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Implications of Mycorrhizae and Seedling Competition for American Chestnut Restoration

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Biology from The College of William and Mary

by

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**Introduction**

The biological conditions present belowground can greatly influence plant performance as well as plant community development (van der Putten 2013). The earth’s soil is host to a myriad of microorganisms that have the ability to interact with neighboring plants. Microbes like protozoa, bacteria, viruses, and fungi can fulfill several different kinds of beneficial and detrimental relationships with neighboring plants in the soil environment. Some microbiota act as parasitic pathogens to plants or even serve as competitors for essential nutrients (Kuzyakov & Xu 2013). Other microbes, like mycorrhizal fungi, can provide certain resources to the plant by transforming nutrients into forms that the plant can naturally metabolize (Emam 2016). Furthermore, some plants have the ability to shape the abiotic and biotic conditions within their own soil environment. These altered conditions can then in turn influence soil-mediated plant success in survival, growth, and reproduction (van der Putten 2013, Kulmatiski & Kardol 2008). These microbial interactions and plant-soil feedback loops demonstrate the highly connective biological relationships present within the soil and underscore the importance of understanding how plants interact with the soil community.

Several studies have found a range of plant responses in varying soil environments. These responses are contingent on the plant’s origin in relation to the soil environment as well as the characteristics of the plant and soil community. Some plants greatly benefit from growing in their natal soil environment, since the soil provides access to mutualistic microbiota like mycorrhizal fungi (Emam 2016).
Local soil can also be important to plant development since mycorrhizal colonization success within plants can vary based on soil origin. Studies have shown that mycorrhizal compatibility to a host can at times be genus specific (Molina et al. 1992, Ji et al. 2012). Other plants experience inhibited performance when grown in their local soil, as they are more susceptible to species-specific pathogen accumulation (Klironomos 2002). The relative magnitude of these costs and benefits from local soil microbes vary amongst different plant species and different soil communities.

Mycorrhiza are a key symbiotic mutualism between a fungus and a root of a living plant that can have a large effect on the soil environment for plants. Nutrient transfer between plant and fungus is beneficial to both partners since many kinds of nutrients given by fungi or plant would not be naturally accessible to the receiving partner (Kahiluoto & Vestberg 1998). Plants with mycorrhizal fungi associations also attain a larger supply of nutrients since mycorrhizal fungi hyphae extends the surface area of the plant’s root system, providing the plant a broader range of soil to access. Mycorrhizal associations can also benefit plants by suppressing the colonization of parasitic fungi and nematodes (Morin et al. 1999). These benefits can give plants associated with mycorrhizal fungi a competitive advantage over plants without mycorrhizal fungi connections, and reports have shown how colonized plants can outcompete non-mycorrhizae plants (Allen et al. 1989a).

Most mycorrhizal fungi species are not specifically associated to one plant species, and have the ability to form associations with multiple hosts (Warner et al. 1984). Some mycorrhizal fungi can form common hyphal networks with multiple
different plant hosts at the same time (Simard and Durall 2004). These common mycorrhizal networks (CMNs) are especially beneficial to seedling introduction, since early mycorrhizal associations can strengthen chances of survival and successful establishment (Simard et al. 1997). Additionally, some seedlings can receive carbon nutrients from established trees through their common mycorrhizal networks, further contributing to seedling recruitment success (Hogberg et al. 1999, Horton et al. 1999).

Progressively more research is examining how established mycorrhizal networks can facilitate fungal colonization within introduced species. One study found that introduced Southern Chinese pine (Pinus tabulaeformis) seedlings experienced more mycorrhizal associations and better growth rates when planted nearby mycorrhizae-colonized hazel hornbeam (Ostryopsis davidiana) seedlings (Bai et al. 2009). Another study observed the influence of established mycorrhizal networks by comparing American chestnuts planted either in an abandoned mine plot, a 10-year pine plot, or a forest-edge plot. The study discovered that chestnuts grown in the forest-edge and pine plots experienced higher colonization rates and, furthermore, that seedling survival and growth was highest for chestnuts with mycorrhizal associations within the pine plots (Bauman et al. 2012).

Though associating with established vegetation can potentially provide mycorrhizal benefits, competitive factors between seedlings and plants can also arise. One greenhouse experiment demonstrated the influence of competition between plants, even with the presence of mycorrhizal colonization. The study reported that though seedlings significantly benefited from mycorrhizal
associations when planted in isolation, seedlings grown with a mycorrhizal plant did not show any difference in growth compared to seedlings grown with non-mycorrhizal plants (Kytoviita et al. 2003).

