

THE EFFICIENCY OF INTRODUCED *PISOLITHUS TINCTORIUS* INOCULUM ON BACKCROSSED CHESTNUT GERMINATION AND SURVIVAL¹

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Abstract: American chestnut was eliminated as a canopy tree from the Appalachian region of North America with the introduction of chestnut blight in the early 1900s. Breeding programs initiated in the 1980s have produced seedling lines that display the pure American morphology with potential resistance to chestnut blight. More work is required to assess their field performance in field sites representative of their native range. This study used American (*Castanea dentata*) and backcrossed hybrid chestnuts (*C. dentata* × *C. mollissima*) on an abandoned coal mine in southeastern Ohio. Half of the seeds were planted with ectomycorrhizal fungus (ECM) *Pisolithus tinctorius* (Pt) as a granular inoculum. Germination, survival, percent ECM colonization, and ECM community were assessed. In addition, soil data such as pH, cation exchange capacity, nutrients, texture, temperature, moisture, and organic matter were evaluated to determine their influence on ECM. After the first and second growing season, germination and survival were not influenced by the Pt inoculum or chestnut genotypes. In addition, ECM root colonization, fungal community composition, and host response were similar between seedling types, regardless of the inoculum added. This indicated: 1) backcrossed breeding produces seedlings similar to pure American chestnuts with regard to growth, establishment, and fungal symbionts, and 2) the granular inoculum had no influence on ECM colonization in this field site. Introducing spore inoculum to a field site greatly deficient in nutrients and organic matter is not an efficient method to ensure ECM symbiosis. However, natural colonization by native ECM fungi, though limited, did result in larger chestnut seedlings. Cation exchange capacity significantly contributed to the percent of root colonization of ECM fungi on chestnut. Determination of planting protocols and factors that influence ECM root colonization will be useful for in future mine restoration projects using backcrossed American chestnut as a restoration tree.

Additional Keywords: ectomycorrhizal species composition, organic matter, chestnut field trials

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Introduction

Soils on reclaimed coal mine sites may have very little microbial activity due to low abundance of host plants, and on some sites, soil compaction and adverse soil chemistry. The low abundance of these microbes may contribute to the limited native tree and shrub survival on certain reclaimed and abandoned mine sites. Ectomycorrhizal (ECM) fungi are important to habitat reconstruction in North American forest systems. *Pisolithus tinctorius* (Pt) is an ectomycorrhizal (ECM) fungus with a broad host range that will form mycorrhizas with a large number of species (Cairney and Chambers, 1997). Methods for nursery inoculation are well understood and extensively used in reforestation programs (Marx, 1979). Pt has the ability to aid in seedling establishment in conditions typical of some mine sites; i.e. high soil temperatures and heavy metals coupled with low soil fertility and pH (Sprouse, 2004). Seedlings inoculated with Pt have been reported to improve survival rates, increase nutrient uptake, and decrease mineral toxicity (Cordell et al., 1999; Walker et al., 2004).

Tree taxa such as Fagaceae and Pinaceae inoculate readily in nursery environments and are commonly used in reforestation projects (Sprouse, 2004). To inoculate seedlings with Pt, the roots are treated and/or the seed beds are fumigated with a spore suspension and nursery grown under natural conditions (Marx et al., 1977; Hopkins, per com.). In eastern Ohio, it has been estimated that 66% of all trees planted in mine reclamation projects in Ohio are inoculated with Pt with reports of survival rates between 75-85% (Cordell et al., 1999; Sprouse, 2004). The formation of Pt mycorrhizas is easy to recognize under a dissecting microscope; roots are characterized by their monopodial morphology with golden hyphal threads (Cairney and Chambers, 1997). Pt is not a good competitor and thrives in stressful conditions in the absence of other fungal species (McFee and Fortin, 1988). The duration of Pt has been reported between two and four years in the field before it is replaced by other fungi (Grossnickle and Reid, 1982).

American chestnut (*Castanea dentata* (Marsh. Borkh.), like other members of Fagaceae, forms ectomycorrhizae with Pt (Hiremath and Lehtoma, 2007; Bauman et al., 2012). Previous studies have reported that Pt inoculated American chestnut seedlings can establish on both abandoned and reclaimed mine sites (McCarthy et al., 2008). The fast growth rate coupled with quality timber makes American chestnut a desired species for use in reforestation projects. Backcrossed breeding programs have resulted in chestnuts that may display blight-resistance

with the desired morphological characteristics of the American chestnut (Hebard, 2005). However, these backcrossed hybrids need proper testing to assess both the field survival and growth habit of these genotypes in areas where American chestnut was once prevalent.

