The American Chestnut Foundation Final Grant Report

Project Title: Plant and Fungal Dynamics in American Chestnut Restoration

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Report Summary: This grant report summarizes the following objectives: 1) fifth year survival and growth data of American and backcrossed chestnuts on a field site in Dresden, Ohio, 2) document natural cankers and report on the field resistance of the backcrossed chestnut lines, vegetation survey in chestnut plots, and 4) ectomycorrhizal (ECM) community analysis on chestnut in restorations. Undergraduate student interns participated in this research project while collaborating with professionals from Ohio institutions: Miami University, Ohio University, Muskingum University, The Ohio Chapter of TACF, and the US Forest Service.

Duration of the Project: May 2012 - May 2013

Manuscripts based on the following report are currently in prep:
Asterisks indicate student research involvement

Bauman, J.M., *Cochran, C., Keiffer, C. and McCarthy, B. Overcoming arrested succession: Field survival of backcrossed American chestnuts in surface coal mine reclamation.

Bauman, J. M., *Francino, S., and Santas, A. 2013. Fungal interactions and their influence on establishing American chestnut (*Castanea dentata*) seedlings in Ohio coal mine restoration.

Bauman, J.M., *Cochran, C., *Chapman, J., and Gilland, K.E. Vegetation spatial and temporal patterns in response to management strategies on reclaimed coal mined landscapes.

Narrative Summary:

American chestnut's fast growth rate, early nut production, and quality of timber make this a desired species for use in reforestation projects (Jacobs et al. 2013). This species tolerates a wide range of ecological conditions, including tolerance to drought and low pH, typical of coal mined sites (Jacobs 2007). Coupled with proper planting methods, chestnuts have the ability to quickly establish on reclaimed coal mine sites (McCarthy et al. 2008). Using backcrossed varieties in mine reclamation projects provides a reliable restoration tree species. This also provides field trials for chestnut lines in the native range of American chestnut. Further assessment of existing sites will provide valuable insight to field growth, long-term survival, and blight-resistance potential of the backcrossed genotypes.

Like other members of Fagaceae, American chestnut forms ectomycorrhizas with certain fungal species (Hiremath and Lehtoma 2007; Palmer et al. 2008; Bauman et al. 2012). Previous studies have documented the benefits that this symbiosis has on many conifers and angiosperms in reforestation projects (Marx 1991). These benefits include greater access to water, nutrients, alleviation of metal toxicity, and protection from root pathogens (Cordell et al.1999). In turn, these fungi receive carbon in the form of photosynthates from their plant host forming a mutualistic relationship between plant and fungi. Developing management strategies that enhance soil microorganism activity is integral to the recovery of soil properties necessary for a resilient landscape (Bradshaw 1984; Allen et al. 2002). Maximizing growth and symbiotic interactions may aid in the long-term survival and recruitment of other ECM tree species.

Chestnut's cultural significance, ecological importance, and value as a timber crop have motivated an enthusiastic pursuit to restore this species (Jacobs 2013). Coupling planting protocols with American chestnut restoration accomplishes two goals; reintroduction of chestnut aids in reforesting marginal lands left from mining into an ecological and economical valuable tree crop and provides additional data for field testing of blight-resistant hybrids within the native range of chestnut. The objective of this study was to evaluate soil sub-surface treatments using various chestnut lines (pure American, BC_2F_1 and BC_3F_1). This paper reports on: 1) the influence mechanical soil preparation has on the targeted restoration tree 2) growth and survival of different chestnut seedling lines, 3) the presence of stem cankers caused by natural infection of chestnut blight fungus, *C. parasitica*, 4) the composition of vegetation per treatment, and 5) the succession of ECM on chestnut in restoration.

Research Methods:

Study Site:

The field site used for this study is located in the Tri-Valley Wildlife Management Area (TVWMA), Muskingum County, central Ohio, USA (40° 11' 32" N, 81° 98' 35" W). This coal surface mine site was reclaimed under SMCRA in 1978. Topsoil that was stockpiled on the site was replaced during reclamation to varying depths. Reclamation records from the county indicate seed mixes that included Birdsfoot-Trefoil *(Lotus corniculatus),* Tall Fescue, *(Festuca arundinacea),* Orchard Grass *(Dactylis glomerata),* Alfalfa *(Medicago sativa),* Red Clover *(Trifolium pratense),* Rye Grass *(Lolium perenne),* Timothy *(Phleum pratense),* Kentucky Blue Grass *(Poa pratensis),* and Chinese Lespedeza *(Lespedeza cuneata).* Small pockets of forest comprised primarily of *Quercus, Pinus,* and *Acer* species were left undisturbed at the time these lands were mined. 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).

This study used one-year-old, bare root chestnut seedlings that were sown in March 2006 at the State Nursery in Marietta, Ohio by the Ohio Department of Natural Resources. Seedlings were germinated in seed beds with soils that were injected with spores of mycorrhizal fungus, *Pisolithus tinctorius.* Seedlings were nursery grown for one year and then lifted as bare root seedlings in March of 2007 (J. Hopkins pers. comm.). The seed lines were comprised of the following: 507 pure American chestnuts (*C. dentata*), 257 backcrossed chestnuts BC_2F_1 (backcrossed to create a progeny that is $7/8^{th}$ *C. dentata* and $1/8^{th}$ *C. mollissima*) and 423 backcrossed chestnuts BC_3F_1 (backcrossed to create a progeny that is $15/16^{th}$ *C. dentata* and $1/16^{th}$ *C. mollissima*; Table 1).

Table 1. Table of the seedling parental origin, cross (Identifier), accession number assigned by The Ohio State Nursery, the purity of the *C. dentata* seed lines, number of seedlings planted as one year- old bare root seedlings, and the seedling code used in this study.

Parental origin	Seedling identifier	Accession number	*C. dentata* purity	Cross Designation	Seedlings Planted	Seedling code
PA	OU	06006A	Pure American	Pure	507	Pure Am
NY	P-11XOPEN	06006B	7/8	BC_2F_1	257	BC_2
NY	SA417XOPEN	06006C	15/16	BC_3F_1	423	BC_3

The study site was initiated in the spring of 2007. Three experimental blocks, each containing the control and three soil treatments, were set-up prior to planting. Each block was 73 x 36 m with four 18 x 36 m treatment plots contained within (Figure 1). Each block was replicated three times. The following treatment plots were established: 1) a control (C) that was left undisturbed, 2) a plot cross-ripped (R) at a depth of approximately 1 m created by a D-6 dozer with a 1.0 m steel ripper bar attachment, 3) a plowed and disked (PD) plot installed by a conventional tractor (PD), and 4) a ripped + plowed and disked plot (RPD). A total of 1200 one year- old chestnut seedlings were planted in the treatment plots (12 plots, approximately 100 seedlings per plot) as bare rootstock in March of 2007 at a spacing of 2.15 x 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 m × 1 m weed mat was used around each seedling to prevent competition from regenerating herbaceous plant species. Also, a 1.5-m tall chicken wire cage was installed to prevent browse (McCarthy et al. 2010). Soil cores were collected to analyze soil chemistry and bulk density.

