ABIOTIC AND BIOTIC FACTORS IN COAL MINE SOILS INFLUENCE ECTOMYCORRHIZAL COMPOSITION AND SYMBIOSIS[1]

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Abstract. Surface mining for coal leads to significant changes in ectomycorrhizal (ECM) fungal community composition by removing host plants and altering the structure and chemistry of the soil environment. These changes impact ECM fungal composition and efficiency of ectomycorrhizal formation. This study investigates the influence of soil abiotic factors on ECM community composition and root colonization. Two different surface coal mine sites in Ohio, U.S.A. were used: an abandoned surface mine and a site reclaimed under the Surface Mining and Control Act of 1977. Within the abandoned site, three unique conditions were delineated: 1) remnant forest edge, 2) restoration plots consisting of *Pinus virginiana*, and 3) non-vegetated sites. American chestnut (*Castanea dentata* Marsh. Borkh) were sown as nuts for use as ECM trap trees. The presence and identity of native ECM were determined by fungal DNA sequencing of the internal transcribed spacer (ITS) region. Relationships between fungal genera and soil characteristics were determined by a non-metric multidimensional scaling (NMDS) ordination. A multiple regression was used to determine which soil variables influenced ECM root colonization. Results illustrated significant differences between the abandoned mine soils when compared to the reclaimed soils in terms of pH, phosphorus, and potassium. ECM fungal composition was dependent upon the levels of phosphorus, organic matter, and magnesium in the soil. Certain ECM genera were associated with higher phosphorus and pH, while some were linked to nutrient impoverishment. Differences existed between two ectomycorrhizal fungal species within the *Scleroderma* genera, suggesting that not all species within a genus share environmental preferences. Organic matter was a significant predictor of ECM root colonization. Knowledge of these factors will be helpful for designing suitable mine reforestation strategies such as prediction of ECM fungi, use of ECM inoculum, and soil amendments.

Additional Keywords: anthropogenic soil disturbances; abandoned coal mines; legacy mines; soil fungi; seedling establishment; mine reclamation; *Castanea dentata*

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Introduction

In temperate forests, approximately 90% of roots on forest trees are colonized by a diverse assembly of ectomycorrhizal (ECM) fungi (Visser, 1995). In undisturbed ecosystems, ECM diversity can be quite rich (Horton and Bruns, 2001), surpassing hundreds of species within a forest stand (Courty et al., 2008; Smith and Read, 2008). Such fungal assembly results from an integration of many abiotic and biotic factors including mineral nutrients, soil depth, O_2 and CO_2 concentrations, amount and quality of organic matter, temperature, moisture levels, host plants, and the age of the forest stand (Blasius and Oberwinkler, 1989; Bruns, 1995; Smith et al., 2002). How these factors interact to promote distribution of ECM species and plant interactions is not well understood (Leake, 2001; Lilleskov et al., 2004; Burke et al., 2009). More information is required to better understand how these symbiotic communities form when ecological conditions are altered by large-scale, anthropogenic disturbances (Buscot et al., 2000).

Surface mining for coal leads to significant changes in ECM community composition by removing host plants and altering the structure and chemistry of the soil environment (Jones et al., 2002; Jasper, 2007; Bauman et al., 2013). Soils left behind can be very difficult to generalize; they are largely heterogeneous with zones of high acidity (Hossner et al., 1997), patchiness of nutrients (Mummey et al., 2002), metals, and organic matter (Boruvka and Kozak, 2001). These differences in the soil structure and chemistry may influence the ECM species composition and efficiency of ectomycorrhizal formation, even at small spatial scales. In addition, the removal of host plants and organic material causes a dramatic decline in fungal populations, promoting the low species richness of ECM fungal genotypes. This tends to favor the selection of a very few, yet important, fungal species that can tolerate drought (Swaty et al., 2004), persist after anthropogenic disturbances (Horton and Burns, 2001), or survive in soils with toxic levels of metals and temperature extremes (Samson and Fortin, 1986; Iordache et al., 2009).

To enhance tree seedlings' ability to absorb water and nutrients and tolerate heavy metals and low pH, ECM inoculum is commonly used in restoration (Castellano, 1996; Walker, 2004; Nara, 2005). Seedlings used in reclamation projects are either pre-inoculated with selected ECM fungi in field nurseries or in greenhouses as potted plants. Bauman et al., (2011) reported that seedlings out-planted on a reclaimed mine site did not maintain their original ECM inoculum.

