PERFORMANCE OF CONTAINER-GROWN SEEDLINGS OF AMERICAN CHESTNUT BACKCROSS HYBRIDS BC_3F_3 GENERATION IN CENTRAL LOUISIANA

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Abstract—Seedlings from two families of the BC_3F_3 backcross generation of the American chestnut (*Castanea dentata*) and Chinese chestnut (*C. mollissima*) were cultured in 2013 in Missouri using the Root Production Method®, a container-based system used to avoid disease problems associated with bareroot nursery production. After culling part of the seedlings at initial leaf formation in a greenhouse, both the culled and the remaining "kept" seedlings were transplanted into containers of 1.33, 1.57, and 2.62 L and grown outdoors. Seedlings, remaining in their containers, were brought to central Louisiana in mid-June 2013 and cultured outdoors. Half of the seedlings were harvested in September, and the other half continued to grow until storage in a cold room in mid-November. Of the seedlings harvested in September, family W4938 (W) seedlings had 90 percent more biomass than family D3862 (D) seedlings. Patterns of biomass allocation from each component within a seedling did not differ between families. All but one seedling had short (less than 6 cm) taproots, whereas the lengths of first-order lateral roots and adventitious roots were similar to the depths of the containers used. In family W, seedlings cultured in the 2.62 L containers were larger in size and biomass than those of the smaller volumes. Seedlings were outplanted in central Louisiana in late March, 2014. First-year survival was only 47 and 33 percent for the W and D seedlings, respectively. High summer temperature and local wet planting spots are suspected of causing the high seedling mortality.

INTRODUCTION

The chestnut blight fungus (*Cryphonectria parasitica* Barr) killed almost all mature American chestnut (*Castanea dentata* Borkh) trees throughout the species' native range in the first half of the 20th century. Today, young trees originating from tree stumps or suppressed seedlings established before the arrival of blight typically do not grow much more than 15 m in height before eventually succumbing to the blight (Paillet 2002). The restoration strategy used by the American Chestnut Foundation is a backcross breeding technique designed to produce trees with the American (timber type) genes while maintaining blight resistant genes from the Chinese (*C. mollissima* Blume) (orchard type) (Burnham and others 1986). In theory, the third generation of the third backcross (BC_3F_3) should have the desired growth habit of the American (94 percent American) and the blight resistance of the Chinese (6 percent Chinese) chestnuts (Hebard 2011).

The artificial regeneration of the blight resistant American chestnut hybrids has been the main approach used in recent years to restore this species to its native range. Clark and colleagues reported the nursery cultural protocol, seedling quality, and field performance of backcross chestnut seedlings (Clark and others 2009, 2012a, 2012b). For example, Clark and others (2012a) reported a 3-cm height increase in bareroot stock of chestnut seedlings for every 1-g increase in the mean nut weight. Thus, the overall nursery seedling quality could be improved by culling small nuts. Pinchot and others (2015) used individual nut weight to predict chestnut seedling quality and found a 1-g increase in nut weight resulted in increases of 6 cm in height and 0.5 mm in root collar diameter, and one additional first-order lateral root.

Between 2009 and 2011, Clark and others (2014) outplanted over 4000 1-0 bareroot seedlings of various generations on several National Forests in Virginia, North Carolina, and Tennessee. They found two 2010 plantings and six 2011 plantings were succumbing to a root rot disease (*Phytophthora cinnamomi* Rands). This fungus was present in the bareroot nursery and in some of the planting sites. *Phytophthora* root rot is thought to have contributed to chestnut dieback prior to the arrival

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of chestnut blight (Anagnostakis 2012). Rhoades and others (2003) cautioned that it is crucial to recognize and avoid sites where soil physical factors such as wetness and compactness promote *Phytophthora* root rot. Growing seedlings in containers with sterile media will reduce the risk of planting root rot-infected bareroot seedlings in the field and will hopefully improve the success of restoring American chestnut in the forests. The objective of this study was to examine growth performance, including root system morphology, of the backcross American chestnut seedlings cultured in containers of various dimensions. Because the native range of this species does not include Louisiana, this study was limited to one year after outplanting.