Ohio mine land reclamation projects compliment the native range of American chestnut and provide opportunities for examining planting methods most conducive to seedling establishment. Although there has been success with planting bare-rooted seedlings, chestnut establishment by direct seeding has not been as successful (McCarthy et al., 2010). The low availability of ECM propagules may have played a role in the low survival. This current study evaluated the efficiency of Pt granular inoculum upon seeding. Seedlings were sampled after 4 and 18 months to assess the ECM fungi found colonizing roots. Fungi were quantified and related to chestnut biomass production. Secondly, this study compared the germination, survival, and ECM colonization of the three different chestnut genotypes. Lastly, soil chemistry was evaluated to determine how the abiotic substrate influences ECM root colonization.

Methods

Field Site

A partially reclaimed mine located in Avondale Wildlife Area in Muskingum County OH (39° 49' 44" N, 82° 7' 38" W), was selected for this study (Fig. 1). 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). This area was surface mined in the 1950s and past reclamation efforts were met with partial success. Areas that were successfully reclaimed are now a 55 year-old forest area comprised mainly of *Acer*, *Pinus*, *Fagus*, *Quercus*, and *Ulmus* species. In 1997, a mix of hardwood and evergreen species were planted in an attempt to restore the areas left barren. The only surviving seedlings were *Pinus virginiana*. This site has remained devoid of plant cover (less than 5%) and has very little topsoil or organic matter. Soil characteristics of these bare areas resemble gob piles (soil mixed with coal debris). Soil samples collected prior to this study were sent to Spectrum Analytic Inc., Washington Court House, OH for analysis and are reported in Table 1. Soil pH ranged from 2.9 to 3.1. Organic matter averaged 2.55% and the mean cation exchange capacity was 32. Mean values for extractable soil nutrients were: P, 1.46 ppm; K, 81 ppm; Mg, 255 ppm; Ca, 607 ppm; S, 967 ppm; B, 0.54 ppm, Zn, 9.43 ppm; Fe 494 ppm, Cu, 6.55 ppm; and Mn, 8.2 ppm.



Figure 1. Avondale Wildlife Area in Muskingum County, OH showing field plot layout. The area was 1 hectare. Fifty-four, 4 m × 3 m plots were established (27 with Pt inoculum and 27 without). A 1.8m (6 ft) high fence was constructed with metal t-posts and plastic snow fencing to discourage deer from grazing on the seedlings.

There were 54 (4 m x 3 m) plots each with 18 chestnut seeds, established for this study (972 seeds total). The seeds were sown in a 2:2:1 ratio of three genotypes: American chestnuts (*C. dentata*), backcrossed chestnuts BC_2F_1 (backcrossed to create a progeny that is 7/8 *C. dentata* and 1/8 *C. mollissima*), and backcrossed chestnuts BC_3F_1 (backcrossed to create a progeny that is 15/16 *C. dentata* and 1/16 *C. mollissima*). The chestnut seed were produced and provided by the American Chestnut Foundation. Seeds were stored at 4° C for 16 weeks and planted in the field in March, spaced 0.50 meters apart. One half the plots (27 plots) had commercial Pt mycorrhizal fungal inoculum, Mycor® Plant Saver® mixed into the backfill upon sowing the seed. To prevent disturbance from seed predators, each seed was caged using aluminum gutter screening. To prevent grazing by deer, a 1.8m (6 ft) high fence was constructed out metal t-posts and plastic snow fencing fastened by plastic zip strips (Fig. 1). Survival was recorded monthly during the growing season for the first two field seasons. Chestnut growth parameters were recorded after

18 months: plant height (from ground to terminal bud in cm), leaf area (using a Li-cor® Area Meter in cm³), and basal diameter (measured with hand-held caliper and recorded mm).

Table 1. Average soil pH, organic matter (OM), cation exchange capacity (CEC), and micro and macronutrients (ppm) sampled during this study.

