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Effect of soil compaction and moisture on incidence of phytophthora root rot on American chestnut (*Castanea dentata*) seedlings

C.C. Rhoades[a,*], S.L. Brosi[a], A.J. Dattilo[a], P. Vincelli[b]

[a]*Department of Forestry, University of Kentucky, Lexington, KY 40546-0073, USA*
[b]*Department of Plant Pathology, University of Kentucky, Lexington, KY 40546-0073, USA*

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Abstract

American chestnut is one of hundreds of plant species plagued by root rot caused by *Phytophthora cinnamomi*. Phytophthora root rot is thought to have contributed to chestnut dieback prior to the arrival of chestnut blight, and it may now present a serious limitation to establishment of blight-resistant hybrid chestnut. We manipulated soil compaction and moisture to evaluate the effect of soil physical factors on incidence of Phytophthora root rot on American chestnut seedlings. Seedlings were grown under three watering regimes, two soil compaction levels and two fungicide levels. Increased soil moisture enhanced seedling growth in loose soil, but irrigation did not impact seedlings growing in compacted soils. Seedling mortality was greatest in wet, compacted soils. Disease incidence was highest in the wettest soils, irrespective of compaction level. Fine root necrosis and Phytophthora infection occurred on 58 and 24% of non-fungicide-treated seedlings, respectively. Presence of ectomycorrhizal fungi declined in compacted soils that were either wetter or drier than optimal. Occurrence of ectomycorrhizal fungal symbionts was unrelated to root rot. This study demonstrates the high susceptibility of American chestnut to this common root pathogen, even under moderate levels of soil compaction and moisture. While overcoming chestnut blight is the first step in restoring chestnut to its original range, to establish successful plantings it will be crucial to recognize and avoid sites where soil physical factors promote Phytophthora root rot.

Keywords: Forest restoration; Soil bulk density; Root disease; Forest pathology; Ectomycorrhiza; Seedling propagation; Nursery production

1. Introduction

American chestnut, *Castanea dentata* (Marsh.) Borkh., was a dominant component of the eastern deciduous forest until the early 1900s, when chestnut blight caused by *Cryphonectria parasitica* (Murr.) Bar, was introduced to North America (Russell, 1987). In less than 50 years, the blight transformed the American chestnut from an abundant overstory species to an occasional understory shrub. Today there is optimism that blight-resistant hybrid chestnuts may soon be available for introduction into North American forests. Hybrid chestnuts are generated through a back-cross breeding program that combines blight-resistant traits of Asian chestnuts (*C. mollissima* or *C. crenata*) with desired characteristics of the American chestnut (Burnham, 1981). The American chestnut research community currently projects that blight-resistant

* Corresponding author. Tel.: +1-859-257-6359; fax: +1-859-323-1031.
E-mail address: ccrhoa2@uky.edu (C.C. Rhoades).

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hybrid chestnuts will be available for outplanting within the next decade (Hebard, 2002; Anagnostakis, Conn. Ag. Expt. Station, pers. commun.).

Unfortunately, chestnut blight is not the only biological impediment to chestnut restoration (Anagnostakis, 1995, 2002). Chief among the obstacles facing chestnut restoration are the oomycete pathogens of the genus *Phytophthora*. Recent plantings of chestnut seedlings in southern Appalachian forests have experienced high mortality attributable through standard diagnostic practices to Phytophthora species, principally *Phytophthora cinnamomi* (Brosi, 2001). *P. cinnamomi* was identified on chestnut seedlings grown at four sites in Kentucky's Knobs and Cumberland Plateau regions. The disease caused root and collar rot, branch dieback, defoliation and resulted in 60% seedling mortality within 4 months of planting. Similar root disease symptoms have been noted in association with chestnut seedling dieback in Pennsylvania, Tennessee and North Carolina plantings (S. Schlarbaum, University of Tennessee, pers. commun.) and *Phytophthora* spp. have been isolated from diseased seedlings where evaluated (T. Hall, forest pathologist, Pennsylvania Bureau of Forestry, unpublished data).