MATERIALS AND METHODS

Seedling Culture

Nuts from two families of the BC_3F_3 generation, D3862 (D) and W4938 (W), came from the open-pollinated B_3F_2 seed orchard of the American Chestnut Foundation in Meadowview, VA (Hebard 2011). Progenies were putative half siblings. Seedlings were grown at the Forrest Keeling Nursery (Elsberry, MO) using a modified version of their Root Production Method® in 2013. This method is designed to produce root systems consisting of many adventitious roots stemming from a shortened taproot. Nuts were sown into 3-cm deep mesh-bottomed trays in a cold room in December of 2012 and then moved to a greenhouse in March 2014 to initiate germination. The taproot was air pruned at around 3 cm, consistent with the depth of the germination tray. The Root Production Method® typically involves culling conducted at the initial leaf formation stage, based on size of shoot, to discard the smallest seedlings. However, we wanted to test the effects of this culling treatment on American chestnut, and seedlings were divided into two groups, "kept" or "culled." After the culling treatments, the germinant seedlings were transplanted into mesh-bottomed, open drainage, square containers of three volumes, namely, small (7.6 cm/22.9 cm/1.32 L, width/depth/volume), medium (10.2 cm/15.2 cm/1.58 L), and large (10.2 cm/25.4 cm/2.64 L). Depth of the small containers is greater than that of the medium containers. Roots were naturally air pruned. The growth media (decayed rice hulls, sand, and pine bark) were amended with time-release fertilizer. Seedlings were cultured outdoors for four weeks after transplanting. Due to low germination rates, only 18 D and 37 W seedlings were transported in containers to the Alexandria Forestry Center in Pineville, LA, in mid-June 2013. Seedlings continued to grow outdoors. In late June, some seedlings began to show signs of heat damage, with brown discoloration and curling of the leaf edge. Two layers of 30 percent shade cloth were applied across the iron frame (3-m tall) over the seedlings to lower the temperature and light intensity.

Seedling Assessment

Growth and photosynthesis—In mid-July, 8 D and 16 W seedlings were randomly selected for photosynthesis measurements with a LiCor® 6400 portable, open-system infrared gas analyzer (LiCor®, Lincoln, NE). Measurements were made between 9 a.m. and 11 a.m. and again between 1 p.m. and 3 p.m. on the same day on the most recently fully expanded leaves. Photosynthetic active radiation was set between 1400 and 1600 $\mu E\ m^{-2}\ s^{-1}$ with a red-blue light source and the CO_2 level for the reference chamber was 400 ppm. The middle section of the attached leaf was enclosed in the measuring chamber (2 x 3 cm). After each photosynthesis measurement, a hole punch was used to obtain a sample of 2.85 cm^2 from the same leaf for chlorophyll analysis. The leaf samples were stored on ice and transported to the laboratory. Chlorophylls a and b were extracted with N,N-dimethylformamide and the absorbance of the extract was read at 664 nm and 647 nm as described in detail by Sung and others (2010). Seedling height and root collar diameter were also measured. Half of the seedlings were harvested in mid-September for biomass and root system morphology. The remaining seedlings were stored in a cold room from December until being outplanted in March 2014.

Root system morphology—Parameters of the root system morphology assessed included the number of first-order lateral roots (FOLR) and adventitious roots (ADV), and lengths and dry weights of taproots, FOLR, and ADV. An FOLR was defined as a lateral root that originated from the taproot, sturdy in structure, and with a diameter, measured 1 cm away from the taproot, of at least 1.5 mm (for D) or 2.0 mm (for W). The ADV initiated near the air-pruned end of the taproot.

Outplanting

The field experiment site is on an open field located on the Palustris Experimental Forest within the Kisatchie National Forest in Rapides Parish of central Louisiana (31°11', -92°41'). The soil is a moderately well-drained, gently sloping Beauregard silt loam (fine silty, siliceous, superactive, thermic, Plinthaquic Paludults). A power auger with a 10-cm bit was used to drill holes large enough to accommodate roots of seedlings cultured in the largest containers. Soil from the holes was used to fill the hole after planting. Seedling mortality was monitored quarterly.

Statistical Analysis

The original study was designed as a 2 by 2 by 3 factorial with 2 nut sizes (large and small) by 2 seedling grades (kept and culled) by 3 container sizes (small, medium, and large) for each of the two backcross families. However, due to low germination rates, only 18 D and 37 W seedlings were used in the analysis.

Seedlings in the D family came from 4 of the original 12 treatments, namely, nut size (large or small) in combination with 2 container sizes (large or medium). All D seedlings were in the kept category. For the W seedlings, each of the 12 treatments had at least 2 seedlings except the small nut, culled, medium-container treatment which had only 1 seedling.

Seedlings from all 12 treatments were combined and comparisons made between the two backcross families for growth, photosynthesis rate, chlorophyll content, and root system morphology. All comparisons between two families were tested for differences at the $\alpha = 0.05$ level using PROC GLM from the SAS Institute (SAS 2004). In order to have a valid statistical test based on the analysis of variance (ANOVA), it is assumed that the data are independent and normally distributed, and that the variance is equal in each group. Because the seedlings were randomly arranged during growth, independence is assumed. All residuals were checked for normality and homogeneity. Often, the assumption of normality was violated, and the assumption of homogeneity was violated occasionally. In cases where either assumption was violated, the data were reanalyzed using PROC NPAR1WAY (SAS 2004) with the Wilcoxon option, but this did not change any results.

