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Establishment of American chestnuts (*Castanea dentata*) bred for blight (*Cryphonectria parasitica*) resistance: influence of breeding and nursery grading

Stacy L. Clark[1] · Scott E. Schlarbaum[2] · Arnold M. Saxton[3] · Frederick V. Hebard[4]

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Abstract European and American chestnut species (*Castanea*) have been decimated by exotic species, most notably chestnut blight (*Cryphonectria parasitica*), since the early nineteenth century. Backcross breeding programs that transfer blight disease resistance from Chinese chestnut (*C. mollissima*) into American chestnut (*C. dentata*) offer promise for chestnut restoration, particularly for the American chestnut which was a keystone species in eastern North America. Nursery prescriptions and conformity to desired American chestnut traits following planting must be tested, however, before blight resistance can even be evaluated. We tested early field performance of American and Chinese chestnut and hybrid seedlings from the third backcross generation (e.g., BC_3F_3) in two-aged regeneration harvests on highly productive sites in the southern Appalachians, USA. We also tested a common nursery prescription of grading seedlings by size prior to planting. BC_3F_3 seedlings had similar 4-year survival to American chestnut seedlings, but generally had smaller stem heights and ground-line diameters (GLD). Although blight had not yet substantially challenged some sites, the BC_3F_3 seedlings had blight incidence similar to the Chinese chestnut which was lower than the American chestnut. Visual seedling grading affected planting shock and stem height and GLD by the end of year 4. Large size-class seedlings had more stem dieback and 5 % lower survival compared to small size-class seedlings, but larger trees exhibited the same height in year 3 as small trees in year 4. Advanced breeding material (BC_3F_3) was successfully established during the stand initiation phase of forest development on highly productive sites, but deviations in

✉ Stacy L. Clark
stacyclark@fs.fed.us

1 Southern Research Station, Forest Service, U.S. Department of Agriculture, 2431 Joe Johnson Dr., Rm 274 Ellington Plant Science, Knoxville, TN 37996-4563, USA

2 Department of Forestry, Wildlife, and Fisheries, The University of Tennessee, 2431 Joe Johnson Dr., Rm 274 Ellington Plant Science, Knoxville, TN 37996-4563, USA

3 Animal Science Department, The University of Tennessee, 208C Brehm Animal Science Building, Knoxville, TN 37996-4574, USA

4 The American Chestnut Foundation, 29010 Hawthorne Dr., Meadowview, VA 24361-3349, USA

desired growth rate of the American chestnut was evident. Visual grading of seedlings affected establishment of breeding material, and should be considered in the restoration process.

Keywords Artificial regeneration · *Cryphonectria parasitica* · Exotic plant disease · Genetic family · Restoration · Visual grading

Introduction

Exotic diseases have been negatively impacting plant species around the world since humans have been able to travel the globe, and this will probably continue for the foreseeable future (Lovett et al. 2006; Liebhold et al. 2013). Similarity in climate between portions of eastern North American, European, and Asian forests often allows exotic pests to thrive without natural controls or evolutionary resistance of the host (Anagnostakis 2012; Fei et al. 2012; Santinia et al. 2012). The American chestnut [*Castanea dentata* (Marsh.) Borkh.] was a keystone species in eastern hardwood forests of North America for thousands of years (Paillet 1982; Delcourt and Delcourt 1997), until devastated by two Asian diseases, ink disease (casual agent—*Phytophthora cinnamomi* Rands) and chestnut blight [causal agent—*Cryphonectria parasitica* (Murr.) Barr] (Anagnostakis 2012). By the mid-1900s the species was extirpated as a functioning component of ecosystems and as a timber tree (Saucier 1973). The loss of American chestnut resulted in large-scale shifts in species composition, particularly on upland, well-drained stands where the species was most competitive (Keever 1953; Stephenson et al. 1991; Burke 2012). In particular, the loss of a sustainable and nutritious hard mast component probably negatively impacted wildlife species (Diamond et al. 2000; Orwig 2002; Gilland et al. 2012). Successful reforestation of blight-resistant American chestnut could restore historical ecosystem functions, including insect and wildlife population dynamics and soil chemical processes (Opler 1978; Ellison et al. 2005; Rhoades 2007; Dalgleish and Swihart 2012). Expertise and technology gained from American chestnut restoration research could potentially assist other programs attempting to restore tree species affected by exotic pests, including chestnut breeding programs in Europe (Míguez-Soto and Fernández-López 2012).

Although no longer a significant component in eastern forests, American chestnut now exists as understory sprouts from extant root systems, occasionally living long enough to flower and bear fruit (Mattoon 1909; Paillet 1988; Griffin 1992). This natural genetic resource is used for hybridization of American chestnut and blight-resistant Asian chestnut species (Hebard 2001; Anagnostakis 2012). The American Chestnut Foundation (TACF) is one program that uses a backcross breeding approach directed towards producing trees with the form, phenology, and growth characteristics of the American chestnut, while incorporating blight resistance from the Chinese chestnut (*Castanea mollissima* Blume) (Burnham et al. 1986; Hebard 2012). The most advanced breeding generation currently available for testing from TACF is the third intercross of the third backcross generation, commonly referred to as the BC_3F_3 generation.

Experimental material with theoretically high levels of blight resistance has only recently become available for testing (Hebard 2012). Research directed toward restoration of American chestnut has been historically focused on production of blight resistant trees through genetic modification by backcross breeding, (Merkle et al. 2007; Jacobs et al. 2013; Newhouse et al. 2014) or in Europe through use of hypovirulent blight strains (Milgroom and Cortesi 2004). Successful restoration, however, will also require proven nursery and silvicultural prescriptions for planting blight-resistant chestnuts in native forests before trees are even challenged by blight (Jacobs 2007; Clark et al. 2014b). Chestnuts have been planted in eastern North America since the time of European settlement (Emerson 1846; Hough 1878; Van Fleet 1914), but empirical research is limited (e.g., McCament and McCarthy 2005; Rhoades et al. 2009; Clark et al. 2009, 2012a).

In a previous study, American chestnut and BC_3F_3 half-sibling families were similar to each other in nut weight and 1-year growth in a commercial nursery study (Clark et al. 2012b), indicating breeding was successful in achieving desired phenotypic characteristics of hybrid nursery seedlings. BC_3F_3 families, however, had lower chestnut blight resistance than Chinese chestnut families in orchard inoculation tests of juvenile hybrids in an orchard setting (Hebard 2012). Forest field trials are important to any breeding program, and will provide information on performance of various breeding lines in 'real-world' conditions. Additionally, results from field tests can be used to complement orchard tests (Hebard et al. 2013). Although young BC_3F_3 seedlings have initially exhibited higher levels of blight resistance and are predicted to have equitable growth characteristics compared to American chestnut seedlings (Diskin et al. 2006; Hebard 2012), this supposition has not yet been evaluated. Inferences from these field trials will be more robust if confounding effects of genetic family and seedling stock type can be controlled (Pinto et al. 2011).

Nursery and grading protocols for eastern hardwood species have been developed to improve field performance by increasing overall size and quality of seedlings (Kormanik et al. 1994; Dey et al. 2008; Grossnickle and El-Kassaby 2015). Compared to conifer species, hardwood species are still largely undomesticated as a nursery crop, however, and uniformity of planting stock and predictions of seedling success cannot be reliably and repeatedly produced (Wilson and Jacobs 2006). American chestnut artificial regeneration practices are nearly completely untested (Clark et al. 2009). Infrastructural and institutional knowledge investments of producing advanced breeding material demands that mangers use proven methods for American chestnut restoration.

In the present study, we used experimental material from Clark et al. (2012b) to establish field tests in commercially harvested stands in national forests managed by the United States Department of Agriculture, Forest Service within the Central Appalachian Broadleaf Forest Province (Bailey et al. 1994) of the southeast US. This study is the first to empirically test the most advanced generation (i.e., BC_3F_3 generation) seedlings bred for blight resistance and desired American chestnut traits in silviculturally managed forests. The goal was to develop predictions of early field performance of breeding material and parental species and to evaluate cultural impacts of nursery seedling grading. A specific objective was to test effects of seedling size class, breeding generation or species, and genetic family on growth, survival, stem dieback, and blight resistance of artificially regenerated seedlings. These same dependent factors have been frequently used to determine success of particular genotypes or breeding lines in tree improvement or breeding programs of Fagaceae species in the US (Kriebel et al. 1988; Hebard 2012) and around the world (Míguez-Soto and Fernández-López 2012, 2015). BC_3F_3 generation seedlings were predicted to grow similar to American chestnuts with stable blight resistance from Chinese chestnut (Burnham et al. 1986; Hebard 2006). We, therefore, focused on specific comparisons between BC_3F_3 generation seedlings, and American and Chinese chestnut species.

