FINAL REPORT

# Mine Land Reclamation and American chestnut Restoration: Bringing Technologies Together

Principal Investigator:
Brian C. McCarthy, Ph.D.

Project Manager:
Corinne L. McCament, M.S.

Department of Environmental and Plant Biology
Ohio University
Athens, OH 45701-2979
E: mccarthy@ohio.edu
T: 740-593-1615
F: 740-593-1130

In cooperation with
Carolyn H. Keiffer, Ph.D. and Jenise Bowman, M.S.
Department of Botany
Miami University
Oxford, OH 45056

Prepared for:
USDI OSMRE

January 30, 2008

## Introduction & Objectives

The goal of this project was implemented primarily to address OSMRE special interest topics of (1) soil development on reclaimed mines, (2) alleviating soil compaction on reclaimed soils, and (3) wildlife conservation & reforestation.

Since seedling growth is often most stunted by soil compaction, this topic may be the most important of this study (Burger et al. 2005). Soil that has been part of the typical reclamation process is usually heavily compacted and has an alkaline or acidic pH, making it unsuitable for tree survival and growth (Torbert and Burger 1990). It also has greater bulk density, decreased porosity, permeability and moisture-holding capacity compared to it pre-mined state (Bussler et. al. 1984). In addition to having adverse soil conditions, reclaimed mine land is also planted with aggressive grass species that make it difficult for succession to proceed past a grassland landscape to a more productive forest (Sprouse 2004). In essence, succession is arrested and unable to proceed to provide the forest cover that existed prior to mining. Previous plantings in these reclaimed mine sites resulted in high seedling mortality and low long-term survivorship (Rathfon et. al. 2004). We utilized four different soil treatments (a control plot, a ripped plot, a plowed and disked plot, and a ripped, plowed, and disked plot), using conventional farming and mining equipment, to assess what method would be most effective at addressing these concerns.

Current research has shown that the adverse growing environment on exposed mine land soils can be overcome to accommodate a range of different hardwood species (Torbert et al. 1994). American chestnut (*Castanea dentata*) has provided a tremendous opportunity for successful reforestation of abandoned and reclaimed mine lands. American chestnut has largely been ignored in mine land reforestation efforts due to its virtual demise caused by the chestnut blight (*Cryphonectria parasitica).* However, hybrids that are resistant to blight and have the stand qualities of native chestnut are becoming increasingly available (Burnham 1988). Recent research shows that native seedlings grow more rapidly in areas with increased light levels (McCament and McCarthy 2005) and at pH's that are moderately acidic (Jacobs 2005) like those of abandoned mine lands. By improving the soil quality with four different treatments in our test plots, we were also able to test if American chestnut is able to adapt to mine soil conditions and if any additional treatment is necessary for their survival. We tested three different varieties of chestnut in this study: pure American chestnut, a 7/8$^{th}$ hybrid American/Chinese chestnut, and a 15/16$^{th}$ hybrid American/Chinese chestnut.

In addition to the structural and chemical changes of the soil, the soil microbial communities responsible for nutrient cycling, soil structure, and biological interactions are severely disturbed on mine land sites (DeGrood et al. 2005). Studies conducted post-reclamation have reported low soil microbial diversity, biomass, and activity (Manchulla et al. 2005). Thus, an additional objective of this research was to examine these communities and assess their potential influence on American chestnut survival and growth.

To aid in seedling establishment, fungi that are often missing from the soil (e.g., *Pisolithus tinctorius* ) of reclaimed mine lands (Sprouse, 2004) were inoculated into the roots of chestnut seedlings prior to planting. In eastern Ohio, hybrid chestnut seedlings inoculated with *P. tinctorius* are being

incorporated into strip-mine restoration projects with 85% survival rates (Herendeen & McCarthy 2006). Current molecular techniques provide a method that can confirm fungal species found colonizing chestnut roots in the field. Thus, this may provide additional information on other ectomycorrhizal fungal populations present in these mine sites that may further benefit the growth and survival of the chestnuts. Our goal was to evaluate what mycorrhizal fungi were present in the soil and what was the survivability of *Pisolithus tinctorius* (Pt) on chestnut under different levels of soil compaction.

This report summarizes the results of our two part study, the method first of which evaluates how best to introduce hybrid chestnut seedlings in the face of heavy soil compaction and grass competition. In essence, what is the most effective planting medium and planting technique for reintroduction of American chestnuts on reclaimed mine land. The second part of the study addresses the belowground assessment of the persistence of *P. tinctorius* and how that relates to the survival and growth of American chestnut seedlings in different soil treatments.

## Methodology

### *Background and experimental design*

The study site used for this project was the Tri-Valley Wildlife Management Area, owned and managed by the Ohio Department of Natural Resources. The refuge is located in Madison County, Ohio, was reclaimed in the early 1980s, and is currently vegetated mainly by *Festuca* sp. (Figure 1) and a variety of forbs.

Figure 1. Tri-Valley Wildlife Area study site prior to soil treatment installation. Photo taken dormant season (February, 2007).

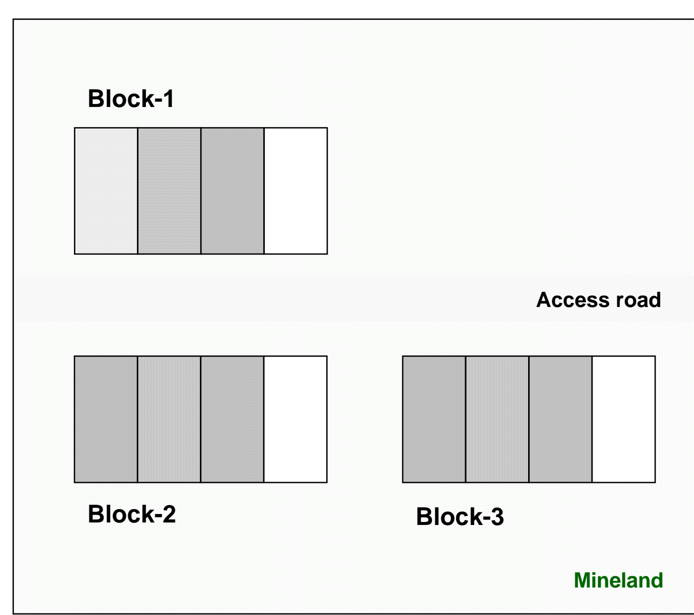
Three experimental blocks (240 × 120 ft; 73.2 × 36.6 m) were established at the site. Each block contains four soil prep treatment plots (each 60 × 120 ft; 18.3 × 36.6 m). The treatments include a ripped (to 32 in; 81 cm) plot, a standard agricultural plowed & disked plot, and a plot that is ripped, plowed and disked. There is also an untreated control plot. Thus, we have a standard 2 × 2 factorial experimental design. A contractor was hired to install the four soil treatments. A bulldozer was used with a ripper attachment designed to penetrate the soil, loosening it ~ 1.0 m deep (Figures 2 & 3).

Figure 2. D-6 grade bulldozer with 32-inch ripper attachment used to rip soil at field site.

Figure 3. Soil shown ripped 1.5 ft. with ripper attachment on a bulldozer.

A conventional tractor with a plow and disc was also used (Figure 4). Treatment blocks were replicated three times and each plot received 100 chestnut seedlings (Figures 5 & 6).

Figure 4. Wjite tractor (240 hp) with plow and disk attachments used to install plots at site.




**Key to treatments:**

CONTROL
Fescue-dominated

RIPPED
Soil deep ripped to 38-in

RIPPED, PLOWED & DISKED
Soil deep ripped; surface plowed & disked

PLOWED & DISKED
Surface soil plowed & disked

**Experimental Design:**

**2 × 2 Crossed-Effects Block Design**

**3 Blocks (each 200 × 100 m) w/ 4 trts**
**Each Trt Plot = 50 × 100 m)**

**Sample Unit: Chestnut seedlings**
**100 per plot (1200 total)**

Figure 5: Field Design schematic

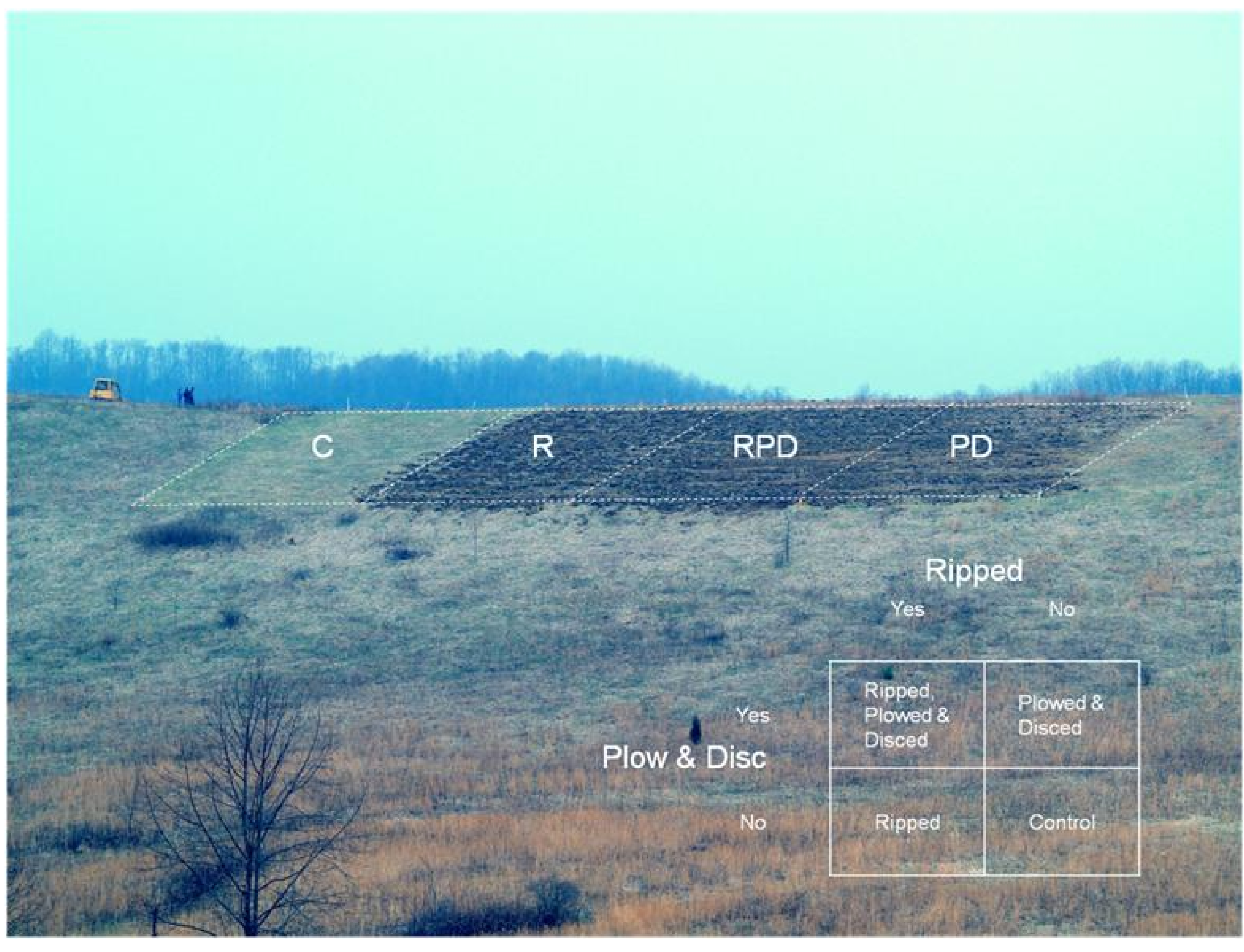



Figure 6. View of field design showing one block with control plot and three treatments: R = ripped, PD = plowed & disked, RPD = ripped, plowed & disked.

In the spring of 2006, 1200 American chestnuts were planted at the ODNR Marietta Tree Nursery. The 1200 seeds were comprised of the following: equal parts were planted of pure American chestnut, Chestnut Hybrids B1-F3 (backcrossed to create a progeny 7/8 American chestnut, 1/8 Chinese chestnut) and Chestnut Hybrids B2-F3 (backcrossed to create a progeny 15/16 American chestnut, 1/16 Chinese chestnut). The 1200 seeds originated from the American Chestnut Foundation, Meadowview, VA. To inoculate seedlings with Pt, the roots of the seedlings were sprayed with the spores of the fungus in May of 2006 (Hopkins, per com.) The seedlings were nursery grown for one year in natural light and watered when needed. In the spring of 2007, two days prior to planting in the field, seedlings were lifted from the inoculation beds, bare root, for planting and stored in moist peat.

*Planting protocol*

The seedlings were planted as bare rootstock in April of 2007 at a spacing of 2 meters, as described by Hebard (2005). The between-row spacing was approximately 2 meters as well. Seedlings were carried in a paper-planting bag with moist peat at the bottom or in a bucket containing sufficient water to cover the roots (Sprouse 2004). Seedlings were planted with the root collar level with the grade of the soil,

backfilled with original soil, moistened with 12 oz. of fully hydrated TerraSorb (water holding gel), and two 20-10-5 fertilizer pellets. Three separate colored flagging were used to decipher between the three types of American chestnut backcrosses being planted (7/8ths, 15/16ths, and pure American chestnut). A 36 in. weed mat was installed to control reemerging previous groundcover. To prevent herbivory, a 12 x 48 in (1.5 m) tall poultry mesh cage was placed around each seedling held by 3 wooden stakes fastened with zip ties (Sprouse 2004) (Figures 7 & 8).

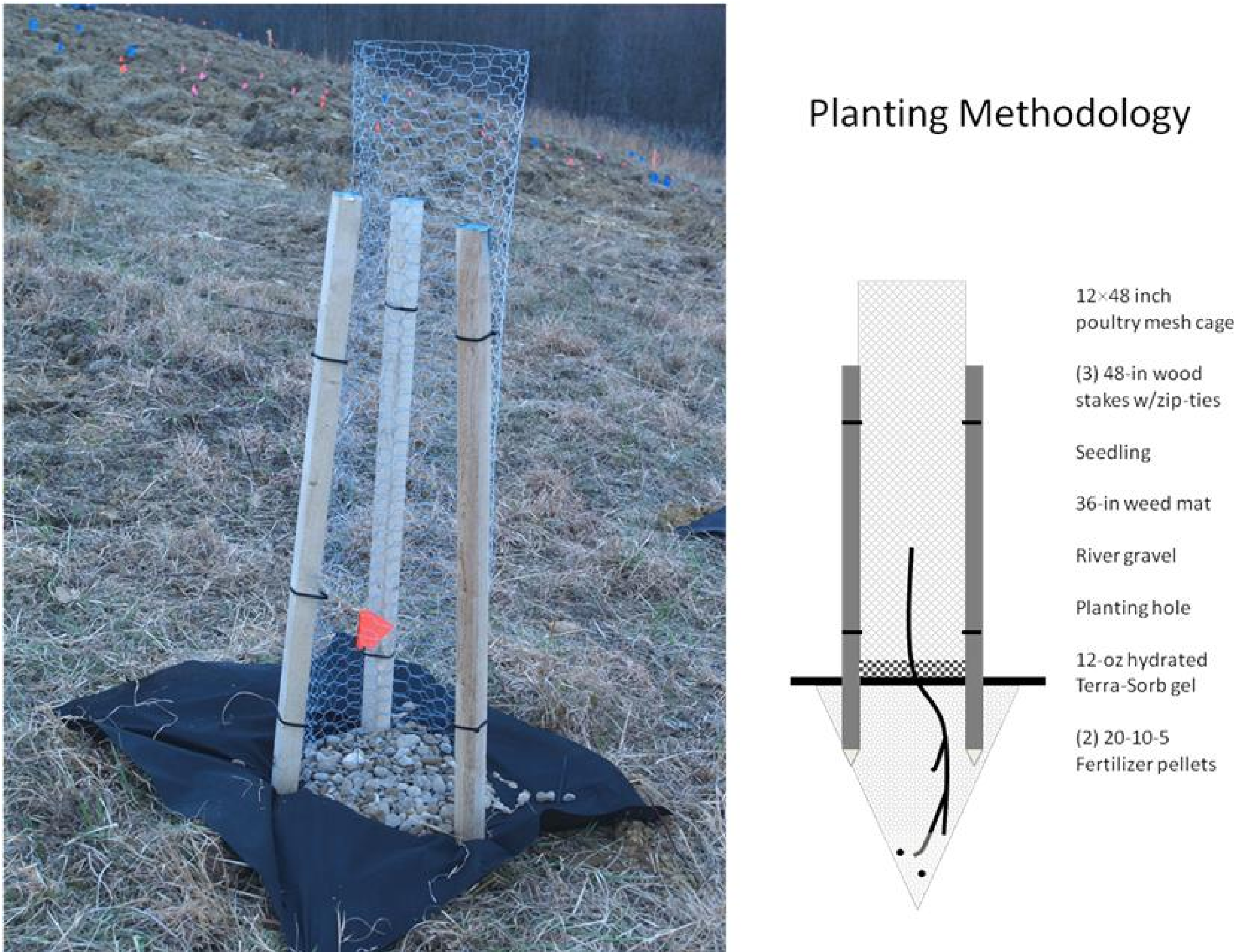



Figure 7. Planting methodology for 1-yr-old bare root American chestnut seedlings.

Figure 8. American chestnut seedling being planted at the Tri-Valley Wilife Management Area.

***Other recorded parameters***

*Data collection of aboveground survival and growth*

Seedling survival data was recorded once a month for the duration of the growing season (April to October 2007). Growth parameters such as stem diameter and seedling height were recorded in April and October. Number of leaves and approximate leaf area were recorded in October.

*Soil methodology*

Soil chemistry parameters were measured either in the lab of the PI or at Spectrum Analytic, Inc. (Washington Court House, OH). Protocols used for internal soil sample processing followed Brian McCarthy's Lab Protocols for the Testing of Eastern Deciduous Forest Soils (http://www.plantbio.ohiou.edu/epb/soils/soils.htm). A soil core was taken in the center of each treatment at three locations within the block totaling thirty-six samples. Soil cores used for chemistry and bulk density were taken prior to soil treatments. Soil bulk density was measured before and after treatment installation. The soil was dried and weighed for calculation of BD on a volumetric basis.

*Climate methodology*

Climate variables were measured by HOBO dataloggers for the duration of the growing season. Relative humidity and temperature were recorded by a HOBO placed in the center of each plot

(totaling 12 dataloggers) from April until September.  Light was recorded in 4 of the blocks equidistant from one another from July until September.

