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Research Article

Facilitation of American Chestnut (*Castanea dentata*) Seedling Establishment by *Pinus virginiana* in Mine Restoration

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This study evaluated the influence of planting sites on the establishment and ectomycorrhizal (ECM) colonization of American chestnut (*Castanea denetata* (Marsh.) Borkh.) on an abandoned coal mine in an Appalachian region of the United States. Root morphotyping and sequencing of the fungal internal transcribed spacer (ITS) region were used to identify the ECM species associated with the chestnut seedlings. Germination, survival, ECM root colonization, and growth were assessed in three habitats: forest edge, center (plots without vegetation), and pine plots (a 10-year-old planting of *Pinus virginiana*). Seedlings in pine plots had higher survival (38%) than the other plot types (center 9% and forest edge 5%; $P = 0.007$). Chestnuts found colonized by ECM within the pine plots were larger ($P = 0.02$), contributed by a larger root system ($P = 0.03$). Forest edge and pine plots had more ECM roots than seedlings in center plots ($P = 0.04$). ITS fungal sequences and morphotypes found among chestnut and pine matched *Scleroderma, Thelephora,* and *Pisolithus* suggesting these two plant species shared ECM symbionts. Results indicated that the presence of *P. virginiana* had a greater facilitative effect on growth and survival of chestnut seedlings.

1. Introduction

Ectomycorrhizal (ECM) fungi play a crucial role in aiding the regeneration of plant communities after industrial disturbances such as coal mining [1, 2]. Typically, coal-mined soils have poor physical and chemical properties which include low water-holding capability, low organic matter, extremes in temperature and pH, and high levels of toxic metals [3]. Extensive research has shown that ECM symbiosis alleviates the impact of highly stressed soils on plant growth by increasing access to water and nutrients, mitigating the effects of metal toxicity, and providing protection from root pathogens [4–7]. In turn, these fungi receive carbon in the form of photosynthates from their plant host, indicating a mutualistic relationship between plant and fungi. However, these fungi are not well adapted to endure severe soil disturbances caused by surface coal mining [8, 9]. The severe decline of these microbes may have contributed to the high mortality of planted tree species observed in past reforestation efforts [2].

Although disturbed soils may be capable of supporting some early-successional plant communities, these conditions are generally not desirable for long-term survival of late-successional species [10]. It has been a common practice in restoration efforts to use an ectomycorrhizal inoculum to compensate for the microbial deficiency in these sites. However, reclamation of highly stressed soils requires integrated approaches to reduce costs and increase the chance of plant establishment and survival. Characterization of ECM communities present in disturbed environments may aid in identifying native ECM species that are more suitable for the establishment of specific hardwood tree species in mine-reclaimed locations. However, early studies have shown that the number of fungal species available to the planted seedling is limited in mined soils [11]. Low ECM species richness dominated by fungi that can tolerate disturbance has been previously described following stand replacing fires, clear cutting associated with logging, and mining for coal and other minerals [8, 12, 13].

Pockets of existing vegetation in these mine sites are reservoirs for indigenous species of fungi. The existence of common mycorrhizal networks (CMNs) associated with existing vegetation may facilitate spread of these networks to the newly introduced seedlings, making them a part of the existing network of ECM hyphae [14]. This has been demonstrated in reforestation projects where shrub patches increased mycorrhizal colonization and overall microbial mass [15, 16]. The success of tree species found along wood lots and forest edges has been attributed to the colonization of ECMs harbored by existing forest trees [17]. New seedlings incorporated into these CMNs may also receive carbon transferred from existing trees, which may be responsible for successful plant establishment [18]. Although most studies of plant interactions focus on competition among species for available resources, the importance of facilitation by non-related species through ECM is of great importance in stressed environments [19].

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 [20, 21]. In addition to harboring ECM fungi, neighboring vegetation may buffer soil temperatures [22], increase water and nutrient availability [23], and increase soil aeration. However, in situations where larger vegetative densities occur, positive effects of facilitation may be overshadowed by the competition for other resources and negatively affect the survival of introduced seedlings [24, 25]. This generally happens with canopy trees which, when present in high densities, can mask advantages of ECM facilitation and interfere with seedling establishment [26].

The objective of this study was to evaluate the influence of two different vegetation types on the establishment of American chestnut (*Castanea dentata* (Marsh.) Borkh.) and hybrid-backcrossed chestnut (*C. dentata x C. mollissima*) planted as seeds in an abandoned mine site in the Appalachian region of the central Ohio, USA. The study evaluated germination, survival, and growth of chestnut seedlings in three types of habitat: forest edge, center (away from the edges), and adjacent to 10-year-old *Pinus virginiana* (pine plots). In addition, ECM colonization and its effect on growth of American chestnut seedlings were documented. Morphotyping and sequencing of the fungal internal transcribed spacer (ITS) region was used to identify the ECM species associated with the chestnut.