The blue or brownish-black root lesions of "ink stain disease" were noted on American chestnut before introduction of the chestnut blight (Corsa, 1896 (in Anagnostakis, 2002)). Root rot was associated with reduced vigor, increased mortality and may have contributed to contraction of chestnut's pre-blight range in the Mid-Atlantic, Southeastern and Gulf States (Anagnostakis, 2002; Crandall et al., 1945). In Kentucky, soil-related chestnut decline was attributed to land use conversion as forestland was cleared for agriculture (Holmes and Bradfield, 1907).

> "In the northern counties, as in so many other parts of the Southern Appalachian region at the lower elevations, chestnut as a commercial tree seems to be dying out... This decadent condition of the chestnut is caused probably by the gradual change in soil-moisture conditions, brought on in part by the clearing up of the country (Holmes and Bradfield, 1907)."

In spite of early awareness of *P. cinnamomi* as an important pathogen of American chestnut, investigation of the specific conditions that favor the disease was preempted by chestnut blight.

The environmental and biological factors controlling incidence and severity of *Phytophthora* infection have been described for a variety of forest hosts. Crandall et al. (1945) conducted experiments designed to isolate the effects of soil texture and soil water content on the incidence of *P. cinnamomi* on red pine (*Pinus resinosa*). Poor soil aeration and prolonged soil saturation promote sporangia formation and zoospore release required for growth, reproduction and dissemination of Phytophthora (Wilcox and Mircetich, 1985; Agrios, 1997). Such conditions are common in fine-textured soils with high clay content, though Phytophthora incidence is widespread on a range of different geologic substrates and soil textures (Jung et al., 2000).

The presence of ectomycorrhizal (ECM) fungi on tree roots has been shown to form a protective barrier (i.e. fungal mantle) against Phytophthora infection (Marx and Davey, 1969). Early work on shortleaf pine (*Pinus echinata*) demonstrated increased resistance of roots to *P. cinnamomi* on seedlings with naturally occurring *Cenococcum graniforme* ectomycorrhizae (Marx and Davey, 1969). European chestnut (*Castanea sativa*) seedlings inoculated with ECM resisted infection by *P. cinnamomi* during a 3-month trial (Branzanti et al., 1999). Pathogen resistance was attributed to an ECM fungal mantle that enveloped most of the root system. The interaction between *P. cinnamomi* and ECM fungi on American chestnut has yet to be studied.

The objectives of this study were to determine the effects of soil compaction and soil moisture on incidence of Phytophthora root rot on American chestnut seedlings. We also evaluated the relationship between seedling vigor, disease incidence and the presence of ECM.

2. Methods

2.1. Soil treatments

To evaluate physical soil controls on the susceptibility of American chestnut to root disease, we conducted a 90-day greenhouse seedling trial using soil from a plantation where high incidence of natural infections of *P. cinnamomi* and other Phytophthora species had been previously confirmed on dead and dying chestnut seedlings (Brosi, 2001). Necrotic root pieces from these seedlings incubated in distilled water consistently produced sporangia of *Phytophthora* spp.;

the morphology of the majority of these matched the description for *P. cinnamomi*. ELISA tests on rotted roots also consistently reacted positively for Phytophthora species (P. Vincelli, University of Kentucky, Plant Pathology Diagnostic Laboratory, unpublished data). No other pathogen was detected in those diagnostic procedures. The plantation was located near Berea, Kentucky on the western edge of the Cumberland Plateau. Sprouts, stumps and logs indicate the historical presence of American chestnut at the site. Soil is developed from mixed shale, sandstone and scattered limestone sediments. The silt loam soils are classified as fine, mixed mesic, Dystrochrepts (Soil Conservation Service, 1973). The top 10 cm contain 9% clay, 36% sand and 55% silt; clay content increases to 14 and 20% in the 10–20 and 20–30 cm soil depths, respectively.

