Journal of Applied Ecology

Journal of Applied Ecology 2013, **50,** 721–729 doi: 10.1111/1365-2664.12070

Soil preparation methods promoting ectomycorrhizal colonization and American chestnut *Castanea dentata* establishment in coal mine restoration

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Summary

1. The objective of this research was to evaluate soil subsurface methods that may aid in seedling establishment and encourage root colonization from a diverse group of ectomycorrhizal (ECM) fungi during restoration projects.

2. American chestnut *Castanea dentata* Marsh. Borkh. and backcrossed chestnuts seedlings were planted on a reclaimed coal mine site in central Ohio, USA. Roots from chestnut seedlings planted in the plots that were cross-ripped, plowed and disked, or a combination of treatments were sampled for ECM fungi and compared with control plots. The presence and identification of native ECM were determined by fungal DNA sequencing of the internal-transcribed (ITS) region.

3. After two growing seasons, mechanical soil treatments resulted in seedlings with significantly more ECM species when compared to seedlings grown in the control plots ($P < 0{\cdot}0001$). A nonmetric multidimensional scaling ordination followed by a permutational MANOVA confirmed significant dissimilarities in community composition between the control and mechanically treated plots ($F = 0{\cdot}24$, $P = 0{\cdot}015$).

4. Ectomycorrhizal root colonization was significantly higher on the chestnut seedlings sampled from the mechanically treated plots when compared to the control plots ($F = 10{\cdot}63$, $P < 0{\cdot}0001$). Differences did not exist among the three mechanical treatments. There was also a significant increase in above-ground seedling growth in the plots that were treated with a surface soil method ($F = 15{\cdot}72$, $P < 0{\cdot}0001$). It is not clear whether ECM activity was the driver of plant growth; regardless, both are strong indicators of healthy tree establishment.

5. *Synthesis and applications.* This study illustrates that the use of soil subsurface methods increased ectomycorrhizal (ECM) activity and seedling growth. Employing methods that encourage the root colonization by beneficial ECM and promote healthy seedling establishment may aid the long-term survival of chestnuts in restoration projects. This can be applied to other hardwood seedlings used in reforestation in soils compacted after anthropogenic disturbances.

Key-words: Appalachian Regional Reforestation Initiative, ecological restoration, ectomycorrhizal fungi, Forestry Reclamation Approach, plant–fungal interactions, soil subsurface treatments

Introduction

Succession is the change in a plant community over time and its progression is of great concern when managing the recovery of landscapes after coal mining. Applying proper methods in the early stages of reclamation promotes a natural rate of forest stand recovery following large-scale operations (Groninger *et al.* 2007). Hardwood seedling recruitment leading to canopy formation has been reported to occur within 15–20 years after initial mine reclamation (Zipper *et al.* 2011). In contrast, reclamation methods enforced by The Surface Mining Control and Reclamation Act (SMCRA) of 1977 have not resulted

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in forest succession. Heavy equipment used to grade lands to the original contour and the use of exotic species as cover crops have resulted in severely compacted soils dominated by non-native herbaceous canopies (Torbert & Burger 2000). In addition, the native soil microbial community has been significantly disturbed resulting in low biomass and activity (Bradshaw 1984). These microbes play primary roles in nutrient cycling, soil structure and biological interactions facilitating plant community establishment (Bever 2002).

Ectomycorrhizal (ECM) fungi are the primary microbial components essential for tree establishment and survival. The presence of these fungal species is required for many forest tree taxa including Betulaceae, Fagaceae, Pinaceae and Salicaceae (Smith & Read 2008). The mycorrhizal symbiosis enhances the seedling's ability to absorb water and nutrients, tolerate heavy metals and low pH, and protect against root pathogens (Marx 1972; Danielson 1985). The formation of ECM roots increases seedling vigour when resources are limited and enhances the competitive ability of seedlings during establishment (Perry *et al.* 1989; Nara 2005). In return, the fungus receives carbon from the host plant in the form of photosynthates. This symbiotic association greatly aids in the amelioration of stressful environmental conditions and in the regeneration of plant communities following disturbances (Izzo, Nguyen & Bruns 2006).

Disturbances such as coal mining cause a significant decline in available ECM propagules by removing host plants, increasing soil compaction and contaminating natural areas with heavy metals and coal spoil (Iordache, Gherghel & Kothe 2009). The severe decline of these microbes may contribute to limited woody tree and shrub survival on these former mine sites (Marx 1991). Additionally, the shading coupled with the densely packed rooting zone imposed by the invasive plant species used as cover crops may limit native tree recruitment (Ashby 1997; Holl *et al.* 2000). The persistence of these non-native forbs greatly reduces the abundance of pioneer shrub and tree species that support the ECM fungi required to facilitate the succession of later arriving woody natives (Amaranthus & Perry 1994). Most ECM fungi do not persist without the presence of their host plants, and low ECM propagules favour non-ECM plant species. Therefore, reclaimed mine sites dominated by non-ECM plant species may be difficult to return to the historic forest conditions (Amaranthus & Perry 1994).