2. Methods

2.1. Study Site and Experimental Design. An abandoned mine site located in Avondale Wildlife Area in Muskingum County, Ohio, USA (39°C 49′ 44″ N, 82°C 7′ 38″ W) 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. Mined in the 1950s, it had very little reclamation work done. In areas where soils were indiscriminately piled, natural tree growth occurred resulting in forest stands that are approximately 50 years old. In other areas where natural recovery did not occur, experimental tree plantations were initiated in the 1990s using *Fraxinus* spp., *Populus* spp., *Robinia pseudoacacia*, and *Pinus virginiana*. Of these plant species, mostly *P. virginiana* survived, creating small monoculture pine stands. This area normally receives about 99 cm of annual precipitation and temperatures average ∼22°C during the growing season (17°C, 28°C, and 11°C, spring, summer, and fall, resp.).

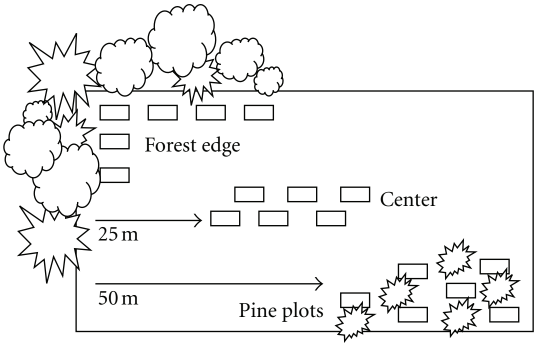


Figure 1: Schematic diagram of field plot layout representing one block (0.80 ha). In each block, three distinct areas were designated as habitat types: forest edge (near the edges), center (away from edges), and in the vicinity of established plantings of 10-year-old *Pinus virginiana* (pine plots). Six plots (4 m × 3 m) were established in each habitat type, each containing 20 chestnut seeds. There were a total of three blocks, each block comprised of 18 plots. In total, there were 54 plots containing a total of 1080 seeds.

The area designated for study had less than 5% herbaceous vegetative cover, very little topsoil or organic matter, and poorly sorted debris. Adjacent forests are ∼55 years old and comprised primarily of *Acer*, *Pinus*, *Fagus*, *Quercus*, and *Ulmus*. The site was divided into three blocks of 0.80 ha. Each block contained 6 plots (4 m × 3 m) belonging to each habitat type: forest edge (near the edges), center (away from edges), and pine plots that were in the vicinity of 10-year-old *Pinus virginiana* (Figure 1). Soil characteristics in each habitat type resembled those found in abandoned gob piles (soil mixed with coal debris). Forest edge plots were placed 4 meters from the edge of the forest canopy spaced 10 m from each other (Figure 1). The areas designated as center plots were completely devoid of trees and were located in the center of the field site, approximately 25 meters from the forest edge (Figure 1). The *P. virginiana* in this area were established as bare-root seedlings in the spring of 1997. These pines averaged 2 to 2.5 m in height with 1 m spread in 2007. These plantings were located about 50 meters from the forest edge and were designated as pine habitat.

Twenty chestnut seeds were planted in each plot. In summary, there were a total of three blocks, each comprised of eighteen 4 m × 3 m plots for a total of 54 plots containing a total of 1080 seeds. The seeds sown consisted of three genotypes: American chestnut, backcrossed chestnut B2-F1 (87% American chestnut genes), and advanced backcrossed chestnut B3-F1 (94% American chestnut genes). Seeds were planted in a 2 : 2 : 1 ratio, respectively. The American and hybrid-backcrossed chestnut seeds were provided to us by The American Chestnut Foundation. 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 plot was fenced with a 2 m high fence constructed from metal *t*-posts and plastic snow fencing. Soil samples (4 samples per plot, spaced 1 m apart) were collected at the time of planting using a soil probe at an 18 cm depth. The four samples 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 analyzed 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). Due to the mobility and fluctuation of nitrogen in the system, soil samples were not analyzed for N. Summer temperatures were recorded on July 8, 2006 between 12:00 and 2:00 PM by soil thermometer with the probe 6 cm into the soil. Two readings were recorded and averaged per plot type. 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 second growing season.

2.2. ECM Sampling, Fungal DNA Extraction, PCR Amplification, and Sequencing. Six months after planting (October 2006), 40 pure American chestnut seedlings representing various treatments were randomly selected for destructive sampling. Because the backcrossed chestnuts (B2-F1 and B3-F1) were not to be destroyed, they were not subjected to destructive sampling. This paper only reports ECM fungi and plant biomass (g) on the pure American seedlings. Seedlings were carefully removed from the field, returned to the lab where root systems were washed and examined under the stereoscope for mycorrhiza formation. To measure plant biomass, roots and shoots were oven-dried for 24 hours at 100°C and then weighed (g). One hundred root tips per seedling were randomly selected (total of 4,000 root tips) and were further characterized with a dissecting microscope for the presence of a fungal sheath. Two samples per morphotype, per seedling, were selected for DNA extraction. A 3 mm segment of root tip was removed, transferred to a microcentrifuge tube, and stored at −70°C until extracting the DNA. After 18 months (October 2007), another 90 seedlings (9000 root tips) were selected and sampled in the same manner. In addition to chestnut seedlings, 25 root samples from *P. virginiana* present in plots were also collected for similar analysis. A 25 cm deep, 45 cm wide trench was excavated at the root zone of the existing pine trees to expose roots for sampling. Roots collected were then stored on ice in the field and returned to the laboratory.

