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An experiment to test for rapid evolution in an introduced ectomycorrhizal symbiosis

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AN EXPERIMENT TO TEST FOR RAPID EVOLUTION IN AN INTRODUCED ECTOMYCORRHIZAL SYMBIOSIS

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ABSTRACT
VALERIE ANNE REWA: An Experiment to Test for Rapid Evolution in an Introduced Ectomycorrhizal Symbiosis
(Under the direction of Jason Hoeksema)

The rapid evolution of introduced ectomycorrhizal-plant partnerships is an under-explored topic that may have immense impacts on ecosystems around the world. This experiment sought to identify and quantify this evolution and its impacts on both fungal colonization as well as plant growth. I used a laboratory experiment to analyze these factors in native and exotic genotypes of *Suillus cothurnatus* and *Pinus* species. Much of the data was not able to be collected, but that which was did not support the presence of rapid evolution in the mutualistic partnership. Pine species was seen to have a significant effect on plant root length, though this did not support either hypothesis. This study supports the need for further exploration of this topic and serves as groundwork for future experiments.

TABLE OF CONTENTS

Introduction

The introduction of invasive exotic species is becoming more common as humans continue migrating and transplanting various organisms to non-native environments. Vitousek et al. (1997) found that many countries now have 20% more alien plant species than were previously present. The presence of these invaders has been seen to alter the biodiversity of their newly established locations, for example by decreasing local plant fitness and growth (Vilà et al., 2011). The success of introduced species may be partially attributed to rapid evolution, which has been shown to improve fitness in novel environments (Hendry, 2016; Thompson, 2013). Rapid evolution has become a widely studied phenomenon in the biological world and has impacts in both ecological and medical realms (Thompson, 2013). While there is previous research demonstrating the rapid evolution of many exotic species, both macro and microscopic, there has not been sufficient research pertaining to the rapid evolution of mycorrhizal fungi (Gladieux et al., 2015). These symbiotic fungi, along with their host plants, are some of the most common co-invaders on Earth; for example, many mycorrhizal fungal species were brought to the Southern Hemisphere when pines were introduced for the establishment and cultivation of tree farms (Vellinga et al., 2009; Richardson & Higgins, 1998). With the overwhelming occurrence of these invasions and the underwhelming focus on their evolution in the scientific world, my experiment sought to identify signs of rapid local evolution in an introduced ectomycorrhizal (EM) fungus, *Suillus cothurnatus.* I organized a reciprocal cross-inoculation experiment using two pine species with which this EM fungus co-occurs, one from its native environment in the southeastern United States and one from its co-introduced environment of South Africa. These pines were then

inoculated with fungal genotypes collected in either the southeastern United States or South Africa.

Rapid evolution is gaining attention for its ability to show on a shorter timescale the changes that have been occurring within the ecological world throughout the history of Earth. This convergence of evolutionary and ecological time allows scientists to witness the impacts that an evolutionary change in phenotype can have on the environment that surrounds the changing population (Hairston et al., 2005; Thompson, 1998). When considering what constitutes "rapid" evolution, it is important to consider the difference in generation time between organisms, as those with shorter generation times will often evolve faster, though mutation rates per generation can be similar (Hafner et al., 1994). This timescale difference is evident in laboratory experiments; for example, the bacterium *Escherichia coli* has been seen to evolve resistance to antibiotics in only 12 days (Baym et al., 2016). Rapid evolution thus adheres to the time frame of ecological processes and may be the cause of observed ecological patterns and dynamics (Thompson, 1998). The ability of a population to rapidly respond evolutionarily to environmental changes may aid the persistence of such high biodiversity on this planet.

There have been many different species and traits found to undergo natural selection on a rapid timescale, ranging from beak size and shape in birds to changed patterns of viral virulence (Grant and Grant, 2008; Nelson and Holmes, 2007; Bhatt et al., 2011; Thompson, 2013). Rapid evolution also acts on animal behaviors, life history, and species interactions – both mutualistic and antagonistic (Thompson, 2013). The rapid evolution of microbial populations is also becoming a focus for scientific studies all around the world due to their prevalence, as well as their quick growth and small size,

making them easy to study in the lab (e.g. Forde et al., 2004). While some examples of rapid evolution, such as pesticide resistance, have gotten repeated attention from ecologists and evolutionists, there are also areas in which studies are hugely lacking, such as antibiotic resistance in the medical field and populations of mutualistic ectomycorrhizal fungi (Restif, 2009; Thompson, 2013; Hoeksema et al., 2020). The importance of these evolutionary events cannot be overlooked, and the impact of humans has only increased the rate at which species must respond to a changing environment (Thompson, 2013).