Soil Variable	Average
pH	3
OM (%)	2.55
CEC	32
P (ppm)	1.46
K (ppm)	81
Mg (ppm)	255
Ca (ppm)	607
S (ppm)	967
B (ppm)	0.54
Zn (ppm)	9.43
Fe (ppm)	494
Cu (ppm)	6.55
Mn (ppm)	8.2

ECM Sampling

To determine existing ECM species in the field site, soil from was collected from the field site, returned to the greenhouse, and used as a growth medium for 40 pure American chestnut seeds. Ten seeds were planted per 30 cm × 30 cm × 7.5 cm plastic seed tray and grown in the greenhouse for six-weeks (four trays total). Seedlings were provided natural light and watered with tap water when needed. ECM fungal sheaths were observed on 10 seedlings after six weeks. ECM species identity was determined by DNA extraction and sequencing of the internal transcribed spacer (ITS) region as described in Bauman et al. (2011).

After 4 months in the field, 40 pure American seedlings were selected for destructive sampling. One hundred root tips per seedling were randomly selected and observed under a dissecting microscope for ECM formation. Roots were sampled, ECM sheath morphology documented, and the ITS region was sequenced to identify fungi. After 18 months in the field, 90 seedlings (45 pure American and 45 *BC₂F₁* seedlings) were selected for sampling. Percent colonization (ECM roots/100) was calculated and the differences in the morphologies of ECM fungal sheaths were used to assess differences in ECM community composition between the pure

American chestnuts and the backcrossed BC_2F_1 seedlings. The BC_3F_1 genotype was not destructively sampled and therefore not included in the ECM analysis. To measure plant biomass, roots and shoots were oven-dried for 24 hours at 100° C and then weighed (g).

Statistical Analysis

Survival among both inoculation and chestnut genotypes was assessed using a logistic regression. To determine significant interactions between inoculation status and seedling genotype with regard to % ECM root colonization, a 2×3 factorial design, mixed model, two-way analysis of variance (ANOVA) was used. To test main effects (inoculation status or seedling genotype) on ECM colonization and biomass (g), a one-way ANOVA was used. To determine differences in plant biomass between ECM seedlings and non-ECM seedlings, ECM presence was nested in genotype and analyzed by an ANOVA followed by Tukey's HSD post hoc. Differences were considered significant when $p \leq 0.05$ according to the F test. Log ($n + 1$) and square root (power = $\frac{1}{2}$) transformations were used to control for unequal variances when needed. All survival, growth, and ECM % colonization were performed using JMP (7.0, SAS Institute, Cary NC, USA).

A non-metric multidimensional scaling (NMDS) ordination followed by a per-mutational multivariate analysis of variance (PERMANOVA) was used to test for differences in ECM community composition between the seedling genotypes (pure American and BC_2F_1 hybrid). 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). NMDS, PERMANOVA, multiple regression, and BIC were performed using R, version 2.9.2 (R Development Core Team 2009).

Results

Seedling Survival

There were no differences when germination and survival were compared between seedlings planted with the granular inoculum to those sown without inoculum (Fig. 2). Germination rates were the same (84%) for both treatments. After eight months there was little difference in

seedling survival when Pt inoculum was compared: without inoculum 63% to with inoculum 62%. After 18 months the same trend continue; no differences existed in survival when inoculation status was compared, 38% inoculated compared to 35% non-inoculated.