Mechanical soil treatments such as deep ripping have been proposed by the Appalachian Regional Reforestation Initiative to accelerate succession (Skousen *et al.* 2009; Burger & Evans 2010). Using the Forestry Reclamation Approach (FRA), Zipper *et al.* (2011) recommends cross-ripping for loosening soil (>1 m depth) and increasing air, water and nutrients available to woody tree roots. Previous studies have shown ripping to increase the size of the root system aiding in seedling establishment (Cleveland & Kjelgren 1994; Ashby 1997). However, it is not known how these mechanical subsurface treatments affect native ECM fungi and their interactions with their plant host. Although mechanical treatments may disturb existing mycelium networks, they may promote the initial synthesis and establishment of early successional ECM species. Soil disturbance may also aid in the recruitment of species by creating a medium for windblown spores. Small-scale root disturbances by mechanical methods mimic natural soil disturbances (burrowing, decomposition and tree fall) and allows for greater niche differentiation and ECM species changes over time (Bruns 1995).

This study evaluated the effects of various subsurface treatment methods on the ECM root colonization and community composition on American chestnut *Castanea dentata* Marsh. Borkh. In addition to pure American chestnuts, backcrossed chestnuts (*C. dentata x C. mollissima*) were used. These seed lines were selected due to their American chestnut morphology and adequate field resistance to chestnut blight (Burnham 1988). The fast growth rate coupled with quality timber makes American chestnut a desired species for use in reforestation projects. Previous studies have reported chestnut to establish on former coal mined sites (McCarthy, Bauman & Keiffer 2008). In addition, areas of Appalachia impacted by surface coal mining (including the site used in this study) correspond to the historic range of chestnut. The ultimate goal of this research is to develop planting methodologies that would maximize the effectiveness of ECM symbiosis that may aid in the establishment of an ECM woody host plant.

Materials and methods

EXPERIMENTAL DESIGN

This study used one-year-old, bare root chestnut seedlings. Bare root seedlings are commonly used for tree planting in reforestation projects in south-eastern Ohio. In the spring of 2006, 1200 American chestnuts were sown at the State Nursery in Marietta, Ohio by the Ohio Department of Natural Resources. The 1200 seeds were comprised of the following: 400 pure American chestnuts *C. dentata*, 400 backcrossed chestnuts BC_2F_1 (backcrossed to create a progeny that is 7/8 *C.dentata* and 1/8 *C. mollissima*) and 400 backcrossed chestnuts BC_3F_1 (backcrossed to create a progeny that is 15/16 *C.dentata* and 1/16 *C. mollissima*). All seedlings were originally inoculated at the Marietta tree nursery with the ectomycorrhizal (ECM) fungus *Pisolithus tinctorius* and observed for root colonization (J. Hopkins pers. comm.). The seedlings were nursery grown for one year and lifted as bare root seedlings in the spring 2007.

The field site used for this study is located in the Tri-Valley Wildlife Management Area, Muskingum County, central Ohio, USA (40° 11' 32" N, 81° 98' 35" W). This coal surface mined site was reclaimed under SMCRA in 1978 and is currently vegetated with the original species used for reclamation (*Festuca* sp. and *Lespedeza* sp.) with trace patches of native ragweeds (*Ambrosia* sp.) and goldenrods (*Solidago* sp.). Small pockets of forest comprising primarily of *Quercus, Pinus* and *Acer* species were left undisturbed at the time these lands were mined (McCarthy,

Bauman & Keiffer 2008). This area received an average of 99 cm 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 2009).

Three experimental blocks, each containing the control and three soil treatments, were set-up prior to planting in the spring of 2007. Each block was comprised of graminoids and forbs (without existing trees) and measured 73 × 36 m. Four 18 × 36 m treatment plots were contained within each block (Fig. 1). In each block, the following treatment plots were established: (i) a control left undisturbed (C), (ii) a plot cross-ripped at a depth of approximately 1 m on 2-m cross spacing by a D-6 dozer with a 1-m steel ripper bar attachment (R), (iii) a plowed and disked plot installed by a conventional tractor (PD), and (iv) a ripped + plowed and disked plot (RPD). A 15-m unplanted buffer zone was maintained between treatments in each block.

Soil cores were collected to analyse soil chemistry and bulk density. No differences existed among blocks. Soil pH ranged from 5·4 to 5·7. Soil texture averaged 61% sand, 23% silt and 16% clay. Organic matter and cation-exchange capacity (CEC) averages were 1·3% and 7·5 CEC, respectively. Mean values for soil nutrients were: aluminium, 3·5 ppm; calcium, 720 ppm; potassium, 78 ppm; magnesium, 182 ppm; manganese, 3·75 ppm; nitrogen, 2 ppm; and phosphorus, 8 ppm. Measurements of bulk densities of soil per treatment showed decreases. Bulk densities (mg m^{-3}) were as follows: R plots from 1·65 to 1·48, PD plots from 1·63 to 1·47 and RPD plots from 1·70 to 1·59. The control plots averaged 1·64 mg m^{-3}.

A total of 1200 chestnut seedlings were planted in the treatment plots (12 plots, 100 seedlings per plot in a 1:1:1 seedling type ratio) as bare rootstock in April 2007 at a spacing of 2·15 × 2·15 m (Hebard 2005). The root system of each seedling was dipped in TerraSorb gel prior to planting. Two fertilizer pellets (20-10-5) were put in each hole, and the seedling was backfilled with original soil. A 1 × 1 m weed mat was used around each seedling to prevent herbaceous competition and a 1·5-m tall chicken wire cage was installed to prevent browse.