As more species are being transported across the globe, rapid evolution is more commonly being studied through invasive populations. These populations often undergo local adaptation in order to survive in new environments, for example, to avoid new predators or to better acquire nutrients (Phillips et al., 2010; Rollins et al., 2015). Rapid evolution occurs commonly within newly introduced species because they are often subject to novel selective pressures, which differ from the pressures experienced by long-established native populations (Brown and Marshall, 1981; Mooney and Cleland, 2001; Sakai et al., 2001; Whitney and Gabler, 2008). In new environments, introduced species often escape competitors from their home range, are not constrained by their own population size, and have more opportunity for hybridization (Abbott, 1992; Dlugosch et al., 2008). When compared to populations that are already well-adapted to their current natural habitat, populations subject to these substantial differences in evolutionary pressures are potential hot spots for rapid phenotypic change. Well-studied examples of rapid local adaptation of invasive species include the soapberry bug (*Jadera hematoloma)* evolving its beak length less than 50 years after the introduction of new host plants (Carol

and Dingle, 1996; Whitney and Gabler, 2008), as well as the appearance of hybridized species of duck in response to the introduction of the Mallard duck (*Anas platyrhynchos*) to many parts of the globe (Rhymer and Simberloff, 1996; Whitney and Gabler, 2008). Microbial species are also commonly introduced throughout the world and can undergo rapid evolution on even shorter time scales due to their quicker generation times (Hoeksema and Forde, 2008). This advantage can be seen in invasive pathogenic fungi, which have been shown to colonize new hosts as well as increase pathogenicity in newly introduced environments (Giraud et al., 2010; Hovmøller et al., 2008). While the impact of the rapid evolution of microbial species on human health is being studied constantly, there are still many gaps in the knowledge regarding how the globalization of these professional adaptors affects the ecological world as a whole.

One widespread invasive microbial organism that is often overlooked due to its mutualistic rather than pathogenic relationship with plants are mycorrhizal fungi. These symbiotic fungi, which are found on the roots of plants, aid their hosts in nutrient uptake and are believed to have facilitated the transfer of the first plants from water to land (Smith and Read, 2008). Mycorrhizal partnerships involve the exchange of nutrients, which are obtained from the soil by the fungus and needed by the plant, for organic carbon, which is created by the plant and needed by the fungus (Smith and Read, 2008). EM fungi are a specific type of mycorrhiza that consists of a mantle, which encloses the plant root tip, and hyphae, which radiate into the substrate as well as between individual cells of the root in order to facilitate nutrient exchange (Smith and Read, 2008). Because of this close relationship, EM fungi are often brought to novel environments on the roots of their hosts (Schwartz et al. 2006), and over 200 different identified EM fungal species

have previously been found in non-native ranges (Hayward et al., 2015; Vellinga et al., 2009). Pines (plant species in the genus *Pinus*) are obligately dependent on EM fungal symbiosis, and thus the two are often introduced to novel environments together (Richardson et al., 1994; Walbert, 2010; Hynson et al., 2013; Hoeksema et al., 2020). While most native populations of pine are associated with over 100 species of EM fungi, invasions cause evident filtering and can reduce EM fungal species richness to fewer than 20 species (Chu Chou and Grace, 1988; Walbert et al., 2010; Hynson et al., 2013; Hoeksema et al., 2020). It has even been found that suilloid EM fungi (fungi in the genera *Suillus* and *Rhizopogon*) can facilitate the successful invasion of a pine species in the absence of any other EM fungal species (Hayward et al., 2015; Policelli et al., 2018). Because of the absence of many competitors and the ongoing demand to adapt to the novel environment, populations of introduced mycorrhizae are likely very vulnerable to the selection pressures of rapid evolution.

There is currently a lack of investigation regarding the evolution of introduced EM fungi, though the knowledge could help us better understand microbial-plant mutualism and their co-introduction and co-invasion. EM fungi have a huge impact on nutrient cycling in the ecosystem surrounding them (Chapela et al., 2001), and evolutionary change in this impact could generate substantial changes and affect the stability of biotic communities (Hoeksema et al., 2020). To address this deficiency, I set up an experiment to answer these questions:

Question 1: Does adaptation of ectomycorrhizal fungi to novel plant hosts affect its compatibility with native plant hosts?

