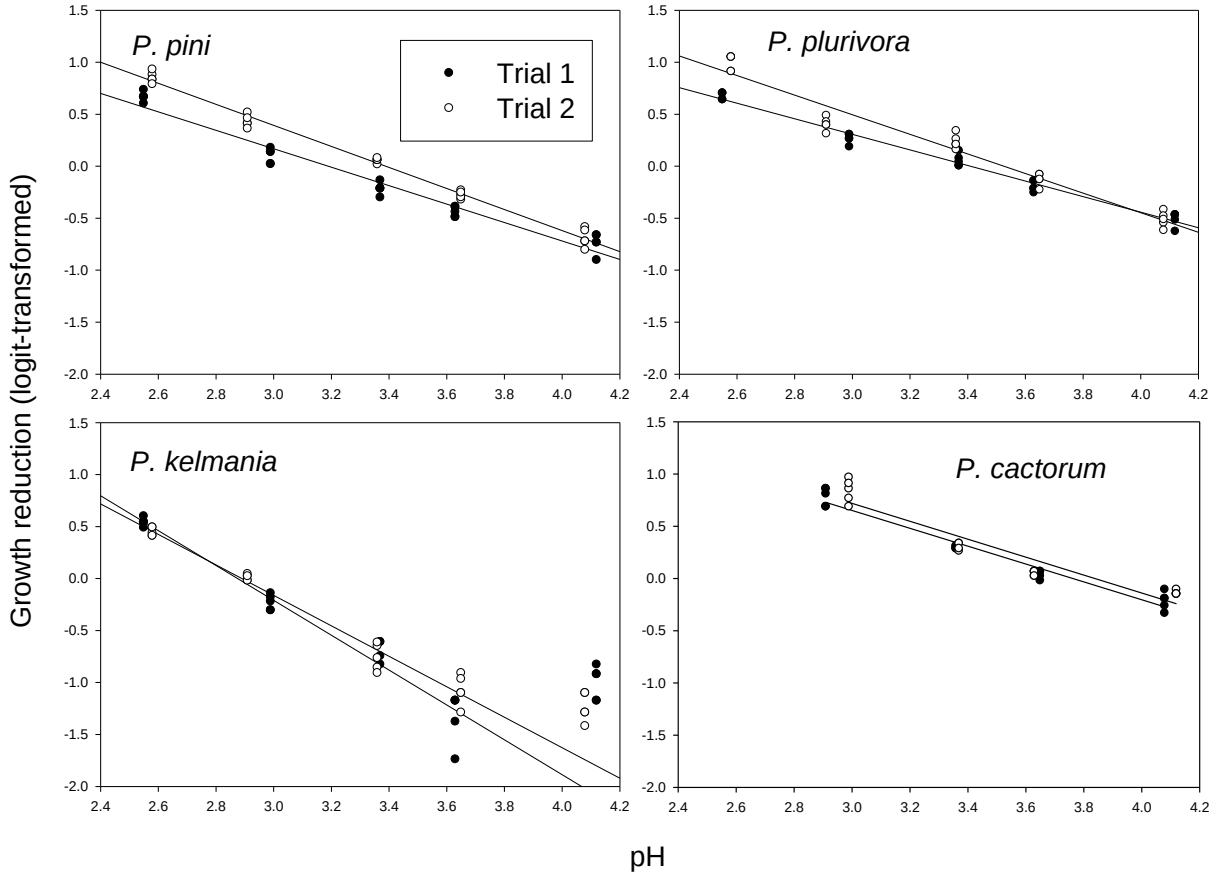

Fig. 1. The effect of pH of V8 medium on the radial growth of mycelium for four *Phytophthora* spp. isolated from Christmas trees in Connecticut. The proportion reduction in radial growth, relative to the growth at pH 6, was subjected to logit-transformation to linearize the relationship. Logit is log(p/(1-p)), where p is the proportional reduction relative to the control group.

