NATIVE MYCORRHIZAL FUNGI REPLACE INTRODUCED FUNGAL SPECIES ON VIRGINIA PINE AND AMERICAN CHESTNUT PLANTED ON RECLAIMED MINE SITES OF OHIO[1]

Shiv Hiremath[2], Kirsten Lehtoma, and Jenise M. Bauman

Abstract. Plant-microbe community dynamics influence the natural succession of plant species where pioneer vegetation facilitates the establishment of a distantly related, later successional plant species. This has been observed in the case of restoration of the American chestnut (*Castanea dentata*) on abandoned mine land where Virginia pine (*Pinus virginiana*) facilitated the establishment of chestnut seedlings. This was apparently due to the natural mycorrhizal networks of pine, which aided the survival and growth of chestnut seedlings. In this study, we assessed the survival and propensity of introduced mycorrhizal fungi on Virginia pine to colonize pure American and backcrossed American chestnut. Seedlings were planted in Perry State Forest located in southeastern Ohio. This area was mined for coal in the 1950s and had very little reclamation done aside from experimental tree plantings. The selected site, with little topsoil or organic matter, was characterized by high concentrations of Al, high soil temperatures, and a pH of 3.6. Virginia pine seedlings were inoculated using ectomycorrhizal (ECM) cultures of *Amanita rubescens, Laccaria laccata,* and *Pisolithus tinctorius* via liquid media. After three months, roots were tested for the presence of mycorrhizae. They were then transplanted and grown for two years in the greenhouse. After verifying mycorrhizal colonization, 600 pines were out planted in May of 2005. Chestnut seedlings (100 one-year-old seedlings) inoculated with *P. tinctorius* by the Ohio state tree nursery had been planted by other researchers at the same time. After eight growing seasons, pines and chestnuts were measured and sampled for ECM colonization. Growth measurements showed that pines and hybrid chestnuts had significantly more aboveground biomass compared to pure American chestnut ($P = 0.01$). Eleven fungal species were detected using DNA sequencing. With the exception of *Amanita,* the inoculum that were out planted with both chestnut and Virginia pine were replaced after 8 field seasons by fungi native to the site. More fungal species were sampled from the Virginia pines than from chestnut roots, which contributed to the significant differences in ECM fungal community composition between the two species ($P = 0.005$).

Additional Key Words: root colonization of fungi, chestnut restoration, American chestnut.

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[2] Shiv Hiremath is a Research Scientist and Kirsten Lehtoma a Biological Technician with USDA Forest Service, Delaware, OH 43015M. Jenise Bauman is a Professor at Miami University, Oxford, OH 45056.

Introduction

Even under normal soil conditions, commercially important forest tree species belonging to Betulaceae, Dipeterocarpaceae, Fagaceae, Myrtaceae and Pinaceae depend heavily on ectomycorrhizal fungi for their survival, health, and vigor. In addition, many forest tree seedlings depend on ectomycorrhizal fungi for establishment and survival after transplanting. Because of their ability to withstand a variety of adverse environmental and edaphic conditions, several ectomycorrhizal fungi have the capacity to help in the establishment of forest tree species in soils with low nutritive value (Moser and Haselwandter, 1983; Smith, 1988; Tinker, 1984). They can also withstand contamination with heavy metals (Cumming and Weinstein, 1990; Denny and Wilkins, 1987; Jones and Hutchinson, 1988; Jongbloed and Borst-Pauwels, 1990; Morselt et al., 1986; Wilkins and Hodson, 1989), as well as marginal and doughty soils (Coleman et al., 1989; Richter and Bruhn, 1989). They can tolerate soils with suboptimal pH's for plant growth (Jongbloed and Borst-Pauwels, 1990; Mcafee and Fortin, 1987), and soils with high incidence of soil borne plant pathogens (Chakravarty et al, 1990; Duchesne et al., 1989; Grahm, 1988; Marx, 1969; Moser and Haselwandter, 1983; Sylvia and Sinclair, 1983; Smith, 1988; Tinker, 1984).