Hypothesis 1: The compatibility of ectomycorrhizal fungi with their native plant hosts will be reduced and result in fewer mycorrhizal colonizations on plant roots.

Question 2: Does adaptation of ectomycorrhizal fungi with novel plant hosts affect the growth of native plant hosts when inoculated with the introduced genotype?

Hypothesis 2: Native plant growth will be decreased when inoculated with the introduced fungal genotype compared to when inoculated with the native fungal genotype.

The system I used to test my hypotheses was a laboratory experiment studying two *Pinus* species – *P. radiata* (production forestry genotypes from New Zealand, often used in exotic pine plantations, including South Africa) and *P. taeda* from native populations in the southeastern USA – inoculated with the EM fungal species *Suillus cothurnatus* from either its native range or its exotic range in South Africa. I then measured plant height, plant root length, and fungal root colonization to quantify the growth of both mutualists and to assess whether any evolutionary change was evident.

Methods

Overview of experimental design

I tested for rapid evolution of introduced ectomycorrhizal fungi and their pine hosts by growing *Pinus* seeds in mycocosms (Rygiewicz et al., 1988) within a growth chamber in the laboratory in order to quantify both mycorrhizal colonization of host plant roots and pine seedling performance. The design involved two experimental EM fungal groups – five genotypes of native *Suillus cothurnatus* from Mississippi, USA, and five genotypes of introduced *Suillus cothurnatus* from South Africa. I used these EM fungi to inoculate two *P. taeda* genotypes from the southeast United States and one *P. radiata* genotype from New Zealand. There were three replicates of each combination of genotypes, except the *P. radiata* control, of which there were four replicates. This resulted in experimental mycocosms and 10 control mycocosms. I used the following abbreviations when labeling each box:

R – *Pinus radiata*

T1 – *Pinus taeda* genotype one (x5409)

T2 – *Pinus taeda* genotype two (x5458)

MS – *Suillus cothurnatus* spores collected from Mississippi

MS1 – *S. cothurnatus* spore genotype #10

MS2 – *S. cothurnatus* spore genotype #12

MS3 – *S. cothurnatus* spore genotype #18

MS4 – *S. cothurnatus* spore genotype #19

MS5 – *S. cothurnatus* spore genotype #29

SA – *Suillus cothurnatus* spores collected from South Africa

SA1 – *S. cothurnatus* spore genotype #6

SA2 – *S. cothurnatus* spore genotype #9

SA3 – *S. cothurnatus* spore genotype #12

SA4 – *S. cothurnatus* spore genotype #70

SA5 – *S. cothurnatus* spore genotype #75

Each replicate was then also labeled with a number (1-3) referring to the replicate number of that mycocosm. For example, T2SA1.1 refers to the first replicate of *Pinus taeda* genotype two inoculated with *Suillus cothurnatus* genotype #6 from South Africa. To ensure the position of treatments did not affect the outcome of the experiment, 100 box positions were labeled in the growth chamber, and each treatment was given a randomly generated number corresponding to its labeled placement.

Preparation of pine seedlings and ectomycorrhizal fungus inoculum

The pine "genotypes" used in the experiment were open-pollinated families of seeds obtained from tree breeding programs. *Pinus radiata* seeds, provided by Sheffield's Seeds in New Zealand, represent a typical genotype commonly used in forestry production in the Southern Hemisphere and specifically in South Africa. Though *Radiata* pines are native to California and Mexico where *Suillus cothurnatus* is not naturally found, they have been planted together throughout the Southern Hemisphere due to tree breeding activity. *Pinus taeda* seeds (having medium growth rates and medium resistance to rust pathogens**)** were acquired from breeding zones of the Weyerhaeuser Southern Tree Improvement Program in central Alabama and Mississippi. To be sure seeds were surface-sterilized, they were soaked in a 10% dilution of bleach for two minutes, and then rinsed thoroughly using deionized water. They were then soaked in water (at 4ºC) for 48

hours. After this time, excess water was removed and a moist paper towel was added to the container. The seeds were then cold stratified for 6 weeks (from 9/12/2020 until 10/25/2020). Mycocosms consisted of two 20x16x3cm chambers with a central divider made of mesh and designed to prevent root growth between the two sides while still allowing fungal growth. They were constructed using clear plexiglass to allow visualization of both root and fungal development. Before planting, each mycocosm was wrapped fully in aluminum foil to discourage algae growth. After stratification was completed, each seed was planted 1 cm below the surface in autoclaved Jolly Gardener Proline C/V Growing Mix (which consists of Canadian Sphagnum Peat Moss, Aged Pine Bark, and vermiculite), and mycocosms were placed in the fluorescently lit growth chamber providing 16-hour days (with 265 μmol m^{-2} s^{-1} of light at plant height) and 8-hour nights at a constant 22.0°C. Plants were watered every two days.

