REPRODUCTION POTENTIAL AND NATURAL CANKER INFECTION ON BACKCROSSED CHESTNUT TREES[1]

J. M. Bauman[2], C. Cochran, J. Chapman, and K.E. Gilland

Abstract: American chestnut (*Castanea dentata* Marsh. Borkh.) is a once-dominant hardwood species with the potential to be a valuable restoration tree for use on surface mined lands in the Appalachian region. Coupling soil ripping and plowing and disking with plantings of American and backcrossed chestnuts has resulted in high seedling survival on a reclaimed surface mine site in southeast Ohio. The objective of this study was to evaluate flowering, chestnut bur production (seed), and natural cankers caused by chestnut blight fungus (*Cryphonectria parasitica*) on three chestnut breeding lines, in three soil tillage treatments after seven field seasons. Pure American (*Castanea dentata*), and two types of *C. dentata* × *C. mollissima* hybrids (BC_2F_1 and BC_3F_1) were documented. Reproduction potential was measured via flower production in June. Canker incidence was recorded as necrotic bark lesions with the presence of orange spore bearing structures. When reproduction potential was compared among seed types, there were no differences; all chestnuts trees were flowering and producing chestnut burs after 7 seasons. Soil treatment had no impact on flowering incidence; however, hybrid breeding lines had a mild effect. Canker incidence and presence of flowers were not related statistically. When natural canker incidence was compared, pure American chestnut exhibited the most infection ($P < 0.0001$). There were also notable treatment effects, plots that applied the deep ripping had greater disease incidence on pure American chestnuts ($P < 0.0001$). Long-term survival and stand stability will depend on chestnut's tolerance to the blight at an age of fruiting and flowering. Results after seven years suggest that hybrids were exhibiting a decrease in blight incidence and were flowering and producing burs. Employing deep ripping methods to backcrossed American chestnut plantings provide a viable method for hardwood seedling establishment in soils impacted by surface mining.

Additional Key Words: Arrested succession, directed plantings, backcross breeding program, soil subsurface methods, reclaimed mine lands, The American Chestnut Foundation.

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Introduction

The introduction of chestnut blight in 1904 resulted in wide spread mortality of American chestnut (*Castanea dentata*) throughout the eastern deciduous forests of North America. Early breeding programs hybridized American chestnuts with blight-resistant Asian species (*Castanea mollissima* and *C. crenata*) to produce some hybrids that were resistant to blight. However, blight resistant progenies did not display the growth form of the American chestnut forest tree. To overcome this, backcrossed breeding methods were initiated in the 1980s by breeding programs including The American Chestnut Foundation (TACF) (Burnham, 1988). This entailed an initial cross either between blight-resistant Chinese chestnut (*C. mollissima*) or Japanese chestnut (*C. crenata*) and pure American chestnut (*C. dentata*). Progenies of these crosses that displayed blight-resistance were selected for subsequent breeding with pure American chestnuts (backcrossing). Continual backcrossing with the American species and intercrossing of hybrids is predicted to isolate the Chinese chestnut genes for blight-resistance into hybrid seed lines that display the morphological characteristics of the American chestnut (Hebard 2005).

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 is reported to tolerate a wide range of ecological conditions, including tolerance to drought and low pH that are typical of disturbed soils associated with coal mining (Jacobs, 2007). TACF is currently assessing the putative resistance of multiple advanced breeding lines (B_3F_3) that are estimated to contain approximately 94% American chestnut genes (Hebard, 2012). Field studies in national forests and mine reclamation sites are in the process of developing planting strategies for chestnut re-introduction into the Appalachian forests of North American (McCarthy et al., 2008; Bauman et al., 2012; Clark et al., 2012a and 2012b; Skousen et al., 2013; Gilland and McCarthy, 2014). Soil placement methods using The Forestry Reclamation Approach (FRA) proposed by The Appalachian Regional Reforestation Initiative (ARRI) call for minimal grading and are most conducive for seedling establishment on mine sites. The thought is that once established, trees can accelerate forest recovery by natural succession (Zipper et al., 2011).