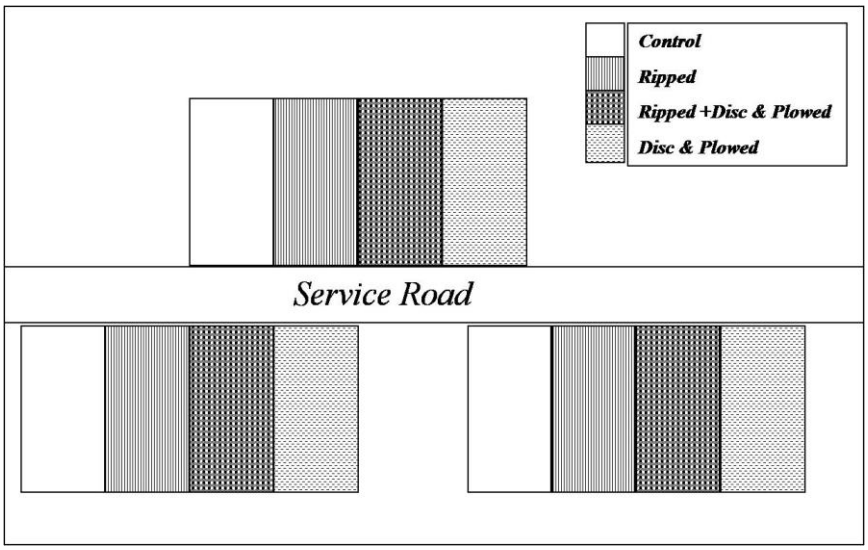

Fig. 1. Field plot block design consisted of four treatments per block: control (C), ripped (R), ripped + plowed and disked (RPD), and plowed and disked (PD). Each block was 73 x 36 m with each treatment 18 x 36 m within. Each block was replicated three times.

Data Collection:

Survival data was recorded as presence and absence in August of 2007, 2008, 2009, 2011, and 2012. In August of the 2007, 2009, and 2012, growth data was collected from each surviving chestnut. Seedlings were measured by height and basal diameter upon planting. Height (cm) was measured using a meter stick from soil level to the tip of the main stem. Basal diameter (mm) was measured 3 cm above the root collar by using a digital caliper and then converted to cm. Growth was then derived from the difference between the original measurements (recorded April 2007) and the measurement recorded at the end of each particular time period (2007, 2009, and 2012). Seedlings that were dead were scored a 0 growth (cm). A volume index (height cm × basal diameter cm^2) was be used to estimate the volume of each chestnut seedling after five field seasons. During the fifth field season, all seedlings were scored for the presence or absence of natural chestnut blight cankers (Fig 2).

Fig. 2. Basal canker produced by pathogen *C. parasitica* on pure American chestnut seedling in a test plot in the field site of the Tri-Valley Wildlife Management Area, Muskingum County, central Ohio, USA (40° 11' 32" N, 81° 98' 35" W).

ECM Collection and Identification

In April 2012 (after five field seasons), 60 chestnuts planted as bare root seedlings and another 60 were selected for root sampling across all mechanical treatments. 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. Between 100 and 300 root tips were randomly selected from each seedling and viewed under a dissecting microscope for the presence of a fungal sheath (120 samples, 31,400 root tips). Each ECM tip was sorted into one of the nine morphotypes based on their surface color, texture, emanating hyphae, and rhizomorphs (Bauman et al. 2013). Two root tips of each morphotype per seedling were selected for DNA extracting and sequencing. A 3-mm section of the root tip was transferred to a microcentifuge tube and stored at -70˚ C until DNA extraction. As described in Bauman et al. (2013), fungi were identified by DNA sequencing of the internal transcribed spacer (ITS) region.

Statistical Analysis:

Cox proportional hazard model was used to determine significant differences in survival among treatments and seedling types using survival data after 5, 18, 30 and 54 months in the field. Growth was derived from the difference between the original measurements (recorded before bud break, a few weeks after planting) minus the current measurement. To avoid negative values, seedlings that were dead were scored as 0 (cm). Growth rates were compared over the 1^{st}, 3^{rd} and 5^{th} growing season using an analysis of variance with repeated measures added as an error-term. A volume index (height cm × basal diameter cm^2) was used to estimate the volume of each chestnut seedling using a log+1 transformation to meet the assumption of equal variances. Volume was compared using a two-way, mixed-model ANOVA using soil treatment and seedling type as main effects and the block as a random effect. For height, basal diameter, and seedling volume, a Bonferroni pairwise t-tests was used as a *post hoc.* To compare presence of chestnut blight cankered trees by seed type, a Pearson's Chi-Square (X^2) was used.

To evaluate the efficacy of our sampling protocol for the vegetation and ECM community analysis, overall species richness was estimated using Chao II and Jackknife II. A

non-metric multidimensional scaling (NMDS) ordination using a Bray–Curtis dissimilarity matrix followed by a permutational multivariate analysis of variance was used to test for significant differences between vegetation and ECM fungal communities. A simple regression was used to determine the effect of plant biomass on ECM root colonization. All statistical tests were performed using R v2.91 (R Development Core Team 2009).

Results:

Soil Properties:

When soil samples were compared there were no differences among treatment plots (Tables 1 and 2). 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. The only differences noted among the treatment plots were in the measurements of bulk densities. Bulk densities (mg m-3) in the treated plots were similar and were as follows: R 1.48, PD 1.47, and RPD 1.59. These were less than the bulk densities measured in the control plots which averaged 1.64 mg m^{-3}. Values for soil nutrients were similar and therefore averaged together: aluminum, 3.5 ppm; calcium, 720 ppm; potassium, 78 ppm; magnesium, 182 ppm; manganese, 3.75 ppm; nitrogen, 2 ppm; and phosphorus, 8 ppm (reported in Tables 2 and 3).

Table 2. Comparison of soil structure among each treatment plot (C = control, PD = plowed and disked, R = ripped, and RPD = ripped + plowed and disked) of the reclaimed surface mine in Central Ohio in 2007. No statistical differences existed among treatment plots.

Treatment	Organic Matter (%)	Sand (%)	Silt (%)	Clay (%)	Bulk Density (g cm-3)
C	1.35	57.13	24.38	18.50	1.64
PD	1.44	60.68	24.09	15.23	1.47
R	1.18	58.85	24.58	16.56	1.48
RPD	1.19	60.42	22.81	16.77	1.59

Table 3. Comparison of soil chemistry among each treatment plot (C = control, R = ripped, PD = plowed and disked. RPD = ripped + plowed and disked) of the reclaimed surface mine in Central Ohio in 2007. No statistical differences existed among treatment plots.

Treatment	pH	CEC (cmolc kg-1)	N (ppm)	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	Mn (ppm)	Al (ppm)
C	5.60	8.37	2.00	9.70	75.80	802.10	220.00	4.31	4.41
PD	5.55	7.72	2.00	8.09	82.36	757.18	180.36	4.04	4.63
R	5.51	8.03	2.00	7.17	80.25	736.42	229.42	3.45	3.15
RPD	5.37	8.07	2.00	8.75	74.33	650.75	212.75	3.74	3.33

Chestnut Seedling Survival:

During the first growing season, improvements in seedling survival in the treatment plots (90-95% survival) were noted when compared to the control plots (40%; Cox proportional hazard model, Likelihood = 273, df = 3, $P < 0.0001$; Fig. 3). This was also the trend after two and three field seasons. After 30 months, seedling survival in the mechanically treated plots was significantly higher (79-85%) than survival in the control plots (32%) (Cox proportional hazard model, Likelihood = 564, df = 3, $P < 0.0001$). Any form of surface treatment increased survival of chestnut in the early stages of establishment. However, after five years in the field differences became apparent among the treatments. The RPD and the R plots had the highest survival (81 ± 1 % and 77 ± 2%, respectively). This was significantly higher than seedlings in the PD plots (70 ± 2%) and seedlings growing in the control plots (21 ± 2%; Cox proportional hazard model, Likelihood ratio test= 1008, df = 3, $P < 0.0001$; Fig. 3).

