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ABSTRACT

ECTOMYCORRHIZAL COMMUNITIES ASSOCIATED WITH RESTORATION PLANTINGS OF AMERICAN CHESTNUT (*CASTANEA DENTATA*) SEEDLINGS ON OHIO MINE LANDS: PLANTING METHODOLOGIES TO PROMOTE ROOT COLONIZATION

by Jenise M. Bauman

Ectomycorrhizal (ECM) fungi form mutualistic symbioses with woody trees and shrubs allowing for an increase in water and nutrient uptake. The absence of these microbes may contribute to seedling mortality and the arrested succession observed in barren landscapes and grasslands in Ohio. The central objective of this dissertation was to develop planting methodologies to accelerate succession by woody tree establishment; specifically by maximizing the effectiveness of ECM root colonization. American chestnut and chestnut hybrids were used to describe host response to root colonization in both abandoned and reclaimed mine sites in central Ohio. A set of experiments was designed to test the influence existing vegetation, site selection, soil modification, and the addition of ECM inoculum may have on seedling establishment in former mine sites.

I investigated the influence existing vegetation had on germination and survival of chestnut in an abandoned mine site. Three areas were assessed: center, areas that had monoculture plantings of *Pinus virginiana*, and forest edges. Small monoculture plantings of pines had a greater facultative effect on the germination and survival of deciduous hardwood seedlings than did the forest edge; presumably by alleviating negative density-dependent factors. Importantly, pine and chestnut shared ECM symbionts. This provided an ECM propagule source to chestnut and resulted in an increase in seedling biomass, which may have contributed to the increase in survival after two years.

In reclaimed mines, heavy equipment and the use of exotic species as cover crops have resulted in severely compacted soils with aggressive herbaceous canopies. I evaluated surface soil treatments, which included deep ripping and traditional plow and disking, as ways to remediate these mine lands in arrested succession. These methods were very successful in alleviating compaction and disturbing the aggressive herbaceous

canopy, thereby promoting chestnut seedling establishment. In addition, mechanical soil treatments resulted in seedlings with significantly more ECM root tips with greater species richness. Further, there was a significant interaction between soil treatment and ECM colonization. Chestnut seedlings naturally colonized by ECM fungi in treatment plots had the greatest shoot production when compared to their non-ECM counterparts.

I assessed the field performance of five different ECM fungi inoculated on hybrid chestnut. These ECM species did not persist on chestnut after one year in the field or impede natural root colonization of native fungi. However, the presence of ECM inoculum greatly contributed to the survival of hybrid chestnut seedlings. Therefore, introduced inoculum that was present in the very early stages of outplanting had persisting effects with regard to seedling development in the field, even if the original inoculum did not persist. Important to chestnut restoration was that native ECM fungi colonized chestnuts and resulted in an increase in seedling growth.

Soil variables and ECM community data were used to determine the influence the soil environment has on ECM community composition and root colonization of American chestnut. Differences in ECM communities were associated with differences in nutrient availability; this may have catalyzed a shift in fungal communities to species better able to persist in acidic soils under nutrient-limited conditions. In addition, certain species appeared not to exist as mycelium on existing vegetation, but have the ability to rapidly recruit after mechanical soil treatments. Results of this study help us better understand whether abiotic soil variables can be used to predict ECM composition and root colonization potential in mine restoration using blight-resistant chestnut hybrids.

Proper site selection and soil surface treatment methods significantly contributed to ECM root colonization on chestnut in abandoned and reclaimed mine sites in central Ohio. Employing methodologies that encourage the formation of native ectomycorrhizas may aid in promoting the long-term survival of woody tree species in mine reclamation and accelerate succession to closed canopy forests.

ECTOMYCORRHIZAL COMMUNITIES ASSOCIATED WITH RESTORATION PLANTINGS OF AMERICAN CHESTNUT (*CASTANEA DENTATA*) SEEDLINGS ON OHIO MINE LANDS: PLANTING METHODOLOGIES TO PROMOTE ROOT COLONIZATION

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Chapter 1
Introduction

Succession is the change in a plant community over time. Its progression is of particular concern when managing the recovery of landscapes after anthropogenic disturbances. Certain trends have been documented; disturbed lands are first colonized by pioneer plant species that are able to establish rapidly with high photosynthetic rates and high seed production (Grime 1979). Over time, plant communities shift to species that are better competitors for diminishing resources; herbaceous plant communities may be eventually replaced by woody shrubs and trees resulting in a closed canopy. Restoration ecology tries to imitate this successional pathway to incorporate the natural process of recovery following large scale disturbances (Lockwood and Pimm 1999). However, nature is not entirely predictable, even when dealing with an intact ecosystem that is recovering from a natural disturbance. After anthropogenic perturbations, the successional pathway may be further complicated. So are the attempts of restoring ecosystems where biological systems are either severely disturbed or altogether missing.

In post strip mine reclamation, grasslands or barren landscapes are indicators of an arrested successional pathway. This ecologically undesirable stable state may be indicative of a much more complex disturbance on a microbial scale. The original vegetation and the majority of the organic matter and nutrients were removed with the top soil prior to coal excavation. The substrate left is heavy in parental rock material and mine spoil. The equipment used for the reclamation results in compacted soils with reduced porosity, permeability, moisture-holding capacity, and nutrient transport (Bussler et. al. 1984; Ashby 1997; Torbert and Burger 2000). In addition to the physical characteristics of the soil, the biological components are drastically disturbed. The soil microbial communities responsible for nutrient cycling, soil structure, and biological interactions are rendered low in biomass and activity (Degrood et al. 2004; Jacobs 2005).

Ectomycorrhizal (ECM) fungi are major players in the microbial community. In nature, ECM fungi include 6,000 species known to form symbioses with woody shrubs and trees (Brundrett 2009). Ectomycorrhizal associations are distinguished from other mycorrhizal interactions by the fungal sheath (Figure 1). In addition, these mycorrhizas

are described by the formation of a modified lateral root (Tagu et al. 2003) and Hartig net (Reddy et al. 2005). Previous studies have documented the benefits that this symbiosis has on many conifers and angiosperms in nature (Smith and Read 2008). These benefits include greater access to water, nutrients, alleviation of metal toxicity, and protection from root pathogens (Marx 1972; Cordell et al. 1999; van der Heijden et al. 2003; Nara 2005). In turn, these fungi receive carbon in the form of photosynthates from their plant host forming a mutualistic relationship between plant and fungi (Smith and Read 2008). However, these fungi are not adapted to endure mining disturbances that destroy hyphal networks and remove host plants (Jasper 2007). The severe decline of these microbes may contribute to the observed arrested succession and the mortality of tree species in past reforestation efforts (Marx 1991; Cordell et al. 1999; Nara 2005).

These conditions are quite similar to what is observed in old fields stalled in a grassland state. The success of the limited tree species found establishing along the edge of wood lots may be due to the formation of mycorrhizas from a diverse community of ECM fungi harbored by the existing vegetation (Jonsson 2001). It has been suggested that seedling establishment during succession is dependent on the availability of a mycorrhizal symbiont (Marx 1991); ECM plants have been found to have a greater access to nitrogen and phosphorus, which may give an establishing plant a competitive advantage when nutrients are limited (Nara 2005). Seedlings may be quickly colonized by a diverse array of existing fungi when in close proximity to established forest trees (Dickie et al. 2005; Dickie and Reich 2005). Additionally, seedlings may be incorporated into an existing network of hyphae that facilitates establishment by carbon and nutrient transfer from existing vegetation (Simard et al. 1997; Selosse et al. 2006).

Artificial inoculation of seedlings is a common practice in areas where ECM host plants are absent (Marx 1991). However, not all plant and fungal combinations result in functional mycorrhizas. Environmental conditions play a role in the maintenance of ECM roots (Taylor 2002; Dickie 2007). Therefore, ECM symbioses that are observed in the laboratory may differ significantly from those sampled in the field (Smith and Read 2008). In these situations, poor root colonization in the field may not provide benefits to an establishing seedling (Haskins and Gehring 2004). In addition, the introduction of

inoculum may deter the colonization of seedlings from indigenous populations of fungi better adapted for the extreme environmental conditions in such sites.

The central objective of this dissertation was to develop planting methodologies that would maximize the effectiveness of ECM root colonization and host response by aiding in the establishment of an ECM woody host plant, thereby accelerating succession. This study used American chestnut (*Castanea dentata*) and blight-resistant hybrids (*C.dentata x C. mollissima*). Pure American chestnut (*C. dentata*) was eliminated as a canopy tree from the eastern North American forests with the introduction of chestnut blight (*Cryphonectria parasitica*) in the early 1900's. This hardwood species, valued for its economic and ecological qualities, was highly susceptible to canker producing *C. parasitica*. By the 1950's, 200 million acres of American chestnut had succumbed to the disease and this once prominent species was relegated to a minor place in the eastern forests (Kuhlman, 1978). Breeding programs described by Burnham (1988) have been successful in incorporating blight resistant genes from Chinese chestnuts, producing hybrids that display pure American morphology with adequate field-resistance to chestnut blight (Figure 2).

Preliminary studies have reported American chestnut and blight-resistant chestnut hybrids can establish on both abandoned and reclaimed mine sites (Herendeen 2007; McCarthy et al. 2008; Jacobs et al. 2009; Rhoades et al. 2009). The fast growth rate coupled with quality timber makes American chestnut a desired species for use in reforestation projects. In addition, chestnut is also a prolific nut producer and their yearly mast is an important protein source for a wide range of wildlife species (Steele et. al. 2005). An established chestnut stand may also provide habitat for other seed hoarding animals that may promote seedling recruitment from native tree species. Chestnut is reported to be a generalist, adapted to a wide range of ecological conditions, including tolerance to drought and low pH (reviewed in Jacobs 2007). Chestnut will survive long periods as an understory tree and act as a superior competitor for light following a canopy disturbance (Latham 1992; McEwan et al. 2006). Like other members of Fagaceae, *C. dentata* forms ectomycorrhizas (Rhoades et al. 2003; Dulmer 2006; Palmer et al. 2008). Because chestnut was eliminated as a canopy tree from the Eastern deciduous forests by the 1950s, very little is known about these microbial interactions.

Ohio mine land reclamation projects provide tremendous opportunities for examining plant and ECM fungal interactions using American and hybrid chestnut. This research will investigate the influence ECM fungi has on chestnut seedlings under management protocols. Further, this research will offer supplementary information identifying ECM fungi that may enhance the establishment of chestnut hybrid seedlings for future mine reclamation projects. This dissertation addresses the following research objectives: 1) evaluates the influence existing ECM vegetation has on germination and survival of chestnut in an abandoned mine, 2) identifies the soil surface treatments most beneficial to increasing ECM species richness and root colonization on a reclaimed mine, 3) determine the influence introduced inoculum has on seedling establishment and subsequent root colonization by native ECM fungi, 4) identity soil environmental variables that may predict ECM species composition, and 5) synthesize the impacts these various planting sites and methods have on maximizing the natural beneficial symbiosis of ECM on chestnut in mine reclamation in central Ohio.

Chapter 2 tests the influence existing ECM vegetation has on germination and survival of chestnut on an abandoned mine (Figure 3). The study site is representative of coal mines excavated prior to the enforcement of The Surface Mining Control and Reclamation Act of 1977 (SMCRA). These former mines make up 600,000 acres of land in Ohio and millions of acres in the United States (Cordell et al. 1999). Similar to the conditions described above, the soil left behind in these sites is severely disturbed and compacted, with extreme alkaline or acidic pH conditions, and low microbial activity (Torbert and Burger 1990). The abandoned mine site used for this study is located in the Avondale Wildlife Area in Muskingum County, Ohio. This site is comprised of three very distinct areas: 1) Small pockets of undisturbed forests (forest edge), 2) plots of 10-year-old *Pinus virginiana* (pine plots), and 3) center areas between the existing vegetation that is devoid of plant material (center). Models of facilitation suggest that the presence of established vegetation may create microclimates more conducive for the establishment of later-successional tree species (Kennedy and Sousa 2006; Sanchez-Gomez et al. 2006; Richard et al. 2009). Germination, survival, ECM colonization, and growth response of American chestnut were assessed in forest edge, pine, and center subplots. This study predicts: 1) that chestnuts adjacent to established vegetation will

have increased growth and survival, 2) chestnuts adjacent to vegetated plots will have a greater proportion of ECM roots, and 3) facilitation of establishment will be density dependent; growth and survival will be greater at lower densities (pine plots).

Initiating proper methodologies directing the successional process in the early stages of reclamation may promote a natural rate of forest stand recovery following large scale disturbances (Groninger et al. 2007). However, minelands reclaimed under The Surface Mining Control and Reclamation Act of 1977 (SMCRA) have not always resulted in forest succession (Figure 4). Heavy equipment and the use of exotic species as cover crops have resulted in severely compacted soils with non-native herbaceous canopies (Bussler et. al. 1984; Torbert and Burger 2000). Many of these habitats remain arrested at the early successional stage with herbaceous plant species non-native to the habitat. Native tree establishment is limited by the shading imposed by the excessive dense covers of the existing herbaceous canopy (Ashby 1997). The persistence of these non-native forbs greatly reduces the abundance of pioneer shrub and tree species that support ECM fungi required to facilitate the succession of later arriving woody natives (Ashby 1997; Amaranthus and Perry 1994).

The Appalachian Regional Reforestation Initiative (ARRI) utilizes surface soil treatment methods to improve the rooting medium for the establishment of a range of different hardwood species (Torbert et al. 1994; Groninger et al. 2007.) Mechanical soil treatments such as deep ripping and traditional plow and disking have been proposed to alleviate soil compaction and disturb the grass canopy, thereby improving the survival of woody trees in reclamation (Rokich et al. 2001). Chapter 3 evaluates the influence these soil surface treatments have on chestnut growth, survival, ECM root colonization, and ECM community composition. There is not much known regarding the native ECM community in these non-native grasslands or how mechanical surface treatments impact their composition or ability to colonize an ECM host plant. Mechanical treatments may disturb existing mycelium networks, but favor the establishment of early successional ECM species by providing a disturbance. Small scale disturbances by mechanical methods may mimic natural disturbances that mix soil horizons and alter pH and nutrient availability that may create additional habitats for ECM fungi (Bruns 1995). Our predictions were as follows: 1) soil surface disturbances will result in differing ECM

community compositions among treatment plots, 2) chestnut seedlings grown in the mechanically treated plots will have more ECM root colonization, and 3) ECM infection will result in a positive growth response in chestnut regardless of treatment.

A common practice in reforestation using hardwood trees on reclaimed mine land is employing ecotmycorrhizal (ECM) inoculum prior to outplanting (Castellano 1996). However, there is evidence in previous studies (Kennedy and Bruns 2005; Kennedy et al. 2009) that an introduced fungal species may be the dominant competitor and inhibit the root colonization of indigenous species. The fourth chapter of this dissertation evaluates the field performance of five ECM fungi inoculated on hybrid American chestnut. The ECM fungi used were *Hebeloma crustuliniforme, Laccaria bicolor, Scleroderma polyrhizum, Amanita rubescens,* and *Suillus luteus.* In this study I examined the persistence of introduced inoculum and the influence various inocula have on the ECM community composition. In addition, I determined whether the introduced inoculum had any effect on the survival and growth of chestnut hybrids planted in a reclaimed mine site. I hypothesized the following: 1) Priority effects of certain species will have a negative influence on native ECM species and 2) ECM infection will not result in a uniform host response; certain species of fungi will have a greater benefit on its host with regard to growth and nutrient uptake than others.

Fungal community assemblage integrates many abiotic and biotic factors including mineral nutrients, soil depth, O_2 and CO_2 concentrations, amount and quality of organic matter, temperature, moisture levels, and age of the forest stand (Bruns 1995; Smith et al. 2002; Blasius and Oberwinkler 1989). The community patterns of ECM fungi may be a response to the soil environmental conditions, but how these factors interact to influence ECM root colonization and community structure is not fully understood (Burke et al. 2009). Fungal communities appear spatially variable in the field and shift in composition as ecological conditions change through disturbances (Buscot et al. 2000). Differences in soil chemistry, especially as they relate to pH and essential nutrient concentrations, may favor selection of fungi most capable of tolerating environmental extremes (Agerer et al. 1998; Gehring et al. 1998; Erland and Taylor 2002). In chapter 5, the soil variables and ECM community data will be used to determine the influence the soil environment has on ECM community composition and

root colonization of American chestnut. Our hypotheses are: 1) variation in the abiotic characters of the soil environment will influence changes in ECM community composition and 2) differences in soil chemistry and structure will influence ECM root colonization.

In the conclusion (Chapter 6) of this study I integrate the findings from these studies to make recommendations to current planting protocols used to introduce blight-resistant hybrids chestnut in mine reclamation projects. Results of these studies will be incorporated in future planting protocols using chestnut, as well as other tree species in future mine restoration projects. In addition, these studies will give insight to biotic and abiotic variables that can be used to predict ECM composition and root colonization in ecosystems disturbed by surface mining. I conclude this chapter discussing how my results can be used to initiate future studies that further evaluate the interactions between ECM fungi and seedling establishment in mined lands.

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Figure 1. Ectomycorrhizal sheath (45x) of a *Scleroderma* species colonizing *Castanea dentata* sampled from Tri-Valley Wildlife Management Area in Muskingum County, Ohio, sampled in May, 2008.

Figure 2. Blight-resistant B3-F3 chestnut hybrid (*Castanea dentata x C. mollissima*).

Figure 3. An abandoned surface coal mine that was mined in the 1950s without proper reclamation. These barren spots lie between undisturbed forest edges and 10 year-old plantings of *Pinus virginiana.* This former mine site is in the Avondale Wildlife Area in Muskingum County, Ohio.

Figure 4. This surface mine was mined in the late 1970s and reclaimed under The Surface Mining Control and Reclamation Act of 1977. Instead of succeeding into forests, these lands have remained in a grassland state. This reclaimed mine site is located in the Tri-Valley Wildlife Management Area, Muskingum County, Ohio.

Chapter 2

Facilitation of American chestnut (*Castanea dentata*) seedling establishment by established *Pinus virginiana* in mine reclamation

This portion of the dissertation is currently under review as:

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Abstract

This study used American chestnut (*Castanea dentata*) and blight-resistant hybrids (*C.dentata x C. mollissima*) as a study system to test seedling establishment near existing vegetation in an abandoned surface coal mine. Germination, survival, ectomycorrhizal (ECM) colonization, and growth response of both chestnut taxa were assessed in three areas of a reclaimed mine: forest edge, center, and adjacent to previously planted 10-year-old *Pinus virginiana* (pine plots). Germination was higher in the pine plots (48%) when compared to center plots (26%) and forest edge (21%) ($P = 0.002$). After two field seasons, chestnuts in pine plots had significantly higher survival (38%) than the other plot types (center 9% and forest edge 5%) ($P = 0.01$). Chestnuts among the pine plots also had a greater seedling biomass ($P = 0.02$) contributed by a significantly larger root system ($P = 0.03$). Forest edge and pine plots were similar with regard to ECM colonization on roots and significantly higher than ECM sampled from seedlings in center plots ($P = 0.04$). ITS fungal sequences and morphotypes found on both chestnut and pine root tips matched *Scleroderma, Thelephora*, and *Pisolithus* suggesting these two plant species share ECM symbionts. These findings indicate that small monoculture plantings of conifers had a greater facultative effect on the germination and survival of deciduous hardwood seedlings than did the forest edge; presumably by alleviating negative density-dependent factors. Utilizing previous plantings as facilitators for seedling recruitment can be used as a management strategy for reclaiming severely debilitated mine sites.

Introduction

Ectomycorrhizal (ECM) fungi play a crucial role in aiding in the regeneration of plant communities after industrial disturbances like coal mining (Schramm 1966; Marx 1991; Walker 2004). Typical of mined lands are soil conditions with poor physical and chemical properties, low water-holding capability, low organic matter, extremes in temperature and pH, and high levels of toxic metals (Marx 1975; Marx 1980). Much work has shown that ECM symbiosis alleviates the impact of highly stressed soils on plant growth by increasing access to water and nutrients, mitigating the affects of metal toxicity, and providing protection on from root pathogens (Marx 1972; Cordell et al. 1999; van der Heijden et al. 2003; Nara 2005). In turn, these fungi receive carbon in the form of photosynthates from their plant host, implying a mutualistic relationship between plant and fungi. However, these fungi are not well adapted to endure severe soil disturbances caused by surface coal mining (Jasper 2007; Iordache et al. 2009). In turn, the severe decline of these microbes may contribute to the high mortality of tree species observed in past reforestation efforts (Marx 1991; Cordell et al. 1999; Nara 2005). Conceptual models of ecosystem development suggest that hardwood succession is dependent on the restoration of the microbial community composition and diversity (Bradshaw 1984).

Disturbed soils may be capable of supporting early-successional plant communities; however, these species are generally not considered desirable for a restoration project to be considered successful (Allen et al. 2002). To compensate for the microbial deficiently in these sites, restoration efforts using ectomycorrhizal inoculum is a common practice. However, reclamation of highly stressed soils requires integrated approaches to reduce costs and increase the chance of plant establishment and survival. Surveys characterizing ECM communities present in disturbed environments may aid in identifying native ECM species better suited as symbionts for the establishment of specific hardwood tree species in mine reclamation. Early studies, however, report the number of fungal species available to incoming plant species to be quite low in mine spoil (Danielson 1985). These anthropogenic disturbances cause a decline in available ECM propagules by removing host plants, increasing soil compaction, and contaminating

natural areas with heavy metals and coal spoil (reviewed in Iordache et al. 2009). Low ECM species richness dominated by "disturbance fungi" has been previously described following stand replacing fires, clear cutting associated with logging, and mining for coal and metals (Horton et al. 1998; Jones et al. 2003; Dulmer 2006, Jasper 2007). These disturbance fungi may contain species better able to adapt to environmental extremities and the conservation of these species may facilitate long-term survival of deciduous tree species historical to these lands. Identifying these naturally occurring species, as well as planting methods that increase root colonization of these fungi, may aid in the survival of late-successional trees species used in reclamation.

Pockets of exiting vegetation in these mine sites provide host plants for indigenous species of fungi. The existence of common mycorrhizal networks (CMN) associated with existing vegetation may facilitate establishment by incorporating arriving seedlings into an existing network of ECM hyphae (Perry et al. 1989; Dickie et al. 2004). This has been demonstrated in reforestation projects; shrub patches increased mycorrhizal infection and overall microbial mass (Allen and Friese 1992; Allen 1993; Bai et al. 2009). The success of tree species found along wood lot and forest edges may be due to the formation of mycorrhizas harbored by the existing forest trees (Jonsson 2001). Seedlings incorporated into these CMNs receive carbon transferred from mature trees that may increase plant establishment (Simard et al. 1997; Selosse et al. 2006). Although most studies of plant species interactions focus on competition among species for available resources, the importance of facilitation by non-related species is of great importance in stressed environments (Callaway 1995).

Models of facilitation suggest that the presence of established early-successional vegetation may create microclimates more conducive for the establishment of late-successional tree species (Kennedy and Sousa 2006; Sanchez-Gomez et al. 2006; Richard et al. 2009). In addition to harboring ECM fungi, neighboring vegetation may buffer soil temperatures (Raffaele and Veblen 1998), increase water and nutrient availability (Flores and Jurado 2003), and increase soil aeration. However, despite the positive effects existing vegetation may provide, establishment may become impaired by larger vegetation densities. These greater tree densities may change the facilitative interaction at low densities to a negative interaction via competition for resources at high densities

(Dickie et al. 2002; Bruno 2003). Facilitation of seedlings by canopy trees may be masked by competition at high densities interfering with seedling establishment (Canham et al. 2006). ECM seedlings rely on the presence of ECM trees for infection, a benefit which may be maximized at lower densities (Dickie et al 2002).

The objective of this study was to evaluate the influence two different vegetation types have on the establishment and ECM colonization of American chestnut (*Castanea dentata*) and blight-resistant hybrids (*C.dentata x C. mollissima*) in mine reclamation. This study evaluated germination, survival, ECM colonization, and growth response of seedlings to ECM colonization in three areas of a reclaimed mine: forest edge, center, and adjacent to 10-year-old *Pinus virginiana* (pine plots). This study hypothesizes facilitation by existing vegetation to be density dependent. This study predicts that chestnuts adjacent to established vegetation will have a greater proportion of ECM roots, however, growth and survival will be increased at lower densities (pine plots). Growth and survival was recorded for two growing seasons. Chestnuts were sampled at the end of both the first and second growing season to determine extent of ECM colonization per treatment. Morphotyping and sequencing of fungal ITS region was used to characterize ECM species found forming ECM with chestnut.

Methods

Study Site and Experimental Design

An abandoned mine located in Avondale Wildlife Area in Muskingum County, Ohio, (39° 49' 44" N, 82° 7' 38" W), USA was selected for this study.
This site is representative of conditions prior to The Surface Mining Control and Reclamation Act of 1977 (SMCRA), when lands were typically strip mined for coal and then abandoned. This site was mined in the 1950s and has had very little reclamation, aside from experimental tree plantations using *Fraxinus* spp., *Robinia pseudoacacia,* and *Pinus virginiana.* Of these plant species, *P. virginiana* survived, creating small monoculture pine stands. Soil characteristics are typical of abandoned gob piles (Steiger 1996). The site is characterized by less than 5% vegetative cover, very little topsoil or organic matter, with poorly sorted debris in center areas. This area receives an average of

approximately 99 cm of precipitation annually with temperatures averaging 22° C during the growing season (17°, 28°, and 11° C, spring, summer and fall, respectively; National Climatic Data Center).

Three 0.80 ha blocks were established in the Avondale Wildlife area. In each block, three distinct areas were designated as plot types: forest edge, center, and established plantings of 10-year-old *Pinus virginiana*. Pockets of 55 year-old forest area comprised mainly of *Acer, Pinus, Fagus, Quercus,* and *Ulmus* tree species formed an existing forest edge. Four meters from the edge of the forest canopy, forest edge plots were established, six per block spaced 10 m apart from each other. Fifty meters from the forest edge, *Pinus virginiana* plantings were previously established. These pines were planted as bare root seedlings inoculated with ECM species *Pisolithus tinctorius* (Pt) in the spring of 1997. Subplots were established amongst the 10-year-old pines and formed the basis of the pine plots in this study. The areas designated as center plots were completely devoid of vegetation located in the center of the field site, approximately 25 meters from the forest edge and pine plots (Figure 1).

In each plot type, six subplots (4 m x 3 m) were established each containing 20 chestnut seeds (Figure 1). A total of three blocks were established, each comprised of 18 subplots for a total of 54 subplots containing a total of 1080 seeds. The seeds sown consisted of three genotypes: American chestnut, American Chestnut Foundation Chestnut Hybrids B1-F3, and B2-F3 (in a 2:2:1 ratio, respectively). Seeds were planted in March of 2006 and spaced 0.50 meters apart. To prevent disturbance from seed predators and deer, each seed was caged using aluminum gutter screening and each subplot was fenced with a two meter high fence constructed from metal t-posts and plastic snow fencing. Soil samples were collected at time of planting using a soil probe at an 18 cm depth, four samples per subplot. The four samples per treatment were mixed thoroughly, allowed to air dry, and 0.50 liters were sent to Spectrum Analytic Inc., Washington Court House, Ohio for analysis. Soil parameters included: pH, cation exchange capacity (CEC), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), sulfur (S), boron (B), zinc (Zn), iron (Fe), copper (Cu), and manganese (Mn) in parts per million (ppm). Summer temperatures were collected on July 8th, 2006 between 12:00 and 2:00 pm by soil thermometer probed 6 cm into the soil. Two readings were

recorded and averaged per subplot. Growth parameters including height (cm), basal diameter (mm), leaf area (cm^2), and dry weight of seedlings destructively sampled were recorded at the end of the first and second growing season.

ECM sampling, Fungal DNA Extraction, PCR Amplification, and Sequencing

After six months (October 2006) 40 pure American seedlings representing all treatments were randomly selected for destructive sampling. Seedlings were carefully removed from the field, returned to the lab where root systems were washed and observed under the stereoscope for mycorrhizal formation. One hundred root tips per seedling were randomly selected from each of the 40 chestnut seedlings. A total of 4,000 root tips were viewed under a dissecting microscope for the presence of a fungal sheath. Two samples per morphotype per seedling were selected for DNA extraction. A three mm segment of root tip was removed and transferred into a microcentifuge tube for storing at -70˚ C.

After 18 months in the field, another 90 seedlings were randomly selected and sampled in the fall (October 2008) and returned to the lab. Root tips (100 per sample, 9,000 total) were inspected and sampled as stated above. All 130 seedlings sampled were used to describe the ECM community. The 90 seedlings selected for sampling after the second season was used to compare biomass (g) between the seedlings found naturally colonized by ECM fungi to seedlings found non-ECM. Natural ECM colonization was confirmed by PCR amplification and sequencing. In addition to chestnut seedlings, 25 root samples were collected from *P. virginiana* in subplots. A 25 cm deep, 45 cm width wide trench was excavated at the root zone of the existing pine trees to expose roots for sampling. Roots were then stored on ice in the field and returned to the laboratory.

At the time of the 18 month harvest, 30 chestnut seedlings were randomly selected and harvested from the pine plots were selected for foliar nutrient analysis. Of these, 15 seedlings had no visibly detectable fungal sheath and the other 15 had *Scleroderma* morphotype (later confirmed with DNA extraction a sequencing of the fungal ITS region). Twenty-five leaves per seedling were harvested in the lab, packaged in paper bags, and sent to Spectrum Analytic Inc., Washington Court House, Ohio, for

tissue analysis. Leaf tissue parameters included N, P, K, Ca(%), B, Cu, Fe, Mn, and Zn (ppm).

To molecularly identify the type of mycorrhizal fungi, fungal DNA was extracted from the collected root tips using QIAgen Dneasy Plant Mini-Prep kit purchased through QIAGEN Inc. Primers ITS1-F (5' cttggtcatttaggaagtaa 3') and ITS4 (5' tcctccgcttattgatatgc 3') were used to amplify internal transcribed spacer sequences (ITS) during polymerase chain reaction *(*PCR*)* (Gardes and Bruns 1993). PCR amplifications were performed in 15 µl reactions consisting of: 9 µl of molecular grade water, 3 µl of 5x Green GoTaq® Reaction Buffer, 0.125 µl of Promega® *Taq* DNA Polermerase, .2 µl of 25µM of each primer, 1µl of dNTPS (200µM each of dATP, dCTP, dGTP, and dTTp) and 1 µl of DNA template. PCRs were performed using thermal cycling heating using a programmable thermal cycler heating block. Times and temperatures were programmed as described by Gardes and Bruns (1993).

The presence of fungal DNA was confirmed via gel electrophoresis and PCR product was cleaned prior to sequencing using Wizard® SV 96 Genomic DNA Purification System by Promega. Samples were prepared for sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit by mixing 10 µl reactions of the following concentrations: 2 µl BigDye Terminator v3.1 Reaction Mix, 3 µl 5 X Sequencing dilution buffer, 1 µl primer (25µM concentration), and 1 µl of template. DNA was labeled for sequencing using a programmable Thermal Cycler for the following cycles: 96ºC for 1 min followed by 25 cycles of 10 s at 96ºC, 5 s at 50 ºC, and 4 min at 60 ºC. Following labeling, products were purified to remove all unincorporated dye-labeled terminators by alcohol precipitation. Sequencing was performed using The Applied Biosystem ABI Prism 3730 DNA Analyzer (Bioinformatics facility, Miami University, Oxford, Ohio).

Sequences were analyzed and edited using Sequencher 4.2 software (Gene Codes, Ann Arbor, Michigan). To identify fungi found on roots, sequences were compared with known species in GenBank using The Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). This program finds regions of local similarity between cataloged sequences of fungal ITS regions and calculates best matches (Altschul et al. 1997). Fungi reported here are named based both on the most similar morphological characteristics coupled with most similar sequences that are available in

GenBank. Sequences are based on statistical analysis that generates both a bit value and an Expect (E) value. The bit score is a value that is indicative of how well the sequenced aligned with the known sequence in the database. The higher the score, the better the match. The E value is a parameter that describes the probability of the number of matches that can be generated by chance. It decreases exponentially as the match increases; a score closest to zero is the most significant. The gap score introduced into an alignment compensates for insertions and deletions in one sequence relative to another. Thus, when deciding the genera to report here, a threshold was decided on that included an E-value of 0, highest ranking bit value, and a gap value of < 4.

Statistical Analysis

Germination and survival among plot types were assessed using an analysis of variance (ANOVA) followed by Tukey's HSD. ECM root colonization was analyzed using a non-parametric Kruskal-Wallis utilizing X^2 test statistic to determine differences among plot types. For growth and soil analysis the 4m x 3m subplots were selected as a sample unit because samples from such a small area are likely strongly autocorrelated and not independent (Taylor 2002). Differences in soil chemistry were detected using a mixed model multivariate analysis of variance (MANOVA) with block as a random effect followed by Tukey's HSD post hoc. To determine differences in seedling biomass (root, shoot, and total dry weight) between chestnuts naturally colonized by native ECM (+ECM) to non-ECM seedlings (-ECM) per plot type, a two-way ANOVA on a 2 x 3 (with or without ECM x three plot types) factorial design was used. Both ECM status on seedlings (+ECM or – ECM) and plot type (forest edge, center, pine plots) were main effects with block as a random effect. Log(n + 1) transformation was used to control for unequal variances in the tissue analysis. Square root (power = ½) transformations were used to control for unequal variances for soil parameters and seedling biomass. Differences were considered significant when $p \leq 0.05$ according to the F test.

At the end of the second growing season, a secondary subset of chestnut seedlings sampled from the pine plots (n=30) were selected for foliar analysis. Fifteen seedlings sampled without native ECM were compared to 15 seedlings sampled naturally inoculated with *Scleroderma*. Subplots were selected as a sample unit to maintain

independence. To determine differences in leaf tissue analysis between *Scleroderma* seedlings and non-ECM seedlings, an independent-samples t-test was used. Log(n + 1) transformations were used to control for unequal variance. All statistics were performed using JMP (8.0, SAS Institute, Cary NC, USA).

Results

Soil properties among treatments

Analyses of the soil samples collected at the beginning of this study indicated that CEC (averaged 31.31-33.61) and pH (2.8 to 3.1) were similar among the treatments (Table 1). Summer soil temperatures were significantly higher in the center plots (38.0º C) than either the forest edge (33.2 º C) or in the pine plots (35.7º C) when recorded in July of the first growing season ($F = 8.44$, df = 3, $P = 0.0007$). Treatment plots were also statistically different when organic matter (OM; $F = 24.80$, df = 3, $P < 0.0001$) was compared: pine plots (1.33%) were lowest when compared to center (2.88%) and plots along the forest edge (3.44%).

Due to the mobility and fluctuation of N, this macronutrient was not tested for in the soil analysis. One-way MANOVA of macro and micronutrients revealed a significant multivariarate effect. Subsequent univariate ANOVA followed by Tukey's HSD of each soil nutrient was used to determine significance among the nutrients per plot type (Table 2). Among the macronutrients detected in the soil analysis, soil K ($F=14.73$, df = 2, $P <0.001$), Ca ($F = 5.92$, df = 2, $P = 0.005$), Mg ($F = 7.91$, df = 2, $P = 0.001$), and S ($F = 5.41$, df = 2, $P = 0.007$) differed significantly among plot types (Table 2). Pine plots had the highest concentrations of K. Pine and forest edge plots contained higher soil Ca then center plots. Mg and Zn were higher in both the pine and center plots. Differences were also recorded in soil concentrations of S and B, both were higher in the center and forest edge plots. Conversely, concentrations of soil P did not differ among the three plot types.