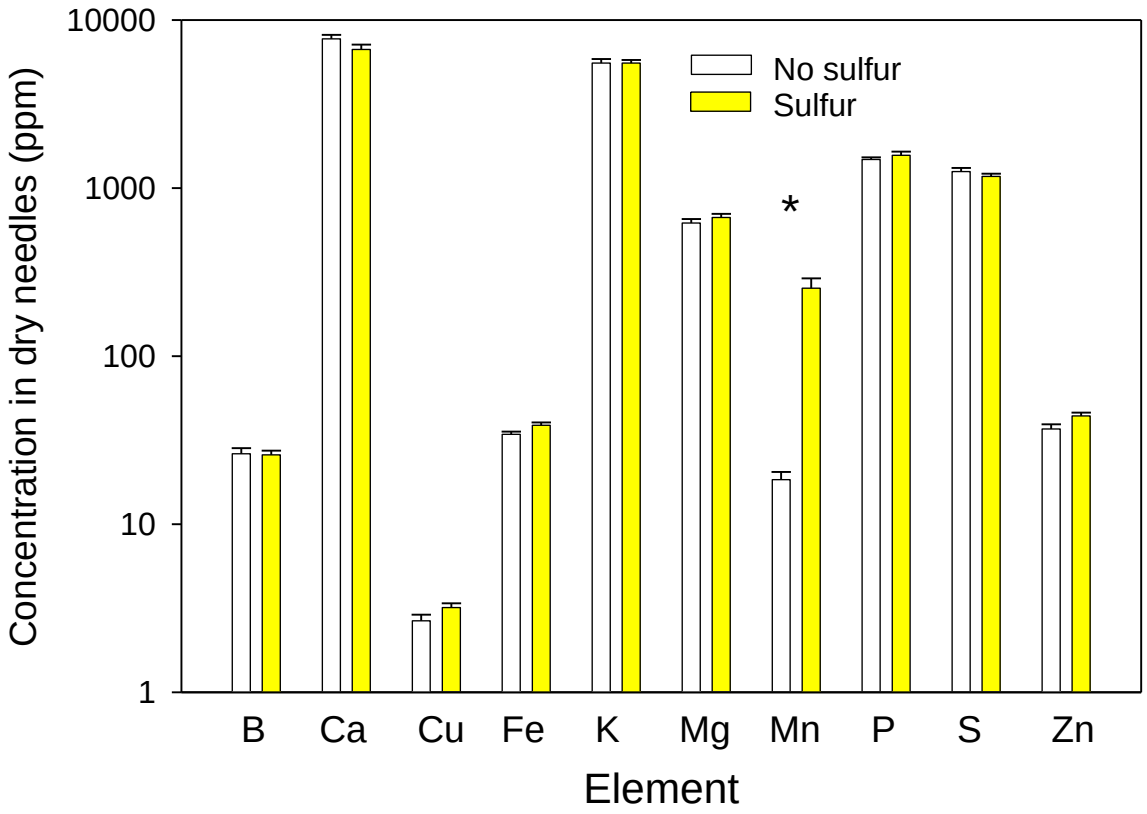

Figure 2. Mineral composition compared for foliage collected from Fraser fir trees grown with soil at pH 4 (sulfur) vs. pH 6 (no sulfur), 5.5 years after the incorporation of elemental pelletized sulfur into the soil. Statistical significance indicated by *, $P < 0.05$. LSD test, n = 3.