Similarly, container size was tested for differences at the $\alpha = 0.05$ level using PROC GLM. Only the W seedlings, which came from containers of all three sizes, were used for the analysis. All residuals were checked for normality and equality. Because the sample sizes were nearly equal, ANOVA is robust to unequal variance. Often, the assumption of normality was violated, but the statistical power lost by this was without effect because the tests that had non-normal residuals either had significant differences or were not close to significance.

RESULTS AND DISCUSSION

Most chestnut seedlings had set a terminal bud and completed their height growth by early August (table 1). This was at least one month sooner in growth termination than the reported height growth termination by bareroot chestnut seedlings grown in Delano, TN (Pinchot and others 2015), probably due to the summer heat in our area. Both W and D family seedlings had at least 40 percent of their leaves suffering from heat damage by late August. Nevertheless, the W seedlings were 72 percent taller than the D seedlings (table 1). The W seedlings harvested in September weighed almost twice as much as the D seedlings (table 1). However, patterns of biomass allocation by each component within a seedling did not differ between the two families.

Mean morning and afternoon photosynthesis rates measured in mid-July were 13.1 and 11.6 μmol m^{-2} s^{-1}, respectively, for the D seedlings and 12.5 and 10.4 μmol m^{-2} s^{-1}, respectively, for the W seedlings. The higher

Table 1—Mean seedling parameters for Root Production Method® container-grown chestnuts from two BC_3F_3 generation families, D3862 and W4938, harvested in September 2013. Height was measured on all seedlings

Parameter[a]	D3862	W4938
Height (cm)		
July 11*	33.0	53.2
July 26*	34.0	57.6
Aug. 8*	34.5	59.4
Aug. 26*	34.2	59.0
Sept. 11*	33.5	58.1
Root collar diameter (mm) Sept. 11	7.8	8.4
Healthy leaves >5 cm long (#) Aug. 26	6.9	11.1
Damaged leaves >5 cm long (#) Aug. 26*	4.9	9.4
Seedling dry weight (g)*	13.0	24.8
Leaf dry weight (g)	4.5 (34.6)[b]	7.7 (31.0)
Stem and branch dry weight (g)	3.9 (30.0)	9.4 (37.9)
Root system dry weight (g)	4.6 (35.4)	7.7 (31.0)

[a]Parameters associated with * have Pr > F values of <0.05.
[b]Numbers in parentheses are percent of biomass allocation within a seedling.

photosynthetic rates measured in this study compared to those of potted American chestnut seedlings grown under full sun (Wang and others 2006) suggested that the two layers of 30 percent shade cloth used in this study did not negatively affect seedling photosynthesis or growth. The chlorophyll a+b content for the D and W seedlings were 60.3 and 55.2 nmol cm^{-2}, respectively. The chlorophyll a-to-b ratio was similar between families (3.70 for D and 3.78 for W). Neither photosynthetic rate nor chlorophyll content of the D seedlings differed from those of the W seedlings. Lower numbers of both healthy and heat-damaged leaves in D than in W may be the main reason for less height growth and biomass accumulation for D seedlings (table 1). Compared to the values of height and root collar diameter reported for bareroot nursery seedlings (Clark and others 2012b, Pinchot and others 2015), container-grown seedlings in this study were much smaller. Family, cultural practices (container versus bareroot), and high temperature and humidity growth conditions in central Louisiana may have contributed to the smaller seedlings in this study.

We used a lower lateral root diameter limit for FOLR in the D seedlings than the W seedlings (1.5 versus 2.0 mm) for three reasons. First, D seedlings were much smaller than W. Second, using a higher diameter limit would render some of the D seedlings without any FOLR, but these seedlings did have some lateral roots with sturdy structure and a diameter ranging between 1.5 and 2.0 mm. Third, the W seedlings' lateral roots with a diameter between 1.5 and 2.0 mm usually were not sturdy in structure. Although not statistically significant, the percentage of biomass allocated to the non-FOLR group was less for the W seedlings, suggesting that only a few potential FOLR, based on the 1.5-mm criterion, were placed in the non-FOLR group with the 2.0-mm criterion. Greater numbers of FOLR have long been associated with larger seedling sizes in pines (*Pinus* spp.), oaks (*Quercus* spp.), and the American chestnut (Kormanik and others 1990, 1998a, 1998b, Pinchot and others 2015). In this study, the greater seedling size of the W seedlings (table 1) cannot be explained by their FOLR number. However, if the 2.0-mm limit had been applied to the D seedlings, their mean FOLR number would have reduced from 4.9 to 2.4, and this difference may have been significant. The W seedlings also had greater ADV diameter than the D seedlings (table 2). The rest of the root system parameters assessed did not differ between families.