Understanding how soil environments influence plant performance is especially relevant to American chestnut (*Castanea dentata*) restoration practices. Recently Coughlin (2015) has shown that American chestnut seedlings experienced reduced growth and survivorship when grown in their local soil, compared to other southeastern tree-specific soils. The study suggests that American chestnuts seedlings may be subject to harmful chestnut-specific soil microbes, and might perform more favorably in non-chestnut-specific soil environments (Coughlin 2015). On the other hand, studies have found that other soil microbes found in native soil contribute to American chestnut success. Bauman et al. (2011) found that native mycorrhizal fungi played a significant role in American chestnut establishment, as fungal colonization had persistent effects on successful seedling recruitment. Understanding how the local soil microbe community influences plant performance is an important aspect of American chestnut restoration, especially since microbial inoculation from local soil can be a more effective colonization method than individual-species inoculation (Emam 2016). Emam (2016) found that the use of local soil to colonize plants with mycorrhizal fungi resulted in greater plant growth compared to plants inoculated with commercial mycorrhizal products.

These studies elucidate how dependence of soil communities may provide benefits to American chestnuts, but also may expose seedlings to harmful pathogens. Further research is needed to attain a deeper understanding of the
microbial relationship that chestnuts have with soil communities and of how this relationship can influence chestnut restoration.

This project aims to identify the potential relationship between mycorrhizal fungi and interspecific seedling competition, and to investigate how these factors influence American chestnut seedling performance. To determine the effects of mycorrhizal fungi and competition on American chestnuts, we performed a greenhouse study with American chestnuts and Northern red oaks (*Quercus rubra*), a natural competitor to the chestnut. Chestnuts and red oaks were grown either in isolation or together in pots enriched with different soil treatments. We hypothesized that seedlings grown without a competitor in mycorrhizae-rich soil would exhibit greater height, diameter, and biomass than seedlings grown with a competitor in non-mycorrhizal soil. We also hypothesized that the different sources of mycorrhizae might cause various performance outcomes.

**Study System**

*American Chestnut*

The American chestnut was, historically, one of the most common tree species in eastern North America. It served as a dominant species in eastern upland hardwood deciduous forests of North America, with an estimated range of over 800,000 square kilometers, making up to 25-50% of the canopy cover in some regions (Braun 1950). They were especially influential as a dominant species in the Appalachian region of eastern North America (Jacobs et al. 2013). The American chestnuts were once defined as a “foundation species” due to its influence on forest
communities and ecological processes. The tree overall was a major contributor to forest productivity, decomposition, and nutrient cycling due to its fast growth rate, high levels of wood tannin, and high leaf nitrogen-to-carbon ratio (Ellison et al 2005). Additionally, it was a consistent seed resource to many species, as the tannin concentration within chestnut seeds was low enough to attract many granivores (Dalgleish & Swihart 2012). American chestnuts also provided important economic services, acting as an accessible source of edible nuts, durable decay-resistant lumber, and leather-producing tannins (Wang et al. 2013). However, populations have experienced dramatic decline over the past century due to the spread of chestnut blight, a crippling disease instigated by infection from an invasive parasitic fungal species.

First reports of Chestnut blight infections on American chestnuts were documented in 1904 at the Bronx Zoological Park, in New York City (Roane et al. 1986). By the middle of the 20th century, chestnut blight had rapidly spread throughout the country (Anagnostakis 1987).

Chestnut blight is caused by Cryphonectria parasitica, an ascomycete fungus that operates as a necrotophic pathogen, killing the host tissue and consuming the dead matter. The fungus invades the bark and establishes within the stem, often creating cankers. This fungal intrusion results in stem degradation and eventual stem tissue death (Beattie and Diller 1954, Griffin et al. 1983). The fungus infects the aboveground stem of the American chestnut, but leaves the root system intact. Infected trees that experience stem dieback often produce new stems asexually from the root system. However, American chestnuts caught in this repetitive cycle of
stem-die back and stem regrowth rarely develop into a seed-producing canopy tree. As a result of chestnut blight, chestnuts are now more commonly identified by stunted growth and woody shrub characteristics (Paillet 2002). Though chestnut blight does not permanently kill a chestnut, the blight’s effect on a chestnut’s life cycle often results in functional loss within the tree. Because many surviving chestnut trees experience blight-induced stunted growth, they cannot compete as a canopy tree. Due to chestnut underdevelopment caused by chestnut blight, the population is no longer capable of performing important ecological services. The loss of competitive ability and ecological influence has left the American chestnut species functionally extinct.

The dramatic decline of a dominant species, like the American chestnut, can often lead to a cascade of other broader effects on an entire ecosystem. For example, American chestnut population decline resulted in the rise of Northern red oak, chestnut oak (*Quercus prinus*), and red maple (*Acer rubrum*) as the dominant replacement tree species (Woods & Shanks 1959). Furthermore, at least 60 insect species relied on American chestnuts as a food resource, with seven of these species acting as specialists towards only American chestnuts (Opler 1978). Dalgleish and Swihart (2012) have also highlighted that the decline of the American chestnut as an influential mast-producing tree may have reduced the abundance of mast resources for several mammalian species.

