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# THE INFLUENCE OF INOCULATED AND NATIVE ECTOMYCORRHIZAL FUNGI ON MORPHOLOGY, PHYSIOLOGY AND SURVIVAL OF AMERICAN CHESTNUT<sup>1</sup>

Jenise M. Bauman<sup>2</sup>, Carolyn H. Keiffer, and Shiv Hiremath

**Abstract.** The objective of this study was to evaluate the influence of five different species of ectomycorrhizal (ECM) fungi on root colonization of native fungi on putatively blight resistant chestnut hybrids (*Castanea dentata* x *C. mollissima*) in a reclaimed mine site 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 identify 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 the 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.

**Additional Keywords:** root colonization of fungi, chestnut restoration.

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## **Introduction**

Employing ectomycorrhizal (ECM) inoculum prior to outplanting is a common practice in restoration projects on reclaimed mine land using hardwood trees (Castellano, 1996). This technique enhances 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 et. al., 2008; 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 observed in the field (Dahlberg and Finlay, 1999). In order for the host plant to receive the benefits from an ECM fungus, it must be able to maintain functional mycorrhiza under the environmental conditions of a specific planting site (Perry et al. 1989). These manipulations might bypass some stages of natural succession and accelerate the establishment of late successional species in initial plantings.

Another issue to be considered is the probability of inhibition of mycorrhization with native fungi present at the site by the introduced fungus. Community composition is often affected by the sequence of species arrival referred to as priority effects. These priority effects can involve "early colonists" negatively affecting the performance of later arrivals, probably through preemption of shared resources (Alford and Wilbur, 1985; Shorrocks and Bingley, 1994). Interspecific species interactions demonstrate that the "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 for disturbed environments, and thus better facilitating the survival of the plant. 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.

Two recent laboratory studies found contrasting results with respect to the role of priority effects in ECM species interactions. Lilleskov and Bruns (2003) found pine seedlings originally colonized with ECM fungus *Rhizopogon occidentalis* to be 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). 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 overall goal of the study was to evaluate the influence of five different species of ECM, introduced through a newly planted seedling, on the root colonization of native fungi on seedlings germinating from seeds in a reclaimed mine site in the central Ohio. More specifically we wanted to investigate: 1) whether the presence of these inoculated ECM nearby has any competitive effect on the native fungi to colonize on new seedlings; 2) whether ECM colonization has any effect on the growth and survival of American chestnut hybrids; 3) the longevity of inoculations; and 4) the differences between the pure American and hybrids with respect to native fungal colonization. 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 & Lehtoma, 2007). *H. crustuliniforme* is a *basidiomycete* 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, species of this fungus 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., 2010). *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).

In the present study, we looked at the competitive effects of ECMs present on a year-old chestnut seedling on native fungal colonization of seedlings germinating from seeds planted in the vicinity. Our studies suggest that persistence of inoculated ECM on the chestnut is variable; fungi with strong priority effects out-compete native species for root colonization. In addition, the observations also suggest that better competitors have an increased benefit in establishing chestnut seedling.

## **Methods and Materials**

### **Study Site**

In the spring of 2006, chestnuts hybrids B1-F3 (obtained from the The American Chestnut Foundation) were planted in the greenhouse at the USDA Forest Service in Delaware, Ohio (Hiremath and Lehtoma, 2007). The B1-F3 hybrids are progeny of initial backcrossing (B1-F1), which were intercrossed two more generations leading to the B1-F3 chestnut hybrid genotype (Hebard, 2005). Statistically, these trees contain an average of 75% American chestnut alleles. Inoculations were done as described earlier (Hiremath and Lehtoma, 2007). Briefly, seeds were planted in a 1:1:1 vermiculite, sphagnum peat, Perlite mix present in a ~ 400ml conical container