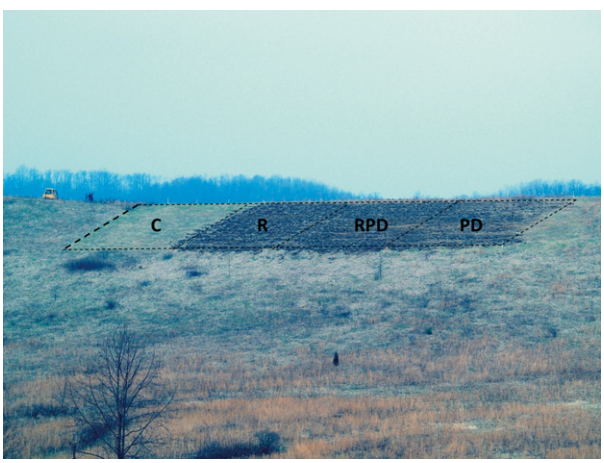

Fig. 1. Field plot design: Each block installed consisted of four treatments: 1) a control left undisturbed (C), 2) a plot cross-ripped at a depth of approximately 1 meter (R), 3) a ripped + plowed and disked plot (RPD), and 4) a plowed and disked plot (PD). Each block is 73 × 36 m, each treatment 18 × 36 m. A 15-m unplanted buffered area was left between each treatment. Each block consisted of 400 chestnut seedlings and was replicated three times for a total of 1200 chestnuts.

At the time the bare root seedlings were planted, 150 additional American chestnut seeds were surface sterilized with 10% bleach solution and planted by direct seeding. An 18 × 8 m plot was included in each RPD treatment plot. There were three plots total and each consisted of 50 chestnuts sown in rows of five on a 1 × 1 m spacing. This was performed to detect and identify ECM native to the field site. This clarified the ECM survey and allowed for determination of those fungi that were transplanted in with the bare root seedlings.

DATA COLLECTION

In April 2007 (before bud break) and October 2008 (end of second field season), growth parameters (basal stem diameter and seedling height) were recorded. Height was measured using a meter stick from soil level to the tip of the main stem. Basal diameter measured 3 cm above the root collar was recorded by using a digital caliper.

After 6 months (October 2007), a total of 60 pure American chestnuts planted as bare root seedlings were selected for root sampling (representing all treatment plots). A similar analysis was under taken after 18 months (October 2008). Roots from 75 pure American chestnuts and 45 BC_2F_1 backcrossed chestnuts (7/8th American) planted as bare root seedlings were investigated. Trees were first randomly selected by a random number generator, and then, two criteria were imposed: 1) selected seedlings were not neighbouring (to avoid root system overlap and ensured independence) and 2) The BC_3F_1 genotype (1/16th American) was not disturbed by sampling and only a small subset of the BC_2F_1 were sampled. Therefore, pure American chestnuts were again randomly selected to compensate for the decision made as a protective measure to ensure undisturbed field testing for the backcrossed seed types. However, this caused a sampling bias favouring the sampling of pure American chestnut.

Three soil cores (10 cm × 10 cm × 10 cm) were collected from the drip line of each seedling. Samples were pooled among cores, per seedling. Roots were stored on ice until returned to the laboratory where they were washed and transferred into a Petri dish containing sterile water. Two hundred and fifty root tips were randomly selected from each seedling and viewed under a dissecting microscope for the presence of a fungal sheath (180 samples, 45,000 root tips). Each ECM tip was sorted into one of the nine morphotypes (Fig. 2) based on their surface colour, texture, emanating hyphae and rhizomorphs (Nara *et al.* 2003). Two root tips of each morphotype per seedling were selected for DNA extracting and sequencing (308 bare-root tips total). A 3-mm section of the root tip was transferred to a microcentifuge tube and stored at -70° C until DNA extraction. In addition, 75 chestnuts that were directly seeded were selected for destructive sampling after 12 months and processed as described previously. Another 75 seedlings (by direct seeding) were selected after 18 months. Of these, 174 root tips representing all ECM morphotypes from chestnuts that were directly seeded were selected for DNA extracting and sequencing.

DNA SEQUENCING AND ECM IDENTIFICATION

Fungal DNA was extracted from the ECM root tips. Fungi were identified by DNA sequencing of the internal transcribed spacer