Work in southeastern Ohio on coal mined sites reclaimed using 'end dump' reclamation yielded impressive survival rates of pure American chestnut seeds and seedlings when appropriate protective measures were utilized (McCarthy et al., 2010). On sites reclaimed using this method, a moderately high degree of microsite specificity, edge effects, and interactions between soil chemistry and the amount of competing herbaceous vegetation present were all found to impact growth and survival rates of chestnut when introduced as bare-root seedlings (Gilland and McCarthy, 2012). Comparisons of multiple hybrid lines of chestnut showed a high degree of morphological and ecological fidelity of late-generation hybrid chestnuts to the American parent even under harsh open-grown field conditions. However, the differences observed among the hybrid genetic lines were disappointing in that the Chinese parents and earlier hybrid genetic lines exhibited better growth and survival in that particular study. The exact mechanism for this performance disparity was preliminarily explored but a further inquiry into photosynthetic performance and the water relations among the lines would aid greatly in further developing the hybrid chestnut for large-scale restoration (Gilland and McCarthy 2014).

Soil characteristics of legacy mine sites reclaimed under The Surface Mining Control and Reclamation Act (SMCRA) are high in compaction with heavy herbaceous competition. These constructed non-native grasslands appear to slow the recruitment of native pioneer species resulting in a ‘legacy’ of arrested succession in otherwise forested landscapes. Deep soil ripping and traditional plowing and disking have been used experimentally to relieve soil compaction for chestnut plantings in central Ohio. In the first and second years of establishment, sub-surface methods significantly increased chestnut growth and biological interactions with beneficial soil fungi (McCarthy et al., 2008; Bauman et al., 2013). After five seasons, differences existed among the soil treatments; plots that applied deep ripping had the highest survival (81–77%) and growth (averaging 2 m; Bauman et al., 2014). Canker incidence caused by chestnut blight pathogen *Cryphonectria parasitica*, was also first documented at this time and compared among two chestnut breeding lines, two backcrossed seedling types (*C. dentata* × *C. mollissima* backcrosses), BC_1F_3 and BC_2F_3, and the pure American chestnut (*Castanea dentata*). After five field seasons, pure American chestnuts had significantly more cankers than BC_1F_3 and BC_2F_3 (Bauman et al., 2014). In addition to increased growth and survival, seedlings in all soil treatment methods were noted producing chestnut burs, spiny cupules surrounding seed, after the fifth field season (Fig 1).

Figure 1. Photograph of chestnut seedlings producing burs after the fifth field season on a legacy surface mine site in central Ohio. Plots were treated with a deep ripping + plowing and disking soil treatment and planted with a mix of pure American chestnuts, BC_1F_3, and BC_2F_3 chestnuts.

The survival, growth, and reproduction recorded from this legacy mine site indicate backcrossed chestnut's ability to establish on certain mine sites when deep soil ripping and traditional plow and disking are implemented, at least in the short-term. The long-term goal of TACF is to form sustainable populations of advanced chestnut hybrids that reproduce blight-resistant progeny (Jacobs et al., 2013). However, this is highly dependent on planting methods that promote healthy establishment and chestnut's ability to tolerate the blight at an age of flowering and fruiting. The objective of this study was to evaluate chestnut seedlings that were growing on a SMCRA site in southeastern Ohio after six and seven field seasons. The following parameters were measured: seed set (bur count), flowering, survival, and presence of natural cankers caused by chestnut blight fungus in (*C. parasitica*). The three chestnut breeding lines (Pure American, BC_2F_1 and BC_3F_1) were documented among the three soil treatments (ripping methods, traditional plowing and disking, and the combination of the two) were also evaluated.

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. Bulldozers were used to grade lands to the original contour and 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)* were used to produce a vegetative cover crop. Small pockets of forest comprised primarily of *Quercus, Pinus,* and *Acer* species were left undisturbed at the time these lands were mined.