Surface mineral soil (0–10 cm depth) was collected from the planting site, then was mixed and passed through a 2.5 cm mesh sieve to remove roots and coarse fragments. Two soil compaction levels were established by hand-tamping different masses of sieved soil into 300 ml planting tubes. Density of the compacted soils was significantly greater than that of the uncompacted soils (0.72 vs. 0.64 g/cm^3, $P < 0.001$). Pure American chestnut seeds from The American Chestnut Foundation's breeding program in western Virginia were planted at 5 cm depth. At the time of planting, half the growing pots were treated with 60 ml of the oomycete-specific systemic fungicide mefenoxam (Subdue MAXX^{TM}, Novartis Crop Production Inc., Greensboro, NC) to suppress Phytophthora (0.4 g ai/l). Fungicide application was repeated after 42 days when seedlings were well established. The fungicide-treated seedlings provided a comparison with untreated seedlings subjected to natural inoculum in the field soils containing *P. cinnamomi*.

Three soil moisture levels (wet, optimal, dry) were established by manipulating irrigation frequency. In previous greenhouse bioassays, we have determined that optimal watering for chestnut seedling growth and root zone aeration was 200 ml on alternate days (Rhoades and Miller, unpublished data). The wet treatment maintained a nearly saturated root environment and the dry treatment was intended to generate drought stress. Wet treatment seedlings received 400 ml of a very low nutrient ''artificial rain'' solution (Lee and Walker, 1979) added in two 200 ml applications, 6 days per week; those in the dry treatment received 100 ml on alternate days. The elemental concentration of the artificial rain was equal to precipitation inputs to forested New Hampshire watershed (Likens and Bormann, 1972) after subtracting out estimated nitric and sulfuric acid inputs. Artificial rain supplied 12 μeq/l concentrations each of NH_4^+ and NO_3^-; a 400 ml irrigation provided a N input of <0.1% of the plant available soil N pool.

2.2. Seedling response

After 90 days, height, root collar diameter and leaf area of fresh excised leaves (Li-300, LiCor Inc., Lincoln, NE) were measured for each seedling. Leaves and stems were then dried at 60 °C for 48 h to determine aboveground biomass. After harvesting aboveground portions, seedling roots were carefully removed from the soil, washed and stored at 5 °C for further analysis. To estimate ECM infection on fine roots ($\leq$1 mm diameter), a subsample of twenty-five 3 cm long root segments were excised from each seedling, suspended in deionized water and distributed evenly within a Petri dish. The presence or absence of ECM root tips was recorded using a line intercept method on 100 root tips per seedling under a dissecting microscope (McGonigle et al., 1990). Following determination of ECM colonization and disease incidence (discussed below), root biomass was determined as described for aboveground tissues.

2.3. Disease assessment

Percent root rot was estimated visually under a dissecting microscope on a subset of fine roots as detailed above for the ECM survey (i.e. twenty-five 3 cm long roots per tree). To determine the cause of the root disease, seedling roots (four replicates per seedling) were also assessed with an enzyme-linked immunosorbent assay (ELISA) with Phytophthora-specific antibodies (Neogen Corp., Lansing, MI). The ELISA was conducted using 2 mm long segments of fine root from each seedling from non-fungicide-treated soils. Half the seedlings grown in fungicide-treated soils also were assayed for the presence of Phytophthora; none tested positive. The presence of *P. cinnamomi* was assessed by inspection of sporangial size and form on rotted root tissue that tested positive to the ELISA

screen (Erwin and Ribeiro, 1996). Prior to inspection, root tissue was incubated for 4 days in deionized water and then was surveyed at 40× magnification under a compound microscope.