Spore prints of native *S. cothurnatus* were acquired from locally collected mushrooms found under *Pinus taeda* in Lafayette County, Mississippi, USA. Those from introduced *S. cothurnatus* were provided by Rytas Vilgalys (Duke University) and were collected from mushrooms found under *Pinus radiata* in South Africa. Spore prints were scraped using a sterilized scalpel and washed using deionized water to create slurries. Spore concentrations in the slurries were counted using a hemocytometer to allow dilution to equal concentrations. Because the spore slurry with the lowest concentration would yield $3.91x10^5$ spores per inoculation, I calculated the number of microliters of each slurry that would contain $3.91x10^5$ spores. Slurries were then stored at 4ºC until inoculation one week later.

Experimental set-up

On 17 December 2020, 53 days after seeds were planted, each experimental seedling was inoculated with a spore slurry of the *S. cothurnatus* genotype matching its treatment label. Volumes of spore slurry previously calculated to contain $3.91x10^5$ spores were pipetted onto the surface of the soil. The plants continued to grow in the growth chamber with 16-hour days (with 265 μmol m^{-2} s^{-1} of light at plant height) and 8-hour nights at a constant 22.0°C. Plants were watered every 2-3 days. Originally, only one seed was planted per box, and 100% germination was not achieved. 40 treatments did not germinate, and 15 of those were eventually replanted. For this experiment, only 59 treatments were developed enough to be included in the final data analysis (**Table 1)**.

Treatment ID	**Number of replicates**	**Treatment ID**	**Number of replicates**	**Treatment ID**	**Number of replicates**
Rc	4	T1c	1	T2c	2
RMS1	2	T1MS1	1	T2MS1	3
RMS2	1	T1MS2	0	T2MS2	3
RMS3	3	T1MS3	2	T2MS3	1
RMS4	2	T1MS4	2	T2MS4	1
RMS5	2	T1MS5	2	T2MS5	1
RSA1	2	T1SA1	2	T2SA1	1
RSA2	0	T1SA2	2	T2SA2	2
RSA3	1	T1SA3	2	T2SA3	3
RSA4	1	T1SA4	0	T2SA4	2
RSA5	2	T1SA5	2	T2SA5	3

Table 1. Replicates of each treatment included in data analysis.

Data Collection

Seven weeks after inoculation, the height of each plant was measured, from the soil surface to the top of the apical bud. Fourteen weeks after inoculation, plant height was measured for a second time and visible root length was estimated on one side of each mycocosm. To quantify visible root growth, each mycocosm was removed from the chamber and one side was affixed with a transparent grid. The grid line intercept method was used to estimate the length of roots visible within each growth box (Tennant, 1975). Furthermore, any presence of mycorrhizal colonization was recorded. To quantify the relative growth rate (RGR) of height, I used the equation $RGR = \frac{\ln(S_2) - \ln(S_1)}{(t_2 - t_1)}$ with ($t_2 - t_1$) being equal to 47 days and S_1 and S_2 representing the initial and final height measurements, respectively.

Data analysis

All analyses were conducted using R software version 4.0.3. For root length and RGR of height separately, I used linear mixed models (in the lmer() function of the lmerTest package in R) to test the influence of pine species (*taeda* or *radiata*), fungal origin (MS vs. SA), and their interaction as fixed effects. Pine genotype and fungal genotype were included as random effects, to account for the hierarchical data structure of genotypes nested within the fixed effects. When significant effects were found, I obtained marginal means and standard errors using the emmeans() function of the emmeans package in R. Both of my hypotheses predicted significant effects of the pine species x fungal origin interaction.

Results

Fungal growth was only visible in three mycocosms. There were definite ectomycorrhizal structures found within the second experimental replication of *Pinus taeda* genotype one inoculated with genotype five of the native Mississippi fungus (T1MS5.2). Notable dichotomous splits were observed on multiple root tips, but no mycelium had formed. There was also an unknown fungal colonization found in mycocosms #89 – *Pinus radiata* inoculated with *S. cothurnatus* genotype four from Mississippi (RMS4.3) – and #11 – *Pinus taeda* genotype two inoculated with *S. cothurnatus* genotype four from South Africa. The colonization did appear to be associated with roots, but no dichotomously branched root tips were visible, and the fungal growth was granular in texture rather than obviously mycelial, which is not expected of ectomycorrhizal fungi such as *S. cothurnatus*. Because only one plant showed obvious signs of mycorrhizal colonization, no statistical analysis was conducted regarding ectomycorrhizal compatibility.