Presence and type of ECM species on the root tip were determined by DNA extraction followed by PCR and DNA sequencing [27]. Briefly, 10 mg tissue was homogenized in extraction buffer using a bead beater and DNA was extracted using the QIAgen's DNeasy Plant Mini Kit. About 10 ng of this DNA was used in PCR reactions using primers ITS1-F (5′ cttggtcatttaggaagtaa 3′) and ITS4 (5′ tcctccgcttattgatatgc 3′), which amplify the highly variable internal transcribed spacer (ITS) region of ECM fungal ribosomal DNA (rDNA) [28]. The PCR products were purified using Wizard SV 96 Genomic DNA Purification System (Promega, USA) and used for DNA sequencing. By comparing these sequences to those present in the GenBank, identity of the ECM fungus was determined [29].

In addition to root sampling, the second set of the American chestnut seedlings (90 seedlings collected after 18 months) were also used to determine differences in biomass between mycorrhizal and nonmycorrhizal seedlings. Also, 30 chestnut seedlings harvested from the pine plots were subjected to foliar nutrient analysis. Of these, 15 seedlings had no detectable fungal sheath when examined under the dissecting scope. The other 15 had *Scleroderma* morphotype and were later confirmed by DNA extraction/sequencing. Twenty-five leaves per seedling were harvested in the lab, packaged in paper bags as fresh samples, and sent to Spectrum Analytic Inc., Washington Court House, Ohio, for tissue analysis. Leaf tissue parameters analyzed included N, P, K, Ca, B, Cu, Fe, Mn, and Zn.

2.3. Statistical Analysis. Germination and survival among treatment types were assessed using an analysis of variance (ANOVA) followed by Tukey's HSD (Honestly Significant Difference). The ECM root colonization was analyzed using a nonparametric Kruskal-Wallis utilizing X^2 test statistic to determine differences among habitat types. For growth and soil analysis, 4 m × 3 m plots were selected as a sample unit because seedlings sampled within a small area are likely to be strongly autocorrelated and not independent. Differences in soil chemistry were detected using an ANOVA and Tukey's HSD post hoc. To determine differences in seedling biomass (root, shoot, and total dry weight) between chestnuts colonized by native ECM (+ECM) and non-ECM seedlings (–ECM), a two-way ANOVA on a 2 × 3 (with or without ECM × three plot types) factorial design was used. Both ECM status (+ECM or –ECM) and habitat type (forest edge, center, pine plots) were the main effects and the block was a random effect. Log (n+1) transformation was used to control for unequal variances in the tissue analysis. Square root transformations were used to control for unequal variances for soil parameters and seedling biomass. An independent samples *t*-test was used to determine differences in leaf tissue analysis between *Scleroderma* seedlings and non-ECM seedlings (Log ($n + 1$) transformations used to control for unequal variance). All statistical analyses were performed using JMP software (8.0, SAS Institute, Cary, NC, USA).

3. Results

3.1. Soil Properties among Habitat Types. Analysis of soil samples collected at the beginning of this study indicated that all the habitat type areas were similar with respect to the CEC (31.31–33.61) and the pH (2.8 to 3.1) (Table 1). Summer temperatures recorded in July of the first growing season were significantly higher in the center plots (38.0°C) than those in the forest edge (33.2°C) and in the pine plots (35.7°C; $F(2) = 5.57$, $P = 0.03$). The organic matter composition was also statistically different ($F(2) = 4.42$, $P = 0.04$) among the 3 areas; pine plots (1.33%) had the

Table 1: Comparison among habitat types (C = center, FE = forest edge, P = pine plots) 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 $\alpha = 0.05$ to Tukey's HSD. Analyses based on data transformed by square root.

Treatment	Summer temp (C)	CEC	OM (%)	pH
C	38.08 ± 1.02^{a}	33.61 ± 0.52^{a}	$2.88 \pm 0.42^{a,b}$	2.82 ± 0.07^{a}
FE	33.18 ± 1.59^{b}	31.31 ± 0.98^{a}	3.44 ± 0.42^{a}	2.90 ± 0.19^{a}
P	35.69 ± 0.86^{b}	31.86 ± 0.76^{a}	1.33 ± 0.21^{b}	3.10 ± 0.06^{a}

Table 2: Comparison among habitat types (C = center, FE = forest edge, P = pine plots) with regard to soil concentrations of macro- and micronutrients obtained from soil samples from plots. Values are expressed as ±1 SE. Means sharing the same letter do not differ significantly according to $\alpha = 0.05$ to Tukey's HSD.

Plot	P ppm	K ppm*	Mg ppm*	Ca ppm	S ppm
C	1.3 ± 0.2^{a}	71.3 ± 13.4^{ab}	334.4 ± 45.7^{a}	401.6 ± 142.6^{a}	1211 ± 130.81^{a}
FE	1 ± 0^{a}	67.4 ± 9.8^{b}	174.4 ± 27.5^{b}	676.4 ± 469.5^{a}	918.8 ± 177.2^{a}
P	2.1 ± 0.8^{a}	103.3 ± 15.4^{a}	254.9 ± 24.4^{ab}	742.5 ± 189.4^{a}	772.1 ± 157.96^{a}
Plot	B ppm*	Zn ppm	Fe ppm*	Cu ppm	Mn ppm
C	0.6 ± 0.03^{a}	12.2 ± 4.0^{a}	476.7 ± 102.4^{ab}	6.9 ± 1.3^{a}	8.4 ± 1.4^{a}
FE	0.5 ± 0.04^{ab}	6.1 ± 2.0^{a}	704.2 ± 225.1^{a}	5.2 ± 1.0^{a}	7.5 ± 2.1^{a}
P	0.5 ± 0.03^{b}	10 ± 3.5^{a}	303.2 ± 76.8^{b}	7.5 ± 1.3^{a}	8.8 ± 2.0^{a}

lowest as compared to the center (2.88%) and plots along the forest edge (3.44%; Table 1).