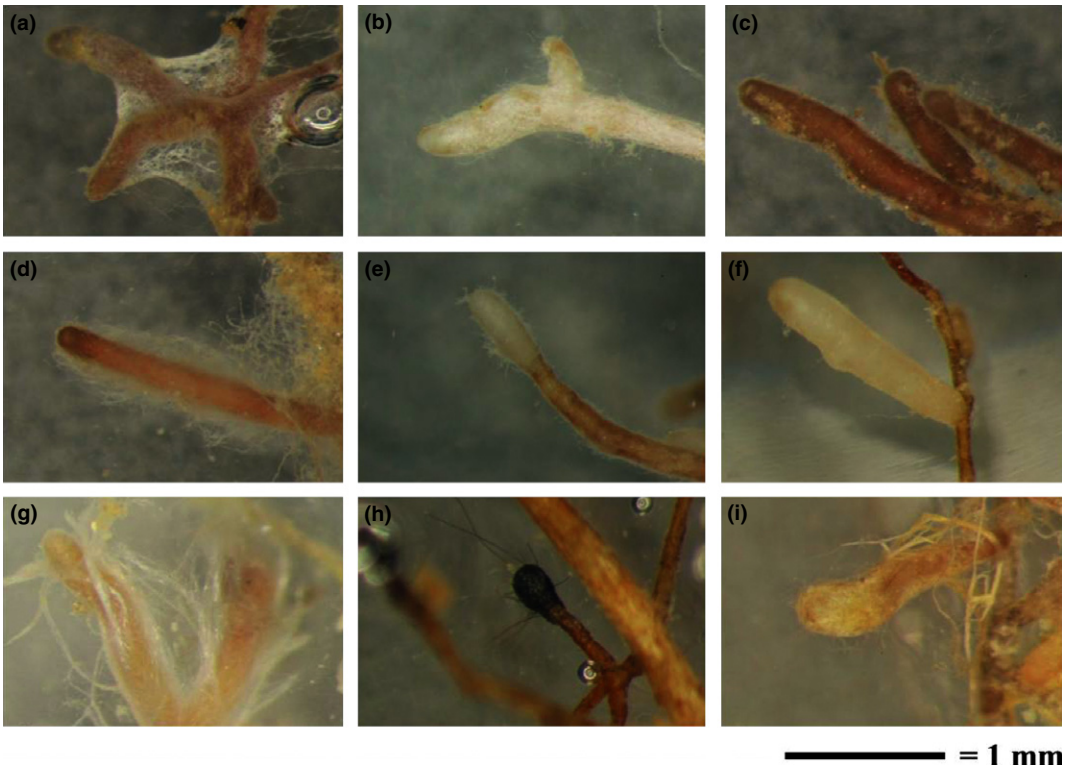

Fig. 2. Photographed (45x) ectomycorrhizal (ECM) morphotypes sampled from root tips from *C dentata*. Panels display fungal species matched to vouchered GenBank sequences: (a) *Scleroderma* sp. 1, (b) *Scleroderma* sp. 2, (c) Unknown ECM 1, (d) *Hebeloma* sp., (e) Thelephoraceae, (f) *Tomentella* sp., (g) *Cortinarius* sp., (h) *Cenococcum* sp. and (i) *Pisolithus* sp.

(ITS) region. For this, root tips were first grouped by similar morphology and one root tip belonging to each morphotype was used. The DNA was extracted by manufactures guidelines using the QIAgen DNeasy® Plant Mini Kit. Primers ITS1-F (5' cttggtcatttaggaagtaa 3') and ITS4 (5' tcctccgcttattgatatgc 3') were used to amplify internal transcribed spacer sequences (ITS) PCR (Gardes & Bruns 1993) and analysed by electrophoresis. Sequencing was performed using 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 fungus found on roots, ITS sequences from samples were compared with those in the GenBank using the BLAST search (Altschul *et al.* 1997). The genera of the fungi reported in this study were based on the best matches of those in the GenBank with a > 97% ITS sequence similarity as a threshold. A total of 324 sequences that were generated were matched to vouchered fungal genera in the NCBI database and used in the subsequent analysis.

STATISTICAL ANALYSES

Description of ECM diversity was quantified by species richness, Shannon–Weiner diversity index and Simpson's index of diversity based on ECM tip counts for each morphotype. A nonmetric multidimensional scaling (NMDS) ordination followed by a permutational multivariate analysis of variance was used to determine whether chestnut seedling type, sampling time, or soil treatments influenced ECM species composition sampled on chestnuts planted as bare root seedlings. Bray–Curtis dissimilarities were employed with maximum number of random starts set at 100 with $k = 2$ stress value. A permutational multivariate analysis of variance was used to test for significant differences among the soil treatments using the Vegan package of R (R Development Core Team 2009).

ECM colonization per treatment was assessed by taking the percentage (#ECM tips/250) of ECM colonized root tips from chestnuts planted as bare root seedlings ($n = 180$) after two field seasons. Arcsine square root transformation was used to control for unequal variances. Growth was derived from the difference between the original measurements of seedling height and basal diameter and the final measurements at the end of the second field season. A volume index (height cm × basal diameter cm^2) was used to estimate the volume of each chestnut seedling using a log + 1 transformation. Differences in % ECM colonization and seedling volume were statistically determined by using a one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test.

Results

ECM SPECIES SAMPLED FROM BARE ROOT AND DIRECT SEEDED CHESTNUTS

A total of 9 distinct morphotypes were detected from the chestnut seedlings while screening under the dissecting microscope (Fig. 2). Six additional ECM species (pictures not available) were detected through DNA sequencing, which identified a total of 15 ECM species (Table 1). Two

Table 1. Ectomycorrhizal (ECM) fungal species sampled from chestnut root tips ranked by relative abundance generated from root tip count data. Roots were collected from 180 chestnut bare root seedlings (Total) from the four treatment plots: control (C), plow and disk (PD), ripped (R), and ripped + plow and disk (RPD). In addition, 150 chestnuts planted by direct seeding (Seed) are also included. This table reports fungal colonization from 324 sequences that were matched to vouchered ECM sequences available in GenBank. The GenBank sequence accession numbers assigned to the ECM fungi described in this study are reported in the last column