Because of the ecological importance of American chestnuts, there have been several projects focused on American chestnut reintroduction. Efforts to restore the American chestnut within its historically native range have included artificially
selecting blight resistant chestnuts for breeding, cross-breeding naturally resistant Asian chestnut species with American chestnuts, treating chestnuts with hypovirulent blight strains to reduce infection from more detrimental blight strains, and genetically engineering blight resistance (Jacobs et al. 2013). There has been consistent success in a number of these projects, and American chestnut restoration is progressively becoming an attainable goal. However, before reintroduction can be fully reached, there must be a more complete understanding of the ecology of the American chestnut as a species, and as an active component within its ecosystem. Information on how American chestnut populations will respond to the current environment, and how other species will interact with introduced American chestnuts must be obtained in order to holistically predict the effectiveness of reintroduction.

Methods

Pure American chestnut seeds were provided by the Maryland Chapter of the American Chestnut Foundation. Northern red oak seeds were collected October 2014 from 8 different trees in Williamsburg, Virginia. Seeds were stored in peat moss-filled, sealed plastic bags and refrigerated at 5 degrees Celsius.

In total, 175 American chestnut seeds and 125 Northern red oak seeds were planted in flats from April 6th through April 16th 2015. The flats were kept in the William and Mary Greenhouse under artificial light and routinely watered. On April 27th 2015, we transported flats to the William and Mary facilities Sullivan.
Greenhouse and stored them in an outdoor shelter to acclimate the seedlings to the local environment.

On May 2\textsuperscript{nd} 2015, 66 American chestnuts and 60 Northern red oaks were transplanted into 6-gallon pots. From May 2\textsuperscript{nd} to Sept 28\textsuperscript{th} 2015, American chestnuts and Northern red oaks were grown at Sullivan Greenhouse. Each pot was assigned a soil origin treatment, and a competition treatment.

Soil treatment included American chestnut-dominant soil, Northern red oak-dominant soil, tulip poplar-dominant soil, fungicide-treated soil, and sterilized soil. The species treatment involved either American chestnut or Northern red oak. Competition treatments were defined as plants either grown alone or with an interspecific seedling competitor.

Seedlings planted together in the same pot were matched to each other in height, but overall, seedlings grown in different pots varied from each other in height through random selection.

\textit{Soil Treatments}

Each pot was assigned an 80 mL soil treatment. All soil treatments were collected in forested sites. Soil sites were collected at Granruth Farm (Marshall, Virginia) and Blandy Experimental Farm (Boyce, Virginia). Soil was collected from sites directly under the edge of the targeted tree canopy. American chestnut dominant soil and Northern red oak dominant soil was collected from Granruth Farm. 10 soil samples were collected in total, with 7 by American chestnuts and 3 by Northern red oaks. Each American chestnut soil sample collection site was
approximately 1.5 to 3.5 meters away from the nearest American chestnut. Each Northern red oak soil sample was approximately 1.5 to 2.5 meters away from the nearest Northern red oak. Tulip poplar dominant soil was collected at Blandy Experimental Farm. Three soil samples were collected approximately 3 to 6 meters away from tulip poplars.

A section of the collected soil was allocated towards creating fungi-free and sterile soil treatments. We applied Topsin fungicide solution to selected soil in order to create a fungi-free treatment soil (Wilson & Williamson 2008). The solution was made by dissolving 1 gram of fungicide powder for every 1 liter of water. Initially, 52.1 mL and 54.0 mL of solution was respectively added to the collective American chestnut- and red oak-dominant soil. Solution volume was relative to the measured weight of each soil type. Fungicide solution volume varied based on weight of each soil type. Once the soil treatments were added to the pots, 140 mL of the fungicide solution was added to each fungicide soil treatment every three weeks.

Sterilized soil was created through mixing equal quantities of American chestnut dominant, red oak dominant, and tulip poplar dominant soil. The mixed soil was then autoclaved, removing all potential fungi, bacteria, and pathogens from the soil.

Growing Period

On May 2nd 2015, 66 American chestnuts and 60 Northern red oak seedlings, of varying sizes, were randomly selected and planted into 6-gallon nursery pots. Seedlings planted alone were centered in the pot. Seedlings in competition
treatments were planted at the same distance from each other. The north-south position of the two seedlings in a competition treatment pot was randomized for each treatment type. Each pot was labeled with a designated number from 1 to 90 and randomly given a position in a 3 by 30 grid.

Each treatment type was repeated 6 times. Overall, there were 15 different treatment combinations, and a total of 90 pots. 126 trees were examined in the study.

Data Collection

On May 6th 2015, height to apical meristem and root collar diameter for each plant were measured. Measurements were then taken every two weeks until September 28th 2015.

After the growing season, the aboveground biomass was separated from the belowground roots, oven-dried at 60 degrees Celsius, and weighed. Belowground roots were gently washed, oven dried at 60 degrees Celsius, and weighed as well. Roots were then randomly allocated for analysis on root colonization abundance or genetic identification.