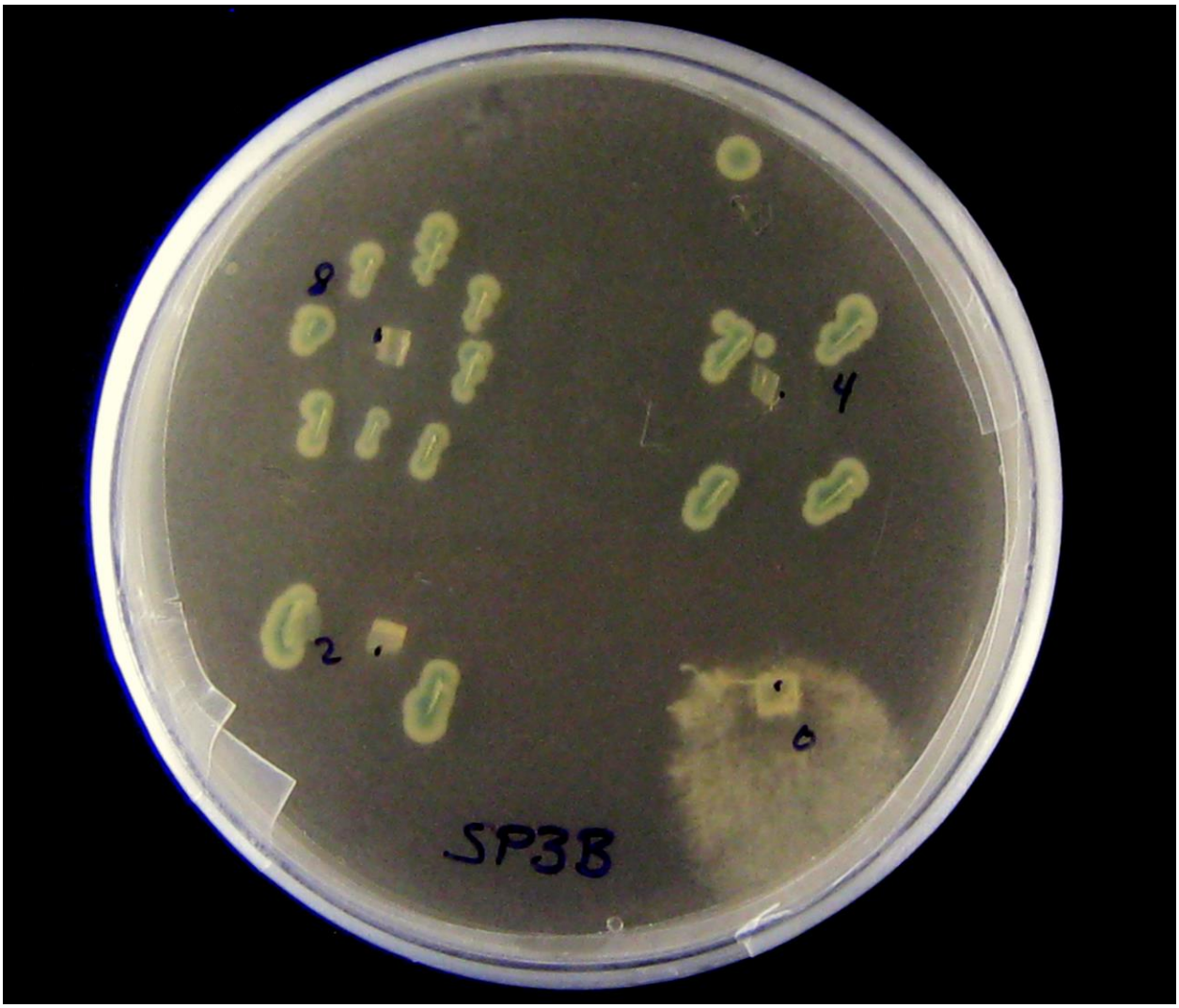

Fig. 3. A representative example demonstrating inhibition of *Phytophthora* growth in the presence of soil rhizosphere bacteria, in a dose-response experiment. The number of colonies the *Phytophthora* was exposed to is indicated by the label on the Petri dish: 0, 2, 4, and 8. For this Phytophthora/bacterial species interaction test, the only growth from the introduced Phytophthora inoculum was from the “0” treatment. Note also that the Phytophthora only grows away from the colonies of bacteria present in the other treatments.