Regardless, the fact that seedlings were colonized prior to planting contributed significantly to the survival of the seedlings within the first few months. Further, subsequent root colonization by certain native ECM fungi improved chestnut seedling growth within the first two seasons (Bauman et al., 2011). Documenting fungal genera that correlate with abiotic soil conditions may contribute to our understanding of how ECM communities reassemble during soil recovery in areas that have been severely degraded (Jasper, 2007). Knowledge of these factors may provide a sense of predictability of these fungi and aid in management decisions when approaching a reforestation project. A better understanding of ECM fungal response to secondary soil disturbances during restoration may also aid in determining whether inoculum is needed, or whether spores or existing mycelium provide propagules for root colonization (Fox, 1986; Deacon and Fleming, 1992; Bauman et al., 2012). 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.

The objective of this study was to use environmental soil data such as pH, soil nutrients, and organic matter to determine the influence of soil environment on ECM community composition and root colonization. American chestnut (*Castanea dentata*) was used as a trap tree to sample ECM in the field due to its affiliation with ECM genera in both forested and reclamation soils (Palmer et al., 2008; Bauman et al., 2013). Two different surface coal mine sites in Ohio, U.S.A. were used, an abandoned surface mine and a site reclaimed under the Surface Mining and Control Act (SMCRA). Within the abandoned surface mine site, three unique conditions were delineated and experimentally replicated: 1) remnant forest edge, 2) restoration plots consisting of *Pinus virginiana*, and 3) non-vegetated sites. This was compared to a fourth site that was reclaimed under SMCRA, which included the addition of stockpiled topsoil, mechanical grading, and seeding with non-native species that resulted in an exotic grassland. This study investigated how variations in the abiotic characteristics of the soil environment influence ECM community composition and the effect of differences in soil chemistry and organic matter on ECM root colonization. The overall goal of this study was to improve our knowledge of how abiotic soil variables influence plant/fungal interactions, which can be taken into consideration when approaching the restoration of disturbed landscapes.

Methods and Materials

Site description and soil sampling:

Two different mines were sampled for ECM fungi. The first is an abandoned coal mine located in Avondale Wildlife Area in Muskingum County, Ohio (39° 49' 44" N, 82° 7' 38" W; Fig. 1A). This site is within an abandoned mine representative of surface mined sites prior to the Surface Mining Control and Reclamation Act of 1977 (SMCRA) in Ohio. This site was mined in the 1950s and has had very little reclamation aside from experimental tree plantings resulting in small monoculture pine stands (*Pinus virginiana*). Soil quality is poor and is comprised of abandoned gob and mining debris. The site is characterized by less than 5% vegetative cover and poorly sorted debris. There is no topsoil, very little organic matter, and presumably low microbial activity. This area receives an average of approximately 99 cm of precipitation annually with temperatures averaging 22° C during the growing season (17°, 28°, and 11° C, spring, summer, and fall, respectively; National Climatic Data Center). Within this site, three distinct areas were sampled: non-vegetated sites, 10-year-old pines that were able to establish in areas adjacent to the non-vegetated sites, and edges of forests that were a product of natural recovery in areas around the perimeter of the abandoned site.

To provide for blocking and replication within this abandoned mine site, the site was divided into three blocks of 0.80 ha (Bauman et al., 2012). Each block contained 6 plots (4 m × 3 m) belonging to each habitat type: forest edge (near the edges), non-vegetated sites (away from edges), and 10-year-old pine plots that were in the vicinity of 10-year-old *Pinus virginiana*. Soil characteristics in each habitat type resembled those found in abandoned gob piles (soil mixed with coal debris; Fig. 1). Forest edge plots were placed 4 meters from the edge of the forest canopy spaced 10 m from each other. 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. 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.

The second coal mine was located in Tri-Valley Wildlife Management Area, Muskingum County, Ohio (40° 11' 32" N, 81° 98' 35" W; Fig. 1B) and reclaimed under SMCRA in the 1980s. This reclaimed mine is typical of post-SMCRA sites (McCarthy et al., 2008) and is primarily vegetated with the original species used for reclamation (*Festuca* spp. and

Lespedeza spp.). These areas were sampled as "grasslands". This area receives an average of approximately 99 cm of precipitation annually. During the 2007 and 2008 growing seasons, 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).