Results of analysis of other macro- and micronutrients are summarized in Table 2. One-way ANOVA followed by Tukey's HSD of each soil nutrient was used to determine significance among the nutrients per habitat type (Table 2). Among the macronutrients detected in the soil analysis, K, Ca, Mg, and S differed significantly with respect to the habitat type (all $P < .05$). Pine plots had the highest concentrations of K while Ca was the highest in forest edge and pine habitat soils. The elements Mg and Zn were highest in both the pine and center plots. Differences were also observed in soil concentrations of S and B; both were highest in the center and forest edge plots. However, concentrations of P were not significantly different among the three habitat types.

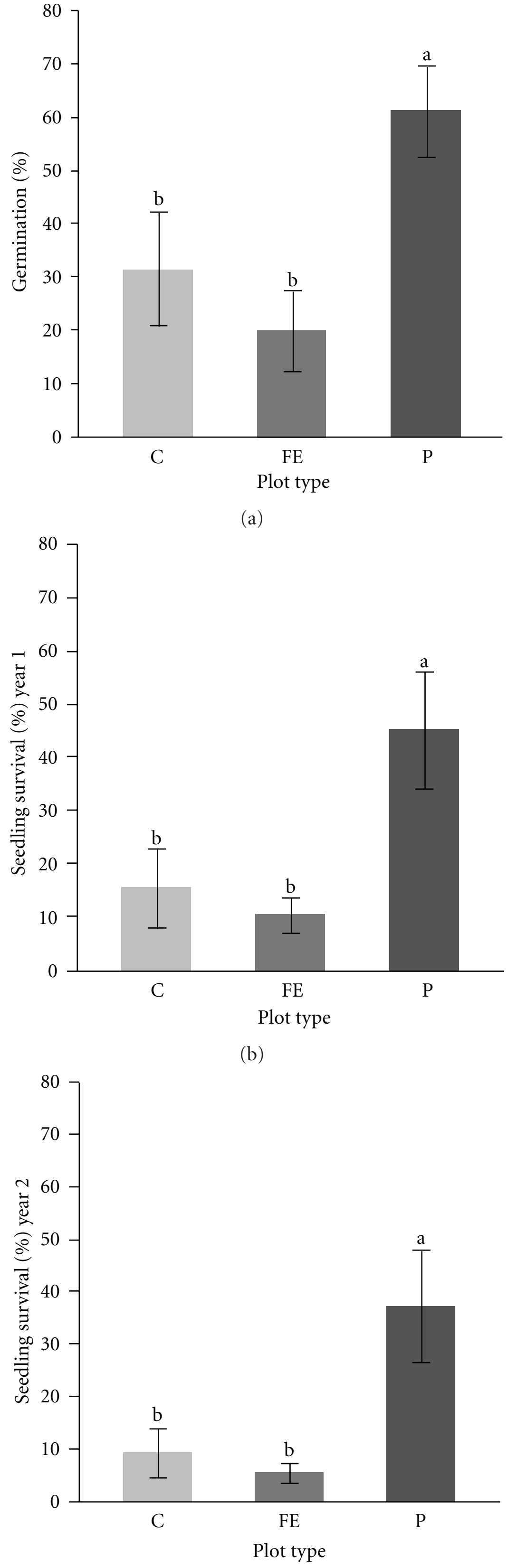
The levels of micronutrients B, Zn, Fe, and Cu were also significantly different among the habitat types (all $P < 0.05$; Table 2). Levels of B were significantly higher in the center and forest edge plots while concentrations of Fe were higher along the forest edge. Levels of Zn and Cu were highest in the pine and center plots. Only in case of Mn levels, there were no differences among the habitat types.

3.2. Seedling Survival and Growth. Germination, recorded three months after chestnut seeds were sown, was significantly higher in pine plots (61%) as compared to the center plots (32%) and forest edge plots (21%) ($F(2) = 5.14$, $P = 0.02$; Figure 2(a)). Greater seedling survival was also recorded in pine plots after the first growing season: pine plots (46%), center plots (17%), and forest edge (12%; $F(2) = 4.92$, $P = 0.02$; Figure 2(b)). After two growing seasons the habitat type effect was still apparent; the seedlings in pine plots had the highest survivorship (38%) compared to those in center plots (9%) and plots in the forest edge (5%; $F(2) = 7.02$, $P = 0.007$; Figure 2(c)).

Results of ECM root colonization are shown in Figure 3. Seedlings growing along the forest edge had the highest incidence of colonization (58%) followed by those in the pine plots (38%). However, the rate of colonization was much lower in the center plots. Both were significantly greater than what was sampled from chestnut seedlings in the center plots (14%; $F\ (2) = 5.28$, $P = 0.04$; Figure 3).