and allowed to germinate. A week after planting they were inoculated using liquid inoculum. The latter was prepared by placing mycelial pieces grown for 2-3 weeks on M4N agar plates at 24<sup>0</sup>C in 1L M4N media in a 2L Erlenmeyer flask and incubating at room temperature in the dark for 2-3 weeks. The inoculum was then macerated with a Tissuemizer homogenizer for about 30 sec. and 30 ml was pipetted into the soil around the seed. After approximately three months, the seedlings were checked for the presence of ECM on roots under a microscope and transferred to a ~ 1 gallon containers. The following ECM fungi were used in the study: *H.crustuliniforme*, *L. bicolor*, *S. polyrhizum*, *A. rubescens*, and *S.luteus* (inoculation was done as described by in Marx and Bryan 1975). Seedlings were grown in the greenhouse under natural light, watered as needed, and fertilized monthly with a 12-12-12 liquid fertilizer for one year. All chestnut hybrids were tested for colonization of the inoculated fungus by sequencing the ribosomal ITS region (see below) prior to planting. Only chestnut seedlings with at least 50% ECM colonization were selected for field planting. Non-inoculated chestnut hybrids were used as control plants.

In the spring of 2007, plants were planted 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 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 among blocks and the averages were as follows: soil pH 5.4, CEC 8.13, organic matter 1.5 %, P 8.7 ppm, K 77 ppm, Mg 155 ppm, Ca 640.3 ppm, N (NO<sub>3</sub>-N) 2, Mn 4.4 ppm, Al 5.29, sand 55%, clay 24%, and silt 21%. Soil chemistry was measured in the laboratory (McCarthy, 1997) as well as at the Spectrum Analytic, Inc, Washington Courthouse, OH.

We used a randomized block design with replicates where placement of seedlings was randomly assigned in each block. Three 20 x 6 meter (m) blocks were installed by plowing and disking using a conventional tractor. Each block contained 36 one year-old chestnut seedlings,

six of each treatment type (five different mycorrhizal + a non-inoculated control). In addition, each block was replicated three times for a total of 108 seedlings. Seedlings were planted in April of 2007 at a spacing of 1.5 m within and in between the rows. Seedlings were planted such that the root collar was at the level with the grade of the soil. They were tagged and backfilled with original soil along with one 20-10-5 slow release fertilizer pellet. A weed mat was installed with all four corners pinned using sod staples for controlling the reemergence of previously present groundcover. To prevent herbivory, a 1.5 m tall fence was constructed around each block using chicken wire and t-posts. Each seedling was planted with three pure American chestnut seeds placed in a single location about 30 cm from the center of the seedling. To insure seed germination, all seeds were stored in the dark in moist peat at room temperature until the radical emerged from the seed.

#### Data Collection

Growth parameters such as plant height, basal diameter, and leaf area (cm<sup>2</sup>) 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, packaged in paper bags and sent to Spectrum Analytic Inc., Washington Court House, OH, 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.

#### ECM Sampling

Two sampling times were selected: 12 months and 18 months after planting in the field. One-hundred and three chestnut seedlings were sampled after 12 months and 108 seedlings were sampled after 18 months, that latter group containing the 60 surviving hybrids and 48 pure American. Root samples were harvested only during the 18 month sampling time. Roots were carefully removed from the field, returned to the laboratory, washed, and observed under the stereoscope for mycorrhizal formation. To quantify ECM root colonization (Percent colonization), 250 root tips per seedling were randomly selected and checked for ECM. Percent colonization (ECM roots per 100 total root tips tested) was calculated and used to describe relative abundance and ECM community composition.

### DNA Extraction and PCR

We used ECM root tips for isolating the fungal DNA for identifying the fungus through DNA sequence. For this, root tips were first grouped by similar morphology (later single root tips were used), and about 10 mg was homogenized in extraction buffer using a bead beater for 3 minutes. DNA was extracted using QIAgen's DNeasy Plant Mini Kit and eluted from the column in 2 x 50 µl AE buffer. PCR primers were used to amplify the highly variable internal transcribed spacer (ITS) region of fungal ribosomal DNA (rDNA) in order to distinguish fungal species (White *et al.* 1990). We performed PCR using primer pair ITS1 and ITS4. PCR reactions were set up in 500 µl tubes using ~10 ng template DNA, 1x Eppendorf Taq buffer (50mM KCl, 10 mM Tris-HCl pH 8.0, 0.15 mM Mg(OAc)<sub>2</sub>), 20 µM each dNTP, 0.2 µM each primer ITS1 and 5 units of the enzyme Taq in 100 µl reactions. Reactions were subjected to 40 cycles in Perkin-Elmer thermal cycler under the following temperature regime: 1.5 min at 96° C, 1 min at 55° C, 2 min at 72° C. Completed reactions were subjected to 10 min incubation at 72° C and then stored at 4° C. Products were subjected to electrophoresis on 0.7% agarose gel and the PCR product bands were isolated from the gel using Bio101 GeneClean Kit and resuspended in 20 µl dH<sub>2</sub>O.