Pure American chestnut seeds (*Castanea dentata* Marsh. Borkh), provided by the American Chestnut Foundation, Meadowview, VA, were planted in the spring of 2007 and sampled at the end of the 2008 growing season. The abandoned mine located in the Avondale Wildlife Area had three distinct areas from which samples were collected. Although 18 plots (six plots in three blocks) were initiated per mine area within the abandoned mine, not all plots had surviving seedlings. Therefore, we were able to sample from six non-vegetated plots, six plots along the forest edge, and 10 pine plots. Each plot was 4m × 3m and each 4m × 3m plot was considered as one sample to avoid pseudo-replication. Six, 4m × 3m grassland sites were sampled from Tri-Valley Wildlife Management Area. Additional preparation was required for planting chestnut seeds in the grassland plots. This included cross-ripping soil at a depth of approximately 1 meter by a D-6 dozer with a 1.0 m steel ripper bar attachment followed by a plow and disk using a conventional tractor (McCarthy et al., 2008).

Figure 1. Two different mines were sampled for ECM fungi. Panel A shows the abandoned coal mine located in Avondale Wildlife Area, location of the non-vegetated, forest edge, and 10-year-old pine plots. Panel B Tri-Valley Wildlife Management Area, location of the grassland treatment.

In the fall of 2008, two harvest dates retrieved 129 surviving seedlings, representing all sample plots: 48 grassland seedlings, 21 seedlings from forested edge, 27 seedlings planted in

non-vegetated plots, and 33 seedlings planted in the pine plots. Seedlings were carefully removed from the field, stored on ice, and returned to the laboratory within 24 hours. In the laboratory, the entire root system was washed using distilled water, roots were cut into 3 cm segments, and stored in Petri dishes at 4˚ C for 7 days. All samples were observed under a dissecting microscope for ectomycorrhizal formation determined by the presence of a fungal sheath. One hundred root tips per seedling were randomly selected from root segments. Using a grid template as a guide, the number of ECM root tips was divided by the total number of roots sampled up to 100 tips to calculate percent ECM. ECM was morphotyped based on the color and texture of sheath, emanating hyphae, and presence of rhizomorphs (Nara et al., 2003). Morphotypes were counted and used for the subsequent multivariate analysis (described below). Two samples per morphotype per seedling were selected for DNA extraction. A three mm segment of root tip was removed and transferred into a microcentifuge tube and stored at -70˚ C until extraction.

Soil samples were collected from a depth of 0-18 cm using a soil probe from both Tri-Valley and Avondale Wildlife Areas, four samples per plot. The four samples per plot were mixed thoroughly, allowed to air dry, and 0.50 liters were sent to Spectrum Analytic Inc., Washington Court House, Ohio for analysis. Soil variables measured were: pH, soil texture, organic matter, P, K, Mg, Ca, cation exchange capacity (CEC), Al, S, Bo, Zn, Fe, Cu, and Mn. All macro and micronutrients were measured using the Mehlich 3 extraction method.

DNA extraction and purification:

ECM present on root tips were identified by DNA extraction followed by PCR of the ITS fingerprint region and DNA sequencing (Hiremath and Lehtoma, 2007). Two samples per morphotype, per seedling, were selected for DNA extraction. As mentioned above, a 3 mm segment of root tip was removed, transferred to a microcentrifuge tube, and stored at -70˚ C to await DNA extraction. Briefly, the 3 mm segment was homogenized using a bead beater, and DNA was extracted using QIAgen's DNeasy Plant Mini Kit per manufacturer's protocol. About 10 ng of this DNA was used in PCR reactions using primers ITS1-F (5' cttggtcatttagaggaagtaa 3') and ITS4 (5' tcctccgcttattgatatgc 3'), which amplify the highly variable internal transcribed spacer (ITS) region of ECM fungal ribosomal DNA (rDNA) (Gardes and Bruns, 1993). PCR 15 µl reactions were mixed based on the following concentrations: 9 µl of molecular grade water, 3 µl of 5x Green GoTaq® Reaction Buffer, 0.125 µl of Promega® *Taq* DNA Polermerase, .2 µl

of 25µM of each primer, 1µl of dNTPS (200µM each of dATP, dCTP, dGTP, and dTTp), and 1 µl of DNA template. Temperature cycling was accomplished using a programmable Thermal Cycler Heating block. Times and temperatures were programmed as described by Gardes and Bruns (1993): The initial denaturation step was 94 ºC for 85 s followed by 35 amplification cycles of denaturation, annealing, and extension. The temperature and times for the first 13 cycles were 95 ºC for 35 s, 55 ºC for 55 s, and 72 ºC for 45 s. Cycles 14-26 and 27-35 repeated the above parameters with lengthened extension steps 120 and 180 s, respectively. When the 35 cycles were completed the samples were programmed to incubate for 10 min at 72 ºC for 45 s.