Mined-land sites, even after initial reclamation work, are generally nutrient poor and can contain higher concentrations of heavy metals and other pollutants that are harmful for establishment and survival of transplanted seedlings. Starting with the pioneering work of J. R. Schramm for reclaimed coal-mined locations, it has been a normal practice to use mycorrhizal fungal inoculums for establishing successful hardwood tree communities on mined sites (Castellano, 1996; Danielson, 1985). Mycorrhizal fungi play an important role in reforestation by providing the plant several benefits that are critical for its survival and growth in a nutrient poor and water deficient environment (Norland, 1993). These fungi improve the 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 (Danielson, 1985; Marx, 1972; Marx, 1991; Nara, 2005). While mycorrhizal relationships occur naturally in undisturbed ecosystems, it will be necessary to reintroduce these fungi in mined sites for reforestation to be successful. Primarily, this is because such sites are chemically, physically, and biologically altered and often lack the necessary quantity of mycorrhizal fungi to sustain replanted tree seedlings (Norland, 1993). In addition, newer and improved strains of fungi may be required initially to alleviate the

harmful effects related to soil disturbances, before the once indigenous strains can take hold in the affected regions.

We have been planting laboratory inoculated mycorrhizal seedlings on reclaimed lands of Ohio in order to test efficacy of different mycorrhizal fungi in supporting survival and growth of planted seedlings (Bauman et al., 2011; Bauman et al., 2012; Hiremath and Lehtoma, 1987). The objectives are to test which fungi help survival and establishment initially after planting, and to understand how indigenous fungi interact and, probably, replace the introduced fungus. We are using two tree species for this work: Virginia pine (*Pinus virginiana*) and American chestnut (*Castanea dentata*). Both the pure American chestnut and the backcrossed variety that is putatively resistant to the blight disease were used (Bauman et al., 2011; Bauman et al., 2012; Hiremath and Lehtoma, 2007). While Virginia pine was chosen because it is native to this region and does well, the latter is part of a project for the restoration of the American chestnut that was once common to this region. Once a major component of the eastern US forests, the American chestnut was almost eliminated by the invasive fungus *Cryphonectria parasitica* (Anagnostakis, 1982). However, breeding research undertaken by the American Chestnut Foundation has produced resistant hybrids by crossing with the Chinese chestnut (*Castanea mollissima*). Furthermore, through backcrossing these hybrids with the American chestnut, they have developed putatively resistant chestnuts having all the timber qualities of the American variety (Burnham et al., 1986; for details see www.acf.org). We have been using these chestnut seedlings for testing efficacy of mycorrhizal fungi for their survival and establishment on reclaimed mined sites.

Our work on reforestation using mycorrhizal fungi has also involved assessing the influence of soil treatments and planting protocols that are beneficial to mycorrhization of planted seedlings resulting in improved survival and growth (Bauman et al., 2012). Our recent work showed that presence of established pine trees in the vicinity facilitated the survival and growth rates of planted chestnut seedlings on reclaimed lands (Bauman et al., 2012). The data presented here describes results of a similar study where Virginia pines inoculated with different mycorrhizal fungi were planted along with chestnut seedlings pre-inoculated with *Pisolithus tinctorius*. The site was in Perry State Forest located in southeastern Ohio. After eight years of growth, we investigated the presence and changes in mycorrhizal associations among these seedlings.

Methods

Sites and Data Collection

This study was conducted on a restoration site that was planted in 2005 in Perry State Forest located in central Ohio (N39.76951° W82.20820°). Virginia pines (600) were inoculated using mycorrhizal cultures of *Amanita rubescens*, *Laccaria laccata*, and *Pisolithus tinctorius* via liquid media (Hiremath and Lehtoma, 2007). One-year-old chestnut seedlings (100) inoculated with *P. tinctorius* by the Ohio state tree nursery were planted by different researchers at the same time. Mycorrhizal pines were planted in a ring surrounding the American chestnut group at a spacing of 10-15 ft from the chestnut group. While the pines were verified for the presence of the fungus before planting, the chestnut seedlings, inoculated by germinating the seeds in the presence of the inoculum, were presumed to be mycorrhizal and not verified for presence of the fungus before planting.