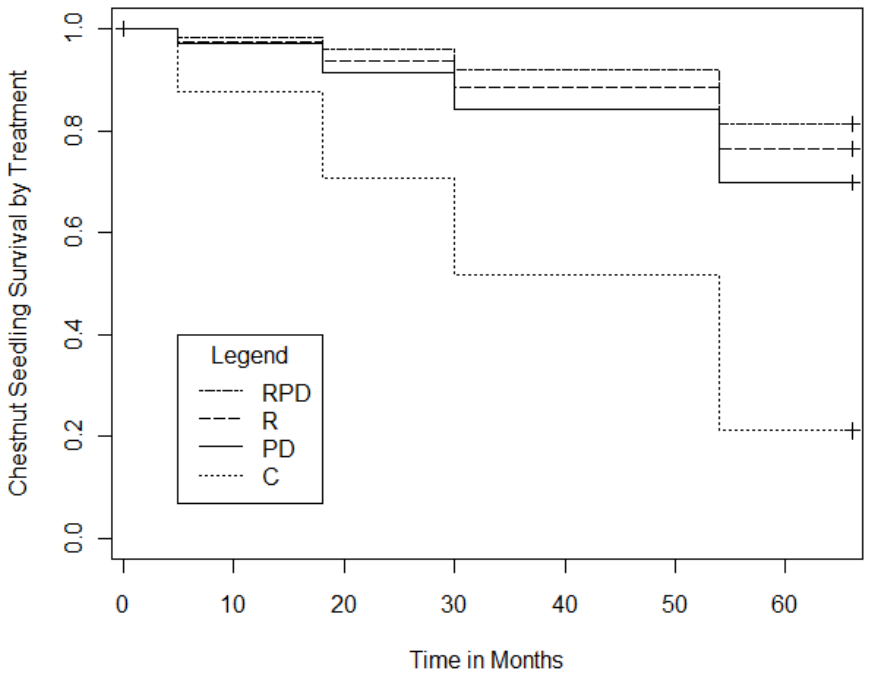

Fig. 3. Seedling survival per soil treatments (C = control, PD = plowed and disked, R = ripped, RPD = ripped + plowed and disked) after five years in the field. The RPD (dotted and dashed line) and the R plots (dashed line) had the highest survival (81 % and 77%, respectively). This was significantly higher than seedlings in the PD plots (70%, solid line) and seedlings growing in the control plots (21 %; indicated by the dotted line).

When comparing survival among the seedling types (pure American, BC_2, and BC_3) after the first two growing seasons, no differences in survival were detected. After three growing seasons (30 months) the BC_3 seedlings (74%) had a significantly higher survival rate than the pure American seedlings (64%) (Cox proportional hazard model, Likelihood ratio test= 20.4, df = 2, $P < 0.0001$; Fig 4). Survival of the BC_2 seedlings (68%) was similar to both seed types. Survival continued to differ among the seed types after five years (Cox proportional hazard model, Likelihood ratio test= 36.4, df = 2, $P < 0.0001$). The most advanced backcrossed line, BC_3 had the highest survival rate (68 ± 1 %) followed by BC_2 (59 ± 1 %) and the pure American seedlings (55± 2%; Fig 4).

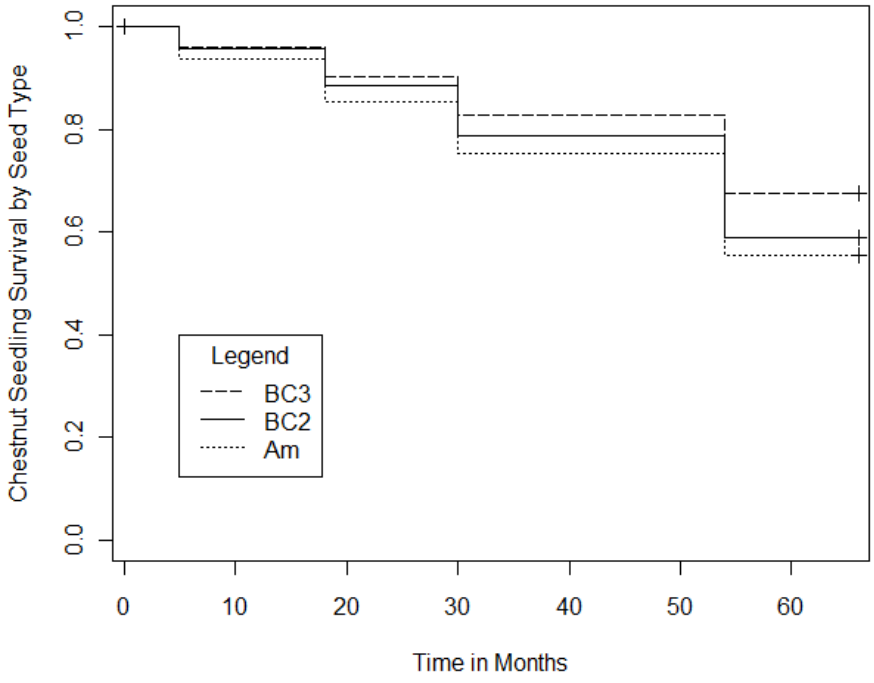

Fig. 4. Survival data for the chestnut seedling types monitored over five growing seasons. BC_3 seedlings (dashed line) had a significantly higher survival rate (68%) than both the pure the BC_2 chestnut (solid line) and pure American chestnut seedlings (dotted line); 59 and 55%, respectively.

Chestnut Seedling Growth by Treatment:

There were no interactions between treatments and seedling types when growth (height, basal diameter, and volume) was compared. The first growing season was most detrimental to the control plots (C). Extreme dieback resulted in seedling height averaging 15.4 (cm) in control

plots, which was significantly smaller than chestnuts growing in the either treatment plot (F = 47.37, *P* < 0.0001; Fig. 5). Average heights of chestnuts in the treatment plots were: PD (52.3 cm), R (57.1 cm), and RPD (60.8 cm). This trend continued into after year three with seedling height averaging C (16.2 cm), PD (75.6 cm), R (89.93 cm), and RPD (100.30 cm) (F = 172.88, P < 0.0001). After five years in the field the treatment affect remained apparent. In addition, differences are recorded among the soil treatments. Seedlings growing in the plots that received a soil treatment differed significantly in height (cm) when compared to the seedlings in the control plots (ANOVA, *f*= 112.8, *P* < 0.0001). Height was maximized both plots that received the ripping: R (123.0 ± 5.4 cm) and RPD plots (14l.7 ± 5.5 cm; Table 4, Fig. 5). PD plots were intermediate with regard to height (105.7 ± 4.8 cm) and significantly taller that chestnuts in the controls plots (0.2 - 0.3 m). Similar results occurred for basal diameter (Table 4).