ECM species	Total	C	PD	R	RPD	Seed	Accession
Hebeloma sp.1	0·31	0·57	0·36	0·21	0·28	0	GU246983
Hebeloma sp. 2	0·20	0·09	0·14	0·27	0·23	0	GU246984
Cortinarius sp. 1	0·16	0	0·15	0·11	0·24	0	GU246986
Scleroderma sp. 1	0·09	0	0·08	0·15	0·05	0·61	GU246989
Thelephora sp.	0·07	0·12	0·10	0·06	0·05	0·07	GU246993
Unknown ECM 2	0·04	0	0·04	0·09	0	0·01	GU246997
Hebeloma sp. 3	0·03	0·01	0	0·05	0·01	0·01	GU246985
Laccaria sp.	0·03	0·04	0·06	0·04	0·01	0·01	GU246994
Unknown ECM 1	0·02	0	0	0	0·01	0	GU246996
Scleroderma sp. 2	0·01	0	0	0	0·02	0·13	GU246990
Cortinarius sp. 2	0·01	0·15	0	0	0	0·01	GU246987
Pisolithus sp.	0·01	0	0·04	0	0	0	GU553367
Tomentella sp.	0·01	0	0·02	0	0	0·03	GU246992
Cenococcum sp.	0·01	0·01	0·01	0·02	0·10	0·11	GU246995
Thelephoraceae	0	0	0	0	0	0·01	GU553376
# seedlings inspected	180	45	44	44	47	150	
# of root tips inspected	45,000	11,500	11,000	11,000	11,750	37,500	
# root tips with ECM	15,060	1,202	4,477	4,197	5,184	13,240	
Proportion of ECM	0·33	0·10	0·41	0·38	0·44	0·35	

of the ECM sequences did not match to known sequences in the GenBank and are reported as Unknown ECM 1 and 2. No differences existed between pure American and BC_2F_1 seedlings ($F = 1{\cdot}5$, $P = 0{\cdot}14$) with regard to ECM community composition. Therefore, bare root seedling data were pooled for the subsequent NMDS analyses.

The most common species found on the bare root seedlings in the field were *Hebeloma* sp. 1, *Hebeloma* sp. 2, and *Cortinarius* sp. 1 (Table 1). *Scleroderma* sp. 1 and *Thelephora* sp. were found moderately frequently throughout the study. The remaining rare species consisted of the Unknown ECM sp. 2, *Hebeloma* sp. 3, *Laccarria* sp., the Unknown ECM sp. 1, *Scleroderma* sp. 2, *Cortinarius* sp. 2, *Pisolithus* sp., *Tomentella* sp. and *Cenococcum*.

Only 10 fungal species were detected from chestnuts planted as direct seeds (Table 1). This sampling significantly differed from those planted as bare root seedlings ($F = 3{\cdot}86$, $P = 0{\cdot}005$). The more abundant species found were *Scleroderma* sp. 1 and *Scleroderma* sp. 2, while *Cenococcum* sp. and *Thelephora* sp. were found moderately throughout. Others that were less common consisted of *Tomentella* sp., *Hebeloma* sp. 3, *Cortinarius* sp. 2, *Hebeloma* sp. 2, Unknown ECM sp. 2, and an uncultured species within the family Thelephoraceae.

SOIL TREATMENT EFFECTS ON ECM COMMUNITY AND ROOT COLONIZATION

The average number of ECM species was significantly greater in the mechanically treated plots when compared to the control; 7 species recorded in the mechanically treated plots compared to an average of 4 ECM species in the control plots ($P < 0{\cdot}05$; Table 2). Diversity indices also revealed a similar pattern. Shannon–Weiner diversity indices in the treated plots ranged from 1·43 to 1·54 compared with 1·01 in the control plots (Table 2). Although species diversity was higher in the mechanically treated plots, this was not significant. Simpson's Diversity, which ranged from 0·66 to 0·72 in the treated plots as opposed to 0·54 in the controls, also was not statistically different.

Table 2. Mean species richness, Shannon–Weiner diversity index, and Simpson's diversity index (1-D) ± 1 SD from chestnuts sampled among the four treatments: control (C), plow and disk (PD), ripped (R) and ripped + plow and disk (RPD) ($n = 12$). Sample size (n) refers to the number of blocks (60 seedlings per block) sampled per treatment. Different letters indicate significant differences at $P < 0{\cdot}05$ determined by Tukey's HSD

Treatment	*N*	Ave. Species Richness	Shannon–Weiner	Simpson's Diversity
C	3	$4{\cdot}3 \pm 0{\cdot}58^{b}$	$1{\cdot}01 \pm 0{\cdot}18^{a}$	$0{\cdot}54 \pm 0{\cdot}13^{a}$
PD	3	$7{\cdot}3 \pm 2{\cdot}31^{a}$	$1{\cdot}54 \pm 0{\cdot}30^{a}$	$0{\cdot}72 \pm 0{\cdot}10^{a}$
R	3	$7{\cdot}3 \pm 2{\cdot}08^{a}$	$1{\cdot}43 \pm 0{\cdot}59^{a}$	$0{\cdot}66 \pm 0{\cdot}24^{a}$
RPD	3	$7{\cdot}3 \pm 2{\cdot}31^{a}$	$1{\cdot}48 \pm 0{\cdot}36^{a}$	$0{\cdot}68 \pm 0{\cdot}16^{a}$