When the micronutrients were compared, differences were detected among the plot types: concentrations of soil B ($F = 6.35$, df = 2, $P = 0.003$), Zn ($F = 10.72$, df = 2, P

< 0.0001), Fe (F = 12.33, df = 2, P < 0.0001), and Cu (F = 7.24, df = 2, P = 0.002; Table 2). Levels of B were significantly higher in the center and forest edge plots. Soil concentrations of Fe were higher along the forest edge. Levels of Zn and Cu were highest in the pine and center plots. Levels of soil Mn did not differ between treatments.

Seedling Survival and Growth

Germination was dramatically higher in pine plots (61%) when compared to center plots (32%) and forest edge (21%) three months after seeds were sown (Pearson X^2 = 60.7, df = 2, P < 0001; Figure 2A). Greater seedling survival was also recorded in pine plots after the first growing season: pine plots (46%), center plots (17%), and forest edge (12%) (Cox proportional hazard model, Likelihood = 104, df = 2, P < 0.0001; Figure 2B). After two growing seasons plot type effect was still apparent, pine plots had the highest survivorship (38%) when compared to center plots (9%) and plots along the forest edge (5%) (Cox proportional hazard model, Likelihood = 297, df = 2, P < 0.0001; Figure 2C).

With regard to ECM root colonization, seedlings growing along the forest edge (58%) and amongst pine plots (38%) were similar. Both were significantly greater than what was sampled from chestnut seedlings in the center plots (14%; X^2=5.95, df 2, p = 0.05; Figure 3).

After two growing seasons, there were no significant interactions between plot type and native ECM colonization with regard to total seedling biomass (F = 0.15, df = 2, P = 0.85). There were no differences among the plot types (ANOVA, F = 1.11, df= 2, P = 0.34). Differences in total seedling biomass did exist between seedlings naturally colonized with ECM fungi (+ECM) and their non-ECM (- ECM) counterparts (ANOVA, F = 5.74, df = 1, P = 0.02) in pine and forest edge plots (Figure 4). In the pine plots, +ECM seedlings (6.9 g) were greater than - ECM counterparts (4.2); this was also seen in the plots along the forest edge, + ECM plants (7 g) were larger than the -ECM plants (3.8 g). Seedlings in the center plots had less biomass than the other plot types and the ECM seedling biomass (4.1 g) and were similar to their non-ECM counterparts (3.5 g) (Figure 4). No significant differences existed between interactions or main effects when shoot biomass was compared (all P > 0.05). Similar to total biomass, differences were significant when root biomass was compared between the + ECM and – ECM seedlings

(F = 5.28, df 1, P = 0.03; Figure 4). +ECM seedlings in the pine plots averaged 4.02 g root dry weight compared to 2.55 g recorded from –ECM. Conversely, seedlings growing along the forest edge did not differ statistically when +ECM was compared to -ECM ; 3.65 g to 2.70, respectively. This was also recorded for center plots, + ECM seedlings (2.69 g) did not differ from their – ECM counterparts (1.68 g; Figure 4).

ECM Survey

One hundred and thirty-one seedlings representing all treatments were sampled and ECM root tips were identified to 12 different genera using ITS sequences and BLAST queries (Table 3). When comparing species richness among the treatments, both the pine and forest edge plots contained eight species each. Only three ECM species were sampled from the center plots. Collectively, *Scleroderma* spp. was the most abundant in this study (51%). This was followed by *Thelephora* spp. (13%), *Pisolithus* (8%), *Oidiodendron* (6%), *Cenococcum* (4%), and *Laccaria* (4%). Genera considered rare in this survey include *Russula,* unknown ECM, Thelephoraceae, *Tomentella* , *Lactarius,* and *Suillus* (Table 3).

Root samples from *P. virginiana* from pine plots were also morphotyped and sequenced. Five matching morphotypes were shared between the chestnut and pine hosts (Figure 5, Table 3); three were later identified by sequencing ITS region to be *Scleroderma*, *Thelephora*, and *Pisolithus*. An additional two were identified by their sequences to match ECM species on chestnut, unidentified Thelephoraceae, and unidentified ECM spp. 1 (photo not available).

Leaf Chemistry:

A subsample of chestnut seedlings from pine plots naturally colonized with *Scleroderma* ECM (+) were compared to non-ECM (-) seedlings with regard to foliar micro and macronutrients and analyzed using an independent –samples *t* test. Although foliar concentrations of macronutrients (N, P, K, and Mg) were slightly elevated in *Scleroderma* inoculated chestnut seedlings (+), no significant differences existed (all P > 0.05; Table 4). There was one exception: chestnuts colonized by *Scleroderma* (+) had significantly less percent calcium when compared to their non-inoculated counterparts (-)

(t = 1.97, df = 20, p = 0.04). Comparison of micronutrients revealed one significant difference between the two groups (Table 4); *Scleroderma* (+) seedlings had significantly lower foliar concentrations of copper (t = 1.98, df = 20, p = 0.03). When Mn was compared, this result was marginally significant; *Scleroderma* (+) were lower in foliar Mn than *Scleroderma* (-) seedlings (t = 1.32, df = 20, p = 0.10).

Discussion

Our results show that chestnut seedlings growing among the pines had higher survival than those in center plots or along the forest edge. Further, chestnut seedlings in pine plots naturally colonized with ECM fungi had greater biomass production. This can be attributed to several factors investigated in this study. Comparison of chemical properties of mine spoil of this study site was typical of mined soils in the eastern United States (Walker et al. 2004). Nutrients essential for plant growth (P, K, and B) were in very low concentrations. This was coupled with toxic levels of S, Zn, Fe, Cu, and Mn. When compared among the plot types, differences existed: pine plots had significantly greater concentrations of K when the soil chemistry was analyzed in the beginning of this study. Neighboring tree species modify the physical and biotic conditions in the surrounding soil, which may facilitate greater seedling establishment. Pine vegetation could influence changes in rhizosphere chemistry (Bai et al. 2009) and increase soil nutrients from litter accumulation (Flores and Jurado 2003). Although not measured in this study, moisture levels are likely to remain higher in the soils under established plants by the reduction of solar energy reducing evaporation and the increase in water availability by hydraulic lift (Richards and Caldwell 1987). These mechanisms contribute to lower soil temperatures (Valiente-Banuet and Ezcurra 1991; Rey and Alcantara 2000), as we measured in this study. In previous reclamation projects, pines have been reported to improve permeability via decreasing soil bulk densities influencing the establishment of later-successional plant species (Ashby 1989).

This study reports significantly higher numbers of ECM root tips and greater species richness in plots along the forest edge and pines plots. Higher ECM root

colonization and species richness have been linked to existing vegetation (Allen and Friese 1992; Dickie et al 2002; Dickie and Reich 2005). Lower species richness and root colonization is a common finding in gaps due to the lack of root contact from other trees (Kranabetter and Friesen 2002), as recorded in the center plots of this study. Kranabetter and Friesen (2002) reported root colonization to decrease in gaps despite initial colonization with ECM fungi suggesting other site factors that may limit ECM growth. During our study significant differences in soil temperature and chemistry may have reduced ECM colonization. Interestingly, this reduced colonization may actually be beneficial to the host plant. It has been suggested that in extremely harsh environments, reduced root colonization by ECM fungi may reduce the carbon cost on the host plant when water stress limits photosynthetic efficiency (Swaty et al. 2004). Regardless of the mechanism, this study documented limited root colonization on seedlings in center plots as well as a neutral response to ECM colonization.

ECM colonization was similar between seedlings growing with the pine plots to those growing along the forest edge. However, survival in the forest edge plots was drastically lower than what was seen in the pine plots. The fact that the forest edge supplies chestnut with an ECM symbiont does not remove the competition imposed by the canopy trees. Taller chestnut seedlings recorded along the forest edge may be indicative of competition for light. This canopy shading effect may have greatly contributed to the diminished germination and seedling survival along the forest edge. Previous studies have shown higher germination and survival rates for American chestnut in areas of high light. Canopy gaps and thinned areas seemed more conducive for chestnut establishment (McCament and McCarthy 2005). These species interactions may be spatially dependent; competition for resources may require seedling establishment to be at a distance from existing larger areas of vegetation (Dickie et al. 2007; Teste and Simard 2008). This study illustrates that as tree densities increase, there is little additional gain from an ECM symbiont (Dickie et al. 2002).

Without the imposed competition from an existing forest canopy, chestnuts growing in the pine plots had higher germination and survival. Important to the survival of ECM seedlings in harsh environments (Callaway 1995), pine plots provided chestnuts with an ECM propagule source. ITS sequence analysis identified five symbionts shared

by both chestnut and established *P. virginiana*: *Scleroderma*, *Thelephora*, *Pisolithus*, unidentified Thelephoraceae, and unidentified ECM spp. 1. This is important to document because past restoration efforts in mine spoil reported functional ectomycorrhizas hindered by lack of available inoculum (Marx 1991). The availability of ECM inoculum from a distantly related plant species demonstrates positive interactions between plants facilitating the establishment of a later successional group (Horton et al. 1999). It is likely that root colonization by these species may have been accomplished by spores existing in the soil or by hyphae or rhizomorphs radiating from the established pines (Jefferies 1999). *Scleroderma* and *Pisolithus* produce rhizomorphs that are capable of long-distance exploration; they spread through the soil several decimeters, resulting in growth increases in their plant hosts (Agerer 2001; Burgess et al. 1993). Although this study did not test common mycorrhizal networks (CMN), previous work has reported net carbon gains for an establishing seedling linked in an existing CMN (Simard 1997; Nara 2005; Bai et al. 2009). In addition, both species are prolific spore producers capable of forming mycorrhizas from spore inoculum (reviewed in Jefferies 1999).

The growth data in our study illustrated a significant increase in root biomass contributing to total seedling biomass (g) in + ECM seedlings adjacent to the pine plots. No differences in above ground growth were noted in this study suggesting ECM seedlings allocated carbon to belowground growth. This type of allocation of resources is essential for plant establishment on mine soils where water stress is high and nutrient availability is low (Lavender 1984; Walker et al. 2004). Stress from lack of water is a common cause of the high mortality observed in mine reclamation projects. Heavy equipment used in industrial operations destroys the air filled pore space, reducing water capture and infiltration (Craul 1992; Watson and Kelsey 2006). Rhizomorph forming ECM species like *Scleroderma* and *Pisolithus* greatly improve seedling-water relations, allowing for greater access to water and generally results in increased photosynthesis rates and net carbon gains (Wu and Nioh 1997).

Because *Scleroderma* was the most abundant ECM species sampled in this study, leaf samples from pine plots were analyzed to determine the influence this species has on nutrient and metal uptake. There was not a significant increase in foliar macronutrients

in the leaf tissue. This may have been an artifact of the overall low nutrient levels seen in the soil analysis or additional tissue analysis of root and stem may have been required to detect a difference in nutrient concentration. Interestingly, the only difference in nutrient uptake was found with regard to Ca; these levels were significantly lower in ECM plants. Although this nutrient was in higher concentrations in the soil around the pines, calcium uptake may have been impeded by the drastically low pH measured in these plots (average of 3.1 in pine plots). At these low levels H+ ions displace Ca+ impeding uptake by the plant (Fitter and Hay 2002). Although previous studies that have demonstrated that ECM colonization remedies the effect low pH levels has on the plant uptake of Ca+ (Kinraide et al. 2004; van Scholl et al. 2005), this was not seen in this study.

Importantly, our results showed significant decreases in only one micronutrient known to be in toxic concentration in leaf tissue in mine spoils on seedlings colonized by *Scleroderma*. Though copper is a micronutrient is essential for plant growth, elevated levels damage photosynthetic apparatus (particularly photosystem I) compromising photosynthetic efficiency (Balsberg Påhlsson 1989; Van Tichelen et al 1999). In addition to copper levels, marginally lower significant levels of Mn were observed in *Scleroderma* colonized seedlings. Toxic levels of this micronutrient also decrease photosynthetic efficiency of plant by causing the oxidation of phenols leading to necrotic tissue on leaf surfaces (Marschner 1995). The presence of *Scleroderma* has been cited as an ECM species that greatly contributes to seedling establishment in mine soils by amelioration of metals (Jefferies 1999). However, previous studies have reported contrasting results with regard to *Scleroderma*'s tolerance of copper (Tam 1995; Howe et al. 1997). Our study documented this species abundance in soils high in copper. This may have contributed to the reduction in foliar Cu in chestnuts naturally colonized by *Scleroderma*. The mechanism that these fungi employ for metal tolerance is not known. Some species can sequester substantial amounts of metal in the hyphae (Massaccesi et al. 2002). Alternatively, other species bind metals to soil particles by the production of fungal polysaccharides (Gadd 2007). Regardless, this propensity to store or bind metals prevents uptake by its plant host enabling plant establishment in sites high in mine spoils.

A total of 12 species of ECM fungi were collected after the first and second growing season: *Scleroderma, Thelephora, Laccaria, Pisolithus, Cenococcum, Oidiodendron, Russula, Tomentella, Lactarius,* and *Suillus.* Collectively, these species contributed to the higher seedling biomass production. The more commonly sampled genera in this study, include basiodiomycetes *Scleroderma*, *Thelephora* and *Pisolithus,* and asomycetes *Oidiodendron*, and *Cenococcum.* Each of these species shares attributes of early stage, stress tolerating (S-selected) mycorrhizal fungi. These adaptations included sclerotia or persistent spores, saprophytic capabilities allowing them to persist without a host plant, ability to utilize difficult forms of N and P, a broad host range, and the ability to tolerate toxic metals (Jones et al. 2003; Dickie and Reich 2005; Gadd 2007; Smith and Read 2008).

Several studies surveying arid soils show the ECM community is dominated by ascomycete fungi thought to tolerate stressful abiotic conditions (Haskins and Gehring 2004; Hubert and Gehring 2008). *Cenococcum* is globally ubiquitous; particularly at farther distances from existing vegetation where environments are stressful, but competition with other ECM fungi is low (Dickie and Reich 2005). It remains unclear under what environmental conditions this species is beneficial to its host (Smith and Read 2008). *Oidiodendron* spp. was once considered specific to plant species in the Ericaceae taxon (Peterson et al. 2004; Cairney and Meharg 2003) Recent findings suggest these ascomycete fungi also form dark septate endophyte infection with other plant taxa (Chambers et. al 2008). The role of these dark septate fungal species is also unknown, although they have been reported on root tips after major disturbance (Horton et al. 1998). Important to restoration, these fungi are found in nutrient poor soils with the propensity to obtain limited N and P and may aid in host plant nutrient uptake (Leake and Read 1989; Peterson et al. 2004). In addition, the mucilage produced by these fungi have been found to bind zinc to soil particles, which may decrease toxic metals in plant tissue (Bradley 1982; Denny and Ridge 1992).

Biological interactions between distantly related plants are of particular ecological interest with regard to restoring disturbed ecosystems. In nature, community dynamics influence the natural successional pathways by pioneer vegetation facilitating the recruitment of later successional tree species (Dickie et al. 2006). To aid in the natural

successional pathway, previously successful restoration plantings may facilitate the establishment of distantly related, later successional species. These earlier plantings result in vegetation that influences soil chemistry, nutrient availability, organic matter, and temperature. In turn, these alterations in soil characteristics influence the fungal species composition and root colonization (Marx 1990; Aggangan et al. 1996; Bakker et al. 2000; Dickie et al 2006). Looking at these ECM groups and their characteristics is suggested to be an important indicator of microbial functioning throughout a reclamation project (Allen et al. 2002). Theoretically, as the plant community succeeds into early forests comprised of a more diverse, mid- to late-stage plant types, the ECM fungal community will shift from disturbance fungi to a more species rich assemblage comprised of ECM species that are better competitors. Therefore, we can measure soil reclamation by the increase in abundance of these later-stage fungi. These species may be more represented of these later-stage species sampled exclusively along the forest edge (*Russula, Laccaria, Lactarius*). Establishing a hardwood like chestnut may provides a host plant to many fungal species. This, in turn, increases the inoculum source for incoming trees and may facilitate seedling recruitment and the restoration of these severely disturbed lands.

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Table 1. Comparisons among plot types of the following soil characteristics: cation exchange capacity (CEC), percent organic matter (OM), pH, and summer temperature (C). Values are expressed as means ± 1 SE. Means sharing common letters do not significantly differ at α = 0.05 to Tukey's HSD.

Treatment	Summer Temp (C)	CEC	OM (%)	pH
Center	38.08±1.02[a]	33.61±0.52 [a]	2.88±0.42 [a,b]	2.82±0.07 [a]
Forest Edge	33.18±1.59 [b]	31.31±0.98 [a]	3.44±0.42 [a]	2.90±0.19 [a]
Pt Pines	35.69±0.86 [b]	31.86±0.76 [a]	1.33±0.21 [b]	3.10±0.06 [a]

Analyses based on data transformed by square root.

Table 2. Comparison among treatments with regard to soil concentrations of macro and micronutrients. Values are expressed as means ± 1 SE. Means sharing common letters do not significantly differ at α = 0.05 to Tukey's HSD.

Treatment	P ppm	K ppm	Mg ppm	Ca ppm	S ppm	B ppm	Zn ppm	Fe ppm	Cu ppm	Mn ppm
Center	1.33±0.24 [a]	71.28±5.67 [b]	334.44±31.87 [a]	401.61±73.52 [b]	1211.00±97.41 [a]	0.61±.023 [a]	12.22±2.57 [a]	476.67±70.94 [a,b]	6.96±0.78 [a]	8.44±0.90 [a]
Forest Edge	1.00±0.1 [a]	67.44±6.28 [b]	174.39±19.85 [b]	676.47±505.05 [a]	918.81±130.77 [a]	0.52±0.024 [a]	6.06±1.24 [b]	704.17±140.74 [a]	5.18±0.63 [b]	7.53±1.92 [a]
Pt Pines	2.06±0.72 [a]	103.28±9.37 [a]	254.94±22.43 [a]	742.50±141.47 [a]	772.11±133.46 [b]	0.49±0.027 [b]	10.00±2.01 [a]	303.22±66.51 [b]	7.53±0.76 [a]	8.83±1.61 [a]

Analyses based on data transformed by square root.

Table 3. ECM species sampled from pure American and chestnut hybrids after 2 field seasons. Species are recorded with their relative abundance, treatment plot they were sampled (C = Center, FE = Forest Edge, and P = Pine Plot), and published GenBank accessions.

ECM Genera	Relative Abundance	Plot Sampled	Sampled on *P. virginiana*	Accession
Scleroderma spp.	**0.52**	**C, FE, P**	**yes**	**GU553366**
***Thelephora* spp.**	**0.13**	**C, P**	**yes**	**GU553377**
Pisolithus tinctorius	**0.08**	**P**	**yes**	**GU553367**
***Oidiodendron* spp.**	**0.06**	**P**	**no**	**GU553368**
***Cenococcum* spp.**	**0.04**	**C, FE, P**	**no**	**GU553373**
***Laccaria* spp.**	**0.04**	**FE**	**no**	**GU553370**
***Russula* spp.**	**0.03**	**FE**	**no**	**GU553374**
Unknown ECM	**0.03**	**FE, P**	**yes**	**GU553372**
Thelephoraceae	**0.03**	**FE, P**	**yes**	**GU553376**
***Tomentella* spp.**	**0.02**	**FE, P**	**no**	**GU553375**
***Lactarius* spp.**	**0.01**	**FE**	**no**	**GU553369**
***Suillus* spp.**	**0.01**	**P**	**no**	**GU553371**

Table 4. Nutrient and metal concentration (± 1 SE) from a subsample of seedling leaf tissue between chestnuts colonized by *Scleroderma* (+) to those found without (-) sampled 18 months after planting (n=26). Asterisk (*) indicates significant differences. ECM plants had significantly lower Ca (%) and Cu (ppm) ($P < 0.05$).

	% N	% P	% K	% Ca	% Mg
Scleroderma					
-	2.06 ± 0.15	0.11 ± 0.01	0.80 ± 0.03	* 0.60 ± 0.05	0.41 ± 0.03
+	2.27 ± 0.09	0.12 ± 0.01	0.81 ± 0.03	* 0.49 ± 0.03	0.44 ± 0.03
	ppm B	**ppm Cu**	**ppm Fe**	**ppm Mn**	**ppm Zn**
Scleroderma					
-	133.87 ± 17.39	* 11.96 ± 1.60	2131.11 ± 334.17	1218.33 ± 272.61	77.56 ± 11.21
+	126.98 ± 8.52	* 9.37 ± 0.61	2090.46 ± 344.78	826.77 ± 159.18	73.46 ± 5.80

Analyses based on data transformed by Log(n + 1).

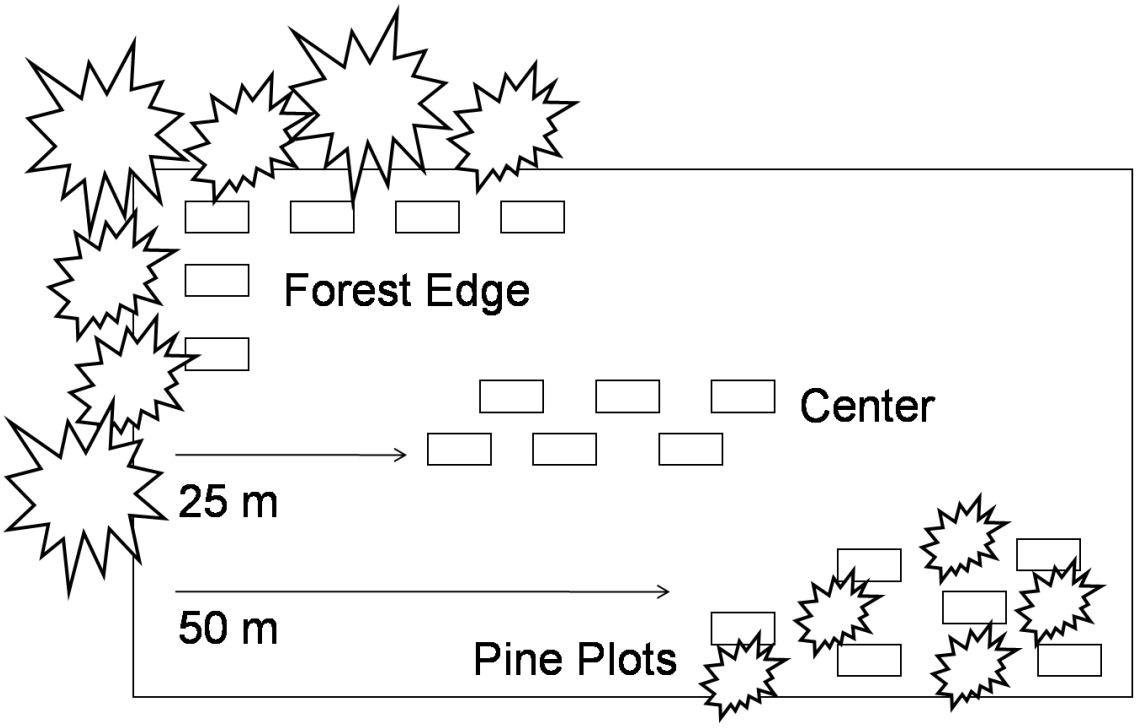

Figure 1. Schematic Diagram of field plot layout. Six subplots were established per treatment. This design was replicated three times (54 subplots total).

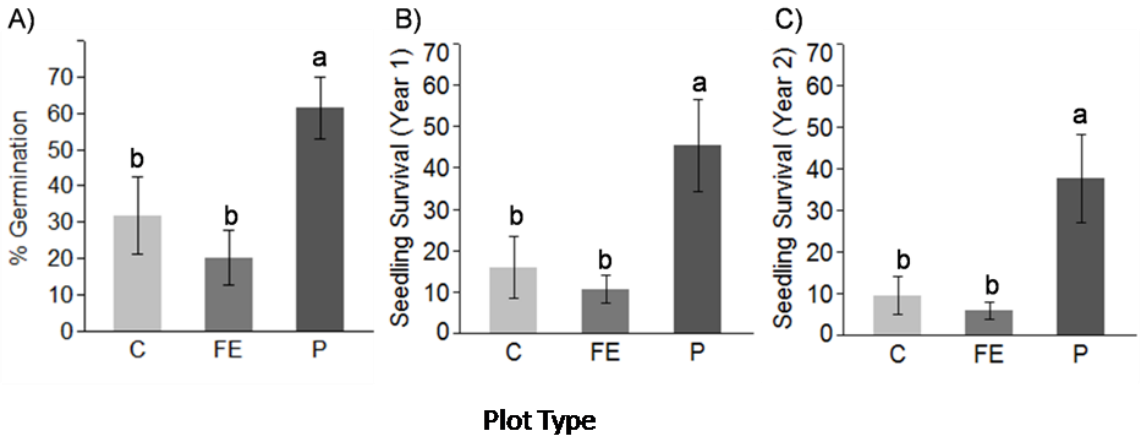

Figure 2. Germination and survival of the three treatment plots: center (C), forest edge (FE), and pine plots (P). Pine plots had a significantly higher germination and survival rate after two growing seasons (all $p < 0.001$). Bars sharing common letters do not significantly differ at $\alpha = 0.05$ determined by Tukey's HSD.

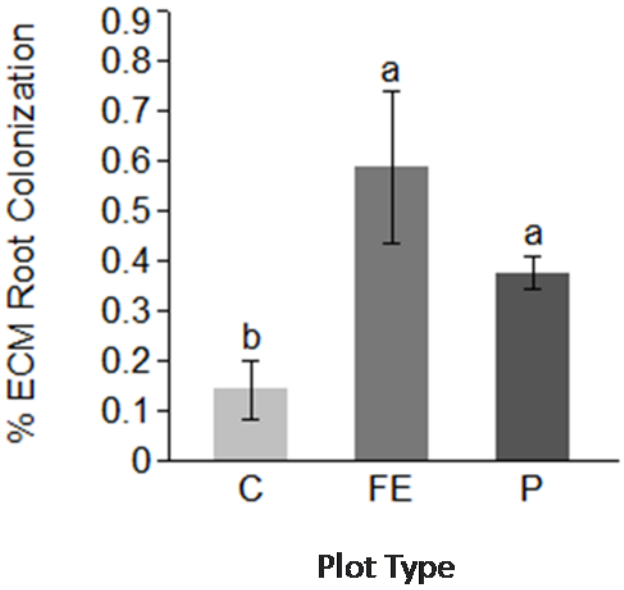

Figure 3. Percent root tips infected (± 1 SE) on American chestnut seedlings after two field seasons. Bars sharing common letters do not significantly differ at α = 0.05 determined by Tukey's HSD. Seedlings in the center plots (C) had less ECM on roots when compared to forest edge (FE) and pine plots (P).

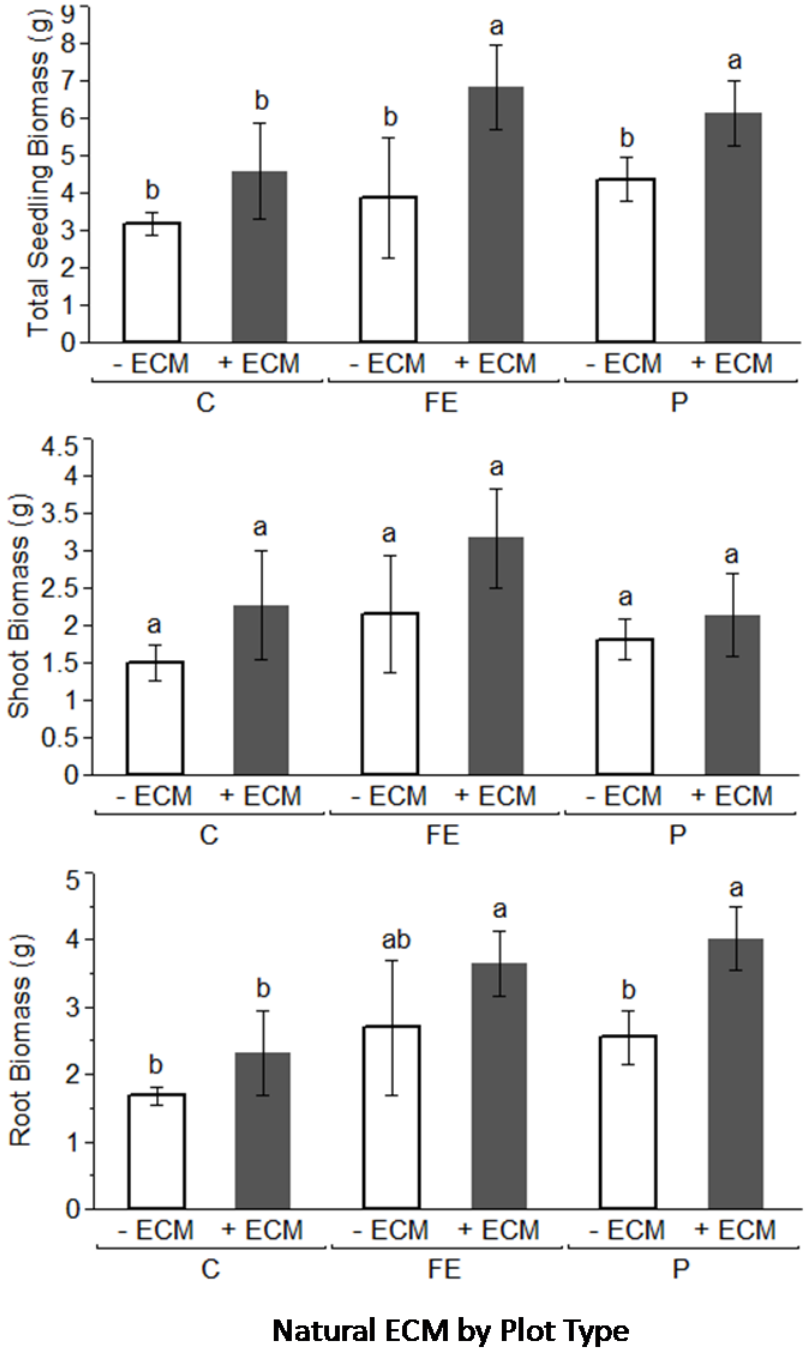

Figure 4. Comparison of chestnut seedling biomass (total, shoot, and root dry weight in grams, ± 1 SE) among treatments with (+ECM) and without (-ECM) native ECM fungi. Bars sharing common letters do not significantly differ at α = 0.05 determined by Tukey's HSD.

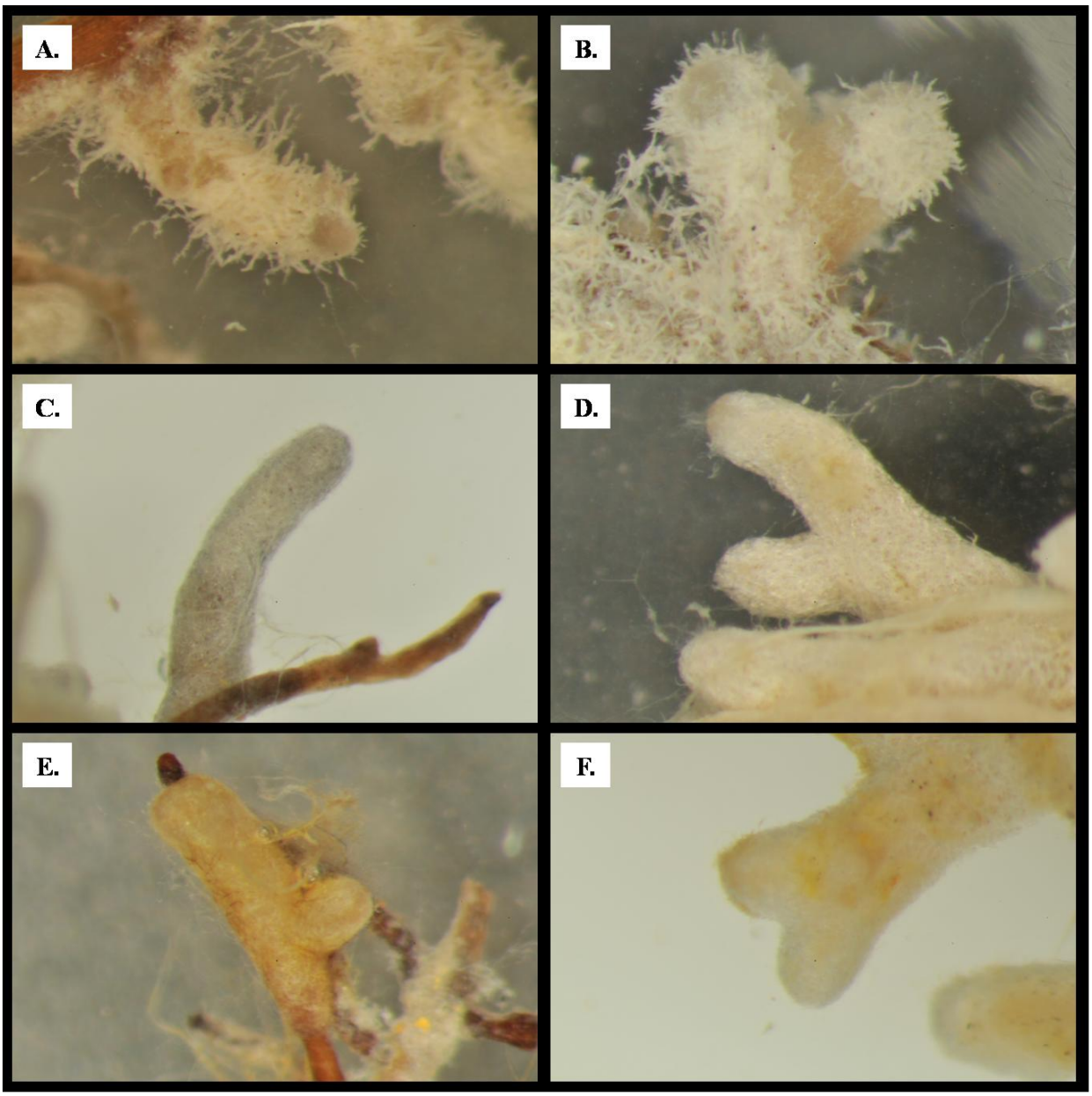

Figure 5. Photographed (45x) ECM morphotypes sampled from American chestnut (*C. dentata*) and *P. virginiana* root tips from pine plots. Panels display the following that were matched to vouchered GenBank sequences: A. *Thelephora* spp. on *C. dentata*, B. *Thelephora* spp. on *P. virginiana*, C. *Scleroderma* spp. on *C. dentata*, D. *Scleroderma* spp. on *P. virginiana*, E. *Pisolithus* spp. on *C. dentata*, and F. *Pisolithus* spp. on *P. virginiana*. Bar = 1 mm.

Chapter 3

Planting methods to promote ectomycorrhizal colonization and species richness on American chestnut (*Castanea dentata*) seedlings in Ohio coal mine reclamation

This portion of the dissertation is currently under review as:

Bauman, J. M., Keiffer, C. H., and Hiremath, S. In Review. Planting methods to promote ectomycorrhizal colonization and species richness on American chestnut (*Castanea dentata*) seedlings in Ohio coal mine reclamation. *Plant and Soil*

Abstract

The objective of this study was to evaluate planting protocols for establishing American chestnut in grasslands that are currently arrested in succession. American chestnut (*Castanea dentata*) and blight resistant hybrid chestnut (*C.dentata x C. mollissima*) were used to evaluate the effects of soil treatments on seedling growth and colonization of beneficial ectomycorrhizal (ECM) fungi on roots. Twelve hundred chestnuts were planted as bare-root seedlings among four soil treatments established on a reclaimed strip mine: 1) a control plot left undisturbed, 2) plots mechanically cross-ripped, 3) plots plowed and disked, and 4) plots ripped + plowed and disked. One hundred and eighty seedlings representing all treatment types were selected for root sampling at the end of the first and second growing season. In addition, 150 trap trees sown as seed around the parameter of the plot were also sampled. The most abundant fungi sampled from chestnuts in the experimental plots (*Hebeloma* spp. 1, *Hebeloma* spp. 2, and *Cortinarius* spp. 1) did not appear on the trap trees. Colonization of these bare-root seedlings likely occurred in the field nursery and greatly inhibited the natural colonization of indigenous *Scleroderma* species. Mechanical soil treatments resulted in greater chestnut survival, more ECM root tips, and greater ECM species richness compared to the control plots ($P = 0.0001$ and 0.01, respectively). In addition, ECM community composition differed between the controls and the mechanically treated plots. There were significant interactions between soil treatments and native ECM infection on seedling height ($P = 0.008$) and basal diameter ($P = 0.03$); chestnut seedlings found naturally colonized by ECM fungi in the mechanically treated plots had the greatest shoot production when compared to their non-ECM counterparts.