2.4. Statistical design and analysis

The bioassay was set up in a factorial design with two compaction levels (dense, loose), two fungicide levels (fungicide-treated, not fungicide-treated) and three watering treatments (dry, optimal, wet) with eight replicate seedlings per treatment. All treatments were considered fixed effects; seedling response and Phytophthora infection were dependent variables (SPSS version 10.1 2000, SPSS Inc., Chicago, IL). Where interactions between the two main effects occurred, variables were analyzed independently by one-way analysis of variance (ANOVA). Where significant treatment differences occurred, means were compared using orthogonal contrasts or Tukey's pairwise tests ($P = 0.05$ significance level). Percentage data was arcsine transformed and seedling variables were log-transformed prior to statistical analysis.

3. Results

3.1. Seedling growth

After 90 days, overall mean seedling height was 32.2 cm and root collar diameter was 3.8 mm (Table 1) and individual maximum height and root collar diameter were 55.8 cm and 6.0 mm, respectively. Seedlings grown in loose, wet soil were significantly taller (43.7 and 36.0 cm for fungicide- and non-fungicide-treated, respectively) than those grown in the other compaction, moisture combinations ($P < 0.0001$).

Watering level explained a significant portion of the variation between treatment means for most seedling variables (Tables 1 and 2). Compaction had a significant effect on aboveground biomass ($P < 0.042$); fungicide treatment had no significant effect on seedling growth or disease parameters ($P > 0.1$). The main effects were not consistent across treatment combinations, however, and factor interactions were significant in many cases (Table 2). For example, while frequent watering increased seedling growth and biomass in loose soil, growth in compacted soils was unaffected by irrigation level (Table 1). The effect of compaction on seedling growth was significant only on aboveground production for seedlings grown in the saturated soil moisture treatment ($P = 0.017$).

Ectomycorrhizal root tips were present on all seedlings. The mean infection rate was 30% of sampled root tips and ranged from 8 to 56% on individual seedlings. *Cenococcum geophilum* was the most abundant ECM species. Both compaction and watering treatment influenced ECM abundance, but the oomycete-specific fungicide had no effect (Table 2). Mean ECM infection was highest in optimal and dry loose soils (Table 1) and lowest in dry and wet compacted soils. Within both compaction treatments,

Table 1
Mean chestnut seedling dimensions and standard error after 90 days growth ($n = 8$)[a]

	Height (cm)	Root collar diameter (mm)	Leaf area (cm^2)	Seedling biomass		Ectomycorrhizal root tips[b] (%)
				Aboveground (g)	Belowground (g)	
Loose soil						
Dry	29.5 b (1.2)	3.6 a (0.2)	469.0 b (57.8)	3.7 b (0.4)	2.9 a (0.3)	33.2 ab (4.9)
Optimal	30.9 ab (2.0)	3.7 a (0.2)	393.3 b (53.3)	3.5 b (0.5)	2.8 a (0.4)	37.4 a (1.9)
Wet	36.0 a (2.2)	3.9 a (0.2)	679.6 a (85.8)	5.6 a (0.7)	3.6 a (0.4)	29.0 abc (1.1)
Compacted soil						
Dry	33.9 ab (1.6)	4.0 a (0.2)	494.1 ab (47.4)	4.1 b (0.2)	3.4 a (0.3)	22.1 c (2.9)
Optimal	34.4 ab (1.6)	4.1 a (0.4)	611.8 ab (112.8)	4.6 ab (0.6)	3.4 a (0.3)	31.7 ab (3.0)
Wet	30.7 ab (3.4)	3.8 a (0.2)	477.9 ab (27.9)	3.8 b (0.2)	3.0 a (0.4)	26.3 bc (2.3)

[a] Fungicide application had no significant effect on seedling growth so for clarity fungicide-treated seedlings are not shown. Within columns, similar letters indicate that vegetation type averages are equal based on Tukey's means separation test ($\alpha = 0.05$).

[b] ECM root tips from $n = 5$ seedlings per treatment.