The seed sources used in this study were from pure American chestnut seeds collected in Pennsylvania and from BC_1F_3 and BC_2F_3 chestnut trees from TACF open-pollinated orchards at Meadowview, VA (Hebard, 2005). Nuts were harvested in September-October 2005 and stored in sphagnum peat moss in closed plastic bags at 3°C. In March 2006, they were sown at the State Nursery in Marietta, Ohio by the Ohio Department of Natural Resources and lifted as bare root seedlings in March of 2007 (J. Hopkins pers. comm.). Three experimental blocks (117 m × 40 m) each containing the control and three soil treatments (each treatment 18 × 36 m) were installed prior to planting in the spring of 2007 (Fig. 2). The following treatments were established. 1) A control left undisturbed (C); 2) A plot cross-ripped at a depth of approximately 1.5 meters created by a D-6 dozer with a 1.0 m ripper bar attachment (R); 3) A plowed and disked plot installed by a conventional tractor (PD); and 4) A ripped + plowed and disked plot (RPD) (Bauman et al., 2014; Fig. 2). In each treatment plot, one-year-old bare root seedlings were randomized and planted on a 2.15 × 2.15 m spacing using 507 pure American chestnuts (*C. dentata*), 257 BC_1F_3, and 423 BC_2F_3 in groups of approximately 100 trees in each treatment plot (12 treatment plots, 1,187 trees total) . BC_1F_3 and BC_2F_3 seedlings are predicted to have on average 75% and 88% inheritance from the American chestnut and the remainder from the Chinese chestnut (*C. mollissima*) parent. A 1 m × 1 m weed mat was used around each seedling to prevent competition from herbaceous plants and a 1.5-m tall chicken wire cage was installed to prevent browse (McCarthy et al., 2010; Fig 3).

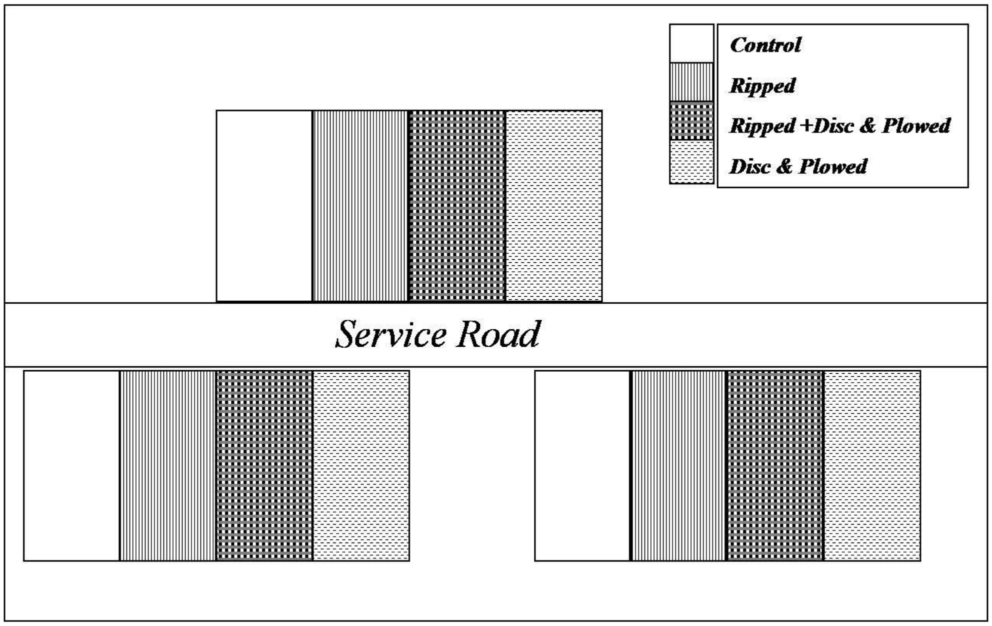

Figure 2. Field plot block design consisted of four treatments per block. Each block was 73 x 36 m with each treatment 18 x 36 m within and replicated three times.

Data Collection:

After seven field seasons all chestnut seedlings were evaluated for reproduction potential and the presence of stem cankers caused by *Cryphonectria parasitica*. Survival was also recorded at this time. Reproduction potential was measured via flower and chestnut bur production. Bur production was surveyed in October of 2013 by bur counts. To assess flower production, chestnut trees were scored either 1 (flowers present) or 0 (flowers absent) in June of 2014 for the presence of either staminate catkins that developed at the base of the flower branch and/or catkins observed near the apex (Botta et al., 1995). Canker incidence was recorded as necrotic bark lesions with the presence of orange spore bearing structures (Fig. 4).