*Statistical analysis of aboveground data*

The general experimental design was established as a randomized complete block factorial experiment. Block (1,2,3) was treated as a random effect and treatments (C, P, R, RPD) as fixed effects. Data were analyzed using analysis of variance procedures following examination of assumption of normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test). All data were analyzed using R statistical and graphics software (http://www.r-project.org/).

***Belowground methodolgy***

Ten percent of pure American chestnuts were randomly selected per treatment group for root sampling (N = 120) In October 2007.  Using a spade, soil was carefully removed to expose the root system (Figure 9).  This was done to ensure that the roots that were sampled were from the chestnut seedling and not from the surrounding vegetation.  Root tips were randomly sampled by coring the soil profile at a 20 to 30 cm depth.  Roots were sifted from soil, washed with distilled water, stored on ice, and returned to the laboratory.

Figure 9.  Exposed roots from chestnut seedlings approximately 20 to 35 cm underground. Root tips were randomly sampled with soil probes.

Once in the lab, roots were washed with autoclaved distilled water, placed into a Petri dish with sterile water, and viewed under a dissecting microscope (Figure 10). Root tips were then observed for mycorrhizal colonization.  Roots tips were selected based on morphological changes characteristic of mycorrhizal infection.  This includes roots with diminished elongation of root hairs, the formation of

short roots, visible fungal hyphae, and the formation of fungal sheath (Figure 11). Root tips approximately 2 mm to 5 mm in length were selected, stored in an autoclaved microcentifuge tube, and stored at -70˚ C.

Figure 10. View of ECM root under the dissecting microscope. Roots are colored white by the presence of hyphae creating the fungal sheath.

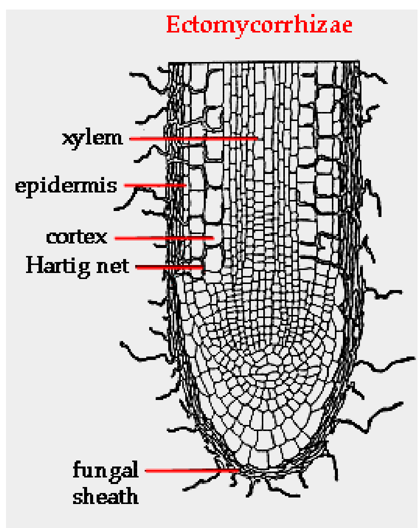



Figure 11. Diagram of characteristic morphology of an ectomycorrhizal root tip.
biology.uwsp.edu/.../symbioses/endomyco.gif

*Fungal DNA extraction, PCR amplification, and sequencing methodology*

To molecularly identify the type of mycorrhizal fungi, fungal DNA was be extracted from the collected root tips using QIAgen Dneasy Plant Mini-Prep kit purchased through QIAGEN Inc. Primers ITS1-F (5' cttggtcatttaggaagtaa 3') and ITS4 (5' tcctccgcttattgatatgc 3') was be used to amplify internal transcribed spacer sequences (ITS) during PCR (Gardes and Burns 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 programmed as described by Gardes and Burns (1993): The initial denaturation step of 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 95ºC for 35 s, 55ºC for 55 s, and 72ºC 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 72ºC for 45 s. PCR reactions were run of 1% argarose gels for 20 min to allow for the visualization of fungal DNA (Figure 12.) Positive controls of known samples were used to ensure PCR amplification. Negative controls lacking template were used to ensure that the DNA amplified was from the root samples and not from contamination form reagents and reaction mixtures.

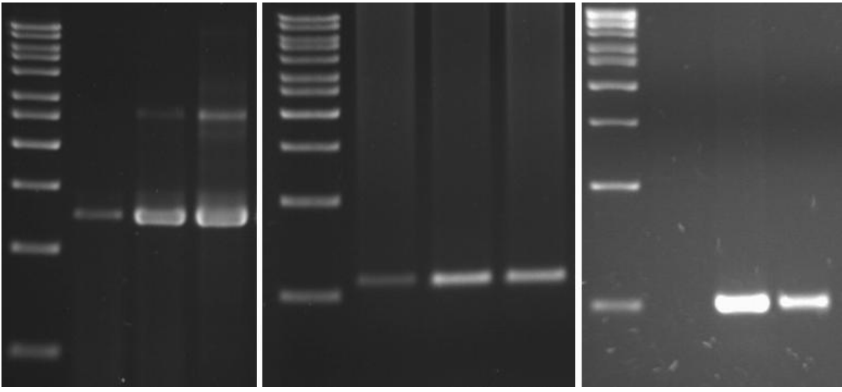


Figure 12. Resulting bands for electrophoresis. The bands are indicative of the presence of fungal DNA from the PCR reaction.

The presence of DNA was confirmed via gel electrophoresis and PCR product was cleaned by using Clean-Gene. Samples were prepared for sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit by mixing 10 µl reactions of the following concentrations: 2 µl BigDye Terminator v3.1 Reaction Mix, 3 µl 5 X Sequencing dilution buffer, 1 µl primer, and 1 µl of template. Sequencing cycle to label DNA for sequencing was performed on a programmable Thermal Cycler for the following cycles: 96ºC for 1 min followed by 25 cycles of 10 s at 96ºC, 5 s at 50 ºC, and 4 min at 60 ºC. Following labeling, products were purified to remove all unincorporated dye-labeled terminators by alcohol precipitation. Sequencing was performed with TheApplied Biosystem ABI Prism 3730 DNA Analyzer (Bioinformatics facility, Miami University, Oxford, Ohio). Sequences were analyzed and edited when applicable using Sequencher 4.2 software (Gene Codes, Ann Arbor, Michigan) (Figure 13). To identify fungi found on roots, sequenced samples were compared with known species in GenBank using BLAST searching. Genera reported here are based on the best match to known Genera based on the similarity to the reported ITS sequences in GenBank.

Characteristics are based on statistical analysis that generates both a bit value and an Expect (E) value. The bit score is a value that is indicative of how well the sequenced aligned with the known sequence

in the database. The higher the score, the better the match. The E value is a parameter that describes the probability of the number of matches that can be generated by chance. It decreases exponentially as the match increases; a score closest to zero is the most significant. Thus when deciding the genera to report here, a threshold was decided on that included an E-value of 0, highest ranking bit value, and a gap value of < 4.

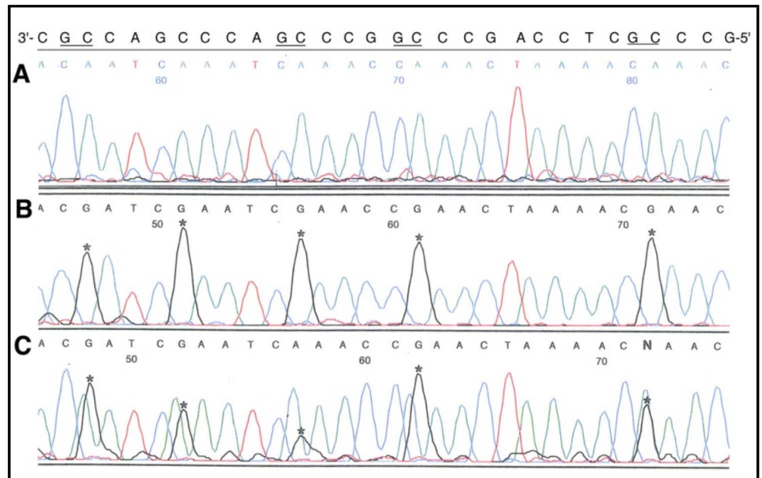



Figure 13. Sequencing results generated from ABI Prism 3730 DNA Analyzer. Peaks (highlighted by the asterisks) indicate base pair. These base pair calls produce the sequence used in GenBank.

*Species diversity calculation*

The Simpson's Index was used to calculate species diversity.

$$Ds = \sum_{i=1}^{s} n_i (n_i - 1)/(N(N-1))$$

Where $n_i$ is the number of individuals in the $i_{th}$ species.

*Statistical Analysis of belowground data*

Analysis of Variance (ANOVA) was performed to determine differences in the measured growth parameters for each treatment and ECM genera group (*Hebeloma, Thelephora*, and Non-ECM). Growth rates were calculated by subtracting the final measurements from the initial height and basal diameter. All data used was from the seedlings selected for sun sampling. Negative values resulted in conditions of negative growth. An ANOVA was also used to determine differences in species diversity. Treatment effects were considered significant when $p \leq 0.05$ according to the F test. Differences among means were further assessed by using multiple comparison test, Tukey's HSD. Chestnut

seedlings were analyzed for correlations between the growth parameters and species diversity. All statistics were performed using JMP (5.0, SAS Institute, Cary NC, USA).

## Results

### *Environmental variables analysis*

*Rainfall*

During the 2007 growing season the local weather was relatively dry, verging on moderate drought from June through August (Figure 14). Fortunately, the seedlings were planted when the weather was somewhat wetter in April and March and combined with the TerraSorb gel were likely to have remained moist enough to establish an adequate root system by mid-summer.

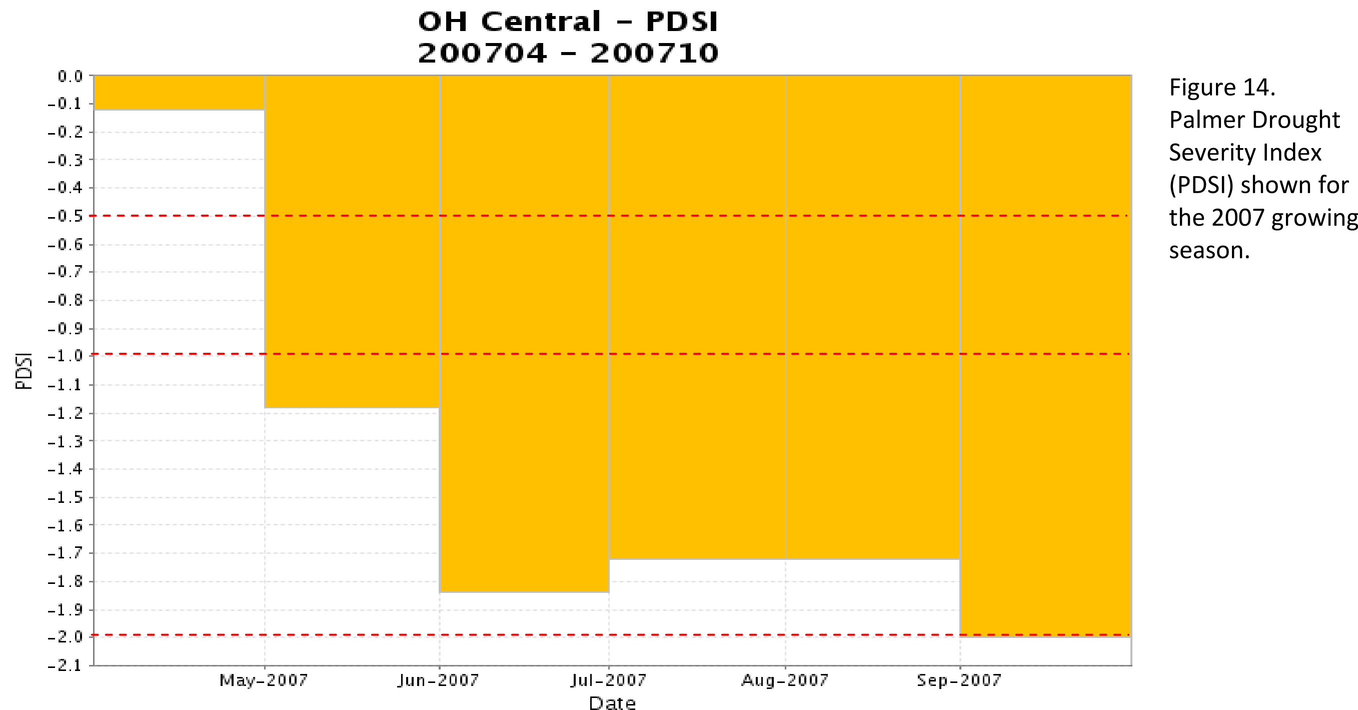



Figure 14. Palmer Drought Severity Index (PDSI) shown for the 2007 growing season.

*Temperature Data*

Temperature data was collected in all twelve soil treatment plots in all 3 replicated blocks. Given that blocks 1&2 had a different slope aspect than block 3, there was concern that there might be a temperature differential among blocks. However, the data are consistent among blocks and treatments (Figures 15-22) and rarely differed statistically (and then only for very short periods of time). They were also consistent on a daily basis (Figure 15). Block 3 shows a slight elevation by one degree (F) in October (when we would expect to see a stronger difference) in all treatments except for the plow and disk treatment when compared to blocks 1 and 2 (Figures 16-19). Block 3 plow and disk is slightly lower than other treatments (Figure 22), but that is the only visible variation among the

treatment temperatures (Figures 20-22). Temperatures in all blocks were highest in August and lowest in October.

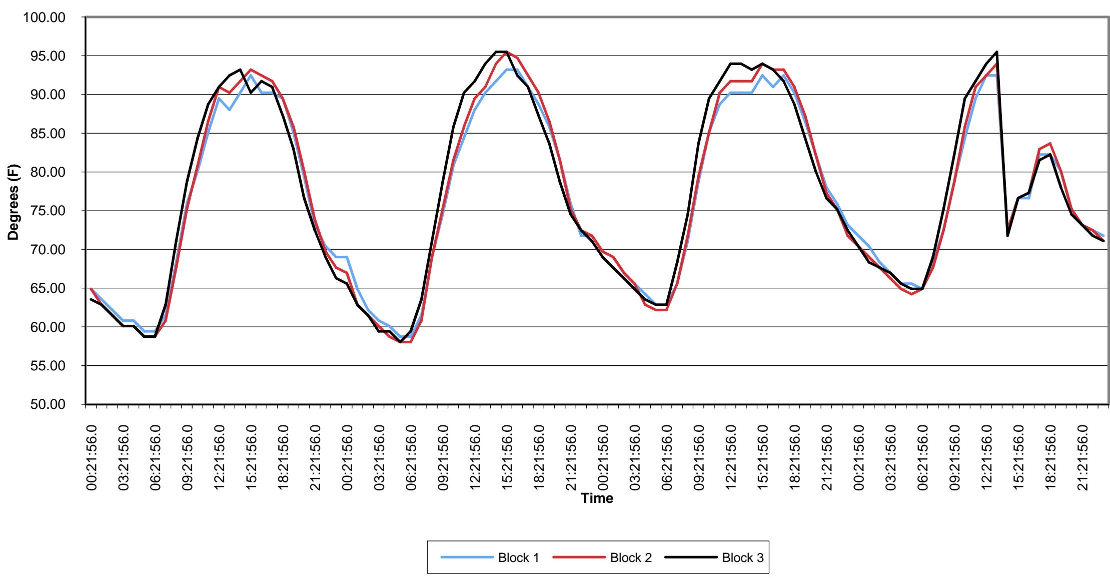



Figure 15. Diurnal temperature readings for all 3 blocks shown in degrees F.

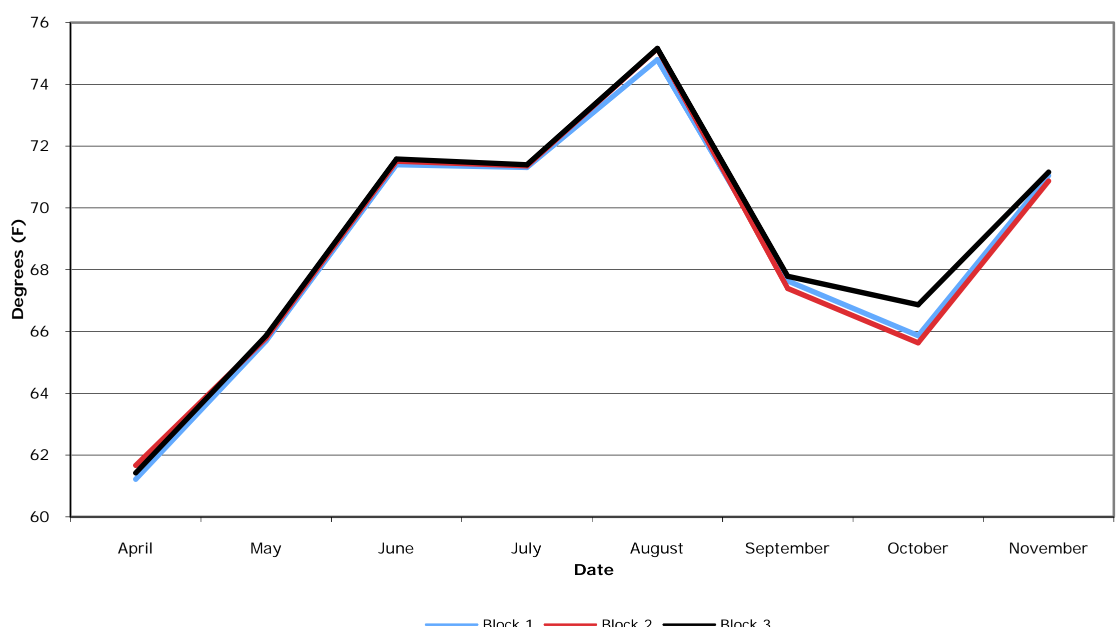



Figure 16. Control temperature monthly means for all 3 blocks shown in degrees F.

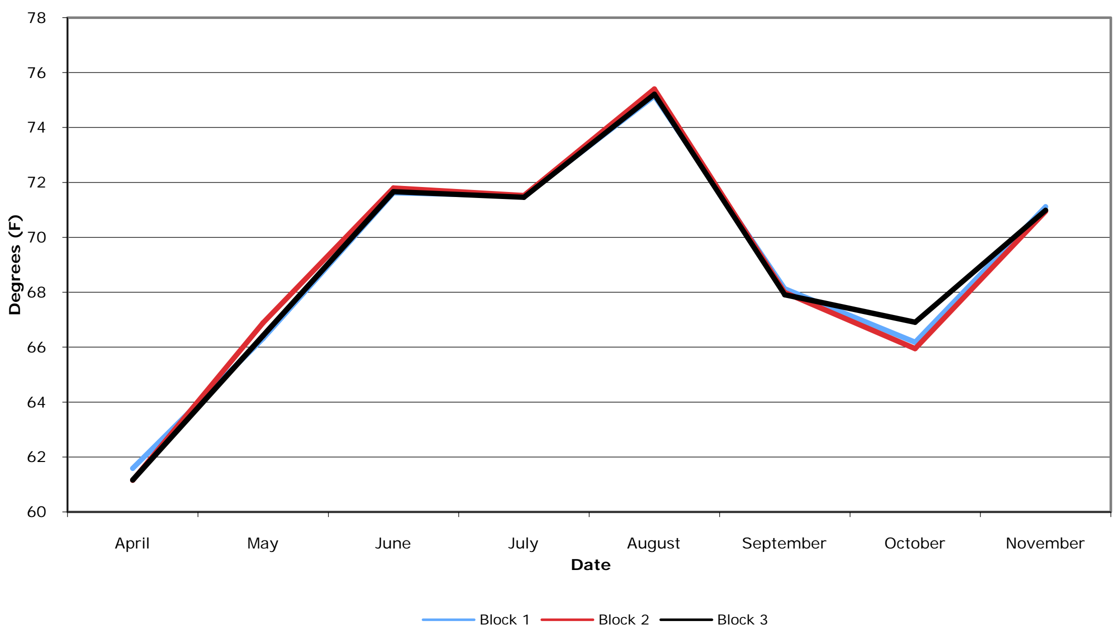



Figure 17. Rip temperature monthly means for all 3 blocks shown in degrees F.