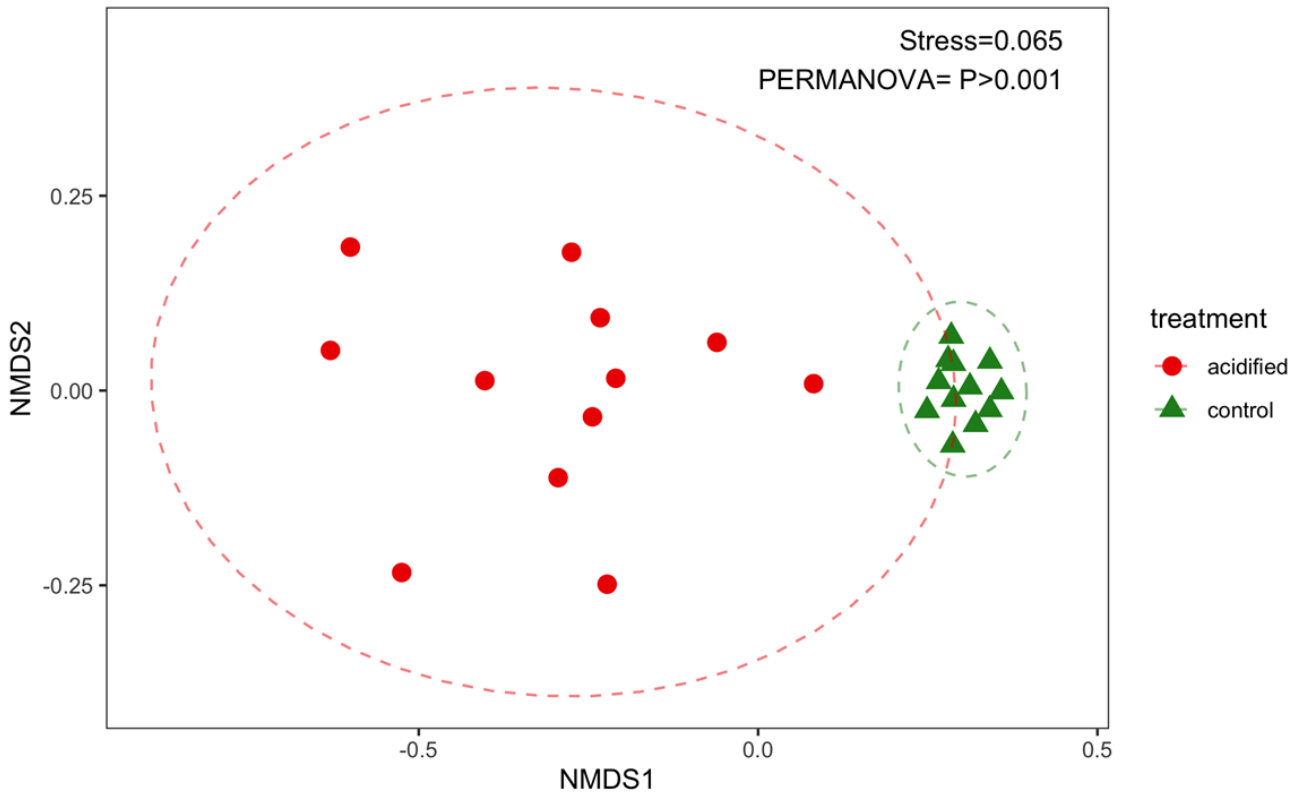

Fig. 4. Analysis of the bacterial populations associated with fir roots for trees growing in higher pH (control) vs. acidified conditions. Note that the populations of the twelve sampled trees in the control group are tightly clustered, meaning that they have very similar bacterial communities. The large distance between points representing trees growing with acidified soil signifies that there are large differences between their communities of bacteria – each tree has a unique microbial community. The method used was 16S rRNA gene sequencing visualized by non-metric multidimensional scaling.