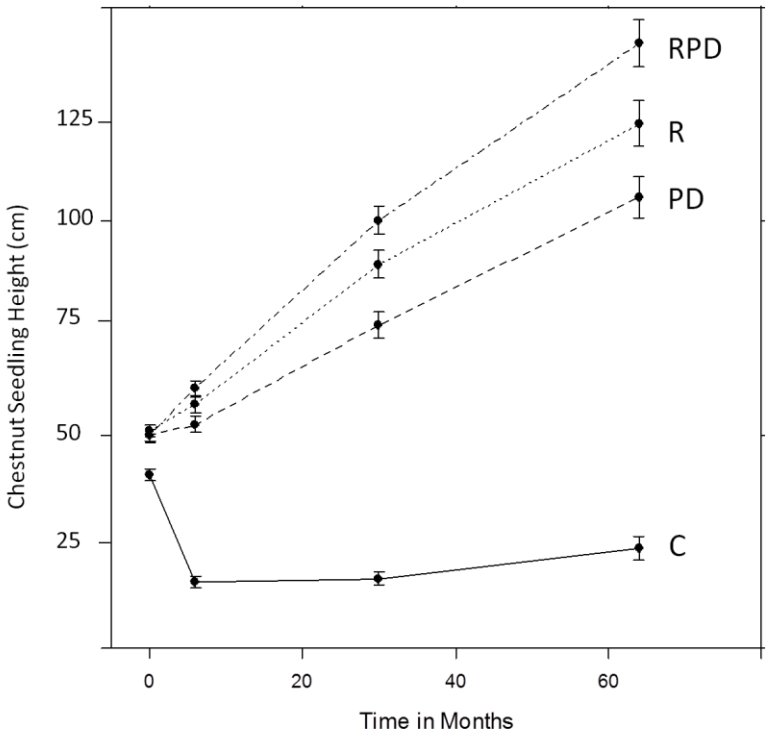

Fig. 5. Comparison of chestnut seedling height per treatment plot (RPD = ripped + plow and disk, R = ripped, PD = plow and disked, and C = control) after five, 30, and 66 months in the field. Soil treatments had a significant effect on seedling height. After three years, differences among the treatments became apparent. After five years (66 months), height was maximized both plots that received the ripping: R (123.0 cm) and RPD plots (14l.7 cm). PD plots were intermediate with regard to height (105.7 cm) and significantly taller that chestnuts in the controls plots (23.4 cm).

Chestnut seed types also differed with regard to height. Pure American seedlings were smaller than the BC_2 and BC_3 seed types when planted (Fig. 6). After the first growing season, both backcrossed suffered dieback but stabilized during subsequent growing seasons. After 5 months, the BC_3 were significantly taller (52.2 ± l.8) than the BC_2 (45.3 ± 2.5) and the pure American seed lines (42.0 ± l.3). After three field seasons, pure American chestnuts (74.4 ± 2.8) were equal to BC_3 (72.2 ± 2.7) in height, which were both taller than BC_2 seedlings (60.0 ± 3.6; ANOV A, df = 2, f = 5.51, P = 0.004; Fig. 6). After five field seasons, BC_3 seedlings (102.6 ± 4.25) were similar to Pure American seedlings (99.4 ± 4.4), and significantly taller than the BC_2 seed types (89.1 ± 5.5 ; Fig. 6). Similar results occurred for basal diameter (Table 4).

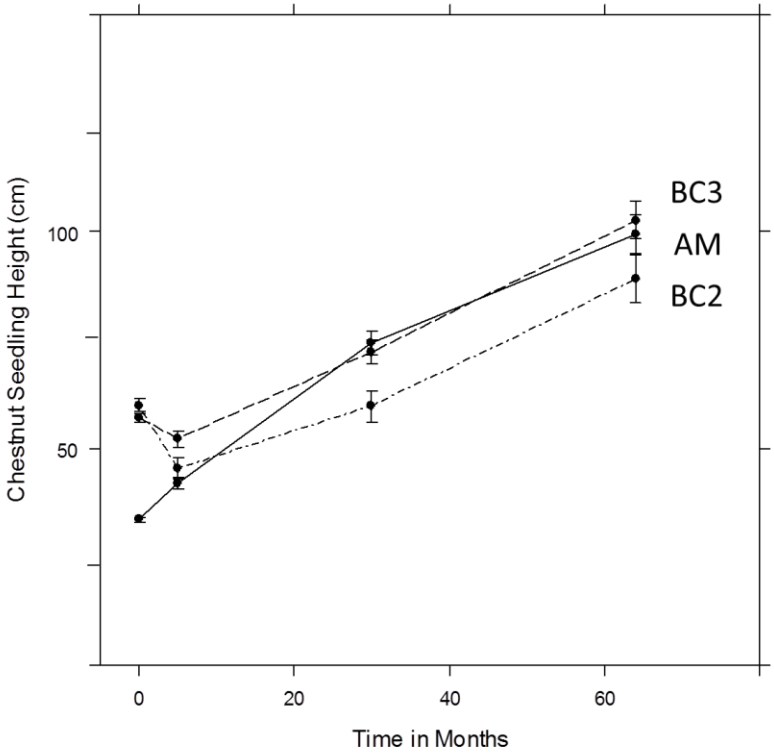

Fig. 6. Comparison of chestnut seedling height per seedling type (BC3= 15116 backcrossed seedlings, AM = pure American chestnuts, and BC2= *7/8* backcrossed seedlings) after five, 30, and 66 months in the field. After 30 months in the field (during the third growing season, BC3 and pure American were similar in height Seedling height was similar among seed types.

Seedling volume cm3 (height cm * basal diameter cm^2) exhibited the same trend. After the fifth growing season, plots that applied the ripping techniques (R and RPD) had significant increases in volume growth when compared to the PD and the control plots (F= 19.73, *P* = 0.002; Fig. 7). Mean seedling volume in the RPD plots (1,824.0 ± 122.7 cm^3) were larger than the seedlings in the R plots (1,469.1 ± 113.2 cm^3; *P* = 0.04). Chestnut seedlings in the RPD plots and the R plots were significantly larger than seedlings in the PO (991.6 ± 86.9 cm^3) plots (P = 0.002 and P< 0.0001). All treatment plots recorded greater seedling volume than the seedlings in the control plots (93.6 ± 17.9 cm^3; all P < 0.0001 ; Fig. 7).

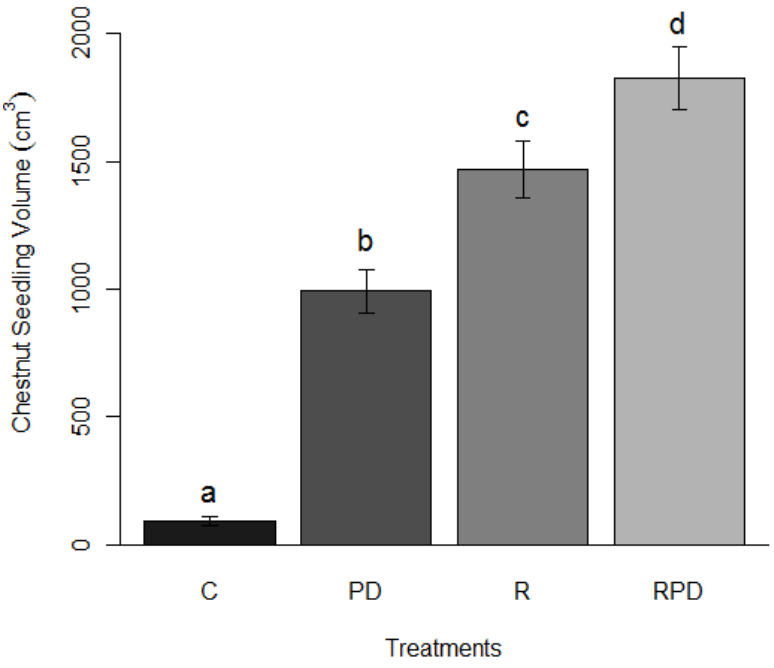

Fig. 7. Seedling volume cm3 (height m * basal diameter cm2) compared among the treatments: (control (C), ripped (R), and ripped + plow and disk (RPO), and plow and disk (PO). Plots that applied the ripping techniques (R and RPO) had significant increases in seedling growth when compared to the PO and the C plots. Error bars are ± 1 SE, bars sharing common letters do not significantly differ at a = 0.05 determined by Bonferroni pairwise t-tests.