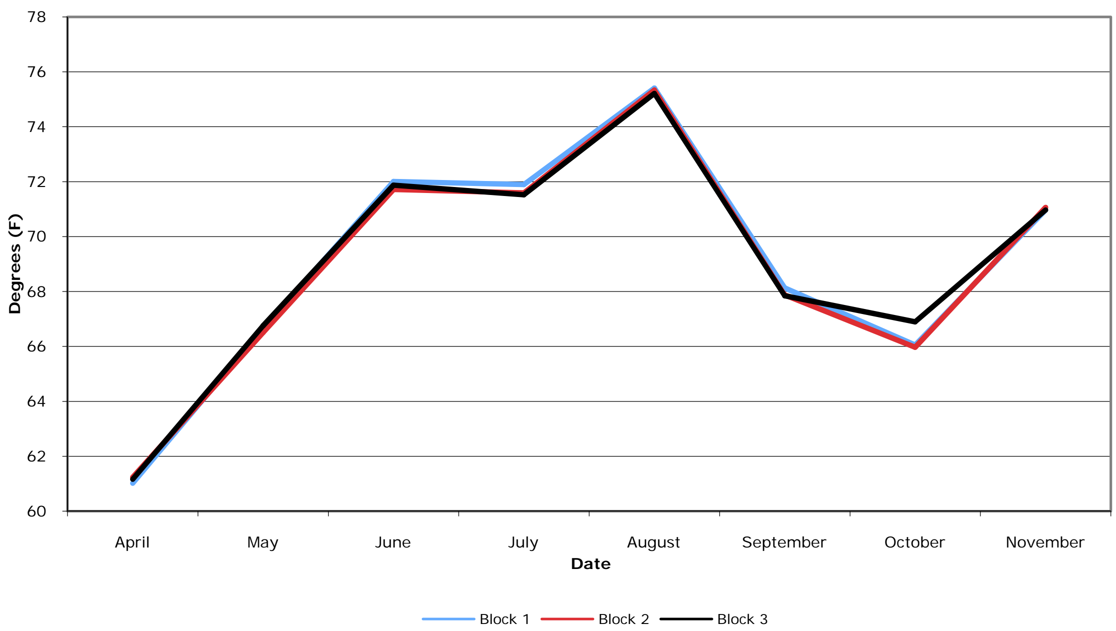



Figure 18. Rip, Plow, and Disk temperature monthly means for all 3 blocks shown in degrees F.

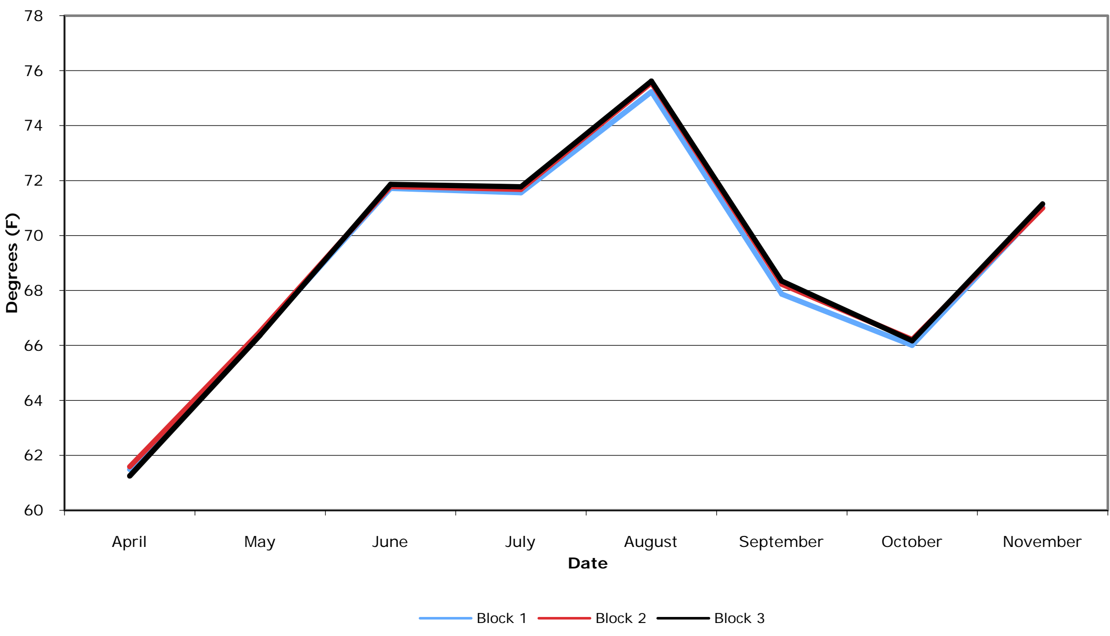



Figure 19.  Plow and disk temperature monthly means shown for all 3 blocks shown in degrees F.

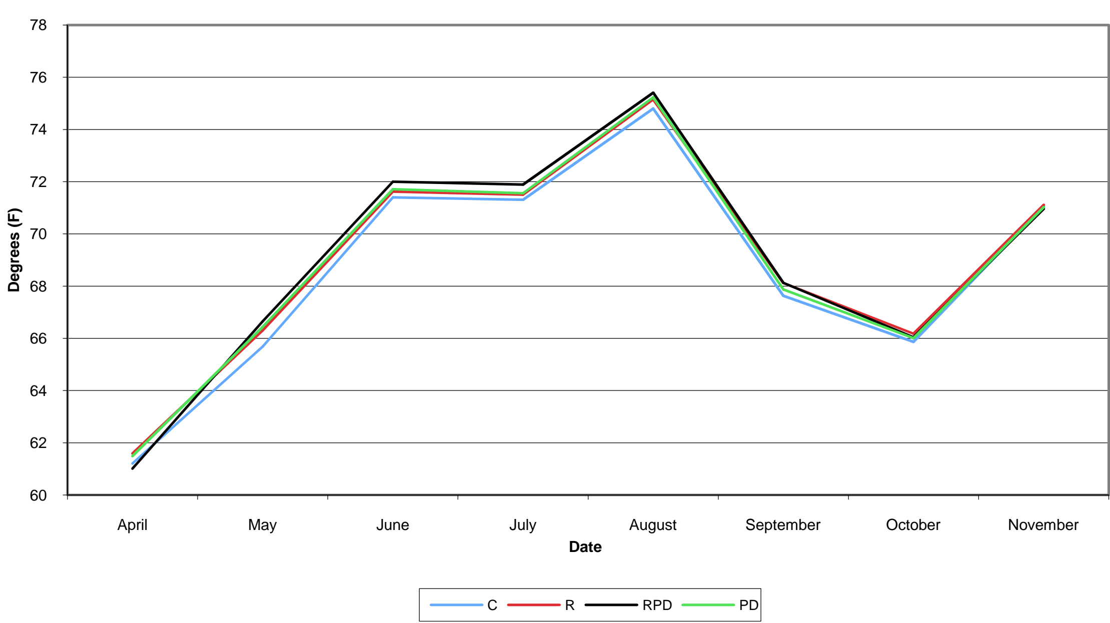



Figure 20.  Block 1 temperature monthly means for all four treatments shown in degrees F.

Figure 21. Block 2 temperature monthly means for all four treatments shown in degrees F.

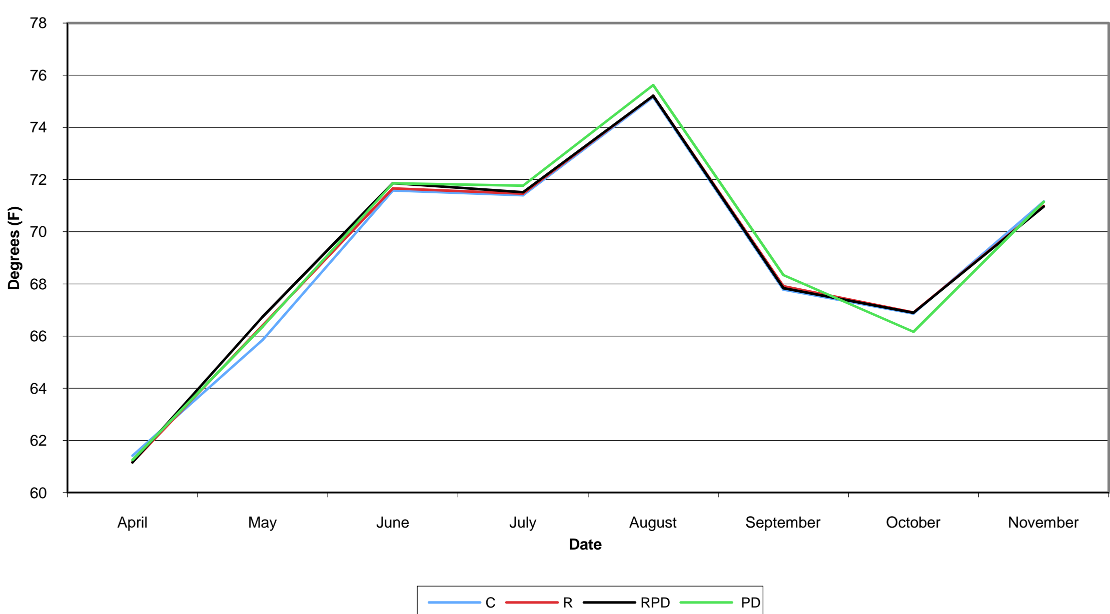


Figure 22. Block 3 temperature monthly means for all four treatments shown in degrees F.

*Relative Humidity Data*

Relative humidity results were also fairly consistent among treatments and blocks (Figures 23-29). Block 3 was higher in October than the other blocks, but only by a percentage, not enough to be biologically significant (Figures 23-25). Relative humidity was highest in August and lowest in November. Block 1 rip was slightly elevated (Figure 27) than the other treatments in that block. And block 2 control was slightly more elevated than other treatments in that block, but again, not enough to be biologically significant. There were however some meters that took faulty readings, so that data was deleted; block 1 plow and disk, block 2 rip, and block 3 plow and disk meters.

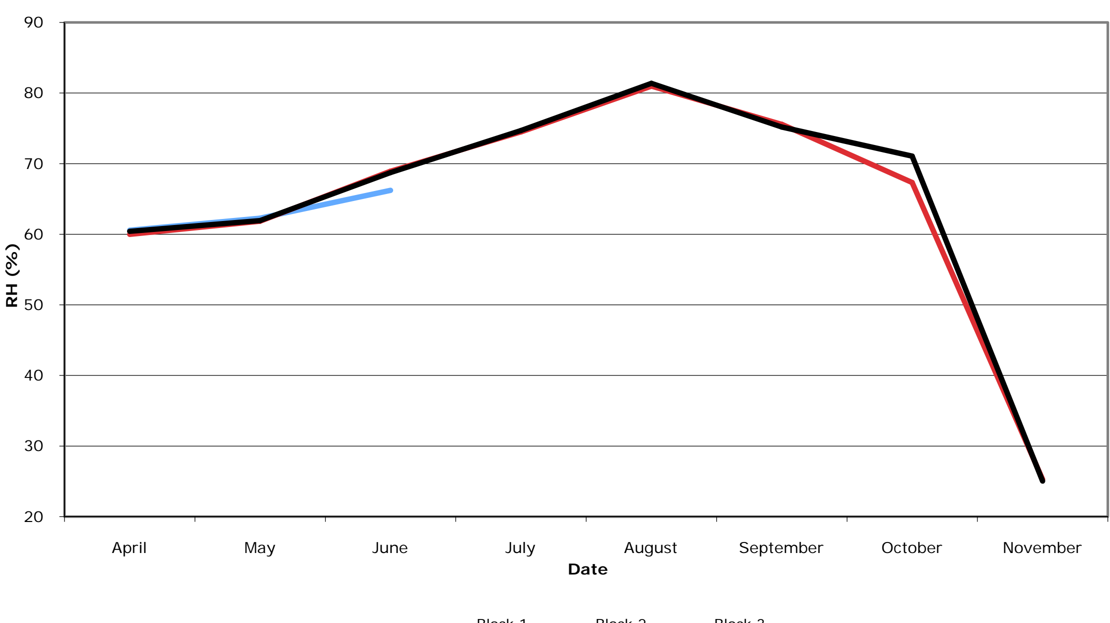



Figure 23. Control RH monthly means for all 3 blocks shown by percentage. RH data probe for Block-1 failed in mid-June, so data are missing for that point forward.

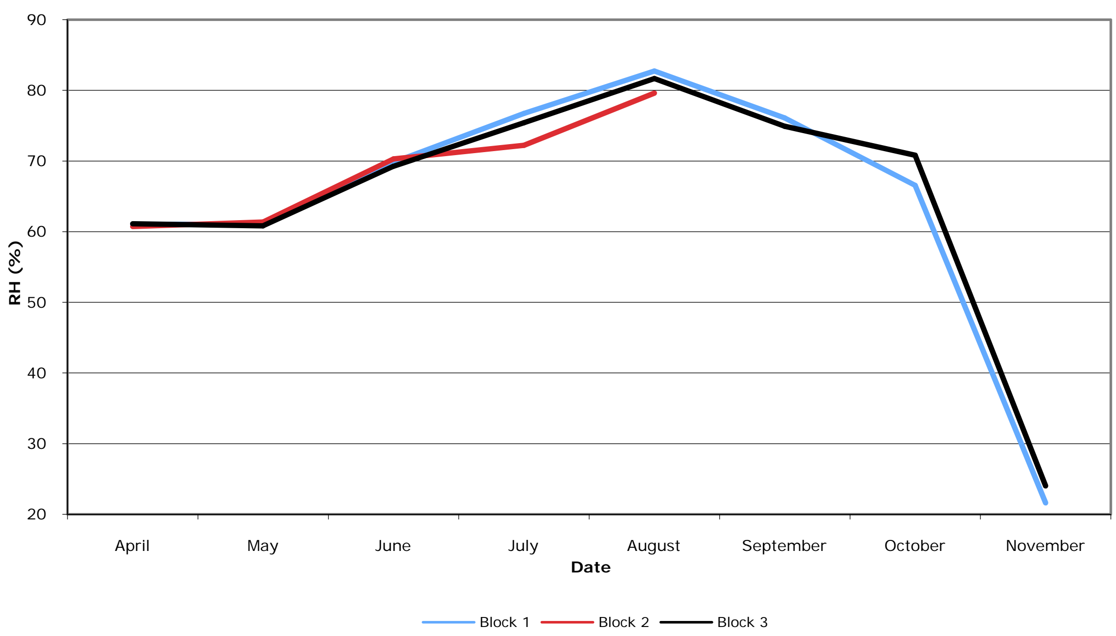



Figure 24. Rip RH monthly means for all 3 blocks shown by percentage.

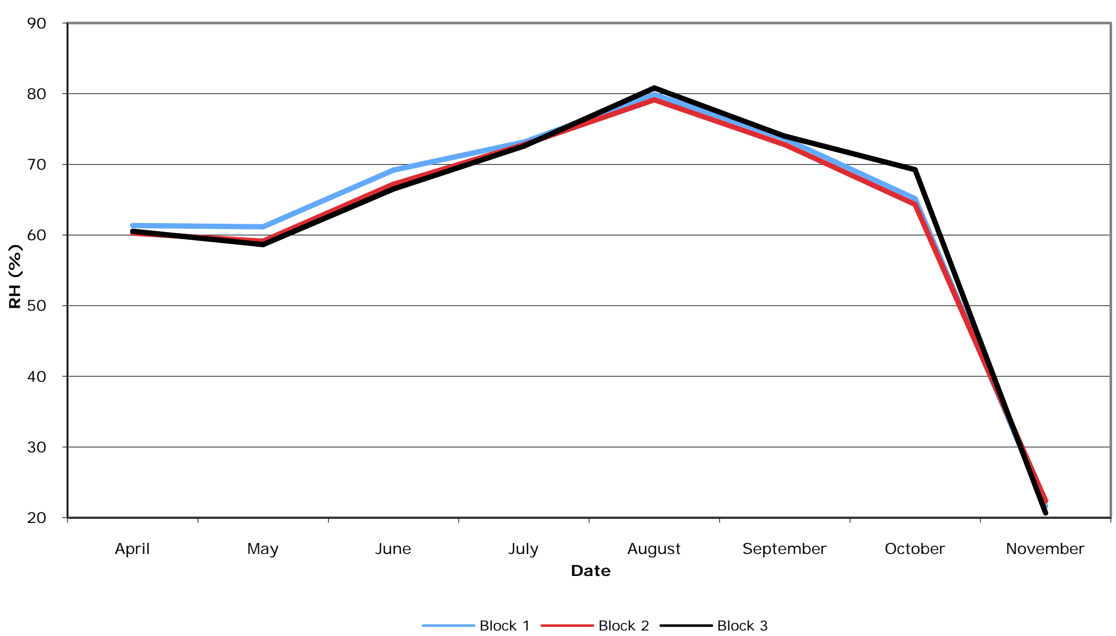



Figure 25. Rip, plow and disk RH monthly means for all 3 blocks shown by percentage.