Biomass variations measured after two growing seasons are shown in Figure 4. After two growing seasons, there were no significant interactions between habitat type and native ECM colonization with regard to seedling biomass. Differences in total seedling biomass did exist between seedlings colonized with ECM fungi (+ECM) and their non-ECM (–ECM) counterparts ($F(5,\ 32) = 2.92$, $P = 0.02$); this was the case for the pine and forest edge plots (Figure 4(a)). In the pine plots, +ECM seedlings (6.9 g) were greater than –ECM counterparts (4.2 g); 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. Also, biomass of +ECM (4.1 g) and –ECM (3.5 g) seedlings were similar (Figure 4(a)). No significant differences existed between interactions or main effects when shoot biomass was compared (Figure 4(b)). Similar to total biomass, differences were significant when root biomass was compared between the +ECM and –ECM seedlings in the pine plots ($F = 2.75\ (5,\ 32)$, $P = 0.03$; Figure 4(c)). +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 g, 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(c)).

3.3. ECM Survey. One hundred and thirty-one seedlings from different habitat types were sampled and ECM fungi related to 12 different genera were found to be associated with root tips. Presence of different fungi by habitat plot is shown in Table 3. The pine and forest edge plot seedlings



(a)

(b)

(c)

Figure 2: Germination and survival of the three habitat types: 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.

Table 3: Species sampled from pure American and backcrossed chestnut after two field seasons. Species are recorded with their percent abundance, habitat type plot from where they were sampled (C : center, FE : forest edge, and P : pine plot), whether they were found on the roots of adjacent *P. virginiana* and published GenBank accessions.

ECM Genera	Relative Abundance	Plot Sampled	Sampled on *P. virginiana*	Accession
Scleroderma sp.	52%	C, FE, P	Yes	GU553366
Thelephora sp.	13%	C, P	Yes	GU553377
Pisolithus sp.	8%	P	Yes	GU553367
Oidiodendron sp.	6%	P	No	GU553368
Cenococcum sp.	4%	C, FE, P	No	GU553373
Laccaria sp.	4%	FE	No	GU553370
Russula sp.	3%	FE	No	GU553374
Unknown ECM	3%	FE, P	Yes	GU553372
Thelephoraceae	3%	FE, P	Yes	GU553376
Tomentella sp.	2%	FE, P	No	GU553375
Lactarius sp.	1%	FE	No	GU553369
Suillus sp.	1%	P	No	GU553371

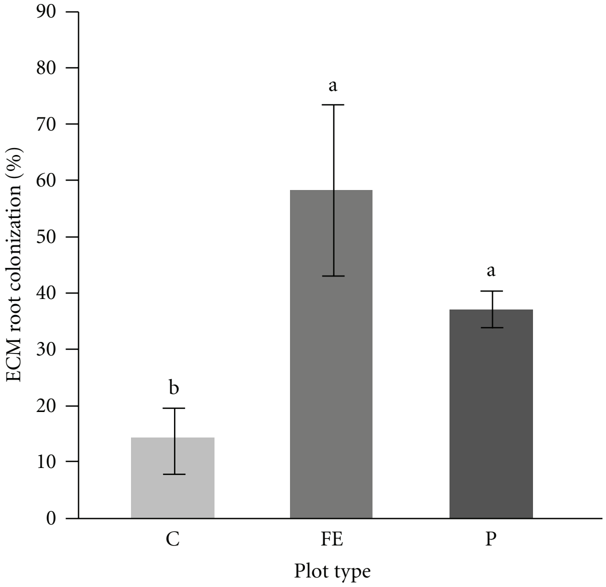


Figure 3: Percent ECM root tips (±1 SE) on American chestnut seedlings after two field seasons. Bars sharing common letters do not significantly differ at $\alpha = 0.05$ determined by Tukey's HSD. Seedlings in the center plots (C) had less ECM on roots when compared to forest edge (FE) and pine plots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

contained 8 species each while only 3 species were found on seedlings in the center plots.