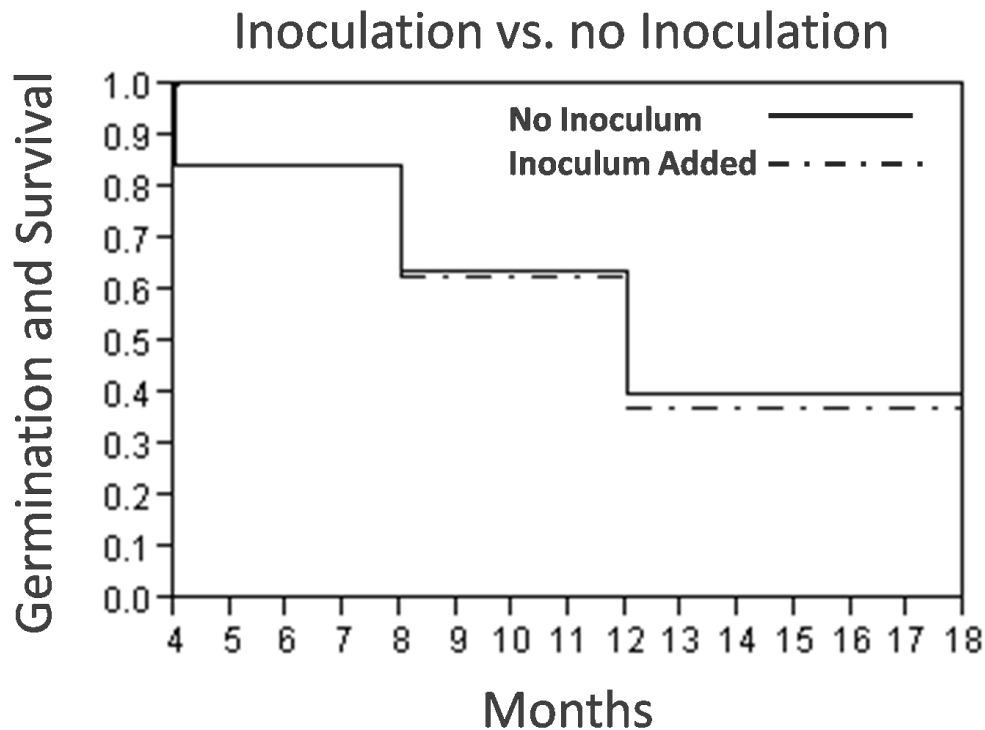


Figure 2. Germination (after 4 months) and survival of chestnuts (recorded after 8, 12, and 18 months in the field). Chestnuts growing in plots without inoculum (solid line) were compared to seedlings in plots that were inoculated with granular Pt inoculum (dashed line). The presence of Pt inoculum did not influence germination or survival.

No differences were detected when germination and survival were compared among chestnut seedling genotypes (Fig. 3). Chestnut hybrids BC_2F_1 , BC_3F_1 , and pure American chestnut seedlings were very similar with regard to germination: 82, 81, and 83%, respectively. After one growing season, this trend continued with regard to survival; BC_2F_1 (61%), BC_3F_1 (59%), and pure American chestnut seedlings (60%). After the second growing season seedling types performed similarly: BC_2F_1 (40%), BC_3F_1 (38%), and pure American chestnut seedlings (35%).

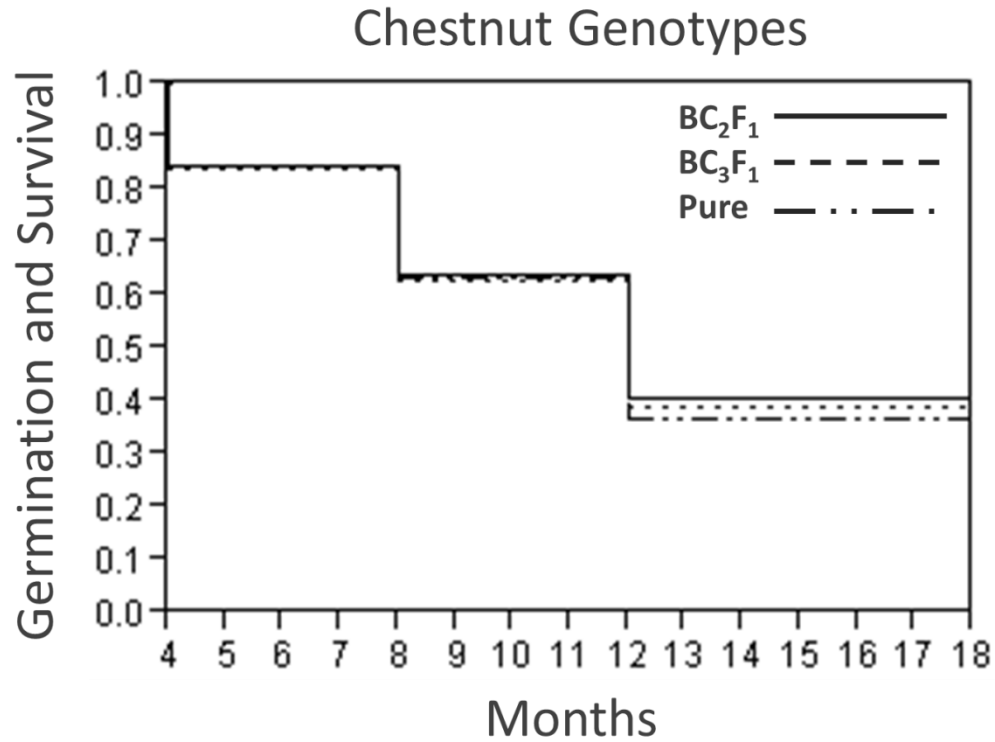


Figure 3. Germination (after 4 months) and survival of chestnuts (recorded after 8, 12, and 18 months) of the three chestnut genotypes: backcrossed chestnuts BC_2F_1 (solid line), backcrossed chestnuts BC_3F_1 (dashed line), and pure American chestnut (broken line), were similar in germination and survival after 18 months in the field.