### Sequencing

The PCR product was cleaned and prepared for sequencing using a 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: 96° C for 1 min followed by 25 cycles of 10 s at 96° C, 5 s at 50° C, and 4 min at 60° C. Sequencing was performed with The Applied Biosystem ABI Prism 3730 DNA Analyzer (Bioinformatics facility, Miami University, Oxford, Ohio).

### ECM Identification

The DNA sequences were analyzed and edited using a Sequencher 4.2 software (Gene Codes, Ann Arbor, Michigan). To identify fungi found on roots ITS sequences from our samples were compared to those in the GenBank using BLAST searches (Altschul *et al.*, 1997). Genera reported here were based on the best match to those reported for the fungi in the GenBank. These species formed the basis of the ECM community described in the results. To add



statistical support to the BLAST identification for multiple species of *Scleroderma* sampled from chestnut, we constructed a phylogenetic tree using our sampled sequences to known *Scleroderma* sequences (Binder and Bresinsky, 2002). The sequences were first auto-aligned using the MUSCLE, and then manually aligned in Se-Align v2.0a11. Maximum-parsimony analyses were carried out using the 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

A permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences in ECM community per season, tree type, and inoculum treatments using ECM species composition as the response variable (Data not shown since no differences were detected). Differences in ECM colonization among inoculum types were determined by using a one-way analysis of variance (ANOVA) using percent ECM colonization from pure American chestnut seedlings (n = 48) and inoculated chestnut hybrids (n = 60). Arcsine square root transformation was used to control unequal variances. Growth parameters such as seedling height (cm), basal diameter, and leaf area (cm<sup>2</sup>) 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 assigning a value of zero to the most negative growth value and using Log+1 transformation (McCarthy, personal comm.). A one-way multivariate analysis of variance (MANOVA) followed by a univariate ANOVA and Tukey's posthoc test were conducted on the growth parameters listed above. The differences were considered as significant when  $p \leq 0.05$  according to the F test. A one-way ANOVA was used to determine differences in macro and micronutrient concentrations in leaf tissue among the ECM and non-ECM chestnut seedlings. All statistics were performed using the R v2.91 (R Development Core Team 2009).

### Results

Nine distinct ECM morphologies were observed on chestnut seedlings (n = 211). The DNA sequencing of samples identified a total of 12 different ECM species (Table 1). *Scleroderma* species 1 and 2 were the most abundant (74% of the chestnut roots sampled) while *Cenococcum*

and *Thelephora* species ranked 3rd and 4th, respectively. This was followed by a *Tomentella* species and two *Hebeloma* species. In addition, some other species were also sparingly seen which included an unidentified species belonging to the *Thelephoraceae* and another from *Cortinarius*. Two unknown ECM and *Pisolithus* species were observed very rarely and only on the hybrids (Table 1).

Table 1. Relative abundance of different ECM species on seedlings after 12 and 18 months. Values represent the percentage of root tips that had the ECM fungus. Molecular identification of ECM root tips ranked by relative abundance sampled from all 211 chestnut seedlings (total) and further separated into hybrids and pure chestnut trap trees at both sampling times. Table includes corresponding GenBank sequence accession number through which the ECM fungus was identified.

ECM Species	All Sampled after 12 and 18 months	Pure after 12 Months	Hybrid after 18 months	Pure after 18 months	GenBank Accession #
<i>Scleroderma</i> spp. 1	63%	52%	70%	72%	GU246983
<i>Scleroderma</i> spp. 2	11%	18%	3%	8%	GU246984
<i>Cenococcum</i> spp.	11%	12%	8%	12%	GU246986
<i>Thelephora</i> spp.	6%	10%	3%	4%	GU246989
<i>Tomentella</i> spp.	3%	5%	3%	0	GU246993
<i>Hebeloma</i> spp. 1	2%	2%	4%	1%	GU246997
<i>Hebeloma</i> spp. 2	1%	1%	2%	2%	GU246985
Unknown ECM 2	1%	1%	3%	0	GU246994
<i>Cortinarius</i> spp. 1	1%	0	1%	2%	GU246996
Thelephoraceae	1%	0	1%	1%	GU246997
Unknown ECM 1	1%	0	2%	0	GU553376
<i>Pisolithus</i> spp.	1%	0	1%	0	GU553367