Methods

Experimental material, seedling measurements, and size sorting

Seedlings were grown from nuts collected at the TACF's Research Farm in Meadowview, VA, USA in autumn 2007 (Table 6 in "Appendix 1"). Hereafter, a genetic family refers to seedlings derived from nuts collected from a single open-pollinated or controlled–pollinated mother tree located at the Meadowview orchard or from a wild population. Seedlings were putative half-siblings, a result of open-pollinated crosses between female (known genetic identity) and male (unknown genetic identity) parents. The American chestnut families were from open-pollinated mother trees growing wild in southeast VA, USA. The Chinese chestnut family was from a controlled pollination of a Chinese chestnut mother tree at the Meadowview orchard. Families from hybrid generations were produced through backcross breeding (Burnham et al. 1986) in seed orchards at Meadowview, and each generation had unique lineages. Different mother trees were used in each backcross of each generation, and the same backcrosses from early generations were not used in later generations (cf. Hebard 2006). Nuts from each family in a backcross generation were a result of open-pollinations in isolated BC_1F_2, BC_2F_2 or BC_3F_2 seed orchards (cf. Hebard 2006). Contamination within each orchard from outside seed sources was minimized by isolating each seed orchard and using close spacing of orchard trees (Burnham et al. 1986; Hebard 2006). Hereafter, the term 'generation/species' refers to the backcross breeding generation (BC_1F_3, BC_2F_3 or BC_3F_3), pure American chestnut, or pure Chinese chestnut from which nuts were collected. Each generation/species constitutes multiple genetic families, with the exception of the Chinese chestnut which was from one genetic family as indicated above. After collection, nuts were stored in slightly moistened sphagnum moss in plastic bags at 4 °C until sowing.

Nursery production of trees and nursery growth measurements were described by Clark et al. (2012b). After lifting from the nursery, each seedling was assigned an individual numbered tag to ensure that family identification was maintained. Lateral roots were clipped to within 15 cm of the main taproot to facilitate planting and to prevent root-tip dieback.

Seedlings from each family were visually assessed and equally divided into two seedling size classes (small or large). Size class distinctions were based on overall tree size with an emphasis on root-collar diameter because it was most highly correlated with other seedling variables in Fagaceae species (Clark et al. 2000, 2009, 2012b). Size class sorting allowed us to examine the effect of seedling quality and reduced potential bias associated with selecting larger seedlings first during sorting into experimental designs (cf. Pinto et al. 2011). Using a general linear model to analyse seedling size effects on nursery data prior to planting, we found significant differences in height ($df = 1127$, $F = 879.2$, $P < 0.0001$), RCD ($df = 1127$, $F = 776.7$, $P < 0.0001$) and FOLR number ($df = 1127$, $F = 192.3$, $P < 0.0001$) between size classes overall and within all families for height and RCD; only two families did not have significant size differences in number of FOLR. These results indicate that the visual separation of size classes was successful.

Study areas

Site selections and site preparation treatments were similar to those that would be conducted by USDA Forest Service, once blight-resistant seedlings become widely available

(Clark et al. 2014b). We divided the experimental material across three sites that were relatively similar in productivity (i.e., site index averaging 23 m for northern red oak (*Quercus rubra* L., base age 50) to ensure that planting failures caused by unpredictable events (e.g., severe drought or disease, Clark et al. 2009) did not result in complete loss of the study. The study was not designed to determine how specific site characteristics influence seedling performance, but to determine if interactions of genetic family or generation/species with location did exist. Seedlings were planted in three national forests in the Southern Region of the United States Department of Agriculture, Forest Service. One planting was located within the Northern Cumberland Mountain physiographic section in Virginia (N36°53′, W-82°32′), and two plantings were located within the Blue Ridge Mountain physiographic section in North Carolina (N35°00′, W-82°37′) and Tennessee (N36°09′, W-82°37′) (Table 6 in "Appendix 1"; Bailey et al. 1994). All three locations were within the Central Appalachian Broadleaf Forest Province (Bailey et al. 1994). Hereafter, locations will be referenced according to the USA postal code abbreviations for the corresponding state (NC, TN, or VA).

Sites were approximately 6 ha in size, and soils were moderate- to well-drained consisting of sandy, sandy-loam, or loamy-sand. Prior to harvest, stands were dominated by mature hardwoods, primarily oak (*Quercus* L.), with at least 18 m^2/ha of basal area. Elevation was 841 m above sea level (ASL) on the NC site, 1036 m ASL on the TN site, and 944 m ASL on the VA site. Slopes averaged 20–40 % from horizontal, and aspect direction of the plantings ranged from North (0°) to Southeast (130°). Natural American chestnut sprouts were found at or within 0.5 km from each site, indicating that planting areas were well-suited for American chestnut.

Stands were harvested using a shelterwood-with-reserve regeneration treatment, which creates a two-age stand and provides adequate sunlight to regeneration (Nyland 1996). Commercial timber harvests were completed within each stand from February 2008 until November 2008. After harvest, basal area averaged 2.3–4.6 m^2/ha of primarily oak and hickory (*Carya* Nutt.) species. Within each stand, site preparation treatments within the planting area included herbicide (triclopyr) treatment of undesirable species [i.e., species except oak, hickory, American chestnut, or cherry (*Prunus* L.) species] originating from stump sprouts of harvested trees that were at least 3 cm in diameter at breast height (DBH). The herbicide was applied pre-harvest using a hack-and-squirt tree injection, or immediately post-harvest using a streamline basal bark application to existing stump sprouts. Some species treated with herbicide, primarily red maple (*Acer rubrum* L.), did not die from the initial herbicide application, and sprouts were retreated either mechanically by cutting them to ground level or by re-spraying the sprouts with herbicide (triclopyr) using a streamline basal bark treatment in the year following planting. Care was taken to avoid herbicide application to the planted seedlings, and no damage from herbicide was detected.

Experimental design and planting methodology

We used an a priori experimental design to empirically test effects of nursery seedling size class, generation/species, and genetic family, on survival, growth, stem dieback, and blight incidence. In the center of each harvested stand, we planted using a resolvable incomplete block design with single tree plots with a nested, factorial treatment arrangement. Incomplete blocks were grouped together within a location to constitute one complete replication of each treatment combination. This design was used to accommodate the relatively high number of family by size class treatment combinations we had at each location (20–24); we could not assume that environmental variation would be the same within a complete replication. Each incomplete block consisted of six trees that represented six different treatment combinations at each location. Treatment combinations within each incomplete block were arranged using Proc Optex in program SAS (2012) to maximize the ability to test for generation/species, family and seedling size class differences.

Planting location was treated as a fixed effect so that we could empirically test location by treatment interactions and quantify treatment mean differences among and within locations. Family was treated as a fixed effect nested within the fixed effect of breeding generation/species and location. All American and BC_3F_3 families could not be planted at each location, due to lack of available material (Table 6 in "Appendix 1"). Identifying family and generation/species as fixed effects allowed computation of means and differences among means, which can be compared to results from previous orchard inoculation tests (Hebard 2012). Furthermore, the number of families within a generation tested was relatively low ($n \leq 5$) due to the lack of available material in TACF's breeding program, making extrapolation of results as a random variable to the general population of the breeding program difficult. Seedling size class was a fixed effect cross-classified with generation/species and family.

Seedlings were planted on a 2.5 m by 2.5 m spacing using modified JIM GEM® KBC bars (modified to increase bar width to 30 cm). Severe deer browse was noted at the VA and TN locations during the first growing season after planting. Tree bark protectors (A.M. Leonard company™) were, therefore, erected on all seedlings at these two locations after the first growing season. The protectors were 1.5 m in height, 10 cm in diameter, had a 2 cm by 2 cm rigid plastic mesh, and were held in place using metal rebar and cable ties. The mesh protectors were preferred over a closed shelter to avoid increasing temperature and moisture conditions around the tree that would be hospitable to blight or other disease infections (Minter et al. 1992; Ponder 1995; Ward et al. 2000). The protectors were left on the trees until year 3 after planting or until the tree grew above the top of the protector. Deer browse to the terminal bud could still occur on the seedlings after the tree protectors were erected because the stem sometimes grew through the 2 cm mesh; however, yearly maintenance of shelters was conducted to position terminal buds back into the tree protector.