After confirmation using gel electrophoresis, the PCR products were purified using Wizard® SV 96 Genomic DNA Purification System (Promega, USA) and used for DNA sequencing. Sanger sequencing was performed with The Applied Biosystem ABI Prism 3730 DNA Analyzer (Bioinformatics Facility, Miami University, Oxford, Ohio). The DNA sequences were analysed and edited using the Sequencher 4.2 software (Gene Codes, Ann Arbor, Michigan). To identify the fungi found on roots to genus, ITS sequences from samples were compared with those in GenBank using the Basic Local Alignment Search Tool (BLAST; Altschul et al., 1997). From 129 seedlings, 205 ECM root tips were sampled, which produced 151 sequences. Of the 151 sequences, 97 matched >95% to our previously GenBank published accessions (Bauman et al., 2012; Bauman et al., 2013). The remaining 54 sequences matched other fungi that represented similar genera. To be conservative, we only reported the 97 sequences that did match >95% of the database. Therefore, authors acknowledge that other genera may have been sampled but lacked the similarity match to be considered additional genera reported in this paper.

Statistical analyses:

Principal component analysis (PCA) was used to extract the initial set of uncorrelated components from the independent soil variables sampled from Tri-Valley and Avondale Wildlife areas. To avoid redundancy in correlated variables, autocorrelated variables were removed before PCA was performed. Linearity and normal distribution assumptions were met by transforming (Log10 + 1) and standardizing (Wisconsin double standardization) the dataset. An eigenvalue threshold of unity (1.0) was used to retain factors in the model. Eigenvalues are the sum of squared correlation between the original independent variables and the principle components obtained, and they represent the amount of variance attributable to the components. Each of the components were rotated to facilitate their interpretation, and then referred to as

factors. An orthogonal rotation (Varimax rotation) was used in this analysis to obtain the factors, maintaining their independence. Factor loadings were used to interpret the resulting factors. Absolute loading value > 0.75 was used to interpret the resulting factor pattern.

Differences in soil chemistry among the sampled plots were identified using a multivariate analysis of variance (MANOVA) followed by ANOVAs. MANOVA significance was evaluated using Wilks' λ test statistic. Description of ECM per site was quantified by species richness based on fungal genera, Shannon-Weiner diversity index, and Simpson's index of diversity based on ECM tip counts for each morphotype. ECM colonization per treatment was assessed by taking the percentage (#ECM tips/100) of ECM colonized root tips from chestnuts planted as bare root seedlings. When significant, an ANOVA followed by Tukey's HSD post hoc was used to assess differences among sites. PCA, ANOVAs, and MANOVAs were performed using JMP (8.0, SAS Institute, Cary NC, USA).

A non-metric multidimensional scaling (NMDS) ordination was used to determine if ECM community composition, quantified by root tips counts on American chestnut, differed among the four sites (metaMDS function in vegan package, version 2.13.0). To improve the NMDS ordinations, the data were square root transformed and standardized via Wisconsin double standardization (Oksanen. 2005). Bray-Curtis dissimilarities were employed due to their preferred analysis for community data due to the restriction within the range of 1 to 0 (Kindt and Coe 2005). The maximum number of random starts in search was set at 100 with k=2 stress value. A permutational multivariate analysis of variance (formerly nonparametric MANOVA) was used to test for significant differences among the soil treatments using the adonis function in vegan package, version 2.13.0. This is a method for partitioning variation in dissimilarity or distance matrices using a "pseudo-F" statistic analogous to MANOVA (Oksanen et al., 2005). All ECM community statistics were performed using Vegan: Community Ecology Package.

To determine whether ECM fungi were correlated with the soil measurements, environmental variables were fit onto the NMDS fungal genera ordination via fitted vectors. These vectors are shown as the strength and direction of the correlations between the ordination and environmental variables (Oksanen et al., 2005). This was accomplished by employing function envfit to the ordination. This results in directional cosines of the vectors along with the squared correlation

coefficient (r^2). The significances are presented by a p-value based on random permutations of the data.

A multiple regression analysis was used to determine which independent variable best predicted percent ECM root colonization. To meet model assumption of normality and equal variance, predictor variables were transformed Log10+1 and standardized, and the dependent variable (ECM % root coverage) was arcsine transformed. The optimal number of variables to include in the models was determined by choosing the best subset regression with the lowest Bayesian information criterion (BIC) using R (version 2.13.0).