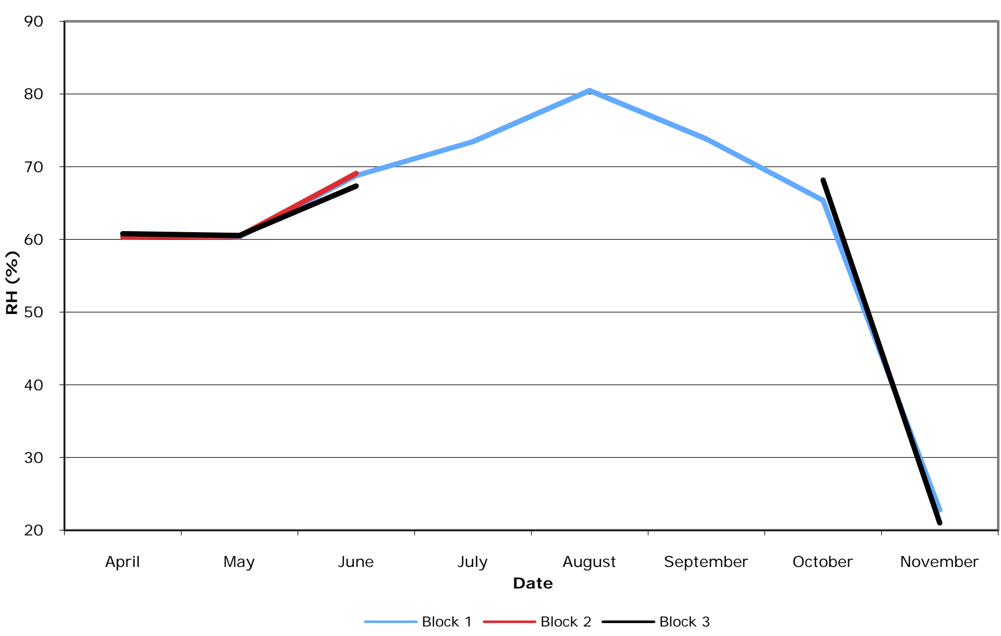



Figure 26. Plow and disk RH monthly means for all 3 blocks shown by percentage.

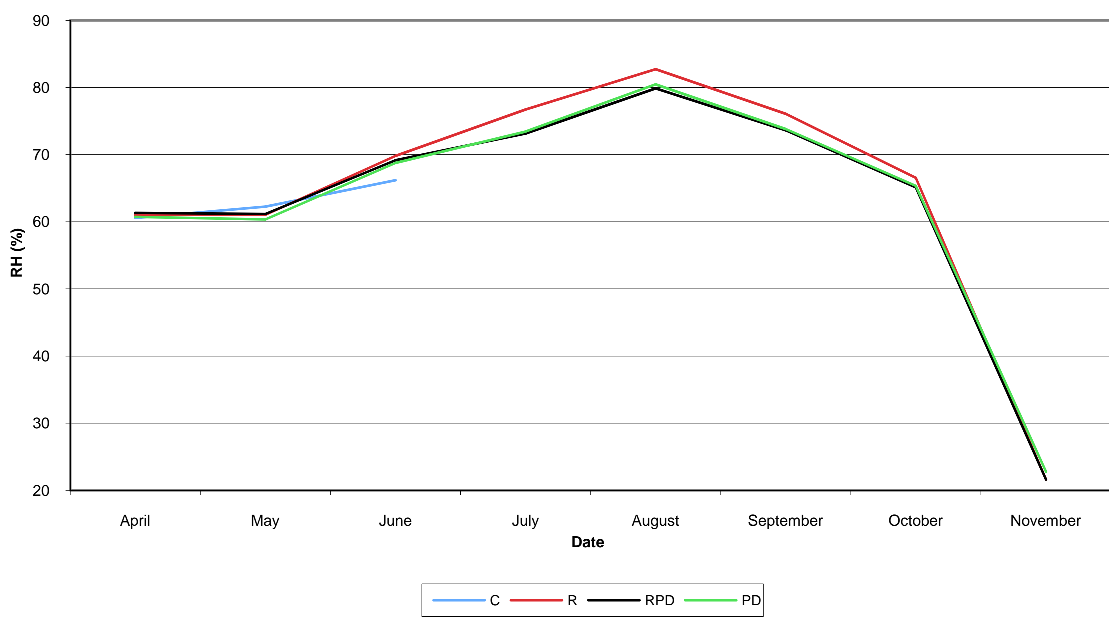



Figure 27. Block 1 RH monthly means for all four treatments shown by percentage.

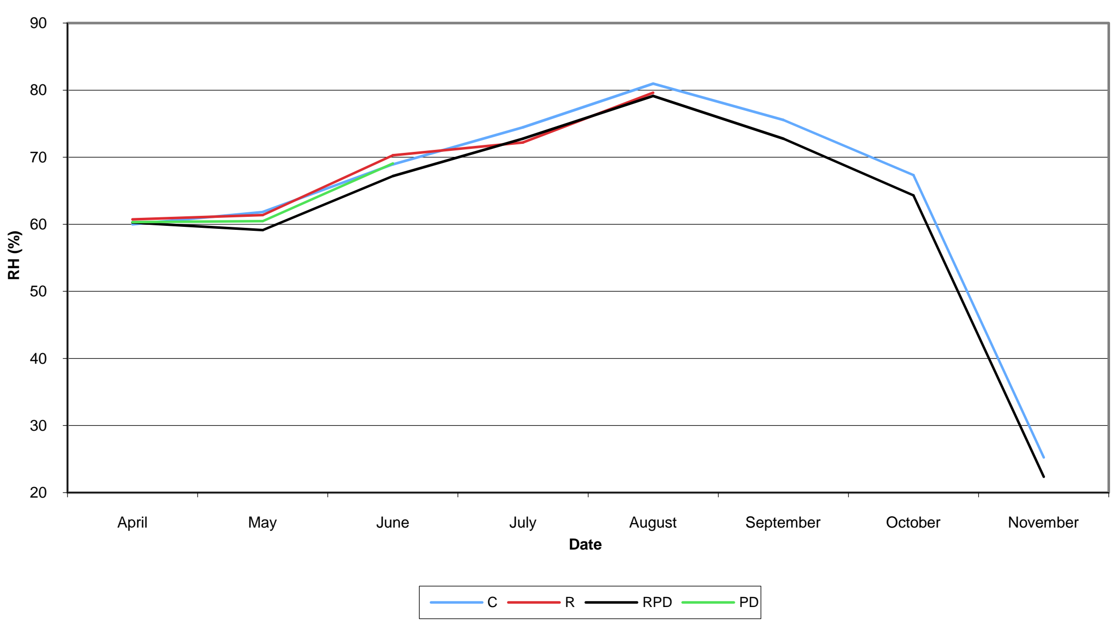



Figure 28. Block 2 RH monthly means for all four treatments shown by percentage.

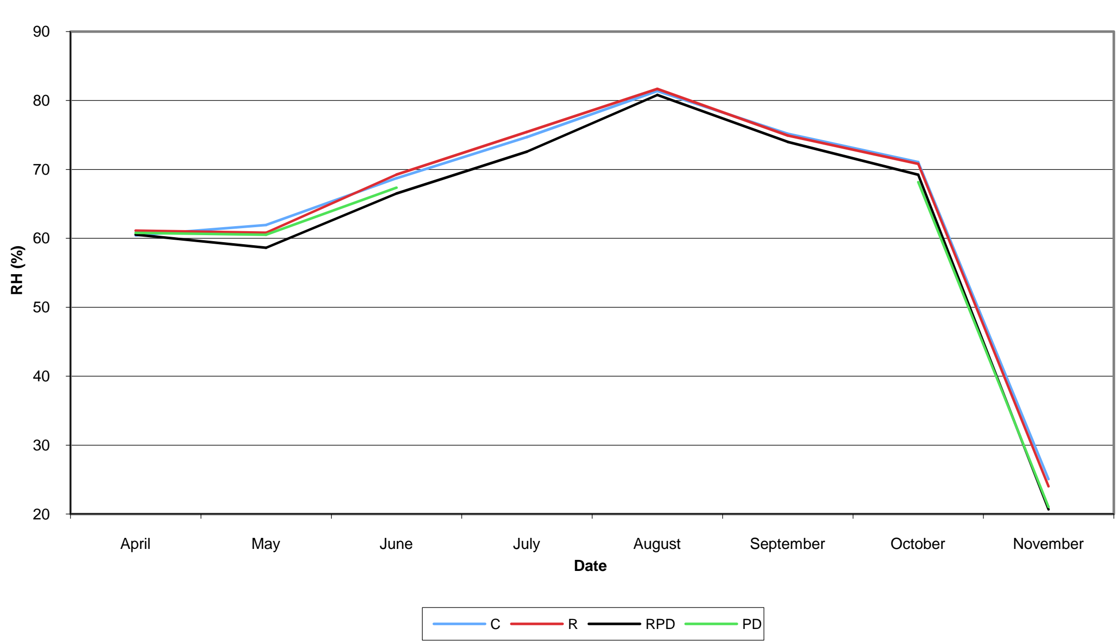



Figure 29. Block 3 RH monthly means for all four treatments shown by percentage.

*Light Intensity*

The light intensity was also very similar among the different plots (blocks and treatments). Plot 2 had the lowest amount of light, but not enough to be significant to the growth of the seedlings (Figures 30-33). The plots located in block 3 (plot 2 and plot 3 control) had slightly more light than plots 1 and 2, but only in July for a few days, therefore not enough to make an impact on the seedling's growth. Because of faulty light meters in the beginning of the growing season, new ones were put out onto the site From July to September to record some measurements. The budget allowed for 4 light meters each spaced equidistant from each other – therefore the meters labeled Plot 1 and Plot 2 are on the SW side of the slope, in blocks 1 and 2 and the meters labeled Plot 3 and Plot 3 control were placed on the opposite side of the slope in block 3.

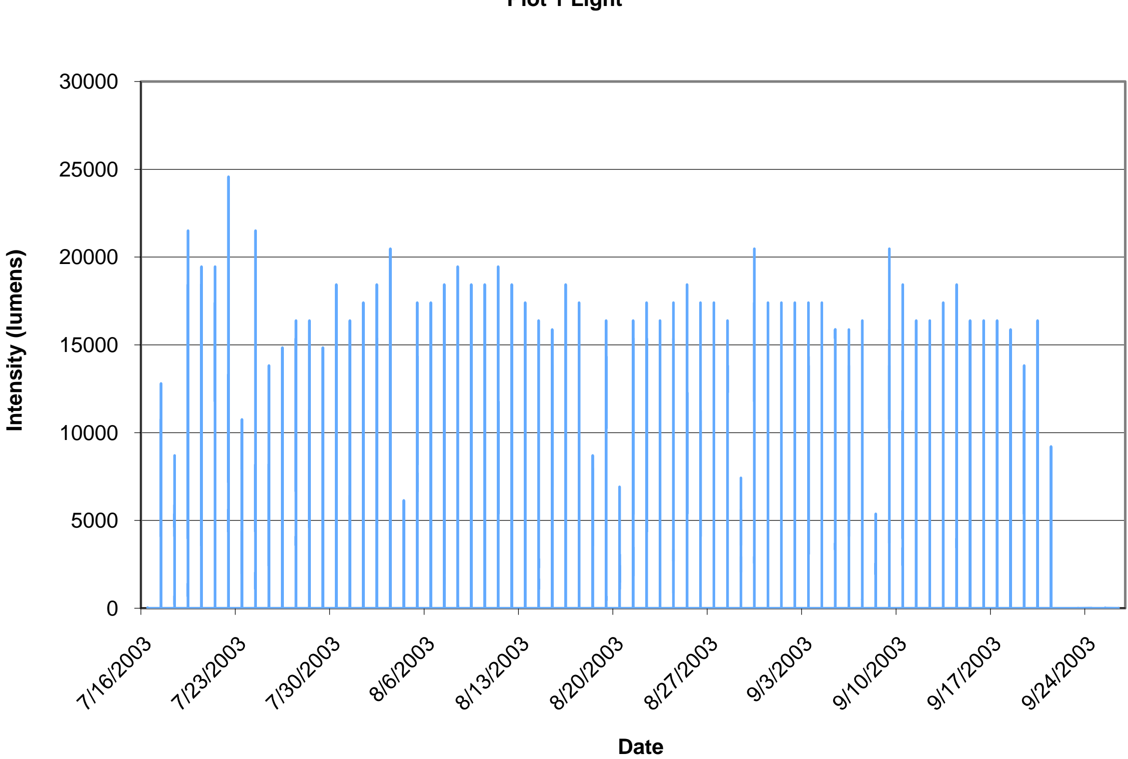



Figure 30. Plot 1 light shown in lumens.

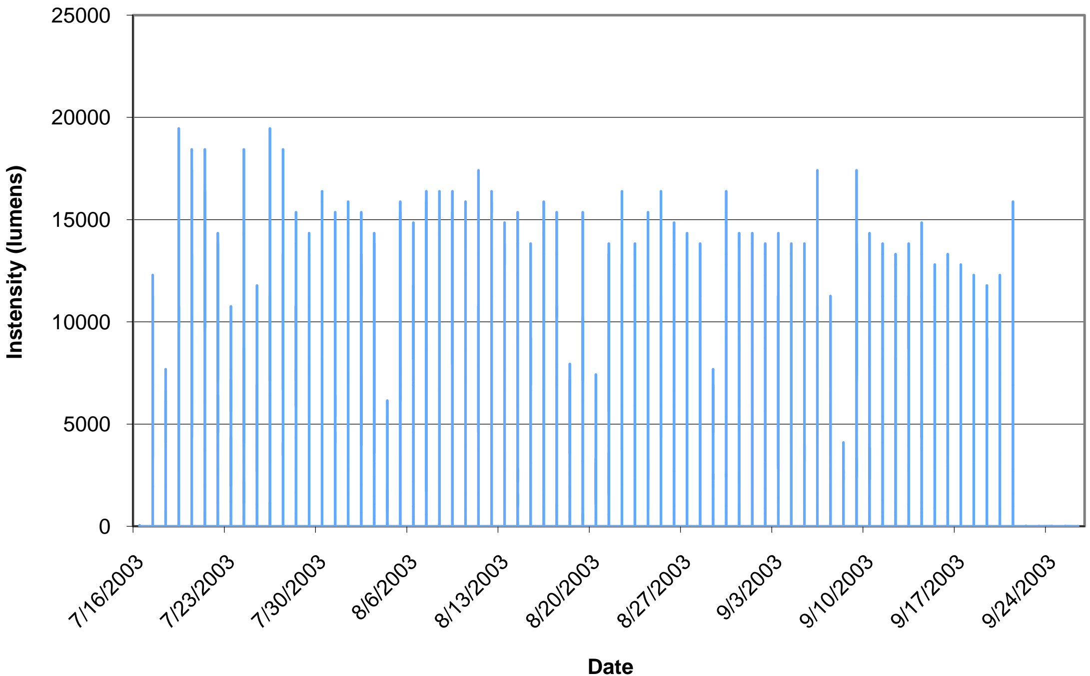



Figure 31. Plot 2 light shown in lumens.

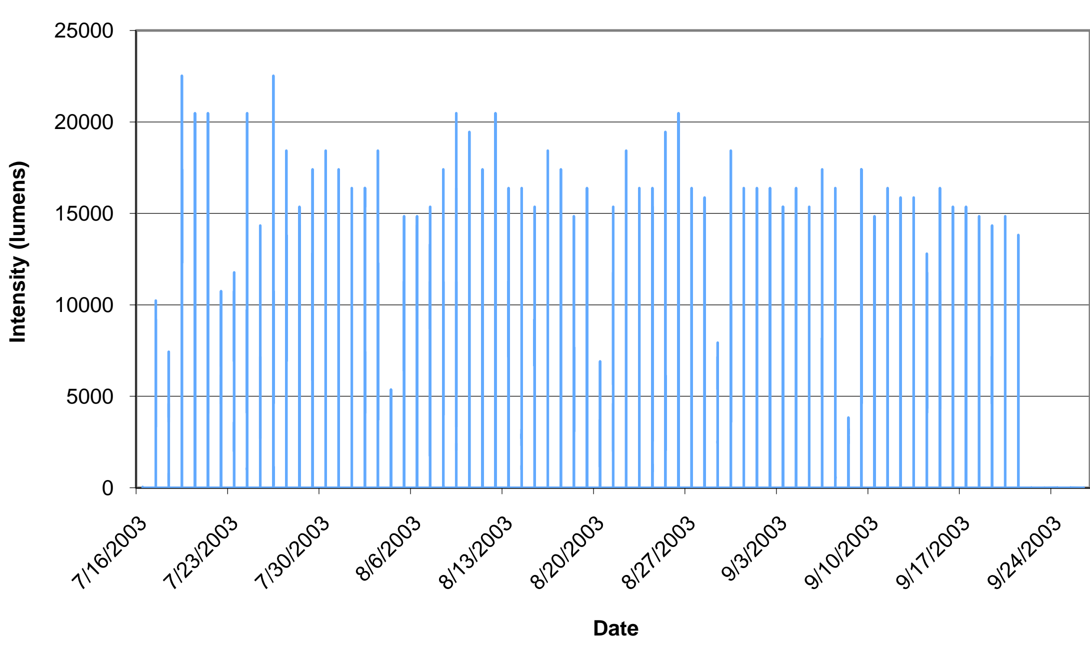



Figure 32. Plot 3 control light shown in lumens.

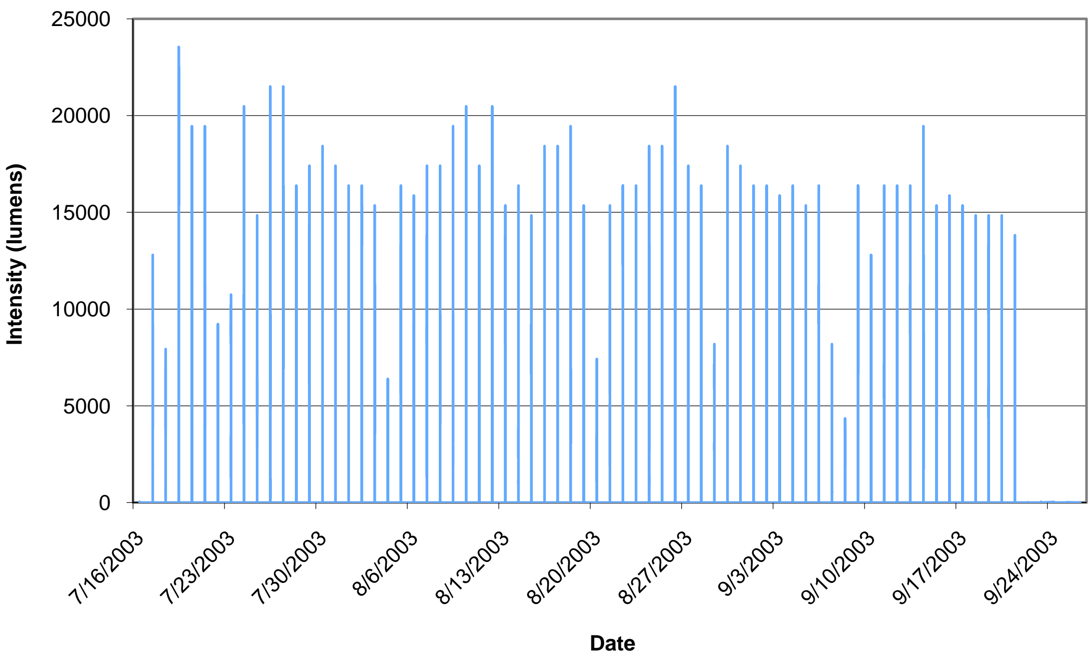



Figure 33. Plot 3 light shown in lumens.