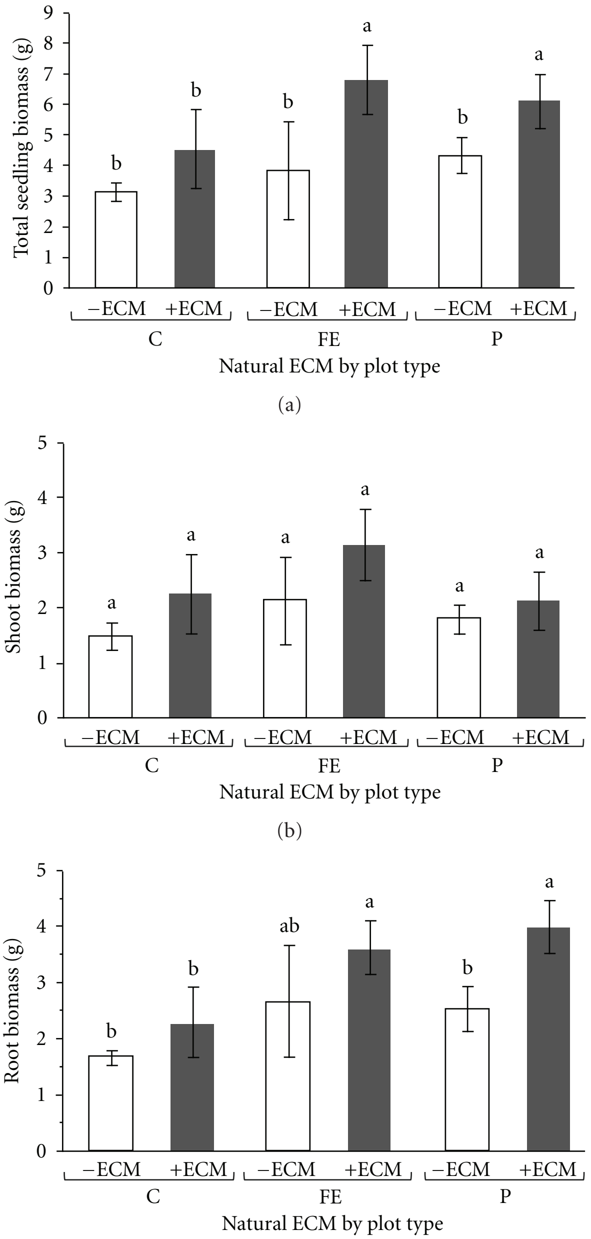
Overall, *Scleroderma* sp. was the most abundant in this study (51%). This was followed by *Thelephora* sp. (13%), *Pisolithus* (8%), *Oidiodendron* (6%), *Cenococcum* (4%), and *Laccaria* (4%). Fungi representing *Russula*, Thelephoraceae, *Tomentella*, *Lactarius*, and *Suillus* were relatively rare (Table 3). In addition, an unknown ECM species was also found that could not be identified through ITS region sequencing and comparison to the sequences in the GenBank.

Root samples from *P. virginiana* in pine plots were also characterized morphologically and by the ITS region sequencing. Five morphotypes appeared to be common between the chestnut and pine hosts (Figure 5, Table 3). Out of these, three were later identified by ITS region sequencing to be *Scleroderma*, *Thelephora*, and *Pisolithus*. Two additional morphotypes were observed which, by ITS region sequencing, matched to the ones on the chestnut and corresponded to an unidentified Thelephoraceae and another unidentified ECM spp. 1 (data not shown).

3.4. Leaf Chemistry. A set of chestnut seedlings from the pine plots were selected for analysis of leaf chemistry to determine whether ECM colonization had any effect (Table 4). Seedlings with ECM (+) and without (–) *Scleroderma* were used for this analysis. Although foliar concentrations of macronutrients N, K, and Mg were slightly elevated in *Scleroderma*-associated chestnut seedlings, no significant differences were observed in the levels of P. However, Ca levels were lower in the ECM-colonized chestnut samples as compared to their counterparts. Comparison of micronutrients showed that the most significant difference was in the levels of Cu, which were significantly lower in the ECM+ samples ($t\ (1)\ =\ 2.01$, $P\ <\ 0.03$). Manganese levels also differed; however, these levels were only marginally lower in the *Scleroderma* + samples.

4. Discussion

Our results show that chestnut seedlings growing among the pines had higher survival rates than those in the center plots or along the forest edge. Furthermore, chestnut seedlings in pine plots that were colonized with ECM fungi had greater biomass. This can be attributed to several factors investigated in this study. The chemical properties of the soil in the study site were typical of mined soils in the eastern United States [30]. Analyses done prior to the beginning of this study showed that the essential nutrients for plant growth, P, K, and B, were in very low concentrations and this was coupled with toxic levels of S, Zn, Fe, Cu, and Mn. Comparison among different habitat soils indicated clearly that soils in pine plots had higher concentrations of K and P



(a)

(b)

(c)

Figure 4: Comparison of chestnut seedling biomass among habitat types (C : center, FE : forest edge, P : pine plots) of the following: total seedling biomass (a), shoot biomass (b), and root biomass (c) reported in grams, ±1 SE. Bars sharing common letters do not significantly differ at $\alpha = 0.05$ determined by Tukey's HSD.

while the levels of Fe and S were much lower. The presence of certain tree species modifies the physical and biotic conditions in the surrounding soil, which may facilitate seedling establishment. For example, pine vegetation has been shown to influence changes in rhizosphere chemistry [16] and increase soil nutrients from litter accumulation [23]. Although not measured in this study, moisture levels are known to remain higher in the soils under established plants by reducing evaporation and increasing the water availability by hydraulic lift [31]. These mechanisms contribute to lower temperatures [32], as observed 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 [33].

Although ECM colonization and species richness were highest among seedlings growing along the forest edge, seedling survival in these plots was the lowest. The forest edge clearly supplies seedlings with an ECM symbiont. However, it also contributes to the harmful effects of competition imposed by the canopy trees. The taller chestnut seedlings recorded along the forest edge may be indicative of