Root Staining

A portion of the total root matter was analyzed to quantify mycorrhizae colonization. From the collection of roots samples, 3 of the 6 replicates from each treatment were randomly selected for further investigation. Roots from each sample were cut into 1 cm lengths, and placed into histology cassettes. Roots were then
soaked in 10% potassium hydroxide solution at 100 degrees Celsius for three hours to clear the natural color of the roots. This process was then followed by soaking the roots in alkaline hydrogen peroxide (10:1 H₂O₂ : NH₄OH) at room temperature for another three hours, in order to further clear the natural root color. Roots were considered cleared of their natural color when they achieved a white, translucent appearance. Afterwards, the roots were gently washed in water, then soaked in a boiling 3% ink-vinegar solution for one minute. The ink-vinegar solution acted as the root stain, giving fungi associations within the roots a bright blue color (Vierheilig et al. 1998). Stained roots were then washed quickly in water and slightly acidified with a brief vinegar rinse.

Root Colonization Abundance Quantification

After the roots were stained, root colonization abundance of mycorrhizal fungi was quantified. From the stained root cohort, we randomly selected 40 1-cm root sections. Under a compound microscope roots were examined for mycorrhizae presence and absence. Mycorrhizal hyphae, vesicles, and arbuscules were noted as signs of mycorrhizal colonization (Image 1, Image 2). Roots were determined void of mycorrhizal fungi if the sections did not exhibit any of the mentioned mycorrhizal features. Root colonization abundance was recorded as a percentage of colonization within the 40 total roots. Roots that did not display obvious mycorrhizal features but were seen with spore-like features were also noted. However, these were observed separately from the colonization observations (Image 3, Image 4). One
replicate of all the treatments was recorded by one observer. A second observer recorded the other two replicates.

Data Analysis

Data collected on height, root collar diameter, above and belowground biomass, as well as percent colonization was statistically evaluated through ANOVA analysis and linear regression models using R. Analysis was performed at an $\alpha = 0.10$ due to the low sample size within each treatment (n=6).

Results

There was no significant difference in the initial apical meristem heights of chestnut and red oak seedlings in the competition treatments ($AC = 10.7\, \text{cm}, RO = 9.9\, \text{cm}; P = 0.341$) (Figure 1). However, red oak seedlings in competition treatments had larger initial root collar diameters than chestnut seedlings ($AC = 1.96\, \text{mm}, RO = 2.24\, \text{mm}; P = 0.00265$) (Figure 2). Overall, mortality in the experiment was low: by the end of September 28th, a total of six plants (out of 126) had died during the growing period (Table 1).

Analysis between Species

Chestnuts grew larger by all measurements of growth examined in this study. Chestnut on average experienced a greater total change in height than red oak. ($AC = 33.77\, \text{cm}, RO = 15.98\, \text{cm}; P < 0.001$) (Figure 3). Chestnuts on average exhibited a
greater total change in diameter than red oaks as well (AC = 8.17 mm, RO = 3.95 mm; P < 0.001) (Figure 4).

Furthermore, we found that chestnut biomass significantly differed from red oak biomass at the end of the experiment. The average aboveground biomass of chestnuts was greater than that of red oaks (AC = 30.96 g, RO = 6.28 g; P < 0.001) (Figure 5). Chestnuts also displayed a greater belowground biomass when compared to red oaks (AC = 20.51 g, RO = 11.68 g; P = 0.00353) (Figure 6).

*Analysis of Competitive Factors*

With respect to change in plant height, competition did not seem to affect either chestnut or red oak growth. There was no significant difference in the change of height between chestnuts grown with a competitor and chestnuts grown without a competitor (P = 0.115) (Figure 3). The same results were also shown for red oaks grown with a competitor and grown without a competitor (P = 0.333) (Figure 3). Furthermore there was not a significant difference in the total change in diameter for chestnuts grown with or without a competitor (P = 0.217) (Figure 4). Likewise, red oaks did not experience a significant difference in total change in diameter when grown with or without a competitor (P = 0.568) (Figure 4).

Aboveground weight revealed an effect of competition for chestnuts only. Chestnuts grown without a competitor exhibited on average a greater biomass than chestnuts grown with a competitor (No Competition = 41.75 g, Competition = 21.97 g; P = 0.062) (Figure 5). On the other hand, there was no significant difference in aboveground weight between red oaks grown with a competitor and red oaks
grown without a competitor ($P = 0.862$) (Figure 5). Similarly, there was no significant difference in belowground weight between chestnuts grown with a competitor and chestnuts grown without a competitor ($P = 0.516$) (Figure 6). However, red oaks grown with a competitor exhibited a greater belowground biomass than red oaks grown without a competitor (Competition = 14.63 g, No Competition = 9.41 g, $P = 0.0902$) (Figure 6).

**Analysis of Soil Treatments**

There was no significant difference in total change in height between chestnuts grown in the different soil treatment types ($P = 0.765$) (Figure 7), which was the case for red oaks as well ($P = 0.739$) (Figure 8). Analysis of the total change in diameter also showed that, for both chestnuts and red oaks, there were no significant differences between trees grown in different soil treatments ($P = 0.765; P = 0.973$) (Figure 9, Figure 10).