## *Soil Analysis*

### *Soil bulk density*

The post soil preparation bulk density results show that there was a significant decrease in density in the plots that were treated; the rip, the rip, plow, and disked and the plow and disked. The ripped plots showed the most difference among the treated plots. The plow and disked the least difference in density among the treated plots. In the control plots there was no significant difference in bulk density (Figure 34).

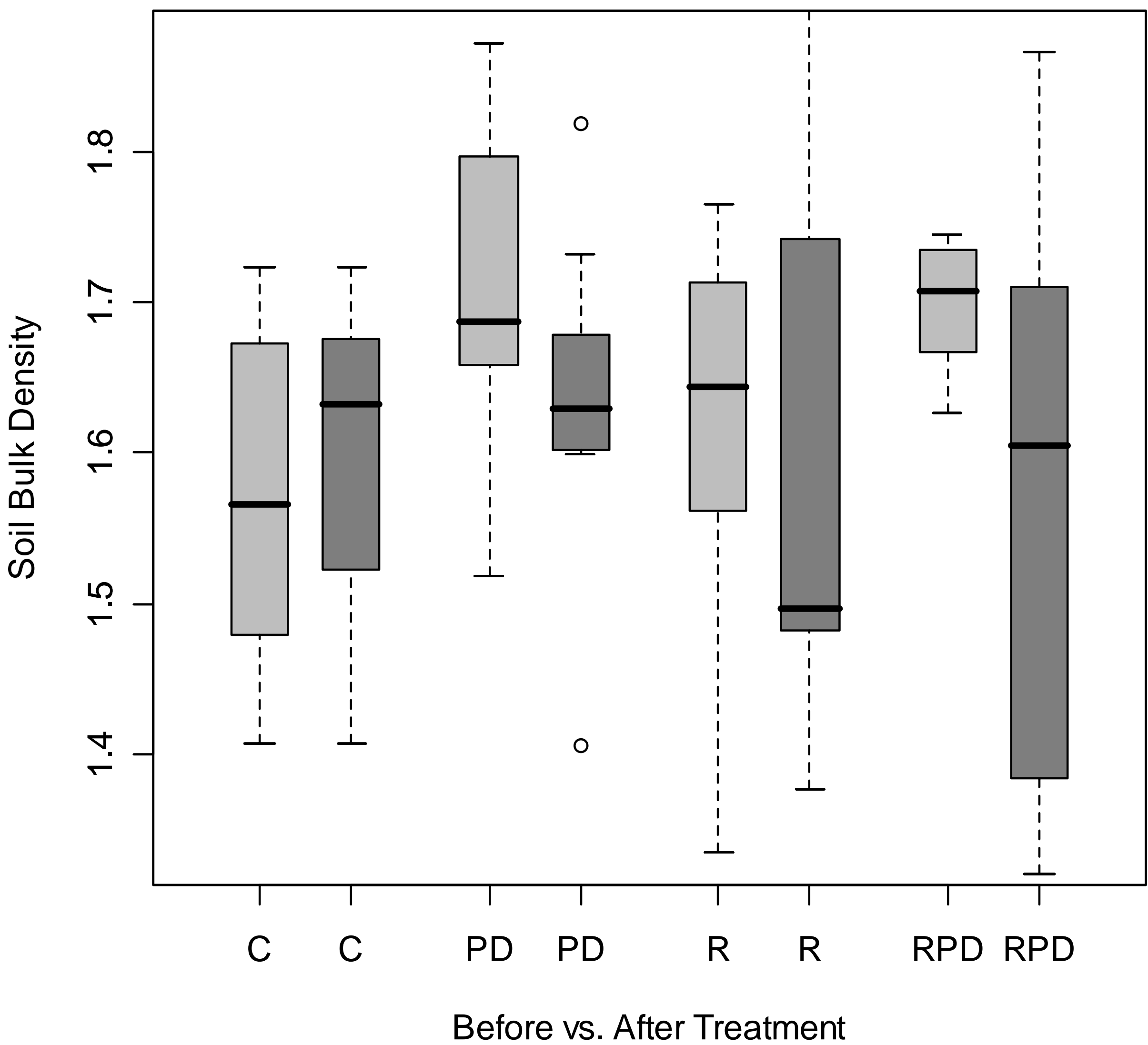



Figure 34. Soil bulk density results before and after soil preparation treatments were completed (C = control, PD = plow & disk, R = rip, RPD = rip, plow & disk). Boxes are interquartile range (middle 50% of data); howizintal line in box is the mean; whiskers extend to 1.5x IQR or min/max, whichever comes first. Light gray is BEFORE measurement, dark gray is AFTER treatment measurement. All treatments decreased in SBD significantly ($P < 0.05$) following treatment.

*Soil Chemistry*

Soil pH was not significantly different across the 3 different planting blocks, although block 3 was the highest of the blocks (Figure 35). Soil manganese and aluminum were also not significantly different across the planting blocks (Figure 36). Although block 2 showed the highest manganese concentrations and block one showed the highest aluminum concentrations. Phosphorous, Calcium, Potassium, and Magnesium concentrations were also not significantly different among blocks (Figure

37). Along there was an elevated level of Calcium in block three, but again, not enough to be statistically significantly different that the other blocks. All of these soil chemistry results illustrate that the concentrations should have been similar and not had a differing affect on plant growth in different blocks.

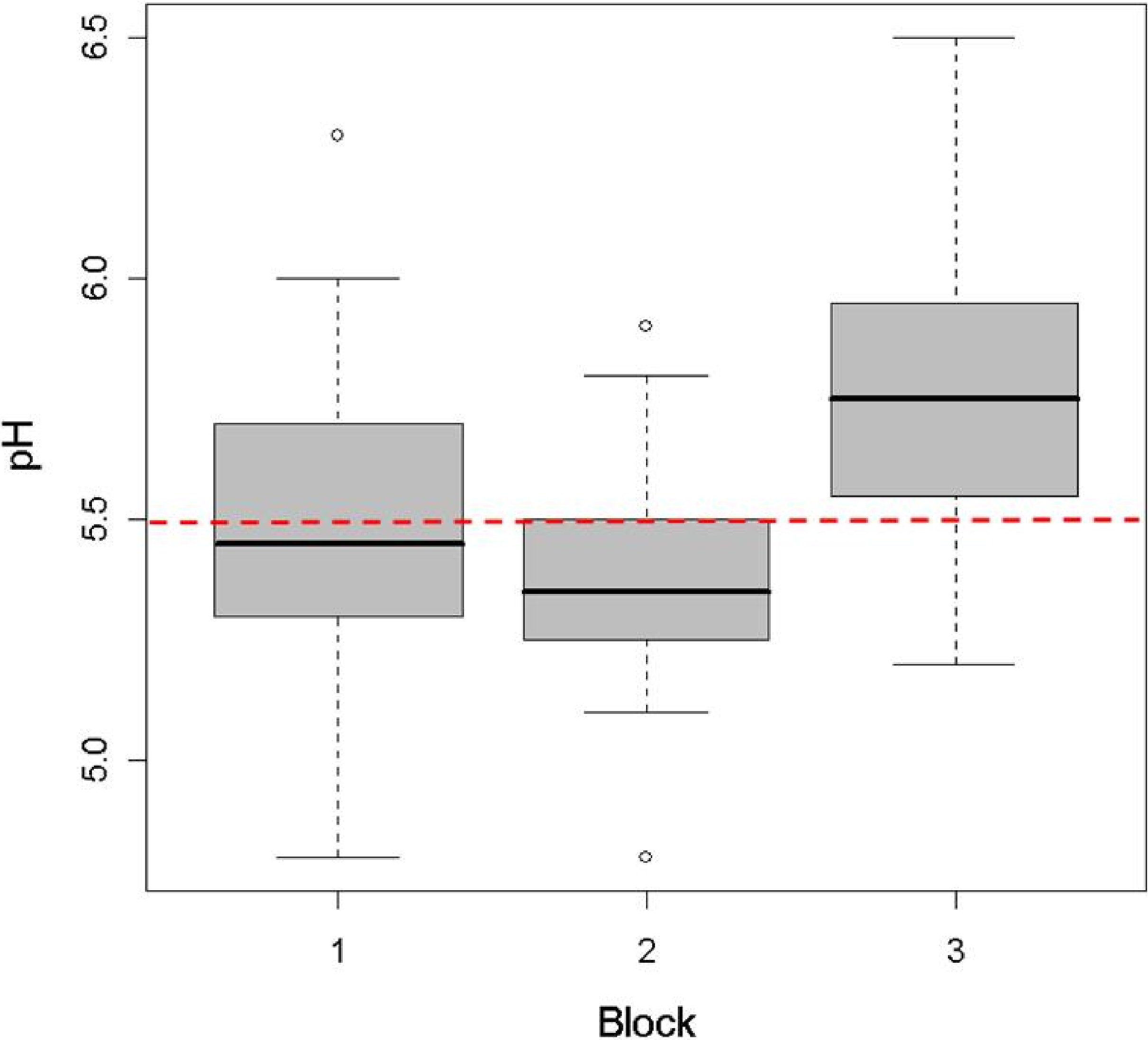



Figure 35. Soil pH results. The red dotted line shows soil pH mean across all blocks. Black lines within each gray box (IQR) show soil pH mean.

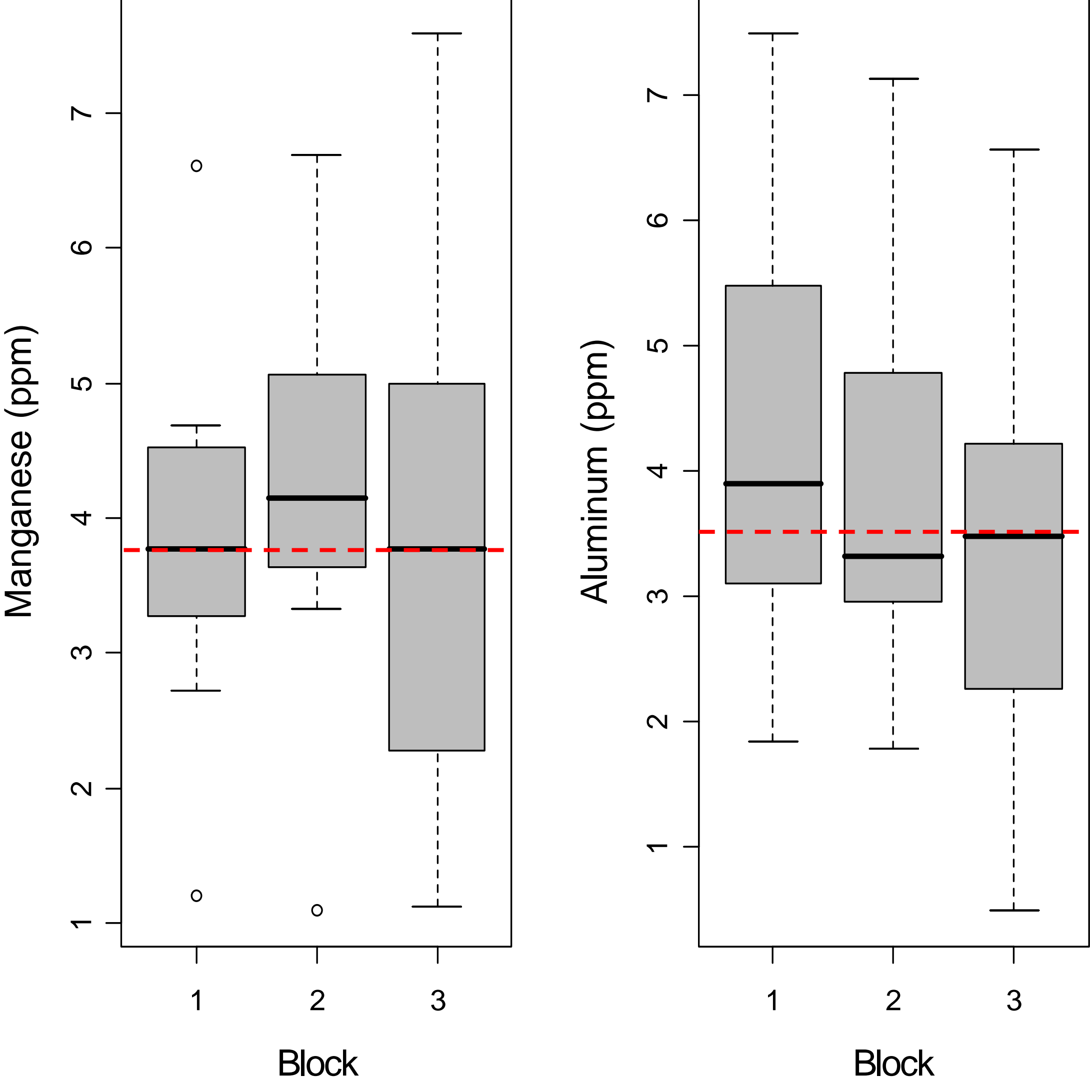
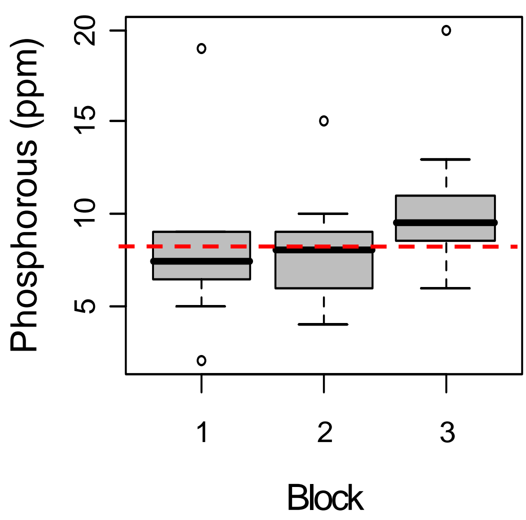



Figure 36. Manganese and Aluminum soil chemistry results. Red lines show the mean across the blocks and black lines show the mean within the blocks.



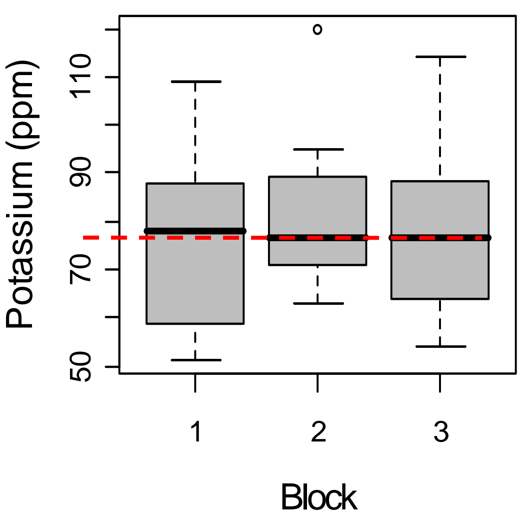
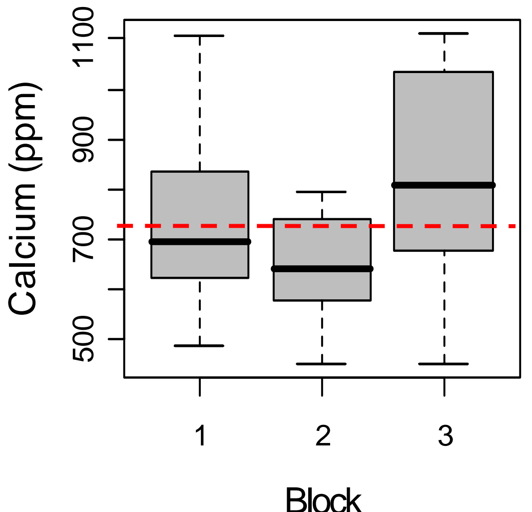
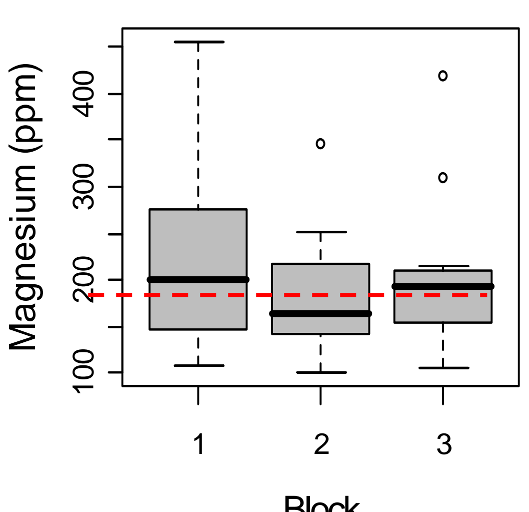




Figure 37. Phosphorous, Potassium, Calcium, and Magnesium soil chemistry results. Red lines show the mean across the blocks and black lines show the mean within the blocks.




*Soil texture*

Soil texture results show that the soil at the site is a sandy loam. Sand, silt, and clay did not significantly differ among blocks (Figure 38). Silt concentrations were a little elevated in block three, clay in block one, and sand in block two, but not enough to be statistically significant (P = 0.16).