Results

Since there were many soil factors (variables) in our study, we used a principal component analysis (PCA) and a factor analysis to identify a smaller set of correlative factors that represent the highest amount of variability. Further, we applied a varimax rotation analysis to this data which provides a new set of factors that explain variances observed. Table 1 reports the varimax rotated factor loadings for soil characteristics of the four mine sites. Three factors contributed to a total of 78% variance. Out of these, factor 1, contributing to 32% of the total variance, presented high loadings for pH and phosphorous. Factor 2 contributing 27% of variance presented high negative loadings for organic matter (%) while showing high positive loading for potassium (K). The factor 3 contributing only 19% of the total variance had high loading for manganese and high negative loading for magnesium.

Table 1. A principal component analysis (PCA) was used to identify a smaller set of factors that represent the highest amount of variability. High factor loadings, representing greatest variability are shown in bold.

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

To further understand how these five soil characteristics (identified by the PCA) differed among the plot types, an ANOVA followed by Tukey's HSD post hoc test was used (Table 2). Soil pH (F= 179.4, df=3, $P < 0.0001$) and phosphorus (F=20.5, df = 3, $P < 0.0001$) were significantly higher in the grassland plots. Soil concentrations of potassium (ppm) were significantly higher in the pine plots (F = 3.26, df = 3, $P = 0.04$). Organic matter (%) and manganese were fairly similar among the sites.

Table 2. An ANOVA followed by Tukey's HSD post hoc test was used on selected soil characteristics that differed among the plot types. Values in the table are the means with ± 1 SE. Significant differences ($p < 0.05$) are shown in bold text. Analyses based on data transformed by $\log_{10}+1$.

Plots	Soil pH	P (ppm)	%OM	K (ppm)	Mn (ppm)
Non-vegetated	3.0 ± 0.1	2.2 ± 1.6	1.4 ± 0.7	77.4 ± 9.0	6.4 ± 1.1
Forest Edge	3.0 ± 0.1	1.0 ± 0	1.9 ± 0.5	83.8 ± 10.1	6.0 ± 1.6
Grassland	**5.3 ± 0.1**	**7.8 ± 1.9**	1.5 ± 0.1	86. 7 ± 7.1	**3.8 ± 0.9**
Pines	3.2 ± 0.03	1.5 ± 0.8	0.9 ± 0.2	**117.2 ± 10.4**	7.0 ± 2.5

ECM Community Composition:

We identified 14 sequences that matched up to our previously published GenBank accessions. These genera included *Cenococcum*, *Cortinarius*, *Hebeloma*, *Laccaria*, *Lactarius*, *Pisolithus*, *Oidiodendron*, *Russula*, *Scleroderma (*sp. 1 and sp. 2*)*, unknown ECM, *Tomentella*, *Thelephora*, and Thelephoraceae (Table 3). The more abundant genera included *Scleroderma* spp. 1 and 2 (44 and 21%, respectively) and *Thelephora sp.* (17%). When richness and Shannon-Weiner Diversity indices were compared, no differences existed. However, differences did exist when percent colonization was compared. Seedlings planted along the forest edge (56%) and in the grasslands (43%) were similar and significantly greater in colonization when compared to the non-vegetated (21%) and the pine plots (24%; F= 4.28, df=3, *P*= 0.02; Table 4).

Table 3. Morphotyped ectomycorrhizal fungi (Sampled ECM) were quantified by proportion of roots colonized by ECM fungi (%Root Colonization) and compared to the four different sites that they were sampled: grasslands, forest edges, pines, and non-vegetated plots (Sampled From) and matched to published accessions (Accession).

Sampled ECM	NMDS code	% Root Colonization	Sampled From	Accession
Scleroderma sp. 1	Scl1	44.50%	Grassland	GU246989
Scleroderma sp. 2	Scl2	20.70%	All Sites	GU553366
Thelephora sp.	Thel1	16.80%	Non-vegetated, Grasslands, and Pines	GU553377
Cenococcum sp.	Cen	3.90%	All sites	GU553373
Pisolithus sp.	Pis	2.60%	Pines	GU553367
Thelephoraceae	Thel2	2.60%	Non-vegetated, Grasslands, and Pines	GU553376
Hebeloma sp.	Heb	2.30%	Grassland	GU246985
Oidiodendron sp.	Oid	2%	Non-vegetated and Pines	GU553367
Laccaria sp.	Lac	1.20%	Forest Edge and Pines	GU246994
Russula sp.	Rus	1.10%	Forest	GU553374
Cortinarius sp.	Cort	< 1.0 %	Grassland	GU246987
Lactarius sp.	Lact	< 1.0 %	Forest Edge	GU553369
unknown ECM	Unkn1	< 1.0 %	Pines	GU553372
Tomentella sp.	Tom	< 1.0 %	Forest Edge	GU553375