Evaluation of the aboveground biomass of chestnuts and red oaks also revealed that the different soil treatments was not correlated with significant differences in chestnut or red oak aboveground weight ($P = 0.661, P = 0.761$) (Figure 11, Figure 12). Moreover, we found that the different soil treatments were not associated with a significant difference in belowground biomass for chestnut or red oaks ($P = 0.853, P = 0.783$) (Figure 13, Figure 14).
Analysis of Percent Mycorrhizal Colonization within Plant Roots

A statistical analysis of all the quantified samples revealed that there was not a significant difference in observations of percent colonization between the two observers ($P = 0.138$). Additionally, there was no significant difference in percent colonization between chestnut and red oak roots ($P = 0.971$).

A comparison of colonization percentages demonstrated significant differences between soil treatment groupings. Root samples from the mycorrhizae-rich soil treatments (field soil collected under red oak, chestnut, or tulip poplar) exhibited on average a greater percentage of root colonization than root samples from sterilized soil ($\text{Mycorrhizae} = 76.33, \text{Sterile} = 54.58, P = 0.00247$)(Figure 15). However, fungicide treated soils were intermediate in root colonization and not significantly different from any other treatment (Figure 15). If the roots exhibiting spore-like features were interpreted as mycorrhizal then similar results were found. Mycorrhizae-rich soil treatments demonstrated higher rates of colonization when compared to solely sterilized soil treatment ($\text{Mycorrhizae} = 91.80, \text{Sterile} = 80.83, P = 0.050$).

An analysis of percent colonization between more specific soil treatment types revealed some significant differences as well. Roots grown from American chestnut dominant soil obtained higher colonization rates compared to roots grown in sterile soil when considering only the presence of hyphae in roots ($\text{ACS} = 77.73, \text{STERILE} = 54.58, P = 0.0421$)(Figure 16). Similarly roots grown in tulip poplar also exhibited greater percent colonization compared to roots grown in sterile soil ($\text{TPS} = 82.22, \text{STERILE} = 54.58, P = 0.0145$) (Figure 16). The inclusion of the spore-like
features into the mycorrhizal percentage resulted in no significant difference in colonization for American chestnut dominant soil and tulip poplar dominant soil when both were compared to sterile soil (P = 0.333, P = 0.595). Roots from American chestnut dominant soil did not differ in colonization percentage from roots grown in fungicide-treated American chestnut dominant or red oak dominant soils (P = 0.447, P = 0.863), as well as tulip poplar soil (P = 0.221, P = 0.560). Roots grown in red oak dominant soil did not display differences in mycorrhizal colonization when compared to roots from fungicide-treated chestnut and red oak dominant soil, or to roots from sterile soil (P = 0.999, 0.860, 0.570, respectively)(Figure 16).

Examination of competitive influence on percent colonization revealed another interesting layer of analysis when comparing the mycorrhizal effect between treatments. Overall, roots grown with a competitor exhibited greater percentage of colonization compared to roots grown without a competitor (Competition = 73.75, No Competition = 62.308, P = 0.0244)(Figure 17). The inclusion of the spore-like features resulted in no difference between the competition treatments (P = 0.271). Analysis of competitive influence over treatment types demonstrated that roots grown with a competitor in sterilized soil experienced greater colonization compared to roots grown without a competitor in sterilized soil (Competition = 69.583, No Competition = 39.583, P = 0.040)(Figure 18). Additionally roots grown without competition in sterilized soil on average expressed a lower colonization percent when compared to roots grown with and without competition in mycorrhizae-rich soil treatments (Sterile/No Competition =
Further investigation of competitive impact over specific soil treatments showed that roots grown with a competitor in American chestnut dominant soil, red oak dominant soil, and tulip poplar dominant soil experienced greater colonization compared to roots grown without a competitor in sterilized soil (ACS = 75.833, ROS = 77.500, TPS = 87.917, STERILE = 39.5833, P = 0.0208, P = 0.0175, P = 0.000678)(Figure 19). Only roots grown without competition from American chestnut dominant soil were significantly different from roots grown without competition in the sterilized soil (ACS = 80.00, STERILE = 39.583, P = 0.0147)(Figure 19).

**Linear Regression Analysis**

Linear regression models for both chestnut and red oak seedlings demonstrated that percent colonization was not significantly correlated with change in height (P = 0.273), change in diameter (P = 0.231), aboveground biomass (P = 0.573), or belowground biomass (P = 0.411).

Based on linear regression models, there was no significant correlation between competition and belowground biomass (P = 0.897) or change in height (P = 0.138). However, linear regression models did find a significant negative correlation among competition treatments when analyzing the average chestnut aboveground biomass (P = 0.00936) as well as average chestnut change in diameter (P = 0.0482). Chestnuts grown in competition were smaller in diameter (Competition = 7.22 mm,
No Competition = 9.39 mm) and lower in aboveground biomass (Competition = 21.97 g, No Competition = 41.75 g) when compared to chestnuts grown without competition (Figure 4, Figure 5).