Data collection

Data were collected just after planting (year 0) and in years 1–4 following planting, unless otherwise noted. Total stem height (nearest 1 cm) and ground-line diameter (GLD) (nearest 0.1 mm) were measured on all seedlings in the dormant season after bud set was complete (October–March). Total height was measured from the base of the tree to the top of the tallest live bud. Total GLD was measured where the stem emerged from the litter layer using a digital or manual dial caliper. GLD was not measured in years 2 and 3 for the VA and TN locations because tree protectors restricted access to the ground-line in these years. Mortality of the top portion of the main stem, with live buds present below the dead portion of the stem, was defined as stem dieback. Dieback associated with damage to the stem from deer browse was not included in estimates of stem dieback.

We recorded the presence or absence of natural chestnut blight cankers on live trees. Each tree was observed three times a year for blight, once in the early spring (April–May), once in the late growing season (August–September), and once in the dormant season (October–March). Blight was identified as an ellipsoid–shaped canker on the stem that was sometimes sunken or slightly swollen and was sometimes accompanied by bark discoloration. We were conservative in our identification of natural blight cankers, and the cankers had to be accompanied by vertical cracking or fissuring of the bark with mycelial fans just below the bark surface (visible with a 5× hand lens), and/or have orange stromata protruding through the bark surface (cf. Griffin and Elkins 1986). Questionable blight cankers were cultured and isolated as described by Anagnostakis (1986). The record of blight incidence recorded for a seedling in any year persisted for each consecutive year, including dead trees. Similarly, the record of blight absence recorded for a seedling also persisted for each consecutive year, including dead trees, until blight was identified.

Statistical analysis

All data were analysed using SAS (2012), and all models included the independent effects of location, seedling size class, family, and breeding generation/species, unless otherwise noted. We used a 5 % alpha level to denote statistical significance for all tests. We used general linear mixed models (PROC MIXED) to analyse dependent variables height and GLD. Models for GLD did not include years from some locations where tree bark protectors prohibited access to the ground line (described above). Models for GLD also did not include trees identified with chestnut blight; reaction to the blight often creates a swollen base that would bias GLD measurements. Replication within location and incomplete block within replication and location were random effects in models. Year was used as a repeated measure in the linear mixed models, and we used an autoregressive covariance structure (Littell et al. 1998). We compared corrected Akaike information criterion (AICc) values for other covariance structures (compound symmetry, unstructured, spatial power) and determined the autoregressive structure had the smallest AICc values and was the most parsimonious model Normality assumptions of residuals were assumed if Kolmogorov–Smirnov D-statistic was <0.05. Homogeneity of variance assumptions were tested for each model by examining plots of residual versus predicted values. Unequal variance was added to the model if plots of residuals versus predicted values indicated unequal variance was present in the dataset; degrees of freedom were accordingly adjusted in the unequal variance models using the Kenward–Roger method. A likelihood ratio test was used to test whether the unequal variance model was justified.

We used PROC GLIMMIX in SAS to conduct generalized linear mixed modelling (GLMM) on binary dependent variables survival (alive = 1 or dead = 0), stem dieback to the main leader (presence = 1 or absence = 0), and chestnut blight (presence = 1 or absence = 0). GLMMs were preferential over linear models for binary response data to accommodate for violation of non-normality assumptions (Bolker et al. 2009; Stroup 2014). We specified a binary response distribution with a logit link function, used LaPlace estimation methods, and GLMMs were modelled on live trees (event = 1). Adequate model fit was sometimes difficult due to inherit complexity of the a priori experimental design. We considered models to have an adequate fit when confidence intervals of the fixed effect estimates were not exceedingly large (e.g., <6 times the SE of the estimate) (Bolker et al. 2009). GLMMs were analysed by year, as opposed to repeated measures analysis, to simplify the GLMMs to improve model fit (Bolker et al. 2009). To further simplify the results, we restricted presentation of GLMM results to year 4 for survival and chestnut blight, and year 1 for stem dieback. (See Table 7 in "Appendix 2" for results for other years). GLMMs were sometimes further simplified to ensure adequate fit by excluding interaction terms of fixed effects or by dropping random incomplete block effects. If the incomplete block variable was dropped from the GLMM, incomplete block means were used as the input variable, and a binomial response distribution with a logit link function was used. GLMMs were produced by location for the chestnut blight response variable to ensure adequate fit. Overdispersion of the residuals was checked using a Pearson Chi square test, and all models had values <1, indicating a lack of overdispersion. For all GLMM or linear mixed models where main effects or interactions were significant, we computed comparisons among least-squares means using Tukey's mean separation, and we used macros (DAWG 2011) to more easily identify differences among the means.

Results

Survival

Mean 4-year survival was 82, 71 and 78 % for the NC, TN, and VA locations, respectively. Survival decreased the most from the time of planting to year 1 at the NC (10 %) and TN (16 %) location, and remained generally stable thereafter. However, the VA planting had a 7 % decrease in survival from year 3 to 4. Generation/species affected 4-year survival (Table 1), but the only significant difference was at the TN location where Chinese chestnut had lower survival (39 ± 7) than the American chestnut (80 ± 5) and all breeding generations (78–82). Seedling size class significantly affected 4-year survival (Table 1), and small size class seedlings had higher survival (82 ± 2) than large size class seedlings (77 ± 2). Family effects on survival were not significant in any year (Table 1 and Table 7 in "Appendix 2"). We could not obtain an adequate fit of the GLMM if size by family or three-way interactions were included in the models.

Height

Height growth was generally negligible from planting to the first growing season, and seedlings decreased in height at the TN location during this period (Fig. 1). Trees grew

Table 1 Generalized linear models for binary response variables (survival, stem dieback)

Source of variation	Survival[a]			Stem dieback[b]		
	DDF	*F*	*P*	DDF	*F*	*P*
Location	198	3.6	*0.0286*	198	9.6	*<0.0001*
Generation	915	2.7	*0.0310*	915	1.9	0.1142
Location × generation	915	3.2	*0.0013*	915	1.8	0.0693
Size	915	4.2	*0.0412*	915	16.9	*<0.0001*
Location × size	915	0.4	0.6554	915	1.3	0.2758
Generation × size	915	1.3	0.2730	915	2.9	*0.0205*
Family (generation location)	915	1.1	0.3489	915	1.0	0.4404
Size × family (generation location)	–	–[c]	–	–	–[c]	–
Location × size × generation	–	–[c]	–	–	–[c]	–