Of the three seedling types, no differences existed with regard to total seedling volume (cm3) after five growing seasons. The pure American chestnuts had a slighter higher seedling volume (1194.7 ± 83.0) when compared to the two backcrossed lines, however, this was not statistically significant. The BC3 had a higher average volume (1056.1 ± 78.0) than the BCs seedlings (949 ± 107.1), but not significantly (Fig. 8).

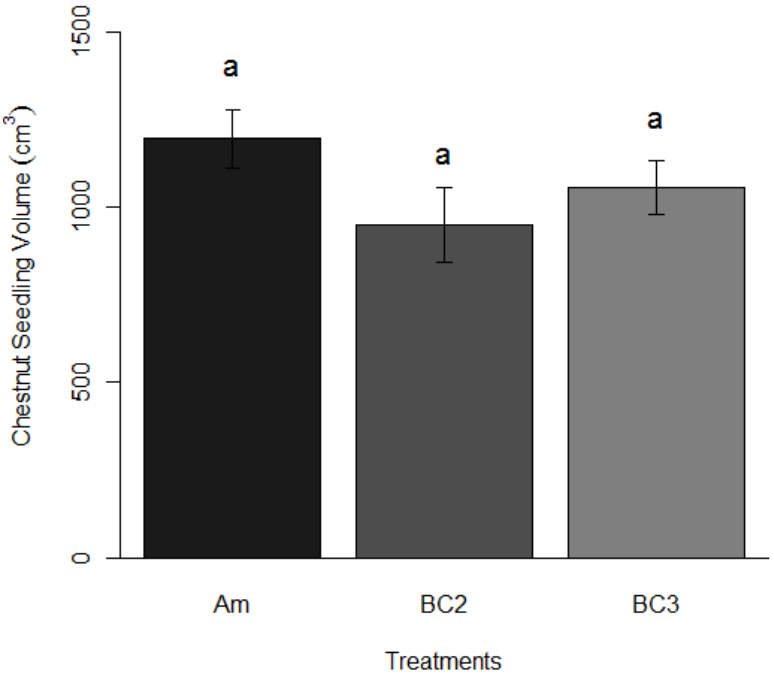

Fig. 8. Seedling volume cm^3 (height * basal $diameter^2$) compared among the chestnut seedling types (pure American chestnut (Am), backcrossed BC_2, and backcrossed BC_3). The pure American chestnuts (Am) had the highest seedling volume after five growing seasons when compared to the two backcrossed lines. Although the BC3 were larger than the BC2 seed types, this did not differ significantly. Error bars are ± 1 SE, bars sharing common letters do not significantly differ at a = 0.05 determined by Bonferroni pairwise t-tests.

C. parasitica Cankers on Chestnut Seedlings

We documented a total of 64 cankers on the chestnuts in the field ranging from those producing obvious stroma (Fig. 9A) to those that appeared calloused (Table 4; Fig. 9C). However, at this time only 42 of these seedlings that were recorded had prominent stroma produced on the field cankers (Fig 9A). Seedlings that were infected with cankers formed by *C. parasitica* were significantly greater on pure American chestnut seed types (38 cankered individuals) when compared to the backcrossed lines (x^2 =28.5, df = 2, P <0.0001). Of the backcrossed seedling types, there were four BC_3 seedlings with evident cankers and no BC2 seedlings with prominent blight.

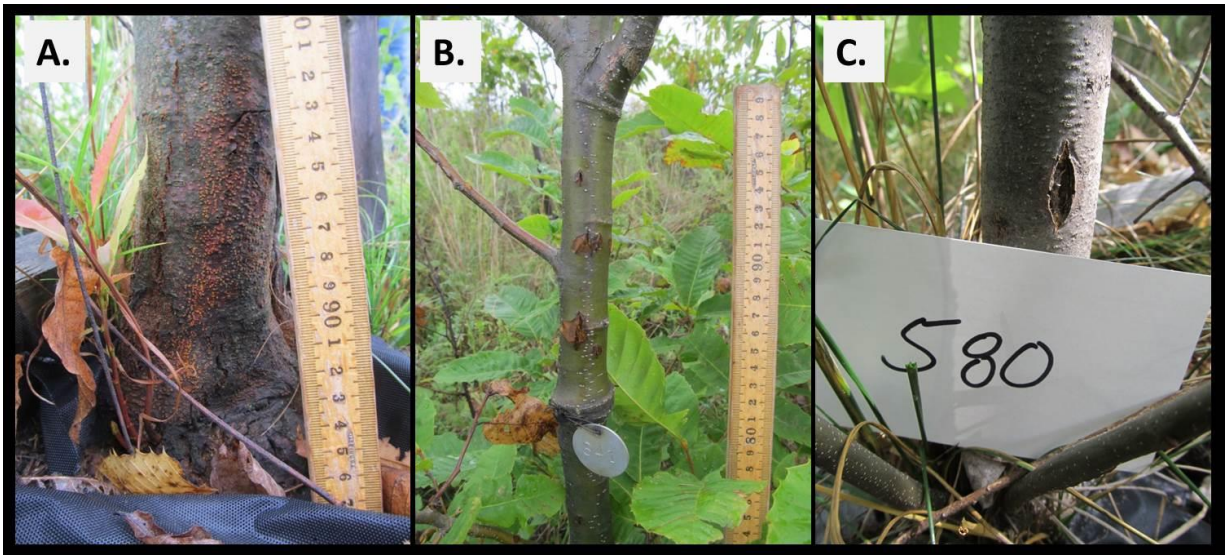

Fig. 9. Panels A-C are field cankers recorded in July 2012 from the test plot in Tri-Valley Wildlife Management Area, Muskingum County, central Ohio, USA (40˚ 11' 32" N, 81˚ 98' 35" W). ***Panel A:*** A basal canker with evident orange stroma protruding from tree bark on a pure American chestnut seedling. ***Panel B:*** BC_3 chestnut with smaller, superficial cankers without visible stroma. ***Panel C:*** is a presumably healed canker found on a BC_3 chestnut which, may be a sign of resistance when naturally challenged in the field. Further testing is currently underway to confirm *C. parasitica* as the infecting pathogen.

Table 4. Comparison of final growth, survival, and number of chestnut trees (pure American chestnut, BC_2 chestnuts, and BC_3 chestnuts) infected with a chestnut blight canker caused by pathogen *C. parasitica* recorded in all treatment plots (C = control, R = ripped, PD = plowed and disked, RPD = ripped + plowed and disked) in 2012. Statistical differences existed among treatment plots, common letters do not significantly differ at $\alpha = 0.05$ determined by Bonferroni pairwise t-tests.