Discussion

An analysis of American chestnut and red oak seedlings subjected to competitive and mycorrhizal factors revealed differences in growth performance. American chestnuts grew more than red oaks by all measures: they had greater change in height, change in diameter, aboveground biomass, and belowground biomass compared to red oaks. Only American chestnuts appeared to exhibit an effect from competition. Although some soil treatments demonstrated differences in percent colonization for both red oak and chestnut seedlings, none of the soil treatments seemed to affect any measure of growth for chestnuts or red oaks. The presence of another tree in the pot was correlated to increased percent colonization for all soil treatments.

The differences in seedling performance between red oak and chestnut that we observed are consistent with several studies. Jacobs and Severeid (2004) found that after a seven to eight year study in Wisconsin, American chestnuts exhibited greater height and diameter at breast height (DBH) compared to red oaks. A one-year greenhouse study performed by Latham (1992) revealed that when exposed to various light and nutrient treatments, American chestnuts displayed greater growth compared to red oaks. Brown et al. (2014) reasoned that American chestnuts show greater gains in aboveground growth compared to red oaks partly due to differences
in biomass allocation strategies between the two species. Juvenile red oaks are known to devote more energy towards belowground biomass development. Our results, however, demonstrated that American chestnut seedlings outperformed red oak seedlings in belowground biomass growth, suggesting that American chestnuts have the potential to outcompete red oaks on both aboveground and belowground fronts, regardless of resource partitioning strategy.

Within this study, American chestnuts grown without a competitor experienced increased aboveground biomass compared to American chestnuts grown with competition. On the other hand, red oaks exposed to a competitor exhibited increased belowground biomass compared to red oaks grown without competition. These variances in growth outcomes might be explained by differences in energy allocation. While American chestnuts dedicate most of their biomass towards aboveground development, red oaks have been shown to partition more energy towards root biomass growth. Wang et al. (2006) analyzed American chestnut growth in various light levels and discovered that one year-old chestnut seedlings distributed over 70% of biomass to the aboveground shoot system regardless of light intensity. Rebbeck et al. (2011) found that during their first year of growth red oaks devoted 53-65% of their growth towards belowground biomass. This allocation of growth to belowground root systems might explain why red oak seedlings on average displayed a greater belowground response when faced with competition. It is common for many plants to express increased root growth when subjected to competition. Plants will occasionally express this plasticity in response to the threat of competition in order to acquire more resources within the soil (Kolb
& Steiner 1990). In several cases, competitive factors can induce an increase in allocation towards belowground biomass often at the expense of the aboveground shoot system (Kolb & Steiner 1990).

Though ANOVA analysis demonstrated species-specific biomass responses to competition, some linear regression models produced mixed results. Linear regression models exhibited consistent results with analysis of change in height and aboveground biomass. However, in contrast to ANOVA analysis, the model did not indicate a belowground biomass response to competition. Additionally, these tests highlighted significant differences in chestnut diameter across the competition treatments. Differences in results may be attributed to the relatively small sample size present within this study, though the conflict between results underlines a need for further analysis on how competition influences seedling performance for both chestnuts and red oaks.

Though American chestnuts and red oaks both uniquely responded to competitive factors present within this experiment, seedling performance was not associated with differences in soil origin. Quantification of mycorrhizal colonization revealed that there were some differences in mycorrhizal associations between roots from some of the mycorrhizae-rich soil treatments and roots from the sterile soil treatments. However, not all mycorrhizal soil treatments differentiated from the non-mycorrhizal treatments, as roots from red oak dominant soil on average did not exhibit distinct rates of colonization compared to other soil treatments. Additionally, roots grown from American chestnut dominant soil and tulip poplar dominant soil were not significantly different in percent colonization when
compared to fungicide-treated soil. This result raises questions over the effectiveness of the fungicide treatment recommended by Wilson & Williamson (2008). Differences in mycorrhizal exclusion success between this study and the study performed by Wilson and Williamson may have arisen due to differences in the plants targeted. Wilson & Williamson (2008) conducted their Topsin fungicide analysis on grass species, *Andropogon gerardii* and *Pasocyrton smithii*, while our study involved only woody species. Their study specified that Topsin is not a universally recommended fungicide, but instead suggested that this new fungicide can serve as an alternative to the fungicide benomyl, a retired biocide primarily utilized for agricultural purposes, not forestry use.

Further analysis of mycorrhizal abundances within different treatments revealed that competitive factors influenced colonization rates amongst roots from several soil types. Within the sterile soil treatment, roots grown with a competitor on average expressed greater percent colonization compared to roots grown without a competitor. Also within the American chestnut dominant, red oak dominant, and tulip poplar dominant treatments, roots grown with a competitor experienced on average more mycorrhizal colonization than roots grown in sterile soil without a competitor. Whereas roots from the three treatments grown without a competitor did not express different rates of colonization compared to sterile soil roots. Differences in colonization levels may be explained by differences in probability of root encounters. Increased root density from an additional plant present in the competition treatments may have provided initial mycorrhizal fungi within the soil an increased chance of colonization. Mycorrhizal colonization within
roots has been shown to positively correlate with increased host plant density (Genney et al. 2001) and even different plant species have been known to share mycorrhizal networks (Horton et al. 1999). The result from this study brings further attention to the mycorrhizal relationship between American chestnuts and red oaks.