DDM denominator degrees of freedom

Italicised *P* values are significant at $P < 0.05$

[a] Results presented for year 4 only

[b] Results presented for year 1 only

[c] Interactions were not included to improve fit of model

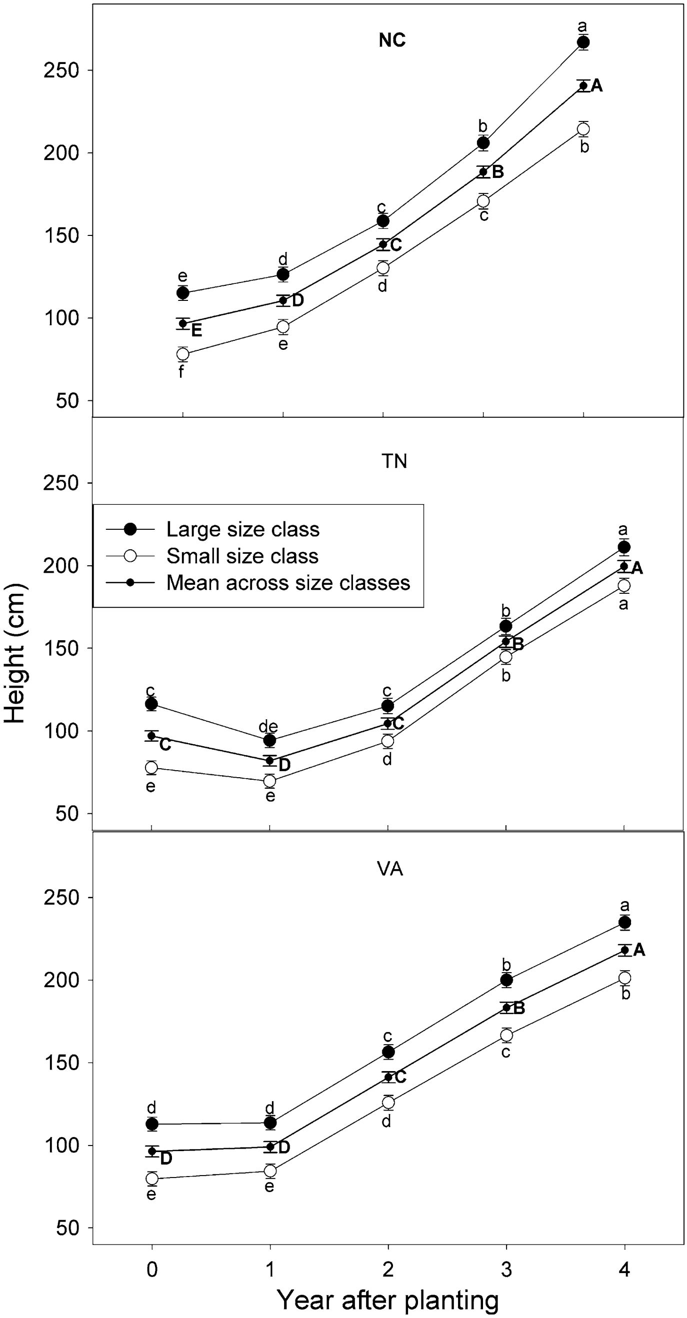

Fig. 1 Least-squares means and associated SEs of height for each location by size class. The *same uppercase letters* are not significantly different among years within the same location. The *same lowercase letters* are not significantly different between size classes and among years within the same location

significantly in annual height growth after year 1 at all locations, and averaged 200–241 cm in height after 4 years. Generation/species affected height (Table 2) beginning in year 2 when American chestnut seedlings were taller than Chinese chestnut seedlings at the VA location (Fig. 2). The American chestnut and BC_3F_3 generation had similar height at all locations until year 4 when the American chestnut was approximately 40 cm taller than the BC_3F_3 generation at the NC and VA locations. The Chinese chestnut was 45 and 80 cm shorter in 4-year height than the BC_3F_3 generation at the VA and TN locations, respectively, but did not differ in height with the BC_3F_3 generation at the NC location. The American chestnut grew 51–54 cm per year in height, while the BC_3F_3 generation grew 40–46 cm per year.

Seedling size grading resulted in a 33–38 cm height advantage for large size class seedlings over small size class seedlings at the time of planting for each location. After planting, size class interacted with year, location, and family to affect height (Table 2). Size grading significantly improved height for only two families (NB1 at NC and D4 at VA), but non-significant differences between large and small size classes were as large as 91 cm (D4 at NC) or −63 cm (D5 at VA) (Table 3). Across all families, large size class seedlings at the NC and VA locations were 53 and 34 cm taller compared to small size class seedlings, respectively, but size class effect on height was generally not significant at the TN location. Large size class seedlings in year 3 had similar height to small size class seedlings in year 4 for all locations except TN (Fig. 1).

Table 2 General linear models for continuous response variables (height and ground-line diameter, GLD)

Source of variation	Height			GLD		
	DDF	*F*	*P*	DDF	*F*	*P*
Location	49	24.3	*<0.0001*	49	18.2	*<0.0001*
Generation	868	13.6	*<0.0001*	868	3.6	*0.0068*
Location × generation	868	1.3	0.2185	868	0.6	0.7499
Size	868	123.1	*<0.0001*	868	174.7	*<0.0001*
Location × size	868	1.4	0.2479	868	0.7	0.5033
Generation × size	868	1.2	0.3000	868	2.8	*0.0244*
Family (generation location)	868	2.3	*0.0027*	868	1.4	0.1565
Size × family (generation location)	868	1.6	0.0706	868	0.9	0.5801
Location × size × generation	868	0.5	0.8871	868	0.2	0.9891
Year	3470	1208.1	*<0.0001*	2186	730.4	*<0.0001*
Year × location	3470	28.0	*<0.0001*	2186	44.1	*<0.0001*
Year × generation	3470	14.5	*<0.0001*	2186	13.3	*<0.0001*
Year × location × generation	3470	1.9	*0.0020*	2186	0.8	0.7216
Year × family (generation location)	3470	1.9	*<0.0001*	2186	3.4	*<0.0001*
Year × size	3470	5.4	*0.0003*	2186	4.5	*<0.0001*
Year × location × size	3470	2.3	*0.0216*	2186	1.5	0.1992
Year × size × generation	3470	1.0	0.4805	2186	2.4	*0.0018*
Year × size × family (generation location)	3470	1.3	*0.0373*	2186	1.6	*0.0066*
Year × location × size × generation	3470	1.0	0.4696	2186	0.7	0.8172

DDM denominator degrees of freedom

Italicised *P* values are significant at $P < 0.05$

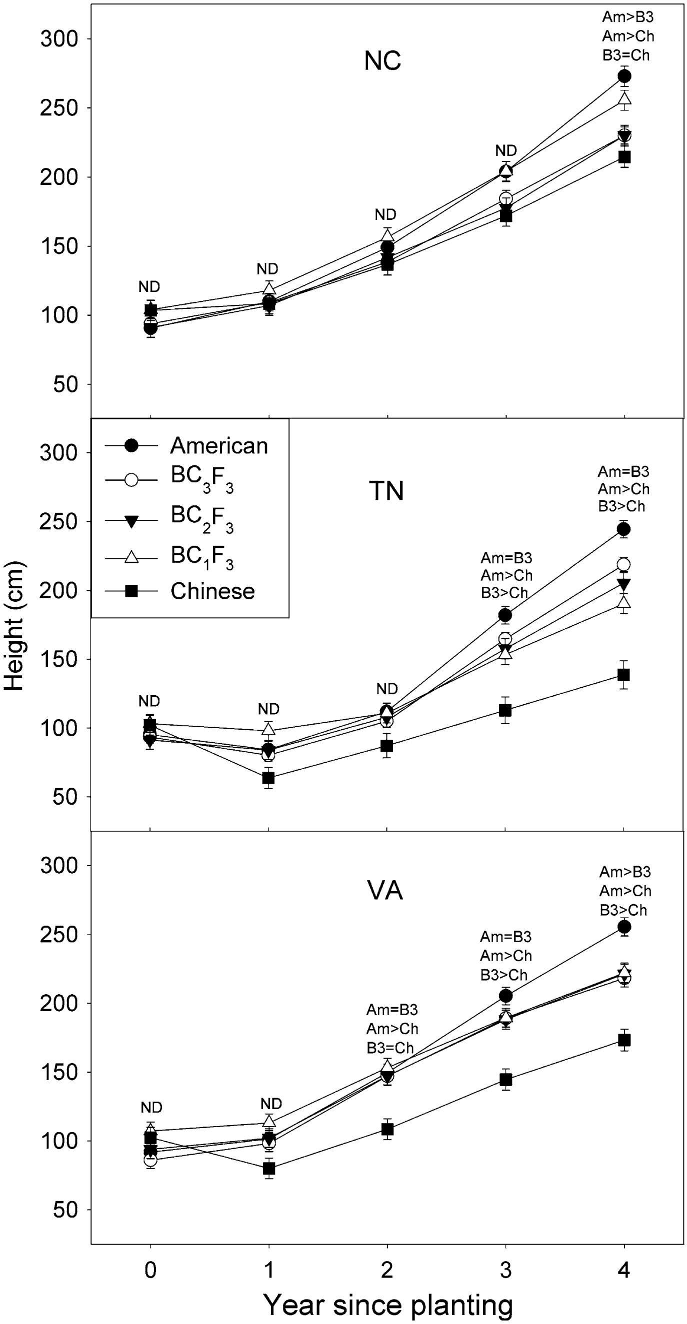

Fig. 2 Least-squares means and associated SEs of height for each location by generation/species. Specific mean comparisons among the American chestnut (Am), BC_3F_3 (B3) generation, and Chinese chestnut (Ch) are shown within each year and location. *ND* indicates differences were not significant

Table 3 Least-squares means of four-year height for each family and between large and small size class seedlings within each family