There were no apparent differences between the pure and hybrid American chestnut seedlings in their ability to associate with different ECM species (Table 1). To verify this, PERMANOVAs were used to test for statistical differences in the ECM community composition with regard to seedling type (hybrid vs. pure American), season in which the seedlings were sampled (spring vs. fall), and the five different ECM species used for inoculum in this study. All the analyses indicated that there were no significant differences with respect to ECM community composition, the time of sampling, the genotype of the seedling, and the ECM species selected as

inoculum (data not shown). In addition, our ECM inoculum did not have any influence on percent root colonization on chestnut seedlings (data not shown).

To confirm that ECM species sampled in the field were not part of the inoculum used in the greenhouse, we used a maximum parsimony tree to illustrate the phylogenetic relationship of our ECM samples as they relate to our greenhouse inoculums. An example of this technique is illustrated using *Scleroderma* sequences in the phylogeny in Fig. 1 (ITS phylogeny of *Scleroderma* species). Sequences sampled from both hybrids and pure American seedlings were

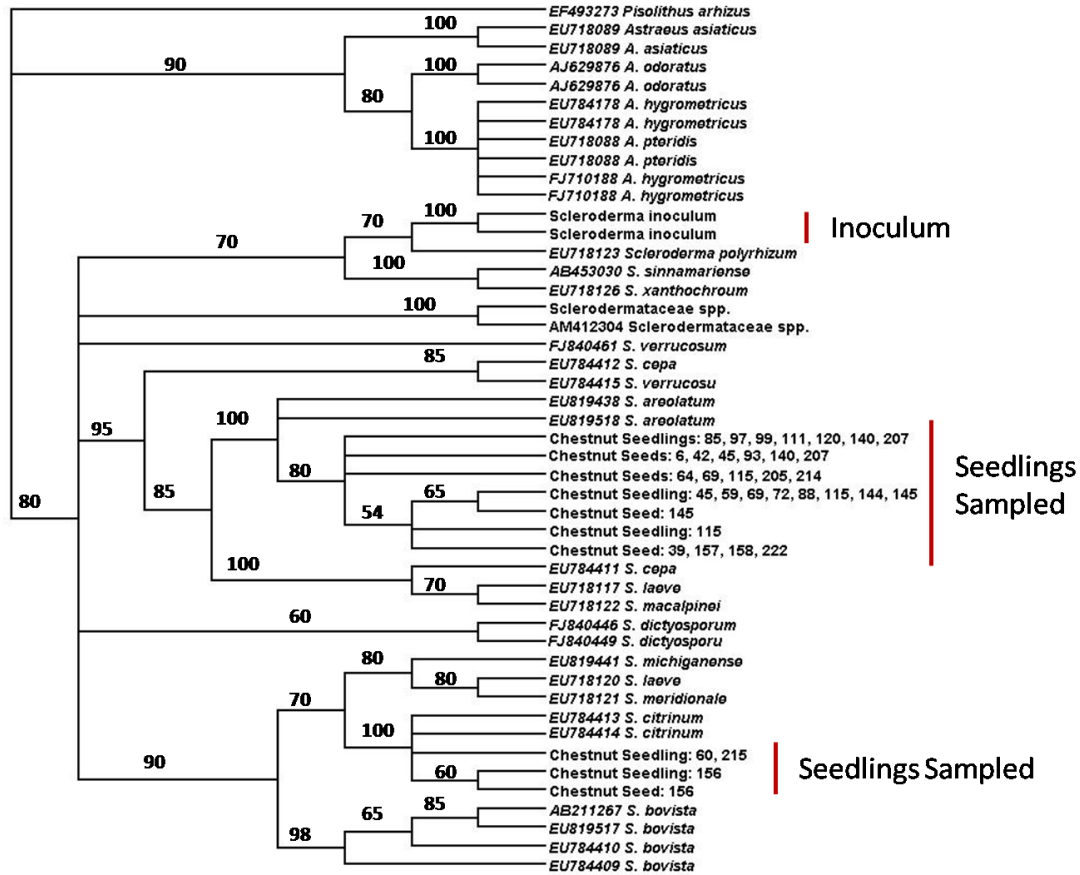


Figure 1. ITS phylogeny of *Scleroderma* species. Maximum parsimony 50% majority rule tree with bootstrap values is shown. Each accession number represents sequence of the ECM species deposited in the Genbank. The positions of *Scleroderma* species 1 are within a clade that is closely allied with the *S. areolatum* species (GenBank accession numbers EU819438 and EU819518). The *Scleroderma* spp. 2 forms a clade with the species *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.