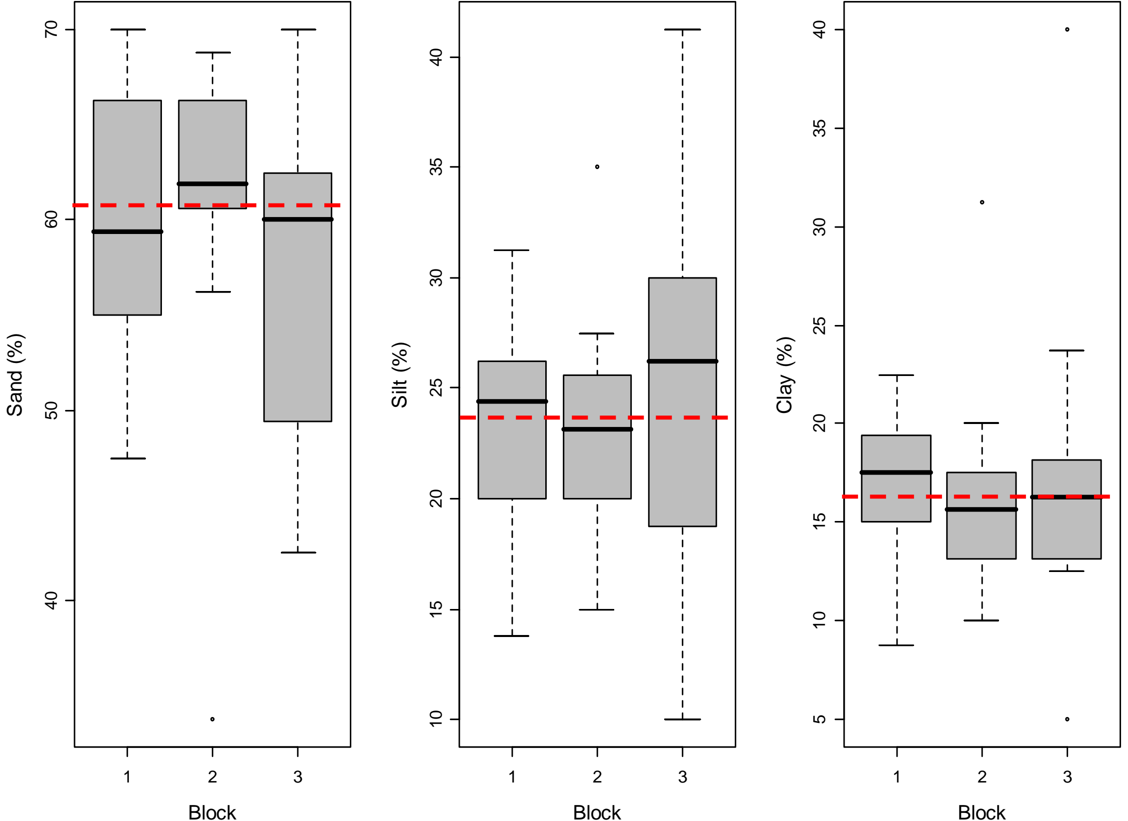



Figure 38. Soil texture analysis. The red lines show the mean across the blocks and the black lines show mean within the blocks.

*CEC and Organic Matter*

Organic matter and CEC was a little higher in block two than the other blocks, but no results were statistically significant among blocks (Figure 39).

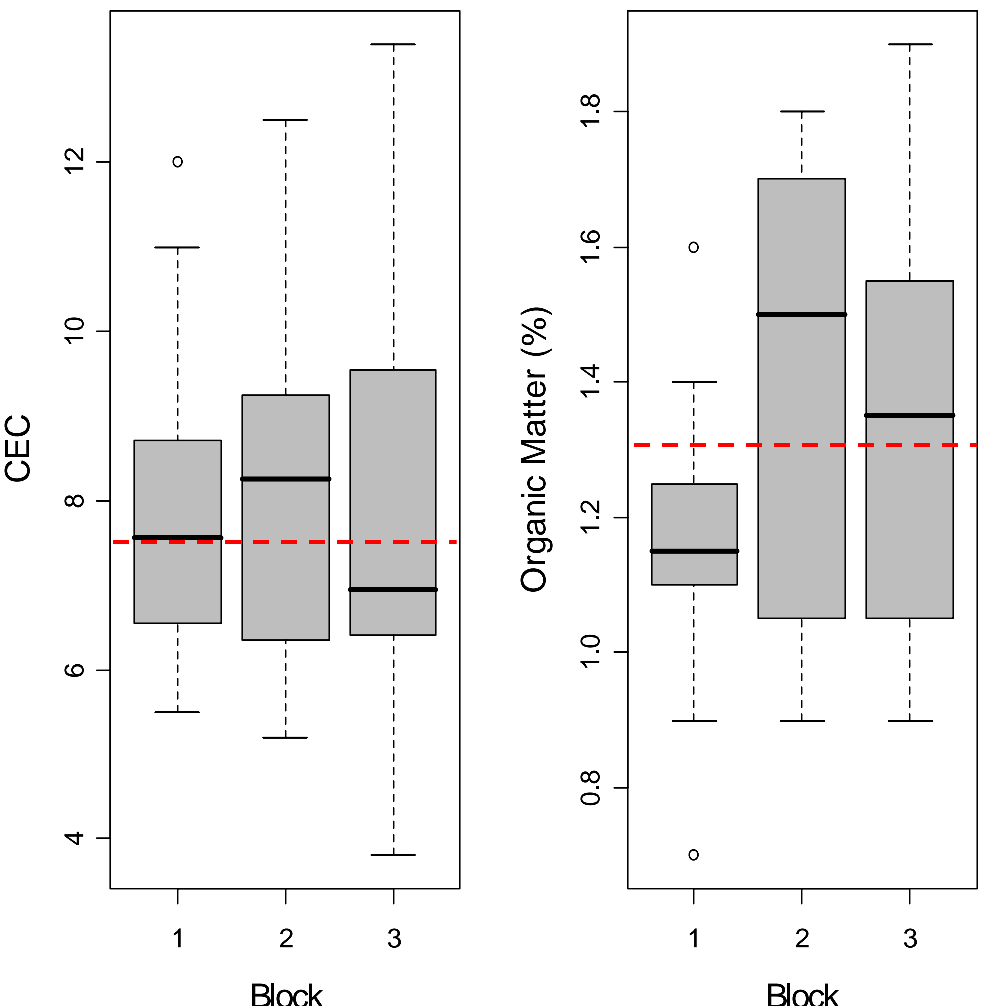



Figure 39. CEC and Organic Matter results. The red lines show the mean across the blocks and the black lines show mean within the blocks.

Blocking was not found to be important in regard to the soil analysis results.

*Survival Analysis*

Survival analysis results show that there was a significant difference ($P < 0.0001$) in survival among genetic type of American chestnut (Figure 40). The pure American seedlings were less viable than their hybrid counterparts, with F2 (7/8ths) performing slightly better than F3 (15/16ths) seedlings. There was also a significant difference ($P < 0.0001$) among soil preparation treatments (Figure 41). Seedling survival was significantly lower in the control plots with seedlings performing the best in the rip, plow, and disc plots.

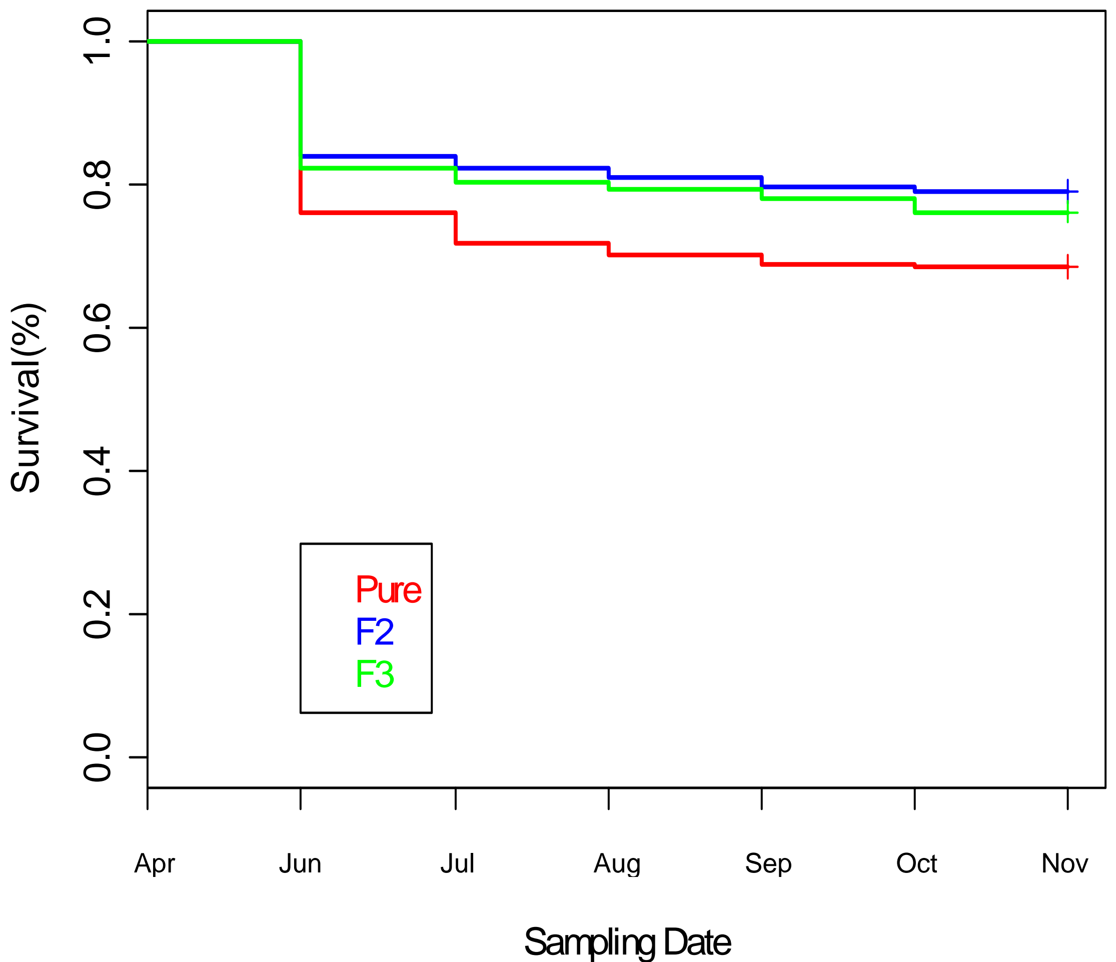



Figure 40. Survival analysis by genetic origin by cox proportional hazards model. Likelihood = 9.32 and df = 3, $P < 0.0001$.

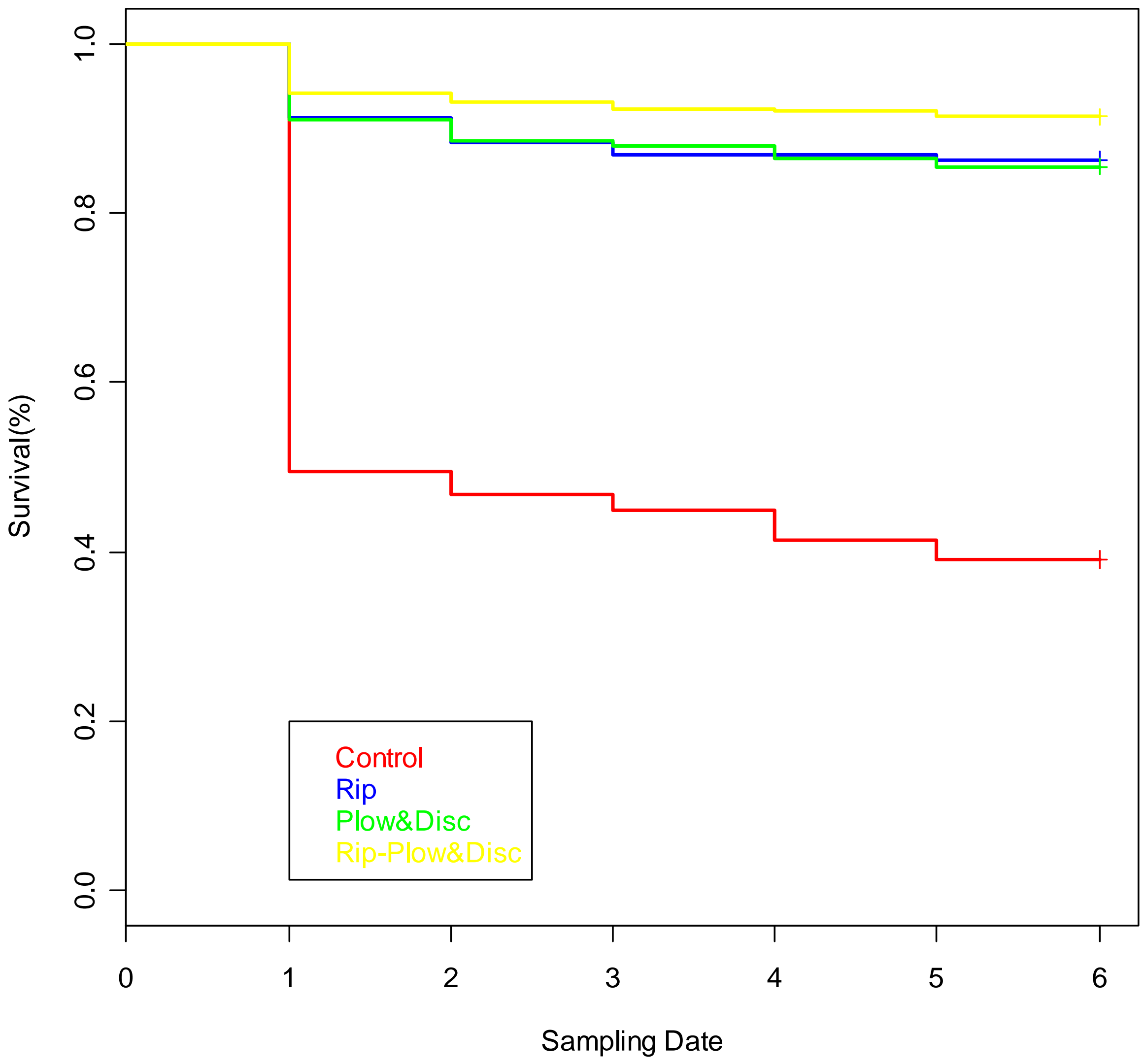



Figure 41. Survival analysis by cox proportional hazard model. Likelihood = 273, df = 3, and $P < 0.0001$.

*Growth analysis*

Seedling height was significantly different ($P < 0.0001$) in respect to the pure American chestnut seedlings. These results were similar to the survival analysis in that the F2 and F3 hybrid seedlings grew taller than the pure American chestnuts (Figure 42). There was also a significant difference ($P < 0.0001$) among treatments with the treatment plots (R, RPD, and PD) growing much taller than the control seedlings (Figure 43).

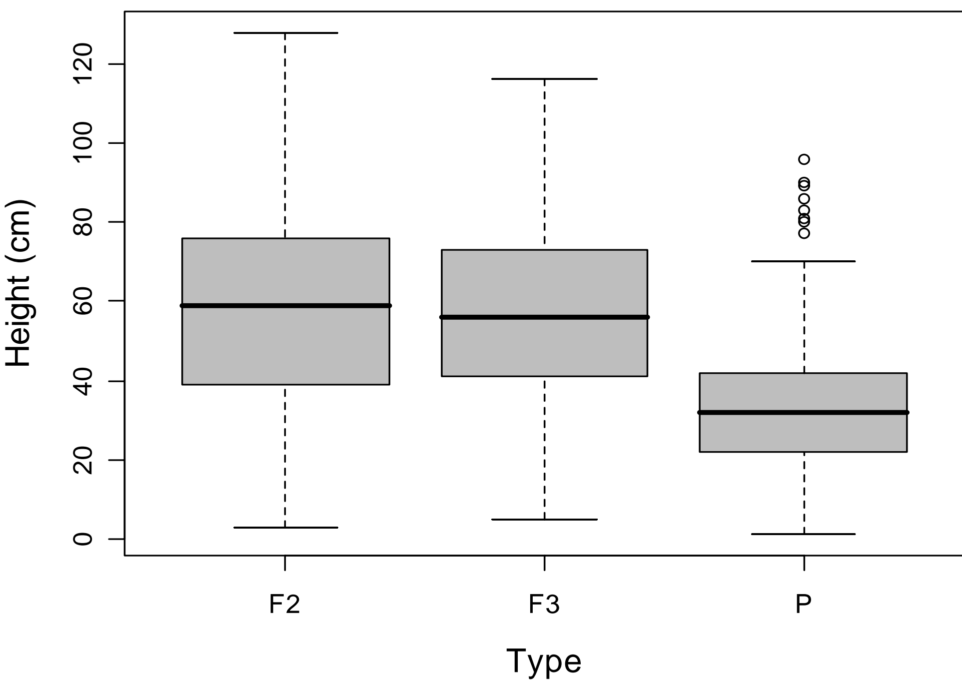



Figure 42. Seedling growth by type. F3 is 15/16$^{th}$, F2 is 7/8ths, P is pure. F = 214, df = 2, $P < 0.0001$.

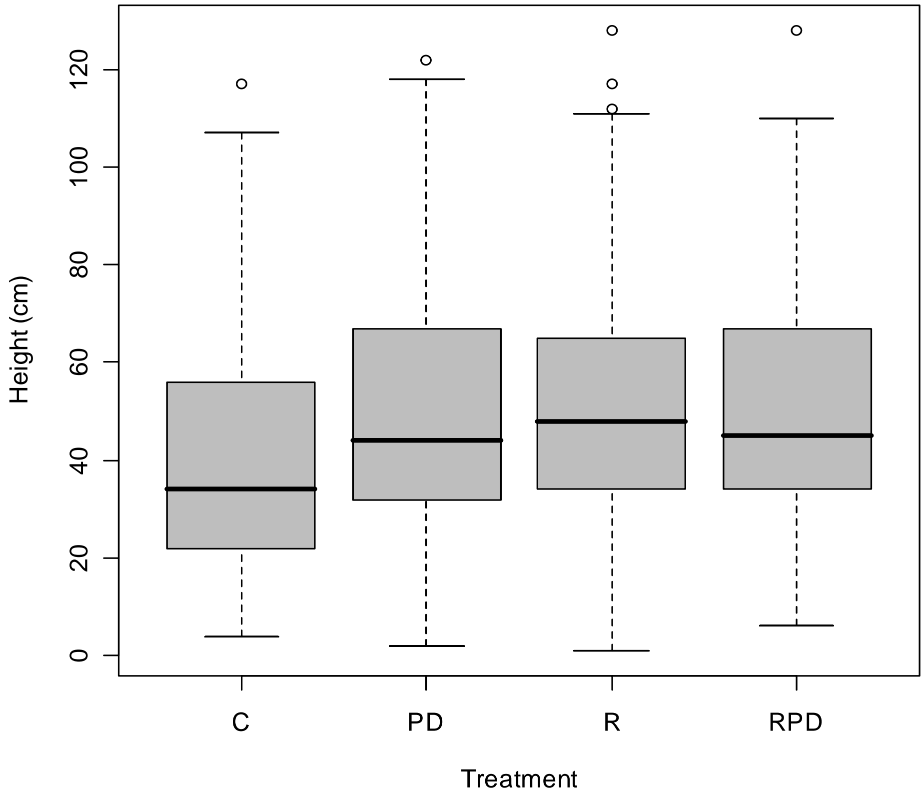



Figure 43. Seedling growth by treatment. F=12.7 df = 3, $P < 0.0001$.

*Field observations*

When walking through the field plots some observations were made on the surrounding herbs and grasses that were emerging during the growing season. In the control plot where there was no soil preparation, the herb/grass layer did come back much more intensely compared to the other treatments that had this growing layer disturbed (Figures 44 & 45). The seedlings in the control plot did however have the weed mat in the immediate growing area beside the seedling.

Figure 44. Seedlings during the growing season in the control plot. Note abundance of Lespedeza and other forbs in addition to Festuca.