Of all collected samples, the first dimension of the ordination was negatively associated with *Cortinarius* sp. 2 (Cort2) and positively associated with *Scleroderma* sp. 2 (Scl2). The second dimension was negatively associated with *Tomentella* sp. (Tom) and positively associated with *Hebeloma* sp. 2 (Heb2; Table 3). Overlapping convex hulls illustrated similarity in ECM community composition among soil treatments; the control plots appeared separately in the ordination (Fig. 3). A permutational MANOVA confirmed significant dissimilarities in ECM community between the control and mechanically treated plots ($F = 0{\cdot}24$, $P = 0{\cdot}015$). Species scores of NMDS coordinates showed strong associations among ECM species and ordination dimensions (Table 3). Cort2 appeared strongly related to the control plots, whereas *Scleroderma* sp. 2 (Scl2), *Tomentella* sp. (Tom) and Heb2 were strongly correlated with the mechanically treated plots (Fig. 3; Table 3). No differences existed between sampling season (spring and fall) on the chestnuts that were directly seeded ($F = 1{\cdot}36$, $P = 0{\cdot}28$).

When ECM root colonization was compared across the treatments, percentage colonization was statistically higher on the chestnut roots sampled from the mechanically treated plots than the controls ($F = 10{\cdot}63$, $P < 0{\cdot}0001$). No differences existed among the subsurface treatment methods: PD (42%), R (40%), and RPD (45%). All were significantly higher than the C (13%) plots (Fig. 4). With regard to above-ground seedling growth, a similar trend was apparent. Seedling volume (height cm * basal diameter cm^2) was significantly higher for chestnuts measured in the mechanically treated plots ($F = 15{\cdot}72$, $P < 0{\cdot}0001$). Again, no differences were detected when methods were compared: PD (23·26 cm^3), R (30·11 cm^3), and RPD (38·18 cm^3). All were significantly higher than the seedlings in the control (10·14 cm^3) plots (Fig. 4). The ECM colonization by mechanical treatment interaction was significant for seedling volume index cm^3 ($F = 4{\cdot}29$, $P = 0{\cdot}006$).

Table 3. Species scores (coordinates) of nonmetric multidimensional scaling (NMDS) dimensions used to plot species on ordination. Strong associations between ectomycorrhizal (ECM) species and NMDS dimensions are shown in bold

ECM Species	Dim1	Dim2
Unknown sp. 1 (Un1)	0·46777544	−0·03724253
Unknown sp. 2 (Un2)	0·26283783	−0·32269375
Cortinarius sp. 2 (Cort2)	**−0·84236024**	0·22229829
Cortinarius sp. 1 (Cort1)	0·28285894	0·01324338
Laccaria sp. (Lac)	0·33661071	0·14928644
Pisolithus sp. (Pis)	0·52713717	−0·31390793
Hebeloma sp. 1 (Heb1)	0·18554867	−0·26631058
Hebeloma sp. 2 (Heb2)	0·39031097	**0·41189741**
Hebeloma sp. 3 (Heb3)	0·24585326	0·0808811
Cenococcum sp. (Cen)	0·21119263	−0·17648915
Scleroderma sp. 1 (Scl1)	0·35614304	−0·02711169
Scleroderma sp. 2 (Scl2)	**0·55132987**	0·38893662
Thelephora sp. (Thel)	0·27126195	−0·07455187
Tomentella sp. (Tom)	0·09156124	**−0·57198265**

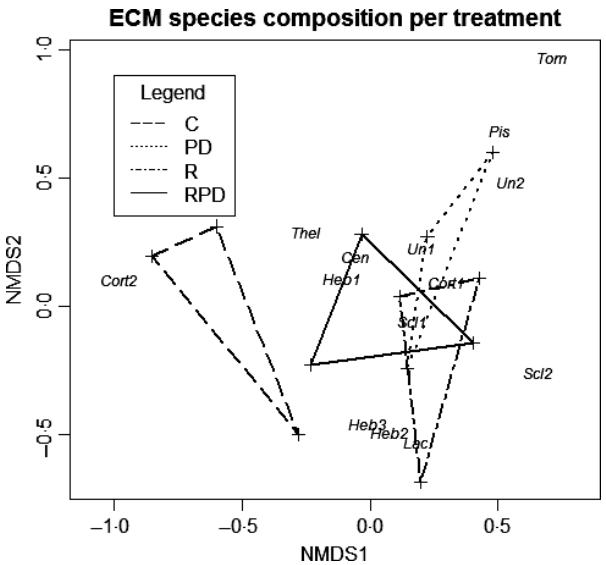

Fig. 3. Nonmetric multidimensional scaling (NMDS) ordination comparing ectomycorrhizal (ECM) fungal species sampled among the soil treatments (C = control, PD = plowed and disked, R = ripped, and RPD = ripped + plowed and disked). There was a significant difference in ECM community composition when mechanical treatments were compared to the control plots (MANOVA, $F = 0{\cdot}24$, $P = 0{\cdot}015$). Crosses (+) represent the sites sampled and are shown with abbreviated ECM species names annotated on the ordination. Convex hulls outline each treatment.