compared with sequences of *Scleroderma* isolates deposited in the GenBank. A maximum parsimony, 50% majority rule tree with bootstrap values is shown. The positions of *Scleroderma* species 1 are within a clade that represents species related to *S. areolatum* (GenBank accession numbers EU819438 and EU819518). Similarly, the *Scleroderma* spp. 2 forms a clade with *S. citrinum* (GenBank accession numbers EU784413 and EU784414). The resulting phylogeny gave support to our finding that the *Scleroderma* species sampled from chestnuts were not part of the original inoculum (*S. polyrhizum*) used to inoculate chestnuts in the greenhouse prior to planting in the field.

PERMANOVAs were used to test for differences in ECM communities per time of sampling, tree source, and inoculum. These analyses showed that there were no community differences existing (data not shown). In addition, ECM inoculum did not have a significant influence on chestnut root colonization (data not shown).

There were significant differences after 18 months in survival among seedlings inoculated with different ECM inoculum (Fig. 2; Cox proportional hazard model, Likelihood = 121, df = 5,  $P < 0.0001$ ). Chestnut seedlings inoculated with *S. lutues* and *S. polyrhizum* had the highest survival rate (87% and 81%, respectively) and was followed by *L. bicolor* (61%), *H. crustuliniforme* (58%), *A. rubescens* (28%). The survival rate for the non-inoculated control plants was 16%.

The effect of native ECM root colonization on the growth rate (RGR) was compared among various genera (ECM species pooled; Fig. 3). The data showed that ECM species in the genus *Scleroderma* and *Thelephoraceae* family significantly improved growth rates on the hybrid chestnuts with regard to height (ANOVA,  $F = 5.65$ ,  $df = 5$ ,  $p = 0.0005$ ) basal diameter ( $F = 4.81$ ,  $df = 4$ ,  $P = 0.002$ ) and leaf area ( $F = 7.72$ ,  $df = 4$ ,  $p < 0.0001$ ). This was not the case for *Cenococcum* or the unknown ECM, whose growth rates were comparable to the chestnuts seedlings without any ECM (Fig. 3).