Table 4. Total number of genera, average richness, Shannon-Weiner diversity index, and percent colonization ± 1 SE from chestnuts sampled among the four mine sites: non-vegetated, forest edge, grassland, and pine plots (n = 12). Different letters indicate significant differences by Tukey's HSD.

Plots Sampled	Total # of Genera	Avg Richness	Avg Diversity	% Colonization
Non-vegetated	4	2.7 ± 0.33 [a]	0.68 ± 0.16[a]	**21 ± 9%** [b]
Forest Edge	7	3.3 ± 1.20 [a]	0.78 ± 0.40[a]	**56 ± 10%**[a]
Grassland	7	4.3 ± 0.33 [a]	0.74 ± 0.16[a]	**43 ± 1%** [a]
Pines	9	5.0 ± 2.08 [a]	1.06 ± 0.53[a]	**24 ± 5%** [b]

A NMDS ordination was used to illustrate how ECM fungi group in correlation with the plot types (Fig. 2). Ordination patterns illustrated a clustering of ECM communities within the two respective sites (grassland and abandoned sites). This pattern was supported by a permutational MANOVA, which showed a significant site effect (F = 4.20, df = 3, P = 0.005). To illustrate how soil chemistry influenced the ECM community composition, the environmental soil data (Mg, Mn, P, pH, and OM) were imposed as vector lines on the NMDS ordination (Fig. 2). Analysis of these correlations is presented in Table 3. Along the first dimension of the

ordination (NMDS 1), P (ppm) correlated significantly with those fungi associated with the grasslands (r^2 = 0.30, *P* = 0.03) and marginally associated with Mg (r^2 = 0.21, *P* = 0.08). These fungi included *Scleroderma* sp. 1 (Scl1), unknown ECM (Unkn1), *Tomentella* (Tom), and *Hebeloma* (Heb).

The second axis of the ordination (NMDS 2) was marginally negatively associated with organic matter (r^2 = 0.21, *P* = 0.08; Fig. 2; Table 5). This correlated with ECM fungi *Pisolithus* (Pis), *Oidiodendron* (Oid), and *Thelephora* sp. 1 (Thel 1) that were associated with the plots among the pines. In addition, *Cenococcum* (Cen) appeared in the ordination with pine plots with higher pH levels. The *Scleroderma* sp. 2 (Scl2) and *Laccaria* (Lac) were associated with both pine and forest edge plots. *Russula* (Rus) were associated with the forest edge and were marginally influenced by the organic matter (r^2 = 0.21, *P* = 0.08).