Though colonization analysis demonstrated some differences in mycorrhizal abundance between treatments, there was no evident seedling reaction to mycorrhizal fungi. A number of factors may contribute towards the observed colonization rates within roots and the lack of response from seedlings. First, certain aspects of the experiment design did not control for consistent mycorrhizal exclusion within the soil. The study was carried out in an outdoors facility, which exposed the treatment soil to various natural elements. The soil treatments could have been compromised by mycorrhizal spore dispersal sourced from outside the bounds of the experiment. Mycorrhizal fungi can rely on the wind as an agent of spore dispersal, and spores have been observed to migrate up to two km from their origin site by air currents, especially during drier seasons (Allen et al. 1989b). There is a possibility that mycorrhizal spores outside of the study could have established within the treatment soils. Secondly, the observed differences in mycorrhizal colonization in the soil treatments might not have been significant enough to elicit a seedling response. Though statistical analysis revealed markedly significant differences in colonization percentages, it is unknown whether the noted differences are dramatic enough to provoke a biological significance for seedling performance. Likewise, the seedlings grown for this study may have experienced enough access to certain resources, allowing the seedlings to develop in an
environment with reduced mycorrhizal significance. For several plants, the expressed benefit of mycorrhizal associations is contingent on the scarcity of resources (Jasper et al. 1993). Some plants often demonstrate greater mycorrhizae-induced changes in performance only when grown with a lack of resources, when they rely more on mycorrhizal associations rather than their surroundings for nutrient absorption (Jasper et al. 1993). Conditional influence of mycorrhizal fungi on plant performance might be a contributing reason for the absence of mycorrhizal impact within this experiment. Further research on how mycorrhizal interactions influence seedling performance should account for the effect of outer mycorrhizal spore dispersal and determine the impact resource availability has on mycorrhizal seedling performance outcomes.

Though this study was able to analyze variances in mycorrhizal colonization abundance within different soil treatments, this study did not investigate the diversity of the mycorrhizal communities present in the roots. It remains unknown for this study whether different species or treatment types harbored similar mycorrhizal associations, or if there were differences in the composition of these mycorrhizal communities. Compositional analysis may contribute towards greater awareness of differences in mycorrhizal fungi interactions between American chestnut and red oak seedlings, as well as variances in mycorrhizal diversity in differing soil localities.

This study was carried out in order to understand the ecological factors involved with American chestnut development. Though American chestnut populations have greatly declined within the past century, recent headway in
chestnut blight resistance research strengthens the possibility of restoration. Artificial selection, crossbreeding, hypovirulence, and genetic engineering all make chestnut reintroduction a future prospect for eastern deciduous forests. Research in American chestnut ecology is critical in order to understand the environmental implications of chestnut reintroduction and to determine the most effective practices for chestnut restoration. Through analysis of soil conditions and competition, this study addressed the potential influence of environmental variables on blight resistant chestnuts.
Images, Tables, & Figures

Image 1 & Image 2. Photographic observations magnified by a compound microscope (400x) of mycorrhizal associations within root samples.

Image 3 & Image 4. Photographic observations magnified by a compound microscope (400x) of spore-like features within root samples.
Table 1. By September 28\textsuperscript{th} a total of six trees had died. The six trees were grown in different kinds of soil and competition treatments. ACS = American Chestnut Soil, NFACS = No-Fungi American Chestnut Soil, No-Fungi Red Oak Soil, STERILE = Sterile Soil, TPS = Tulip Poplar Soil. The wide distribution of the characteristics of the dead trees suggests that no singular treatment trait contributed to seedling death.

<table>
<thead>
<tr>
<th></th>
<th>ACS</th>
<th>NFACS</th>
<th>NFROS</th>
<th>ROS</th>
<th>STERILE</th>
<th>TPS</th>
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<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>No Competition</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Figure 1. The initial height (cm) of chestnut (AC) and red oak (RO) seedlings at the beginning of the growing period, May 6\textsuperscript{th} 2015. The average height of AC = 10.7 cm and RO = 9.9 cm. The initial average heights were not significantly different (\(P = 0.341\)).
Figure 2. The initial diameter (mm) of chestnut (AC) and red oak (RO) seedlings at the beginning of the growing period, May 6th 2015. The average diameter of AC = 1.96 mm and RO = 2.24 mm. The average diameter of RO was greater than AC (P = 0.00265).

Figure 3. The total change in height (cm) of chestnuts (AC) and red oaks (RO) in different competition treatments. The average change in height of AC = 33.77 cm and RO = 15.98 cm. The average change in height of AC was greater than RO (P < 0.001). There is no significant difference between competition treatments for AC and RO.
Figure 4. The total change in diameter (mm) of chestnuts (AC) and red oaks (RO) in different competition treatments. The average change in diameter of AC = 8.17 mm and RO = 3.95 mm. The average change in diameter of AC was greater than RO (P < 0.001). There is no significant difference between competition treatments for AC and RO.