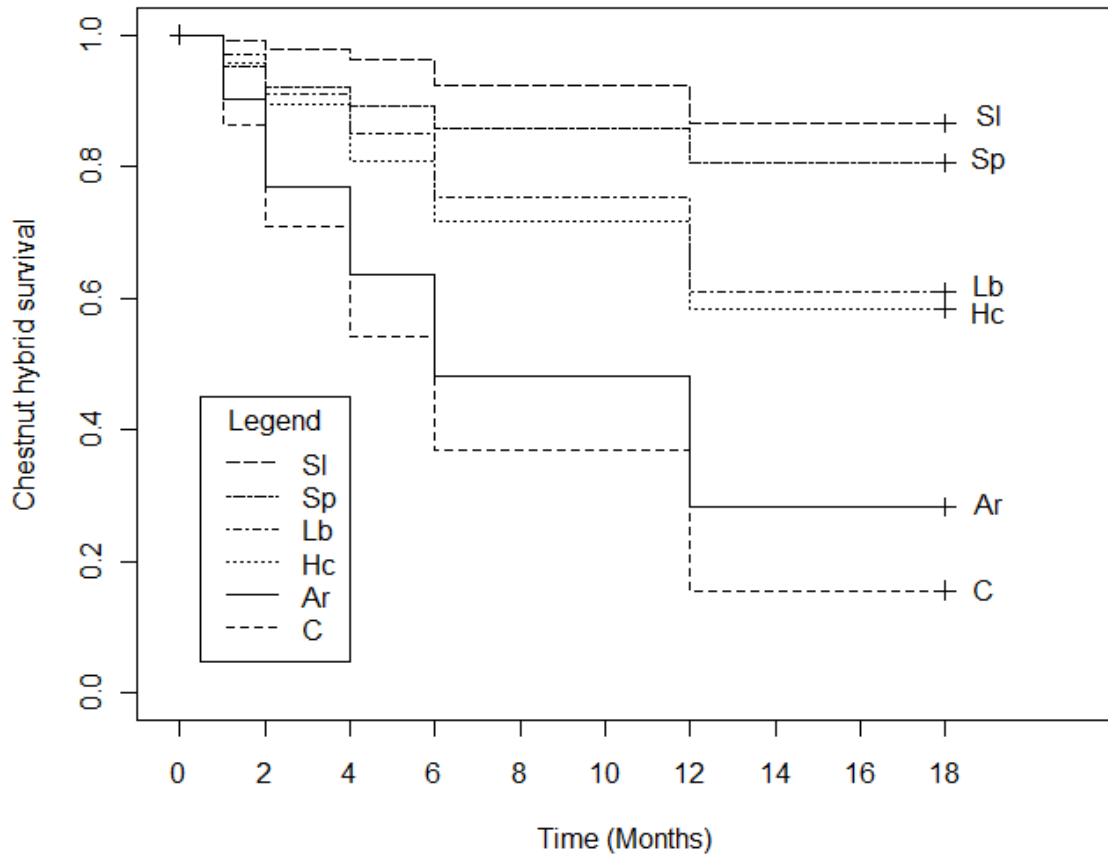


Figure 2. Survival data for hybrid chestnuts among the six different inoculum treatments (SI = *S. luteus*, Sp = *S. polyrhizum*, Lb = *L. bicolor*, Hc = *H. crustuliniforme*, Ar = *A. rubescens*, and C = Control). ECM species had a significant effect on survival (Cox proportional hazard model, Likelihood = 121, df = 5,  $P < 0.0001$ ).

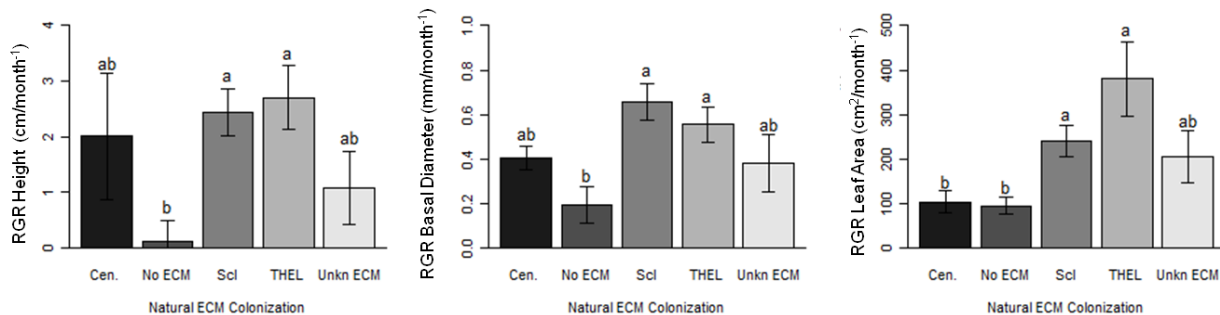


Figure 3. Growth rates of chestnut seedlings with and without the naturally colonized ECM fungi. *Scleroderma* (Scl) and *Thelephoraceae* (THEL) species significantly increased growth rates of chestnut hybrids (all  $P < 0.05$ ). *Buty*, *Cenococcum* (Cen) and an unknown ECM fungus (Unknown ECM) had no effect on the growth and were similar to the non ECM seedlings (No ECM). Bars represent mean  $\pm$  SE. Bars sharing common letters did not differ significantly at  $\alpha = 0.05$  as determined by Tukey's HSD.