Introduction

Succession is the change in a plant community over time and its progression is of particular concern when managing the recovery of landscapes after anthropogenic disturbances such as coal mining. Proper methodologies that direct the successional process in the early stages of reclamation promote a natural rate of forest stand recovery following large scale disturbances (Groninger et al. 2007). Hardwood seedling recruitment and subsequent canopy closure have been reported to occur within 15-20 years after initial mine reclamation (Burger et al. 2005). In contrast, reclamation methods enforced by The Surface Mining Control and Reclamation Act of 1977 (SMCRA) have not resulted in forest succession. Heavy equipment used to grade lands to the original contour and the use of exotic species as cover crops have resulted in severely compacted soils with non-native herbaceous canopies (Bussler et al. 1984; Torbert and Burger 2000). In addition, the native soil microbial community has been highly disturbed and is rendered low in biomass and activity (Degrood et al. 2004; Jacobs 2005; Manchulla 2005). These microbes play primary roles in nutrient cycling, soil structure, and biological interactions facilitating plant community establishment (Bever 2002).

Microbial interactions essential for hardwood tree establishment consist of ectomycorrhizal (ECM) fungi. The presence of these fungal species is required for the establishment of many forest tree taxa including *Betulaceae*, *Fagaceae*, *Pinaceae*, and *Salicaceae* (Smith and Read 2008). This symbiosis enhances the establishing seedling's ability to absorb water and nutrients, tolerate heavy metals and low pH, and protect against root pathogens (Marx 1972; Danielson 1985; van der Heijden et al. 2003; Nara 2005). The formation of ectomycorrhizae associations increases seedling vigor when resources are limited, enhancing the competitive ability of establishing seedlings (Perry et al. 1989; Nara 2005). In return, the fungus receives carbon from the host plant in the form of photosynthates. This symbiotic association greatly aids in the amelioration of stressful environmental conditions and in the regeneration of plant communities following disturbances (Izzo et al. 2006). A plant can obtain carbon transferred from existing mycelia networked to mature trees, which may aid in seedling establishment (Simard et al. 1997). Anthropogenic disturbances such as coal mining cause a decline in

available ECM propagules by removing host plants, increasing soil compaction, and contaminating natural areas with heavy metals and coal spoil (reviewed in Iordache et al. 2009). The severe decline of these microbes may contribute to the limited woody tree and shrub survival on these former mine sites (Marx 1991; Dickie and Reich 2005; Nara 2006).

Although microbes facilitate the formation of plant communities, certain plant/microbe combinations can lead to improved growth rates. This positive feedback aids the competitive ability of one host plant but causes a decline in plant species diversity (Bever 2002). Thirty years after the initial reclamation, mine sites in central Ohio remain vegetated by the non-native herbaceous species originally used as cover crops. *Lespedeza* is a legume used as a quick cover crop in past mine reclamation projects. The importance of positive feedback dynamics between plants and nitrogen (N)-fixing microbes on infertile lands are well known (Reynolds et al. 2003). The N-fixing symbiont proves particularly advantageous to its host plant in nutrient poor soils enabling an increase in host fitness that inhibits the recruitment of native plant species. Tall fescue *(Festuca arundinacea)* is also a common cover crop used in mine reclamation. This species of fescue associates with an endophyte, *Neotyphodium coenophialum,* which resides in the vegetative tissues and seeds of its host plant. This particular endophyte produces insect-deterring ergot alkaloids that provide systemic protection to the grass plant. This symbiont provides protection from herbivores while increasing the herbivory pressure on neighboring plant species, contributing to endophyte plant dominance in grasslands (Clay and Holah 1999; Rudgers et al. 2007). These two plant taxa, *Lespedeza* and *F. arundinacea,* have flourished on these former mine sites and remain the more dominant plant species 30 years after the initial reclamation in central Ohio (McCarthy et al. 2008b).

Shading imposed by the dense cover of herbaceous canopies is one factor that limits native tree recruitment (Ashby 1997; Holl et al. 2000). The persistence of these non-native forbs greatly reduces the abundance of pioneer shrub and tree species that support the ECM fungi required to facilitate the succession of later arriving woody natives (Ashby 1991; Amaranthus and Perry 1994). Most ECM fungi will not persist without the presence of their host plants and low ECM propagules will favor non-ECM

plant species. Reclaimed mine sites dominated by non-ECM plant species may be difficult to return to natural forest conditions (Amaranthus and Perry 1994; McCarthy et al. 2008b).

Mechanical soil treatments such as deep ripping and traditional plow and disking have been proposed by Appalachian Regional Reforestation Initiative (ARRI) to accelerate succession (Sweigard et al. 2007). These methods disturb the grass canopy and alleviate soil compaction to improve the survival of woody trees in mine restoration (Rokich et al. 2001). However, not much is known about how certain mechanical surface treatments impact native ECM fungi. Mechanical disturbance may disturb existing mycelium networks, but favor the establishment of early successional ECM species via spores or vegetative propagules. Additionally, small scale disturbances by mechanical methods may mimic natural disturbances that mix soil horizons and alter pH and nutrient availability that may create additional habitats for ECM fungi (Bruns 1995).

The objective of this study was to evaluate surface treatment methods with regard to ECM community composition and ECM root colonization. This study used American chestnut (*Castanea dentata*) and blight-resistant hybrids (*C.dentata x C. mollissima*). Preliminary studies have reported American chestnut as a tree species that can establish in these harsh field sites (McCarthy et al. 2008b, Jacobs 2009; Rhoades et al. 2009). The fast growth rate coupled with quality timber makes American chestnut a desired species for reforestation projects. Chestnut is reported to be a generalist, adapted to a wide range of ecological conditions including tolerance to drought and low pH (reviewed in Jacobs 2007). Chestnut has the propensity to survive long periods as an understory tree, however, will be a superior competitor for light following a canopy disturbance (Latham 1992; McEwan et al. 2006).

This study evaluated ECM root colonization of pure American and chestnut hybrids under varying soil treatments: 1) control, 2) cross-ripped at a depth of approximately 1.5 meters, 3) plowed and disked, and 4) combination of cross-ripping and plowed and disked. Survival data were recorded for three field seasons for all seedlings. Root samples from 180 chestnuts were assessed for root tip colonization and ECM species composition after two field seasons. This experiment tested the hypothesis that disturbances employed by mechanical techniques will accelerate succession by aiding in

hardwood tree establishment and increased ECM activity. An ECM survey conducted on American chestnut seedlings quantified ECM species diversity and root colonization. Growth data were recorded to determine the influence of the mechanical soil treatments, the native ECM, and the interaction of both variables on chestnut establishment after two years in the field. Our predictions were as follows: 1) soil surface disturbances will result in differing ECM community compositions among treatment plots, 2) chestnut seedlings grown in the mechanically treated plots will have greater survival and ECM root colonization, and 3) ECM infection will result in a positive growth response on chestnut regardless of treatment.

Methods

Experimental Design

In the spring of 2006, 1200 American chestnuts were sown at the State Nursery in Marietta, Ohio by the Ohio Department of Natural Resources. The 1200 seeds were comprised of the following: 400 pure American chestnuts, 400 Chestnut Hybrids B1-F3 (backcrossed to create a progeny 7/8 American chestnut, 1/8 Chinese chestnut) and 400 Chestnut Hybrids B2-F3 (backcrossed to create a progeny 15/16 American chestnut, 1/16 Chinese chestnut). The seedlings were nursery grown for one year in the Ohio Division of Natural Resources State Field Nursery in Marietta, OH.

The field site is located in the Tri-Valley Wildlife Management Area (TVWMA), Muskingum County, central Ohio (40° 11' 32" N, 81° 98' 35" W). This mine site was reclaimed in the 1980s and is primarily vegetated with the original species used for reclamation (*Festuca* spp., and *Lespedeza* spp.) with small patches of ragweeds (*Ambrosia* spp.) and goldenrods (*Solidago* spp.). Small pockets of forest comprised primarily of *Quercus, Pinus,* and *Acer* species were left undisturbed at the time these lands were mined (McCarthy et al. 2008b). This area receives an average of approximately 99 cm of precipitation annually. During the 2007 and 2008 growing season, the summer climate was relatively dry to moderate drought with annual temperatures averaging 22° C during the growing season (17°, 28°, and 11° C, spring, summer and fall, respectively; National Climatic Data Center).

Three experimental blocks each containing the control and three soil treatments were installed prior to planting in the spring of 2007. Each block is 73 x 36 m with four 18 x 36 m treatment plots contained within (Figure 1). The following treatments were established: 1) a control left undisturbed (C), 2) a plot cross-ripped at a depth of approximately 1.5 meters created by a D-6 dozer with a 1.0 m ripper bar attachment (R), 3) a plowed and disked plot installed by a conventional tractor (PD), and 4) a ripped + plowed and disked plot (RPD). The statistical design of this experiment was a block design suitable for analysis using both one-way and two-way analysis of variance (ANOVA).

Soil was collected using soil cores prior to planting to analyze soil chemistry and bulk density. Preliminary data analysis revealed no differences among blocks with regard to the soil environment (McCarthy et al. 2008a). Soil pH ranged from 5.4 to 5.7. Soil texture averaged 61% sand, 23% silt, and 16% clay. Organic matter and cation exchange capacity (CEC) averages were 1.3% and 7.5 CEC, respectively. Mean values for minerals were: Al 3.5 ppm, Ca 720 ppm, K 78 ppm, Mg 182 ppm, Mn 3.75, and P 8 ppm. Bulked densities per treatment were as follows: control 1.65, R plots 1.48, PD plots 1.63, and RPD 1.6. Soil chemistry was measured at both Brian McCarthy's laboratory (described by McCarthy 1997) and at Spectrum Analytic, Inc, Washington Courthouse, Ohio. Twelve hundred American chestnut seedlings were planted into treatment plots (12 plots, 100 seedlings each) as bare rootstock in April of 2007 at a spacing of 2.15 m by 2.15 m, as described by Hebard (2005). Holes were dug with a hand shovel and seedlings were planted with the root collar level to soil grade. To prevent desiccation, each seedling was planted with TerraSorb gel. One fertilizer pellet (10-10-10) was dropped in each hole and the seedling was backfilled with original soil. A 1 m x 1 m weed mat was installed to prevent the reemergence of the herbaceous canopy around the root collar. To prevent herbivory, a 1.5 m tall chicken wire cage was placed around each seedling held by three wooden stakes (Sprouse 2004).

At the time the bare root seedlings were planted, 150 American chestnut seeds were planted to be used as trap trees around the perimeter of the treatment plots. This provided additional sampling of ECM fungi native to the field site and was used to

compare with the fungal species sampled from the roots of the bare root seedlings to determine if any ECM was transplanted inadvertently from the bare root seedlings.

Data Collection

Seedlings survival was recorded at the end of each growing season for 30 months. After 6 months (October 2007) and 18 months (October 2008) in the field, 180 pure American chestnuts representing all treatment plots were selected for root sampling (60 and 120, respectfully). Pure chestnuts were randomly sampled with one criteria, seedlings did not neighbor one another. This avoided root system overlap and ensured that each root system sampled was an independent unit. To ensure roots sampled were chestnut and not a part of the surrounding vegetation, soil was carefully removed with a spade shovel to expose the chestnut root system at a depth of 25 cm and a width of 45 cm. Roots were carefully sifted away from the soil and stored on ice. Once in the laboratory, roots were washed with autoclaved distilled water and transferred into a Petri dish with sterile water. Two hundred and fifty root tips were randomly selected from each seedling and viewed under a dissecting microscope for the presence of a fungal sheath (180 samples, 45,000 root tips). Three mm of root tip was transferred into a microcentifuge tube and stored at -70˚ C until DNA extraction. One or two root tips of each morphotype per seedling were sampled for DNA extraction and sequencing.

After 12 and 18 months in the field, 150 of the pure American seedlings planted as trap trees around the perimeter of the study site were destructively sampled (75 seedlings at each sample date). The reason for this sampling, as stated above, was to have a sample of seedlings sown as seeds. This would aid in distinguishing ECM species native to the field site from ECM species that may have come in with the bare rooted seedlings. For root sampling, each seedling was carefully removed from the field and returned to the lab were roots where washed with distilled water. Two hundred and fifty root tips per seedling were randomly selected from each of the 150 chestnut seedlings (37,500 root tips total) and viewed under a dissecting microscope and sampled as explained above, and stored at -70˚ C. In the following sections these seedlings will be referred to as trap trees.

In April 2007 (before bud break) and October 2008 (end of second field season) growth parameters such as stem diameter and seedling height were recorded. Height (cm) was measured using a meter stick from soil level to the tip of the main stem. Basal diameter (mm) was recorded by using digital caliper, 3 cm above root collar.

Fungal DNA Extraction, PCR Amplification, and Sequencing

To molecularly identify the types of mycorrhizal fungi, BLAST searches were employed on the ITS region of the fungal DNA. DNA was extracted from the collected root tips using QIAgen Dneasy Plant Mini-Prep kit purchased through QIAGEN Inc. Primers ITS1-F (5' cttggtcatttaggaagtaa 3') and ITS4 (5' tcctccgcttattgatatgc 3') was used to amplify internal transcribed spacer sequences (ITS) during PCR (Gardes and Bruns 1993). PCR 15 µl reactions were mixed based on the following concentrations: 9 µl of molecular grade water, 3 µl of 5x Green GoTaq® Reaction Buffer, 0.125 µl of Promega® *Taq* DNA Polermerase, .2 µl of 25µM of each primer, 1µl of dNTPS (200µM each of dATP, dCTP, dGTP, and dTTp) and 1 µl of DNA template. Temperature cycling was accomplished using a programmable Thermal Cycler Heating block. Times and temperatures were programmed as described by Gardes and Bruns (1993): The initial denaturation step was 94 ºC for 85 s followed by 35 amplification cycles of denaturation, annealing, and extension. The temperature and times for the first 13 cycles were 95 ºC for 35 s, 55 ºC for 55 s, and 72 ºC for 45 s. Cycles 14-26 and 27-35 repeated the above parameters with lengthened extension steps 120 and 180 s, respectively. When the 35 cycles were completed the samples were programmed to incubate for 10 min at 72 ºC for 45 s. PCR reactions were run in 1% argarose gels for 20 minutes to allow for the visualization of fungal DNA. Negative controls lacking template were used to ensure that the DNA amplified was from the root samples and not a contaminate from reagents and reaction mixtures.

PCR product was cleaned by using Clean-Gene. Samples were prepared for sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit by mixing 10 µl reactions of the following concentrations: 2 µl BigDye Terminator v3.1 Reaction Mix, 3 µl 5 X Sequencing dilution buffer, 1 µl primer, and 1 µl of template. Sequencing cycle to

label DNA for sequencing was performed on a programmable Thermal Cycler for the following cycles: 96ºC for 1 min followed by 25 cycles of 10 s at 96 ºC, 5 s at 50 ºC, and 4 min at 60 ºC. Following labeling, products were purified to remove all unincorporated dye-labeled terminators by alcohol precipitation. Sequencing was performed with The Applied Biosystem ABI Prism 3730 DNA Analyzer (Bioinformatics facility, Miami University, Oxford, Ohio). Sequences were analyzed and edited using Sequencher 4.2 software (Gene Codes, Ann Arbor, Michigan). To identify fungi found on roots, sequenced samples were compared with known species in GenBank using BLAST searches (Altschul et al. 1997). Genera reported here are based on the best match of vouchered fungi based on the similarity to the reported ITS sequences in GenBank. Characteristics are based on statistical analysis that generates a bit value, gap score, and an Expect (E) value. The bit score is a value that is indicative of how well the sequenced aligned with the known sequence in the database. The higher the score, the better the match. The gap score introduced into an alignment compensates for insertions and deletions in one sequence relative to another. The E value is a parameter that describes the probability of the number of matches that can be generated by chance. It decreases exponentially as the match increases; a score closest to zero is the most significant. To decide on the genera to report here, we selected an E-value of 0 coupled with the highest ranking bit value and low gap value (< 4).

Statistical Analyses

To quantify species richness of ECM per treatment and to estimate the sufficiency of sample size, species accumulation curves were constructed based on the 180 chestnuts root samples that were taken at the end of the first and second growing seasons. To compare differences in species diversity between the soil treatments, area-based rarefaction curves with confidence intervals calculated from ± 1 standard deviation were calculated by using BiodiversityR version 1.2 (Kindt and Coe 2005). Species accumulation curves were constructed based on exact calculations of the average species richness for the combination of the treatments with 1000 permutations for each sample size. The exact method was selected over the random method because it is faster and more precise (Kindt and Coe 2005; Appendix 1). Area-based rarefaction was used over

individual-based methods because ECM root tips do not represent fungal individuals and therefore better reflect the true distribution of species (Colwell et al. 2004; Tedersoo et al. 2006). Description of ECM diversity was followed by calculating both Shannon-Weiner diversity index and Simpson's index of diversity using BiodiversityR version 1.2 (Kindt and Coe 2005).

A non-metric multidimensional scaling (NMDS) ordination was used to determine if these soil treatments influenced ECM species composition sampled on chestnuts planted as bare root seedlings. To improve the NMDS ordinations, the data were square root transformed and standardized via Wisconsin double standardization (Oksanen 2005). Bray-Curtis dissimilarities were employed due to their preferred analysis for community data due to the restriction within the range of 1 to 0 (Kindt and Coe 2005). The maximum number of random starts in a search was set at 100 with k=2 stress value. A permutational multivariate analysis of variance was used to test for significant differences among the soil treatments. The sites were plotted on an ordination graph using convex hulls (sensitive to outliers) to outline the various treatments (Kindt and Coe 2005). Therefore, hulls that did not overlap illustrate that species composition was dissimilar. Ordinations were performed using Vegan: Community Ecology Package version 1.6.9. (Oksanen et al. 2005; Appendix 2).

ECM colonization per treatment was assessed by taking the percentage (#ECM tips/250) of ECM colonized root tips from chestnuts planted as bare root seedlings (n=180) after two field seasons. Arcsine square root transformation was used to control for unequal variances. Differences in colonization between the ECM colonization were statistically determined by using a one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. Growth parameters were derived from the difference between the original measurements of seedling height (cm) and basal diameter (mm) and the final measurements at the end of the second field season. Stem dieback resulted in negative values indicating biomass lost. Data were transformed by setting the most negative growth value to zero, adding accordingly to the samples, and using Log+1 transformation (McCarthy per comm.). To determine significant interactions between ECM colonization by treatment, a full factorial two-way ANOVA was used (ECM colonization * soil treatments). The differences were considered significant when $p \leq 0.05$ according

to the F test. Cox proportional hazard model was used to determine significant differences in survival among treatments and seedling types using survival data from all 1200 seedlings. All ANOVAs and Cox proportional hazard model were performed using R v2.91 (R Development Core Team 2009; Appendix 3).

Results

ECM Species Sampled from Bare Root Seedlings

Accumulation curves were used to evaluate the sufficiency of sample size and compare species richness between treatments. The curve did not plateau for the control (C) or the rip+plow and disk (RPD) plot indicating that not all rare species were sampled. However, for plow and disk (PD) and ripped (R) plots, the curves appear to become less steep at a sample size of 35 seedlings per treatment, which constituted 8,750 root tips (Figure 2).

The soil treatments RPD and PD were similar with regard to the expected number of species as indicated by the overlapping confidence intervals (11 and 10, respectively). Species richness in the control plots (8) was significantly lower than in the treatment plots (Figure 2). Number of species averaged per block followed that trend; 7 species recorded in the mechanically treated plots compared to an average of 4 ECM species in the control plots (Table 1). Diversity indices also revealed a similar pattern. Shannon-Weiner diversity indices in the treatment plots ranged from 1.43 to 1.54 compared to 1.01 in the control plots (Table 1). Although species diversity was higher in the treatments, this was not significant. Simpson's Diversity ranged from 0.66 to 0.72 in the treatments to 0.54 in the controls but this, too, was not statistically different (Table 1).

When samples from all 180 bare-root chestnut seedlings sampled from the treatment plots were compiled, 14 ECM fungal species were recorded (Table 2). This diversity sampling consisted of a few dominant species and several rare species. Of these, the more abundant species were *Hebeloma* spp. 1, *Hebeloma* spp. 2, and *Cortinarius* spp. 1 (Table 2). *Scleroderma* spp. 1 and *Thelephora* spp. were sampled moderately throughout the study (Table 2). The remaining rare species consisted of Unknown ECM spp. 2, *Hebeloma* spp. 3, *Laccarria* spp., Unknown ECM spp. 1,

Scleroderma spp. 2, *Cortinarius* spp. 2 and 3, *Tomentella* spp. , and *Cenococcum* (Table 2).

Eleven fungal species were sampled from chestnuts planted as seeds and used as trap trees in this study (Table 2). This sampling did not find the more abundant species sampled from the chestnuts planted as bare root seedlings. The more abundant species found on roots of the trap trees were *Scleroderma* spp. 1 and *Scleroderma* spp. 2. *Cenococcum* spp. and *Thelephora* spp. were sampled moderately throughout the trap trees. The remaining less common species consisted of *Tomentella* spp., *Hebeloma* spp. 3, *Cortinarius* spp. 2, *Hebeloma* spp. 2, Unknown ECM spp. 2, and an uncultured species within the family Thelephoraceae (Table 2).

ECM Colonization and Soil Treatment Effects

Non-metric multidimensional scaling (NMDS) analysis of all collected samples determined that the first dimension of the ordination was negatively correlated with *Cortinarius* spp. 2 (Cort2) and positively correlated with *Scleroderma* spp. 2 (Scl2). The second dimension was negatively correlated with *Tomentella* spp. (Tom) and positively correlated with *Hebeloma* spp. 2 (Heb2; Table 3). Overlapping convex hulls illustrated similarity in ECM community composition (Figure 3). There were no differences detected among the three soil treatments. Conversely, a permutational MANOVA reveled significant differences when the mechanically treated plots were compared to the control plots ($f = 0.24$, $P = 0.015$). *Cortinarius* spp. 2 (Cort2) in particular appeared strongly specific to the controls plots, where as *Scleroderma* spp. 2 (Scl2), *Tomentella* spp. (Tom), and *Hebeloma* spp. 2 (Heb2) were strongly correlated with the mechanically treated plots (Figure 3).

When the percentage of ECM root tips were compared per treatment, root colonization was statistically higher on the chestnut seedlings sampled from the mechanically treated plots (ANOVA, $f = 10.63$, $P < 0.0001$). No differences existed among the surface treatment methods, PD (42%), R (40%), and RPD (45%); all were significantly higher that the C (13%) plots (Figure 4).

Soil treatments and ± ECM had significant main effects on seedling height and basal diameter. The soil mechanical treatments caused an increase in both height (ANOVA, $f = 5.38$, $P = 0.0015$) and basal diameter (ANOVA, $f = 8.34$, $P < 0.0001$). Although differences were not detected among soil treatment methods; each soil method caused a significant increase in stem height and basal diameter when compared to the chestnut seedlings in the control plots. There was also a significant trend when +ECM seedlings were compared to – ECM seedlings. The presence of ECM on chestnut seedlings had a highly significant increase in height (ANOVA, $f = 30.85$, $P < 0.0001$) and basal diameter (ANOVA, $f = 9.37$, $P = 0.003$).

Seedling height (cm) and basal diameter (mm) were analyzed using two-way ANOVA. The ECM colonization by mechanical treatment interaction was significant for seedling height ($f = 4.02$, $P < 0.008$; Figure 5) and basal diameter ($f = 2.88$, $P = 0.03$; Figure 6). This synergistic effect is apparent when comparing ECM colonized chestnut seedlings (+ ECM) with regard to height in R and RDP plots (Figure 5) and basal diameter in RPD treatments (Figure 6). Subsequent analyses using Tukey's post hoc tests demonstrated significant differences between + ECM and - ECM in the rip + plow disk (RPD) plots with regard to height ($f = 21.20$, $P < 0.0001$) and basal diameter ($f = 8.06$, $P = 0.005$). There were no significant differences when growth of + ECM seedlings were compared to - ECM seedlings in the control plots (Figures 5 and 6). Seedling in the C plots were similar, despite the presence of ECM on chestnut roots.

Survival among Treatments and Seedling Types:

After three growing seasons, seedling survival in the mechanically treated plots (79-85% survival) was significantly higher than control plots (32%) (Cox proportional hazard model, Likelihood = 564, df = 3, $P < 0.0001$; Figure 7). When comparing the seedling types there was a significant difference; the B2 hybrids (74%) had a significantly higher survival rate than the B1 hybrids (64%) (Cox proportional hazard model, Likelihood ratio test= 20.4 on 2 df, $P < 0.0001$; Figure 8). Survival of the pure American seedlings (68%) was intermediate and not significantly different from either.

Discussion

The results of this study found: 1) ECM species richness increased in mechanically treated plots, 2) ECM community composition was influenced by soil disturbance; however, no differences were observed among the soil surface treatments, 3) mechanical soil treatment greatly improved ECM root colonization, 4) ECM root colonization resulted in an increase in height and basal diameter in the R and RPD plots, and 5) survival in the treated plots was significantly increased.

Collectively, this study reported 14 different ECM species on chestnut seedlings at the end of two growing seasons. Only 11 ECM species were sampled from the trap trees (chestnuts planted as seed). The most abundant fungi sampled from chestnuts in the experimental plots (*Hebeloma* spp. 1, *Hebeloma* spp. 2, and *Cortinarius* spp. 1) did not appear on the trap trees. This indicates that colonization of roots by these three species most likely occurred in the field nursery and not in these experimental plots.

The remaining 11 species were detected on trap trees and may be most representative of native ECM present on these grasslands. The 11 species recorded here are relatively low when comparing our study to other surveys revealing a more diverse ECM fungal community in monoculture stands (Danielson 1985; Bruns 1995; Jones et al. 1997). With regard to other surveys documenting ECM fungi associating with *Castanea* species, Dulmer (2006) reported 38 species of ECM fungi on American chestnut in oak dominated forest sites in New York State. This comparison shows the stark decline in species richness from developed forest soils to those that have been severely disturbed by surface mining for coal. Surveys in Italy on European chestnut (*C. sativa*) sampled between 23 and 39 ECM fungi from forest stands (Peintner et al. 2007; Blom et al. 2009). Although the soils differed significantly in our study, our ECM species survey used younger, even-aged seedlings. Therefore, ECM colonization of later stage fungi, if present, may have gone undetected due to insufficient host carbon supply and root density produced by an immature seedling (Deacon and Fleming 1992). Regardless, the 11 species sampled after two field seasons illustrates very low ECM species richness and diversity. This supports our original prediction and concurs with other studies that reported low ECM diversity in non-ECM habitats like grasslands (Chapela et al. 2001)

and other environments recovering from natural and anthropogenic disturbances (Baar et al. 1999; Jasper 2007).

There were differences when ECM species recorded from bare-root chestnuts were compared to species documented on the trap trees. *Scleroderma* species were only reported on 9% of the chestnut seedlings planted as bare root seedlings. Conversely, this species was the most abundant genera (74%) on the trap trees. Species of *Scleroderma* are valued for their ability to tolerate mine soils while enhancing the growth and establishment of their host (Beckjord and McIntosh 1983; Chen et al. 2006). *Scleroderma* has a worldwide distribution, a wide host range, and a high infinity for *Castanea* spp. (Newton 1991; Meotto et al. 1998; Jefferies 1999; Bauman et al. in review). These species produce abundant rhizomorphs for long distance exploration (Agerer 2001) and water transport vital for seedling establishment in times of drought (Parlade et al. 1996). Although it is not known how long these fungal structures persist in the soil, *Scleroderma* is a reported saprobe able to tolerate stressful conditions far from existing trees (Jefferies 1999). The saprotrophic capability, persistent sclerotia and rhizomorphs, and prolific spore dispersal may contribute to *Scleroderma*'s abundance in openings that are at a distance from existing ECM hosts (Ingleby et al. 1998; Taylor and Bruns 1999; Jones et al. 2003: Dickie and Reich 2005).

Hebeloma and *Cortinarius* were more abundant on the bare-root chestnut seedlings. This may have inhibited the natural colonization of *Scleroderma* species that are native to these soil types. This type of inhibition has been previously described for *Hebeloma* on oak two years after outplanting (Garbaye and Churin 1997). The successful colonization of new root tips from introduced inoculum illustrates the propensity introduced fungi have as effective competitors on sites where they are not native (Jones et al. 2002). It remains unclear whether the presence of an established mycorrhizal species influences ECM colonization from native species in the field. There is evidence in this study and others (Kennedy and Bruns 2005; Kennedy et al. 2009), that established root colonization may competitively exclude or inhibit the colonization of indigenous ECM. In this study, it is difficult to ascertain whether the better competitor for host tissue translated into a better symbiont on chestnut.

Treatment effects

Plots treated with the mechanical surface treatments differed significantly from the control plots with regard to the ECM community composition. For example, *Cortinarius* spp. 2 was rather unique to the control plots and may be an example of a fungus poorly adapted to mechanical soil disturbances. ECM species richness was greater in plots that were treated with a mechanical disturbance. The arrested succession observed in these grasslands may require a disturbance that promotes regeneration of both the microbial community and native plant recruitment. Ripping has been reported to provide varying microsites for the coexistence of two different species of ECM. Jasper (2007) reports *Scleroderma* species were able to colonize seedlings establishing on the crest of the rip lines that were devoid of organic matter. *Cortinarius* species were found adjacent to *Scleroderma*, established in the ripped furrows higher in organic matter produced by the accumulation of litter (Jasper 2007).

In addition, mechanical soil disturbances may provide recruitment for airborne spores such as *Thelephora* spp., *Scleroderma* spp. 2, and unknown ECM species sampled from treatment plots and trap trees. Existing pockets of forests consisting of ECM tree species were left undisturbed during the original mining operation and that may provide a spore reservoir for dispersing fungal species. Although ECM are not well adapted to survive mining operations, these fungi can re-invade in a few years given the presence of ECM host plants and the availability of nearby propagule sources (Allen et al. 2002). Both ripping and plow and disking soil surface methods seem to aid in ECM fungal recruitment by encouraging ECM seedling establishment while creating a small scale disturbance for fungal recruitment via airborne spores. Chestnut hybrids may be a very important restoration hardwood species that can establish in disturbed grasslands and offer a niche to incoming ECM fungal species.

Seedlings in the mechanically treated plots had a dramatic increase in ECM root colonization after two growing seasons. Previous studies have shown ripping to increase the size of the root system aiding in seedling establishment (Cleveland and Kjelgren 1994; Ashby 1997). Although root biomass was not measured during our study, it can be presumed that the soil treatments promoted an increase in fine root production that provided an increase in host tissue for an ECM fungal symbiont. Also, chestnuts that

grew better in response to the improved rooting medium may have provided more carbon exudates from their roots, promoting greater fungal colonization (Salonen et al. 2000). Increased soil porosity generated by the soil treatments may have contributed to the diffusion of signaling molecules such as host plant root exudates and fungal auxins that initiate the primary synthesis of mycorrhizal roots (Podila 2002).

We found a significant reduction in ECM root colonization in the compacted control plots. Compacted soils with high bulk densities hinder hyphal growth (Skinner and Bowen 1974) and root colonization (Amaranthus et al. 1996; Jordon et al. 2003). Previous work with chestnut in compacted mine soils revealed a significant reduction in ECM colonization and that correlated with greater root disease caused by *Phytophthora* spp. (Rhoades et al. 2003). Other studies have identified that the presence of ECM suppresses disease development from soil-borne pathogens (Branzanti et al. 1999; Whipps 2004). Future restoration efforts using blight resistant hybrids may benefit from field data identifying silvicultural methods that alleviate soil compaction and increase ECM root colonization.

In addition to alleviating soil compaction, soil treatments employed during this study tilled the existing forbs/graminoid canopy belowground (J. M. Bauman pers. obs) providing chestnuts a temporary release from competition. Previous studies have shown that mechanical soil treatments modify the soil structure and drastically disturb the grassland canopy encouraging seedling establishment (Holl et al. 2000; Aston et al. 2001; Hooper et al. 2005). In addition to disrupting herbaceous canopies, mechanical soil disturbances have also been reported to disrupt AM fungal networks and reduce colonization by AM fungi (McGonigle and Miller 1996). With regard to ECM root colonization, chemical and mechanical treatment to disturb grass canopies has been found to increased ECM diversity (Jones et al. 1996). In our study, the mechanical disturbance with the establishment of an ECM tree species encouraged ECM colonization that greatly increased host growth. Our ultimate goal is to offer planting protocols that promote long term survival of chestnut, which may facilitate the shift from non-native herbaceous plants to woody native ECM trees and shrubs. It can be hypothesized that the establishment of woody trees and shrubs may limit light availability, imposing a high energetic cost to maintain the N-fixing (Gutschick 1981) and/or arbuscular mycorrhiza

(AM) fungal symbionts of herbaceous plants. Theoretically, a shift from non-ECM plant species to those obligatory to ECM fungi may occur if succession progresses in response to the mechanical surface treatments promoting the survival of ECM host plants (Janos 1980; Reynolds et al. 2002; Smith and Read 2008).

The presence of ECM did not significantly improve shoot growth in the control plots. Several mechanisms may have contributed to the neutral response resulting from ECM colonization in control plots. Earlier work by Marx et al. (1982) suggests that root colonization must exceed 50% in order to invoke a positive host response. However, we found that the limited ECM colonization may have been due to soil compaction, diminishing hyphae growth and limiting ECM formation. The diminished hyphal expansion and root competition with herbaceous plants may have decreased the amount of resources supplied by the fungi to its plant host. In addition, shading by competing vegetation decreases photosynthetic potential, thereby reducing the carbon gain (Janos 1980). Therefore, the decrease in ECM colonization can be the result of diminishing carbon transfer from the host plant, an adaptation of plant control over the symbiosis preventing parasitism (Swaty et al. 2004). This has also been documented following herbivory (Saikkonen et al. 1999); in that study less extensive hyphae development correlated to photosynthetic tissue lost, suggesting carbon limitation as a mechanism driving the decrease in ECM colonization.

Treatment effect on chestnut survival over three seasons:

This study reports that soil surface treatments, particularly using soil ripping methods, improved American chestnut and chestnut hybrid survival after three field seasons. Aerating compacted soils has been found to enhance tree survival and growth in other projects in Appalachia (Burger et al. 2005; Groninger et al. 2007). Studies utilizing mechanical soil treatments found that these treatments aid in increasing the size of the root system that correlated with the higher survival rate (Cleveland and Kjelgren 1994; Ashby 1997). In addition to promoting deep rooting, the mechanical treatment temporarily removed the competing vegetation. This greatly aided in the B2-F3 hybrids (15/16 American chestnut) establishment. This hybrid genotype has the growth habit of the pure American chestnut. This is in contrast to the more spreading habit of the B1-F3

hybrid (7/8 American) that has a growth habit resembling the Chinese chestnut. The taller chestnuts were successful in outcompeting the re-establishing *Lespedeza* and *Fescue* species; this correlated with greater survival rates reported for the pure Americans and B2-F3 hybrid chestnuts. Importantly, the strong survival of the B2-F3 hybrids suggests that this chestnut type makes an excellent restoration tree species, agreeing with other reports (McCarthy et al. 2008b; Jacobs et al. 2009).