Generation	Family	NC			TN			VA		
		Overall mean	Large	Small	Overall mean	Large	Small	Overall mean	Large	Small
American	GMNEW	301 a	321 a	281 abc	258 a	278 a	238 abcde	260 a	297 a	222 abc
American	PL1S					234 abcde	228 abcde	251 a	273 a	230 abc
American	TOWERS1	245 a	273 abcd	216 bcde	231 a					
BC_1F_3	NB1	256 a	*311 a*	*201 bcde*	208 a	252 abc	164 cdef	226 a	240 abc	212 abc
BC_1F_3	NB35	255 a	287 abc	223 abcde	172 a	171 bcdef	174 bcdef	218 a	237 abc	200 abc
BC_2F_3	SA330	230 a	235 abcde	224 abcde	216 a	217 abcdef	216 abcdef	220 a	232 abc	209 abc
BC_2F_3	SA417	230 a	255 abcde	206 bcde	195 a	228 abcde	162 def	222 a	245 ab	200 abc
BC_3F_3	D1				228 a	227 abcde	230 abcde			
BC_3F_3	D2	180 a	191 cde	168 e	174 a	176 abcdef	172 abcdef	190 a	206 abc	174 bc
BC_3F_3	D3				246 a	248 abcd	244 abcde			
BC_3F_3	D4	245 a	291 ab	200 bcde	236 a	259 ab	213 abcdef	227 a	*276 a*	*179 bc*
BC_3F_3	D5	265 a	278 abc	253 abcde	210 a	212 abcdef	209 abcdef	238 a	206 abc	269 ab
Chinese	Chinese	215 a	240 abcde	189 de	139 a	142 ef	135 f	173 a	184 bc	163 c

Overall means followed by the same letter are not significantly different among families within the same location. Size class means followed by the same lowercase letter are not significantly different between size classes within the same family and location. Italicised values indicate seedling size class differences within the same family and location

Despite significance of family main effects and interaction with year (Table 2), and relatively large differences (>100 cm) between overall family means, mean differences in height among families were not significant at any location (Table 3). Family effects interacted with year and size class, however to affect height (Table 2). BC_3F_3 family D2 was 100 cm shorter in 4-year height compared to BC_3F_3 family D4 within the large size class and was at least 113 cm shorter than American family GMNEW within both size classes at the NC location (Table 3). Differences between family D2 and the Chinese chestnut family were not significant at any location and within any size class.

Ground-line diameter

Trees grew 1.5, 0 and 1.7 mm in GLD in the first growing season, and averaged 30.8 (±0.4) mm, 24.1 (±0.5), and 23.9 (±0.5) by the fourth year, for the NC, TN, and VA locations, respectively. Generation/species affected GLD each year after planting, and these differences were not dependent on location (Table 2; Fig. 3). At the time of planting, the Chinese chestnut was approximately 3.0 mm larger in GLD than the American chestnut and the BC_3F_3 generation (Fig. 3). By the fourth growing season, however, the American chestnut was 8.6 mm larger than the Chinese chestnut and 5.9 mm larger than the BC_3F_3 generation. Differences between the BC_3F_3 generation and the Chinese chestnut in 4-year GLD were not significant. The American chestnut and the BC_1F_3 generation were the only generation/species to increase significantly in GLD from the time of planting to the first growing season (data not shown). From the first to the fourth year after planting, the American chestnut, the Chinese chestnut, and the BC_3F_3 generation averaged approximately 6.2, 2.7 and 4.3 mm of GLD growth per year, respectively.

At the time of planting, large size seedlings were 4.7 mm larger in GLD than small size seedlings. After planting, size class interacted with generation/species to affect GLD

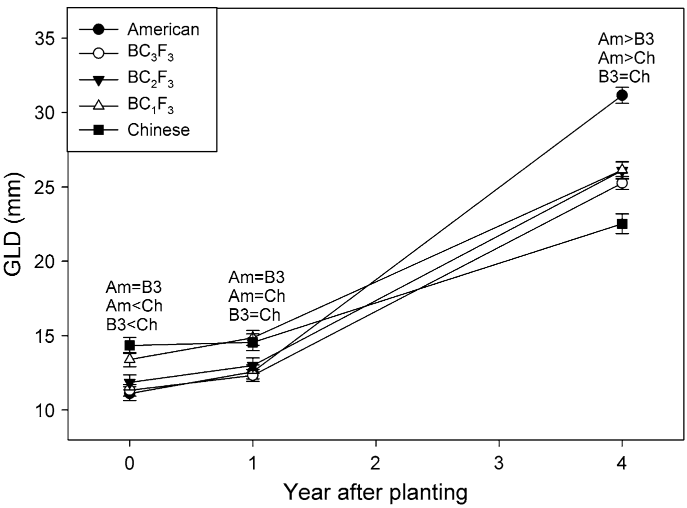

Fig. 3 Least-squares means and associated SEs of ground-line diameter (GLD) for each generation/species across all locations. Specific mean comparisons among the American chestnut (Am), BC_3F_3 (B3) generation, and Chinese chestnut (Ch) are shown within each year. *ND* indicates differences were not significant

(Table 2). By the fourth growing season, the large size class seedlings within the American and Chinese chestnut species and the BC_3F_3 generation were 10.5, 5.7 and 3.9 mm larger in GLD, respectively, compared to small size class seedlings (Fig. 4). Size class also interacted with year and family (Table 2). Only one BC_3F_3 family (D4 at TN) and two American chestnut families (TOWERS1 at NC and GMNEW at VA) had significant 4-year height differences between large and small size classes (Table 4). Size grading did not affect GLD for Chinese chestnut if analysed as a family (not species) effect. Non-significant differences between large and small size classes within a family, however, were as large as 11.3 mm (D4 at NC) or −2.3 mm (D3 at TN) (Table 4).

Family differences in GLD were significant at the NC and TN locations. BC_3F_3 family D2 had smaller GLD in year 4 compared to most other BC_3F_3 families (Table 4). Family D2 had similar 4-year GLD to Chinese chestnut at each location, and was significantly smaller in GLD to American chestnut family GMNEW at all locations.

Stem Dieback

First year stem dieback averaged 22 (±2), 9 (±1), and 19 (±2) % at the NC, TN, and VA locations, respectively. TN was the only location to show an increase in dieback from years 1 to 2 (15 ± 2 %). Dieback in the fourth growing season at the NC and TN locations averaged less than or equal to 10 % while the VA location had 17 (±2) % dieback. Generation/species interacted with size class to affect stem dieback in year 1 (Table 1). The only significant breeding effect on first-year dieback was within the large size class, and Chinese chestnut had 33 (±6) % dieback compared to the BC_2F_3 generation, which had 9 (±3) % dieback. Across generations, the large size class seedlings had significant more dieback (17 ± 2 %) compared to small size class seedlings (8 ± 1 %). Dieback was not affected by family, but interactions of size and family could not be computed in the GLMM for years 1 and 2. GLMM models could not be fit for dieback in years 3 and 4.

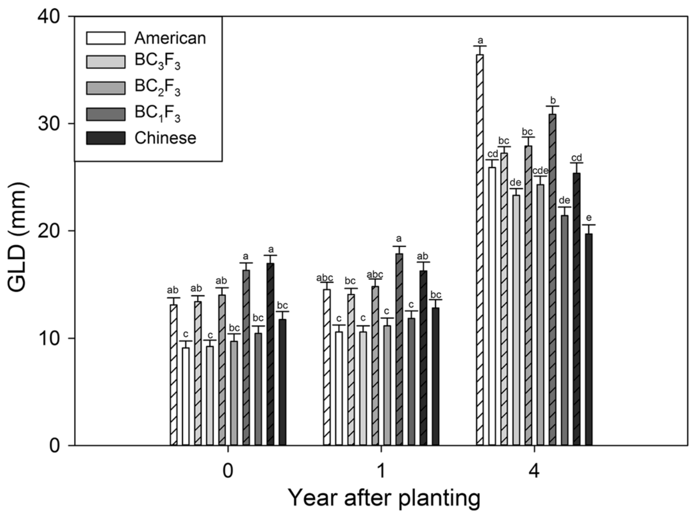

Fig. 4 Least-squares means and associated SEs of ground-line diameter (GLD) for each generation/species for large size class (*striped bars*) and small size class (*solid bars*) seedlings each year after planting. Means with the *same letter* are not significantly different within a year

Table 4 Least-squares means of four-year ground-line diameter for each family and between large and small size class seedlings within each family