Leaf tissues from native ECM and non-ECM seedlings were analyzed for nutrient concentration. Thirty seedlings were sampled representing ECM and non-ECM plants. Of these 30, only 22 analyses were conclusive. Our sample size was not large enough to direct comparisons among ECM species. Furthermore, since we did not detect our original inoculum used, all ECM colonized seedlings were pooled into a single group and compared with non-ECM samples (ECM and non-ECM in Table 2). We did not observe any significant differences in the nutrient content and metal composition between the two groups (all  $P > 0.05$ ).

Table 2. Nutrient and metal concentration ( $\pm$ 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 $\pm$ 0.24	0.25 $\pm$ 0.05	0.69 $\pm$ 0.20	1.12 $\pm$ 0.14	0.48 $\pm$ 0.10	1793.0 $\pm$ 1145.93
ECM	1.39 $\pm$ 0.32	0.28 $\pm$ 0.07	0.65 $\pm$ 0.13	1.22 $\pm$ 0.19	0.45 $\pm$ 0.07	1374.75 $\pm$ 541.67

### **Discussion**

Although ECM colonization was verified prior to outplanting, chestnut hybrids did not maintain this association once they were planted in the field. Our results showed: 1) the original ECM inoculated before outplanting did not persist after 18 months, however, contributed significantly to the survival of the seedling for the first few months, 2) the introduced ECM species did not influence the final ECM community composition after two growing seasons, 3) the presence of the inoculated ECM species did not impede natural root colonization by native fungi, and 4) root colonization by certain native fungi resulted in positive effects on chestnut seedling growth.

None of the five ECM species (*H. crustuliniforme*, *L. bicolor*, *S. polyrhizum*, *A. rubescens*, and *S. luteus*) maintained 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 several 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 had no influence on the ECM community composition on pure American or hybrid chestnuts 18 months after planting. This was in contrast to our earlier observations (Bauman, unpublished data) where we had noticed the 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. Such negative 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 to introduced fungi 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 the current study. Root colonization on the average was not above 50%, which indicates that competitive dominance was not the factor.

Previous studies have speculated that the host plant can decrease mycorrhizal receptivity towards 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). Assuming the host plant increases carbon allocation to the most beneficial fungal symbiont, 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* contributed significantly in increasing the growth of the hybrid chestnuts. Although we did not see any differences in foliar nutrient concentrations in ECM plants, benefits may have been increased in 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. It is worth noting that Dulmer (2006) reported the presence of *Cenococcum geophilum* caused or was seen only on unhealthy chestnuts, suggesting that this species may have a negative impact on the health of the chestnut seedlings. Species like *Cenococcum* have been shown to increase in abundance in the absence of a better competitor (Dickie and Reich, 2005). If competition between different species of fungi is strongly mediated

by plant feedbacks, carbon allocated to a less productive symbiont like *Cenococcum* may decrease 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*. A similar situation existed in our study where 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 observed 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 been all a factor 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). The only exception was in the case of chestnuts inoculated with *A. rubescens*, which had survival rates 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 plant's fitness under certain ecological conditions.

It may be of greater importance that this inoculum did not interfere with root colonization from the native ECM community. *Scleroderma* species were the most abundant and provided chestnuts with significant growth increases during this study. *Scleroderma* species 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 (Jefferies, 1999; Lu et al., 1998). Indigenous *Scleroderma* species has a high affinity for *Castanea* (Meotto et al., 1999) and previous studies report a positive growth response in the field (Bauman, 2010). Planting methods that promote colonization of indigenous ECM species may increase the success rate of colonization of these microbes on planted seedlings. 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 seasonal differences. Seasonal dynamics in above-ground sporophore production has been well documented (Deacon and Fleming 1992), however, not much is known in case of below-ground communities, although a recent study reported temporal partitioning among species in ECM communities (Walker et al., 2008; Koide et al., 2007). In the present case, although there were no significant differences in ECM community composition between spring and fall samples, the relative abundance of *Scleroderma* species 2 increased from 3% in the spring to 18% in the 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). More sampling and analysis is needed to determine if these different *Scleroderma* species indeed display a temporal variability over time.

Lastly, this study explored whether the observed ECM community changes were related to the chestnut genotype, i.e., the hybrid variety or the pure American type. Our data clearly indicated that the genotype did not matter. This is not surprising since it has been shown that 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 observe a similar ECM community composition among pure American (*C. dentata*) and hybrids (*C. dentata* x *C. mollissima*).

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