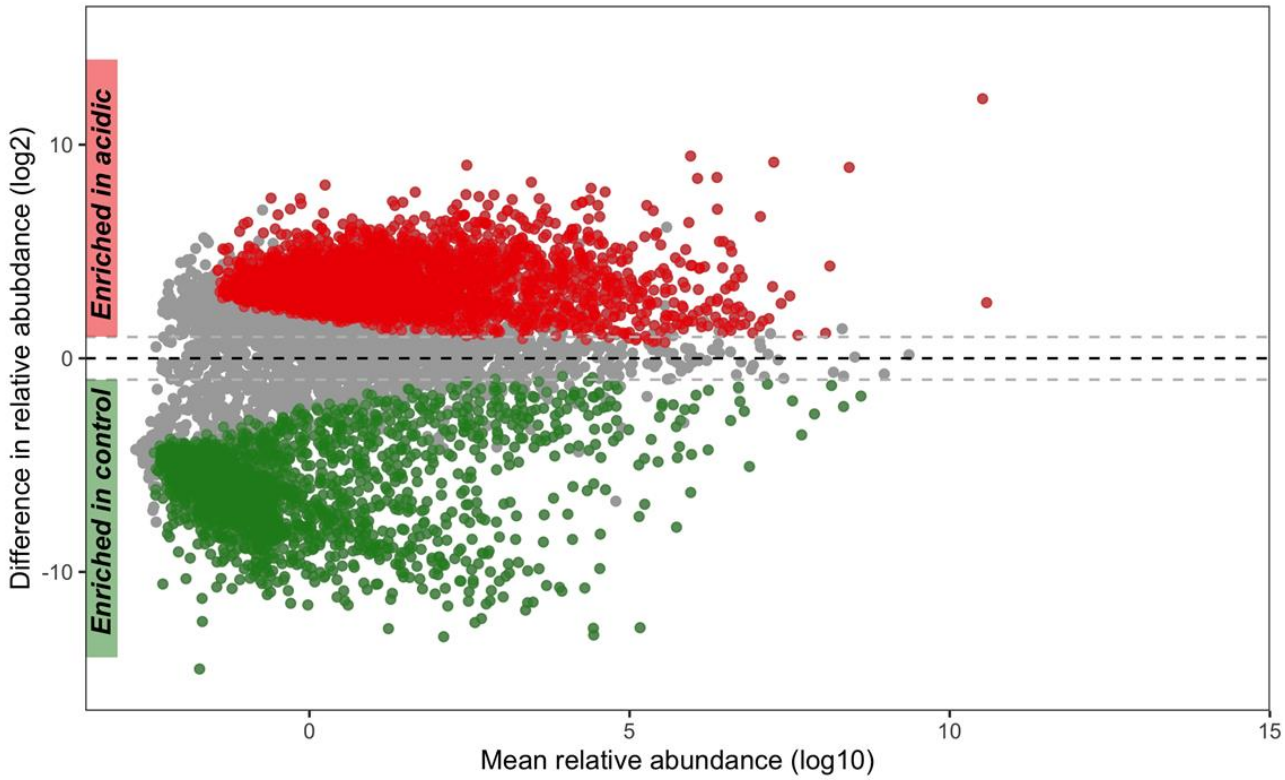

Fig. 5. This Bland-Altman plot shows the changes in bacterial species found under non-acidified (control) plot rhizosphere soil vs. the rhizosphere soil from Fraser firs growing under acidified conditions. The 16S rRNA was extracted from the environmental samples, and the genes sequenced to determine species identity and abundance in each sample. Points further to the right on the graph are those species that were very highly abundant. The abundance of any particular species was compared between control and acidified plot samples. If there was no difference in the RNA signal for a particular species, then the point would lie on the X-axis. Points colored gray are not statistically significantly different between control and acidified conditions. Those species that differed in abundance between soil pH conditions are colored. There are greater than 5,000 species of soil rhizosphere bacteria that were affected by soil acidification.