Generation	Family	NC			TN			VA		
		Overall mean	Large	Small	Overall mean	Large	Small	Overall mean	Large	Small
American	GMNEW	37.3 a	41.5 a	33.1 abc	31.4 a	36.0 a	26.8 abcdef	30.4 a	*37.4 a*	*23.4 bc*
American	PL1S					29.1 abcde	21.6 cdef	27.8 ab	32.8 ab	22.8 bc
American	TOWERS1	34.6 ab	*41.6 a*	*27.7 bc*	25.4 abcd					
BC_1F_3	NB1	33.7 ab	*41.0 a*	*26.4 bc*	25.1 abcd	*32.0 abc*	*18.2 ef*	24.4 ab	28.3 abc	20.4 c
BC_1F_3	NB35	29.4 abc	33.7 abc	25.0 bc	22.9 abcd	24.8 abcdef	21.0 cdef	21.4 b	25.4 abc	17.4 c
BC_2F_3	SA330	31.4 ab	33.5 abc	29.4 abc	26.8 abc	26.0 abcdef	27.7 abcde	23.6 ab	24.0 abc	23.1 abc
BC_2F_3	SA417	28.6 bc	32.5 abc	24.8 c	22.6 bcd	25.0 abcdef	20.1 def	23.6 ab	26.5 abc	20.7 c
BC_3F_3	D1				24.2 abcd	24.2 bcdef	24.1 bcdef			
BC_3F_3	D2	22.2 c	22.5 c	22.0 c	17.1 d	18.0 ef	16.2 ef	20.3 b	23.8 abc	16.9 c
BC_3F_3	D3				30.3 ab	29.1 abcde	31.4 abcd			
BC_3F_3	D4	31.4 ab	37.0 ab	25.7 bc	27.8 ab	*34.5 ab*	*21.1 cdef*	22.6 ab	26.6 abc	18.6 c
BC_3F_3	D5	32.4 ab	33.2 abc	31.6 abc	21.8 bcd	22.2 bcdef	21.4 cdef	25.8 ab	25.1 abc	26.5 abc
Chinese	Chinese	27.6 bc	31.1 abc	24.1 c	19.0 cd	22.4 bcdef	15.6 f	20.9 b	22.6 bc	19.3 c

Overall means followed by the same letter are not significantly different among families within the same location. Size class means followed by the same lowercase letter are not significantly different between size classes within the same family and location. Italicised values indicate seedling size class differences within the same family and location

Blight

GLMM for chestnut blight had to be computed for each location separately to ensure adequate model fit, but the GLMM for the TN location could not be adequately fit at all. Raw means indicated that 4-year chestnut blight was least abundant (3 ± 1 %) at the TN location and most abundant (14 ± 2 %) at the VA location. Generation/species significantly affected blight incidence at the VA location ($df = 291$, $F = 4.5$, $P = 0.0015$), where American chestnut had 29 (±6) % blight, which was significantly more than the BC_3F_3 generation (7 ± 3 %) and the Chinese chestnut (3 ± 3 %). The Chinese chestnut did not differ in blight incidence from the BC_3F_3 generation at this location.

The GLMM for the VA location could not be fit if generation by size interactions were included, and GLMMs could not be fit if size by family interactions were included for any location. Main effect of size class was not significant for the VA ($df = 291$, $F = 0.8$, $P = 0.3805$) or the NC location ($df = 312$, $F = 0.5$, $P = 0.4616$)). At the NC location, size by generation interactions were significant ($df = 312$, $F = 3.1$, $P = 0.0154$). Within the large size class, American chestnut had significantly more blight than the BC_2F_3 and BC_1F_3 generations, but generation/species did not differ within the small seedling size class (Fig. 5).

Blight incidence was not affected by family at the VA location ($df = 291$, $F = 1.5$, $P = 0.1990$), but families did differ in blight incidence at the NC location ($df = 312$, $F = 2.5$, $P = 0.0293$). American chestnut family GMNEW had more blight than American chestnut family TOWERS1 and BC_3F_3 families D2 and D5 (Table 5). Differences between BC_3F_3 families and the Chinese chestnut family were not significant.

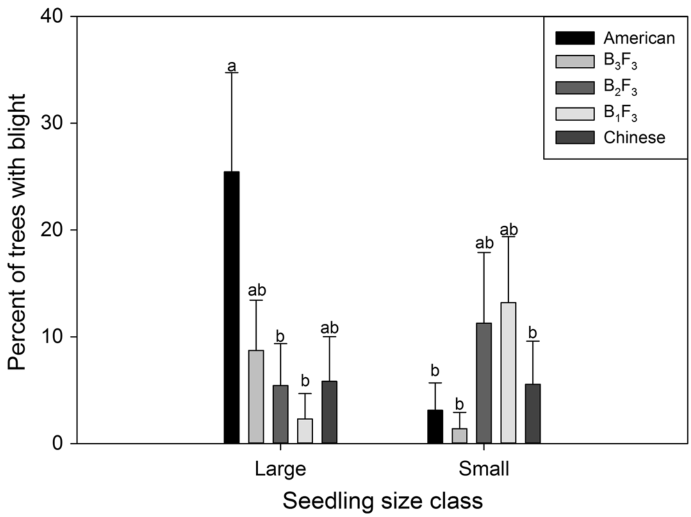

Fig. 5 Least-squares means and associated SEs of chestnut blight incidence for each generation/species and size class in the fourth year after planting at the NC location. Means with the *same letter* are not significantly different

Discussion

Overall field performance

Tree restoration programs, like those for American chestnut, will require seedlings to survive in a competitive environment in the early years (Jacobs et al. 2013), when competition is most intense (cf. Oliver 1981), even before chestnut blight substantially challenges trees. Unfortunately, information is lacking on performance in productive environments with minimal site preparation treatments similar to our study. Genetic tests on European chestnut (*C. sativa* Mill.) have been established (Bazzigher 1981; Míguez-Soto and Fernández-López 2012, 2015), but the majority of these tests are conducted using rather intensive site preparation methods to establish breeding populations, and have not been established in competitive environments, Asian chestnuts (*C. crenata* and *C. mollissima*) have been predominately studied in Europe and Asia for nut production and resistance to ink disease (Bazzigher 1981; Santos et al. 2015), and thus, have little current applicability to forest restoration of American chestnut.

Fortunately, decades of research on artificial and natural regeneration exists for a related Fagaceae species group, *Quercus*, in the US (Korstian 1927; Loftis 1990; Dey et al. 2008; McEwan et al. 2011) and in Europe (Tsakaldimi et al. 2013) which can be utilized to improve restoration efforts of the American chestnut. Slower growth relative to competition on productive sites (i.e., site index ≥23 m for northern red oak) has been a major deterrent to planting success (Carpenter and Guard 1954; Loftis 1979; Buckley et al. 1998) and to natural regeneration (Loftis 1990; Schweitzer and Dey 2011) of Fagaceae species for several decades. The relatively rapid height and GLD growth of American chestnuts and BC_3F_3 generation after the first year in this study suggests that restoration activities should not be hindered by slow growth of seedlings. Seedlings in this study, however, were grown with specific protocols to maximize seedling quality (cf. Kormanik et al. 1994; Clark et al. 2012b), which probably contributed to relatively fast growth rates after planting for some seedlings. Although highly variable, individual trees from the BC_3F_3 generation were capable of growing up to 135–180 cm per year in height and 16.9–27.4 mm per year in GLD after the first growing season, and reached a maximum of 439 cm in total height and 70.5 mm in GLD by year 4. In addition to improved growth that could have derived from advanced nursery protocols, American

Table 5 Least-squares means and associated SEs of chestnut blight by family at the NC location in the fourth year after planting

Generation	Family	Blight (%)
American	GMNEW	23 (8.6) a
American	TOWERS1	4 (3.0) b
BC_1F_3	NB1	7 (4.4) ab
BC_1F_3	NB35	5 (3.8) ab
BC_2F_3	SA330	20 (7.6) a
BC_2F_3	SA417	3 (2.8) b
BC_3F_3	D2	2 (2.2) b
BC_3F_3	D4	10 (6.0) ab
BC_3F_3	D5	2 (2.4) b
Chinese	Chinese	6 (3.0) b

Means followed by the same letter are not significantly different chestnut is inherently a fast growing tree (Mattoon 1909; Ashe 1911, 1913). For example, relatively small seedlings planted under varying competition control regimes reported similar height growth and GLD growth rates to our study (Rhoades et al. 2009; Clark et al. 2012a). When compared to high-quality northern red oak seedlings planted on productive sites in North Carolina (Schuler and Robison 2010), annual growth rates of the BC_3F_3 seedlings in this study were 50–78 % greater, and were 11 % greater than high-quality northern red oak seedlings planted in a commercial shelterwood in western Tennessee (Oswalt et al. 2006). Higher growth rates of American chestnut compared to northern red oak were also reported in an inter-species field trial in Wisconsin and in laboratory studies (Latham 1992; Jacobs and Severeid 2004). In the present study, advanced generation hybrid seedlings exhibited a level of competitiveness similar to the previously extant tree in early years after planting.