Implications for practice:

Soil compaction, competition with non-native herbaceous plants, and the absence of an ECM symbiont all act as mechanisms inhibiting seedling recruitment in reclaimed mines in central Ohio. These human-induced grasslands can remain arrested in succession even decades after mines have been reclaimed (McCarthy et al. 2008b). Soil treatment by ECM colonization interaction was significant indicating that the combination of the mechanical treatments and colonization of ECM fungi had a synergistic effect on the growth of chestnut seedlings. What was demonstrated at the end of the second growing season was the influence proper site preparation has on ECM symbioses during a critical time of seedling establishment. After three field seasons, survival was significantly higher in the plots that received a soil treatment. Important to restoration using hybrid chestnut, the B2-F3 genotype had survival rates comparable to the pure American. Sivilcultural methods of site preparation aided in the success of using chestnut hybrids as a restoration tree species, at least on a short term basis. The two soil treatment methods employed in this study, ripping and plowing with a conventional tractor, were similar in both ECM species richness and ECM community composition. However, when comparing operating costs of the two methods, traditional plow/disc involve only one eighth the cost ($20.00 per acre) of a D-6 dozer with ripper attachment ($150.00 per acre). This study only assessed these hybrids on the short term basis. Further studies will be required to determine how these soil surface methods encourage long-term survival.

Lastly, the large ECM host range of American chestnut and chestnut hybrids provides a method of quantifying the ECM community. Having a base-line documentation of the first two field seasons can be used as a tool to measure ecosystem

recovery by documenting the increase of ECM diversity through time. A better understanding of native fungi whose interactions may be promoted by various site preparation methods may aid in future management strategies in restoring reclaimed mines. Establishing a hardwood provides a host plant to many fungal species, increasing the inoculum source for incoming trees and increasing the probability of the facilitation of ECM inoculation from existing vegetation. In addition, chestnut is a prolific nut producer and will attract hoarding seed dispersers that can contribute to increased hardwood seed dispersal. Developing protocols that elevate soil compaction, encourage root colonization by a diverse population of ECM fungi, and identify the native ECM symbiont that elicits the greatest host response may aid in the natural succession of these grasslands into mature forest ecosystems.

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Table 1. Mean species richness, Shannon-Weiner diversity index, and Simpson's diversity index (1-D) ± 1 SD among the four treatments (n=12). Sample size (n) refers to the number of blocks.

Treatment	N	Ave. Species Richness	Shannon-Weiner	Simpson's Diversity
C	3	4.3 ± 0.58	1.01 ± 0.18	0.54 ± 0.13
PD	3	7.3 ± 2.31	1.54 ± 0.30	0.72 ± 0.10
R	3	7.3 ± 2.08	1.43 ± 0.59	0.66 ± 0.24
RPD	3	7.3 ± 2.31	1.48 ± 0.36	0.68 ± 0.16

Table 2. Molecular identification of ECM root tips ranked by relative abundance from chestnut seedlings among the four treatments (n=180). Table includes relative abundance of fungal taxa on seedlings in each of the four treatments and corresponding GenBank sequence accession numbers.

ECM species	Total	C	PD	R	RPD	Trap	Accession
Hebeloma spp.1	0.31	0.57	0.36	0.21	0.28	0	GU246983
Hebeloma spp. 2	0.20	0.09	0.14	0.27	0.23	0	GU246984
Cortinarius spp. 1	0.16	0	0.15	0.11	0.24	0	GU246986
Scleroderma spp. 1	0.09	0.03	0.08	0.15	0.05	0.61	GU246989
Thelephora spp.	0.07	0.12	0.10	0.06	0.05	0.07	GU246993
Unknown ECM 2	0.04	0	0.04	0.09	0	0.01	GU246997
Hebeloma spp. 3	0.03	0.01	0	0.05	0.01	0.01	GU246985
Laccaria spp.	0.03	0.04	0.06	0.04	0.01	0.01	GU246994
Unknown ECM 1	0.02	0	0	0	0.01	0	GU246996
Scleroderma spp. 2	0.01	0	0	0	0.01	0.13	GU246990
Cortinarius spp. 2	0.01	0.13	0	0	0.01	0.01	GU246987
Cortinarius spp. 3	0.01	0	0.04	0	0	0	GU246988
Tomentella spp.	0.01	0	0.02	0	0	0.03	GU246992
Cenococcum spp.	0.01	0.01	0.01	0.02	0.10	0.11	GU246995
Thelephoraceae	0	0	0	0	0	0.01	GU553376
# seedlings inspected	180	45	44	44	47	150	
# of root tips inspected	45,000	11,500	11,000	11,000	11,750	37,500	
# root tips with ECM	15,060	1,202	4,477	4,197	5,184	13,240	
Proportion of ECM	0.33	0.10	0.41	0.38	0.44	0.35	

Table 3. Species scores (coordinates) of NMDS dimensions used to plot species on ordination. Strong associations between ECM species and NMDS dimensions are shown in bold.

ECM Species	**Dim1**	**Dim2**
Unknown sp. 1 (Un1)	0.46777544	-0.03724253
Unknown sp. 2 (Un2)	0.26283783	-0.32269375
Cortinarius sp. 2 (Cort2)	**-0.84236024**	0.22229829
Cortinarius sp. 1 (Cort1)	0.28285894	0.01324338
Laccaria sp. (Lac)	0.33661071	0.14928644
Cortinarius sp. 3 (Cort3)	0.52713717	-0.31390793
Hebeloma sp. 1 (Heb1)	0.18554867	-0.26631058
Hebeloma sp. 2 (Heb2)	0.39031097	**0.41189741**
Hebeloma sp. 3 (Heb3)	0.24585326	0.0808811
Cenococcum sp. (Cen)	0.21119263	-0.17648915
Scleroderma sp. 1 (Scl1)	0.35614304	-0.02711169
Scleroderma sp. 2 (Scl2)	**0.55132987**	0.38893662
Thelephora sp. (Thel)	0.27126195	-0.07455187
Tomentella sp. (Tom)	0.09156124	**-0.57198265**

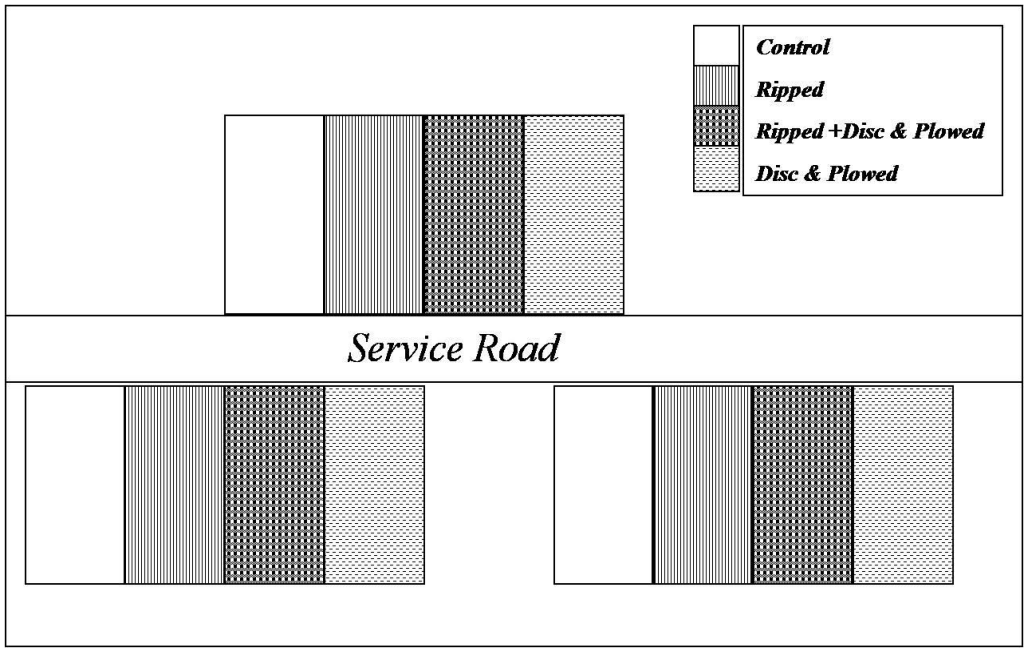

Figure 1. Field plot design: Three blocks installed consisted of four treatments: control, ripped, plowed and disked, and ripped + plowed and disked. Each block is 73 x 36 m, each treatment 18 x 36 m. A 15 m buffered area was left between each treatment (not shown).

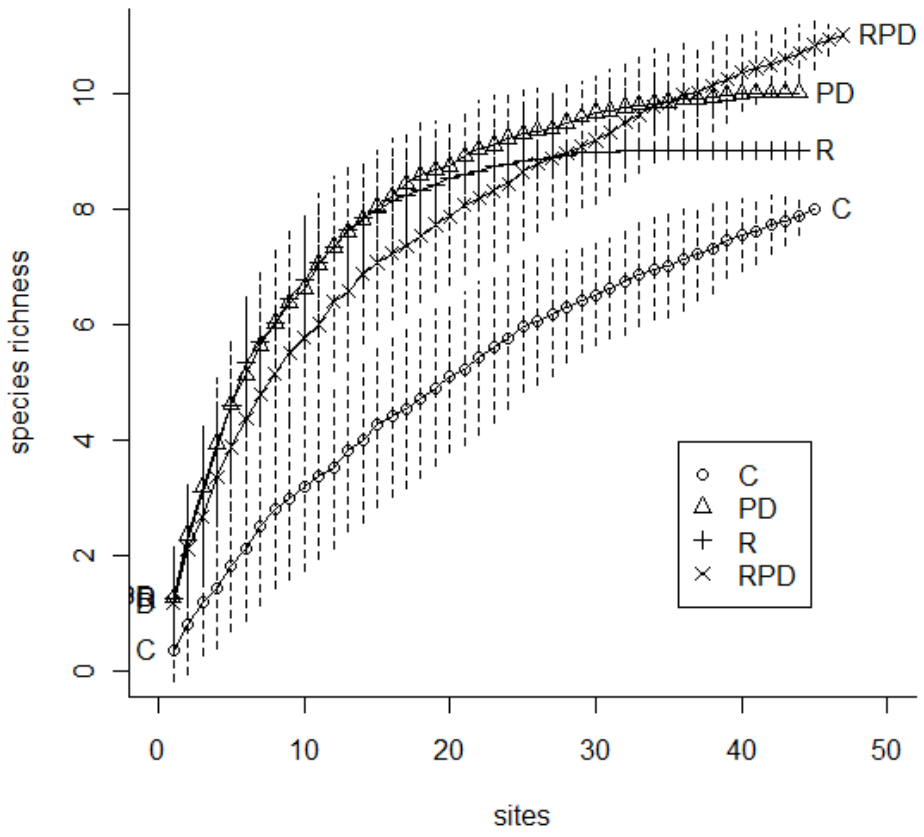

Figure 2. ECM species accumulation curve for each treatment with confidence intervals (vertical bars) generated from ±1 standard deviation sampled randomly using 1000 permutations. The number of chestnut seedlings sampled from the treatment plots is on the x-axis and the accumulative ECM species are shown on the y-axis.

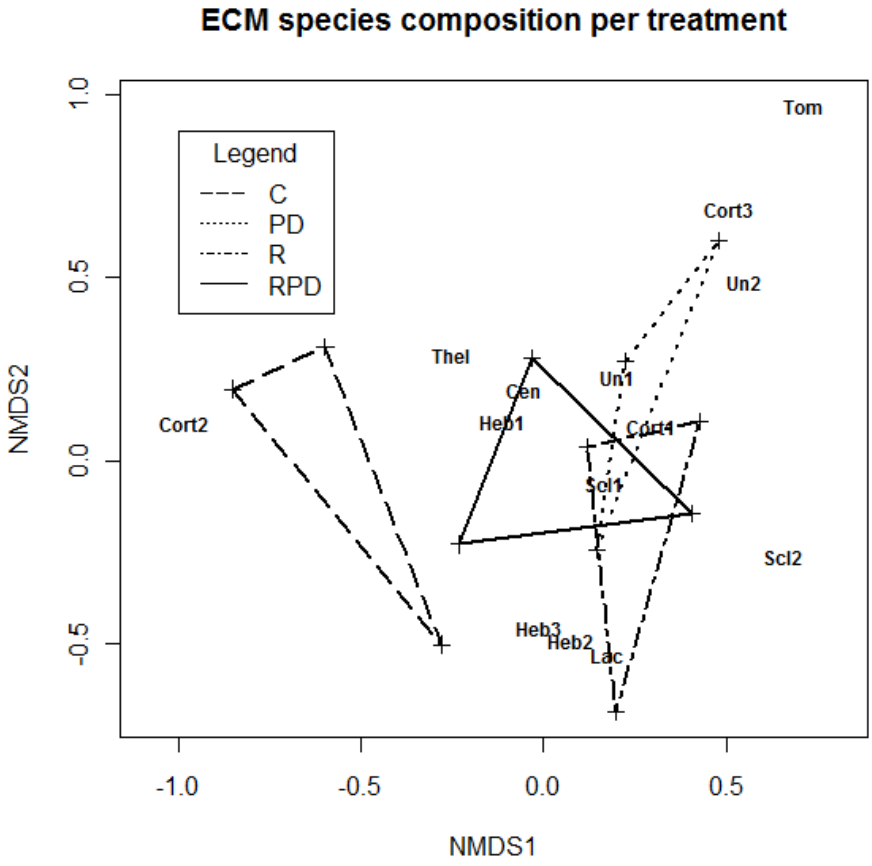

Figure 3. NMDS ordination comparing ECM species sampled among the soil treatments (C = control, R = ripped, PD = plowed and disked, RPD = ripped + plowed and disked) two years after planting. There was a significant difference when mechanical treatments were compared to the control plots (MANOVA, f = 0.24, p = 0.015).

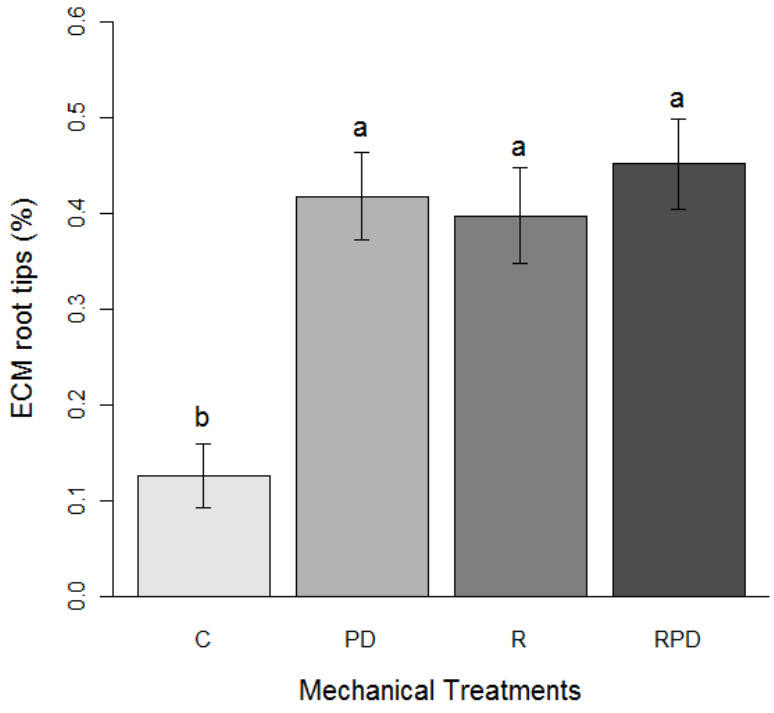

Figure 4. ECM root colonization (%) of chestnut seedlings among the treatment plots (C = control, R = ripped, PD = plowed and disked, RPD = ripped + plowed and disked). Error bars are ± 1 SE, bars with different letters are different at P < 0.05 determined by Tukey's HSD.

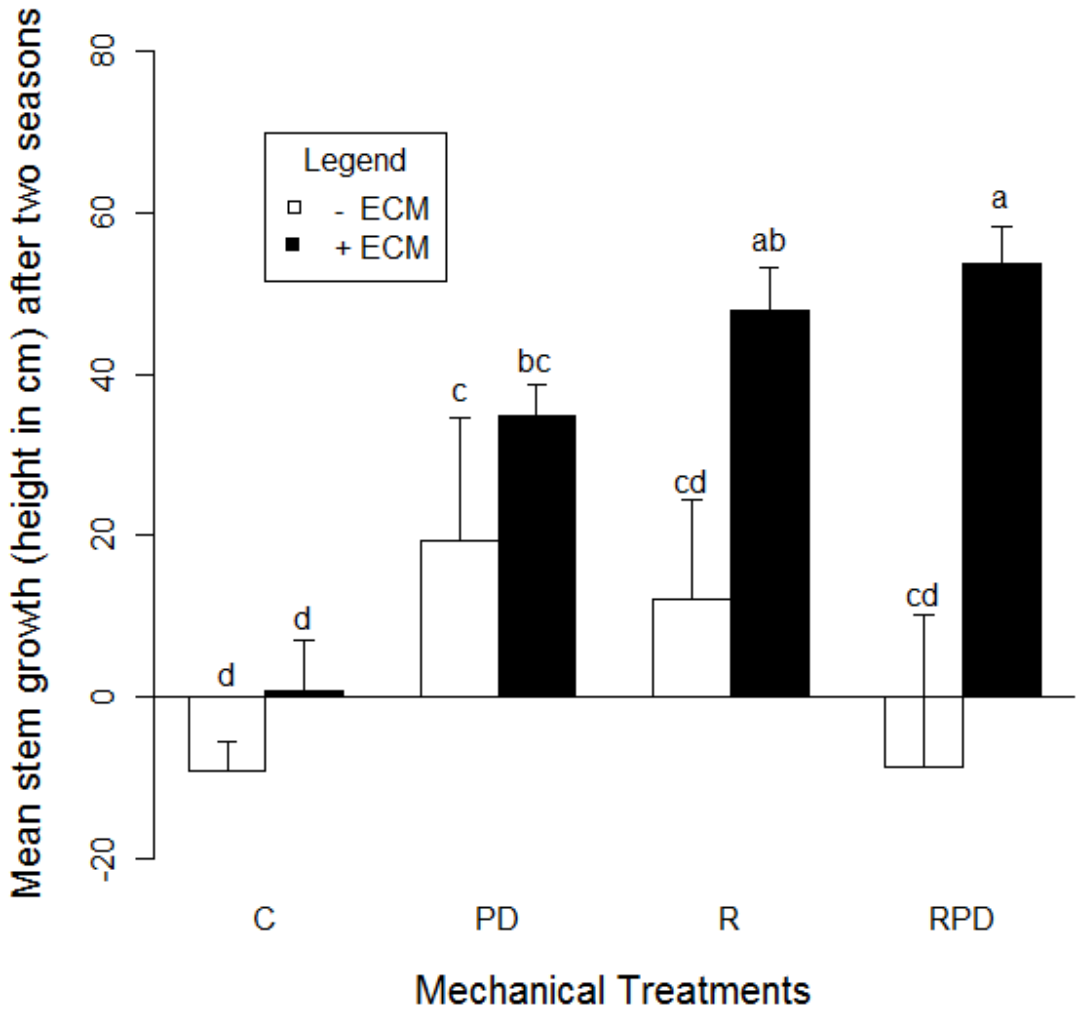

Figure 5. Mean (± 1 SE) chestnut seedling height (cm) of non-ECM inoculated seedlings (white bars) and seedlings that were naturally inoculated with native EM fungi (black bars) per soil treatment (C = control, R = ripped, PD = plowed and disked, RPD = ripped + plowed and disked). Bars sharing common letters do not significantly differ at $\alpha = 0.05$ determined by Tukey's HSD.

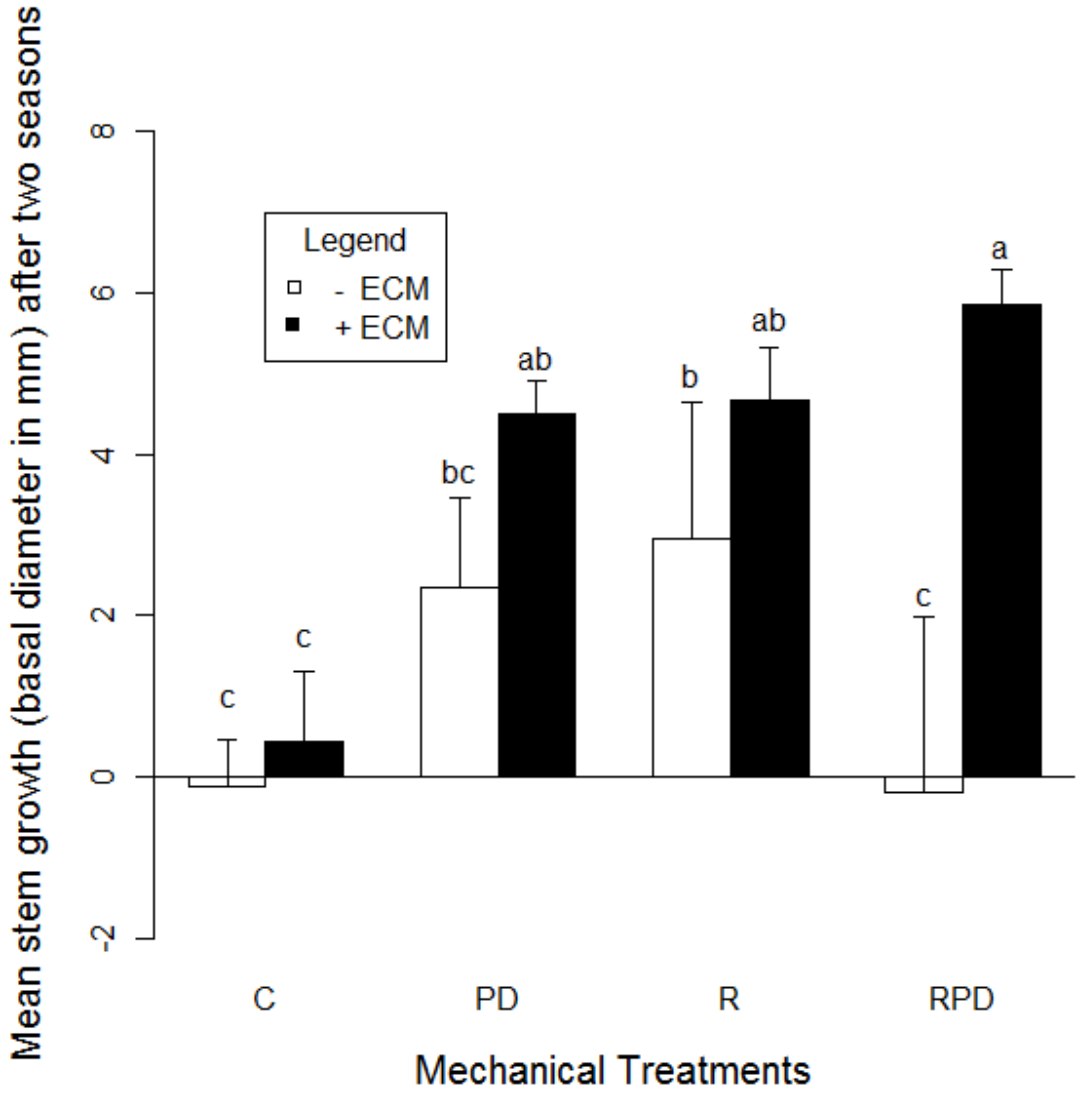

Figure 6. Mean (± 1 SE) basal diameter increase (mm) between non-ECM inoculated chestnut seedlings (white bars) and seedlings that were naturally inoculated with native EM fungi (black bars) per soil treatment (C = control, R = ripped, PD = plowed and disked, RPD = ripped + plowed and disked). Bars sharing common letters do not significantly differ at α = 0.05 determined by Tukey's HSD.

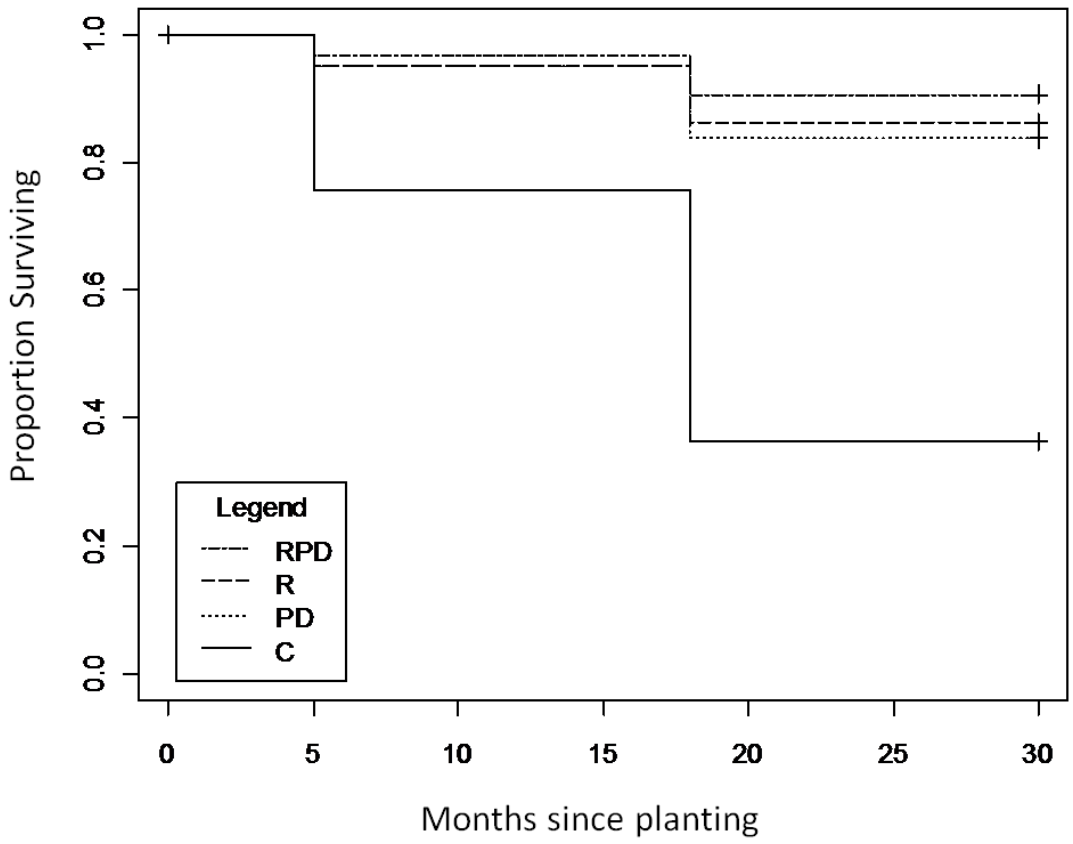

Figure 7. Survival data for chestnut and chestnut hybrids recorded after six, 18, and 30 months for chestnuts in the mechanical treatments (dashed lines) relative to the control (solid line). Soil treatments had a significant effect on survival (Cox proportional hazard model, Likelihood = 564, df = 3, $P < 0.0001$).

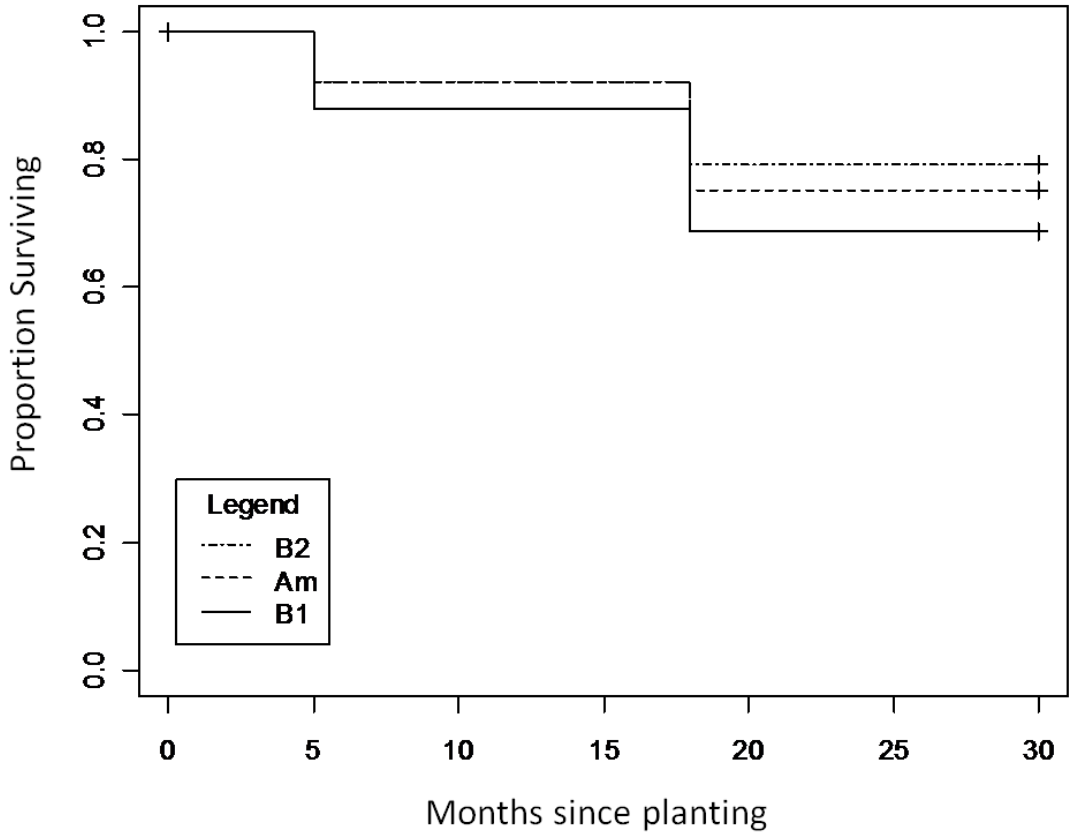

Figure 8. Survival data for the chestnut seedling types monitored over 30 months. B2 hybrid (dotted and dashed line) had a significantly higher survival rate than B1 hybrid chestnut (solid line) (Cox proportional hazard model, Likelihood ratio test= 20.4 on 2 df, *P* < 0.0001). Pure American chestnuts (dashed line) was intermediate, statistically similar to both hybrid lines.

Chapter 4

The influence of introduced fungal inoculum on root colonization potential and community composition of native ectomycorrhizal species on blight-resistant chestnut hybrids on reclaimed mine sites.

Abstract

The objective of this study was to evaluate the influence five different species of ectomycorrhizal (ECM) fungi have on the root colonization of native fungi on blight resistant chestnut hybrids (*Castanea dentata x C. mollissima*) in a reclaimed mine in central Ohio. The five species were *Hebeloma crustuliniforme, Laccaria bicolor, Scleroderma polyrhizum, Amanita rubescens,* and *Suillus luteus.* We used a combination of DNA sequencing of the ITS region and phylogenetic analyses to indentify fungi found on roots after 12 and 18 months in the field. Non-metric multidimensional scaling (NMDS) ordinations were used to determine if ECM community composition was influenced by the fungal inoculum used. The results of this study demonstrated that these selected ECM species do not persist on chestnut after one year in the field. In addition, these selected ECM species did not impede natural root colonization of native fungi or influence ECM community composition after two growing seasons. Although these species did not persist in the field, the presence of ECM inoculum (with the exception of *Amanita*) greatly contributed to the survival of hybrid chestnut seedlings. Therefore, introduced inoculum that was present in the very early stages of outplanting had persisting effects with regard to seedling establishment in the field, even if the original inoculum did not persist. ECM fungi native to the area colonized chestnuts resulting in increased growth rates. These native assemblages may contain species better able to form functional mycorrhizas under these environmental extremes. Therefore, the conservation of these species may be necessary to facilitate long-term survival of deciduous tree species historically native to these lands.

Introduction

Employing ectomycorrhizal (ECM) inoculum prior to outplanting is a common practice in restoration projects using hardwood trees on reclaimed mine land (Castellano 1996). This technique enhances the establishing seedling's ability to absorb water and nutrients, tolerate heavy metals and low pH, and protect against root pathogens in the early stages of plant establishment (Marx 1972; Danielson 1985; Walker 2004; Nara 2005). Seedlings used in reclamation projects are either pre-inoculated with selected ECM fungi in field nurseries or in greenhouses as potted plants. In many instances, field or greenhouse seedlings can become inoculated by fungi either native to that particular field or greenhouse environment. Ensuring maximized root colonization by the target fungal species is a resource-consuming endeavor. Therefore, great effort is taken to select the best ECM fungi suited for a certain host tree species. In addition to host specificity, abiotic and biotic factors may influence a functional, persistent ectomycorrhizae in the field. Ecological specificity is a phenomenon that recognizes that environmental conditions may play a direct role in determining host specificity (Molina et al. 1992). This explains why the EMC syntheses observed in the laboratory may differ from what is sampled in the field (Dahlberg and Finlay 1999). In order for the host plant to receive the benefits of an ECM symbiont, it must be able to maintain functional mycorrhizas under the environmental conditions at a specific planting site (Perry et al. 1989). These manipulations might bypass some stages of natural succession and accelerate the establishment of late successional tree species in initial plantings.

A second consideration is whether introduced fungi will inhibit the root colonization of ECM fungi native to a particular field site. Community composition is often affected by the sequence of species arrival; this is referred to as priority effects. These priority effects can involve early colonists negatively affecting the performance of later arrivals through preemption of shared resources (Alford and Wilbur 1985; Shorrocks and Bingley 1994). Interspecific species interactions demonstrate that early arrivals may exert strong inhibitory priority effects on later species. With regard to using inoculum in tree plantings, ECM species already colonizing tree roots have the potential to completely exclude later ECM species. However, these "later arrivals" may be native

fungi better suited to facilitate the survival of native plant species in disturbed environments. Certain environments may contain species or genotypes of organisms that can better survive human-caused environmental stresses (Gerhing et al. 1998) and better facilitate the establishment of native plant species. Therefore, careful attention should be given to the order in which species are introduced in disturbed systems so that priority effects and direct species interactions do not interfere with plant and fungal species that may play pivotal roles in ecosystem function (Palmer et al. 1997).

Two recent lab studies found contrasting results with respect to the role of priority effects in ECM species interactions. Lilleskov and Bruns (2003) found roots of pine seedlings originally colonized with ECM fungus *Rhizopogon occidentalis* were displaced by a second ECM fungus, *Tomentella sublilacina*. In contrast, Kennedy et al. (2009) found a priority effect; the first colonizing species became the competitively dominant. It has been reported that introduced inoculum may persist a couple of years and eventually become displaced by native species (Jones et al. 1997). Other studies have reported introduced inoculum to persist many years after the initial planting (Selosse et al. 1998; Sawyer et al. 2001; Di Battista et al. 2002). Prior studies using American chestnut on reclaimed mine lands indicate that ECM species present on root systems may deter the colonization of species present on mine sites (Bauman unpublished data, dissertation chapter 3). It is not clear whether the better competitor translates into the better symbiont for an establishing seedling. In addition, environmental conditions may play a very important role in maintaining the beneficial status of ECM root colonization (Kennedy and Bruns 2005). Because differing plant-fungal pairings can result in significant variations in host response (Bever 2002; Nara 2006), evaluating the best plant-fungal combination for a specific site becomes an important management strategy in mine reclamation.