Figure 3. A total of 1,187 chestnut seedlings consisting of 507 pure American chestnuts (*C. dentata*), 257 BC_1F_3, and 423 BC_2F_3 were planted as one – year-old bare root seedlings that were nursery grown and inoculated with spores of mycorrhizal species *Pisolithus tinctorius* in March of 2007 on a legacy mine site in central Ohio. Photo was taken in April of 2007 and illustrates a ripping+ plowing/disking subplot that used a 1 m × 1 m weed mat around each seedling with a 1.5-m tall chicken wire cage (McCarthy et al., 2010).

Figure 4. Only stem and basal cankers that had evident orange stroma (as seen in photograph) were scored positive for chestnut blight in June in 2014.

Statistical Analysis:

Logistic regression was used to examine the influence of treatment and hybrid genetic line (predictor variables) on survival, canker incidence, flowering, and bur production by the trees in our study (response variables; Zuur et al., 2009). Separate regression analyses were conducted for each response variable. For analysis of canker incidence, non-surviving trees were removed from the data set. It was not, however, determined if any trees removed from the data set due to mortality were killed by chestnut blight. A Wald test was performed on the overall effect of each the categorical predictor variables in each model to determine if the predictor variables and their interactions as a whole had a significant effect on bur or canker production (Draper and Smith, 1998) using the Wald Test command implemented via the aod package in R (Lesnoff and Lancelot, 2012). To analyze canker incidence further, treated plots and genetic lines were compared using a Fisher's exact test (Zar, 1999). In addition, bur counts and canker incidence were summed by treatment (block), and significance was determined using a one-way ANOVA followed by a Tukey's HSD.

Results

Survival:

All soil treatments had a significant positive effect on survival compared to the control (Wald Test: X^2 = 17.2, df = 3, P < 0.001; Table 1). Hybrid genetic line did not significantly impact survival (Wald Test: X^2 = 5.1, df = 2, P = 0.078; Table 1). There were also no significant interactive effects between hybrid type and soil treatment on seven-year survival (Wald Test: X^2 = 7.0, df = 6, P = 0.32).

Table 1. Summary of odds-ratio effect of soil treatments and hybrid breeding lines on seven-year survival of American chestnut and hybrids at TVWMA.

Factor	Odds ratio change	*P*	% survival
Treatments	Compared to control		
C	N/A	N/A	18.1
PD	4.60	0.00251	66.6
R	7.11	0.000252	78.6
RPD	7.24	0.000136	82.1
Breeding lines	Compared to Pure Am.		
Pure Am.	N/A	N/A	67.7
BC_1	0.435	0.11398	37.6
BC_2	1.48	0.41391	65.9

Canker Production:

There were no interactive effects between hybrid line and soil treatment on canker incidence (Wald Test: X^2 =2.8, df = 4, P =0.59). There were differences in canker incidence among breeding lines. Pure American chestnuts (26%) had an increased incidence of blight when analyzed (X^2 = 23.3, df = 2, P < 0.0001), but there were no significant differences between the hybrids BC_1 (6%) and BC_2 (9%) (Fig. 5; Table 2). When soil treatments were compared differences existed; ripped plots had more canker incidence (X^2 = 29.5, df = 2, P < 0.0001). Stem basal diameter did have a significant positive impact on canker incidence (odds ratio=1.03, P = 0.000306) and trees in the control plots had significantly smaller basal diameters than other treatments (F=21.0, P < 0.005).