Planting shock, exhibited by dieback and negligible height and GLD growth in year 1, was prevalent across generation/species and size classes and was probably caused by inevitable damage to the root system from nursery lifting and processing (Johnson et al. 1984). Planting shock has been widely reported for oak genera in the US and in Europe (Johnson et al. 1984; Struve et al. 2000; Dey et al. 2008; Tsakaldimi et al. 2013; Woolery and Jacobs 2014), but has only received limited attention for American chestnut (Clark et al. 2009). Transplanting bare-root seedlings results in substantial root loss (Struve and Joly 1992), which reduces shoot growth (Larson 1975; Beckjord and Cech 1980). Planting in the winter when soil temperatures are relatively cold can exacerbate planting shock if budbreak occurs prior to initiation of root growth (Farmer 1975; Struve 1990), but winter planting is necessitated by commercial nursery logistical constraints (Johnson et al. 1984). Additional processing necessary to achieve research objectives (e.g., counting roots, tagging seedlings) could have further exacerbated planting shock for seedlings in this study. Planting shock appeared to largely dissipate by year 2, and was worse for the TN and VA locations, which had the most recorded deer browse (Clark et al. 2014a) prior to erection of tree shelters after year 1. Bud or leaf removal from deer browse would have reduced the seedling's ability to produce and maintain roots, shoot, and cambium, thereby intensifying planting shock (Wareing 1951; Larson 1975; Woolery and Jacobs 2014). Based on our results, planting shock was probably unavoidable, but managers should limit damage from deer browse to avoid exacerbating the effect. We could not identify genetic differences in planting shock in this study, but fast recovery from planting stress would be a desirable trait for plant breeders to consider in their breeding programs.

The importance of seedling quality

Numerous evidence exists to support the hypothesis that quality of nursery stock significantly influences establishment of hardwood seedlings (Villar-Salvador et al. 2004; Grossnickle 2012; Na et al. 2013; Grossnickle and El-Kassaby 2015), but specific data are limited to refine American chestnut establishment methods (cf. Clark et al. 2009, 2012a). This previous research was similar to the present study by finding larger size seedlings remained larger after planting and size grading had minimal effects on survival. The reduced planting shock found in smaller seedlings, exhibited by increased survival and less dieback compared to large seedlings, was also similar to previous studies of northern red oak (Thompson and Schultz 1995; Struve et al. 2000), but is in contrast to other studies of cork oak (Tsakaldimi et al. 2013). Small size trees with a more balanced root to shoot ratio may have advantages over large trees in their ability to withstand stress that can hinder growth and even kill trees (Struve et al. 2000; Villar-Salvador et al. 2004). However, the advantages exhibited by small size seedlings was ephemeral and should be balanced against the benefits of using larger size seedlings, which resulted in increased overall height and GLD by year 4. Specially, a 1 year gain in height was found for large size class seedlings for two of three locations; large size seedlings had the same height in a given year compared to small size seedlings the following year. We predict that the larger size seedlings will maintain competitive advantages over time based on results of other studies with northern red oak (Johnson et al. 1986; Spetich et al. 2002).

Effects of deer browsing would have further exacerbated planting shock for large size class seedlings (not yet above the browse line) compared to small size seedlings. Larger seedlings under stress have exhibited a decreased ability to maintain an appropriate balance between above- and below-ground structures compared to smaller seedlings (Jacobs et al. 2009; Grossnickle 2012). The seedlings at the TN location did not show increases in 4-year height related to size grading, but heavy browse pressure in the first growing season (80 %; Clark et al. 2014a) may have negated positive effects. Browse was common for large size seedlings at the TN location (75 %) in year 1 even though browse was slightly reduced through size grading (85 % browse for small size class seedlings) (unpublished data). Size grading can decrease the time needed to get trees above browse line, and decrease the time and resources required to protect seedlings from deer browse (Dey et al. 2008). Furthermore, costs associated with competition control can be reduced by using larger seedlings that more quickly occupy competitive canopy positions compared to smaller seedlings (Spetich et al. 2002, 2009; Grossnickle 2012).

Large size seedlings develop bark fissuring more quickly than small size seedlings, which may have increased blight incidence of the large size American chestnut at the NC location, which was the only location to have significant generation by size class interactions. Growth cracks can serve as entry points for disease (Griffin and Elkins 1986). Growth rate has been positively correlated to blight induced mortality because of resource allocations that reduced trees' abilities to withstand diseases as growth rates accelerated (Reynolds and Burke 2011). Our results are too premature to make direct correlations between growth rate and blight incidence, but do suggest that faster growing (i.e., larger) and blight susceptible American chestnut trees at the time of planting will contract blight more quickly than slower growing trees. The effect of seedling size on blight incidence was not significant for the slower growing and more resistant breeding generations or Chinese chestnut. Despite the apparent positive relationship between tree size and blight incidence found in this and previous studies, more vigorous trees have better potential for blight control from natural development of hypovirulent cankers compared to smaller suppressed trees (Griffin et al. 1991, 1993). Hypovirulent strains of blight contain a virus that reduces disease virulence on susceptible American chestnut trees (Anagnostakis 1986). The 'ecological succession' hypothesis postulated by Griffin et al. (1993) states that blight incidence increases for faster growing trees, but bio-control of blight through hypovirulence also simultaneously increases. Seedling size grading at planting to improve tree growth in combination with proper site selection and forest management could play an important role in improving blight resistance of American chestnut and hybrid seedlings (Griffin 2000).

Performance of advanced breeding material

Recovery of American chestnut morphological and growth traits in the BC_3F_3 generation was expected (Diskin et al. 2006), and is a primary goal of hybrid chestnut breeding programs (Burnham et al. 1986; Hebard 2001; Anagnostakis 2012). Seedlings from the BC_3F_3 generation performed more similar to American chestnut than Chinese chestnut in all response variables, excluding blight incidence. Such similarity concurs with a study conducted at the NC location that found BC_3F_3 generation seedlings had photosynthetic light–response curves more similar to the American chestnut than the Chinese chestnut (Knapp et al. 2014). Differences between seedlings from the American chestnut parent and BC_3F_3 generation in height and GLD growth, particularly in the last growing season, was primarily related to the poor performance of one BC_3F_3 genetic family (D2) that was planted at all locations and greatly influenced the generational mean (Table 2). However, most BC_3F_3 families at each location displayed reduced height and GLD growth compared to the American families, even though these differences were not necessarily significant. With respect to growth, the results indicate that the BC_3F_3 generation was similar to American chestnut, but not identical at this early stage of growth.

Despite the size advantage at the time of planting due to increased nut size exhibited by the Chinese chestnut (Clark et al. 2012b), this species generally had poorer performance over time when compared to the American chestnut and the BC_3F_3 generation (Figs. 1, 3, 4). However, results indicate that interactions with site are probably affecting Chinese chestnut's ability to establish. For example, Chinese chestnut had reduced survival at only one location (TN), but had comparable height growth to the BC_3F_3 generation at the NC location. Our study was not designed to allow direct inferences of how specific site factors affect performance of generation/species or family, but genotype by environmental interactions are occurring and have been well documented for many plant species (Campbell and Waser 2001). Although Chinese chestnut is performing moderately well at the NC site and has exhibited fecundity on a low-quality low elevation site in the north-eastern US (Miller et al. 2014), widespread tests established in the 1930s–1950s indicate that Chinese chestnut has poor competitive ability over time in most eastern North American forests (Schlarbaum et al. 1994).

The relatively low numbers of families and seedlings in some families (Table 6 in "Appendix 1") was a hindrance in identifying superior families, particularly for survival, stem dieback, and blight. For these characteristics, family SEs could be relatively high and fit of the GLMM was problematic. Our results do indicate that BC_3F_3 family D2 was a poor performer in growth, despite its high level of blight resistance in inoculation tests (Hebard 2012) and in this study. Family rankings will need to be followed over time and at more field tests, however, to determine if certain families need to be rogued from TACF breeding program due to inadequate performance (Hebard 2012). Previous studies using Fagaceae species have shown that family growth rates will change from juvenile to adult stages, and growth rates at various life stages are significantly affected by provenance of mother trees (Schlarbaum and Bagley 1981; Kriebel et al. 1976, 1988; Schlarbaum 1993; Míguez-Soto and Fernández-López 2015). We anticipate that field testing will be more robust in the future, as experimental material is becoming increasingly more available (Hebard 2012; Anagnostakis 2012).