Figure 5. The aboveground biomass (g) of chestnuts (AC) and red oaks (RO) in competition treatments. The average aboveground biomass of AC = 30.96 g and RO = 6.28 g. The average aboveground biomass of AC was greater than RO (P < 0.001). AC grown without competition had a greater aboveground biomass than AC grown with competition (No Competition = 41.75 g, Competition = 21.97 g; P = 0.062).
Figure 6. The belowground biomass (g) of chestnuts (AC) and red oaks (RO) in competition treatments. The average belowground biomass of AC = 20.51 g and RO = 11.68 g. The average belowground biomass of AC was greater than RO (P < 0.005). RO grown without competition had a lower belowground biomass than RO grown with competition (Competition = 14.63 g, No Competition = 9.41 g, P = 0.0902).

Figure 7. The total change in height (cm) of chestnuts (AC) in different soil treatments. There was no significant difference in total change in height between AC grown in the different soil treatments (P = 0.765)
Figure 8. The total change in height (cm) of red oaks (RO) in different soil treatments. There was no significant difference in total change in height between RO grown in the different soil treatments ($P = 0.739$).

Figure 9. The total change in diameter (mm) of chestnuts (AC) in different soil treatments. There was no significant difference in total change in diameter between AC grown in the different soil treatments ($P = 0.765$).
Figure 10. The total change in diameter (mm) of red oaks (RO) in different soil treatments. There was no significant difference in total change in diameter between RO grown in the different soil treatments (0.973).

Figure 11. The aboveground biomass (g) of chestnuts (AC) in different soil treatments. There was no significant difference in aboveground biomass between AC grown in the different soil treatments (P = 0.661).
Figure 12. The aboveground biomass (g) of red oaks (RO) in different soil treatments. There was not a significant difference in aboveground biomass between RO grown in different soil treatments (P = 0.761).

Figure 13. The belowground biomass (g) of chestnut (AC) in different soil treatments. There was not a significant difference in belowground biomass between AC grown in different soil treatments (P = 0.853).
Figure 14. The belowground biomass (g) of red oaks (RO) in different soil treatments. There was not a significant difference in belowground biomass between RO grown in different soil treatments ($P = 0.783$).

Figure 15. The percentages of mycorrhizal colonization observed within 40 1-cm root sections in different soil treatment groups (Mycorrhizae, Fungicide, Sterile). Roots grown in myorrhizae treatment soil had greater percent colonization than roots grown in sterile soil (Mycorrhizae = 76.33, Sterile = 54.58, $P = 0.00247$). Roots grown in fungicide treated soils were intermediate in root colonization and not significantly different from any other treatment.
Figure 16. The percentages of mycorrhizal colonization observed within 40 1-cm root sections in the different soil treatments. Roots grown from ACS had greater percent colonization compared to roots grown in sterile soil (ACS = 77.73, STERILE = 54.58, P = 0.0421). Roots grown in TPS had greater percent colonization compared to roots grown in sterile soil (TPS = 82.22, STERILE = 54.58, P = 0.0145).

Figure 17. The percentages of mycorrhizal colonization observed within 40 1-cm root sections in different competition treatments. Roots grown with a competitor had greater percent colonization compared to roots grown without a competitor (Competition = 73.75, No Competition = 62.308, P = 0.0244).
Figure 18. The percentages of mycorrhizal colonization observed within 40 1-cm root sections in varying soil treatments and different competition treatments. In sterile soil, roots grown with a competitor had greater percent colonization compared to roots grown without a competitor (Competition = 69.583, No Competition = 39.583, P = 0.040). Roots grown without competition in sterilized soil expressed lower percent colonization compared to roots grown with and without competition in mycorrhizae-rich soil treatments (Sterile/No Competition = 39.583, Mycorrhizae/Competition = 80.417, Mycorrhizae/No Competition = 71.071, P = 0.0000675, P = 0.00500).
Figure 19. Percentages of mycorrhizal colonization observed within 40 1-cm root sections in different soil and competition treatments. Roots grown with a competitor in ACS, ROS, and TPS had greater colonization compared to roots grown without a competitor in sterilized soil (ACS/Competition = 75.833, ROS/Competition = 77.500, TPS/Competition = 87.917, STERILE/No Competition = 39.583, P = 0.0208, P = 0.0175, P = 0.000678). Only ACS roots grown without competition had significantly greater percent colonization compared to roots grown without competition in the sterilized soil (ACS/No Competition = 80.00, STERILE/No Competition = 39.583, P = 0.0147).
Works Cited


Kolb, E.T., Steiner, K.C. 1990. Growth and Biomass Partitioning of Northern Red Oak and Yellow-Poplar Seedlings: Effects of Shading and Grass Root Competition. Forest Science. 36. 34-44