Discussion

The results of this study indicated that: 1) ECM species richness increased in the mechanically treated plots, 2) ECM community composition was influenced by mechanical subsurface treatment; however, no significant differences were observed among the different soil treatment methods, 3) there were differences in ECM composition between chestnuts planted as bare root seedlings to those directly seeded, and 4) mechanical soil treatment greatly improved ECM root colonization and seedling growth. We did not find a difference in ECM community composition between the pure American and the BC_2F_1 chestnut seeding types. This was not unexpected because ECM communities are generally similar on host plants with related taxonomic groups (Ishida, Nara & Hogetsu 2007). Collectively, this study reports 15 ECM species on chestnut seedlings at the end of two growing seasons.

Despite the initial inoculation of *Pisolithus*, this fungus was rarely sampled in our survey. Though this fungus forms ECM with chestnut (Bauman, Keiffer & Hiremath 2012), it is described as a poor competitor and is commonly displaced by other ECM fungi shortly after field planting (McAfee & Fortin 1988). The most abundant fungi sampled from bare root chestnuts were *Hebeloma* sp. 1, *Hebeloma* sp. 2 and *Cortinarius* sp. 1. These fungi were not detected on seedlings sown as seed. They may have been transplanted along with the bare root seedlings and their abundance facilitated by both the mechanical

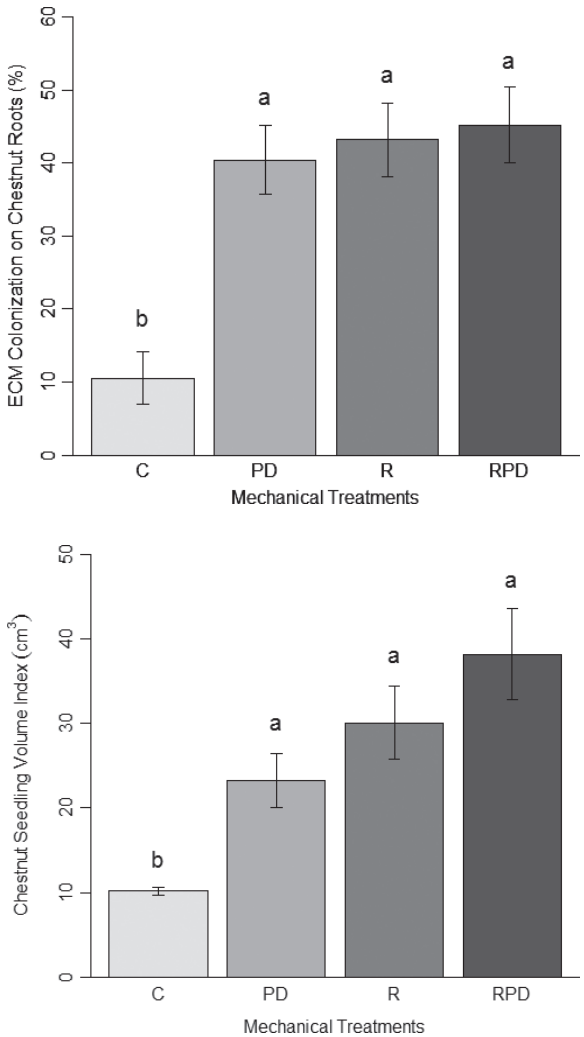

Fig. 4. Comparison of ectomycorrhizal (ECM) root colonization (%) and above-ground seedling growth (cm^3) of chestnuts among the treatment plots (C = control, PD = plowed and disked, R = ripped, RPD = ripped + plowed and disked). The top panel illustrates a significant increase in ECM roots on chestnut seedlings in the mechanically treated plots (PD, R, RPD). The bottom panel shows a similar trend, plant growth is significantly increased in the plots that were mechanically treated. Error bars are ± 1 SE, bars sharing common letters do not significantly differ at $\alpha = 0{\cdot}05$ determined by Tukey's HSD.

treatments and soil conditions at this site. *Scleroderma* were reported on only 9% of the chestnut seedlings planted as bare root seedlings, but was the most abundant ECM genus (74%) on those planted as seeds. This may have been due to the difference in age between chestnuts sown as seed and those planted as one-year-old seedlings. Alternatively, colonization of native *Scleroderma* species may have been inhibited by the initial root presence of *Hebeloma* and *Cortinarius* (Garbaye & Churin 1997; Kennedy, Peay & Bruns 2009). Although ECM are not well adapted to survive mining operations, *Scleroderma* can re-invade within a few years given the presence of ECM host plants and the availability of nearby propagule sources (Allen, Jasper & Zak 2002; Bauman, Keiffer & Hiremath 2012).

Therefore, the 10 species found on chestnuts from direct seeding may best represent the ECM fungi available to chestnut in these grasslands. However, the authors acknowledge that it is likely that a few species may have been missed during sampling. In addition, one host that was relatively even aged can also cause a bias when surveying ECM. Regardless, when comparing American chestnut, this is a small number when compared to 38 species of ECM fungi reported on one-year-old chestnut seedlings in forested sites (Dulmer 2006). Our study is consistent with reports of low ECM diversity in non-ECM habitats like grasslands and other soils recovering from anthropogenic disturbances (Jasper 2007). When ECM community composition was compared among treatments, mechanically treated plots differed significantly from the untreated control plots. Mechanical soil treatments may have increased ECM species richness by improving soil contact for windblown spores (Jones, Durall & Cairney 2003) and by mixing strata layers, which increased the number of fungal species in the rooting zone (Tedersoo *et al.* 2003).