Figure 45. Seedlings during the growing season in the plow and disk plot. Note decreased cover and herbaceous competition with chestnut seedlings.

***Belowground Results***

*ECM Community Composition*

Roots from 120, 1-year-old Pt inoculated chestnut seedlings were sampled in October 2007. Of these 120 samples, 102 of the root samples were morphologically identified as having ECM. Sixty of these root tips were successfully sequenced using ITS primers to genera. The other 42 were sequenced but were determined inconclusive (Table 1). This group was comprised of 19 sequences that were of moderate quality but whose similarities when compared to the GenBank were lacking certain characteristics that would provide a confident identification. Twenty-three of these sequences were of too low of quality for alignment with known sequenced ITS regions. These include sequences that were below 60%.

Table 1. Outline of the root samples from the 120 seedlings randomly sampled.

| n | ECM Presence | Result |
|---|---|---|
| 60 | Conclusive | Sequences of high quality, E-value = 0, high similarity, 0 gaps |
| 19 | Inconclusive | Sequences of moderate quality but similarity of low confidence |
| 23 | Inconclusive | Sequences of low quality (includes multiple bands) |
| 18 | No mycelium | No evidence of fungal DNA after PCR reaction |

In all samples successfully sequenced, Pt was never found (Figure 20), despite seedlings having been inoculated with Pt at the nursery! Eleven different ECM fungal genera were characterized by comparing fungi sampled, sequenced, and aligned with published sequences in Genbank. The resulting genera and the GenBank isolates that best matched the BLAST search are presented in Table 1. After the first growing season, the ECM fungal community was dominated by *Hebeloma* and *Thelephora* species (Figure 46). Of these two species, the sequencing data indicates that there may be several different strains of these genera in these plots, 8 different sequences for *Hebeloma* and 4 for *Thelephora* (Table 2).

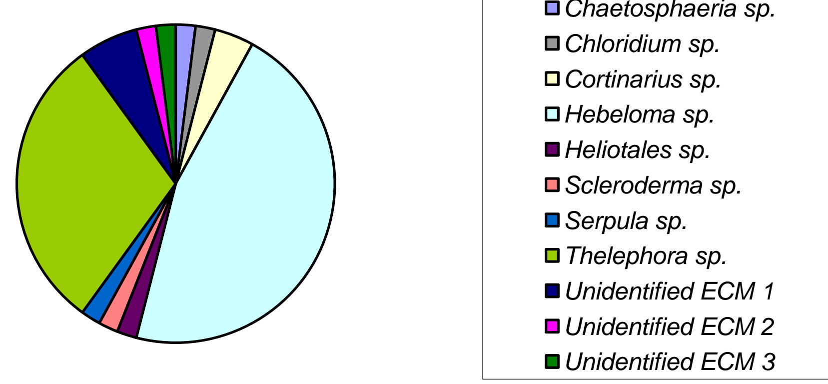



Figure 46. Frequency of ECM found during this study. This pie chart is reporting all genera that were successfully sequenced. Blocks and treatments plots are pooled for this comparison.

Table 2. All ECM genera in order of occureences (N) giving basis of identification and best similarity match to known species in BLAST of ITS sequence data.

| Identified Genera | n | Assession # | Best BLAST Match in GenBank |
|---|---|---|---|
| *Hebeloma* | 13 | EF411103 | Uncultured ectomycorrhiza (*Hebeloma*) clone L4AC8 |
| *Hebeloma* | 5 | AY748853 | Uncultured ectomycorrhiza (*Hebeloma*) isolate NEU13 |
| *Hebeloma* | 2 | DQ974696 | *Hebeloma* cf. src875 voucher src875 |
| *Hebeloma* | 2 | AY320387 | *Hebeloma* sp. GLM 43488 |
| *Hebeloma* | 1 | AY320382 | *Hebeloma sp*. GLM 42698 |
| *Hebeloma* | 1 | AY32387 | *Hebeloma sp*. Ectomycorrhizal Clone |
| *Hebeloma* | 1 | AY311525 | *Hebeloma oculatum* specimen-voucher GLM 42741 |
| *Thelephora* | 17 | EF218819 | Uncultured ectomycorrhiza (*Thelephora*) isolate UBCOCS4F |
| *Thelephora* | 1 | AF272923 | *Thelephora terrestris* specimen-voucher TAA162083 |
| *Thelephora* | 2 | DQ068970 | *Thelephora terrestris* clone NS103 |
| *Thelephora* | 2 | EF218819 | Uncultured ectomycorrhiza (*Thelephora*) isolate UBCOCS4F |
| Unidentified ECM 1 | 4 | EF484935 | Uncultured ectomycorrhizal fungus clone Riv-5 |
| *Cortinarius* | 2 | AY669693 | *Cortinarius balaustinus* voucher TUB 011894 |
| *Chloridium* | 1 | AM262403 | *Chloridium sp*. isolate 1798 |
| *Chaetosphaeria* | 1 | AF178542 | Uncultured *Chaetosphaeria* clone 4S1.21.S05 18S |
| Unidentified ECM 1 | 1 | EF484935 | Uncultured ectomycorrhizal fungus clone Riv-5 |
| *Scleroderma* | 1 | EU202691 | Uncultured Sclerodermataceae clone 92M19 |
| *Scleroderma* | 1 | AM087282 | Uncultured ectomycorrhizal Sclerodermataceae |
| *Scleroderma* | 1 | EF517491 | *Scleroderma bovista* genes (ITS region) |
| *Helotiales* | 1 | DQ914727 | *Helotiales sp*. EXP0409F |

*ECM Community per Experimental Blocks*

After evaluating the experimental blocks established for this field study, *Hebeloma* was the most abundant ECM genus found per block (Figures 47-49). This genus was identified on chestnut roots 40% to 50% of the time in each block. *Thelephora* was also evenly distributed (~30%) among each of the three blocks (Figures 47-49). With the exception of the Unidentified ECM 1, the rare species appeared specific to the experimental block. The following blocks harbored the rare species: *Scleroderma* and Unidentified ECM2 in Block 1 (Figure 47), *Chloridium* and *Cortinarius* in Block 2 (Figure 48), and *Chaetosphaeria* and *Helotiales* in Block 3 (Figure 49). Each block had similar species richness of 5, 6, and 6 different species of ECM, respectively (Figure 50). The Simpson's Index accounts for both species richness and abundance and was used to calculate species diversity. With regard to the three experimental blocks, Block 1 and 3 had a slightly higher diversity index than Block 2 (Figure 50). However, this was not statistically significant. All blocks had similar indices and ranged between 0.65 and 0.72 (Figure 51).

**Frequency of ECM Distribution in Experimental Block 1**

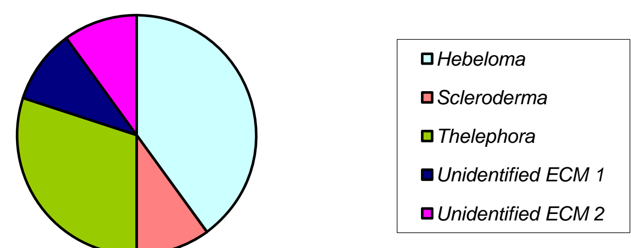



Figure 47. Frequency of ECM distribution in experimental Block 1. Genera reported here are based on the best match to known Genera based on their similarity to the reported ITS sequences in GenBank.

**Frequency of ECM Distribution in Experimental Block 2**

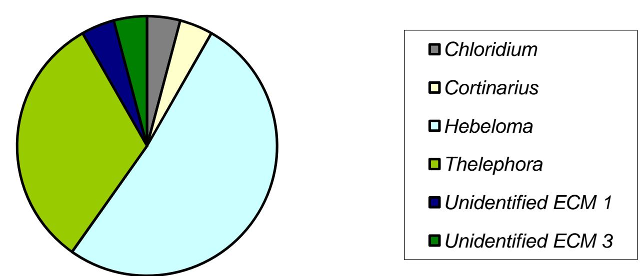



Figure 48. Frequency of ECM distribution in experimental Block 2. Genera reported here are based on the best match to known Genera based on their similarity to the reported ITS sequences in GenBank.

**Frequency of ECM Distribution in Experimental Block 3**

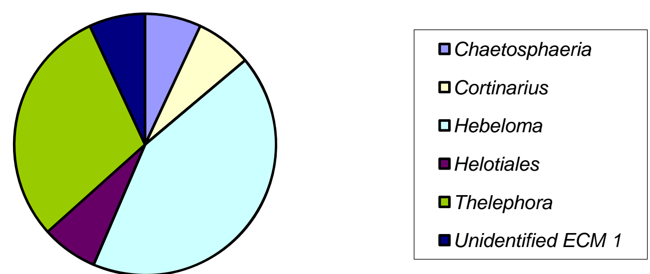



Figure 49. Frequency of ECM distribution in experimental Block 3. Genera reported here are based on the best match to known Genera based on their similarity to the reported ITS sequences in GenBank.

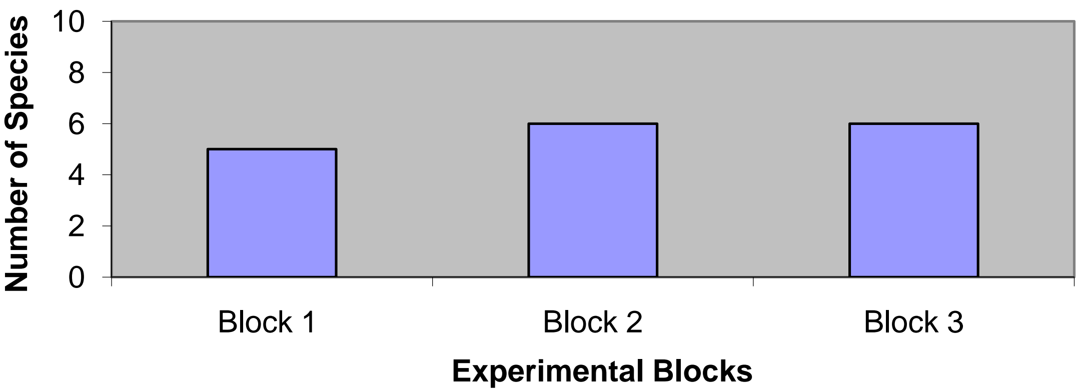



Figure 50. Species richness based on the number of different species found in each experimental block.

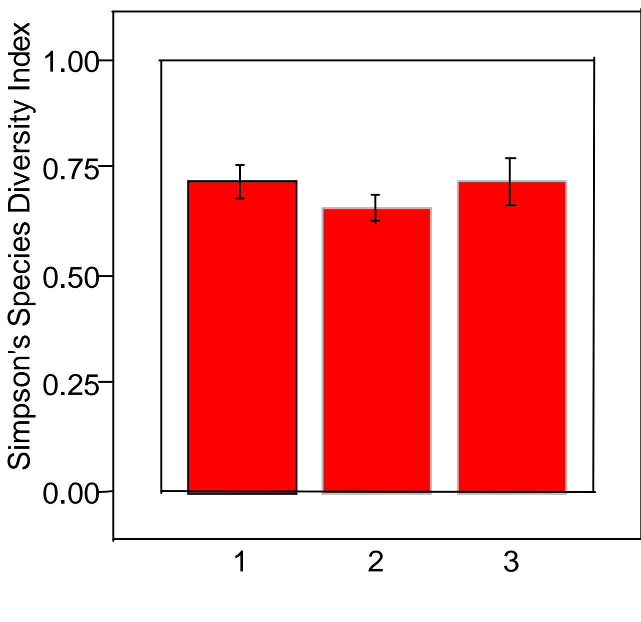



Figure 51. Comparison of species diversity per experimental block by Simpson's Index

ECM Community per Experimental Blocks

Due to the similarity of ECM species distribution among the experimental blocks described above, data for the 4 treatments were pooled. *Hebeloma* was again the most abundant ECM species found consistently throughout the treatment plots (35% - 56%) (Figures 52-55). *Thelephora*, the second most abundant species recorded in this filed study, was not found in the control plots (C) (Figure 48). It was found in larger frequencies (27 – 47%) in the ripped plot (R), ripped + plowed and disked (RPD), and plowed and disked (PD) (Figures 53-55). In the ripped + plowed and disked, *Thelephora* was found in greater abundance that *Hebeloma* 47% and 35%, respectively (Figure 54). With regard to species richness, each of the treatments had a similar number of species 4 or 5 species (Figure 56). When ECM diversity was compared, the control plots had a lower index when compared to R, RPD, and PD (Figure 57). However, this was not statistically significant.

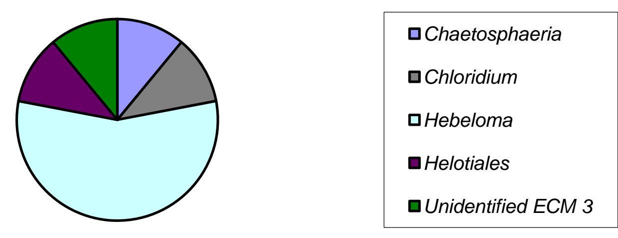



Figure 52. The frequency of all genera found in the 3 control plots.

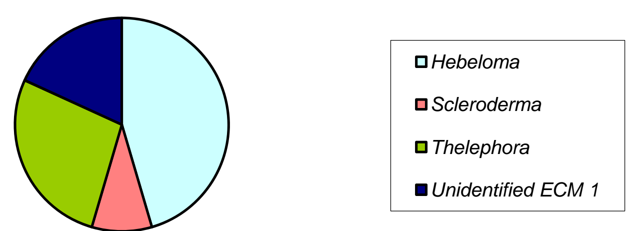



Figure 53. The frequency of all genera found in the 3 R plots.

Figure 54. The frequency of all genera found in the 3 RPD plots.

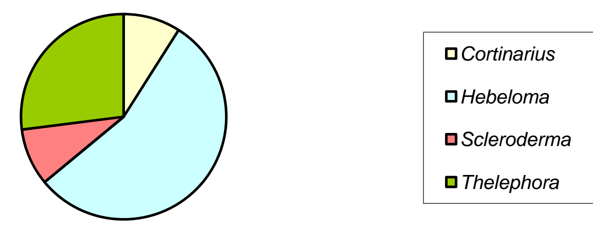



Figure 55. The frequency of all genera found in the 3 PD plots.

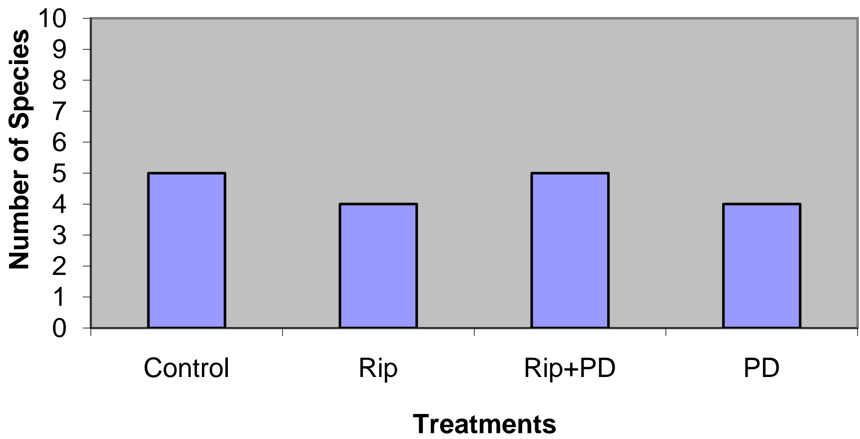



Figure 56. Number of ECM species found per treatment plot.

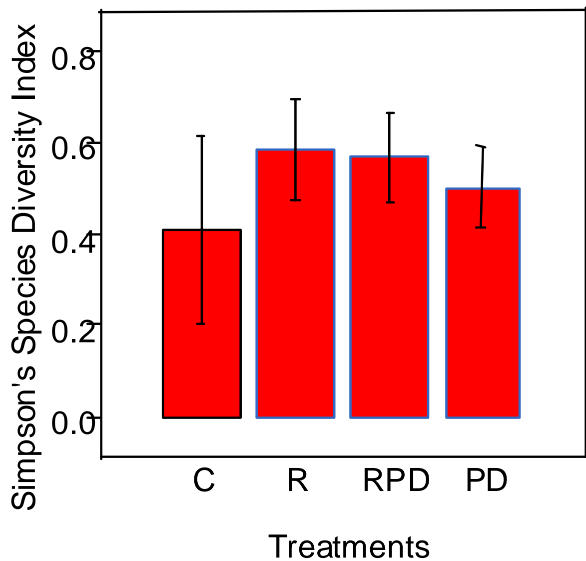


Figure 57. Comparison of species diversity per treatment by Simpson's Index

*ECM Species Richness and Diversity*

All plots were analyzed for differences in species richness and diversity among treatments. R and RPD treatment plots had a greater species richness and a higher diversity index when compared to the PD and control plot (Figure 58-59). However, these differences were not significant. Chestnut seedlings were then analyzed for correlations between the growth parameters and species diversity. With regard to survival, height (cm), basal diameter (mm), and leaf area ($cm^2$), no significant correlations existed.

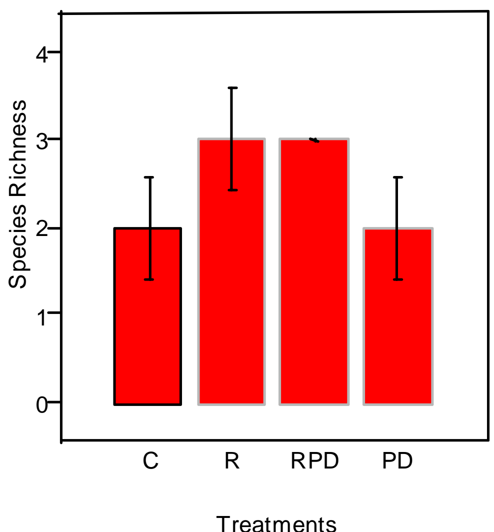


Figure 58. Number of ECM species recorded per treatment plot.

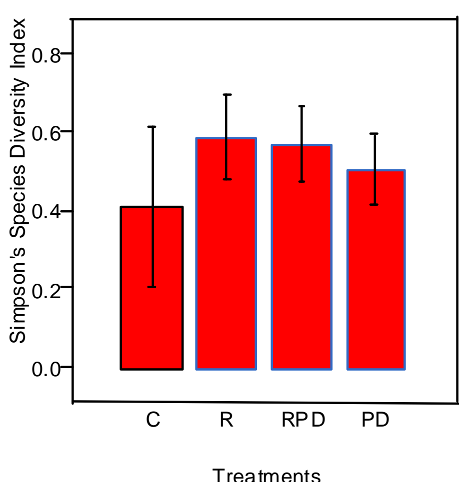



Figure 59. Comparison of species diversity per treatment by Simpson's Index

*ECM Species Effects on Host Response*

Growth rates of *Hebeloma* and *Thelephora*, the most abundant ECM species identified in this study, were compared with the non-ECM samples. Seedlings with root tips inoculated with *Thelephora* had a greater growth rate with regard to height (cm) (Figure 60.) and basal diameter (mm) (Figure 61) and leaf area ($cm^2$) (Figure 62). However, this was not statistically significant.