Taproots in all but one seedling were shorter than 6 cm (table 2). A W seedling cultured in a large container had a curved taproot of 25 cm and was excluded from the analysis. Otherwise, taproot lengths were similar between the two families. The short taproots and the presence of adventitious roots (table 2, fig. 1) that formed near the end of the taproot met one of the objectives of the modified Root Production Method® used. The taproot was air-pruned in the 3-cm tray prior to culling and transplanting into individual containers. However, 32 percent of the seedlings assessed had taproots longer than 3.5 cm and had more than one curve along their length. It is probable that these taproots extended horizontally in the tray before reaching the tray bottom and eventually being air pruned. It would be interesting to find out if growing chestnut seedlings in deeper germination trays increases the number of FOLR, decreases the number of ADV, or both. The Root Production Method® is in contrast to the general practices of container nurseries. Tree seedlings that have been cultured in containers from seeds without being transplanted in the middle of the culture period generally have taproot lengths consistent with their container depths. For example, Sung and others (2009) reported that 58, 36, and 6 percent of FOLR originated from the top 5 cm, middle 5 cm, and bottom 4.4 cm of taproots, respectively, in container-grown longleaf pine (*P. palustris* Mill). In other words, taproot lengths for most longleaf pine seedlings cultured in 12- to 15-cm deep containers were greater than 10 cm.

Lengths of FOLR and ADV were similar between the two families. Furthermore, lengths for FOLR and ADV were closely associated with container depths. For example, lengths of FOLR from seedlings grown in small (23-cm deep), medium (15-cm deep), and large (25-cm deep) containers were 15.9, 13.1, and 23.0 cm, respectively. Similarly, lengths of ADV were 17.1, 12.0, and 22.4 cm for the seedlings grown in small, medium, and large volume containers, respectively. Some FOLR and ADV were longer than the container depths, as shown by the ranges in table 2, indicating root spiraling inside the containers (fig. 1).

Effects of container size on W seedling size and root system are presented in table 3. The large-container seedlings were larger in all growth parameters than the small-container seedlings except for leaf dry weight and a few root system parameters. The medium-container seedlings had values similar to those of the small-container seedlings for most parameters assessed. Container size did not affect seedling photosynthetic rate (a.m. or p.m.), chlorophyll a+b content, or chlorophyll a-to-b ratio. Taproot length, FOLR diameter, ADV number and diameter, and the FOLR and ADV origination points on the taproot were also unaffected (table 3). As shown previously, lengths of FOLR and ADV were significantly greater for the large-container seedlings than for the rest of the seedlings. Since the small containers are deeper than the medium containers, the small-container seedlings had greater

Table 2—Mean root system morphology of Root Production Method® container-grown chestnut seedlings from BC_3F_3 generation families, D3862 and W4938, harvested in September 2013

Parameters[a]	D3862	W4938
Taproot dry weight (g)	1.3 (28.3%)[b]	1.9 (25.0%)
FOLR[c] dry weight (g)	1.0 (21.7%)	2.3 (29.0%)
Non-FOLR[d] dry weight (g)	0.6 (13.0%)	0.6 (7.9%)
ADV[e] dry weight (g)	1.7 (37.0%)	2.9 (38.2%)
FOLR (#)	4.9 (1-17)	5.9 (1-14)
ADV (#)	3.7 (1-10)	4.7 (2-8)
Taproot length (cm)	2.8 (1.3-4.2)	3.2 (1.3-5.5)[f]
FOLR diameter (mm)*	2.0 (1.5-4.8)[g]	3.4 (2.0-6.7)
ADV diameter (mm)*	3.1 (1.7-6.4)	4.2 (2.0-8.4)
FOLR length (cm)	18.4 (4.1-28.7)	16.7 (1.9-29.3)
ADV length (cm)	17.9 (3.2-26.1)	16.5 (3.9-30.2)
FOLR origination point on taproot (cm)	1.7 (0.6-3.3)	1.6 (0.1-4.2)
ADV origination point on taproot (cm)	2.4 (0.2-3.8)	2.6 (0.3-4.5)

[a] Parameters associated with * have Pr > F values of <0.05.
[b] Numbers in parentheses are percent of biomass allocation within the seedling root system or range for a given parameter.
[c] Lateral roots originated from the taproot and with a diameter at least 1.5 and 2.0 mm for D and W seedlings, respectively.
[d] Lateral roots originated from the taproot but not sturdy or large enough to be counted as FOLR.
[e] Adventitious roots initiated near the taproot end.
[f] One large container-grown seedling with a 25-cm long taproot was excluded.
[g] Range in this section covered all individual roots in each family.