The statistical analysis of the RGR showed no significant effects. Analysis of visible root length showed no significant interaction of pine species and fungal origin. However, there was a significant main effect of pine species, resulting in *Pinus radiata,* at 99.5 cm ± 16.5 SE, exhibiting ~69% longer roots than *Pinus taeda,* at 58.8 cm ± 11.8 SE (**Figure 1**).

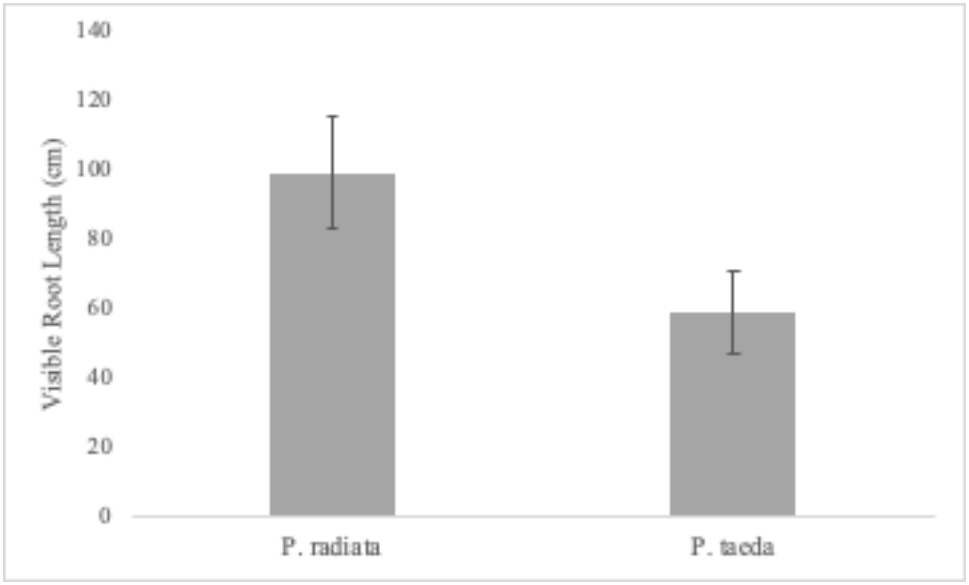

Figure 1. Visible root length of *Pinus radiata* (n=20) and *Pinus taeda* (n=38). Data are the marginal mean ±SE of all experimental replicates of each pine species. ($p = 0.02786$)

Discussion

This experiment was designed to test for rapid evolution within an introduced population of the ectomycorrhizal fungus, *Suillus cothurnatus*, in an attempt to better understand the co-introduction and co-invasion of microbe-plant mutualists. This research also served as a test of experimental methods, which can be used in the future to promote breakthrough in this field. The methods used in this experiment can be repeated and improved in order to uncover the most effective approach to studying relationships between introduced ectomycorrhizal fungi and their hosts.

Fungal colonization

The lack of fungal growth during this experiment prevents the discussion of Question 1. Without significant EM colonization data for analysis, it is impossible to test Hypothesis 1 due to the absence of information regarding an evolutionary change in compatibility between *Suillus cothurnatus* and native versus introduced pines. The lack of fungal growth could be attributed to the timeline within which this experiment was conducted. Based on previous experience in the Hoeksema lab, visible ectomycorrhizal colonization of plant roots can take upwards of three months, and data collection for this experiment occurred just over 14 weeks after inoculation. There is a potential the fungus was simply not yet well enough established on the plant roots to have grown mycelium visible to the naked eye. Another possible explanation for the lack of colonization is the use of an inadequate inoculation technique, whether during the wrong period of the plant life cycle or with insufficient spore density. Ectomycorrhizal fungi typically will begin to colonize pine roots when compatible spores are present in the vicinity of roots at least two months after plant germination when roots are just becoming susceptible to fungal

partnership. Plants were inoculated 7.5 weeks after seeds were planted, and seeds often did not germinate for several weeks, meaning that plants may have not yet been compatible at the time of inoculation. Furthermore, some spore prints used to create spore slurries were very light and resulted in standardized concentrations yielding only $3.91x10^5$ spores per inoculation. This number is low compared to other experiments utilizing spore inoculum of *Suillus* or other closely related EM fungi, which often use anywhere between 10^6 to 10^9 spores per inoculation (Piculell et al., 2008; Lazarevic et al., 2012; Lamb and Richards, 1974; Van Nuland & Peay, 2020). It has also been shown that increasing inoculum spore count by 10^3 spores can increase ectomycorrhizal colonization of plant roots by 62.3% (Lamb and Richards, 1974). After comparing the methods of this experiment to those of other studies, it is plausible that the relatively low spore density of this inoculum could have reduced or delayed *S. cothurnatus* colonization of plant roots.