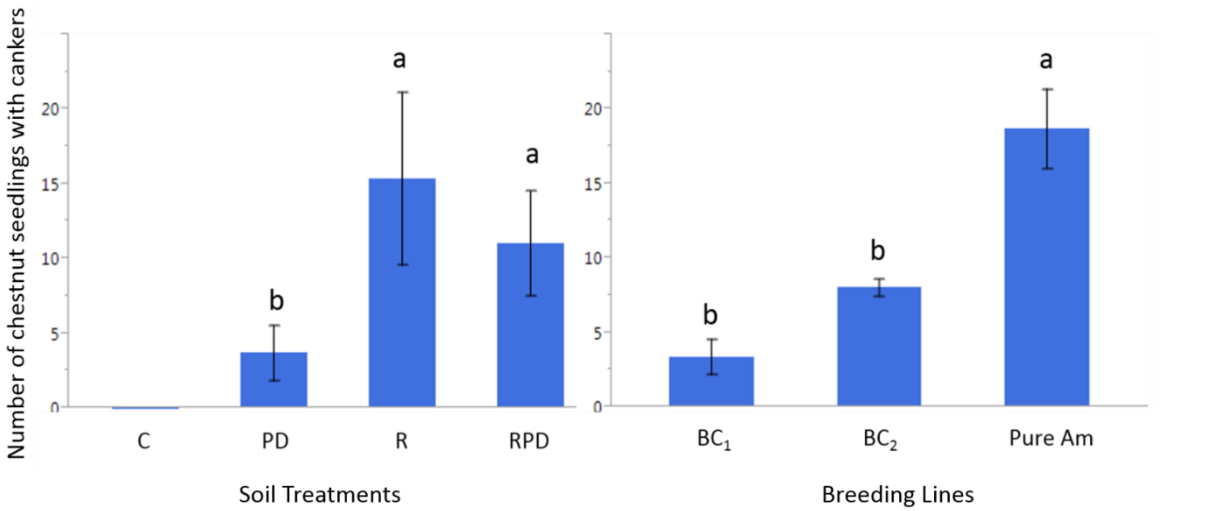

Figure 5. When soil treatments were compared differences existed: ripped plots (R) and ripped + plow disked (RPD) had more canker incidence than plow and disked plots (PD). There were differences in canker incidence among genetic lines, pure American (Pure Am) chestnuts had an increased incidence of blight when analyzed, but there were no significant differences between the hybrids BC_1 and BC_2 seedlings. Sample sizes are reported in Table 2.

Table 2. Summary of odds-ratio effect of soil treatments and hybrid genetic line on canker incidence of surviving American chestnut (Pure Am.) and hybrids at TVWMA. Control plots were removed from this analysis due to low survivorship and insufficient tree size for canker presence (For reference: $n_{control}$ =54, 6% infected). For reference, seedling log-odds ratios for canker incidence are compared to the plow and disked plots (PD) for treatments and Pure American trees for baselines.

Factor	Odds ratio change	P	% infected trees	*n* surviving trees
Treatments	Compared to PD			
PD	N/A	N/A	6.06	198
R	6.86	<0.0001	25.4	185
RPD	2.92	0.002	13.7	248
Breeding lines	Compared to Pure Am.			
Pure Am.	N/A	N/A	20.7	270
BC_1	2.42e-07	0.9796	6.62	151
BC_2	0.58	0.4003	9.13	263

Bur Production

Bur production among the treatment plots was significantly less in the controls plots (*F*= 6.11, P = 0.02; Fig. 6). No statistical differences were found for mean bur production among plowed and disked (PD), ripped (R), and ripped + plowed and disked (RPD) plots. Also, no significant differences existed among the seed types (Table 3).