Table 2
Treatment effects on chestnut seedling growth and belowground properties[a]

	Height (cm)	Root collar diameter (mm)	Leaf area (cm^2)	Seedling biomass		ECM root tips[b] (%)	Fine root necrosis[b,c] (%)
				Aboveground (g)	Belowground (g)		
Main effects							
Watering (Water)	5.4***	4.0***	3.5**	5.3***	6.9***	2.8*	6.5***
Compaction (Comp)	1.7	0.2	1.2	4.3**	3.1*	6.0**	5.6**
Fungicide (Fung)	0.4	0.0	0.5	1.3	0.8	1.7	–
Interactions							
Comp × Water	6.2***	2.5*	5.8***	8.2****	3.6**	0.8	1.5
Comp × Fung	3.8*	5.3**	2.2	2.8*	7.5***	0.0	–
Water × Fung	2.9*	5.6***	0.2	0.6	4.6**	1.2	–
Comp × Water × Fung	0.0	0.3	1.8	0.4	0.2	0.4	–

[a] ANOVA *F*-test and significance levels for main effects and interactions.
[b] Arcsine transformed data.
[c] Fungicide treatment not included in statistical analysis of root necrosis.
* Significant at ≤ 0.1.
** Significant at ≤ 0.05.
*** Significant at ≤ 0.01.
**** Significant at <0.001.

ECM infection was numerically highest at optimal soil moisture and declined both under dry and wet conditions. Combined across compaction treatments, ECM root tips averaged 34.6% at optimal moisture compared to 30.5 and 27.7% in wet and dry soils, respectively ($P = 0.025$). Loose soils supported a significantly higher abundance of ECM root tips (33.2%) compared to compacted soil (26.6%; $P = 0.011$).

3.2. Disease development

Seedling mortality was highest in wet, compact soils in both fungicide-treated (1 dead of 8) and untreated (2 dead of 8) seedlings (Table 3). Mortality was light or non-existent in other treatment combinations; one seedling died in loose soil (dry, fungicide-treated) and one seedling died at optimal watering (compact, untreated). There were no visual symptoms

Table 3
Seedling mortality, spatial extent (mean, maximum), and proportional occurrence (frequency) of necrosis and Phytophthora on non-fungicide-treated live chestnut roots ($n = 8$ seedlings per treatment)

	Seedling mortality (%)	Fine root necrosis			Tap root necrosis			Phytopthora frequency[a] (%)
		Mean (%)	Maximum (%)	Frequency (%)	Mean (%)	Maximum (%)	Frequency (%)	
Loose soil								
Dry	0.0	8.1	30.0	50.0	0.6	5.0	12.5	37.5
Optimal	0.0	6.3	10.0	87.5	0.6	5.0	12.5	0.0
Wet	0.0	35.4	80.0	75.0	36.9	90.0	75.0	50.0
Compacted soil								
Dry	0.0	0.6	5.0	12.5	0.0	0.0	0.0	12.5
Optimal	12.5	3.1	10.0	57.1	0.7	5.0	14.3	28.6
Wet	25.0	11.9	50.0	66.7	5.8	20.0	33.3	16.7

[a] Presence of Phytopthora on fine roots is based on ELISA test.