The objective of this study was to evaluate the influence five different species of ECM have on the root colonization of native fungi on a reclaimed mine in central Ohio. The five species were *Hebeloma crustuliniforme, Laccaria bicolor, Scleroderma polyrhizum, Amanita rubescens,* and *Suillus luteus.* Each of these species was selected because they have been reported to be early colonizers and form mycorrhizas with American chestnut and chestnut hybrids in the laboratory and greenhouse (Hiremath per

comm.). *H. crustuliniforme* is a basidomycete fungus that is a proficient root colonizer of young trees. Perrin and Garbaye (1983) reported that this fungus has the ability to protect seedlings against root pathogens. *L. bicolor* has been used extensively as a commercial inoculum, particularly on Douglas fir in both nurseries and plantations (Le Tacon et al. 2005). It has been reported to improve biomass production and K and Mg assimilation by increasing the mineral weathering and uptake of these nutrients (Christophe et al. 2010); a desirable attribute sought out for mine sites low in available nutrients and high in parent material. *S. polyrhizum* will readily form mycorrhizas by either mycelium or spore propagules and is used in nursery inoculums (Duñabeitia et al. 1996). More notably, *Scleroderma* spp. tolerates stressful environments and have been reported to increase growth and survival of its host plants in highly disturbed mine sites (Jefferies 1999; Bauman et al. in reveiw). *A. rubescens* has the ability to accumulate heavy metals in its tissues (Demirbas 2001) and may aid in plant establishment and growth by alleviating toxic amounts of metal absorbed by the plant. Species in the genus *Suillus* exhibit a high degree of host specificity to *Pinus* spp. with few exceptions (Dahlberg and Finlay 1999). *S. luteus* is a pioneer ECM fungus found on young seedlings (usually pine) in soil polluted with heavy metals (Muller et al. 2007).

Each one year-old chestnut seedling was planted with an accompanying chestnut seed to determine the movement of our introduced inoculum. This allowed us to describe the inoculums' propensity to outcompete native ECM for the germinating chestnut root system. To determine if seasonal dynamics influenced species competition for chestnut, we sampled in the spring (12 months after planting) and again in the fall (18 months after planting). Lastly, we measured host response to determine whether introduced inoculum influenced survival and growth of chestnut hybrids. We hypothesize differential persistence of ECM on chestnut and those that have strong priority effects will out-compete native species for root colonization. In addition, we hypothesize that better competitors will have an increased benefit to the establishing chestnut.

Methods and Materials

Study Site

In the spring of 2006, blight-resistant chestnuts hybrids B1-F3 were planted in a greenhouse in Delaware, Ohio by USDA Forest Service(Lehtoma per comm.). B1-F3 hybrids are progeny of initial backcrossing (B1-F1). Trees of the B1-F1 that exhibit blight-resistance were then intercrossed two more times to result in the B1-F3chestnut hybrid genotype (Hebard 2001). Statistically, these trees average 87% American chestnut alleles. Seeds were germinated in a peat/vermiculite medium that included one of the following ECM fungi: *H.crustuliniforme, L. bicolor, S. polyrhizum, A. rubescens,* and *S.luteus* (inoculation techniques describe in Marx and Bryan 1975). Seedlings were grown in a greenhouse under natural light, watered as needed, and fertilized monthly with 12-12-12 liquid fertilizer for one year. All chestnut hybrids were sampled and ITS region sequenced (DNA sequencing described below) to verify ECM colonization prior to planting. Only chestnut seedlings with ≥ 50% ECM colonization were selected for field planting. Non-inoculated chestnut hybrids were used as control plants.

In the spring of 2007, plants were installed as one-year old potted seedlings in the Tri-Valley Wildlife refuge in Madison County, Ohio. This mine site was reclaimed in the 1980s. However, this grassland is primarily vegetated with the original species used for reclamation (*Festuca* spp., and *Lespedeza* spp.) with small patches of ragweed (*Ambrosia* spp.), and goldenrod (*Solidago* spp.). Small pockets of forest comprised primarily of *Quercus*, *Pinus*, and *Acer* species were left undisturbed at the time when these lands were mined. This area receives an average of approximately 99 cm of precipitation annually. During the 2007 and 2008 growing season the summer climate was relatively dry to moderate drought with annual temperatures averaging 22° C during the growing season (17°, 28°, and 11° C, spring, summer and fall, respectively; National Climatic Data Center). Soil chemistry was similar between plots and the averages are as follows: soil pH 5.4, CEC 8.13, organic matter 1.5 %, phosphorus 8.7 ppm, potassium 77 ppm, magnesium 155 ppm, calcium 640.3 ppm, nitrogen (NO3-N) 2, manganese 4.4 ppm, aluminum 5.29, sand 55%, clay 24%, and silt 21%. Soil chemistry was measured at both Brian McCarthy's laboratory (described by McCarthy 1997) and at Spectrum Analytic, Inc, Washington Courthouse, Ohio.

Three 20 x 6 meter (m) blocks were installed by plowing and disking using a conventional tractor. Each plot contained 36, one year-old chestnut seedlings, six of each treatment type (five different mycorrhizal + non-inoculated control). Each plot was replicated three times for a total of 108 seedlings. Seedlings were planted in April of 2007 at a spacing of 1.5 m with the between-row spacing of 1.5 m. Seedlings were planted with the root collar level with the grade of the soil, tagged, and backfilled with original soil and one 20-10-5 slow release fertilizer pellet. A weed mat was installed with all four corners pinned using sod staples to control reemerging previous groundcover. To prevent herbivory, a 1.5 m tall fence was constructed out of chicken wire and t-posts. Each seedling was planted with three pure American chestnut seeds (trap trees), 30 cm from center of one-year-old seedling (Figure 1). To insure seed germination, all seeds were stored in the dark in moist peat at room temperature until radicles had emerged from all seeds.

ECM Sampling and identification

Growth parameters such as plant height, number of leaves per seedling, basal diameter, and leaf area (cm^2) were recorded after 12 and 18 months in the field. After 18 months, 30 chestnuts were randomly selected for leaf tissue analysis. Twenty-five leaves per seedling were harvested in the field. They were returned to the lab where they were immediately packaged in paper bags and sent to Spectrum Analytic Inc., Washington Court House, Ohio, for tissue analysis. Survival data were recorded monthly for the duration of the first growing season and once again at the end of the second growing season. At 12 months (May 2008) 103 trap trees were destructively sampled. Seedlings were carefully removed from the field, returned to the lab where root systems were washed, and observed under the stereoscope for mycorrhizal formation. Two hundred and fifty root tips per seedling were randomly selected from each of the 103 chestnut seedlings (25,750 root tips total for spring sampling) and viewed under a dissecting microscope for presence of fungal sheath and separated into morphotypes. Each morphotype per seedling was selected for DNA extraction by removing three mm of root tip and transferring this ECM tip into a microcentifuge tube for storage at -70˚ C. This was repeated in the fall (October 2008), 48 trap trees were destructively sampled and

returned to the lab. Root tips (250 per sample, 12,000 total) were inspected and sampled as above. In addition, 60 chestnuts that were planted as potted plants representing all inoculum treatment types were randomly selected and non-destructively sampled. To accomplish this, soil was carefully removed with a spade shovel to expose the chestnut root system at a depth of 25 cm, 45 cm width. Roots were sampled from the seedlings carefully sifting away soil. Samples were then stored on ice in the field and returned to the laboratory. Once in the lab, roots were washed with autoclaved distilled water, and placed into a Petri dish with sterile water. Two-hundred and fifty roots per sample (15,000 root tips total) were inspected for the presence of a fungal sheath and sampled for DNA extraction as stated above.

To molecularly identify the type of ECM fungi, a combination of BLAST searches and phylogenetic analyses was employed on the ITS region of the fungal DNA. DNA was extracted from the collected root tips using QIAgen Dneasy Plant Mini-Prep kits purchased through QIAGEN Inc. Primers ITS1-F (5' cttggtcatttaggaagtaa 3') and ITS4 (5' tcctccgcttattgatatgc 3') were used to amplify internal transcribed spacer sequences (ITS) during PCR (Gardes and Bruns 1993). PCR 15 µl reactions were mixed based on the following concentrations: 9 µl of molecular grade water, 3 µl of 5x Green GoTaq® Reaction Buffer, 0.125 µl of Promega® *Taq* DNA Polermerase, .2 µl of 25µM of each primer, 1µl of dNTPS (200µM each of dATP, dCTP, dGTP, and dTTp), and 1 µl of DNA template. Temperature cycling was accomplished using a programmable Thermal Cycler Heating block. Times and temperatures were programmed as described by Gardes and Burns (1993): the initial denaturation step of 94ºC for 85 s followed by 35 amplification cycles of denaturation, annealing, and extension. The temperature and times for the first 13 cycles were 95ºC for 35 s, 55ºC for 55 s, and 72ºC for 45 s. Cycles 14-26 and 27-35 repeated the above parameters with lengthened extension steps 120 and 180 s, respectively. When the 35 cycles were completed the samples were programmed to incubate for 10 min at 72ºC for 45 s. PCR reactions were run of 1% argarose gels for 20 minutes to allow for the visualization of fungal DNA. Negative controls lacking template were used to ensure that the DNA amplified was from the root samples and not from contamination from reagents and reaction mixtures.

PCR product was cleaned by using Clean-Gene. Samples were prepared for sequencing using BigDye Terminator v3.1 Cycle Sequencing Kits by mixing 10 µl reactions of the following concentrations: 2 µl BigDye Terminator v3.1 Reaction Mix, 3 µl 5 X Sequencing dilution buffer, 1 µl primer, and 1 µl of template. Sequencing cycle to label DNA for sequencing was performed on a programmable Thermal Cycler for the following cycles: 96ºC for 1 min followed by 25 cycles of 10 s at 96ºC, 5 s at 50 ºC, and 4 min at 60 ºC. Following labeling, products were purified to remove all unincorporated dye-labeled terminators by alcohol precipitation. Sequencing was performed with TheApplied Biosystem ABI Prism 3730 DNA Analyzer (Bioinformatics facility, Miami University, Oxford, Ohio). Sequences were analyzed and edited using Sequencher 4.2 software (Gene Codes, Ann Arbor, Michigan). To identify fungi found on roots, sequenced ITS region of our samples were compared to known species in GenBank using BLAST searches (Altschul et al. 1997). Genera reported here are based on the best match of vouchered fungi in GenBank. Characteristics are based on statistical analysis that generates a bit value, gap score, and an Expect (E) value. The bit score is a value that is indicative of how well the sequenced aligned with the known sequence in the database. The higher the score, the better the match. The gap score introduced into an alignment compensates for insertions and deletions in one sequence relative to another. The E value is a parameter that describes the probability of the number of matches that can be generated by chance. It decreases exponentially as the match increases; a score closest to zero is the most significant. Thus when deciding the genera to report here, a threshold that included an E-value of 0, highest ranking bit value, and a gap value of < 4. To verify that the *Scleroderma* species sampled were not part of the inoculum we introduced, a phylogeny was built consisting of known vouchered sequences in the NCBI public database that would align to the sampled fungi and known phylogenies (Binder and Bresinsky 2002). The sequences and were first auto-aligned using MUSCLE then manually aligned in Se-Al v2.0a11. Maximum-parsimony analyses were carried out using PAUP* 4.0b10 (Swofford 1998) using the heuristic search mode with 1000 additional sequence replicates, tree bisection-reconnection branch swapping, and zero-length branches. Fifty percent majority rule consensus trees were calculated and branch support was assessed by bootstrapping with simple taxon addition with 100 replicates.

Statistical Analyses

To compare ECM community composition, a non-metric multidimensional scaling (NMDS) ordination was used to determine if ECM community composition was influenced by season sampled, chestnut tree type sampled, and/or inoculum used prior to planting. To improve the NMDS ordinations, the data were square root transformed and standardized via Wisconsin double standardization (Oksanen 2005). Bray-Curtis dissimilarities were employed due to their preferred analysis for community data due to the restriction within the range of 1 to 0 (Kindt and Coe 2005). The maximum number of random starts in search at was set at 100 with k=2 stress value. The sites were plotted on an ordination graph and convex hulls were used to outline the various treatments in the study. A permutational multivariate analysis of variance was used to test for significant differences among treatments. All ECM NMDS ordinations were performed using Vegan: Community Ecology Package version 1.6.9. (Oksanen et al. 2005; Appendix 5).

ECM colonization per treatment was assessed by taking the proportion (#ECM tips/250) of ECM colonized root tips from trap trees (n= 48) and inoculated one-year-old chestnut hybrids (n= 60) sampled in the Fall after two field seasons. Arcsine square root transformation, commonly used for proportions (0 to 1), was used to control for unequal variances. Differences in colonization among inoculum types and between chestnut tree types (pure American trap trees verses hybrid potted plants) were determined using a one-way analysis of variance (ANOVA). Growth parameters such as seedling height (cm), basal diameter, and leaf area (cm^2) measured at the end of the second field season were subtracted by the original measurement and divided by the number of months of the growing season to calculate relative growth rate (RGR) per month. Data were transformed by setting the most negative growth value to zero, adding accordingly to the samples, and using Log+1 transformation (McCarthy per comm.). The differences were considered significant when $p \leq 0.05$ according to the F test. One-way ANOVAs were used to determine differences in macro and micronutrient concentrations in leaf tissue between the ECM and non-ECM chestnut seedlings. All ANOVAs were performed using R v2.91 (R Development Core Team 2009; Appendix 6).

Results

Nine distinct morphotypes were described and photographed from the 211 sampled seedlings (60 hybrid chestnuts and 151 pure American trap trees; Fig 2.) Three additional ECM species (photos not available) were detected when the ITS region was sequenced, revealing a total of 12 ECM species sampled in this study (Table 1). Two of the ECM sequences match to different "uncultured ectomycorrhizae" when compared to GenBank using BLAST (Unknown ECM 1 and 2). The other 10 matched existing sequences.

Scleroderma spp. 1 and 2 were most abundant in this survey (74% relative abundance; Fig. 2 panels a and b; Table 1). *Cenococcum* and *Thelephora* species ranked 3rd and 4th, respectively; followed by *Tomentella* and two *Hebeloma* spp. Singletons unique to the hybrids include the two unknown ECM and *Pisolithus* spp. Singletons shared by both the chestnut hybrids and the pure American trap trees included an unidentified member of Thelephoraceae and a *Cortinarius* spp.

To confirm that the species sampled were not part of the inoculum, a maximum parsimony tree was used to demonstrate the phylogenetic position of the *Scleroderma* ECM root tips. Root tip sequences sampled from both hybrids and trap trees were compared among vouchered sequences and the inoculum sequence. The resulting phylogeny illustrates the *Scleroderma* root tips were not part of the inoculating strain, rather, were closer in relation to *S. areolatum* and *S. citrinum* than to the inoculating *S. polyrhizum* (Fig. 3).

Non-metric multidimensional scaling (NMDS) ordinations followed by permutation MANOVAs were used to test for the influence time of sampling, tree source, and inoculum on ECM community composition. The ECM community composition of fungi on the roots of pure American trap trees sampled in the spring was not significantly different than those sampled in the fall (Fig. 4; $F = 1.36$, $df = 1$, $p = 0.28$). No significant differences existed between the pure American trap trees and chestnut hybrids (Fig. 5; $F = 0.73$, $DF = 1$, $p = 0.57$). Lastly, no differences in ECM community composition were found among the inoculated treatment groups (Fig. 6; $F = 0.85$, $df = 5$; $p = 0.62$).

ECM root colonization and growth response:

There were significant differences in survival over 18 months (Fig. 7; Cox proportional hazard model, Likelihood = 121, df = 5, $P < 0.0001$). Chestnuts inoculated with *S. lutues* and *S. polyrhizum* had the highest survival rates (87% and 81%, respectively). This was followed by *L. bicolor* (61%), *H. crustuliniforme* (58%), *A. rubescens* (28%), and the non-inoculated control plants (16%).

ECM inoculum did not have a significant influence on chestnut root colonization; all chestnut hybrids had similar natural colonization (Fig. 8; ANOVA $F = 0.84$, df = 5, P = 0.52,). The effect of native ECM root colonization was compared per genus, therefore multiple species were pooled by genus (*Scleroderma* species and *Thelephora* spp., *Tomentella* spp. and ECM identified in the family a Thelephoraceae were pooled and referred to as Thelephoraceae). When relative growth rates were compared for height (cm), basal diameter (mm), and leaf area (cm^2), the following trend emerged: ECM species in genus *Scleroderma* and Thelephoraceae family significantly improved growth rates on the hybrid chestnuts (ANOVA: height F = 5.65, df = 5, $p = 0.0005$, basal diameter F = 4.81, df = 4, $p = 0.002$, leaf area F = 7.72, df = 4, $p < 0.0001$, Figure 9). This was not the case for *Cenococcum* spp. or Unknown ECM, their growth rates were comparable to chestnuts that were not naturally inoculated (Figure 9).

Leaf tissue was analyzed for nutrient concentration between the ECM and non-ECM seedlings. Seedlings found with ECM colonization did not have higher nutrient content than seedlings without native ECM colonization (all P > 0.05; Table 2). There were also no differences in heavy metal composition between seedlings colonized by ECM versus those not colonized (all P > 0.05; Table 2).

Discussion

Although ECM colonization was verified prior to outplanting, chestnut hybrids did not maintain their association with the fungal species used to inoculate the seedlings in the greenhouse. The results of this study show: 1) this method of inoculation with these selected ECM species does not persist in the field after 18 months, however the inoculation did contribute to a higher survival within the first few months, 2) these introduced ECM species do not influence ECM community composition after two

growing seasons, 3) the presence of these ECM species does not impede natural root colonization by native fungi, 4) root colonization by certain native fungi resulted in positive effects on chestnut seedling growth.

ECM species *H. crustuliniforme, L. bicolor, S. polyrhizum, A. rubescens*, and *S. luteus* did not maintain their mycorrhizal associations on chestnuts after 12 or 18 months in the field. This was in contrast to previous studies that have reported introduced inoculum persisting on their host plant years after planting (Garbaye and Churin 1997). Species of *Suillus* have been reported to persist for four years in Mediterranean pine plantations (El Karkouri et al. 2006). *Laccaria bicolor* had maintained functional mycorrhizas for over 10 years in Douglas fir plantations (Selosse et al. 1998; Di Battista et al. 2002). *Amanita* strains have persisted for over 30 years on Monterey pine in Australian plantations (Sawyer et al. 2001). *H. crustuliniforme* has been reported to persist over two years after introduction and significantly impede the root colonization by native fungi (Jones et al. 2002; Bauman unpublished data).

We found that the presence of introduced ECM fungi did not have an influence on the ECM community composition on pure American or hybrid chestnuts 18 months after planting. Conversely, Bauman (unpublished data, dissertation chapter 3) reported that bare root chestnuts that were naturally inoculated in a field nursery by *Hebeloma* and *Cortinarius* species appeared to inhibit the colonization of indigenous *Scleroderma* species when transplanted to a reclamation site. This priority effect has been previously documented on pine seedlings inoculated with *H. crustuliniforme* (Garbaye and Churin 1997) and *Rhizopogon* species (Kennedy et al. 2009), demonstrating a competitive advantage introduced fungi have over indigenous fungi in the field. This inhibition of native colonization could be caused by direct antagonistic interactions by means of mycelia overgrowth (Wu et al. 1999). However, this was not observed during this current study. Root colonization did not average above 50% colonized, which indicates that competitive dominance was not a factor.

Previous studies have speculated that the host plant can decrease mycorrhizal receptivity of roots to less productive symbionts to minimize below-ground carbon loss if they are receiving sufficient benefits from another species (Kennedy and Bruns 2005). The ability of a plant to decrease colonization in high nutrient settings indicates that the

host plant may have substantial control over both root colonization and ECM species interactions (Johnson et al. 1997). If the plant host increases carbon allocation to the most beneficial fungal symbiont, it can be predicted that the best fungal competitors are the species that provide the greatest benefit to the plant (Kennedy and Bruns 2005). In our study, species of *Scleroderma* and *Thelephora* – type species (pooled with Thelephoraceae) showed significant growth increases on the hybrid chestnuts. Although we did not see any differences in foliar nutrient concentrations in ECM plants, benefits may have been increased water uptake, an attribute associated with the rhizomorph production of species such as *Scleroderma*. In contrast, chestnuts colonized by *Cenococcum* and unknown ECM species 1 had similar growth rates to the non-ECM controls. Dulmer (2006) reported the presence of *Cenococcum geophilum* correlated with unhealthy chestnuts and speculated that this species of fungi may have a negative impact on the health of these seedlings. Species like *Cenococcum* have been shown to increase in abundance in the absence of a better competitor (Dickie et al 2004). If competition between different species of fungi is strongly mediated by plant feedbacks, carbon allocated to a less productive symbiont like *Cenococcum* may decreased when a better competitor is present.

Lilleskov and Bruns (2003) found that pine seedlings inoculated with *Rhizopogon occidentalis* were completely replaced by *Tomentella sublilacina*. In their study, *R. occidentalis* was more effective at colonizing roots when nutrients were not limiting. However, when nutrients became a limiting factor, *R. occidentalis*, an ECM species that tends to colonize effectively under resource-rich conditions, was displaced by the better competitor (*T. sublilacina*). What was similar in our study was the shift in resource availability from greenhouse conditions, where both macro and micronutrients were supplied without interspecific root competition, to a resource poor soil environment with competing vegetation. Therefore, we selected ECM fungi that colonized hybrid chestnut in the greenhouse under controlled conditions, and not for fungal species with the ability to persist under low nutrients and water availability. This then undermines ecological specificity, which takes in to consideration all of the abiotic and biotic variables that may influence a functional, persistent ectomycorrhizae in the field (Molina et al. 1992; Dahlberg and Finlay 1999; Taylor 2002; Dickie 2007). Temperature, drought, soil

chemistry, and competition may have all been factors contributing to the demise of the introduced inoculum.

Although the introduced inoculum may not have been able to extend beyond the original rhizosphere into the bulk field soil, all inocula present in the very early stages of outplanting had persisting effects with regard to seedling establishment in the field, presumably due to the ability of ECM to buffer transplant shock (Menkis et al. 2007). There was one exception, *A. rubenscens*. Chestnuts inoculated with this ECM species had survival similar to the control plants. This illustrates that ECM infection may not create symbioses that are uniform in all biological characteristics. Rather, these interactions may result in symbioses with varying attributes to the plants fitness under certain ecological conditions.

It may be of greater importance that this inoculum did not interfere with root colonization by the native ECM community. *Scleroderma* spp. were the most abundant and provided chestnuts with significant growth increases during this study. *Scleroderma* spp., such as *S. bovista*, *S. cepa*, *S. citrinum,* and *S. verrucosum,* have been used in commercial inocula due to their large host range and ability to colonize roots in disturbed environments where water availability is low (Marx 1969; Lu et al. 1998). Indigenous *Scleroderma* spp. has a high affinity for *Castanea* spp. (Meotto et al. 1999) and previous studies report a positive growth response in the field (Bauman in review). Planting methods that promote the colonization of indigenous ECM species may increase the presence and inoculum potential of these microbes to incoming plants. These native ECM assemblages may contain species better able to persist in these disturbed environments and provide greater benefit to its plant host. The conservation of these ECM species may be an important factor for the recruitment and long-term survival of tree species historically native to these lands.

This study sampled roots for ECM in both spring and fall to account for season differences. Seasonal dynamics in above-ground sporophore production has been well documented (Deacon and Fleming 1992). This has been less explored below-ground, however, recent studies have reported temporal partitioning among species in ECM communities (Walker et al. 2005; Koide et al. 2007). Although there were no significant differences in ECM community composition between spring and fall samples,

Scleroderma species 2 increased in relative abundance from 3% to 18% from spring to fall. It has been proposed that these seasonal dynamics may provide a mechanism allowing the coexistence of species (Koide et al. 2007). However, this mechanism may be more applicable in later succession when resources become limiting and temporal partitioning is required for stable species coexistence (Koide et al. 2007). This increase in abundance of *Scleroderma* species 2 may require further sampling to determine if these different *Scleroderma* species display a temporal variability overtime.

Lastly, this study explored whether ECM community differed between hybrid and pure American host genotype. There were no differences between the pure American trap seedlings and the B1-F3 hybrids with regard to ECM community. ECM communities are generally similar on host plants with comparable taxonomic and successional groups (Ishida et al. 2007). Further, proportion of ECM root tips and number of species sampled were similar between seedling types (pure American and B1-F3 hybrid; data not shown). ECM fungi generally exhibit intermediate-to-low host specificity; intermediate may restrict associations to a single host family (Molina et al. 1992) or host genus level (Malajczuk et al. 1982). Therefore, it was not unusual to document a similar ECM community composition between pure American (*C. dentata*) and hybrids (*C. dentata x C. mollissima*).

Implications for Practice:

- Our findings suggest that chestnuts inoculated with these ECM species in the greenhouse did not maintain their ECM symbiosis in the field after one growing season.
- These introduced ECM species did not impede natural root colonization of native fungi or influence ECM community composition after two growing seasons.
- Although these species did not persist in the field, the presence of ECM inoculum greatly contributed to the survival rates of hybrid chestnut seedlings. Therefore, introducing inoculum in the very early stages of outplanting aids in seedling establishment.
- ECM fungi native to the area colonized chestnuts resulting in increased growth rates. These native assemblages may contain species better able to persist under environmental extremities and the conservation of these species may be what is necessary to facilitate long-term survival of deciduous tree species historically native to these lands.

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Table 1. Molecular identification of ECM root tips ranked by relative abundance. Table includes relative abundance of fungal taxa on total seedlings (211 chestnuts) followed by one year old hybrids, and each of the two sampling periods. Table includes corresponding GenBank sequence accession numbers.

Species	Total	Hybrids	Spring Trap	Fall Trap	Accession
***Scleroderma* spp. 1**	**0.63**	**0.70**	**0.52**	**0.72**	**GU246983**
***Scleroderma* spp. 2**	**0.11**	**0.03**	**0.18**	**0.08**	**GU246984**
***Cenococcum* spp.**	**0.11**	**0.08**	**0.12**	**0.12**	**GU246986**
***Thelephora* spp.**	**0.06**	**0.03**	**0.10**	**0.04**	**GU246989**
***Tomentella* spp.**	**0.03**	**0.03**	**0.05**	**0**	**GU246993**
***Hebeloma* spp. 1**	**0.02**	**0.04**	**0.02**	**0.01**	**GU246997**
***Hebeloma* spp. 2**	**0.01**	**0.02**	**0.01**	**0.02**	**GU246985**
Unknown ECM 2	**0.01**	**0.03**	**0.01**	**0**	**GU246994**
***Cortinarius* spp. 1**	**0.01**	**0.01**	**0**	**0.02**	**GU246996**
Thelephoraceae	**0.01**	**0.01**	**0**	**0.01**	**GU246997**
Unknown ECM 1	**0.01**	**0.02**	**0**	**0**	**GU553376**
***Pisolithus* spp.**	**0.01**	**0.01**	**0**	**0**	**GU553367**

Table 2. Nutrient and metal concentration (±SE) from a subsample of seedling leaf tissue sampled 18 months after planting (n=11 per treatment). No significant differences were detected (all, $P > 0.05$).

Treatment	N ppm	P ppm	K ppm	Ca ppm	Mg ppm	Mn ppm
No ECM	1.42 ± 0.24	0.25 ± 0.05	0.69 ± 0.20	1.12 ± 0.14	0.48 ± 0.10	1793.0 ± 1145.93
ECM	1.39 ± 0.32	0.28 ± 0.07	0.65 ± 0.13	1.22 ± 0.19	0.45 ± 0.07	1374.75 ± 541.67

Treatment	S ppm	B ppm	Cu ppm	Fe ppm	Zn ppm	Na ppm
No ECM	0.14 ± 0.01	113.7 ± 53.67	3.14 ± 0.80	188.80 ± 91.0	39.40 ± 26.76	24.0 ± 3.39
ECM	0.13 ± 0.02	90.62 ± 28.93	3.06 ± 0.84	159.31 ± 68.42	27.63 ± 10.03	24.13 ± 8.19

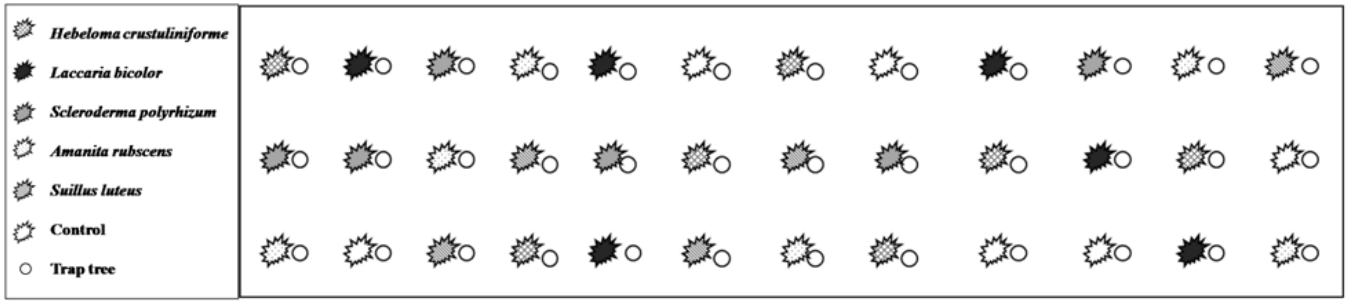

Figure 1. Plot design. Each treatment was randomly placed 1.5 meters apart with 1.5 meters provided for between the rows. Three pure American chestnuts were planted as seed placed 30 cm from one-year-old potted hybrids. This design was replicated three times.

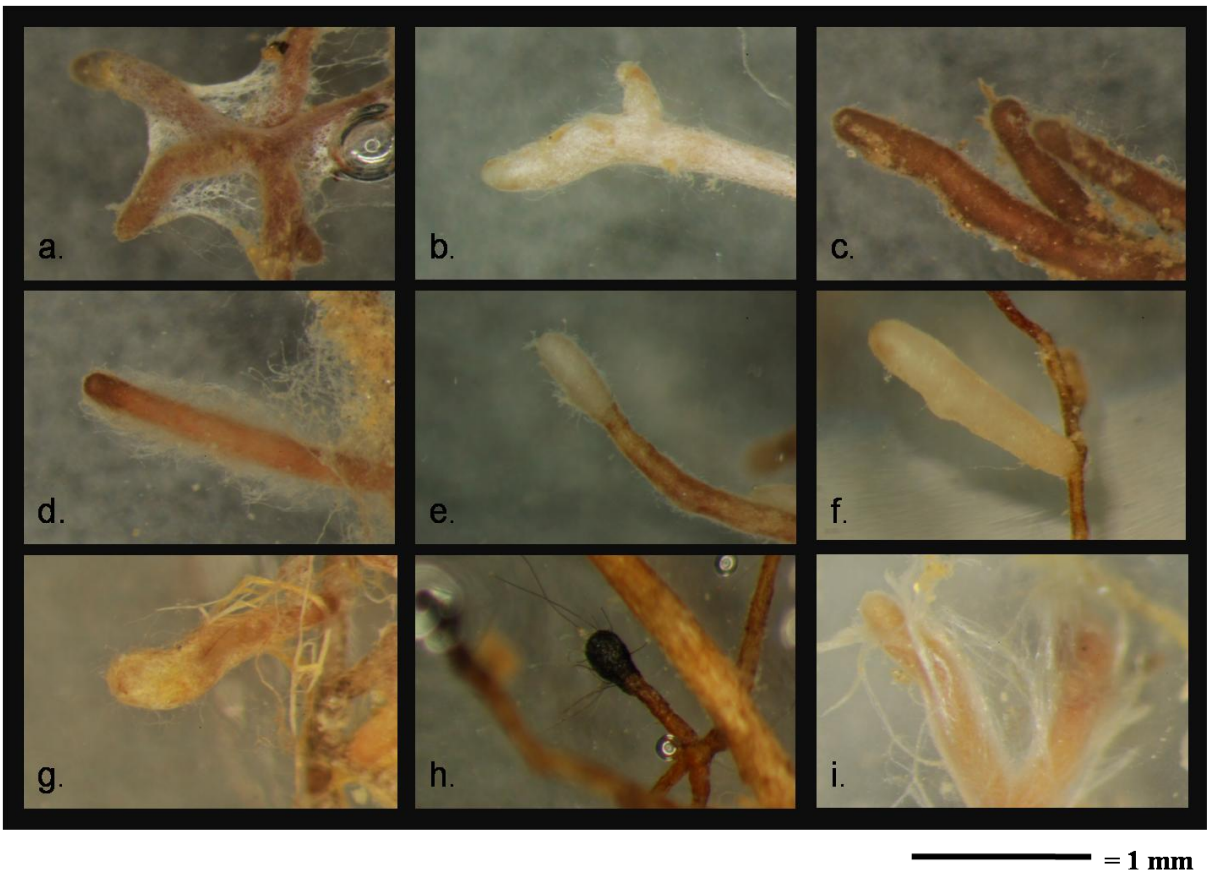

Figure 2. Photographed (45x) ECM morphotypes sampled from root tips from chestnut hybrids and pure American trap trees. Panels display the following that were matched to vouchered GenBank sequences: (a) *Scleroderma* spp. 1, (b) *Scleroderma* spp. 2, (c) *Thelephora* spp., (d) *Hebeloma* spp., (e) Thelephoraceae, (f) *Tomentella* spp., (g) *Pisolithus* spp., (h) *Cenococcum* spp., (i) *Cortinarius* spp..

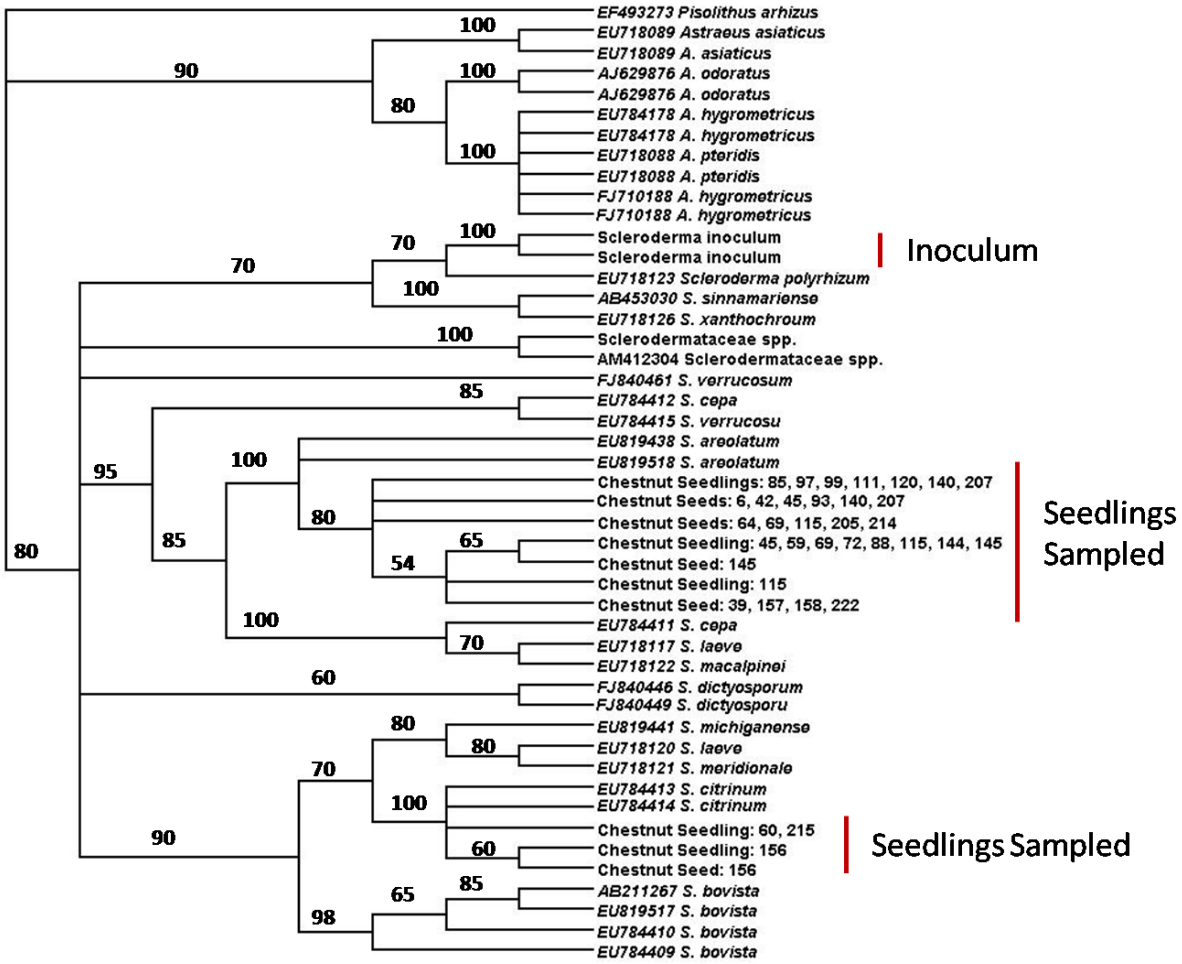

Figure 3. ITS phylogeny of *Scleroderma* species. Maximum parsimony 50% majority rule tree with bootstrap values shown. Each accession number represents vouchered ECM specimen. The positions of *Scleroderma* species 1 are within a clade that is closely allied with vouchered specimens *S. areolatum*, GenBank accession numbers EU819438 and EU819518. *Scleroderma* spp. 2 forms a clade with vouchered *S. citrinum* GenBank accession numbers EU784413 and EU784414. This indicates that the ECM fungi sampled from the root tips were not part of the original *Scleroderma* inoculum.