In the fall of 2012, 62 seedlings were randomly selected for the present study: 31 Virginia pines and 31 chestnut seedlings. Of the chestnuts, 17 were pure American and 14 were 7/8 backcrossed chestnut (BC_2F_1). Height (cm) was measured using a meter stick from soil level to the tip of the main stem. Basal diameter (cm) was measured 3 cm above the root collar. A volume index (height × basal diameter^2) was calculated to estimate the volume of each seedling (Oskarsson et al., 2006).

Roots were sampled from each selected seedling. To ensure sampled roots were that of chestnut or pine and not a part of the surrounding vegetation, soil was carefully removed with a spade to expose the chestnut root system at a depth of 25 cm and 45 cm away from the stem. Roots were carefully sifted away from the soil and samples were taken without destroying the seedling and stored on ice. Once in the laboratory, roots were washed with autoclaved distilled water and then transferred to a Petri dish containing sterile water. One hundred root tips were randomly selected from each seedling and viewed under a dissecting microscope for the presence of a fungal sheath. One or two root tips of each morphotype per seedling were selected for DNA extraction and sequencing the internal transcribed spacer region of the nuclear ribosomal DNA (Bauman et al., 2011; Bruns et al, 1998). A total of 90 samples were sequenced which generated 79 fungal sequences for analysis.

Statistical Analysis

Aboveground seedling biomass was compared using a one-way analysis of variance (ANOVA) followed by a Tukey's HSD post hoc test using relative increase in seedling volumes (height cm * basal diameter cm^2) calculating $cm^3/year^{-1}$. Description of ECM diversity was quantified by species richness based and species abundance on ECM tip counts for each sequenced morphotype. A permutational multivariate analysis of variance was used to test for significant differences between pine and chestnut seedlings with regard to ECM community composition using the Vegan package of R. All statistics were performed using R v2.91 (R Development Core Team, 2009).

Isolation of fungal DNA, PCR analysis, and DNA sequencing

Identification of the fungus was done initially through morphological characteristics and then through sequencing of the internal transcribed spacer region of the ribosomal DNA. The sequence obtained was compared with sequences in the GENBANK using BLAST analysis (Hiremath and Lehtoma, 2007; Bauman et al., 2011; Hiremath et al., 2012; Gardes and Brubs, 1993; Bruns et al., 1998; Altschul et al., 1997). This was achieved by extracting the fungal DNA from root tips using the QIAgen Dneasy Plant Mini-Prep kits. Primers ITS1-F (5' cttggtcatttaggaagtaa 3') and ITS4 (5' tcctccgcttattgatatgc 3') were used to amplify internal transcribed spacer sequences (ITS) through PCR. The products were purified by gel electrophoresis and subjected to nucleotide sequencing. The identification of the fungus was made by comparing the sequence obtained through PCR to known sequences deposited in the GENBANK using BLAST searches (Hiremath et al., 2012).

Results and Discussion

The present study describes changes happening in ECM composition after 8 years of growth of Virginia pines and American chestnuts planted in a reclaimed mine location. The chestnut plantings were done for a different study (Brian McCarthy, Ohio University, Ohio) and were planted as a group. These were pre-inoculated with *P. tinctorius* by germinating the seeds in the presence of the inoculum. The seedlings produced were presumed to be mycorrhizal, and it is not clear whether they were tested for the presence of the fungus before planting. The Virginia pines were planted all around this group of chestnuts. The pines were pre-inoculated with either *A. rubescens, L. laccata,* or *P. tinctorius* and were tested for the presence of the fungus before

planting. Since the chestnuts were in the center of the plot surrounded by the pines, we wanted to find out the ECM distribution and probable contribution of symbionts by the pines to the chestnuts.