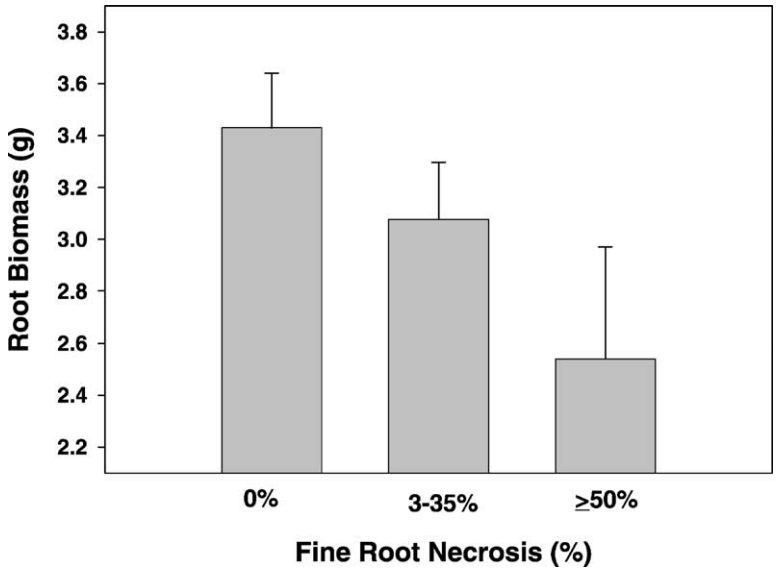

Fig. 1. Relationship between fine root necrosis and root biomass on American chestnut seedlings. Bars report mean ± 1 S.E. Root rot classes were determined by visual inspection of seedling root systems beneath a dissecting microscope.

of Phytophthora infection (i.e. ink stain) on stems or root collars of either healthy or dead seedlings.

In contrast to the aboveground seedling health, necrosis occurred on belowground tissues in all compaction and watering treatments (Table 3). Black rot with cinnamon-colored edges, characteristic of Phytophthora root rot, was present on fine roots ($\leq$1 mm diameter) as well as on coarser lateral roots (>1 mm diameter) and taproots. With the exception of a small amount of abiotic root damage (i.e. necrosis where root tips protruded from planting pots) that was uniform across all soil treatments, roots of fungicide-treated seedlings were free of necrosis.

On non-fungicide-treated seedlings, root rot occurred on $\geq$50% of seedlings in all treatments with the exception of the dry, compact treatment where only 1 of 8 seedlings exhibited root rot. Root necrosis covered up to 80% of fine roots and 90% of taproots. Within both compaction levels, root necrosis was most severe in the saturated soil treatment and less frequent in dry soils. Watering level explained the dominant portion of the variation between treatment means for root necrosis (Table 2).

Root biomass was lowest on trees that suffered the most severe root rot (necrosis on $\geq$50% of fine roots; Fig. 1); the belowground biomass these seedlings was one-third that of necrosis-free seedlings. Overall, root biomass was 15% lower on seedlings with visible root necrosis compared to those roots with no visible rot (one-way ANOVA contrast $P = 0.08$). Aboveground biomass, seedling height and root collar diameter were not consistently or significantly reduced by root rot during the course of the study.

The ELISA test confirmed that Phytophthora was present on 24% of all seedlings grown in untreated soil (Table 3); none of the fungicide-treated seedlings tested positive for Phytophthora. All seedlings with severely necrotic root systems ($\geq$50 of fine roots) as well as 53% of seedlings that did not exhibit visible root necrosis tested positively for Phytophthora. Sporangia of *P. cinnamomi* were identified by microscopy on all seedlings that had both fine root necrosis and that tested positive for the genus-level Phytophthora-specific ELISA test.

4. Discussion

Our greenhouse study demonstrates the susceptibility of American chestnut to Phytophthora root rot. During the 3-month trial period, root necrosis occurred within all soil moisture and compaction treatments, affecting 58% of the seedlings overall (Table 3). *Phytophthora* spp. were confirmed on the

majority of the necrotic root systems and *P. cinnamomi* was verified diagnostically. Though root rot had little impact on seedling height or diameter within the duration of the trial, it contributed to a 15% loss of root biomass on average (Fig. 1).

These findings are consistent with the pattern of rapid disease development that affected recent chestnut plantations in Kentucky (Brosi, 2001). The first indications of disease became evident 90 days after plantation establishment when seedling leaves began to yellow and wilt. Similar to our greenhouse results, belowground symptoms were well advanced at the time that aboveground symptoms first appeared; fine root necrosis and decay was widespread and the disease had begun to girdle seedling root collars. *P. cinnamomi* was positively identified on the roots of symptomatic seedlings at four distinct planting sites.