This is the first study to examine genetic interactions with seedling size class for chestnut species. Although only one BC_3F_3 family (D4) and no American chestnut families had significant differences between size classes in 4-year height, some families had biologically meaningful differences. For example, American chestnut family GMNEW had a 75 cm difference in height between large and small size trees at the VA location, but the difference was not statistically significant, probably due to high within family variability and relatively low seedling numbers (Table 6 in "Appendix 1"). A 75 cm increase in height resulting from seedling grading, however, would increase competitive ability, particularly on high-quality sites (Johnson et al. 2002). Although our data were too few to make empirical comparisons of size class interactions with family on binary variables that define planting shock (e.g., survival and stem dieback), selection of families with less planting shock in large size seedlings could improve restoration efforts by increasing growth rates in the first year after planting (Struve et al. 2000; Woolery and Jacobs 2014). Genetic interactions with cultural prescriptions may change over time and across locations, similar to studies of northern red oak (Kriebel et al. 1988) and chestnut (Míguez-Soto and Fernández-López 2015). Of particular interest will be to determine if the effect of seedling size grading at planting on subsequent growth continues to remain more effective for American chestnut compared to Chinese chestnut and the BC_3F_3 generation.

The plantings are too young to assess durable blight resistance (Anagnostakis 2012), but BC_3F_3 seedlings did have lower blight incidence than American chestnut seedlings at the NC location (large size class seedlings only) and VA location (across size classes). Furthermore, the BC_3F_3 seedlings had blight frequencies similar to the Chinese chestnut seedlings at both of these locations indicating the presence of blight resistance genes from Chinese chestnut in a primarily American chestnut genome. These results are paralleled by inoculations of BC_3F_3 mother trees by Hebard (2012) who found that three of the BC_3F_3 families used in this study (D2, D4, and D5) had resistance better than the American parent when inoculated at the juvenile stage with a low virulent strain of blight, SG2-3. We have not yet identified which blight strains are naturally infecting trees in this study, but this will be important in assessing the overall performance of the advanced breeding generations. Specifically, blight control from hypovirulent cankers could affect survival and growth rates of experimental material, as has been shown in studies of natural American chestnut sprouts (Griffin 2000; Anagnostakis 2001) and of European chestnut (Ježic et al. 2014).

Although not a significant problem in this study, the presence of root rot caused by *P. cinnamomi* (Rands) will be a major consideration for restoration using bare-root nursery seedlings produced in the southern US (Crandall et al. 1945; Clark et al. 2012b). Resistance screening to native and non-native pests, other than blight, will be important to restoration success of American chestnut (Orwig 2002; Jeffers et al. 2009; Anagnostakis et al. 2011; Pinchot et al. 2011), as has been for the European chestnut restoration program (Bazzigher 1981; Santos et al. 2015). We observed two non-native insects in the plantings, Asiatic oak weevil [*Cyrtepistomus castaneus* (Roelofs)] and chestnut gall wasp (*Dryocosmus kuriphilus* Yasumatsu), and native insects, chestnut sawfly [*Craesus castaneae* (Rowher)] and periodical cicadas (*Magicicada* spp.) (Clark et al. 2014a), indicating impacts from pests other than blight may be of importance to successful chestnut restoration (Pinchot et al. 2011; Case et al. 2014; Clark et al. 2014b). When trees reach the stem exclusion stage of forest development, approximately 8–10 years after planting (cf. Oliver 1981), we expect to have more definitive inferences regarding the recovery of American chestnut growth traits and Chinese chestnut blight resistance in the BC_3F_3 generation progeny.

Conclusions

This study will provide baseline information on how early establishment traits (e.g., growth rate, survival, planting shock) of American chestnut seedlings bred for blight resistance may affect resistance and growth once blight becomes more prevalent. Information collected on the variability among individuals and families will be important to understanding temporal dynamics of disease resistance and field performance and will provide predictions for restoration programs, as has been shown with European chestnut (Míguez-Soto and Fernández-López 2015) and other host genera (Jules et al. 2014). In more general terms, successful establishment of American chestnut and BC_3F_3 generation seedlings on productive forest sites was achieved in this study by planting bare-root nursery seedlings grown for maximum growth potential (cf Kormanik et al. 1994), chemically treating stump sprout competition, and mitigating deer browse pressure.

The BC_3F_3 generation exhibited growth and survival more similar to the American chestnut than the Chinese chestnut, but some discrepancies in recovery of favoured American chestnut characteristics were significant. Notably, the American chestnut grew 40 cm more in height and 5.9 mm more in GLD than the BC_3F_3 generation across all locations. Blight resistance of the BC_3F_3 generation seedlings was not different from the Chinese chestnut, but long-term data are needed to adequately access durability of blight resistance before restoration activities are conducted. A poor performing BC_3F_3 generation family was identified based on 4-year height and GLD, but this family also had lower blight incidence at one location (2 %) than an American chestnut family (23 %). Genotype by environment interactions were apparent, as is typical (Míguez-Soto and Fernández-López 2015), but families were too few and data too premature to make definitive recommendations for the breeding program. In the next few years, we will test predictions that BC_3F_3 seedlings currently being produced can survive long enough to reach sexual maturity and naturally reproduce as blight becomes increasingly more prevalent on the sites (Hebard 2012; Hebard et al. 2013).

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Appendix 1

See Table 6.

Table 6 Number of trees in each generation/species and genetic family that were planted (P) and survived (S) until the fourth year of data collection at each location

Generation/species	Field family ID	TACF mother tree	Planting location/year planted							
			VA		NC		TN		Total	
			P	S	P	S	P	S	P	S
American	GMNEW	GMNEW	35	25	34	28	34	29	103	82
	PL1S	PL1S	54	37			53	37	107	74
	Towers1	Towers1			32	26			32	26
BC_1F_3	NB1	NB1	40	30	39	29	39	31	118	90
	NB35	NB35	29	25	29	23	30	22	88	70
BC_2F_3	SA330	SA330	32	26	32	23	32	22	96	71
	SA417	SA417	32	29	31	26	32	28	95	83
BC_3F_3	D1	D5-17-89					39	34	39	34
	D2	D5-17-130	26	18	27	24	26	17	79	59
	D3	D5-25-49					35	26	35	26
	D4	D5-26-131	32	25	33	29	33	26	98	80
	D5	D6-26-27	27	25	26	22	27	19	80	66
Chinese	AD	AD	62	46	61	52	62	25	185	123
Total			369	286	344	282	442	316	1155	884

Appendix 2

See Table 7.

Table 7 Generalized linear mixed models for survival and stem dieback for each years 1–4 after planting

Source of variation	Year 1		Year 2		Year 3		Year 4	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Survival								
Location	1.4	0.2541	65.9	*<0.0001*	8.2	*0.0004*	3.6	*0.0286*
Generation	2.1	0.0782	30.8	*<0.0001*	3.9	*0.0041*	2.7	*0.0310*
Generation × location	1.0	0.4376	–[a]	–	2.8	*0.0051*	3.2	*0.0013*
Size	3.7	0.0558	9.6	*0.0020*	9.9	*0.0017*	4.2	*0.0412*
Location × size	0.1	0.8848	–[a]	–	0.1	0.9061	0.4	0.6554
Generation × size	–[a]	–	–[a]	–	1.7	0.1409	1.3	0.2730
Family (generation location)	0.7	0.8410	1.2	0.2694	0.8	0.6947	1.1	0.3489
Size × family (generation location)	–[a]	–	–[a]	–	–[a]	–	–[a]	–
Location × size × generation	–[a]	–	–[a]	–	–[a]	–	–[a]	–
Stem dieback								
Location	9.6	*<0.0001*	1.4	0.2441	–[b]	–	–[b]	–
Generation	1.9	0.1142	2.4	0.0514	–[b]	–	–[b]	–

Table 7 continued

Source of variation	Year 1		Year 2		Year 3		Year 4	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Generation × location	1.8	0.0693	0.9	0.5481	–[b]	–	–[b]	–
Size	16.9	*<0.0001*	10.0	*0.0017*	–[b]	–	–[b]	–
Location × size	1.3	0.2758	0.7	0.4918	–[b]	–	–[b]	–
Generation × size	2.9	*0.0205*	0.3	0.8819	–[b]	–	–[b]	–
Family (generation location)	1.0	0.4404	1.1	0.3528	–[b]	–	–[b]	–
Size × family (generation location)	–[a]	–	–[a]	–	–[b]	–	–[b]	–
Location × size × generation	–[a]	–	–[a]	–	–[b]	–	–[b]	–

Italicised cells indicate significant effects ($P < 0.05$)

[a] Interactions were not be included to improve fit of model

[b] Model would not fit with all of fixed effects included

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