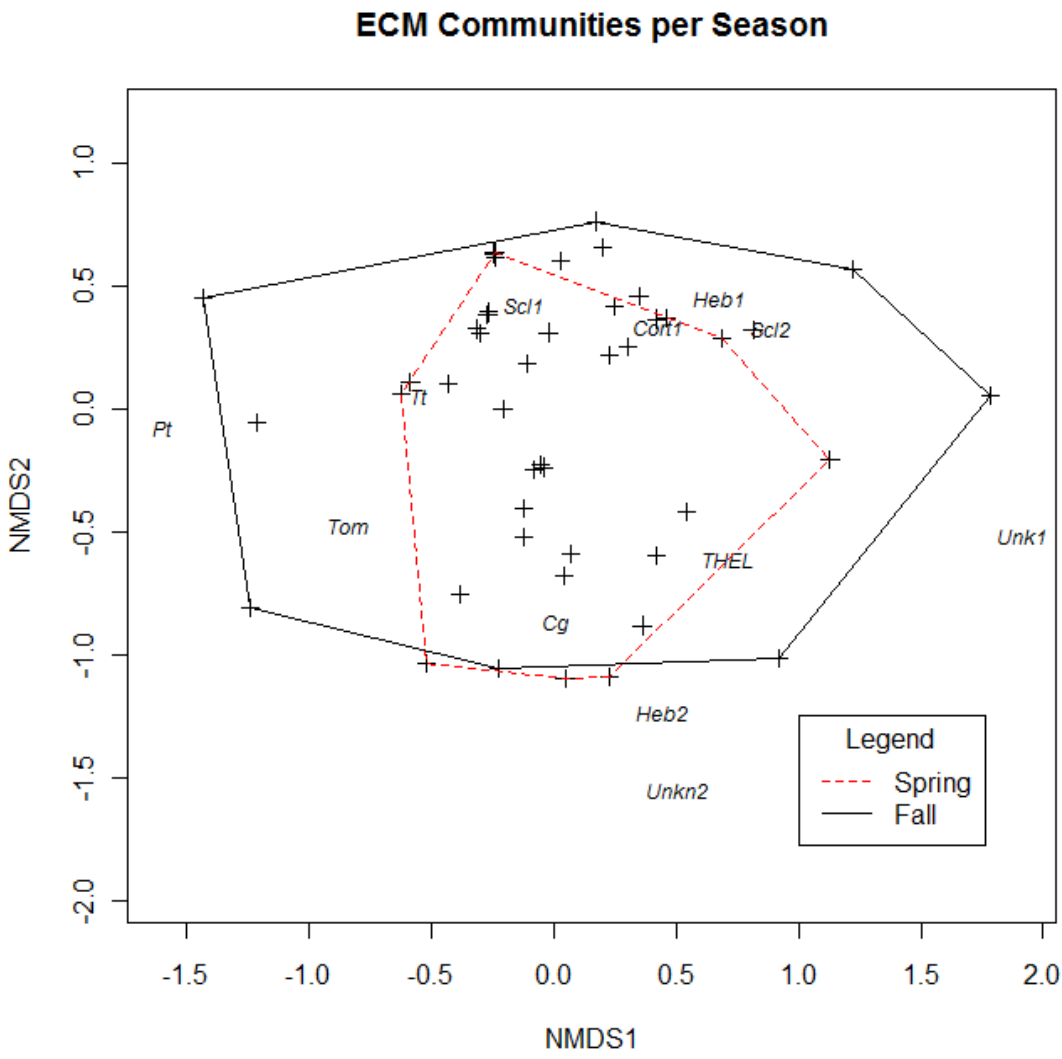

Figure 4. Non-metric multidimensional scaling (NMDS) ordination illustrating the ECM communities of pure American trap trees sampled in the spring and fall. Groupings are outlined and the overlap in the spring sample (dotted red) and fall sample (solid black) suggest no differences in ECM community between the two sampling periods.

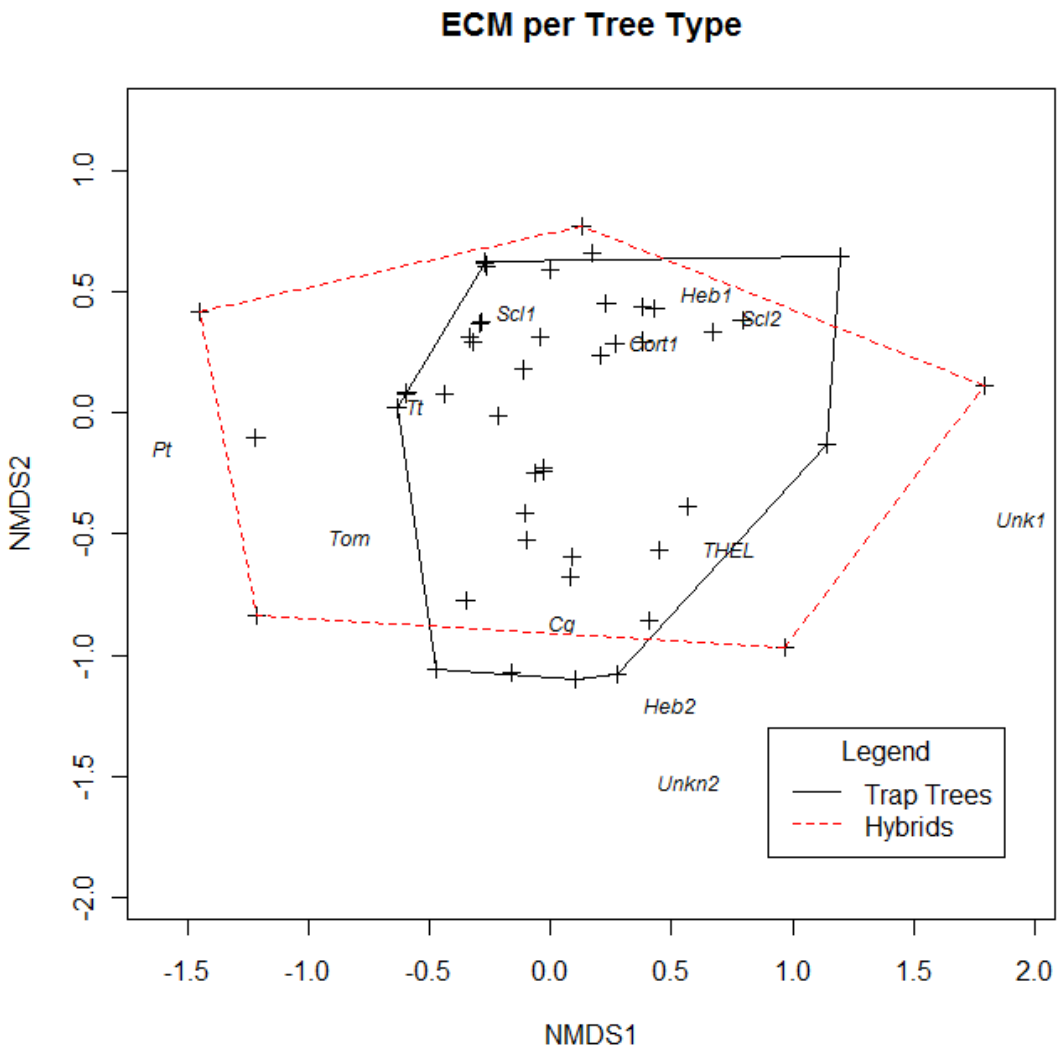

Figure 5. Non-metric multidimensional scaling (NMDS) ordination illustrating the ECM communities of pure American trap trees compared to the ECM communities sampled from chestnut hybrids. Groupings are outlined and the overlap between the pure American chestnut trap trees sample (solid black) and the chestnut hybrids (dotted red) suggest no differences in ECM community between the tree types.

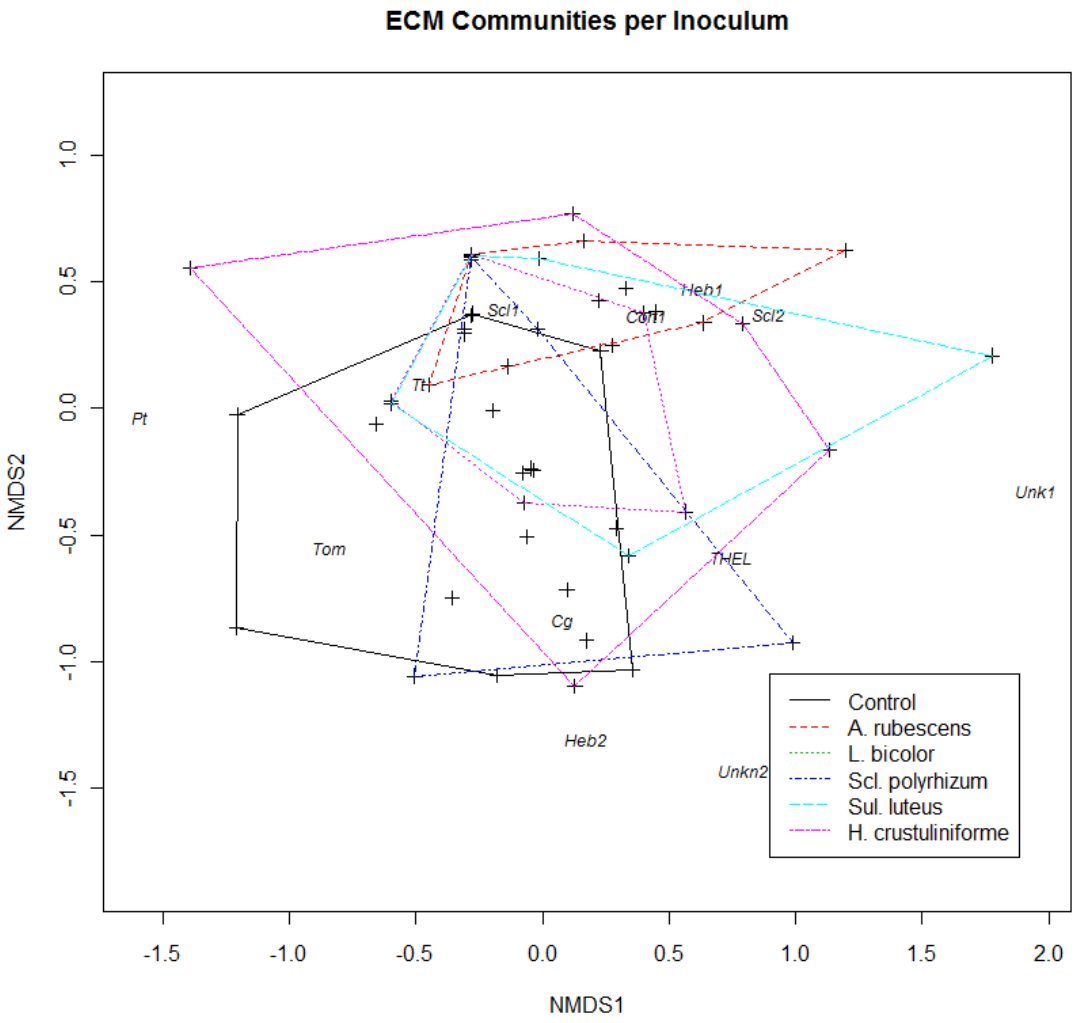

Figure 6. Non-metric multidimensional scaling (NMDS) ordination illustrating the ECM communities of inoculated chestnut hybrids and trap trees. Groupings are outlined per inoculum treatment: Control (solid black), *A. rubescens* (dotted red), *L. bicolor* (dotted green), S. polyrhizum (broken blue), S. luteus (dotted aqua), and *H. crustuliniforme* (broken purple) *Laccaria bicolor, Scleroderma polyrhizum*, *Amanita rubescens*, and *Suillus luteus.* The overlap among the treatment groups illustrates no difference in ECM community composition.

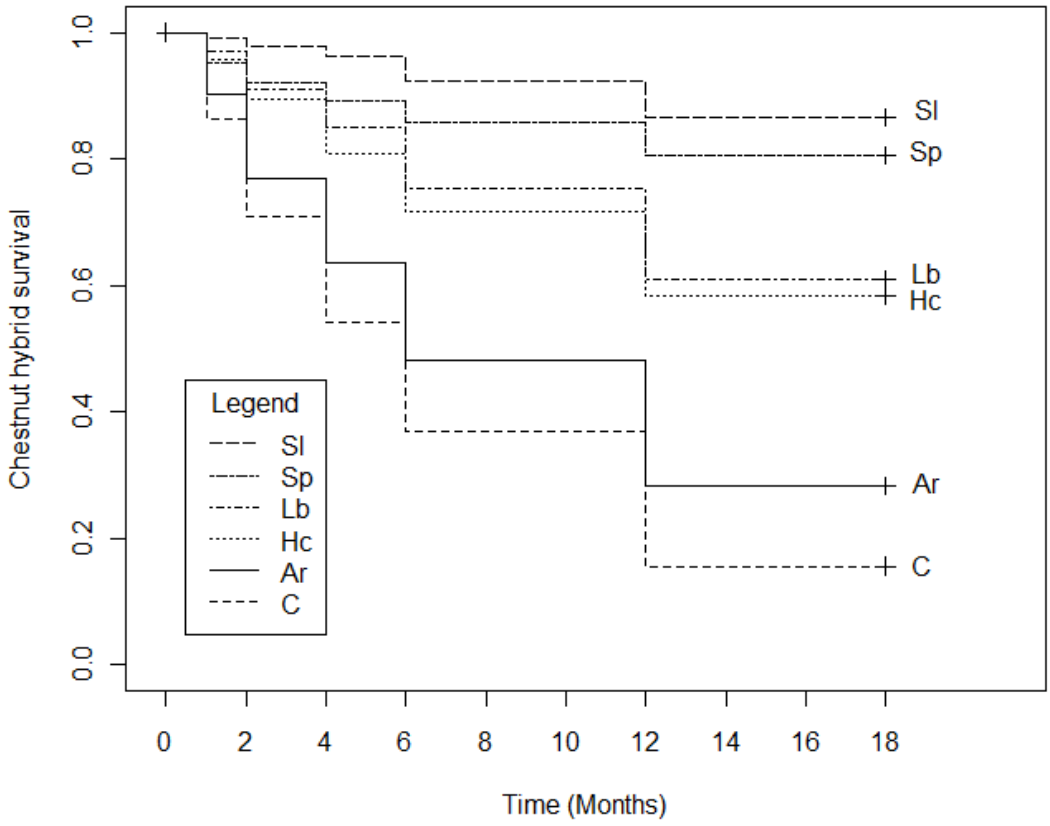

Figure 7. Survival data for hybrid chestnuts among the six different inoculum treatments (Sl = *S. luteus*, Sp = *S. polyrhizum*, Lb = *L. bicolor*, Hc = *H. crustuliniforme*, Ar = *A. rubescens*, and C = Control). ECM species had a significant effect on survival (Cox proportional hazard model, Likelihood = 121, df = 5, *P* < 0.0001).

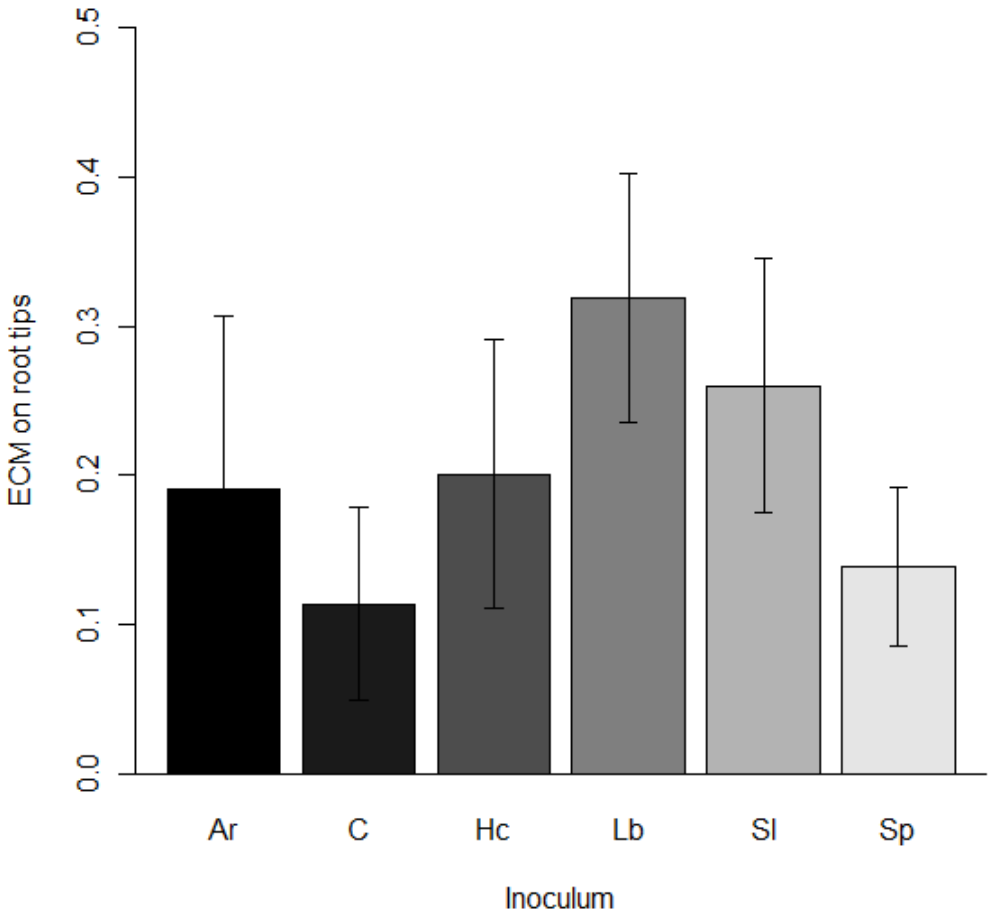

Figure 8. ECM root colonization (%) of hybrid chestnut seedlings among the inoculum treatments (Ar = *A. rubscens*, C = Control, Hc = *H. crustuliniforme*, Lb = *L. bicolor*, Sl = *S. luteus*, Sp = *S. polyrhizum*). No differences were detected.

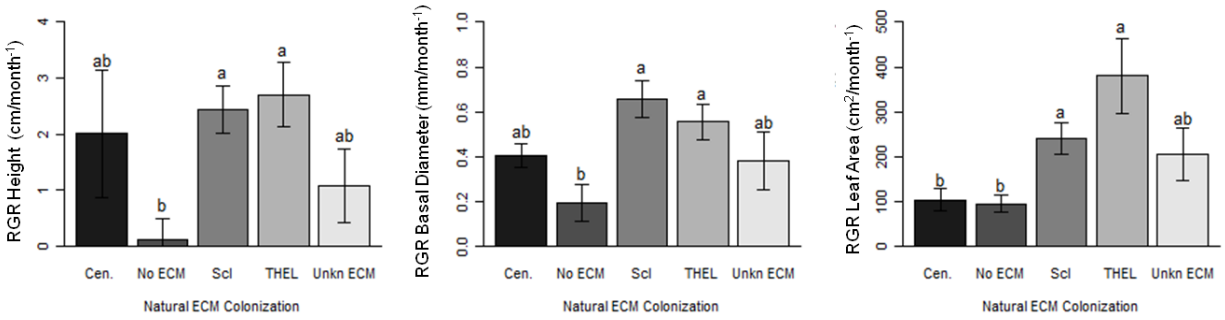

Figure 9. Mean (± 1 SE) of the relative growth rates of chestnuts naturally colonized by one of the following indigenous ECM species: *Cenococcum* (Cen.), *Scleroderma* (Scl), Thelephoracea (THE), and an unknown ECM (Unkn ECM). This comparison included seedlings found non-ECM (no ECM). *Scleroderma* (Scl) and Thelephoracea (THE) species significantly increased growth rates on chestnut hybrids (ANOVA, all $P < 0.05$). Bars represent the mean ± SE. Bars sharing common letters do not significantly differ at $\alpha = 0.05$ determined by Tukey's HSD.

Chapter 5

Using soil variables as predictors for ectomycorrhizal fungi in mine reclamation

Abstract

Ectomycorrhizal fungal (ECM) community assemblage is an integration of many abiotic and biotic factors. The community patterns of ECM fungi may be a response to the soil environmental conditions, but how these factors interact to influence ECM root colonization and community structure is not fully understood. The objective of this study was to use environmental data such as pH, soil nutrients, soil texture, soil temperature, moisture, and organic matter to determine their influence on ECM community composition and root colonization of American chestnut (*Castanea dentata*). Two different surface mines in central Ohio were used; one site is an abandoned surface mine (mined in the 1950's) and the other was reclaimed under The Surface Mining Control and Reclamation Act of 1977 (SMCRA). Four distinct sites within these mines were examined: center, forest edge, pine plots, and grasslands. Differences in species composition per soil characteristics were determined by fitting environmental vectors onto a non-metric multidimensional scaling (NMDS) ordination. Multiple regressions were used to determine which, if any, soil variables influenced ECM root colonization. We were unable to identify a soil variable that contributed to the percent of root colonization of ECM fungi on American and hybrid chestnuts. When soil variables were compared, our analysis demonstrated a clear separation between abandoned mines and mines reclaimed under SMCRA. ECM species were strongly associated with the four different mine sites. In addition, species composition was driven significantly by the levels of soil phosphorus and with marginal significance by levels of organic matter and magnesium. In the ordination certain ECM species appeared associated with higher resource availability of phosphorus and higher pH, while some were linked with nutrient impoverishment. Differences existed between ECM species in the same genera demonstrating that not all species within a genus share environmental preferences. Documenting environmental variables may be useful for predicting native ECM root colonization in future reclamation projects in central Ohio.

Introduction

In temperate forests, approximately 90% of roots of tree species are colonized by a diverse assemblage of ectomycorrhizal (ECM) fungi (Visser 1995). In undisturbed ecosystems, ECM diversity can be quite rich (Horton and Bruns 2001) surpassing 100 species within a forest stand (Courty et al. 2008; Smith and Read 2008). Very little is known about what directs community structure, distribution, and diversity of ECM fungi in plant systems (Leake 2001; Lilleskov et al. 2004). Fungal community assemblage is an integration of many abiotic and biotic factors including mineral nutrients, soil depth, O_2 and CO_2 concentrations, amount and quality of organic matter, temperature, moisture levels, and age of the forest stand (Bruns 1995; Smith et al. 2002; Blasius and Oberwinkler 1989). The community patterns of ECM fungi may be a response to the soil environmental conditions, but how these factors interact to influence ECM root colonization and community structure is not fully understood (Burke et al. 2009). Increased attention is given to the spatial variability of fungal community composition in the field, particularly how these communities assemble as ecological conditions change through disturbances (Buscot et al. 2000). ECM fungi may vary in their tolerance to drought (Swaty et al. 2004), resistance to natural and anthropogenic disturbances (Horton and Burns 2001), soil toxicity (Iordache et al. 2009) and temperature (Samson and Fortin 1986). Changes in soil chemistry due to surface mining, especially as they relate to pH and essential nutrient concentrations, may favor selection of fungi most capable of tolerating degraded landscapes (Agerer et al. 1998; Gehring et al. 1998; Erland and Taylor 2002).

Surface mining for coal significantly catalyzes changes in ECM community composition by altering the structure and chemistry of the soil environment (Jones et al. 2002; Durall and Cairney 2003; Jasper 2007). The removal of host plants and organic material causes a dramatic decline in the populations of these fungi. ECM communities are generally low in species richness promoting the existence of few ECM fungal genotypes by the exclusion of species sensitive to pollution by heavy metals (Kunito et al. 1998). The soils are generally nutrient poor in both abandoned and reclaimed mines and are generally heterogeneous and difficult to characterize (Boruvka and Kozak 2001). This heterogeneity stems from partial mixing and irregular spreading of topsoil over

chemically and mineralogically variable overburden and mine spoil (Jacinthe and Lal 2006). The spatial variability has been characterized with zones of high acidity (Hossner et al. 1997), patchiness of nutrients, (Mummey et al. 2002) metals, and organic matter (Boruvka and Kozak 2001). These differences in the soil structure and chemistry may influence ECM species composition and subsequent mycorrhizal formation, even at a smaller scale.

Mycological studies are rare in post mining landscapes. Surveys of the community composition of ECM fungi and the factors that may influence species composition and richness are needed to better understand the successional dynamics of these organisms. Earlier paradigms centered on the concept of early and late succession of ECM species may be important when measuring ecosystem recovery by means of indicator species that may represent later-stage fungi. However, this dichotomy may not always be appropriate to describe ECM under all conditions. This is because some early-stage fungi can colonize new roots of mature trees and new seedlings near mature trees can be naturally inoculated by late stage fungi. Later studies de-emphasized temporal explanations and related early and late succesional ECM species to r vs. K selection theory and to Grimes's ruderal/stress-tolerant/competitive model of plant life-history strategies (Dighton and Mason 1984; Last et al. 1987). Important to restoration is the prediction that ECM species that respond to soil disturbance appear to do so via resistant propagules or wind-blown spores, while those that dominate undisturbed habitats appear to do so through vegetative expansion from existing mycelium (Fox 1986; Deacon and Fleming 1992; Taylor and Bruns 1999; Lilleskov and Bruns 2003). What may have been considered early-stage fungi can be equated to ruderals, while late-stage fungi are comparable to either K-selected, stress-tolerant, or superior competitors (Dighton and Mason 1984).

The above ground plant community appears to be a highly influential factor driving the composition of the ECM fungal community. The genetic diversity of a plant population and level of host specificity may determine the species diversity of associated ECM symbionts (Lankau and Strauss 2007; Ishida et al. 2007). The importance of feedback becomes apparent with the mutual influence both plant and fungi have on the succession trajectory of each other (Kernaghan 2005). Though ECM symbionts have

been found to associate with certain host plants, variations in root colonization has been documented within the host's distribution (Nantel and Neumann 1992), indicating that abiotic factors may have independent effects on colonization. Variations in root colonization of a single host also indicate that microsites within soils may influence ECM root colonization. Root colonization has been documented to decrease despite initial colonization with ECM fungi, suggesting other site factors that may limit ECM growth (Kranabetter and Friesen 2002; Bauman unpublished data, dissertation chapter 4). Variation in colonization among stressed sites has been reported due to soil properties including soil moisture, temperature, and fertility (Gehring 1998; Swaty et al. 2004).

The objective of this study was to use environmental data such as pH, soil nutrients, soil texture, soil temperature, moisture, and organic matter to determine the influence the soil environment has on ECM community composition and root colonization of American chestnut. Four sites in two different former surface mines in central Ohio were used. One mine is considered an abandoned surface mine that was mined in the 1950s, prior to the initiation of The Surface Mining Control and Reclamation Act of 1977 (SMCRA). In this mine three sites were examined: center, forest edge, and a previous pine planting. The second mine site was reclaimed under SMCRA which resulted in a non-native grassland in arrested succession. Our hypotheses were: that 1) variation in the abiotic characters of the soil environment influences ECM community composition and 2) differences in soil chemistry and structure influences ECM root colonization. Morphological characteristics coupled with the sequencing the ITS region were used to identify ECM. Differences in species composition per soil characteristics were determined by fitting environmental vectors onto a non-metric multidimensional scaling (NMDS) ordination. Multiple regressions were used to determine which, if any, soil variables influenced ECM root colonization. Our overall goal of this study is to better understand the abiotic soil variables in order to predict ECM composition and root colonization in mine restoration projects using blight-resistant chestnut hybrids.

Methods and Materials

Site description and soil sampling:

Two different mines were sampled for ECM fungi. The first is an abandoned mine located in Avondale Wildlife Area in Muskingum County, Ohio (39° 49' 44" N, 82° 7' 38" W). This site is representative of surface mined sites prior to the Surface Mining Control and Reclamation Act of 1977 (SMCRA) in Ohio. Prior to passage of this act, lands were typically strip mined for coal and then abandoned. This site was mined in the 1950s and has had very little reclamation, aside from experimental tree plantations using *Fraxinus* spp., *Robinia pseudoacacia,* and *Pinus virginiana.* Of these plant species, *P. virginiana* survived creating small monoculture pine stands. Soil characteristics are typical of abandoned gob piles. The site is characterized by less than 5% vegetative cover and poorly sorted debris. There is no topsoil, very little competition, and very little organic matter. This area receives an average of approximately 99 cm of precipitation annually with temperatures averaging 22° C during the growing season (17°, 28°, and 11° C, spring, summer and fall, respectively; National Climatic Data Center). In this mine three distinct areas were sampled from: center sites, forest edge, and plots adjacent to 10 year-old pines.

The second mine was located in Tri-Valley Wildlife Management Area, Muskingum County, Ohio (40° 11' 32" N, 81° 98' 35" W) and reclaimed under SMCRA in the 1980s. It is primarily vegetated with the original species used for reclamation (*Festuca* spp., and *Lespedeza* spp.) with small patches of ragweeds (*Ambrosia* spp.), and goldenrods (*Solidago* spp.). Small pockets of forest comprised primarily of *Quercus, Pinus,* and *Acer* species were left undisturbed at the time these lands were mined. This area receives an average of approximately 99 cm of precipitation annually. During the 2007 and 2008 growing season the summer climate was relatively dry to moderate drought with annual temperatures averaging 22° C during the growing season (17°, 28°, and 11° C, spring, summer and fall, respectively; National Climatic Data Center). Each site sampled in Tri-Valley was a grassland.

Chestnut root samples were collected during the 2008 growing season from seedlings established as seed in 4m x 3m subplots. A total of 140 seedlings that were

planted as seeds from 29 subplots (representing all treatments)were randomly sampled at the end of the first season. The distribution of the subplots were: six grassland sites sampled from Tri-Valley Wildlife Management Area and six center, six forest edge, and 10 pine subplots sampled from Avondale Wildlife Area. Seedlings were carefully removed from the field, returned to the lab where root systems were washed, and observed under the stereoscope for mycorrhizal formation. One hundred root tips per seedling were randomly selected from each of the 140 chestnut seedlings to determine ECM root colonization. A total of 14,000 root tips were viewed under a dissecting microscope for the presence of a fungal sheath. Two samples per morphotype per seedling were selected for DNA extraction. A three mm segment of root tip was removed and transferred into a microcentifuge tube and stored at -70˚ C.

In addition to the sown seed, 142 seedlings from chestnuts planted as bare-root seedlings in the grasslands of the Tri Valley Wildlife reclaimed mine were sampled. These were not used to describe ECM community because they harbored ECM species that were transplanted in from the field nursery where they were grown. Instead, these were used to describe ECM root colonization separately from the chestnuts sown as seed. A total of 14,200 root tips total (100 per seedling) were viewed under a dissecting microscope to be observed for presence of fungal sheath. Two samples per morphotype per seedling were selected for DNA extraction. A three mm segment of root tip was removed and transferred into a microcentifuge tube and stored at -70˚ C.

Soil samples from both Tri-Valley and Avondale Wildlife Areas were collected in the spring of 2008 using a soil probe at an 18 cm depth, four samples per subplot. The four samples per subplot were mixed thoroughly, allowed to air dry, and 0.50 liters were sent to Spectrum Analytic Inc., Washington Court House, Ohio for analysis. Soil variables measured were: pH, organic matter, phosphorus, potassium, magnesium, calcium, cation exchange capacity (CEC) , sulfur, boron, zinc, iron, copper, and manganese. Soils variables measured for seedlings planted as bare root seedlings were: soil moisture, pH, CEC, organic matter, phosphorus, potassium, magnesium, calcium, nitrogen (NO3-N), manganese, aluminum, sand, silt, and clay. In addition to soil variables, height of seedling at time of planting was also used as a variable.

DNA extraction and purification:

To molecularly identify the types of mycorrhizal fungi, BLAST searches were employed on the ITS region of fungal DNA. DNA was extracted from the collected root tips using QIAgen Dneasy Plant Mini-Prep kit purchased through QIAGEN Inc. Primers ITS1-F (5' cttggtcatttaggaagtaa 3') and ITS4 (5' tcctccgcttattgatatgc 3') were be used to amplify internal transcribed spacer sequences (ITS) during PCR (Gardes and Bruns 1993). PCR 15 µl reactions were mixed based on the following concentrations: 9 µl of molecular grade water, 3 µl of 5x Green GoTaq® Reaction Buffer, 0.125 µl of Promega® *Taq* DNA Polermerase, .2 µl of 25µM of each primer, 1µl of dNTPS (200µM each of dATP, dCTP, dGTP, and dTTp) and 1 µl of DNA template. Temperature cycling was accomplished using a programmable Thermal Cycler Heating block. Times and temperatures programmed as described by Gardes and Bruns (1993): the initial denaturation step of 94ºC for 85 s followed by 35 amplification cycles of denaturation, annealing, and extension. The temperature and times for the first 13 cycles were 95ºC for 35 s, 55ºC for 55 s, and 72ºC for 45 s. Cycles 14-26 and 27-35 repeated the above parameters with lengthened extension steps 120 and 180 s, respectively. When the 35 cycles were completed the samples were programmed to incubate for 10 min at 72ºC for 45 s. PCR reactions were run of 1% argarose gels for 20 minutes to allow for the visualization of fungal DNA. Negative controls lacking template were used to ensure that the DNA amplified was from the root samples and not from contamination form reagents and reaction mixtures.