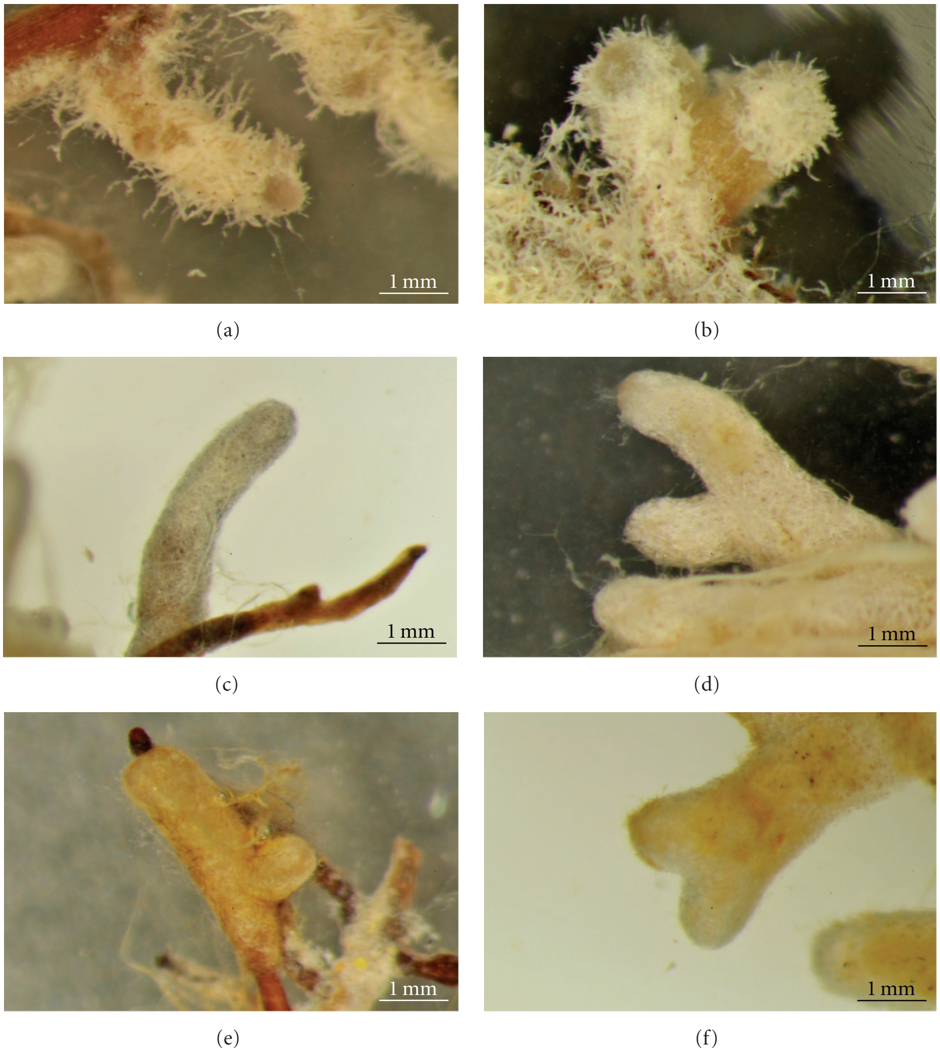


Figure 5: Photographed (45x) ECM morphotypes sampled from American chestnut (*C. dentata*) and *P. virginiana* root tips from pine plots. Panels display the following that were matched to vouchered GenBank sequences: (a) *Thelephora* sp. on *C. dentata*, (b) *Thelephora* sp. on *P. virginiana*, (c) *Scleroderma* sp. on *C. dentata*, (d) *Scleroderma* sp. on *P. virginiana*, (e) *Pisolithus* sp. on *C. dentata*, and (f) *Pisolithus* sp. on *P. virginiana*. Bar = 1 mm.

such competition for light. This canopy shading effect may have greatly contributed to the diminished germination and seedling survival along the forest edge. Although the pine plots may have caused similar problems, they were significantly smaller in size and lacked canopies that would cast predominant shade. The germination and survival rates of American chestnut seedlings are higher in canopy gaps and thinned areas that offer more sunlight [34]. Others have reported that the interactions may be spatially dependent; competition for resources may require seedling establishment to be at a distance from existing larger areas of vegetation [35, 36]. Our results appear to be in agreement with the fact that, as tree densities increase, there is little additional gain from an ECM donor [24].

Without the imposed competition from an existing forest canopy, chestnuts growing in the pine plots had higher germination and survival. Pine plots provided chestnuts with an ECM propagule, which is a needed for survival of ECM plants in harsh environments [19]. The ITS sequence analysis identified five ECM fungi shared by both chestnut and established *P. virginiana*: *Scleroderma*, *Thelephora*, *Pisolithus*, unidentified Thelephoraceae, and unidentified ECM sp. 1. Past restoration efforts in abandoned coal mines high in mine spoil reported significantly reduced incidences of functional ectomycorrhizas due to a lack of available inoculum [2]. The availability of ECM inoculum from a distantly related plant species demonstrates positive interactions between plants facilitating the establishment of a later successional group [37]. Root colonization by these species may have been accomplished by hyphae or rhizomorphs radiating from the established pines [38]. *Scleroderma* and *Pisolithus* produce rhizomorphs that spread through the soil several decimeters away and are capable of long-distance exploration resulting in growth increases of their plant hosts [39]. Although this study did not test common mycorrhizal networks (CMNs), previous work has reported that there are

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

	%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
+	2.06 ± 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

net carbon gains for an establishing seedling linked in an existing CMN [7, 16, 18]. In addition, both ECM species are prolific spore producers capable of forming mycorrhizas from spore inoculum [38].