Plant Growth

After analyzing plant height RGR and root length, there was no significant interaction between pine species and fungal origin, nor any significant main effect of fungal origin on either response variable. Thus, when considering Question 2, my main hypothesis is not supported. I did not see a significant difference in the impact of non-native fungal genotypes on the RGR or root length of either *Pinus* species, meaning plant growth was not affected by the *Suillus* variant with which the plant was inoculated. While this result could be due to a lack of rapid evolution in introduced ectomycorrhizal fungi, it could have also occurred due to some aspect of the lab experiment. For example, plants grown in the lab may not develop enough to show significant differences due to their fungal partners. If evolutionary advantages or disadvantages caused by introduced

mycorrhizal fungi take shape only later in a plant's life, there would be no evidence of this interaction in a lab setting during a relatively short experiment. Additionally, the lab is a fabricated and idealized environment, which lacks competitors of the pine plant and the fungi. The rapid evolution of fungal-pine mutualism could provide an advantage in a competitive environment, but this experiment would not be equipped to reveal such a relationship. The laboratory environment also lacks certain microbes, such as bacteria, which have been seen to positively affect the development of ectomycorrhizal symbiosis (Hoeksema et al., 2010; Piculell et al.; 2008). There is also a possibility the use of potting soil rather than field soil affected the outcome of the experiment, as using field soil has been seen to stimulate greater genetic variation in outcomes of ectomycorrhizal interactions (Piculell et al., 2008). This could be due to plants relying on their microbial partners more in demanding environments and less when the environment is rich. Potting soil is meant to stimulate plant growth, and could have resulted in more uniform plant growth of all experimental treatments, thus obscuring the variations caused by differing fungal sources.

The significant effect of plant root length revealed in this experiment is consistent with the growth patterns of both *Pinus radiata* and *Pinus taeda.* Studies of *Pinus* species have shown a vast distribution of growth patterns and growth rate (Grotkopp et al., 2001). Specifically, the growth of *P. radiata* was found to be 35.1% faster than that of *P. taeda* (Grotkopp et al., 2001), which here may have presented itself as a difference in root length between the species. My experiment has reaffirmed the distinction between these two different species of pine and serves as an example of how plants often differ in the growth allocation of roots.

Potential Limitations

This experiment utilized mycocosms with two compartments of soil and intended to keep plants confined to only one side of the growth medium. However, I found some pine roots were able to cross the physical barrier between the chambers and grow on both sides of the mycocosm. Furthermore, because mycocosms were wrapped entirely in aluminum foil, water tended to pool just below the growing medium and resulted in some replicates developing roots able to pass through the confines of the mycocosm in order to take advantage of the available resource. While this unintended growth did not affect the validity of my own experiment, it is worth noting as it has the potential to affect studies using this growth technique in the future. It should also be mentioned that the temperature under which seedlings were grown for this experiment (22.0°C) was slightly higher than best suits pine growth (Guo et al., 2020). In fact, a recent study found that increasing *Pinus* growing temperature from 20.0°C to 25.0°C has the ability to significantly lower germination percentage – from 92.67% to less than 5% germination (Guo et al., 2020). My experiment was also limited by an external timeline, putting a deadline on my data collection regardless of the breadth of obtainable data at the time. Ideally, I could have delayed my collection of data to allow for further potential mycorrhizal development and treatment interactions, and to cultivate more comprehensive records for analysis.

Conclusion

Though my experiment showed no significant evidence supporting the rapid evolution of ectomycorrhizal fungi and plant mutualism, my work still serves a vital role in expanding the knowledge of microbe-plant interactions and advancing the broader research goals of our laboratory group. Through this research, I reaffirmed the distinctive growth patterns of *P. radiata* and *P. taeda* root systems and uncovered important experimental considerations and concerns that can be applied to investigations performed in the future. The time I have spent in this lab will aid those who continue future exploration in the field of ectomycorrhizal rapid evolution.

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