Treatment	Seedling type	Seedling Height (cm)	Basal Diameter (mm)	Percent Survival	Number of seedlings cankered	Cankers with stroma
R	Pure Am	119.6(9.2)^{ab}	19.38(1.6)^{a}	63% ^{a}	16	15
	BC2	115.2(11.1)^{b}	19.5(1.9)^{ab}	75%^{b}	1	0
	BC3	131.4(7.9)^{a}	23.4(1.6)^{a}	83%^{c}	2	0
RPD	Pure Am	150.1(9.0)^{ab}	24.0(1.6)^{ab}	75%^{a}	16	14
	BC2F1	121.0(11.5)^{ab}	21.9(2.2)^{ab}	74% ^{a}	1	0
	BC3F1	145.6(8.2)^{ab}	23.9(1.4)^{ab}	86% ^{a}	6	1
PD	Pure Am	106.4(7.7)^{b}	17.1(1.4)^{b}	65% ^{a}	12	8
	BC2	99.2(9.8)^{b}	15.9(1.8)^{b}	68% ^{a}	0	0
	BC3	107.7(7.7)^{b}	18.5(1.4)^{ab}	75% ^{a}	3	1
C	Pure Am	21.6 (4.3)^{c}	3.1 (0.6)^{c}	19% ^{a}	3	1
	BC2	20.4(5.4)^{c}	3.4 (1.0)^{c}	22% ^{a}	0	0
	BC3	27.7(4.6)^{c}	4.6(0.8)^{c}	29% ^{a}	4	2

Vegetation Sampled in Treatment plots

Overall, 34 species were documented across treatments this study (Table 5). One very interesting find was chestnut seedlings in the field plots that were the result of seed production (Fig 10). Sampling efficacy was verified using the Chao II and Jackknife I richness estimators, both of which indicated that a slightly greater number of species likely in the plots, with Chao II approximating 39 (± 4.4 SE) species, and Jackknife I indicating a slightly greater number (42 ± 3.4 SE). When the vegetation species richness and diversity was compared per treatment, no significant differences existed (Table 6). Total plant species per treatments were as follows: C=20, R = 25, RPD = 20 and PD = 19. Shannon Diversity Index (*H´*) and Simpson's Index were also similar among plots and ranged from 2.1-2.3 and 0.84-0.88, respectively.

Five plant species made up 67% of the vegetation sampled. Of these, *Poa pratensis* (23%), *Lespedeza cuneata* (16%), *Solidago canadensis* (11%), *Rudbeckia hirta* (11%), and *Festuca arundinacea* (7%), *Achillea millefolium* were the most abundant species in all the treatment plots. No differences existed among the treatment plots based on a permutational MANOVA that compared vegetation community composition per species ($F = 0.67$, $P = 0.92$). No changes in species ranks were apparent when comparing rank abundance per treatment. *Achillea millefolium* was one of the more abundant species in the RPD plots, a difference noted but not significant to the vegetation community analysis.

Fig. 10. One-year-old chestnut seedling that was observed in one of the ripped test plots in 2012 located in test plot in Tri-Valley Wildlife Management Area, Muskingum County, central Ohio, USA (400 II' 32", 81° 98' 35" W). Based on its position in plots that had a documented account of chestnut burs, it is likely to be an offspring of the test trees.

Table 5. Species list that was generated from sampling 36, 1 m^2 quadrats sampled in the study plots of the field site located in Tri-Valley Wildlife Management Area, Muskingum County, central Ohio, USA (40° 11' 32" N, 81° 98' 35" W). Species are listed by their species name, common name, and native status as described by the United States Department of Agriculture, Natural Resources Conservation Service. Plants are listed by their relative abundance calculated from percent cover for the entire field site and listed in their appropriate functional groups: forbs, graminoids, legumes and woody species (vines shrubs and trees). Plots where they were sampled from are indicated are indicated: control (C), ripped (R), and ripped + plow and disk (RPD), and plow and disk (PD).

Latin Name	Common Name	Function	Native Status	% Cover	Plots sampled from
Forbs					
Achillea millefolium L.	Yarrow	Forb	Native	4.1	C, R, RPD, PD
Allium vineale L.	Field Garlic	Forb	Naturalized	0.1	RPD
Cirsium arvense (L.) Scop.	Canada Thistle	Forb	Invasive	0.1	C
Cirsium vulgare (Savi) Ten.	Bull Thistle	Forb	Invasive	0.4	C, PD
Daucus carota L.	Queen Anne's Lace	Forb	Invasive	3.5	C, R, RPD, PD
Dianthus armeria L.	Deptford Pink	Forb	Invasive	0.2	R, PD
Echinacea purpurea	Eastern Cone Flower	Forb	Native	0.7	C, RPD
Erigeron annuus (L.) Pers.	Eastern Fleabane	Forb	Native	1.1	C, R, PD
Leucanthemum sp.	chrysanthemum	Forb	Invasive	4.4	C, R, RPD, PD
Plantago lanceolata L.	English Plantain	Forb	Invasive	0.3	R, RPD
Rudbeckia hirta L.	Black Eyed Susan	Forb	Native	10.7	C, R, RPD, PD
Solidago canadensis L.	Canada Golden Rod	Forb	Native	10.8	C, R, RPD, PD
Solidago nemoralis Aiton	Grey Goldenrod	Forb	Native	0.4	R, PD
Taraxacum officinale Weber ex F.H. Wigg.	Common Dandelion	Forb	Naturalized	0.2	R
Graminoids					
Bromus inermis Leyss.	Smooth Brome	Grass	Invasive	1.3	C, RPD, PD
Dactylis glomerata L.	Orchard Grass	Grass	Naturalized	0.3	PD
Festuca arundinacea Schreb.	Tall Fescue	Grass	Naturalized	6.5	C, R, RPD, PD
Panicum sp.	Witch Grass	Grass	Native	0.2	C, R, RPD
Phleum pratense L.	Timothy Grass	Grass	Invasive	0.4	R

Poa pratensis L.	Kentucky Bluegrass	Grass	Introduced	23.2	C, R, RPD, PD
Schizachyrium scoparium (Michx.) Nash	Little Bluestem	Grass	Native	2.7	C, R, RPD, PD
Unknown Grass 4		Grass		1.1	C, R, RPD
Legumes					
Lespedeza cuneata (Dumont) G. Don	Chinese Lespedeza	Legume	Invasive	16.3	C, R, RPD, PD
Lotus corniculatus L.	Birdfoot-Trefoil	Legume	Invasive	1	C, R, RPD
Woody Vines, Shrubs, and Trees					
Parthenocissus quinquefolia (L.) Planch.	Virginia Creeper	Vine	Native	0.1	RPD
Toxicodendron radicans (L.) Kuntze	Poison Ivy	Vine	Native	1.6	R, RPD
Unknown sp 1 vine		Vine		0.1	PD
Vitis labrusca L.	Fox Grape	Vine	Native	0.1	RPD
Acer rubrum L.	Red Maple	Woody	Native	0.3	R
Rhus glabra L.	Smooth Sumac	Woody	Native	2.2	C, RPD, PD
Rubus allegheniensis Porter	Common Blackberry	Woody	Native	3.2	C, R, RPD, PD
Rubus occidentalis L.	Black Raspberry	Woody	Native	0.5	R, RPD
Spiraea latifolia (Aiton) Borkh.	Meadowsweet	Woody	Native	0.1	R
Castanea dentata Marsh. Borkh.	American Chestnut	Woody	Native	1.1	R, PD
Populus sp.	Hybrid popular	Woody	Naturalized	0.8	R

There was no difference among plots with regard to the native status of the plants sampled ($F = 0.69$, $P = 0.87$). Therefore, vegetation data were pooled for species list for this site that is reported in Table 7. No interactions existed between the treatment plots and the native status of the vegetation. Plots were pooled and vegetation was compared by native status. A difference that existed based on native status of the vegetation (df = 2, F = 71.22, P < 0.0001; Fig. 11). Invasive species (17 ± 0.9%) were the most predominant when averaged per plot followed by native species (13 ± 0.9%) and naturalized plants (3 ± 0.9%).