ECM Colonization and species sampled

Soil was collected from field sites and used as medium for germinating chestnut seedlings in the greenhouse. *Scleroderma citrinum* and *Pisolithus tinctorius* were detected on greenhouse grown chestnut roots. Chestnut roots from the chestnut seedlings generated by direct seeding in the field were evaluated after four months for ECM root colonization. The most common species found on the roots were *Scleroderma citrinum* (35%) and *Thelephora terrestris* (24%; Table 2). *Oidiodendrin maius* (18%) and *Pisolithus tinctorius* (12%) were found in moderate frequency throughout this survey. The remaining species were found in lesser amounts and consisted of *Cenococcum geophilum* (5%), unknown endophyte (3%), and *Cortinarius* sp. 1 (3%).

Table 2. ECM species sampled four months after American chestnut seedling germination in the field. Fungal species are shown ranked by their relative abundance when all root samples were pooled.

ECM Fungi	Relative Frequency
<i>Scleroderma citrinum</i>	35%
<i>Thelephora terrestris</i>	24%
<i>Oidiodendrin maius</i>	18%
<i>Pisolithus tinctorius</i>	12%
<i>Cenococcum geophilum.</i>	5%
<i>Unknown endophyte</i>	3%
<i>Cortinarius sp.</i>	3%

No differences existed with regard to percent ECM root colonization between the two inoculum treatments (with and without) when roots were evaluated after 4 months: without inoculum (8% ECM) compared to seedlings with the granular inoculum (13% ECM; Fig 4A). After one year, ECM colonization increased significantly ($P < 0.05$). However, differences were not detected based on inoculum status after 18 months in the field: chestnut seedlings that were inoculated with granular Pt inoculum were similar to those not inoculated; 23% to 18%, respectively (Fig. 4B).

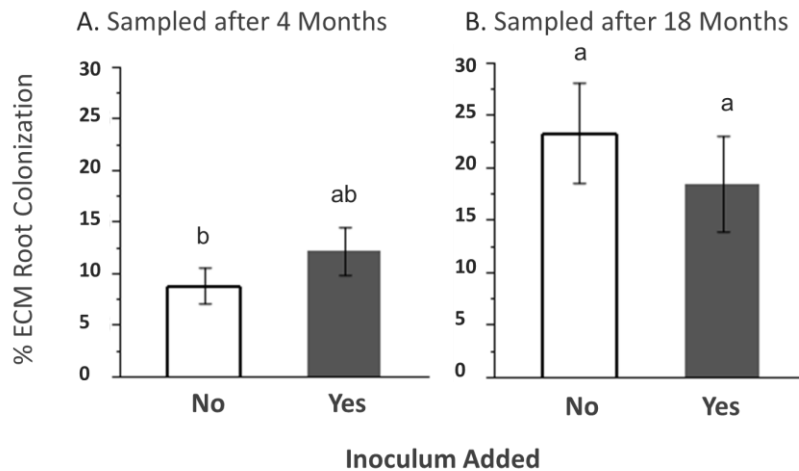


Figure 4. Panel A: Percent ECM colonization of chestnut roots averaged 8% in plots without Pt inoculum (No) compared to 13% on seedlings sown with the Pt inoculum (Yes) sampled after 4 months in the field. No statistical difference in ECM colonization existed. After 18 months, percent ECM increased. However, there was no difference in percent ECM colonization when inoculation status was compared, 23% (No) to 18% (Yes). Bars represent the mean $\pm$ SE. Bars sharing common letters do not significantly differ at $\alpha = 0.05$ determined by Tukey's HSD.

There were also no differences between the chestnut genotypes. ECM root colonization on pure American seedlings was similar when compared to BC_2F_1 after 18 months in the field, (20% to 19% respectively, Fig. 5A). In addition, no differences in ECM community composition existed when treatments were compared. The overlap of the two ellipses indicates community similarity for both chestnut genotypes (Fig. 5B).