PCR product was cleaned by using Clean-Gene. Samples were prepared for sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit by mixing 10 µl reactions of the following concentrations: 2 µl BigDye Terminator v3.1 Reaction Mix, 3 µl 5 X Sequencing dilution buffer, 1 µl primer, and 1 µl of template. Sequencing cycle to label DNA for sequencing was performed on a programmable Thermal Cycler for the following cycles: 96ºC for 1 min followed by 25 cycles of 10 s at 96ºC, 5 s at 50 ºC, and 4 min at 60 ºC. Following labeling, products were purified to remove all unincorporated dye-labeled terminators by alcohol precipitation. Sequencing was performed with TheApplied Biosystem ABI Prism 3730 DNA Analyzer (Bioinformatics facility, Miami University, Oxford, Ohio). Sequences were analyzed and edited using Sequencher 4.2

software (Gene Codes, Ann Arbor, Michigan). To identify fungi found on roots, sequenced samples were compared with known species in GenBank using BLAST searching (Altschul et al. 1997). Genera reported here are based on the best match of vouchered fungi based on the similarity to the reported ITS sequences in GenBank. Characteristics are based on statistical analysis that generates both a bit value and an Expect (E) value. The bit score is a value that is indicative of how well the sequenced aligned with the known sequence in the database. The higher the score, the better the match. The E value is a parameter that describes the probability of the number of matches that can be generated by chance. It decreases exponentially as the match increases; a score closest to zero is the most significant. Thus when deciding the genera to report here, a threshold was decided on that included an E-value of 0, highest ranking bit value, and a gap value of < 4.

Statistical analyses:

Principal component analysis (PCA) was used to extract the initial set of uncorrelated components from the independent soil variables sampled from Tri-Valley and Avondale Wildlife areas. To satisfy the assumptions of independence, scatter plot matrices of all soil variables were analyzed to determine which variables were strongly correlated. These variables were removed before PCA was performed. Linearity and normal distribution assumptions were met by transforming (Log10 + 1) and standardizing (Wisconsin double standardization) data set. Eigenvalue threshold of unity (1.0) was used to retain factors in the model. Eigenvalues are the sum of squared correlation between the original independent variables and the principle components obtained, and they represent the amount of variance attributable to the components. Each of the components were rotated to facilitate their interpretation, and then referred to as factors. An orthogonal rotation (Varimax rotation) was used in this analysis to obtain the factors, maintaining their independence (Lehman et al. 2005). Factor loadings were used to interpret the resulting factors. Absolute loading value > 0.75 was used to interpret the resulting factor pattern.

Differences in soil chemistry among the four mine sites (center, forest edge, pine plots, grasslands) were identified using a multivariate analysis of variance (MANOVA)

followed by ANOVAs. MANOVA significance was evaluated using Wilks' λ test statistic. When significant, an univariate ANOVA followed by Tukey's HSD post hoc was used to assess differences among sites. PCA, ANOVAs, and MANOVAs were performed using JMP (8.0, SAS Institute, Cary NC, USA).

A non-metric multidimensional scaling (NMDS) ordination was used to determine if ECM community composition sampled from American chestnuts differed among the four sites using metaMDS function in vegan package, version 1.12-5. To improve the NMDS ordinations, the data were square root transformed and standardized via Wisconsin double standardization (Oksanen 2005). Bray-Curtis dissimilarities were employed due to their preferred analysis for community data due to the restriction within the range of 1 to 0 (Kindt and Coe 2005). The maximum number of random starts in search at was set at 100 with k=2 stress value. A permutational multivariate analysis of variance (formerly nonparametric MANOVA) was used to test for significant differences among the soil treatments using adonis function in vegan package, version 1.12-5. This is a method for partitioning variation in dissimilarity or distance matrices using a "pseudo-F" statistic analogous to MANOVA (Oksanen 2005). Confidence ellipses were employed to differentiate among treatments by indicating where 95% of the sites if the same category are expected to occur (Kindt and Coe 2005). All ECM community statistics were performed using Vegan: Community Ecology Package version 1.6.9. (Oksanen et al. 2005; Appendix 7).

To determine whether ECM fungi were correlated with the soil measurements, environmental variables were fit onto the NMDS species ordination via fitted vectors. These vectors are shown as arrows pointing in the direction of most rapid change in the environmental variable with the length proportional to the correlation between ordination and environmental variable (Oksanen 2005). This was accomplished by employing function envfit to the species ordination. This results in directional cosines of the vectors along with the squared correlation coefficient (r^2). The significances presented by a p-value based on random permutations of the data.

A multiple regression analysis was used to determine which independent variable best predicted percent ECM root colonization. To meet models assumption of normality and equal variance, predictor variables were transformed Log10+1 and standardized and

the dependent variable (ECM % root coverage) was arcsine transformed. The optimal number of variables to include in the models was determined by choosing the best subset regression with the lowest Bayesian information criterion (BIC) using R (version 2.9.2; Appendix 8).

Results

The resulting varimax rotated factor analysis from the PCA is displayed in Table 1. Three factors were identified that contributed to 78% of the total variance. Factor 1 explained 32% of variance and presented high loadings for soil pH and phosphorus (Table 1). Factor 2 explained 27% of the variation and presented high negative loading of organic matter (%) and positive loading of potassium (ppm; Table 1). Factor 3 explained 19% of the variance and contained high loading of ppm manganese. PCA ordination illustrates separation among the sites with regard to soil variables; grassland plots (+) grouped together along PCA axis 1 (Figure 1).

Variables reduced by the PCA were used in a subsequent ANOVA followed by Tukey's HSD. Soil pH ($F= 179.4$, $df=3$, $p < 0.0001$) and phosphorus ($F=20.5$, $df = 3$, $p < 0.0001$) were significantly higher in the grassland plots (Table 2). Soil concentrations of potassium (ppm) was significantly higher in the pine plots ($F = 3.26$, $df = 3$, $p = 0.04$). Organic matter (%), magnesium (ppm), and manganese were similar among the sites.

Ordination patterns showed a clustering of ECM communities within respective sites (center, grasslands, pines, and forest edge). This pattern was supported by the adonis analysis, which showed a strong site effect ($F = 4.20$, $df = 3$, $p = 0.005$). With regard to ECM species sampled, *Scleroderma* spp. 1 (Scl1), uncultured ECM 1 (Unkn1), *Tomentella* (Tom), and Hebeloma species (Heb 4 and 5) were more abundant in the grasslands (Figure 2). In contrast, *Pisolithus* (Pis), *Oidiodendron* (Oid), and *Thelephora* spp. 1 (Thel 1) were associated with the subplots among the pines (Figure 2). *Cenococcum* (Cen) appeared in the ordination with pine plots with higher pH levels. *Scleroderma* spp. 2 (Scl2) was associated with both pine and forest edge plots. *Laccaria* (Lac) and *Russula* (Rus) were associated with the forest edge (Figure 2). Analysis of ECM collected determined that the first dimension of the ordination was significantly

associated with ppm of P ($r^2 = 0.30$, $p = 0.03$) and marginally negatively associated with magnesium ($r^2 = 0.21$, $p = 0.08$). The second axis of the ordination was marginally negatively associated with organic matter ($r^2 = 0.21$, $p = 0.08$; Figure 2; Table 3).

ECM colonization:

Chestnut seedlings were separated into two different categories for these analyses: 1) those planted as one year-old bare root seedlings and 2) those sown as seeds. For the bare root seedlings, the environmental variables used in the regression based on lowest BIC were organic matter, magnesium, manganese, and aluminum. The regression model indicated that these parameters did not explain a significant amount of the variation in ECM root colonization ($F = 1.91$, $df = 39$, $p = 0.11$, $R^2 = 0.20$; Table 6). This was also the result for seedlings sown as seeds. The model with the lowest BIC included pH, organic matter, calcium, and manganese. These did not result in statistically significant values ($F = 2.29$, $df = 23$, $p = 0.09$, $R^2 = 0.29$; Table 7).

Discussion

Our analysis of soil variables demonstrated a clear separation between two different types of surface mines, abandoned and reclaimed under SMCRA. Grasslands in the SMCRA reclaimed mine site were significantly higher in soil pH and phosphorus. In addition, they were lower in concentration of manganese in the soil, however not significantly. Although SMCRA reclamation methods have been criticized for resulting arrested succession, soil characteristics are improved when compared to abandoned mine lands, specifically in deterring soil erosion and eliminating the incidence of extreme soil pH (Davison et al. 1984). Despite these improvements, soil conditions in this reclaimed mine sites remain low in fertility, deficient in organic matter, and prone to drought conditions (Steiger 1996). With regard to organic matter, these sites are drastically lower (0.9 – 1.9) when compared the soils in adjacent woodlots and remnant forests (13 – 23%; Cavender unpublished data).

ECM species were strongly associated with the four different mine sites. In addition, species composition was significantly linked with soil phosphorus. Levels of percent organic matter and soil magnesium were also noted as potential drivers of species

composition. This provided evidence to support our first hypothesis that variation in the abiotic characters of the soil influence ECM community composition. Certain ECM species (*Hebeloma, Cennoccum, Tomentella*) appeared in the ordination associated with higher resource availability of phosphorus and higher pH. Negatively associated with all other species was ascomycete *Oidiodendron* spp. This species of fungus forms ericoid mycorrhizas with plant species in the Ericaceae taxa (Peterson et al. 2004; Cairney and Meharg 2003). Further, these types of mycorrhizas tend to increase in nutrient impoverished sites and the presence of these fungi allow ericaceous plant to access nutrients that would be otherwise be unavailable (Read 1983; Read 1996; Peterson et al. 2004). Recent findings suggest these ascomycete fungi form dark septate mycorrhizas with other plant taxa (Chambers et al. 2008; Burke et al. 2009). This is confirmed in this study, where this fungal species formed mycorrhizas with chestnut in areas low in nutrients and high in metals, specifically associated with higher levels of manganese. It is not clear what host effects these fungi have on non-ericaceous plants. Important for plant establishment in sites with toxic levels of metals, these fungi have the ability to bind metals, thereby decreasing transport to their plant host (Denny and Ridge 1995). This group represents a mycorrhizal species that is not easily placed temporally in either early or late stage; rather, it fits more the model of stress tolerant strategy (Grime 1979). Placement of *Oidiodendron* in these microsites low in nutrients and higher in metals is consistent with the concept of trade-offs between traits that allow species to tolerate stressful environmental conditions. Nutrient input to soils will cause a shift in ericoid populations to favor fungi (AM and ECM) that are better competitors when resource levels are high (Read 1996).

ECM fungi associated with the forest edges consisted of *Russula, Laccaria,* and *Cortinarius*. Fungi of these genera are ECM colonizers of woody tree and shrubs found in temperate forest ecosystems. These species appear to be more representative of undisturbed habitats (Redecker et al. 2001) and have been considered to be later-stage ECM fungi dominant on mature roots (Lilleskov and Bruns 2003). Collectively, these genera associated with mature trees along the forest edge represent species with long-lived clonal populations that are better competitors under lower resource levels in undisturbed habitats (Taylor and Bruns 1999). The genus *Russula* is capable of

producing enzymes that degrade organic matter in the soil (Agerer 2001). Therefore, it is possible that the correlation between *Russula* and the forest edge may be a response to the presence of litter from the leaves of deciduous trees in these small pockets of forests. The presence of mature roots from trees along the forest edge offer host tissue in areas higher in organic matter and may select for fungi that have the ability to exploit these organic resources. Important to restoration goals geared to the establishment of later successional tree species, these edges may provide for inoculum of later-stage fungi. However, negative density effects such as competition for light or the build-up of soil pathogens and seed predators may limit the success of targeting such areas when using chestnut as a restoration tree (Bauman et al. in review).

Differences existed between *Scleroderma* species with regard to site and soil chemistry, demonstrating that not all species within a genus share environmental preferences. *Scleroderma* spp. 1 was found primarily in the grasslands positively associated with phosphorus and potassium. Previous phylogenic analysis indicates this species of *Scleroderma* is most closely related to *S. areolatum* (Bauman unpublished data, dissertation chapter 4). This *Scleroderma* species performs poorly at lower phosphorus levels (Brady and Weil 1996). *Scleroderma* spp. 2 (closely related to *S. citrinum*) was most abundant in subplots along the forest edge and pines, highly associated with lower pH and lower levels of phosphorus. Differences in enzyme production drives a shift in community toward species better adapted for acidic, phosphorus-limited conditions (Bending and Read 1995; Bidartondo and Bruns 2001; Lilleskov et al. 2002). This is important for future restoration projects with regard to selecting a more effective ECM symbiont for inoculum on chestnut for given site conditions. *S. citrinum* is a species available in commercial inoculums and would be a better choice for chestnut in mine sites experiencing low soil pH (pH <5.0) and high aluminum concentrations that further reduces the availability of phosphorus in soils.

It is interesting to note that current studies have isolated bacterial communities that associate with *S. citrinum* within the rhizosphere. Calvaruso et al. (2007) demonstrated that *S. citrinum* selects for bacterial communities that possess the highest efficiency for phosphorus mobilization in nutrient poor soils. From this research, the authors proposed a new hypothesis to explain the sustainability of tree species on

nutrient-poor soils; the plant selects ECM and bacteria that are more efficient in obtaining nutrients that would have otherwise been unavailable (Calvaruso et al. 2007; Calvaruso et al. 2009). Koele et al. (2009) demonstrated that *S. citrinum* provides a niche for the mineral-weathering bacterium, *Burkholderia*. This microbial symbiosis enables the ECM fungus to take up more bound potassium and magnesium, allowing for greater transfer of nutrients to the plant host, hence improving growth (Koele et al. 2009).

Existing vegetation is very important to the ecology of ECM fungi (Dickie et al. 2006). The presence of forest trees and established pines influenced the presence of one *Scleroderma* species over the other. *Scleroderma* spp. 2 (closely related to *S. citrinum*) was dominant in subplots adjacent to mature trees in our study which corresponds with other reports of the ecology of this species (Newton 1991). Unsuccessful and/or inconsistent ECM formation by spore inoculum of *S. citrinum* (Fox et al. 1986; Chen et al. 2006) indicates that existing mycelium improves root colonization potential in the field. In contrast, *S. areolatum* has been reported to associate with young trees (Keizer and Arnolds 1994) as a pioneer fungus in disturbed habitats, presumably by wind dispersed spores. The dominance of *Scleroderma* spp. 1 (closely related to *S. areolatum*) in grasslands could indicate that this species has the ability to rapidly recruit after a disturbance. Documenting differences in colonization strategies as well as existing vegetation that may harbor a particular symbiont may be useful for predicting root colonization in future mine reclamation projects in central Ohio. Importantly, knowledge of these species may also aid in planning planting strategies that better encourage root colonization by proper site selection, appropriate inoculum choice for chestnut, and soil surface treatments that ensure root colonization by native species.

ECM Root Colonization

We were unable to identify a soil variable that may have contributed to the percent of root colonization of ECM fungi on chestnut. Although this refuted our second hypothesis, it would be difficult to make the conclusion that the soil environment does not have an effect on colonization. Other studies have reported otherwise. In particular, past studies have identified mycorrhizal infection to change in response to changes in

organic matter in forest systems (Baar and deVries 1995; Dickie et al. 2006). Because mine reclamation operations often use the soft rock shale overburden materials in the soil substrate upon reclamation, soils are left very deficient in organic matter. Detectable levels of heterogeneity in these soil types with regard to organic matter may depend on controlled amendments rather than natural patchiness of the soil profile. For example, Lunt and Hedger (2003) reported a significant increase in mycorrhizal root colonization on *Quercus* seedling in mine soils with organic amendments in a greenhouse study. This also resulted in an increase in host response in soils with such amendments, specifically *Hebeloma* species (Lunt and Hedger 2003). This demonstrated that soils with adequate indigenous mycorrhizal propagules may benefit from management practices that incorporate organic matter to field sites, which could increase the colonization capacity of existing ECM communities.

With regard to nutrients, ECM root colonization has been reported to decrease with high levels of nutrient additions (Lilleskov et al. 2002; Avis et al. 2003). This implies host-plant control over the symbiosis under conditions of abundant soil resources (Johnson et al. 1997). It is hypothesized that when plant growth is limited by soil nutrients, more carbon should be allocated to mycorrhizal symbionts to increase nitrogen and phosphorus uptake (Smith and Read 2008). Under nutrient deficient conditions, carbohydrate allocation to the roots increases, which also increases the release of carbon compounds into rhizosphere. This carbon enhancement to the rhizosphere has been correlated with an increase in ECM biomass and activity (Morgan et al. 2005). Therefore, ECM infection potential should be maximized in mine soils deficient in essential nutrients with selection for ECM fungi that can maximize nutrient uptake and transfer. Because we did not identify soil characters that may contribute to root colonization, we can presume that ECM root colonization potential from the host was maximized and fluctuations could have been due to limited ECM propagule availability or carbon allocation from the host.

Included in the predictors was the original size of chestnut planted as bare rooted seedlings. ECM colonization was not influenced by height or basal diameter of original seedling. This aided in confirming that differences in root colonization seemed to be related to certain management practices such as site selection near existing vegetation and

the alleviation of soil compaction and herbaceous competition. For example, after comparing all sites with regard to ECM root colonization, chestnuts in the grasslands, in pine plots, and along the forest edge were all similar with seedlings in the center plots being the least colonized. Chestnuts in soils highly compacted and under competition with surrounding vegetation have also been documented as being sparsely colonized (Bauman et al. in review; Bauman unpublished data, dissertation chapter 3). Decreased colonization may be less dependent on the soil variables and more dependent on the supply of carbon from the host plant when resources are limiting. ECM fungal growth may decline with decreasing carbon allocation from the host plant (Treseder and Allen 2002), and it has been suggested that it may apply when soil nutrients do not contribute to ECM colonization (Swaty et al. 2004).

Documenting environmental variables may be useful in order to predict native ECM root colonization in future mine reclamation projects. Knowledge of existing ECM fungi and may prove to be a cost-effective and an ecologically conscious alternative to introducing fungal inoculum in areas that harbor indigenous fungi that may be better adapted to these local sites. This study demonstrated shifts in ECM community composition in response to lower nutrient availability and life history strategies of the fungi. Sites selected for sampling represent very different successional stages that harbors fungi with differing strategies. Within the abandoned mine there were nutrient impoverished sites that selected for non-ectomycorrhizal species such as ericoid fungus, *Oidiodendron.* Similar to events following ECM colonization in primary succession, chestnut seedlings in the bare ground of center sites were sparsely colonized by either these ericoid fungi or presumably ECM by chance association with spores. In contrast, subplots adjacent to existing vegetation harbored ECM species that exist as vegetative mycelium and are better competitors under lower nutrient levels. These existing fungal species may be able to incorporate establishing chestnut seedlings into an existing mycelium network, thereby facilitating their establishment in the abandoned mines. In contrast, the grasslands that have been treated with a soil surface treatment seem to model secondary succession. Disturbed soils may select for fungi that are better dispersers, high spore producers, and have the ability to colonize roots from spores under slightly higher nutrient levels. Knowledge of these factors may aid in the predictability of these fungi,

or in some cases anticipating their absence that may merit the use of ECM inoculum or additional soil amendments to aid in chestnut seedling establishment.

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Table 1. Varimax rotated factor loading from PCA for soil characteristics of the four mine sites. High factor loadings are shown in bold.

Soil Variable	Factor 1	Factor 2	Factor 3
Soil pH	**0.96**	-0.03	-0.26
Organic Matter (%)	-0.05	**-0.88**	-0.11
Manganese (ppm)	-0.11	-0.12	**0.76**
Phosphorus (ppm)	**0.97**	-0.04	-0.07
Calcium (ppm)	0.41	0.60	0.58
Potassium (ppm)	-0.27	**0.78**	-0.30
Magnesium (ppm)	-0.23	0.06	0.80

Analyses based on data that was standardized and square root transformed.

Table 2. Soil characteristics of the three plot types (center, forest edge, and pines) sampled in Avondale and the grassland plots in Tri-Valley Wildlife Area. Values are expressed as means within a column ± 1 SE. Means sharing common letters do not significantly differ at α = 0.05 to Tukey's HSD. Significantly higher values shown in bold.

Plots	ECM (%)	Soil pH	P (ppm)	%OM	K (ppm)	Mg(ppm)	Mn (ppm)
Center	14 ± 0.1 [c]	3.0 ± 0.1 [b]	2.2 ± 1.6 [b]	1.4 ± 0.7 [a]	77.4 ± 9.0[b]	323 ± 17.1 [a]	6.4 ± 1.1[a]
Forest Edge	**58 ± 0.2 [a]**	3.0 ± 0.1 [b]	1.0 ± 0 [b]	1.9 ± 0.5[a]	83.8 ± 10.1 [b]	240 ± 44.0 [a]	6.0 ± 1.6[a]
Grassland	**38 ± 0.02 [ab]**	**5.3 ± 0.1[a]**	**7.8 ± 1.9 [a]**	1.5 ± 0.1[a]	86. 7 ± 7.1 [b]	201 ± 34.4[a]	3.8 ± 0.9 [a]
Pines	**38 ± 0.03 [ab]**	3.2 ± 0.03 [b]	1.5 ± 0.8 [b]	0.9 ± 0.2[a]	**117.2 ± 10.4[a]**	226 ± 24.5 [a]	7.0 ± 2.5[a]

Analyses based on data transformed by $\log_{10}+1$.

Table 3. Relationship between soil variables and NMDS dimensions of ECM community. Column with r^2 gives the squared correlation coefficient. The P-values (Pr(>r)) are based on random permutations of the environmental variables as they relate to NMDS1 and NMDS2. Significant values are shown in bold.

Soil Variables	NMDS1	NMDS2	r^2	Pr(>r)
pH	0.97	-0.24	0.16	0.14
Organic Matter	-0.38	-0.93	0.21	0.08
K (ppm)	-0.56	0.83	0.01	0.88
Mn (ppm)	-0.39	0.92	0.07	0.46
P (ppm)	**0.99**	0.15	0.30	**0.03**
Mg (ppm)	-0.93	0.36	0.21	0.08

Table 4. Multiple regression relating ECM root colonization (%) on chestnut seedlings planted as bare root seedlings to environmental variables.

Predictor	β Estimate	SE	t-value	*p*
Intercept	1.04	0.38	2.73	0.009
Original Seedling Ht (cm)	2.4	3.53	0.68	0.5
Organic Matter (%)	**-6.15**	**3.72**	**-1.65**	**0.11**
Magnesium (ppm)	2.18	3.23	0.68	0.5
Manganese (ppm)	-2.21	3.03	-0.73	0.47
Aluminum (Al)	-2.85	2.58	-1.1	0.28

Table 5. Multiple regression relating ECM root colonization (%) on chestnut seedlings sown as seeds to environmental variables.

Predictor	β Estimate	SE	t-value	*p*
Intercept	0.03	0.75	0.04	0.96
Soil pH	4.63	3.21	1.44	0.16
Organic Matter (%)	-1.72	1.52	-1.13	0.26
Calcium (ppm)	-3.05	2.30	-1.33	0.97
Magnesium (ppm)	0.95	1.97	0.48	0.63

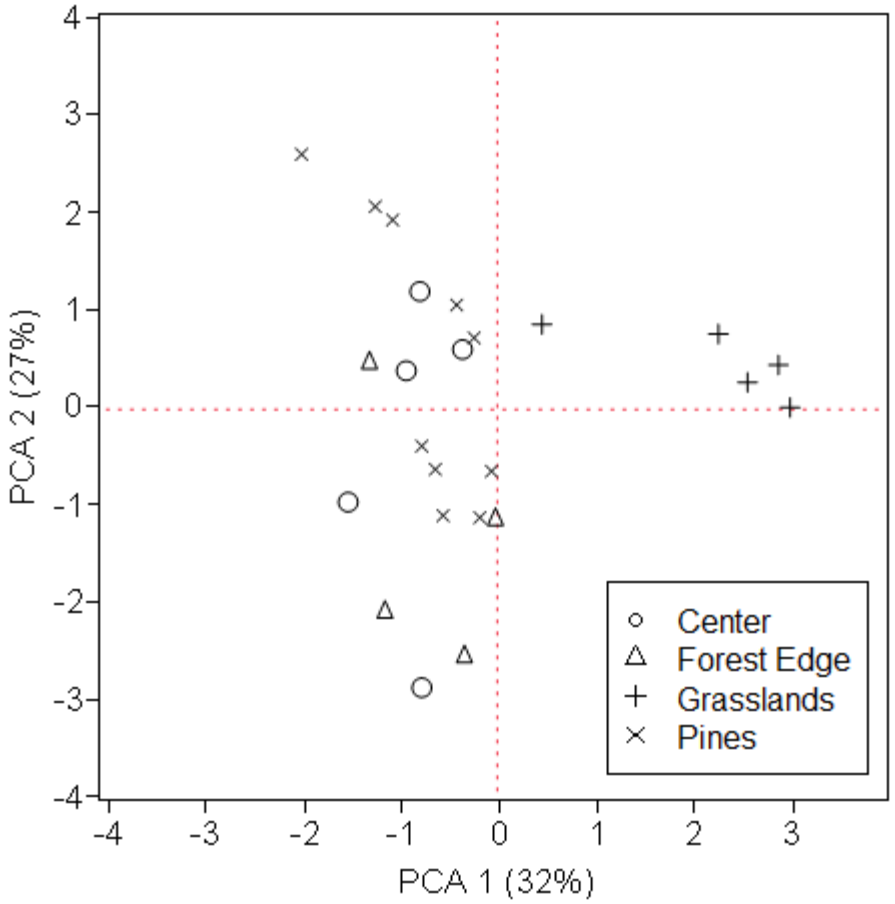

Figure 1. Principle components analysis of soil characteristics in the four different areas of both abandoned and reclaimed mine sites: Center (circles), forest edge (triangles), grasslands (+), and pine plots (X). PCA axis 1 and 2 represent 59% of the variance. Analysis illustrates clear separation between grasslands (+), strongly associated with PCA 1.

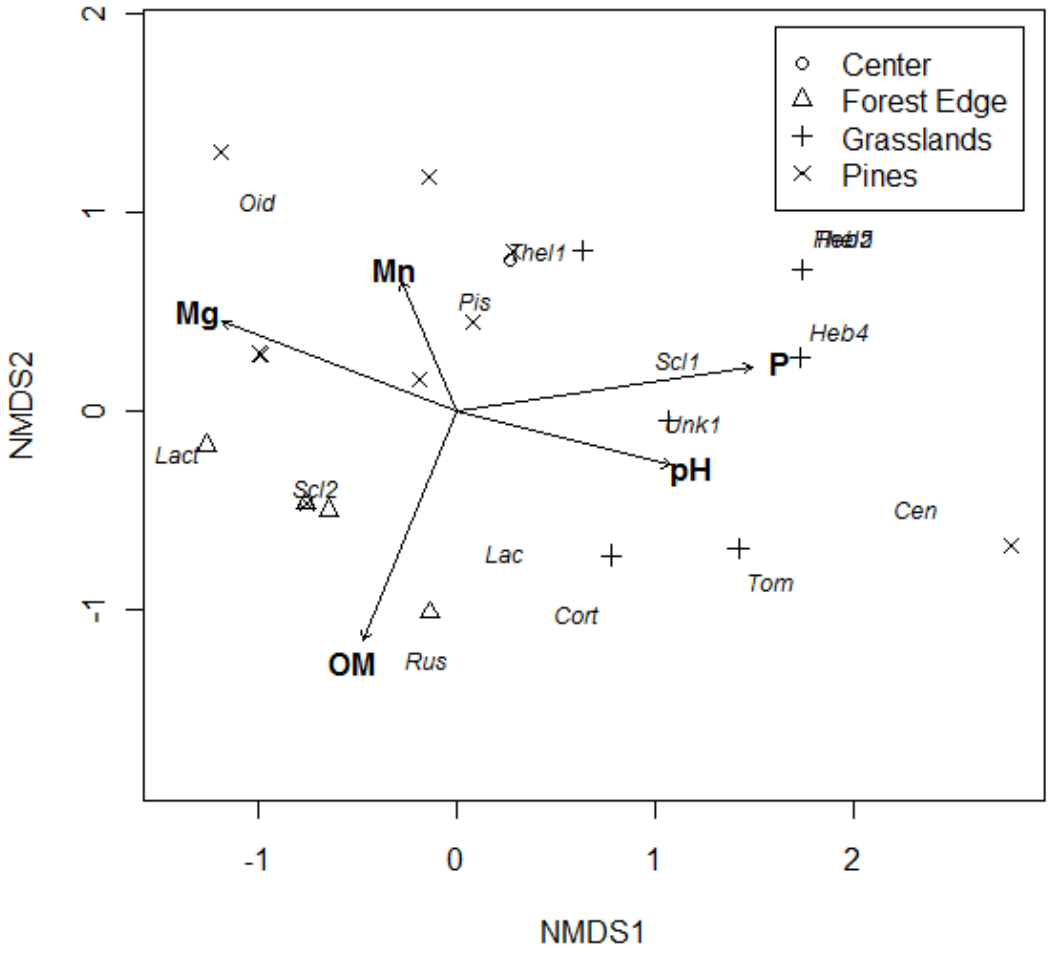

Figure 2: NMDS ordination of ECM fungi with joint plots of environmental variables. The joint-plot vectors indicate strength and direction of the strongest correlations.

Chapter Six

Conclusion

Soils left behind after surface mining are severely compacted, deficient in organic matter, and extremely acidic. These characteristics negatively impact the regeneration of native forest tree species (Torbert and Burger 2000). In addition, the soil microbial communities responsible for nutrient cycling, soil structure, and biological interactions are severely altered (DeGrood et al. 2005). Soil microbes important for woody tree establishment consist of ectomycorrhizal fungi (ECM). This symbiosis enhances the host plant's ability to absorb water and nutrients, tolerate heavy metals, and protects against root pathogens (Marx 1972; van der Heijden et al. 2003; Nara 2005). Studies conducted post-mining have reported significantly lower soil microbial diversity, biomass, and activity when compared to undisturbed ecosystems (Machulla et al. 2005). It has been postulated that barren landscapes and grasslands resulting from extreme disturbances may be preserved partially due to the lack of ECM inoculum available to incoming plant species (Marx 1991; Dickie and Reich 2005; Nara 2006).

In nature, ectomycorrhizal fungi (ECM) include 6,000 species known to form symbioses with many hardwoods trees, including species in the Fagaceae family (Brundrett 2009; Marx 1972). Potential restoration trees within this family are American chestnut (*Castanea dentata*) and blight-resistant chestnut hybrids (*C.dentata x C. mollissima*). Preliminary studies have reported American chestnut as a tree species that can establish in abandoned and reclaimed mine sites (McCarthy et al. 2008; Jacobs et al. 2009; Rhoades et al. 2009). Its fast growth rate coupled with high quality timber and large annual seed masts, makes American chestnut a desired species for use in reforestation projects. Members of the genus *Castanea* has been reported to form ectomycorrhizas (Rhoades et al. 2003; Dulmer 2006; Palmer et al. 2008). However, because American chestnut was eliminated as a canopy tree from the Eastern deciduous forests by the 1950s, very little is known about the microbial interactions essential for establishment.

The central objective of this dissertation was to develop planting methodologies that would maximize the effectiveness of ECM root colonization and host response for seedling establishment in mine reclamation projects in central Ohio. This project accomplished three goals: 1) characterized ECM communities in mine sites in central Ohio and related colonization to chestnut growth, 2) evaluated planting methods that may accelerate succession and aid in establishing hybrid chestnut, and 3) provided a venue for field testing blight-resistant hybrids to add to current chestnut research that will help evaluate the performance of these hybrids for the future public release.

Summary of Dissertation Results

My dissertation used chestnut as a reclamation tree in abandoned mine sites in central Ohio. These mines are typically difficult to reclaim due to high spoil content contributing to drastically low pH levels, essential nutrients, and diminished microbial populations. Utilizing previous plantings of 10-year-old *Pinus virginiana* as "nurse plants" significantly contributed to the establishment of these seedlings as evidenced by increased germination, survival, and growth when compared to center plots and forest edges after two field seasons. Forest edge and pine plots were similar with regard to ECM colonization on roots and significantly higher than ECM sampled from seedlings in center plots. However, small monoculture plantings of pine had a greater facilitative effect on chestnut establishment than did the forest edge. This is presumably because of the facilitative effect these pines have without the negative density-dependent factors such as competition for light and habitat creation for seed predators. ITS fungal sequences and morphotypes found between chestnut and pine matched *Scleroderma*, *Thelephora*, and *Pisolithus*, suggesting these two unrelated tree species share ECM symbionts. Utilizing previous plantings as nurse plants for seedling recruitment may be a method of reclaiming severely debilitated mine sites. Further research is required to determine the long term survival of these chestnuts and whether the establishment of a hardwood will facilitate the recruitment of other hardwood tree species.

The Surface Mining Control and Reclamation Act of 1977 (SMCRA) was developed out of environmental concern and to prevent the abandonment of mine lands.

However, reclamation strategies have not always resulted in forest succession. The Appalachian Regional Reforestation Initiative (ARRI) proposes mechanical soil treatments such as deep ripping and traditional plow and disking in order to alleviate soil compaction, thereby accelerating succession by promoting healthy tree establishment (Torber et al. 1994; Groninger et al. 2007.) These methods were very successful with regard to chestnut growth and survival. In addition, these mechanical treatments increased ECM root colonization and species richness. Further, there was a significant interaction between both soil treatments and ECM colonization; chestnut seedlings found naturally colonized by ECM fungi in the mechanically treated plots had the greatest shoot production when compared to their non-ECM counterparts. Soil compaction, competition from non-native forbs, and the absence of ECM symbionts seem to act synergistically as mechanisms inhibiting seedling establishment. This may, at least in the short-term, be eliminated by soil conditioning that alleviates compaction and competition while encouraging ECM colonization.

ECM colonization increased host plant growth and survival, a finding well supported in the literature (Marx 1972; van der Heijden et al. 2003; Nara 2005; Smith and Read 2008). However, this was not the case in all treatment plots. Chestnut seedlings growing in both center plots in the abandoned mine and in control plots of grasslands demonstrated a neutral host response to ECM root colonization. Compaction may have been a major component shared by these subplots. Soil compaction hinders signaling molecules initiating mycorrhizal formation (Podila 2002), decreases ECM root colonization (Amaranthus et al. 1996; Jordon et al. 2003), and inhibits hyphal growth (Skinner and Bowen 1974). Therefore, the diminished hyphal expansion may have decreased the amount of resources supplied by the fungus to its plant host. In that situation, the plant may have limited the carbon transferred to the fungal partner before the ECM symbiont became parasitic. Therefore, the decrease in ECM colonization may also have been the result of diminishing carbon transfer from the host plant, an adaptation of plant control over the symbiosis preventing parasitism (Swaty et al. 2004). It can be hypothesized that carbon transfer from the host would be correlated to root colonization. Mine reclamation projects provide opportunity for future studies to test these plant and fungal dynamics across levels of compaction. Field tests utilizing applications of labeled

carbon (pulsed as $^{13}CO_2$ and $^{14}CO_2$, as described by Simard et al. 1997) could be used to determine carbon transfer from leaf tissue to fungal sheath by calculating labeled carbon content per set quantity of fungal sheath or sporocarp.

While the research in this dissertation focused primarily on ECM community and root colonization, chestnut also in associate with arbuscular mycorrhizal (AM) fungi (Dulmer 2006). During the course of my dissertation, I observed thousands of root samples. Of these, only a few samples appeared AM (Figure 1). However, AM colonization may have gone unnoticed due to staining methods required for consistent visual observations and phyla specific primers for molecular identification. AM fungi may be an important symbiont for chestnut establishment and it would be interesting to target this group of fungi in future reclamation studies. The versatility of dual microbial associations may aid in establishing chestnut on these sites and further merit chestnut's importance as a restoration species in grasslands comprised of AM forbs and gramminoids.