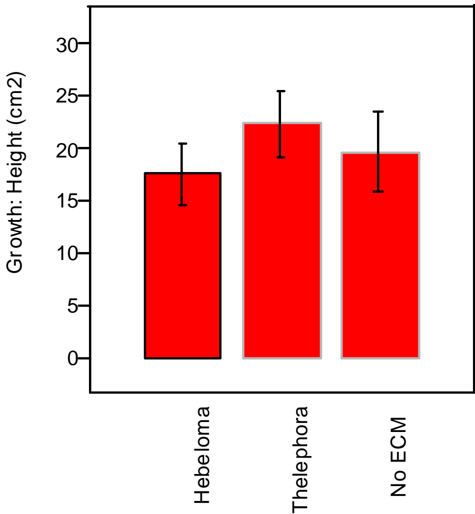



Figure 60. A comparison of growth rates (height cm) among *Hebeloma* inoculated, *Thelephora* inoculated, and non-ECM seedlings.

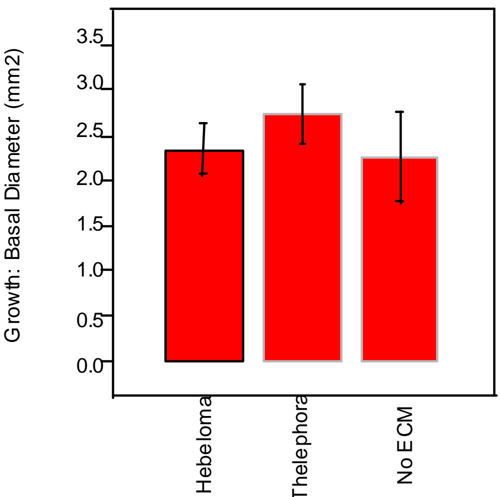



Figure 61. A comparison of growth rates (basal diameter mm) among *Hebeloma* inoculated, *Thelephora* inoculated, and non-ECM seedlings.

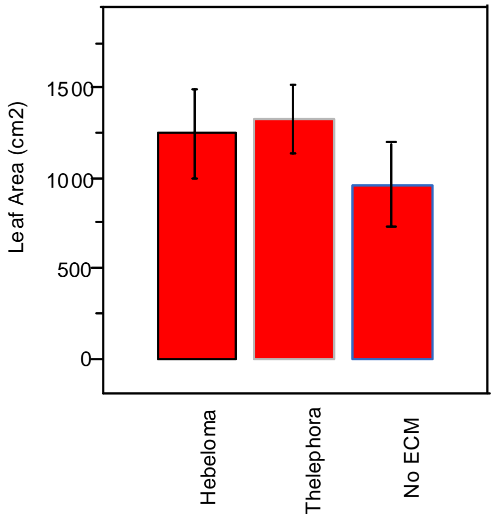



Figure 62. A comparison of leaf area ($cm^2$) among *Hebeloma* inoculated, *Thelephora* inoculated, and non-ECM seedlings.

## Discussion

### *Aboveground Dynamics*

In the first year of seedling survival and growth, American chestnut seedlings performed well on mineland sites with adequate soil preparation. Reducing soil bulk density by any method of loosening and aeration of the soil is not only beneficial to American chestnut seedling survival but also their growth. Using a soil ripper or even conventional farming equipment such as a plow and disk will also ensure an appropriate planting medium according to this study's results. All three methods (rip, plow, and disk) seem to produce the high survival rates. The effects of deep ripping were not likely seen in this study due to its short duration. Roots did not have enough time to develop and grow to the depths provided by ripping. We will likely see a treatment divergence in upcoming years between those treatment plots that were ripped and those that were not. Soil treatment also seems to have the benefit of additionally controlling grass and herb species that compete with newly planted seedlings in the first year by turning them into the soil and slowing reproduction.

The planting protocol we employed most likely had a very positive impact on survival and height. As we observed during the growing season, herbs and grasses eventually grew back quite vigorously, and for that reason the weed mat was probably another variable that decreased competitiveness, soil drought, and increased seedling growth. The planting method implemented in this experiment also used an agent (Terra Sorb) that increased moisture in the root zone thus lessening the impact of soil drought, giving the seedlings a healthy start and a cushion to make it through the dry periods we had on the site that summer. The soil chemistry results revealed that nitrogen was very low on site, in fact, it was below the 2 ppm threshold limit (thus why not reported) so using the fertilizer pellets may also be important, especially to provide nitrogen. Interestingly, the data also suggest that there is likely a hybrid vigor effect with F2 and F3 progeny exhibiting higher growth and survival. As a result, when planting American chestnuts, if hybrids are available they should be used. Considering all these factors it would be advantageous to use these same methods when planting on similar mineland sites when using the expensive American chestnut hybrids.

The site soil conditions and environmental conditions were not significantly different among blocks, and therefore blocking shouldn't have had an effect on seedling growth. There were slight differences, but not enough to be biologically significant. Interestingly, pH, CEC, and most measures of major ions were well within standards for normal agricultural soils which suggest that these soils have likely matured with age following reclamation. Only nitrogen was below levels that were expected for healthy plant growth. Both Lespedeza (a nitrogen fixer) and Festuca grass (low N requirement) will do well under these conditions.

This project accomplished all of its major objectives. It showed that alleviating soil compaction on minelands can be done quite easily with equipment typically used by mine owners and even farmland owners. By manipulating SBD and aeration, seedlings were able to grow vigorously and resulted in dramatically increased survival and growth. These are all factors necessary for successful reforestation. These newly planted seedlings are already showing signs of reproduction – a bur was noticed on one of the hybrid seedlings during the summer months. Soon these seedlings will become a

forest, producing nuts for wildlife and providing cover and shelter to many different species. Hopefully the fruits of this labor will be seen for generations to come. Not only will this project have forested relatively barren mineland, but also will have brought back the American chestnut forest as well.

***Belowground***

One hundred and twenty American Chestnut seedlings were randomly selected for root sampling at the end of the 2007 growing season. Of those, 60 of the root tips were successfully sequenced using ITS primers to genera based on high similarity of known sequences in GenBank. The other 42 were sequenced but were determined inconclusive; nineteen samples generated sequences that were lacking in certain characteristics that would provide a confident identification. What was most curious with regard to the ECM sampling data was the complete absence of Pt from this field site. Pt was the fungus used to inoculate the chestnut seedlings in the state nursery one year prior to planting. It has been previously reported to persist in mine sites several years after being introduced; therefore at least some survival of this ECM fungus was anticipated (Marx et al. 1977; Grossnickle and Reid 1982). McFee and Fortin (1988) reported *Pisolithus* to be a slow grower and may be a poor competitor. Thus, the lack of Pt in the root samples could be reflective of competitive exclusion by the more abundant *Hebeloma* and *Thelephora*. Pt has been reported to grow relatively fast via mycelial threads in soils that have been fumigated. However in non-sterile soil Pt has been reported to be out competed by *Thelephora terrestris* (Ruehle 1983).

*Hebeloma* was the most abundant fungi reported in this field study. The sequencing data indicates that there may be 8 different strains of these genera in these plots. *Hebeloma* species have been reported to associate readily with a taxonomically diverse group of plants species including *Pinus*, *Quercus*, and *Populus* (Marmeisse et al. 2004). *Hebeloma crustuliniforme* has been described on American chestnut root tips in Wisconsin (Volk 2007) and under sterile conditions in the greenhouse (Hiremath per. comm.). Its ecological preference has been reported to be in sandy soils with little organic matter, thriving in recently disturbed soils with little humus accumulation (Guidot et al. 2002). The current field site is recently disturbed, sandy, well drained, with surrounding *Pinus* species. Several *Hebeloma* basidiocarps (fruiting bodies) were collected underneath chestnut seedlings, which provided additional evidence confirming the sequencing data.

*Thelephora* was the second most abundant genera found in the field plots. *Thelephora* species are reported to be a generalist and early succession ECM fungi commonly found infecting root systems in the early stages (Deacon and Fleming 1992). In addition, this genus has been previously reported on chestnut in mine reclamation projects (Bauman et al. unpublished data). *T. terrestris* has been described as a successful colonizer particularly in greenhouse and nursery environments where spore concentrations are in high abundance (Ingleby and Mason 1996). Interestingly, this field study *Thelephora* was not found in the control plots. Considering the competition-colonization hypothesis, *Thelephora* appears to be a good colonizer in open sites but as a tradeoff may be displaced by other species when resources become limited (Hastings 1980). The sequencing results identifying *Thelephora* as the dominant genus in the treatment plots suggest that this colonizing strategy is being employed.

There were no statistical differences with regard to species richness or ECM species diversity. There were no statistical correlations between diversity and growth parameters of the chestnut seedlings (data not reported). However, a trend existed that may suggest that a greater ECM biodiversity may aid in seedling establishment. Previous research has suggested that plots with higher diversity and species richness may have an increased chance of harboring a species more disturbance-resistant (Tilman, 1996). In addition, increased diversity may contain an ECM species more efficient at extracting a greater assortment of nutrients (Van der Heijden et al. 2003). Further, a diverse mycorrhizal community should have an increased inoculum potential translating into increased root colonization and possible greater host response (Kernaghan 2005). However, this study did not statistically demonstrate this theory.

Growth rates of *Hebeloma* and *Thelephora* colonized seedlings were not significantly greater than their non-ECM counterparts. There was a slight trend indicating that *Thelephora* inoculated seedlings had both a greater growth and survival rate. However, it has been well documented that ECM inoculated seedlings are better competitors for water and nutrients as well as more tolerant to heavy metal soils and soil pathogens (Marx 1972; Van der Heijden et al. 2003; Sprouse 2004; Walker et al. 2004; Nara 2005). Thus for this symbiosis to be beneficial to the host plant more time maybe required for mycorrhizal development. Conversely, it has been speculated that certain plant-fungus combinations and environmental conditions could relegate a once beneficial symbiosis to one that is parasitic on the plant host (Graham and Miller 2005; Selosse et al 2006). In an area as depleted as post-mining soils soil fertility is extremely limited and the fungal symbiont may become a greater carbon cost to the plant. However, this was not the case in this study, non-ECM seedlings where comparable to ECM seedlings.

What was demonstrated at the end of the first growing season was the influence proper site preparation has on seedling establishment. Plots that were treated with ripping, plow and disking, or the combination of the two resulted in significantly higher survival and growth rates when compared to the control plots. Statistically teasing out the influence various ECM species have on the chestnut seedling may require more time for mature mycorrhizas to develop in the field. In addition, *P. tinctorius* may require more time to colonize roots when under competition with indigenous superior competitors. The slight trends observed with regard to ECM species diversity merits further investigation into the influence species diversity may have on seedling establishment. Better understanding of native fungi that may be promoted by various site preparation methods may aid in future management strategies for hardwood seedlings in reforestation projects.

**<u>Literature cited</u>**

Barker, S.J., Tagu, D., and Delp, G. 1998. Regulation of root and fungal morphogenesis in mycorrhizal symbioses. Plant Physiology. 116: 1201-1207

Bradshaw, A. D., 1984. Ecological principles and land reclamation practice. Landscape Planning. 11: 35-48

Brundrett, M. C. 2002. Coevolution of roots and mycorrhizas of land plants. New Phytologist 154: 275-304.

Burnham C.R. 1988. The restoration of the American chestnut. American Scientist 76: 478-486.

Bussler, B.H., Byrnes, W.R., Pope, P.E., and Caney, W.R. 1984. Properties of minesoil reclaimed for forest land use. Soil Sci. Soc. A,. J. 48:178-184.

Deacon J. W. and Fleming, L. V. 1992. Interactions of ectomycorrhizal fungi. In: Allen MF (eds) Mycorrhizal functioning: an integrative plant-fungal process. Chapman and Hall, New York, pp. 249-300.

Degrood, S. H., Claassen, V. P., Scow, K. M. 2004. Microbial community composition on native and drastically disturbed serpentine soils. Soil Biology and Biochemistry 37: 1427-1435.

Dickie, I. A. and Reich, P. B 2005. Ectomycorrhizal fungal communities at forest edges. Journal of Ecology. 93: 244-255

Gardes, M., and Burns, T. D. 1993. ITS Primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113-118

Grahm, J. H. and Miller, R. M. 2005. Mycorrhizas: Gene to function. Plant and Soil 274: 79-100

Grossnickle, S. C., and Reid, C. P. P. 1982. The use of ectomycorrhizal conifer seedlings in the revegetation of a high-elevation mine site. Canadian Journal of Forest Research 12: 354-361.

Guidot, A. Gryta, H., Gourbiere, F., Debaud J-C., Marmeisse, R. 2006. Forest habitat characteristics affect balance between sexual reproduction and clonal propagation of the ectomycorhizzal mushroom, *Hebeloma cylindrosporum*. Oikos 99: 25-36.

Hart, M. M., Reader, R. J., and Klironomos, J. N. 2003. Plant coexistence mediated by arbuscular mycorrhizal fungi. Trends in Ecology and Evolution 18: 418-423

Hastings, A. 1980. Disturbance, coexistence, history, and competition for space. Theoretical Population Biology 18: 363-373

Herendeen, R., Keiffer, C., and McCarthy, B.C. 2006. Status Report on Chestnut Restoration Project on Eastern Ohio Strip Mines. Ohio Plant Biotechnology Consortium, Columbus, Ohio.

Ingleby, K., and Mason, P. A. Ectomycorrhizas of the *Thelephora terrestris* formed with *Eucalyptus globulus*. Mycologia 88: 548-553

Izzo, A., Nguyen, D. T., and Bruns, T. D. 2006. Spatial structure and richness of ectomycorrhizal fungi colonizing bioassay seedlings from resistant propagules in a Sierra Nevada forest: comparisons using two hosts that exhibit different seedling establishment patterns. Mycologia 98: 374-383

Kernaghan, G. 2005. Mycorrhizal diversity: Cause and effect? Pedo Biologia 49: 511-520

Manchulla, G., Bruns, M. A., and Scow, K. M. 2005. Microbial Properties of Mine Spoil Materials in the Initial Stages of Soil Development.

Marmeisse, R., Guidot, A., Gay, G. Lambilliote, R. Sentenac, H., Combier, J. P., Melayah, D., Fraissinet-Tachet, L., and Debaud, J. C. 2004. *Hebeloma cylindrosporum* – a model species to study ectomycorrhizal symbiosis from gene to ecosystem. New Phytologist. 163: 481- 498.

Marx. D. H. 1972. Ectomycorrhizae as biological deterrents 3558 to pathogenic root infections. Annual Review of Phytopathology 10: 429-454

Marx, D. H. 1991. The practical significance of ectomycorrhizae in forest establishment. Ecophysiology of Ectomycorrhizae of Forest Trees, Marcus Wallenberg Foundation Symposia Proceedings. 7: 54-90

McCament, C.L. and McCarthy, B.C.2005. Two-year response of American chestnut (*Castanea dentata*) seedlings to shelterwood harvesting and fire in a mixed-oak forest ecosystem. Can. J. For. Res. 35:740-749.

McFee, B. J. and Fortin, J. A. 1988. Comparative effect of the soil microflora on ectomycorrhizal inoculation of conifer seedlings. New Phytologist 108: 443-449

Nara, K. 2005. Ectomycorrhizal networks and seedling establishment during early primary succession. New Phytologist. 169: 169-178

Nara, K. 2006. Pioneer dwarf willow may facilitate tree succession by providing late colonizers with compatible ectomycorrhizal fungi in a primary successional volcanic desert. New Phytologist171: 187-198

Rathfon, M. K., Griffin, G. L., and Groninger, J. 2004. Status of reforested mine site in southwestern Indiana reclaimed under the Indiana mining regulatory program. Purdue University Cooperative Extension Service, West Lafayette, IN. FNR-251.

Reddy, S. M., Pandey, A. K., Melayah, D. Marmeisse, R., and Gay, G. 2003. The auxin response gene Pp-C61 is up-regulated in *Pinus pinaster* roots following inoculation with ectomycorrhizal fungi. Plant, Cell and Environment. 26: 681-691.

Reddy, S. M., Hitchin, S., Melayah, D., Pandey, A. K., Raffier, C., Henderson, J., Marmeisse, R., and Gay, G. 2005. The auxin-inducible GH3 homologue Pp-GH3.16 is down-regulated in *Pinus pinaster* root systems on ectomycorrhizal symbiosis establishment. New Phytologist. 170: 391-400

Reynolds, H. L., Packer, A., Bever, J. D., and Clay, K. 2003. Grassroots ecology: Plant-microbe-soil interactions as drivers of plant community structure and dynamics. Ecology: 84: 2281-2291

Ruehle, J. L. 1983. The relationship between lateral-root development and spread of *Pisolithus tinctorius* ectomycorrhizae after planting of container grown loblolly pine seedling. Forest Science 29: 519-526

Taylor, A. F. S. 2002. Fungal diversity in ectomycorrhizal communities: sampling effort and species detection. Plant and Soil 244: 19-28

Selosse, M., Richard, F., He, X., and Simard, S. W. 2006. Mycorrhizal networks: des liaisons dangereuses? Trends in Ecology and Evolution 21:621-628

Sprouse, J. C. 2004. Reforestation on abandoned mine land (AML) and bond release grassland. www.marcc.osmre.gov

Tilman, D. 1996 Biodiversity: Population verses ecosystem stability. Ecology. 77: 350-363.

Torbert, J.L., Burger, J.A., Johnson, J.E., and Andrews, J.E., 1994. Indices for indirect estimates of productivity of tree crop. Final Report, OSM Cooperative Agreement GR996511. Virginia Polytechnic Institute and State University Blacksburg, VA. 22p.

Torbert, J. L. and Burger J. A. 2000. Forest land reclamation. In: Reclamation of Drastically Disturbed Lands. pp. 371-378 Agronomy Monograph No. 41. Soil Science Society of America, Madison, WI.

Van der Heijden, E. W. and Kuyper, T. W. 2003. Ecological strategies of ectomycorrhizal fungi of *Salix repens*: root manipulation versus root replacement. Oikos 103: 668-680

Volk, T. 2007 http://tomvolkfungi.net/

Walker, R. F., McLaughlin, S. B., and West, D.C. 2004. Establishment of sweet birch on surface mine spoil as influenced by mycorrhizal inoculation and fertility. Restoration Ecology. 12: 8-19