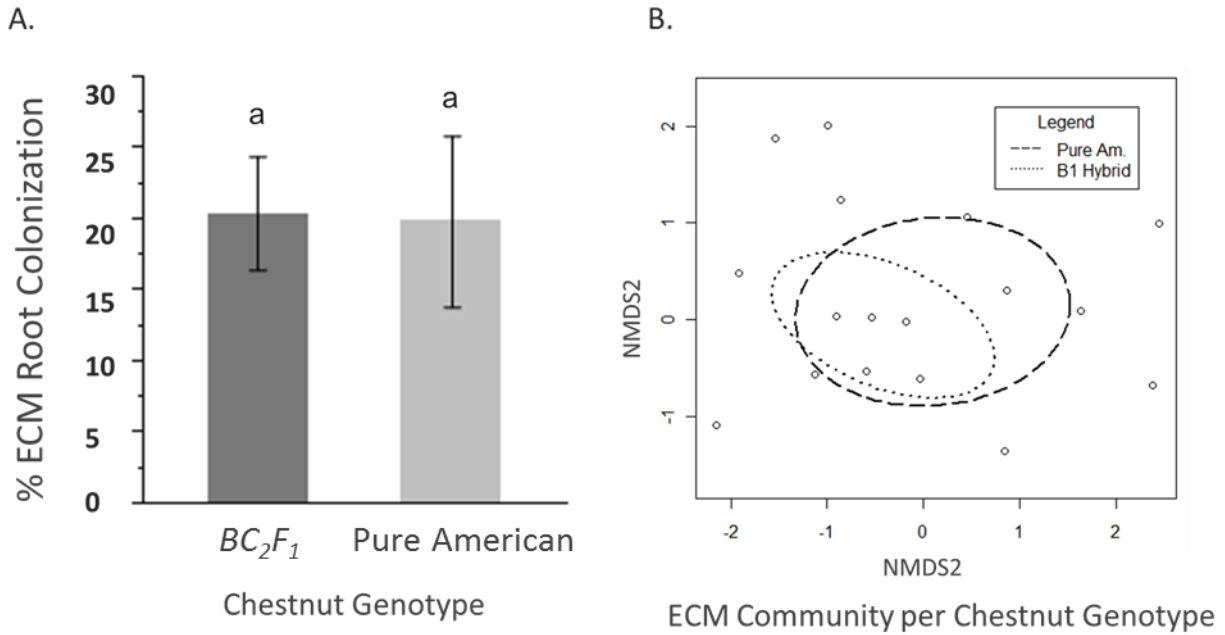


Figure 5. Panel A. After 18 months in the field, two chestnut genotypes were compared. There was no difference in ECM root colonization between the two chestnut lines: backcrossed chestnuts BC_2F_1 (20%) and pure American chestnut (19%). Bars represent the mean $\pm$ SE. Panel B. NMDS ordination comparing ECM communities between Pure American (dashed line) and backcrossed chestnut BC_2F_1 (dotted line) based on morphotypes after 18 months in the field. The overlap in the ellipses indicates community similarity for both chestnut genotypes. The best configuration out of 100 is shown.

Chestnut genotype had no influence on seedling biomass, which was 7.4 and 6.5 g, American to hybrid BC_2F_1 , respectively (Fig. 6). ECM presence resulted in a positive host response, as evident by the significant increase in plant biomass: pure American ECM seedlings (7.4 g) were greater in biomass than non-ECM seedlings (3.7 g); ECM inoculated BC_2F_1 seedlings (6.5 g) were larger than non-ECM seedlings (4.0 g) ($F = 12.68$, $P = 0.01$).