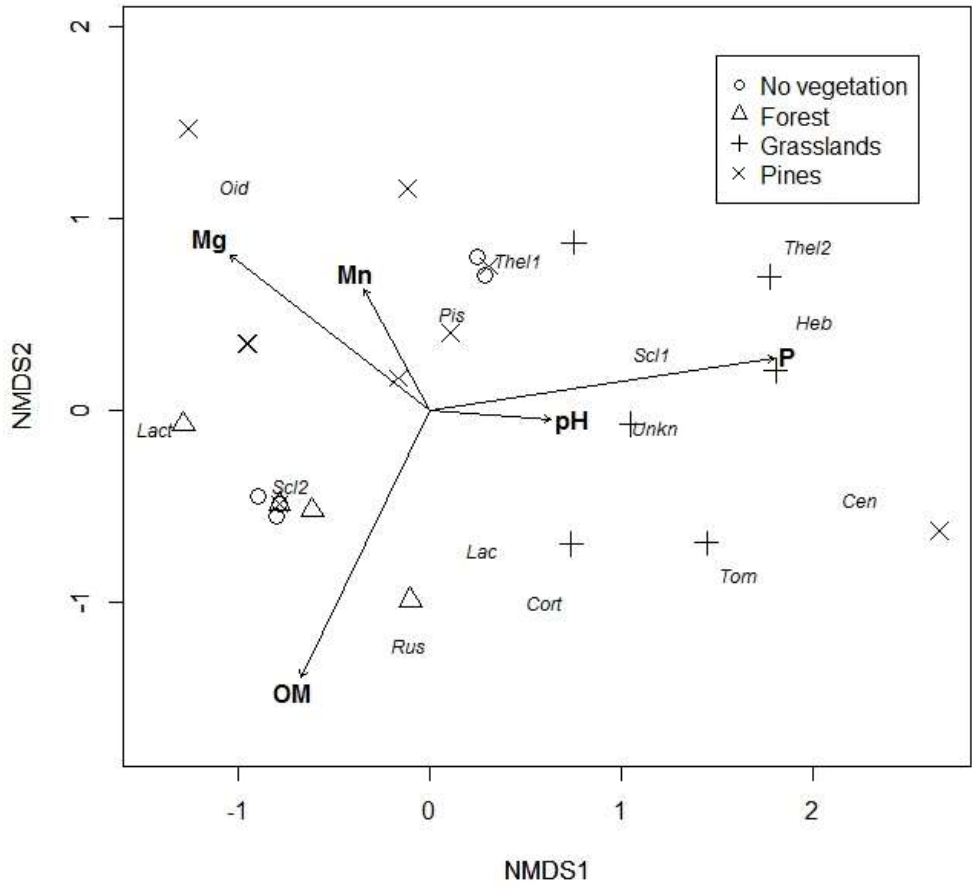

Figure 2: NMDS ordination illustrating ECM community composition (based on root tip counts) with environmental soil data (Mg, Mn, P, pH, and OM) imposed as vector lines indicating strength and direction of the strongest correlations. Ordination patterns

(Caption continued on next page.)

illustrated a clustering of two ECM community groups: 1) genera sampled from the grasslands (+) formed a distinct group and 2) the abandoned plots illustrating an overlap among the plots that represented no vegetation (○), plots along the forest edge (+), and pine plots (×). Along the NMDS 1 axis, phosphorus (P) was correlated with those fungi associated with the grasslands and with fungi associated with magnesium (Mg) within the pine plots. The NMDS 2 axis of the ordination was associated with organic matter (OM) correlated with fungi within the forested plots. Fungal genera codes are listed in Table 3.

Table 5. Relationship between soil variables and NMDS dimensions of ECM community. Columns NMDS1 and NMDS2 give direction cosines of the vectors illustrated in Fig. 2. Phosphorus (ppm) correlated significantly with the first dimension of the ordination (NMDS 1). Organic matter and Mg were marginally correlated with the second dimension of the ordination (NMDS 2). Column with r^2 gives the squared correlation coefficient. The P-values (Pr(>r)) are based on random permutations of the environmental variables as they relate to NMDS1 and NMDS2. Significant values are shown in bold.

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

Soil Factors Related to ECM Root Colonization:

To assess the effects of the soil variables on the percentage of ECM root tips ECM, a multiple regression was used. To find the optimal subset of predictors, the Bayes' Information Criterion (BIC) was used. The BIC identified four soil variables (organic matter, Mg, Mn, and Al) to be used in a multiple regression model. Using these variables, the regression model indicated that organic matter explained a significant amount of the variation and, therefore, could be used as a predictor of variable for ECM root colonization ($F = 1.91$, $df = 39$, $P = 0.03$, $R^2 = 0.20$; Table 6).

Table 6. The multiple regression related the ECM root colonization (%) on chestnut seedlings to the soil environment. This table reports the best model selected by the BIC which included a sub-set of variables (organic matter, magnesium, manganese, and aluminum). The regression analysis shows that organic matter explained a significant amount of the variation and could be used as a predictor for ECM colonization.

Predictor	**β Estimate**	**SE**	**t-value**	***p***
Organic Matter (%)	-7.30	3.30	-2.21	0.03*
Magnesium (ppm)	1.81	3.16	0.58	0.57
Manganese (ppm)	-2.09	3.01	-0.69	0.49
Aluminum (Al)	-3.31	2.48	-1.33	0.19