There was a clear increase in both ECM root activity and seedling growth in the mechanically treated plots. Although it is well recognized in the literature that ECM fungi have beneficial effects on plant growth, it was not clear whether ECM activity was the driver of plant growth, or if, plant fitness contributed to ECM colonization. Chestnuts seedlings that performed poorly in compacted soils may not have been able to produce enough carbon to support its fungal symbiont. Carbon limitation as a mechanism behind the decrease in ECM colonization has been previously described (Saikkonen *et al.* 1999; Swaty *et al.* 2004). In addition to decreasing carbon allocation, soil compaction has a direct negative effect on functional mycorrhiza formation. Decreased soil porosity, characteristic of SMCRA landscapes, may inhibit the diffusion of signalling molecules such as host plant root exudates and fungal auxins that initiate the primary synthesis of mycorrhizal roots (Podila 2002). Compacted soils, such as those in the untreated control plots, equate with high bulk densities that has been shown to hinder ECM hyphal growth (Skinner & Bowen 1974) and root colonization in the field (Amaranthus *et al.* 1996).

Other modifications in soil structure, organic composition and interactions with other microbes (i.e. arbuscular mycorrhiza (AM), plant pathogens) are factors that influence plant and ECM interactions. Competition between AM and ECM may have accounted for limited root colonization contributing to the low survival of pioneer shrub and tree species (Amaranthus & Perry 1994). Interactions with soil pathogens, specifically in soils that are compacted with poor drainage have been noted. Chestnut seedlings on compacted mine soils in southern Appalachian regions of North America reported a greater incidence in root disease caused by *Phytophthora* sp. and a significant reduction in ECM root colonization (Rhoades *et al.* 2003). ECM formation provides a physical barrier

coupled with antimicrobial secondary metabolites that inhibit infection by root pathogens (Branzanti, Rocca & Pisi 1999; Whipps 2004). However, this relationship is dependent on drier soils and soil structural conditions that promote healthy root growth (Marx 1972; Cleveland & Kjelgren 1994; Ashby 1997).

Lower ECM colonization correlates with lower ECM inoculum levels in the soil leading to conditions less conductive for the succession of ECM plants hosts (Swaty *et al*. 2004). Similar to our study, mechanical soil treatments have been reported to modify the soil structure and drastically disturb the grassland canopy, which encouraged seedling establishment (Holl *et al*. 2000; Hooper, Legendre & Condit 2005). The disturbance imposed by the mechanical treatment, coupled by the successful establishment of an ECM host, may play a pivotal role in facilitating the natural successional trajectory leading to timely woody recruitment. This may facilitate a shift from non-native herbaceous plants to woody native ECM trees and shrubs that are able to compete under changing light conditions. Therefore, it can be hypothesized that the establishment of woody trees and shrubs that limit light availability may impose a high energetic cost to maintain the N-fixing (Gutschick 1981) and/or AM fungal symbionts. As forest succession progresses and light becomes the limiting resource in this system, a shift from non-ECM plant species to those obligatory to ECM fungi will occur (Janos 1980; Reynolds *et al*. 2003; Smith & Read 2008).

Employing methods outlined by the FRA encouraged the formation of ectomycorrhizas and promoted healthy seedling growth in the early years of establishment. Developing management strategies that enhance soil microorganism activity is integral to the recovery of soil properties necessary for a resilient landscape (Bradshaw 1984; Allen, Jasper & Zak 2002). Maximizing growth and symbiotic interactions may aid in the long-term survival and recruitment of other ECM plants. Chestnut is a fast grower (Jacobs & Severeid 2004) and under proper planting methods will produce chestnut seed early in its establishment (approximately 5–7 years; J.M. Bauman Per. Obs.). Yearly masts produce a consistent protein source that benefits wildlife and attracts seed dispersers that increase the recruitment of native trees and shrubs in these restored sites.

Planting methods that promote beneficial ECM fungal interactions with chestnut can be applied more broadly to native hardwood seedlings used in restoration. The FRA recommends a suitable planting medium, a deep rooting zone (>1 m deep), valuable tree species, appropriate herbaceous vegetation and proper planting methods to enable a faster return of native forests (Zipper *et al*. 2011). It is hypothesized that established trees will add organic matter to the soil, attract seed-carrying wildlife and provide inoculum for incoming species leading to forest recovery by natural succession. Field assessments that measure establishment and plant vigour will contribute to the development of restoration techniques for land managers in Appalachia regions of North America. These protocols can then be expanded to address land-use and restoration policies in other regions where active mineral extraction creates disturbances to native forests.

Acknowledgements

This study was supported by National Technology and Transfer funds from the US Department of Interior (Office of Surface Mining), part by a Joint Research Venture grant 06-JV-11242300-093 from the US Forest Service and by the Academic Challenge Grant from Miami University's Department of Botany. We thank Steve Castellano, Dr. Erin Douglas, Ryan Homsher, Keith Gilland, Aaron Kennedy, Kirsten Lehtoma, Corie McCament, Jennifer Seabaugh and Steve West.

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Received 25 September 2012; accepted 8 February 2013
Handling Editor: Paul Kardol