The soil physical conditions of the greenhouse bioassay, designed to mimic the conditions encountered in field trials, presented a moderate risk of Phytophthora attack. The silt loam planting substrate had 10% clay and was compacted to a bulk density of 0.7 g/cm^3. In contrast, forest sites with high risk of Phytophthora attack are typically associated with dense clay layers, hardpans, or high traffic areas with restricted drainage or periodic saturation (Campbell and Copeland, 1954; Erwin and Ribeiro, 1996). Root disease would probably advance more rapidly on chestnut seedlings grown in higher clay or higher density soils. Additionally, more aggressive root disease would be expected on seedlings grown in substrate intentionally inoculated with *P. cinnamomi* (Wilcox and Mircetich, 1985; Crandall et al., 1945) rather than in forest soil with a confirmed but non-quantified level of *P. cinnamomi* inoculum.

There was no significant relation between ECM and either root necrosis or Phytophthora occurrence across the range of ECM infection measured in our study ($r^2 = 0.00$, $P = 0.986$; $r^2 = 0.03$, $P = 0.733$). ECM infection rates averaged 30% across treatments and were reduced to 20% by soil compaction; though statistically significant, the biological relevance of this reduction on seedling growth is doubtful. In previous chestnut bioassays, we have found similar ECM infection rates on seedlings grown in a variety of Kentucky soil types without ECM spore inoculation (Rhoades and Miller, unpublished data). When amended with spores of the ECM species *Laccaria laccata*, *Hebeloma sinapizans* or *Paxillus involutus*, European chestnut root tips became densely covered by ECM mantles and hyphal strands, and resisted Phytophthora infection (Branzanti et al., 1999). The *C. geophilum* that we observed on American chestnut roots was scattered and the ECM fungi did not form dense or extensive hyphal sheaths. The protective effect of ECM against *P. cinnamomi* attack probably requires higher overall ECM infection and may require presence of certain ECM species that form dense networks of fungal mycelium.

4.1. Implications for chestnut restoration

It is uncertain how the high susceptibility of pure American chestnut to Phytophthora will influence efforts to establish blight-resistant chestnut hybrids in eastern North America. The resistance of Asian chestnut (*C. mollisima* and *C. crenata*) to Phytophthora root rot has long been recognized (Crandall et al., 1945). Phytophthora resistance is not however a selected trait within current chestnut-blight resistance breeding efforts, and may not be inherited by blight-resistant chestnut hybrids (F. Hebard, American Chestnut Foundation, pers. commun.).

Experience gained from southern Appalachian seedling trials indicates that heavy Phytophthora-related dieback can occur on relatively well-drained forest sites that previously supported American chestnut. Site selection for European chestnut orchards and other Phytophthora-susceptible forest species, typically avoids poorly drained sites (Erwin and Ribeiro, 1996). In contrast, site selection for reintroduction of chestnut to American forests, may be restricted to extremely well-drained sites; such locations represented a small portion of the American chestnuts' original landscape distribution (Braun, 1935; Rhoades and Park, 2001).

The high level of root disease pressure on American chestnut justifies experimentation with strategies to effectively produce and establish disease-free and disease-resistant root systems. In order to minimize root damage and disease associated with transplanting bare-root seedlings, The American Chestnut Foundation currently establishes its breeding orchards by direct seeding (Hebard, 2002). The success of soil fungicide at eliminating root disease in this short-term controlled greenhouse study warrants field trials to

evaluate operation-scale disease control protocols with bare-root seedlings. Future nursery and plantation trials should address the potential to establish and maintain high ECM colonization on American chestnut seedlings. Finally, cultural treatments to ameliorate soil compaction and improve soil drainage may be cost-prohibitive for widespread chestnut plantations, but may be justified in experimental plantings, breeding orchards and nut production orchards.

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