The most abundant fungi sampled from chestnuts in experimental plots of the mechanical treatment study were not found on trap trees (*Hebeloma* spp. 1, *Hebeloma* spp. 2, and *Cortinarius* spp. 1). Colonization of these seedlings likely occurred in the field nursery and greatly inhibited the natural colonization of indigenous *Scleroderma* species. Future root sampling is required to determine if the introduced *Hebeloma* and *Cortinarius* species are eventually displaced by native ECM species. This displacement of ECM was documented when we introduced greenhouse inoculated seedlings. The results of this study demonstrated that these selected ECM species do not persist on chestnut after one year in the field. In addition, the introduced species did not impede natural root colonization of native fungi or influence ECM community composition after two growing seasons. Although these species did not persist in the field, the presence of ECM inoculum greatly contributed to the survival rates of hybrid chestnut seedlings. Therefore, introduced inoculum that was present in the very early stages of outplanting had persisting effects with regard to seedling development in the field, even if the original inoculum did not persist. ECM fungi native to the area colonized chestnuts resulting in increased growth rates. These native assemblages may contain species better able to adapt to environmental extremities and the conservation of these species may be

what is necessary to facilitate long-term survival of deciduous tree species historically native to these lands.

My final study compared the environmental data in each of the sites to determine the influence the soil environment has on ECM community composition and root colonization of American chestnut. When soil variables were compared, our analysis demonstrated a clear separation between two different types of surface mines: abandoned verses reclaimed under SMCRA. ECM species were strongly associated with the four different mine sites driven significantly by the difference in levels of soil phosphorus and marginally significant differences in organic matter and magnesium. Certain ECM species appeared in the ordination associated with higher resource availability of phosphorus and higher pH, while some were associated with nutrient impoverishment. Documenting these differences in species may be useful for predicting ECM community composition in future mine reclamation projects. In addition, anticipating ECM absence can be useful when planning restoration projects in areas that require the additions of inoculum. We were unable to identify a soil variable that may have contributed to the percent of root colonization of ECM fungi on chestnut. Future work investigating the influence certain organic and nutrient amendments have on ECM development is required to identify specific variables that influence root colonization.

The ECM survey conducted in this dissertation provided a thorough description of ECM community composition immediately after a mechanical disturbance. Further studies are required to describe the sequence of ECM species succession after disturbance. Future research frameworks can hypothesize that these ECM community dynamics lead to an increase in the recruitment of other native ECM tree species through time. The large ECM host range of American chestnut and chestnut hybrids provides a method of quantifying the ECM community. Further, this can be used as a tool to measure ecosystem recovery by documenting the increase of ECM diversity and "late successional fungi" through time. Better understanding of native fungi whose interactions may be promoted by various site preparation methods may aid in management strategies for restoring reclaimed mines. Establishing a hardwood provides a host plant to many fungal species, increasing the inoculum source for incoming trees and increasing the probability of the facilitation of ECM inoculation from existing vegetation.

In addition, chestnut is a prolific nut producer and will attract hoarding seed dispersers and may contribute to hardwood seed dispersal. Developing protocols that alleviate soil compaction, encourage root colonization by a diverse population of ECM fungi, and identify the native ECM symbiont that elicits the greatest host response, may aid in directing the succession of these grasslands into mature forest ecosystems.

Restoration Recommendations

Restoration on Abandoned Mine Lands:

Restoration using chestnut without amendment was not a sufficient restoration practice for severely disturbed and compacted soil conditions, particularly in sites without pre-existing vegetation. Seedlings in center sites had limited ECM root colonization and exhibited a neutral response to the sparse ECM colonization. We would recommend amending these sites with organic material and applying some type of surface soil treatment to alleviate compaction prior to replanting. *Scleroderma citrinum* was the most abundant ECM fungus sampled. This indicates the high affinity chestnut and *S. citrinum* share with one another contributing to the abundance of functional mycorrhizas sampled in the field. We recommend using this ECM species coupled with proper soil amendments in future restoration attempts. One cost effective inoculation technique is to use to soil collected from the target site to mix in planting mediums for nursery or greenhouse inoculations. This technique would provide the chestnut seedling with a site-specific ECM symbiont to ensure functional mycorrhizal formation in the field and help preserve locally adapted fungal genotypes (Lesica and Allendorf 1999; Dulmer 2006). If this technique is not logistically feasible, this species is available in commercial inocula that can be applied as spore or mycelium granules to the soil. However, when working with sites with soil pH < 4.5, using the soil from the actual site may ensure genotypes that can form mycorrhizas at these low pH levels.

In contrast to the center sites, chestnuts growing in the pine plots had higher germination and survival. The low specificity of *Castanea* spp. proved beneficial in an area of low ECM species diversity, which may have been the factor contributing to the higher survival and growth rate of ECM chestnut seedlings in the pine plots. The

availability of ECM inoculum from a distantly related plant species demonstrates positive interactions between plants facilitating the establishment of a later successional taxon, possibly by connecting a seedling into an established mycorrhizal network (Horton et al. 1999). It was very encouraging to observe natural seedling establishment along with the chestnuts we introduced in pine plots after the third year (J. M. Bauman pers. obs; Figure 2). I randomly sampled one of the poplars from this plot and it too was colonized by *S. citrinum* (Figure 3). It is interesting to speculate that establishing a hardwood like chestnut may increase fungal biomass activity and inoculum potential of *S. citrinum*. Although the chestnut survival was not as high as we would have aimed for in a restoration attempt, this potential fungal network activity could be tested using Simard et al. (1997) as a method guide. Simard and her research team had experimental evidence that pine and birch exchanged carbon mediated by ECM fungi. Future restoration projects using this model could add greater insight to the facilitative mechanism of ECM common networks and their importance in reclaiming sites without the added costs of amendments or inoculum.

Restoration on Reclaimed Mine Lands:

Soil characteristics in mine sites reclaimed under SMCRA are improved when compared to abandoned mine lands, specifically in reducing soil erosion and buffering the incidence of extreme soil pH (Davison et al. 1984). However, soil conditions in these reclaimed sites are still considered low in fertility, with low organic content, low in ECM propagules, high in compaction, invasive non-native forbs and graminoids, and subject to drought conditions (Steiger 1996). The soil treatment methods proposed by Appalachian Regional Reforestation Initiative (ARRI) were very successful in encouraging healthy chestnut establishment while increasing ECM root colonization. At the end of the third year, chestnuts in treated plots that were found to be ectomycorrhizal in the field were taller than the regenerating herbaceous competition (Figure 4). The two soil treatment methods employed in this study, ripping and plowing with a conventional tractor, were similar with respect to chestnut growth, ECM species richness, and ECM community composition. These similarities are important from an economical perspective. The

average rate for excavating mine sites using a D-6 dozer with a 1.0 m ripper bar attachment costs $150.00 per acre. In contrast, the equipment used to plow and disk using a conventional tractor and plough board averages $20.00 per acre. Future studies recording growth and survival are required to determine if ripping is required for long term survival. Until those conformational studies, we would recommend the initial investment of applying a plow and disking technique prior to planting chestnuts in compacted grasslands.

Introduced inocula differed with regard to persistence depending on how the seedlings were inoculated. Chestnuts growing in the field nursery became naturally colonized by ECM species of *Hebeloma* and *Cortinarius* that persisted on the chestnuts after two field seasons. It appeared that these introduced species may have had an inhibitory effect on root colonization from indigenous *Scleroderma* species. This was not the case for the inoculum that was used to inoculate chestnuts that were greenhouse grown. However, our results also indicated that our inoculum did not influence the subsequent root colonization from a number of native ECM, but was very important for establishment. During the course of this dissertation, *Scleroderma* species were the most abundant ECM fungi to colonize chestnut (Figure 5). This demonstrates the high affinity this ECM species has for chestnut seedlings and its availability in these landscapes. As stated above, using soils from these sites may greatly aid in producing an ECM seedling with a native fungal species. Alternatively, employing commercial inocula that use species such as *S. bovista, S. cepa, S. citrinum,* and *S. verrucosum* may be easier than hauling soil from these sites.

In closing, proper site selection and soil surface treatment methods significantly contributed to the beneficial symbiosis of this natural mutualism, aiding in chestnut establishment in mine sites in central Ohio. For work in abandoned mines, selecting sites with vegetation present will encourage seedling establishment. When this vegetation is not available, additional surface preparation and organic amendments may be required. In mines reclaimed under SMCRA, planting methods that encouraged rooting will also encouraged ECM formation. This could be due the increased signaling between the two symbionts, increased hyphal expansion translating into greater resources supply to the plant host, increased carbon allocation from the host to the ECM fungus, or a

combination of all these factors. Soil compaction and competition from non-native forbs seem to act synergistically as mechanisms inhibiting seedling establishment and ECM root colonization. Employing methods of surface conditioning that alleviate compaction and competition while encouraging native ECM colonization may be the catalyst required to facilitate the natural successional pathway into a closed canopy forest.

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Figure 1. Unknown arbuscular mycorrhizal fungi on roots of *Castanea dentata* sampled in Tri-Valley Wildlife Management Area in Muskingum County, Ohio, sampled in August, 2008.

Figure 2. Subplot (3Pt-5) in *Pinus virginiana* treatment plot in August 2008 in Avondale Wildlife Area in Muskingum County, Ohio, 30 months after chestnut seeds were sown. Photo notes regeneration of native seedlings *Acer saccharum*, *Liriodendron tulipifera*, and *Prunus serotina*.

Figure 3. *Scleroderma* spp. on roots of *Liriodendron tulipifera* seedling establishing adjacent subplot 3Pt-5 in *Pinus virginiana* treatment plot. Sample was collected in August 2008 from Avondale Wildlife Area in Muskingum County, Ohio.

Figure 4. American and blight resistant chestnut hybrids in ripped + plowed and disked treatment plot in Tri-Valley Wildlife Management Area, Muskingum County, Ohio. Picture used with permission by photographer, Alex Snyder, Ohio University.

Figure 5. Fruiting body of *Scleroderma citrinum* growing at the base of a six month old, blight-resistant B3-F2 hybrid chestnut (*C.dentata x C. mollissima*). Seedling was growing in subplot 3FE-2 planted along the forest edge in Avondale Wildlife Area in Muskingum County, Ohio.

Appendix 1. R code for community data in chapter 3 sampled from Tri-Valley Wildlife Management Area, Muskingum County, Ohio. Species accumulation curves were constructed based on exact calculations of the average species richness for the combination of the treatments with 1000 permutations for each sample size.

Sp Accumulation Curve
getwd()
dat2 <- read.csv(file="ECMDiv2.csv")
dat2
head(dat2)

#checking for NA
index <- which(is.na(dat2), arr=T)
index

#checking no. rows and columns
dim(dat2)

#creating the species data
spdata2 <- dat2[,11:24]
dim(spdata2)
head(spdata2)

#creating envir data
envdata2 <-dat2[,1:10]
envdata2
head(envdata2)

curve <- specaccum(spdata2, method="exact", permutations = 1000)
plot(curve, ci=1, xlab="Number of Chestnut Seedlings", xaxt="n", ylab="Number of species", ci.type = "line", ci.lty=2)
axis(1, at=seq(0, 170, 20))

Appendix 2. R code for community data in chapter 3, evaluating ECM community per mechanical treatment in Tri-Valley Wildlife Management Area, Muskingum County, Ohio. A non-metric multidimensional scaling (NMDS) ordination used Bray-Curtis dissimilarities with application of square root transformation and standardized via Wisconsin double standardization. The maximum number of random starts in a search was set at 100 with k=2 stress value. A permutational multivariate analysis of variance was used to test for significant differences among the soil treatments. Lines for code are in bold. Lines that begin with ### are for descriptive purposes only.

####NMDS

```
getwd()
NM <- read.csv(file="Tri NMDS.csv")
NM
head(NM)

#creating the species data
spdataNM <- NM[ ,4:17]
dim(spdataNM)
head(spdataNM)

#creating envir data
envdataNM <-NM[ ,1:3]
envdataNM
head(envdataNM)

NMDS <- metaMDS(spdataNM, zerodist="add", trymax=100)
plot(NMDS, main= "ECM per Treatment", cex.family="sans", font=1, type = "n")
points(NMDS, display = "sites", col = "black", pch = 3, lwd = 1.75)
text(NMDS, display="species", cex=.75, col = "black", font=3, pch = 3, lwd = 1.75)

##Treatment
summary(envdataNM$Treatment)
ordihull(NMDS, groups=envdataNM$Treatment, show.groups="C", lty=5, col=1,
lwd=2)
ordihull(NMDS, groups=envdataNM$Treatment, show.groups="PD", lty=3, col=1,
lwd=2)
ordihull(NMDS, groups=envdataNM$Treatment, show.groups="R", lty=6, col=1,
lwd=2)
ordihull(NMDS, groups=envdataNM$Treatment, show.groups="RPD", lty=1,
col=1, lwd=2)
legend(-1.0, .90, c("C", "PD", "R", "RPD"), lty = c(5,3,4,1), title="Legend")
```

###Testing Significance
##Permutation using adonis
betad <- betadiver(spdataNM, "z")
adonis(betad ~ Treatment, envdataNM, perm=200)

###Species scores
distmatrix <- vegdist(spdataNM, method ='bray')
initNMS <- NMSrandom(distmatrix, perm=100, k=2)
OrdM <- postMDS(initNMS, distmatrix)
OrdM <- add.spec.scores(OrdM, spdataNM, method="wa.scores")
OrdM
plot5<- ordiplot(OrdM)

Appendix 3. R code for % ECM root colonization, growth, and survival in chapter 3, evaluating mechanical treatments in Tri-Valley Wildlife Management Area, Muskingum County, Ohio. Arcsine square root transformation was used to control for unequal variances. Differences in colonization between the ECM colonization were statistically determined by using a one-way analysis of variance (ANOVA) followed by a Tukey's post hoc. Growth parameters (seedling height (cm) and basal diameter (mm)) were transformed by setting the most negative growth value to zero, adding accordingly to the samples, and using Log+1 transformation. To determine significant interactions between ECM colonization by treatment, a full factorial two-way ANOVA was used (ECM colonization * soil treatments). Lines for code are in bold. Lines that begin with ### are for descriptive purposes only.

###ANOVA for %ECM per treatment
anovaECMnb<-aov(asin(sqrt(Proportion))~Treatment, ECMbar)
anovaECMnb
summary(anovaECMnb)
TukeyHSD(anovaECMnb)
plot(anovaECMnb)

###Barplot for ECM on roots. Standard Error Bars
###ECM % roots
ECMbar <-read.csv(file="Tri Bar.csv")
head(ECMbar)
index <-which(is.na(ECMbar),arr=T)
index
dim(ECMbar)
ECMbar

attach(ECMbar)
ECMm <- tapply(Proportion, Treatment, mean)
ECMm

ECMse <- tapply(Proportion, Treatment, std.error)
ECMse

x.s<-barplot(ECMm, ylim= c(0,.60),beside=T, xlab="Mechanical Treatments", cex.lab=1.25, ylab="ECM root tips (%)", col=c("grey90", "grey70", "grey50", "grey30"))
arrows(x0 = x.s, y0=ECMm , x1=x.s, y1= ECMm +ECMse, angle = 90, length = 0.05)
arrows(x0 = x.s, y0=ECMm , x1=x.s, y1= ECMm -ECMse, angle = 90, length = 0.05)
abline(0,0)

```
text(.7, .19, "b", cex=1.25)
text(1.9, .49, "a", cex=1.25)
text(3.1, .47, "a", cex=1.25)
text(4.3, .52, "a", cex=1.25)

ECMbar <-read.csv(file="Tri Bar.csv")
head(ECMbar)
index <-which(is.na(ECMbar),arr=T)
index
dim(ECMbar)
ECMbar

### 2-way ANOVA testing for interactions
anovaTbyP<-aov(HT~Treatment*Pres, ECMbar)
summary(anovaTbyP)
TukeyHSD(anovaTbyP)
plot(anovaTbyP)

###Calculating Means and Standard Error
#### TREAT is Treatment*Pres on my Excel Sheet
attach(ECM)
ECMm <- tapply(HT, TREAT, mean)
ECMm

ECMse <- tapply(HT, TREAT, std.error)
ECMse

####Making Martix for my Bargraph
### These numbers came from the above calculations
barmatrix <-matrix(c(-9.20, 19.25, 12, -8.75, 0.69, 34.86, 47.87, 53.79), nrow=2,
byrow=T)
barmatrix
colnames(barmatrix)<-c("C", "PD", "R", "RPD")
rownames(barmatrix)<-c("-ECM", "+ECM")
barmatrix

errorbars<- c(3.54, 6.29, 15.22, 3.79, 12.35, 5.33, 18.89, 4.47)
errorbars

x.s<-barplot(barmatrix, ylim= c(-20,80),beside=T, xlab="Mechanical Treatments",
cex.lab=1.25, ylab="Mean stem growth (height in cm) after two seasons",
col=c("white", "black"))
arrows(x0 = x.s, y0=barmatrix , x1=x.s, y1= barmatrix +errorbars, angle = 90,
length = 0.05)
abline(0,0)
legend(2, 70, c("-  ECM", "+ ECM"), pch=c(22,15), title="Legend")
```

```
text(1.5, 3, "d", cex=1.)
text(2.5, 10, "d", cex=1.)
text(4.5, 38, "c", cex=1.)
text(5.5, 42, "bc", cex=1.)
text(7.5, 27, "cd", cex=1)
text(8.5, 57, "ab", cex=1)
text(10.5, 13, "cd", cex=1)
text(11.5, 62, "a", cex=1)

#### This is for Basal Diameter
####Making Martix for my Bargraph
### These numbers came from the above calculations
barmatrix2 <-matrix(c(-0.13,  2.35, 2.96, -.20, 0.43, 4.50, 4.67, 5.86), nrow=2,
byrow=T)
barmatrix2
colnames(barmatrix2)<-c("C", "PD", "R", "RPD")
rownames(barmatrix2)<-c("-ECM", "+ECM")
barmatrix2

errorbars2<- c(0.5844870, 0.8702909, 1.1109305, 0.4159363, 1.6828062, 0.6543038,
2.1744731, 0.4380802)
errorbars2

x.s2<-barplot(barmatrix2, ylim= c(-2,8),beside=T, xlab="Mechanical Treatments",
cex.lab=1.25, ylab="Mean stem growth (basal diameter in mm) after two seasons",
col=c("white", "black"))
arrows(x0 = x.s2, y0=barmatrix2 , x1=x.s, y1= barmatrix2 +errorbars2, angle = 90,
length = 0.05)
abline(0,0)
legend(2, 7, c("-  ECM", "+ ECM"), pch=c(22,15), title="Legend")
text(1.5, 1, "c", cex=1.)
text(2.5, 1.75, "c", cex=1.)
text(4.5, 3.8, "bc", cex=1.)
text(5.5, 5.2, "ab", cex=1.)
text(7.5, 5, "b", cex=1)
text(8.5, 5.7, "ab", cex=1)
text(10.5, 2.25, "c", cex=1)
text(11.5, 6.55, "a", cex=1)
```

Appendix 4. R code for Cox proportional hazard model for seedling survival among soil surface treatments (R, RPD, PD, C) and chestnut seedling types (pure American, B1, B2) in Tri-Valley Wildlife Area, Muskingum County, Ohio. Lines for code are in bold. Lines that begin with ### are for descriptive purposes only.

Cox proportional hazard model

####Survival Curves By Mechanical Treatment

```
getwd()
SUR1 <- read.csv(file="Tri 0 Alive1.csv")
SUR1
head(SUR1)

#checking for NA
index <- which(is.na(SUR1), arr=T)
index

#checking no. rows and columns
dim(SUR1)

library(survival)
attach(SUR1)
names(SUR1)

windows (height=7, width=8)
plot(survfit(Surv(Date,Status)~Treatment), lty=c(1,3,5,6), xlim=c(-1,31),
ylab="Survival % ", xlab="Time(Months)")
legend(0.3, 0.3, c("C", "PD", "R", "RPD"), lty = c(1,3,5,6), title="Legend")
```

####Survival Curves by Seedling Type

```
getwd()
SURseed <- read.csv(file="Tri 0 Alive1.csv")
SURseed
head(SURseed)

#checking for NA
index <- which(is.na(SURseed), arr=T)
index

#checking no. rows and columns
dim(SURseed)
library(survival)
```

```
attach(SURseed)
names(SURseed)
windows (height=7, width=8)
plot(survfit(Surv(Date,Status)~Seedling), lty=c(1,3,5), xlim=c(-1,31), ylab="Survival
%", xlab="Time(Months)")
legend(0.3, 0.275, c("B1", "Am", "B2"), lty = c(1,3,5), title="Legend")
```

Appendix 5. Chapter 4 R code for community data describing rank abundance and testing differences in ECM community composition among season sampled, seed type sampled, and inoculum treatment sampled. Non-metric multidimensional scaling (NMDS) ordinations were used with Bray-Curtis dissimilarities and application of square root transformation and standardized via Wisconsin double standardization. The maximum number of random starts in a search was set at 100 with k=2 stress value. A permutational multivariate analysis of variance was used to test for significant differences for each research question stated above. Lines for code are in bold. Lines that begin with ### are for descriptive purposes only.

####ECM Community Composition
getwd()
dat3 <- read.csv(file="TriSeeds3.csv")
dat3
head(dat3)
dat3
#checking for NA
index <- which(is.na(dat3), arr=T)
index

#checking no. rows and columns
dim(dat3)

#creating the species data
spdata3 <- dat3[,8:17]
dim(spdata3)
head(spdata3)

#creating envir data
envdata3 <-dat3[,1:8]
dim(envdata3)
head(envdata3)

RankAbun3 <-rankabundance(spdata3)
RankAbun3

MDSb <- metaMDS(spdata3, zerodist="add", trymax=100)
plot(MDSb, main= "ECM Communities per Inoculum")
points(MDS, display = "sites", col = "black", pch = 3, lwd = 1.75)
text(MDS, display="species", cex=.75, col = "black", font=3, pch = 3, lwd = 1.75)

##Inoculum
ordihull(MDSb, groups=envdata3$Inoculum, show.groups="Amanita", lty=1, col=1)
ordihull(MDSb, groups=envdata3$Inoculum, show.groups="Control", lty=2, col=2)
ordihull(MDSb, groups=envdata3$Inoculum, show.groups="Laccaria", lty=3, col=3)
ordihull(MDSb, groups=envdata3$Inoculum, show.groups="Scleroderma", lty=4, col=4)
ordihull(MDSb, groups=envdata3$Inoculum, show.groups="Suillus", lty=5, col=5)
ordihull(MDSb, groups=envdata3$Inoculum, show.groups="Hebeloma", lty=6, col=6)
legend(-1.0, .90, c("A. rubescens", "Control", "L. bicolor", "Scl. polyrhizum", "Sul. luteus", "H. crustuliniforme"), lty = c(1,2,3,4,5,6), title="Legend")

###permutation
beta <- betadiver(spdata3, "z")
adonis(beta ~ Inoculum, envdata3, perm=200)

seedling type
MDS <- metaMDS(spdata3, zerodist="add", trymax=100)
plot(MDS, main= "ECM per Tree Type", cex.family="sans", font=1, type = "n")
points(MDS, display = "sites", col = "black", pch = 3, lwd = 1.75)
text(MDS, display="species", cex=.75, col = "black", font=3, pch = 3, lwd = 1.75)
ordihull(MDS, groups=envdata3$Source, show.groups="Seeds", lty=1, col=1)
ordihull(MDS, groups=envdata3$Source, show.groups="Tree", lty=2, col=2)

legend(0.90, -1.3, c("Trap Trees", "Hybrids"), lty = c(1,2), col = c(1, 2), title="Legend")
beta <- betadiver(spdata3, "z")
adonis(beta ~ Source, envdata3, perm=200)

######permutation
beta <- betadiver(spdata3, "z")
adonis(beta ~ Source, envdata3, perm=200)
plot(MDSb, main= "ECM Communities per Treatment")

###Season sampled
plot(MDS, main= "ECM per Season", cex.family="sans", font=1, type = "n")
points(MDS, display = "sites", col = "black", pch = 3, lwd = 1.75)
text(MDS, display="species", cex=.75, col = "black", font=3, pch = 3, lwd = 1.75)
ordihull(MDSb, groups=envdata3$Season, show.groups="Fall", lty=1, col=1)
ordihull(MDSb, groups=envdata3$Season, show.groups="Spring", lty=2, col=2)

beta <- betadiver(spdata3, "z")
adonis(beta ~ Season, envdata3, perm=200)

Appendix 6. R code for % ECM root colonization, growth, and survival in chapter 4, evaluating inoculum in Tri-Valley Wildlife Management Area, Muskingum County, Ohio. Arcsine square root transformation was used to control for unequal variances. Differences in colonization between the ECM colonization were statistically determined by using a one-way analysis of variance (ANOVA) followed by a Tukey's post hoc. Growth parameters (seedling height (cm), and basal diameter (mm), and leaf area (cm^2)) were transformed to meet the assumption of equal variances. Lines for code are in bold. Lines that begin with ### are for descriptive purposes only.

```
##### ECM Root Tips and RGR
getwd()
RGR <- read.csv(file="RGRch3.csv")
RGR
attach(RGR)

####Note:  Package Plotrix for standard Error
#### ECM on Roots
ECMse <-tapply(ECM_Por, INOCULUM, std.error)
ECMse

ECMm <- tapply(ECM_Por, INOCULUM, mean)
ECMm

ECMx.s<- barplot(ECMm, ylim = c(0,.5), ylab = "ECM on root tips",
xlab="Inoculum", cex.lab=1, col=c("black", "grey 10", "grey30", "grey50",
"grey70", "grey 90"))
abline(0,0)
arrows(x0 = ECMx.s, y0=ECMm , x1=x.s, y1= ECMm +ECMse, angle = 90, length =
0.05)
arrows(x0 = ECMx.s, y0=ECMm , x1=x.s, y1= ECMm -ECMse, angle = 90, length =
0.05)

ANOVA <-aov(asin(sqrt(ECM_Por))~INOCULUM)
summary(ANOVA)

#### RGR among naturally inoculated seedling
getwd()
RGR <- read.csv(file="RGRch3.csv")
RGR
attach(RGR)

ANOVAHT <- aov(RGR_HT~Blast)
```

```
summary(ANOVAHT)
TukeyHSD(ANOVAHT)

ANOVABD <- aov(RGR_BD~Blast)
summary(ANOVABD)
TukeyHSD(ANOVABD)

ANOVALeaf <- aov(RGR_Leaf~Blast)
summary(ANOVALeaf)
TukeyHSD(ANOVALeaf)

HTse <-tapply(RGR_HT, Blast, std.error)
HTse

HTm <- tapply(RGR_HT, Blast, mean)
HTm

BDse <- tapply(RGR_BD, Blast, std.error)
BDse

BDm<- tapply(RGR_BD, Blast, mean)
BDm

Leafse <- tapply(RGR_Leaf, Blast, std.error)
Leafse

Leafm<- tapply(RGR_Leaf, Blast, mean)
Leafm

windows (height=3, width=10)
layout(matrix(c(1:3), nrow=1, byrow=T))
HTx.s<- barplot(HTm, ylim = c(0,4), ylab =
expression(Relative~Growth~Rate~(cm/month^-1)), xlab="Natural ECM
Colonization", cex.lab=1, col=c("grey 10", "grey30", "grey50", "grey70", "grey
90"))
abline(0,0)
arrows(x0 = HTx.s, y0=HTm , x1=HTx.s, y1= HTm +HTse, angle = 90, length =
0.05)
arrows(x0 = HTx.s, y0=HTm , x1=HTx.s, y1= HTm -HTse, angle = 90, length = 0.05)
text(.7, 3.35, "ab", cex=1.25)
text(1.9, 0.75, "b", cex=1.25)
text(3.1, 3.1, "a", cex=1.25)
text(4.3, 3.5, "a", cex=1.25)
text(5.5, 2, "ab", cex=1.25)
```

```
BDx.s<- barplot(BDm, ylim = c(0,1), ylab =
expression(Relative~Growth~Rate~(mm/month^-1)), xlab="Natural ECM
Colonization", cex.lab=1, col=c("grey 10", "grey30", "grey50", "grey70", "grey
90"))
abline(0,0)
arrows(x0 = BDx.s, y0=BDm , x1=BDx.s, y1= BDm +BDse, angle = 90, length = 0.05)
arrows(x0 = BDx.s, y0=BDm , x1=BDx.s, y1= BDm -BDse, angle = 90, length = 0.05)
text(.7, 0.52, "ab", cex=1.25)
text(1.9, 0.33, "b", cex=1.25)
text(3.1, 0.8, "a", cex=1.25)
text(4.3, 0.68, "a", cex=1.25)
text(5.5, 0.56, "ab", cex=1.25)

Leafx.s<- barplot(Leafm, ylim = c(0,500), ylab =
expression(Relative~Growth~Rate~(cm^2/month^-1)), xlab="Natural ECM
Colonization", cex.lab=1, col=c("grey 10", "grey30", "grey50", "grey70", "grey
90"))
abline(0,0)
arrows(x0 = Leafx.s, y0=Leafm , x1=Leafx.s, y1= Leafm +Leafse, angle = 90, length
= 0.05)
arrows(x0 = Leafx.s, y0=Leafm , x1=Leafx.s, y1= Leafm -Leafse, angle = 90, length
= 0.05)
text(.7, 152, "b", cex=1.25)
text(1.9, 140, "b", cex=1.25)
text(3.1, 300, "a", cex=1.25)
text(4.3, 490, "a", cex=1.25)
text(5.5, 290, "ab", cex=1.25)

getwd().
RGR <- read.csv(file="RGRch3.csv")
RGR
attach(RGR)

####Note:  Plotrix for standard Error
HTse <-tapply(RGR_HT, INOCULUM, std.error)
HTse

HTm <- tapply(RGR_HT, INOCULUM, mean)
HTm

windows (height=3, width=10)
layout(matrix(c(1:3), nrow=1, byrow=T))
x.s<- barplot(HTm, ylim = c(-6,10), ylab =
expression(Relative~Growth~Rate~(cm/month^-1)), xlab="Inoculum", cex.lab=1.5,
col=c("grey 10", "grey30", "grey50", "grey70", "grey 90", "black"))
abline(0,0)
```

Appendix 7. R code for community data in chapter 5, evaluating soil variables to characterize species composition using data from both mine sites (Avondale Wildlife and Tri-Valley Wildlife Management Area, Muskingum County, Ohio). A non-metric multidimensional scaling (NMDS) ordination used Bray-Curtis dissimilarities with application of square root transformation and standardized via Wisconsin double standardization. The maximum number of random starts in a search was set at 100 with k=2 stress value. Environmental variables were fit onto the NMDS species ordination via fitted vectors by employing function envfit to the species ordination. A permutational multivariate analysis of variance was used to test for significant differences among the soil treatments. Lines for code are in bold. Lines that begin with ### are for descriptive purposes only.

```
getwd()
dat5 <- read.csv(file="CH4_5.csv")
dat5
head(dat5)
#checking for NA
index <- which(is.na(dat5), arr=T)
index
#checking no. rows and columns
dim(dat5)

#creating the species data
spdata5 <- dat5[ ,4:19]
dim(spdata5)
head(spdata5)

#creating envir data
envdata5 <-dat5[ ,20:25]
envdata5
head(envdata5)

#standardize
enStand5 <-wisconsin(envdata5)
enStand5

vare.mds5 <- metaMDS(spdata5, zerodist="add", trymax=100)
vare.mds5

ef5 <- envfit(vare.mds5, enStand5, permu = 1000)
ef5
```

```
treatment5 <- dat5[ ,1:3]

plot(vare.mds5, cex.family="sans", font=1, type = "n")
ordisymbol(vare.mds5, treatment5,'Treatment', rainbow=FALSE, )
text(vare.mds5, display="species", cex=.75, col = "black", font=3, pch = 3, lwd =
1.75)
plot(ef5, col = "black", family="sans", font = 2, p.max = 0.46)

betad <- betadiver(spdata5, "z")
adonis(betad ~ Treatment, treatment5, perm=200)
```

Appendix 8. R code for multiple regressions in chapter 5, evaluating soil variables to predict ECM root colonization on chestnut using data from both mines (Avondale Wildlife and Tri-Valley Wildlife Management Area, Muskingum County, Ohio). To meet models assumption of normality and equal variance, predictor variables were transformed Log10+1 and standardized and the dependent variable (ECM % root coverage) was arcsine transformed. The optimal number of variables to include in the models, were determined by choosing the best subset regression with the lowest Bayesian information criterion (BIC). Lines for code are in bold. Lines that begin with ### are for descriptive purposes only.

####Multiple regressions
getwd()
TVmreg <- read.csv(file="Ch4BAREMultReg.csv")
TVmreg
head(TVmreg)

#checking for NA
index <- which(is.na(TVmreg), arr=T)
index

#checking no. rows and columns
dim(TVmreg)
attach(TVmreg)
names(TVmreg)

###scatter plot matrix to eliminate correlations
pairs(TVmreg, panel=panel.smooth, gap = 0)

##varifying assumptions
mod1 <- lm(asin(sqrt(Proportion)) ~ T_OrgHT07 + T_Moisture + T_pH + T_OM + T_P + T_K + T_Mg + T_Ca + T_NO3_N + T_Mn + T_Al, data = TVmreg)
plot(mod1)

###R2 and BIC to determine best model

library(car)
library(leaps)
gsub <-regsubsets(asin(sqrt(Proportion)) ~ T_OrgHT07 + T_Moisture + T_pH + T_OM + T_P + T_K + T_Mg + T_Ca + T_NO3_N + T_Mn + T_Al, data = TVmreg, nbest=5, nvmax=11)
subsets(gsub, statistic="adjr2")

```
subsets(gsub, statistic="bic")
subsets(gsub, statistic="bic", min.size=3, max.size=5, legend = F)

mod2 <- lm(asin(sqrt(Proportion)) ~ T_OM + T_Mg + T_Mn + T_Al, data =
TVmreg)
summary(mod2)

mod3 <- lm(asin(sqrt(Proportion)) ~ T_OrgHT07 + T_OM + T_Mg + T_Mn + T_Al,
data = TVmreg)
summary(mod3)

anova(mod2, mod3)

slrfit <- lm(T_OM ~ asin(sqrt(Proportion)
slrfit
summary(slrfit)
plot(slrfit)
plot(Proportion, T_OM, main ="ECM colonization vs Organic Matter")
abline(slrfit)

##################on seeds trivalley and avondale

getwd()
SEEDmreg <- read.csv(file="Ch4MulReg.csv")
SEEDmreg
head(SEEDmreg)

#checking for NA
index <- which(is.na(SEEDmreg), arr=T)
index

#checking no. rows and columns
dim(SEEDmreg)

attach(SEEDmreg)
names(SEEDmreg)

###scatter plot matrix to eliminate correlations
pairs(SEEDmreg, panel=panel.smooth, gap = 0)

##varifying assumptions
Smod1 <- lm(asin(sqrt(PercentECM)) ~ T_soilpH + T_CEC + T_OM + T_P + T_K
+ T_Mg + T_Ca + T_Mn, data = SEEDmreg)

plot(Smod1)
```

```
library(car)
library(leaps)
gsub2 <-regsubsets(asin(sqrt(PercentECM)) ~ T_soilpH + T_CEC + T_OM + T_P +
T_K + T_Mg + T_Ca + T_Mn, data = SEEDmreg, nbest=5, nvmax=8)
subsets(gsub2, statistic="adjr2")

subsets(gsub2, statistic="bic")
subsets(gsub2, statistic="bic", min.size=3, max.size=5, legend = F)

Smod <- lm(asin(sqrt(PercentECM)) ~ T_soilpH + T_OM + T_Ca +T_Mg, data =
SEEDmreg)
summary(Smod)

Smod2 <- lm(asin(sqrt(PercentECM)) ~ T_soilpH + T_CEC + T_OM + T_P + T_K
+ T_Mg + T_Ca + T_Mn, data = SEEDmreg)
summary(Smod2)
```