Discussion

Our analysis of soil variables demonstrated significant differences between the two types of surface mined sites: an abandoned site with little remediation work and the site that was reclaimed under SMCRA. The grassland plots in the SMCRA-reclaimed mine site were higher in soil pH and P and lower in Mn than the abandoned mine site. These differences were related to the SMCRA reclamation policy that requires stockpiled topsoil to be mechanically graded back to contour and non-native cover crops to be seeded on compacted soils (Zipper et al., 2011). While SMCRA reclamation methods are critiqued for reclaiming historically forested sites into grasslands, soil characteristics are generally improved when compared to the abandoned mine lands, as seen in this study. Such reclamation has led to improved water quality, deterred soil erosion, and buffered soil pH (Davison et al., 1984), as the SMCRA policy intended. However, despite these improvements, soil conditions on reclaimed mine sites remain low in fertility, high in compaction, prone to drought conditions, and subject to invasive herbaceous species (Steiger, 1996; Bauman et al., 2015). Our study only sampled from one SMCRA site and, therefore, our results did not reflect the regional changes in the fungal species pools or illustrate variations of soil chemistry among different sites within the region (Cavender et al., 2014). In addition, soil feedbacks, influenced by parent material, age of restoration, and the reclamation vegetation can ultimately influence both soil chemistry and below-ground soil fungal communities (Dickie et al., 2006; Burke et al., 2009).

ECM genera formed strong associations with seedlings in the respective sites from which they were sampled. Fungal composition was significantly linked to the soil phosphorus content.

Levels of organic matter and soil Mg also appeared to be potential drivers of fungal composition. These findings support the hypothesis that variations in abiotic factors influence ECM community composition. Certain ECM genera (*Hebeloma, Cenococcum, Tomentella*) appeared in the ordination within the grassland plots, which had higher pH and more availability of P. One fungus that spatially separated from all other fungi in the ordination was the ascomycete *Oidiodendron*. These genera of fungus form ericoid mycorrhizas with plant species in the Ericaceae taxa (Peterson et al., 2004). Recent findings suggest these ascomycete fungi form dark septate mycorrhizas with other plant taxa (Chambers et al., 2008; Burke et al., 2009; Bauman et al., 2012). This was the case in our study where these genera formed mycorrhizas with chestnut in areas low in nutrients and high in metals, specifically associated with higher levels of Mn. In contrast, ECM fungi associated with the forest edge consisted of *Russula*, *Laccaria*, and *Cortinarius*. Fungi of these genera are ECM colonizers of woody trees and shrubs found in temperate forest ecosystems. Generally, these are more prevalent in undisturbed habitats (Redecker et al., 2001) and have been considered to be later-stage ECM fungi that are dominant on mature roots (Lilleskov and Bruns, 2003). Collectively, they represent fungi with long-lived clonal populations that are better competitors in undisturbed habitats having low levels of resources (Taylor and Bruns. 1999).

ECM diversity and richness were similar among the sites sampled. Percent colonization was significantly lower on sites that were non-vegetated. ECM colonization appeared to be more affected by soil organic matter, which was identified as a significant predictor of root colonization. Past studies have shown the rates and types of mycorrhizal associations to vary in response to changes in organic matter in forest systems (Baar and deVries, 1995; Dickie et al., 2006). Studies in a greenhouse, where organic matter was added to the mine soil, resulted in a significant increase in the rates of colonization by *Hebeloma* on *Quercus* seedlings (Lunt and Hedger, 2003). Furthermore, the colonization resulted in significant increase in seedling growth. Because mine reclamation operations often use the soft rock shale overburden materials in the soil substrate upon reclamation, soils are left very deficient in organic matter. In remote sites, addition of soil amendments can be logistically or economically prohibitive, and is currently not mandated under SMCRA. Instead, it would be more feasible to conserve organic matter by leaving stumps and other large organic elements in the field to aid soil fertility, improved soil structure, and ECM reservoirs (Skousen et al., 2011).

In summary, this study demonstrated ECM community composition changes in response to selection pressures imposed by soil conditions and neighboring vegetation. The soil conditions were drivers of vegetation, which ultimately influenced resulting ECM genera that could form symbiosis under the varying soil chemistry. Within the abandoned areas, rock overburden mixed with mineral soils devoid of vegetation were similar to primary succession that selected stress tolerating ericoid fungi that could persist under nutrient and organic impoverishment. In contrast, plots adjacent to existing woody vegetation harbored ECM fungi that may exist as vegetative mycelium, able to compete under lower nutrient levels. This provides reservoirs of inoculum on existing vegetation (Bauman et al., 2012). Existing fungi can incorporate establishing seedlings into a common mycelial network, thereby facilitating their establishment in the abandoned mine sites. In contrast, the grasslands that had been reclaimed with topsoil modeled secondary succession. ECM genera can rapidly disperse into a site when restoration introduces an ECM host plant (Bauman et al., 2013). Disturbed soils may select for fungi that are high spore producers, better dispersers, and have the ability to colonize roots from spores under slightly higher nutrient levels.

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