Relative growth rates $cm^3/year^{-1}$ (RGR/YR) were compared among the three seedling types: Virginia pine, pure American chestnut, and $7/8^{th}$ backcrossed chestnut (BC_2F_1). A significant difference was recorded after seven field seasons (F = 5.1, df = 2, P = 0.01). The BC_2F_1 chestnuts (206.0 cm^3/yr) and the Virginia Pine (208.0 cm^3/yr) seedlings had significantly more aboveground biomass than the pure American chestnuts (28.8 cm^3/yr; Fig 1). This difference between the two chestnut lines was presumably due to stem dieback caused by the presence of

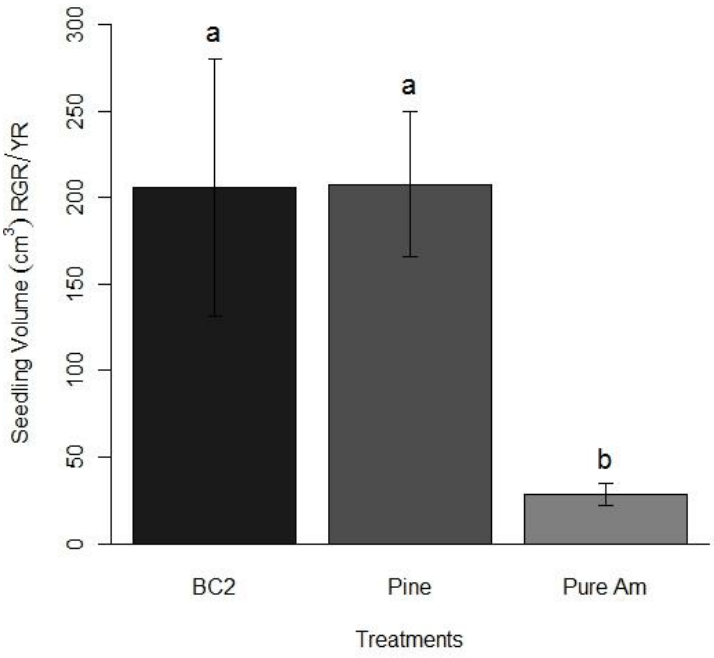

Figure. 1. After eight growing seasons, differences in growth existed among the seedlings. Backcrossed Chestnuts (BC2) and Virginia Pines accumulated significantly more aboveground biomass measured by the Seedling Volume by Relative Growth Rate per Year (RGR/YR) when compared to the pure American chestnut seedlings (P = 0.01).

chestnut blight cankers (*Cryphonectria parasitica*) observed on the stems of the pure American chestnut. It is known that the hyphae of the pathogen penetrate the vascular cambium of the chestnut, followed by enzymatic degradation leading to the dieback (Anagnostakis, 1982). Once

the stem becomes girdled, tissues above the infected canker decay resulting in the loss of seedling biomass. The BC_2F_1 chestnuts displayed field resistance to the canker causing *C. parasitica* and produced stem growth equal to the Virginia Pine.

While only *A. rubescens*, *L. laccata,* and *P. tinctorius* were used during pine planting, the present analysis detected the presence of eleven fungal species through DNA sequencing (Table 1). Among the ones used originally only *A. rubescens* was still present on some seedlings. This was confirmed using the sequence data, which matched the original fungal inoculum (*Amanita rubescens*) used to inoculate pines prior to outplanting. It was only present on pine roots and not on neighboring chestnuts suggesting that it was able to persist, but unable to cross over to colonize chestnut seedlings. There were no significant differences in growth of Virginia pines when seedlings originally inoculated with different mycorrhizal fungi were compared. However, after years of growth in the field, the most abundant mycorrhizal fungi sampled from roots of both Virginia pine and chestnut were *Russula* (30%), *Oidiodendron* (26%), and *Cantharellales* (11%). Some *Helotiales* (9%) species were also found on few seedlings. The Helotiales are not

Table 1. Ectomycorrhizal (ECM) fungal species sampled from Virginia Pine and Pure and BC2F1 chestnuts root tips ranked by relative abundance generated from root tip count data. Roots were collected from 63 seedlings (Total). This table reports fungal colonization from 79 sequences that were matched to vouchered ECM sequences available in GenBank. ECM species shown with an asterisk were part of the original inoculum used to inoculate Virginia Pine prior to outplanting.