Table 6. Total species richness, mean species richness, Shannon-Weiner diversity index, and Simpson's diversity index (1-D) ± 1 SD from vegetation sampled among the four treatments: control (C), ripped (R), and ripped + plow and disk (RPD), and plow and disk (PD), ($n = 12$). Sample size (n) refers to the number of blocks (with 12, 1**× 1** quadrats per block). No differences existed at P < 0.05 determined by Tukey's HSD

Treatment	*N*	Total Species	Ave. Species Richness	Shannon-Weiner	Simpson's Diversity
C	3	22 [a]	12 ± 2.5[a]	2.1 ± 0.1 [a]	0.84 ± 0.01 [a]
R	3	25 [a]	15 ± 1.0[a]	2.3 ± 0.03 [a]	0.88 ± 0.01 [a]
RPD	3	22 [a]	13 ± 2.6[a]	2.1 ± 0.2[a]	0.85 ± 0.03 [a]
PD	3	20 [a]	12 ± 1.0[a]	2.1 ± 0.1 [a]	0.85 ± 0.02 [a]

Table 7. Average percent cover of native status of herbaceous plants sampled among the four treatments: control (C), ripped (R), and ripped + plow and disk (RPD), and plow and disk (PD), ($n = 12$). Sample size (n) refers to the number of blocks (with 12, 1× 1 quadrats per block). No differences existed at P < 0.05 determined by Tukey's HSD

Treatment	*N*	Invasive	Native	Naturalized
C	3	15.8 ± 0.9[a]	12.3 ± 2.1 [a]	2.3 ± 1.5 [a]
PD	3	17.4 ± 1.7 [a]	12.5 ± 2.6 [a]	2.4 ± 1.2 [a]
R	3	18.0 ± 1.5 [a]	14.2 ± 2.9 [a]	3.3 ± 1.7 [a]
RPD	3	15.9 ± 1.4 [a]	12.6 ± 3.2 [a]	2.2 ± 1.4 [a]

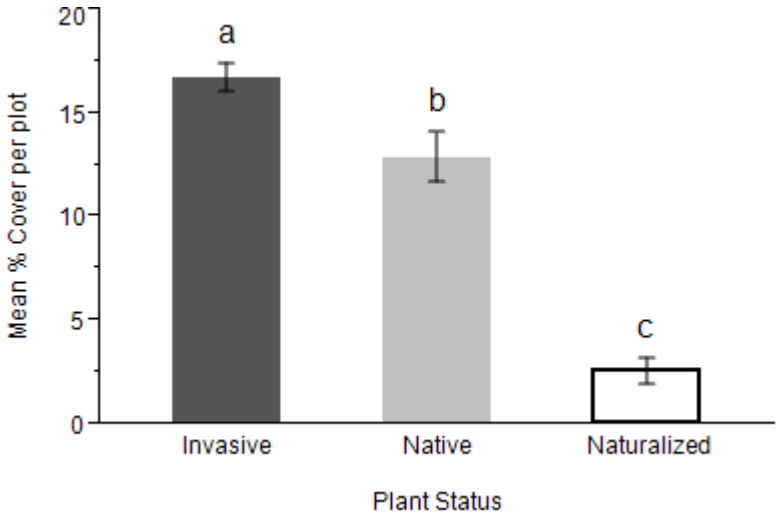

Fig. 11. Native status of plants based on United States Department of Agriculture, Natural Resources Conservation Service plant descriptions. A difference that existed: invasive species (17 ± 0.9%) were the most predominant followed by native species (13 ± 0.9%) and naturalized plants (3 ± 0.9%). Error bars are ± 1 SE, bars sharing common letters do not significantly differ at $\alpha = 0.05$ determined by Tukey's HSD.

ECM Community Composition

Through DNA sequencing, 16 fungal species were documented in ectomycorrhizal association with chestnut seedlings in 2012 (Table 8). Sampling efficacy was verified using the Chao II and Jackknife I richness estimators, both of which modeled similar species richness, with Chao II approximating 20 (± 5.3 SE) species, and Jackknife I (19 ± 2.0 SE). When the fungal species community composition was compared between sampling season and soil treatments, no differences existed. However, differences did exist between ECM species sampled in 2008 when compared to those sampled in 2012 ($F = 2.46$, $P = 0.04$). Two *Hebeloma* species were the most abundant fungi in 2008 and data illustrate a shift in 2012 to a *Cortinarius* sp. and *Cenococcum* dominated community (Table 8; Fig. 12). There is a significant correlation between plant biomass and ECM colonization ($R^2 = 0.08$, $P = 0.001$; Fig. 13). At this time there were significant differences in ECM with regard to blighted seedlings due to sample size, however, blight does decrease ECM colonization on roots, and increases the presence of *Cenococcum*.

Table 8. Ectomycorrhizal (ECM) fungal species sampled from chestnut root tips ranked by relative abundance generated from root tip count data. Roots were collected from 120 chestnut bare root seedlings from the treatment plots: plow and disk (PD), ripped (R), and ripped + plow and disk (RPD). This table reports fungal colonization from 238 sequences that were matched to vouchered ECM sequences available in GenBank.

ECM species	Total %
Cortinarius sp. 1	44
Cenococcum sp.	20
Cortinarius sp. 3	10
Scleroderma sp. 1	6
Cortinarius sp. 4	4
Russula sp.	3
Scleroderma sp. 2	3
Thelephora sp.	2
Inocybe sp.	2
Cortinarius sp. 2	2
Unknown 1	1
Sebacinales sp.	< 1
Laccaria sp	< 1
Tomentella sp.	< 1
Lactarius sp.	< 1
Cantherellaceae	< 1
# seedlings inspected	120
# of root tips inspected	31,400
# root tips with ECM	18,098
# ECM DNA sequences generated	238
Average ECM Colonization	58%

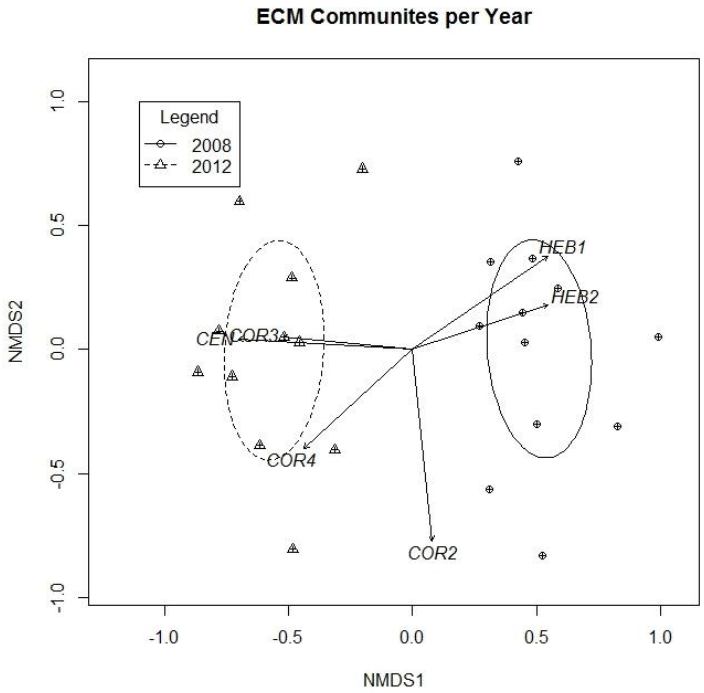

Figure 12. Non-metric multidimensional scaling (NMDS) ordination of ECM community composition. Larger circles (○) symbolize plots sampled in 2008 and triangles (Δ) symbolize the same plots sampled in 2012. The pattern reveals that plant communities differed between the 2 years. Plot vectors indicate strength and direction of the strongest correlations between sampling period and ECM species detected. Two *Hebeloma* species were the most abundant fungi in 2008 and shifts to a *Cortinarius* sp. and *Cenococcum* dominated community in 2012.