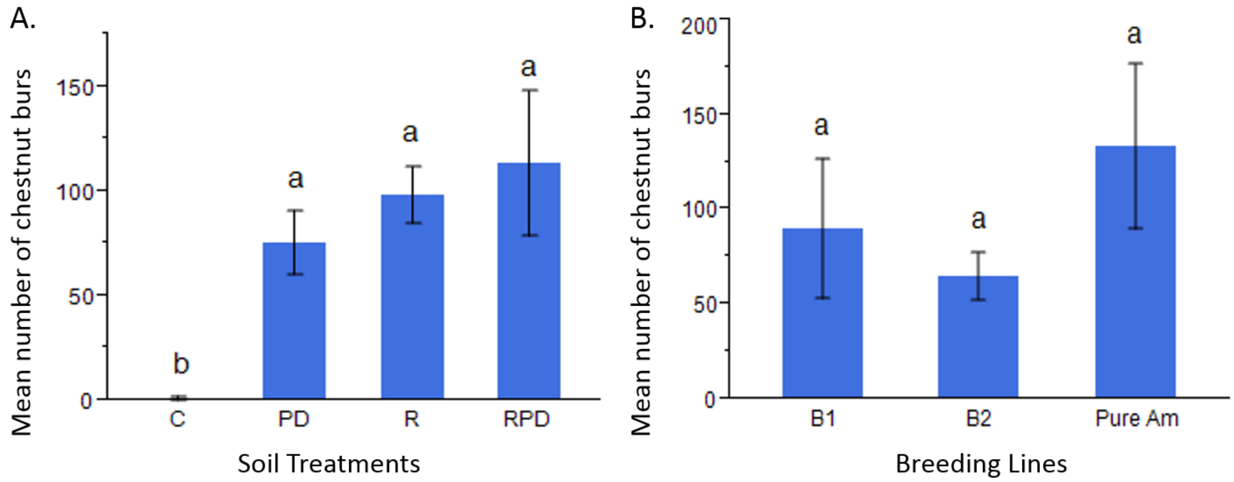

Figure 6. Panel A. Ripped plots (R), ripped + plow disked (RPD), and plow and disked plots (PD) had significantly more burs than the control plots (C) (*F*= 6.11, P = 0.02). Panel B. No differences existed when the production of burs were compared among the genetic lines.

<u>Flower Production</u>

Soil treatment had no impact on flowering incidence (Wald Test: X^2 = 0.38, df = 3, P = 0.94; Table 4). Hybrid genetic line had a mild effect when both were compared with the pure American trees (Wald Test: X^2 = 4.3, df = 2, P = 0.12). There were no significant interactive effects between hybrid line and soil treatment on canker incidence line (Wald Test: X^2 = 5.6, df = 6, P = 0.46). Of the surviving trees, 52% of cankered trees were observed to be flowering while 48% were not. Canker incidence and presence of flowers were not related in a significant way (Fisher's exact test odds ratio = 0.82, P= 0.419).

Table 3. Bur counts by treatments: control (C), plow and disked plots (PD), ripped plots (R), and ripped + plow disked (RPD). There was significantly less bur production in the control plots. Pure American (Pure Am) and BC_2 seedlings were similar with regard to bur production after six field seasons. Note: more Pure American chestnuts were planted, due to seed availability, when plots were initiated in 2007 (see under methods).

Treatment	Breeding Lines	*n*	Bur Count
C	B1	64	0
C	Pure Am	127	3
C	B2	109	0
PD	B1	65	71
PD	Pure Am	129	111
PD	B2	105	44
R	B1	62	126
R	Pure Am	124	103
R	B2	105	65
RPD	B1	67	72
RPD	Pure Am	128	182
RPD	B2	105	85

Table 4. Summary of odds-ratio effect of soil treatments and hybrid genetic line on flowering incidence of surviving American chestnut (Pure Am.) and hybrids at TVWMA.

Factor	Odds ratio change	*P*	% flowering trees	*n* surviving trees
Treatments	Compared to Control			
C	N/A	N/A	38.8	50
PD	0.7962835	0.6547	50.7	199
R	0.7333003	0.5520	55.4	186
RPD	0.7597241	0.5840	47.7	247
Breeding lines	Compared to Pure Am.			
Pure Am.	N/A	N/A	50.3	270
BC1	1.17	0.213	57.8	142
BC2	0.788	0.179	47.6	252

Discussion

Deep soil ripping, plowing, and disking, and the combination of the two methods had a positive effect on survival when compared to the control plots after seven field seasons. When natural canker incidence was compared among the breeding lines, pure American chestnut had greater canker incidence than the two hybrid seed lines. There were also notable treatment effects, plots that applied the deep ripping had greater disease incidence on pure American chestnuts. The extremely small number of remaining trees in the control plots (51 trees) in which none showed canker incidence likely skewed the canker incidence results. The lack of canker incidence in control-treatment trees is most likely due to their small stature. Increased growth rates in soil treatment plots may result in cracks in the bark, which is required for pathogen entry, resulting in increased pathogen infection and canker incidence (Griffin, 1989; Reynolds and Burke, 2011; Bauman et al., 2014).