Total Species	Total Percent	ECM on Pine	ECM on Chestnut
Russula	30%	33%	24%
Oidiodendron	26%	15%	45%
Cantharellales	11%	0%	29%
Helotiales	9%	14%	0%
*Amanita**	6%	10%	0%
Scleroderma	5%	8%	1%
Suillus	4%	7%	0%
Hygrocybe	3%	5%	0%
Unknown sp.	2%	4%	0%
Rhizopogon	2%	3%	0%
*Laccaria**	1%	1%	0%

known to be mycorrhizal symbionts and are generally classified as saprophytes. It is unclear how this species interacts with living roots, or if a functional symbiosis is formed under certain

soil and environmental conditions. The species belonging to *Amanita* (6%), *Scleroderma* (5%), and *Suillus* (4%) were found less frequently. In our earlier studies, we have seen the *Scleroderma* to be dominant in locations similar to the present one (Bauman et al., 2011). The *Laccaria laccata* was another fungal species used as inoculum in the original inoculum. However, the DNA sequencing indicated that the *Laccaria* sampled from roots did not match completely the original inoculum used to inoculate the pines, suggesting that this may be a different species. It appears that the *Laccaria* species we used initially was not suitable to persist longer in soil conditions present in this location. Although it was replaced by other ECM fungi, it may have helped in the initial phase of the seedling establishment and survival. Previous studies have shown that the presence of ECM inoculum greatly contributes to seedling establishment and will have persisting effects with regard to seedling development in the field (Menkis et al., 2007; Bauman et al., 2011).

No differences in ECM community composition were detected between BC_2F_1 chestnut and the pure American chestnuts. A similar observation was made in our earlier studies (Bauman et al., 2013). Therefore, these two seed types were pooled for the following analysis of community composition. A total of 10 fungal species were sampled from the Virginia pines while only four were detected on the chestnut roots, indicating a significant difference in ECM fungal community composition between Virginia pine and chestnut seedlings ($F = 2.46$, $df = 1$, $P = 0.005$). The ITS region DNA sequence analysis identified three symbionts shared by both chestnut and Virginia pine: *Russula*, *Oidiodendron,* and *Scleroderma.* The most abundant species sampled from Virginia pine at this location was *Russula* (Fig 2.). This fungal genera is a known ECM colonizer of woody tree and shrubs found in temperate forest ecosystems. Also, they are shown to be later-stage ECM fungi, which are representative of undisturbed habitats (Lilleskov and Bruns, 2003; Redecker et al., 2001). In contrast, *Oidiodendron* was the most abundant fungal species found on chestnut roots (Fig 2.). This fungus was also found on chestnut seedlings in another reclaimed coal mine site, where soil conditions were similar (Bauman et al., 2012). Although *Oidiodendron* spp. were once considered specific to plant species in the Ericaceae taxon, recent findings suggest that these fungi associate with other plant taxa. However, their role in plant establishment is not well understood (Horton et al., 1998; Chambers et al., 2008). The only ECM fungal species unique to the chestnuts was a fungus from *Cantharellales* (Fig 2.).