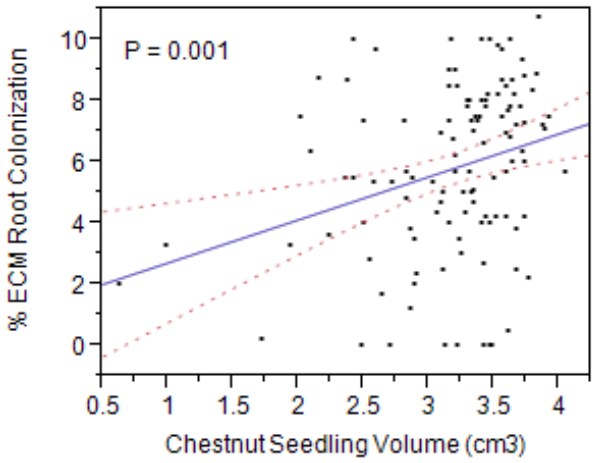

Figure 13. A simple linear regression illustrating the correlation between seedling biomass (chestnut volume cm3) and percent ECM root colonization. There was a significant relationship between the two variables; ECM colonization and seedling volume were correlated.

Summary:

The results of this study indicate that after five field seasons: 1) growth and survival was increased in plots that had some type of soil surface mechanical treatment, 2) as time progressed, plots that applied deep ripping had higher survival and growth when compared to traditional plowing, 3) no differences existed among chestnut seedling types with regard to growth and more importantly, blight-resistance was observed on backcrossed seed types, 4) ECM community composition is succeeding from what was previously reported (Bauman et al. 2013) and stem dieback results in a decrease in fungal root colonization, and 5) no differences existed with regard to the vegetation community among the plots.

Continued monitoring may show that deep ripping has a pronounced effect in later years on both chestnut persistence and recruitment of native tree species that may be both facilitated by the deep rooting zone and microsites produced by the presence of chestnut (i.e., organic matter, increased soil microbial activity, beneficial mutualisms). Data reported here suggest when implementing the proper methods and site selection, American chestnut is a valuable tree for use in coal mine restoration on reclaimed mine sites (Figures 14 – 16). The value that is created is multidimensional; overcoming arrested succession on marginal sites by using chestnut as a pioneer forest tree aids in natural forest recovery, provides a reliable seed source for population sustainability as well as wildlife protein source, aids in bond release to coal companies, and provides a valuable timber commodity in marginal landscapes that are in a state of recovery. Secondly, the usefulness of this tree species brings together a multitude of stakeholders that will ultimately provide additional data to the overall goal of restoring American chestnut to its native range in areas of Appalachian that is currently impacted by both natural and anthropogenic disturbances.

Under Current Investigation:

We recorded 64 chestnut seedlings after 5 field seasons. These included both cankers that had evident stroma production (Fig. 9A), small stem cankers without apparent stroma (Fig. 9B), and what appeared to be healed cankers, possible evidence of genetic resistance (Fig 9C). Of these 64 cankers, only 42 cankers displayed evident stroma (Figs. 2 and 9A) and therefore this study can only confirm this subsample as definite chestnut blight cankers. Further testing is underway to isolate and culture the pathogen from cankers *in vivo* that appeared either small

and/or those that appeared healed (W. MacDonald and M. Double pers. comm.). Of the 42 cankers that we can confirm at this time, 38 were pure American chestnut, 4 were BC_3 seedlings, and none were BC_2 chestnuts. Therefore, we can infer that the backcrossed seedlings are displaying some resistance to the natural infection in this mine restoration site. Prior to publishing this canker data, this site will be sampled again for cankers in July 2013.

Figure 14. Chestnut seedlings in a test plot in the field site of the Tri-Valley Wildlife Management Area, Muskingum County, central Ohio, USA (40° 11' 32" N, 81° 98' 35" W). Growth was comparable among seed types. Fast, upright growth of chestnut is a crucial attribute for survival on these sites because survival is dependent on the ability of chestnut to grow above the deer browse line and outcompete (and impose shade) on the re-establishing vegetation.

Figure 15. Chestnut seedlings were noted forming burrs as early as the fourth field season in this site and one year-old chestnut seedlings were documented in the test plots after the fifth field season (J. M. Bauman measuring chestnut seedling). Theoretically, intercrossings involving both BC_3 parents segregate the genes for resistance and result in progeny that may be homozygous for blight-resistance (Burnham, 1988). However, the theorized blight resistance will not be 100% and mortality is expected. For long-term study, we hypothesize that natural selection will remove chestnuts that are highly susceptible to chestnut blight and select offspring with adequate blight resistance and fast growth rate forming a sustainable restoration planting.

Figure 16. To accomplish this study, this project formed the basis of a summer training program for undergraduate interns. This program coupled hands-on field training with intern project ownership for the summer of 2012 (June 11, 2012 – March, 2013). Interns: from left to right: Shannon Wise, Andrea Renshaw, Jason Capello, Dana Dudra, Caleb Cochran, Samantha Zelenka, and Jessica Spencer. Intern Sarah Francino not shown but assisted in the molecular work for the ECM survey. Three specific research objectives were assigned to three interns and allowed training in the TACF history and breeding programs, concepts of disease ecology, field applications of chestnut seedlings, and DNA extraction and sequencing of ECM fungi (See presentation citations below).

Research Presentations funded by this TACF grant:

***Asterisks indicate student research involvement:**

Bauman, J. M. 2013. Fungal interactions and their influence on establishing American chestnut (*Castanea dentata*) seedlings in Ohio coal mine reclamation. To be presented: Herndon, VA, October 19-20.

Bauman, J. M., Santas, A., C.J., Keiffer, C.H., and McCarthy, B.C. 2013. Growth, blight-resistance, and fungal mutualisms of backcrossed American chestnuts in ecological restoration To be presented at the Society for Ecological Restoration International. To be presented: Madison WI, October 5-9.

Bauman, J. M., *Cochran, C.J., Keiffer, C.H., and McCarthy, B.C. 2013. Assessing growth and blight-resistance of backcrossed chestnuts under natural infection of *Cryphonectria parasitica* in restoration. North American Forest Ecology Workshop. Bloomington IN, June 16-20.

Hiremath, S., Lehtoma, K., and Bauman, J.M. 2013. Mycorrhizal species for the improvement of plant establishment on mine lands. The American Society of Mining and Reclamation – Reclamation Across Industries. Laramie WY, June 2-6.

*Cochran, C.J., Keiffer, C.K., McCarthy, B.C., and Bauman, J.M. 2013. Overcoming arrested succession: Field survival of backcrossed American chestnuts in mine reclamation. Society for Ecological Restoration - Midwest Chapter. Wooster OH, April 12-14.

* Renshaw, A. and Bauman, J.M. 2013. Ectomycorrhizal composition on American chestnut during establishment on restored landscapes West Virginian Academy of Science. April 6, Davis WV.

Acknowledgements:

This work was supported by National Technology and Transfer funds from the US Department of Interior (Office of Surface Mining) and by The American Chestnut Foundation Research Support Grant. Thank you to Dr. Amy Santas from Muskingum University for donating her time, laboratory, and expertise for the molecular work used in the fall ECM survey. We also thank interns Sarah Francino, Jessica Spencer, Andrea Renshaw, Jason Capello, Samantha Zelenka, Dana Dudra, Shannon Wise, and the Ohio Chapter of The American Chestnut Foundation for their field support.

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