When reproduction potential was compared among seed types used in this study, BC_1 trees flowered at a higher rate than pure American trees but BC_2 trees flowered at lower rates. This current study documented simultaneous flower production among the three different genetic lines. This coincidental flowering may suggest the ability for natural backcrossing in the field; however, more work is required to document synchronous flower receptive stigma and viable pollen. An increase in flowering rates in the soil treatment plots was expected but the increases were not significant. Although some chestnut trees may exhibit flowering as a stress response in the presence of blight (Gilland et al., 2012), this was not a statistically significant interaction in this current study. Other factors that could invoke a stress response on mine sites include drought, poor soil nutrition, and herbaceous competition. Therefore, the soil treatments may have mitigated these stressors and allowed root system exploration required to prevent below-ground resource limitation while producing photosynthetic aboveground biomass that allowed for viable offspring production.

Established trees will flower, and the larger trees will more likely have the carbon resources available for production of viable seed. Evidence of viable chestnut offspring has been documented during a vegetation survey conducted in the plots during the fifth growing season (Bauman et al., *in press*). Larger basal diameters is a predictor for canker incidence, therefore, some seedlings were both cankered and producing flowers. Our data shows that cankers often begin to appear on chestnut trees around the same time they begin to produce nuts, although, that interaction was not significant. As chestnut blight spreads through the stand, we predict increased mortality of pure American seedlings and anticipate some loss of the backcrossed seed types. Eventually, chestnut blight will drive selection of breeding lines that display varying levels of blight-resistance; seed types that lack genes for resistance will eventually fail to reproduce while trees with adequate blight resistance will increase in population.

Historical records indicate that chestnut was a native species of the Appalachian forests in eastern Ohio prior to the introduction of chestnut blight (Braun, 1950). The survival and growth reported here indicate soil chemistry and texture that complements chestnut’s reintroduction in this area (Bauman et al., 2014). Recent field studies report chestnut's ability to rapidly replace resident tree species, both in and out of its native range (Paillet and Rutter, 1989; Jacobs and Severeid 2004; McEwan et al., 2006). Future studies will determine the landscape patch dynamics of this establishing site located 80m to 160m from fragmented forest patches. Studies of chestnut seed dispersal and predation patterns in environments are still lacking (Steele et al. 2005). It will be necessary to document migration patterns of chestnut seed dispersed via avian or mammals into surrounding forests as part of long-term population monitoring at these types of reclamations. Previous studies have modeled chestnuts ability to disseminate into forests, regenerate in the understory in areas of light gaps, and be released following a large-scale canopy disturbance (Paillet and Rutter, 1989). As chestnut mortality increases in the plots, nutrient pools from dead plant material and active microorganisms may aid in seedling recruitment of other native forest species. A few woody seedlings have been recorded in the treated plots, which may suggest that these soil treatments will aid in the establishment of other plant species over time.

In summary, the results of this study indicate that after seven field seasons. 1) chestnut survival was increased in plots that had some type of soil surface mechanical treatment; 2) chestnut seed types were similar with regard to survival; 3) decreased canker production was documented on BC_1 and BC_2 chestnut seedlings compared to pure American chestnut; 4) no difference in flowering was detected among treatment plots, but a significant decrease in bur production was found in control plots; and 5) slightly lower flower production was observed from the BC_2 seedlings. Results after seven years suggest that employing deep soil ripping and intensive planting methods to backcrossed American chestnut plantings may provide a viable method for hardwood seedling establishment in soils impacted by surface mining. However, the evaluation of current plantings of hybrid chestnut seedlings will be needed to determine growth and survival under less intensive planting methods. The stipulation in this study is that the treatments may have increased opportunity for pathogen infection and canker incidence due to higher growth rates

(Bauman et al., 2014), which could be a benefit to determine resistance to the blight at an earlier age.

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