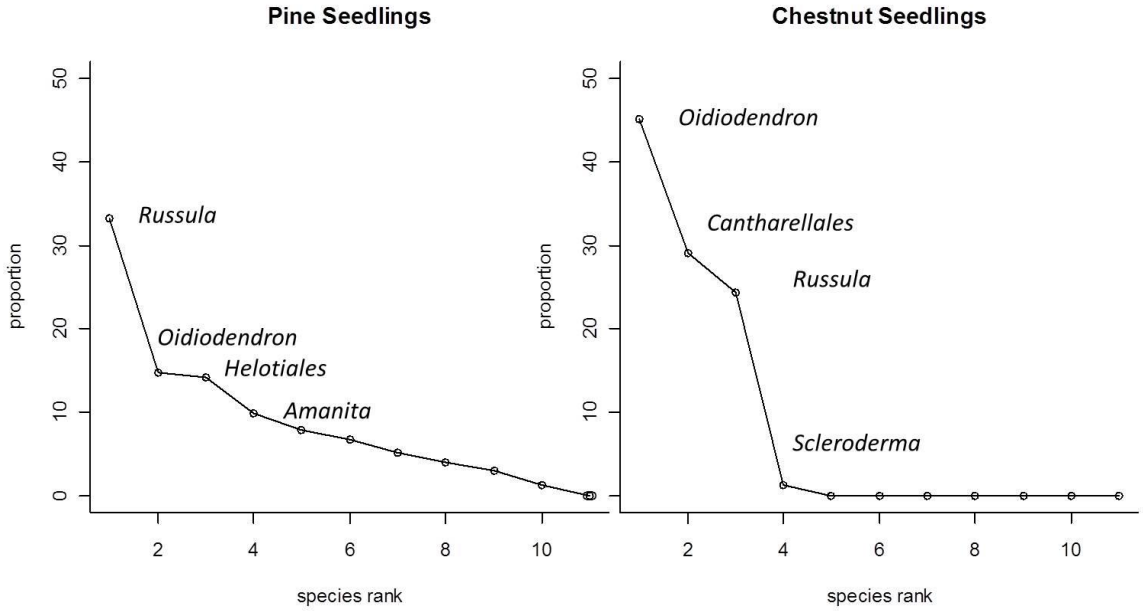

Figure 2. Differences in ECM community composition differed between the two tree species (P = 0.01). *Russula* sp. was the most abundant ECM species on Virginia pine and *Oidiodendron* was the most abundant species on chestnut. Graph shows 10 species of ECM fungi were recorded on the roots of Virginia pine and only four ECM species were collected from chestnut roots.

It is known that the plant-microbe community dynamics significantly influences the natural succession of plant species where pioneer vegetation facilitates the establishment of a distantly related, later successional plant species (Dickie et al., 2006). However, it appears that in the present location the introduced ECM on pines did not provide inoculum for neighboring chestnuts. This was different from our earlier findings in a separate study where established pines were found to act as providers of symbionts to chestnuts (Bauman et al., 2012). However, in that study, the pines were well established and chestnut seedlings were produced by germinating seeds in close vicinity. Although there was no early sharing of ECM inoculum in the present case, it is likely that both species derived mutual benefits from one another for their establishment over time by influencing changes in rhizosphere chemistry (Bai et al., 2009), increasing soil nutrients from litter accumulation (Flores and Jurado, 2003), increasing soil moisture (Richards and Caldwell, 1987), and/or decreasing bulk densities (Ashby, 1989). Over time, both seedling types were colonized by native fungal species that were either in the soil as spores, or more likely, mycorrhizal with existing surrounding vegetation. It has been shown in

many cases that available ECM from an unrelated plant species aids in facilitating the establishment of a later successional group (Callaway, 1995; Horton et al., 1999).

It is also likely that root colonization by these species may have been accomplished by spores existing in the soil or by hyphae or rhizomorphs radiating from the established pines from the forest edge (Jefferies, 1999). In the present case, some established pines were present in the forest edge that were within 4.6-15.4 m (15-50 ft). The genus *Russula* is capable of producing enzymes that degrade organic matter in the soil (Agerer, 2001), and correlates to forest edges and can inoculate via hyphae or spore production. The present study suggests that the forest edge may have provided the inoculum that replaced what was introduced on both pine and chestnut. This is very important for future restoration efforts for the establishment of later successional tree species on reclaimed mined lands. The *Scleroderma,* which was found on both pine and chestnut, are known to produce rhizomorphs that are capable of long-distance exploration for water and minerals that result in growth benefits to their plant hosts (Agerer, 2001). This can also provide an inoculum source to an unrelated plant species, thereby facilitating its establishment on mine soils (Bauman et al., 2012).

There was significant decline of the pure American chestnuts after eight field seasons, presumably due to the blight fungus. Further study is being conducted on these and other chestnuts planted on other sites in the region to document the resistance potential of the hybrid chestnuts to *C. parasitica*. However, the backcrossed hybrid chestnuts were performing very well in the field, at least at this site, and showing biomass accumulation comparable to Virginia pines.

In summary, with the exception of *Amanita,* the inoculum that were out planted with both chestnut and Virginia pine were replaced after 8 field seasons by fungi native to the field site. The inoculated ECM species did not persist or impede natural root colonization of native fungi, which may be better suited for the long-term survival of plant species in disturbed environments. Because *Amanita* did not appear on chestnut seedlings, it appears the fungus may not be compatible with the chestnut and/or the soil conditions present at this location were not conducive for its migration from pines to colonize the chestnut roots.

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