We observed significantly higher numbers of ECM root tips and greater species richness in forest edge and pine habitats. Higher ECM root colonization and species richness have been linked to existing vegetation [24]. Lower species richness and root colonization are a common finding in large tree gaps due to the lack of root contact from other trees [40], which was the case with the center plots in this study. Kranabetter and Friesen [40] also reported that root colonization decreased in gaps despite initial colonization with ECM fungi, suggesting that other site factors may contribute to ECM growth. Some of these factors could be related to the soil temperature, chemistry, other microbes, and organic matter; however, more work is required to identify these variables. Reduced ECM colonization may actually be beneficial to the host plant in arid environments where water stress may limit photosynthetic efficiency. In such situations a decrease in ECM colonization will reduce the carbon cost on the plant [41]. Alternatively, ECM contact without a CMN may not be enough to support seedling establishment in these soils and the carbon production from the seedling is insufficient to support an ECM symbiont. Therefore, in the present study the observed limited root colonization on seedlings in center plots resulted in a neutral response with regard to improving plant biomass.

The growth data in our study illustrated a significant increase in root biomass. This contributed to an increase in total seedling biomass (g) in ECM seedlings within the pine habitats. No significant differences in above ground growth were noted suggesting ECM seedlings allocated carbon mainly to the 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 [30]. 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 [42]. Rhizomorph-forming ECM species like *Scleroderma* and *Pisolithus* are known to improve seedling-water relations, allowing for greater access to water, which results in increased photosynthesis and net carbon gains [43].

Because *Scleroderma* was the most abundant ECM species sampled in this study, we chose leaf samples from pine plots to analyze the influence of this ECM genera on nutrient and metal uptake. Contrary to our expectations, there was no significant increase in foliar macronutrients in the leaf tissue due to ECM colonization. This may have been an artifact due to the overall low nutrient levels seen in the soil. It is also possible that additional tissue analysis of root and stem was needed to detect some differences in nutrient concentrations. The only difference in nutrient uptake worth noting was with regard to Ca, whose 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 such low pH, the H^+ ions displace Ca^+ impeding uptake by the plant [44]. Although previous studies have demonstrated that ECM colonization remedies the effect of low pH on the plant uptake of Ca^+ [45], this was not seen in this study.

Our results showed that *Scleroderma* colonization caused significant decreases in some micronutrients known to be in toxic concentration in leaf tissues. Though copper is a micronutrient essential for plant growth, elevated levels damage the photosynthetic apparatus (particularly photosystem I) compromising photosynthetic efficiency [46, 47]. In addition to copper levels, levels of Mn were also lower in *Scleroderma*-colonized seedlings. Higher levels of Mn are known to decrease photosynthetic efficiency of the plant by causing the oxidation of phenols leading to necrotic tissues on leaf surfaces [48]. Our results are in agreement with those that have shown *Scleroderma* as an ECM species that greatly contributes to seedling establishment in mine soils by amelioration of metals [40]. However, previous studies have reported contrasting results with regard to *Scleroderma*'s tolerance of copper [49]. This study suggests that *Scleroderma* sp. survives in soils high in copper and contributes to the reduction of foliar Cu in chestnuts. The mechanism(s) by which these fungi can tolerate metals is not known. Some species are known to sequester substantial amounts of metal in the hyphae [50] while other species bind metals to soil particles by the production of fungal polysaccharides [51].

A total of 12 species of ECM fungi were detected after the first and second growing season. Overall, colonization of these species contributed to the higher seedling biomass.

The more commonly sampled genera in this study include basidiomycetes *Scleroderma*, *Thelephora* and *Pisolithus*, and ascomycetes *Oidiodendron*, and *Cenococcum*. Each of these species shares attributes of early stage, stress tolerating (s-selected) mycorrhizal fungi. These adaptations included persistent spores or sclerotia, saprophytic life capabilities, ability to utilize difficult forms of N and P, a broad host range, and the ability to tolerate toxic metals [13, 51, 52].

Several past studies surveying arid soils have shown that the ECM community is dominated by ascomycete fungi thought to tolerate stressful abiotic conditions [53]. *Cenococcum* is globally ubiquitous, particularly at farther distances from existing vegetation where environments are stressful and competition with other ECM fungi is low [52]. It remains unclear what environmental conditions make this species beneficial to its host. *Oidiodendron* spp. was once considered specific to plant species in the Ericaceae taxon [54]. Recent findings suggest that these ascomycete fungi also form dark septate endophytes with other plant taxa [55]. The role of these dark septate fungal species is unknown, although they have been reported on root tips after major disturbance [39]. The propensity of these fungi to obtain limited N and P, and bind metals helped increase nutrient uptake and decrease metal toxicity in the plant tissue [54, 56]. Our analysis showed that these fungi contributed to the increased biomass observed in ECM seedlings suggesting that ascomycetes are beneficial to chestnut in stressed soil environments.

5. Conclusion

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 [57]. 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 composition of fungal species and root colonization [3, 57]. Examination of these ECM groups and their attributes can be an important indicator of microbial functioning throughout a reclamation project [10]. 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 assembly containing ECM species that are better competitors. A hardwood such as chestnut hosts several ECM fungal species as it establishes. This, in turn, increases the inoculum source for incoming trees and may facilitate seedling recruitment leading to the eventual recovery of these severely disturbed lands.

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