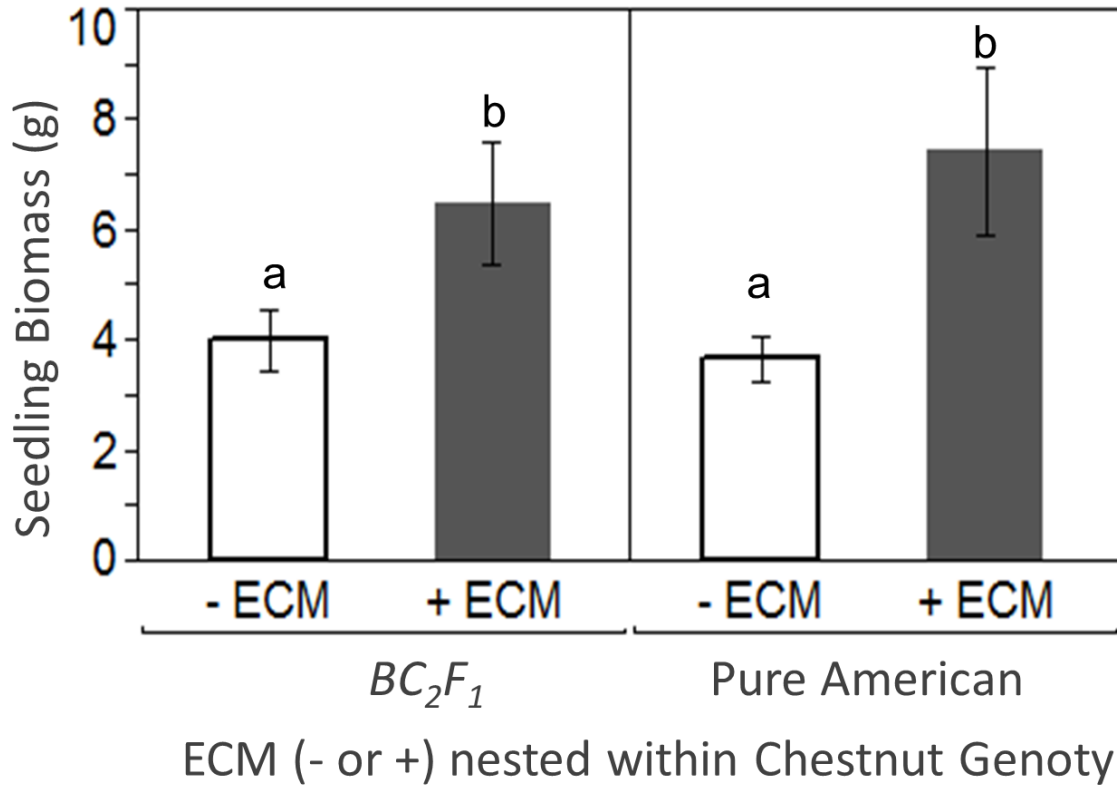


Figure 6. Biomass (g) between backcrossed chestnut genotype BC_2F_1 and pure American chestnut was compared after 18 months in the field with regard to seedlings that were not colonized by ECM fungi (-ECM, white bars) to seedlings that were (+ECM, grey bars). Seedlings sampled with the ECM on root tips (+ECM) were larger than seedlings without ECM colonization (-ECM). Bars represent the mean $\pm$ SE. Bars sharing common letters do not significantly differ at $\alpha = 0.05$ determined by Tukey's HSD.

In the best subset regression for the percent of ECM colonization as a function of soil variables, chestnut seedling infection was best described (lowest BIC) by soil pH, cation exchange capacity, and soil potassium ($P = 0.01$, $R^2 = 0.37$; Table 3). Of these, the cation exchange capacity was the strongest predictor for ECM colonization.

Table 3. Multiple regression relating ECM root colonization (%) on chestnut seedlings sown as seeds to the subset of environmental predictor based of the lowest BIC.

Predictor	β Estimate	SE	t-value	<i>p</i>
Intercept	-1.35	0.51	-2.65	0.01
Soil pH	-4.62	4.31	-1.07	0.29
Cation exchange capacity	11.42	4.32	2.64	0.02
Potassium (ppm)	2.75	1.68	1.63	0.12

Discussion

ECM Sampled from Field Site

The granular inoculum did not appear to have an influence on germination, survival, or percent ECM colonization. Pt appeared in 12% of all root samples when chestnut seedlings were pooled. However, the seedlings that were observed with Pt were sampled from plots that were not inoculated with the Pt granular inoculum. The colonizing fungi most likely germinated from spores produced by a local source population. These sequences matched the Pt that was sampled from the field site prior to planting. It is reasonable to conclude that field inoculation via granular inoculum at the time of seed sowing is not a reliable way to ensure Pt mycorrhizas in such conditions; at least in sites like these where soils are high in spoil, low in pH, and low organic matter. Rather, ECM colonization appears dependent on the availability of local fungal propagules that are adapted to specific site conditions and can form functional mycorrhizas with ECM host plants in drastically disturbed soils.

After four months *Scleroderma citrinum* was the most abundant ECM species sampled. This species was also found in the soil prior to adding inoculum and appeared in each treatment plot regardless of inoculation status. *Scleroderma* species have been described as early succession fungi common to coal mined soils (Newton, 1991) and has been found on chestnut in similar restoration projects (Bauman et al., 2011). In this study, *S. citrinum* was encountered with a greater frequency than Pt on sampled roots. This indicates the high affinity chestnut and *S. citrinum* have for one another, contributing to the abundance of functional mycorrhizas sampled in the field. Importantly, there was a positive host response to this colonization. Various *Scleroderma* species have been selected for this attribute; both reclamation plantings and nursery inoculations with this fungus have reported an increase in host biomass (Beckjord and McIntosh, 1983; Chen et al., 2006).