Figure 1—(L) Root system of a small nut, culled, large-container W4938 seedling of the BC_3F_3 backcross generation. It had a 1.3-cm long taproot, 1 first-order lateral root, and 5 adventitious roots with one of them (arrow) spiraling. (R) Root system of a large nut, kept, medium-container W4938 seedling pictured before the bottom root plug media was rinsed off for root assessment. It had a 2.2-cm long taproot, 8 first-order lateral roots, and 6 adventitious roots located near the taproot end.

Table 3—Effects of container size on chestnut seedling parameters of the BC_3F_3 generation family W4938 harvested in September 2013. Height was measured on all seedlings

	Container Volume		
Parameters	Large	Medium	Small
Height (cm)			
July 11	59.7A	52.6B	47.2B
July 26	68.6A	55.1B	48.9B
Aug. 8	73.0A	55.3B	49.3B
Aug. 26	73.1A	55.0B	48.5B
Sept. 11	72.0A	53.7B	48.3B
Root collar diameter (mm) Sept. 11	9.5A	8.1B	7.6B
Healthy leaves >5 cm long (#) Aug. 26	15.3A	9.5AB	7.3B
Damaged leaves >5 cm long (#) Aug. 26	12.3A	6.7AB	9.1B
Seedling dry weight (g)	38.6A	20.4B	21.5B
Leaf dry weight (g)	11.3A	6.1A	7.0A
Stem and branch dry weight (g)	15.8A	7.5B	7.5B
Root system (g)	11.4A	6.8B	6.9B
TAP dry weight (g)	2.8A	2.0AB	1.4B
FOLR dry weight (g)	3.6A	2.3AB	1.8B
Non-FOLR dry weight (g)	0.7A	0.7A	0.4A
ADV dry weight (g)	4.4A	1.7B	3.3AB
FOLR (#)	6.6AB	8.8A	4.1B
ADV (#)	5.8A	4.0A	4.9A
FOLR diameter (mm)	3.8A	3.1A	3.8A
ADV diameter (mm)	4.8A	3.8A	4.4A
TAP length (cm)	3.0A	3.7A	2.5A
FOLR length (cm)	22.8A	13.0B	16.6B
ADV length (cm)	22.8A	12.4C	17.1B
FOLR origination point on TAP (cm)	1.8A	1.6A	1.4A
ADV origination point on TAP (cm)	2.5A	3.0A	2.1A

[a]One large container-grown seedling with a 25-cm long taproot was excluded.

[b]Numbers followed by the same letter are not different significantly at α = 0.05 by Duncan's multiple range test.

lengths in FOLR and ADV compared to the medium-container seedlings.

Nine D (mean height 36 cm) and 15 W (mean height 59 cm) seedlings were stored in a cold room from December 2013 until outplanted to a site at the Palustris Experimental Forest in central Louisiana in late March 2014. Most seedlings had some fully expanded leaves by early May. However, by September, only 47 and 33 percent of the W and D seedlings, respectively, had survived. Although the topographic change was not visually evident, all seedlings planted in lower-lying areas died. Apparently, the hot and humid conditions in central Louisiana were not conducive to the normal growth of the American chestnut hybrid seedlings.

CONCLUSIONS

The idea of planting container-grown, blight-resistant chestnut seedlings to reduce the risk of *Phytophthora* root rot infection and to enhance the artificial regeneration of the American chestnuts in the forests was sound. Although the environmental conditions in central Louisiana were not conducive to the growth and survival of the American chestnuts in the forest, culturing seedlings under shade cloth for the first growing season still provided some vital information regarding the Root Production Method® for American chestnut, which, to our knowledge, had never before been used. The W4938 seedlings were larger than the D3862 seedlings. Seedlings cultured in large containers (2.64 L in volume) had greater values in size and various root system parameters than seedlings from medium (1.58 L) and small (1.32 L) containers. Seedlings produced had short taproots and several adventitious roots, as expected with the Root Production Method®. Furthermore, lengths of the first-order lateral roots and adventitious roots were consistent with the depths of the containers. To validate the benefits of culturing American chestnut seedlings using this method, a greater number of families and more nuts in each family are needed for future testing.

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