This study also identified *Thelephora terrestris* and *Oidiodendron maius* colonizing chestnut roots in the field. *T. terrestris* has been previously reported on chestnut roots (Palmer et al., 2008). Further, this fungus tends to be a generalist and an early succession fungus, found infecting root systems in both field plots and nursery settings (Deacon and Fleming, 1992). Ruehle (1983) reported that *T. terrestris* out-competed Pt on seedling roots grown in soils that were not fumigated. This was clearly the case in this field study. *O. maius* is an ericoid fungus

that has been isolated from plant roots growing in soils of extremely high concentrations of zinc and cadmium (Perotto et al., 2005). Similar to *S. citrinum*, these fungi appeared in our root samples with a greater frequency than did *Pt*. Collectively, these species made up an assemblage that contributed to an increase in biomass.

Comparison of Chestnut Genotypes

American chestnut's fast growth rate and tolerance to extreme conditions associated with mine soils suggest this species is a suitable hardwood for reclamation projects (Jacobs, 2005). The economic and ecological benefits of these potentially blight-resistant genotypes merit further study to determine requirements for successful establishment and their role as a restoration tree. The low survival recorded during this study (~ 30%) does not reflect the high survival rates (~85%) reported in other studies that planted one-year old bare root seedlings on a reclaimed surface mine (McCarthy et al., 2008). Rather, this study concurs with other studies that report low survival on mine sites that were directly seeded with chestnut (McCarthy et al., 2010). However, important to this study was the pH conditions; pH values were 50 to 100 × more acidic than conditions evaluated by Jacobs (2005). With regard to microbial interactions, successful seedling establishment may require abundant native ECM propagules, adapted to low pH levels, for the formation of ECM roots shortly after seedling germination. These high ECM areas are generally associated with pockets of ECM host plants that may act as a reservoir for ECM species, thereby facilitating seedling establishment (Allen and Friesse, 1992; Bauman et al., 2012).

There were no differences in germination or survival when the seedling types were compared, thus hybridization seemed to have no influence on the growth habit in these sites. There were no differences between the pure American chestnut seedlings and the BC_2F_1 hybrids with regard to ECM community or colonization potential. ECM communities are generally similar on host plants with comparable taxonomic and successional groups (Ishida et al., 2007); therefore, differences were not anticipated. Percentages of ECM root tips and number of morphotypes sampled were similar between seedling types (pure American chestnut and BC_2F_1). Natural ECM colonization had a significant influence on biomass accumulation regardless of the genotype of the chestnut seedlings.

Abiotic Soil Parameters Influence ECM Root Colonization

Of the soil parameters measured, the cation exchange capacity had a significant influence on ECM colonization. Other studies have also reported base-cation and humus-related soil traits as the best predictors of ECM colonization (Dickie et al., 2006). 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 shale and sandstone overburden materials as a topsoil substitute, soils are often very deficient in organic matter. However, organic matter and exchangeable base cations can be modified through the application of soil amendments. In a greenhouse study, Lunt and Hedger (2003) reported a significant increase in mycorrhizal root colonization on *Quercus* seedlings in mine soils to which organic amendments were added. This also resulted in an increase in host response to the amended soils, specifically when colonization by *Hebeloma* species occurred (Lunt and Hedger 2003). Though it would be expensive and logistically difficult to bring in organic matter, it would be of great benefit to leave stumps and other large organic elements in the field to aid in soil fertility and ECM plant regeneration overtime (Skousen et al., 2011).

Conclusions

Introducing spore inoculum to a field site greatly deficient in nutrients and organic matter was not an efficient method to ensure ECM symbiosis. At the end of the first and second growing season, ECM inoculation occurred naturally in the field via spore and/or mycelium propagules in the soil, influenced by base-cation and humus-related soil traits. More importantly to restoration ecology, native ECM fungi particularly *S. citrinum*, freely colonized chestnut seedlings. Documenting native mycorrhizas that readily colonize target tree species provides information that can be used in planning nursery and greenhouse inoculations for future plantings. Additionally, the consideration of using only native ECM may prove to be cost-effective as well as ecologically conscious when restoring forest ecosystems. Future research from this field site will use *S. citrinum* coupled with carefully selected soil amendments. One cost effective inoculation technique is to use 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 and help preserve locally adapted fungal genotypes (Lesica and Allendorf, 1999). If this technique is not logistically feasible for a future chestnut planting, *Scleroderma* species are available in commercial inoculum 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 functional mycorrhizas at these low pH levels.

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