SHORT COMMUNICATION

Leaf physiology and morphology of *Castanea dentata* (Marsh.) Borkh., *Castanea mollissima* Blume, and three backcross breeding generations planted in the southern Appalachians, USA

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Abstract Backcross breeding programs have been used to transfer disease resistance and other traits from one forest tree species to another in order to meet restoration objectives. Evaluating the field performance of such material is critical for determining the success of breeding programs. In eastern North America, The American Chestnut Foundation has a backcross breeding program that uses Chinese chestnut (*Castanea mollissima* Blume) to introduce resistance of the fungal pathogen chestnut blight [*Cryphonectria parasitica* (Murr.) Barr.] to the native American chestnut [*Castanea dentata* (Marsh.) Borkh.]. We compared physiological and morphological characteristics among seedlings of American chestnut, Chinese chestnut, and BC_1F_3, BC_2F_3, and BC_3F_3 hybrid chestnuts during their fourth growing season after field-planting. American chestnut and the BC_3F_3 breeding generation displayed photosynthetic light-response curves that were similar to each other but different from Chinese chestnut. Rates of photosynthesis were higher for American

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chestnut and the BC_3F_3 breeding generation when compared to Chinese chestnut for light levels ≥ 800 μmol m^{-2} s^{-1} photosynthetic photon flux density and for maximum photosynthetic capacity. Leaf morphology variables were not different between American chestnut and any of the breeding generations, but leaf area (on a per leaf basis) of Chinese chestnut was lower than that of any other chestnut type. Our results suggest that backcross breeding can be used to transfer desirable traits for restoration of native species threatened by non-native pathogens.

Keywords Backcross breeding · *Castanea dentata* · Light response curve · Species restoration · Tree improvement

Introduction

Non-native pests and pathogens present considerable threats to forest ecosystems (Lieb-hold et al. 1995). In the eastern deciduous forests of North America, the extirpation of American chestnut [*Castanea dentata* (Marsh.) Borkh.] as a canopy species following the introduction of *Cryphonectria parasitica* (Murr.) Barr., a fungal pathogen from Asia, was one of the most extensive ecological changes of the past century. At the beginning of the twentieth century, American chestnut was a dominant species throughout a range that stretched from Maine to Mississippi, extended into Canada, and encompassed the Appalachian region (Braun 1950; Wang et al. 2013). The introduction of *C. parasitica* resulted in chestnut blight disease, and most mature American chestnuts were dead or dying by the 1950s.

Breeding programs provide a means to introduce resistance into populations of native species following the introduction of non-native pathogens (Sniezko 2006; Ingwell and Preisser 2010). In an effort to produce material for restoring chestnut as a canopy tree within its natural range, The American Chestnut Foundation (TACF) initiated a backcross breeding program that introgresses blight resistant genes of an Asian chestnut parent, primarily Chinese chestnut (*Castanea mollissima* Blume), into American chestnut through multiple backcrosses to the American parent (Burnham et al. 1986; Hebard 2005). After the third generation of the third backcross (BC_3F_3), the resultant progeny should exhibit high levels of blight-resistance (from the Chinese chestnut parent) but maintain the phenology, morphology, and growth characteristics of the American parent. The TACF breeding program recently produced the BC_3F_3 breeding generation, which should theoretically retain almost 94 % of the American chestnut genes and be highly blight-resistant (Hebard 2005; Jacobs 2007).

The production of the BC_3F_3 breeding generation has brought chestnut restoration in the eastern United States closer to fruition; yet, as with any breeding program, evaluation of the resultant material is critical (Sniezko 2006). Early research on the morphology of nursery-grown BC_3F_1 hybrid saplings indicated that leaf, stipule, twig, and bud characteristics were around 96 % similar to American chestnut and were different from Chinese chestnut (Diskin et al. 2006). However, other tests of the breeding generations showed greater dissimilarity from American chestnut. For example, the seedlings used in our study are from populations for which growth and morphological traits were compared after being grown for 1 year in a commercial tree nursery, and the BC_3F_3 generation differed from the American chestnut seedlings in half of the tests conducted for nut weight, seedling growth,

and stem and root morphology (Clark et al. 2012a). More testing is required to determine if differences in the nursery will develop into growth or physiological differences after seedlings are field-planted.

Direct measurements of photosynthesis provide important information for understanding plant growth strategies and tolerance to stress. Generally, shade-tolerant species have lower photosynthetic rates, lower dark respiration rates, lower light compensation points, and higher quantum efficiencies than shade-intolerant plants (Loach 1967; Boardman 1977). Recent research on the physiology of American chestnut shows characteristics similar to shade-tolerant species in studies conducted in rainout shelters (Wang et al. 2006) and in the field (Joesting et al. 2009). However, Wang et al. (2006) reported a light saturation point of American chestnut that was higher than that of red maple (*Acer rubrum* L.), suggesting the potential for American chestnut to respond to high light levels with rapid growth. Moreover, Joesting et al. (2007) found that the maximum photosynthetic rate was greater for American chestnut seedlings growing under a thinned canopy (i.e., increased light) than for seedlings growing under an intact canopy. These results, combined with growth measurements from other studies (McCament and McCarthy 2005; Rhoades et al. 2009; Griscom and Griscom 2012, Clark et al. 2012c), suggest that American chestnut may respond to canopy disturbance with rapid growth.

To date, no studies have compared the photosynthetic characteristics of the BC_3F_3 breeding generation with those of American chestnut, Chinese chestnut, or other breeding generations. Although early tests of morphology suggest similarity between American chestnut and the BC_3F_3 breeding generation (Diskin et al. 2006; Clark et al. 2012a), field testing will be critical for evaluating success of the backcross breeding program and informing management prescriptions for chestnut restoration. This study was designed to compare American chestnut, Chinese chestnut, and the BC_1F_3, BC_2F_3, and BC_3F_3 breeding generations (hereafter collectively referred to as chestnut types). Specifically, our objectives were to (1) compare photosynthesis characteristics among chestnut types and (2) compare leaf morphology and nitrogen concentration among chestnut types.

Materials and methods

This study was conducted in western North Carolina, USA, within the Blue Ridge mountain physiographic province (Fenneman 1938), which is located within the southeastern portion of the American chestnut range (Little 1977). Extant sprouts indicated that this site previously supported American chestnut as a canopy species. Prior to the installation of this study, the site consisted of a mix of hardwood species, including scarlet oak (*Quercus coccinea* Münchh.), northern red oak (*Quercus rubra* L.), black oak (*Quercus velutina* Lam.), white oak (*Quercus alba* L.), chestnut oak (*Quercus prinus* L.), tulippoplar (*Liriodendron tulipifera* L.), and basswood (*Tilia americana* L.), with a basal area of approximately 25 m^2/ha. The site was located on a north-facing slope at an elevation of 840 m, and site index for northern red oak at base age 50 was 24 m, as determined by Forest Service personnel prior to harvest. Soils on this site were of the Evard–Cowee complex, which are classified as fine-loamy, parasesquic, mesic typic hapludults, and slopes ranged from 5 to 30 % (NRCS Web Soil Survey; http://websoilsurvey.sc.egov.usda.gov).

The study area was part of a 4 ha stand that was harvested in winter 2007/2008 using a two-age shelterwood system, leaving a residual basal area of 5 m^2/ha distributed approximately uniformly throughout the stand. In early summer 2008, a mixture of 20 %

triclopyr and mineral oil was used as a basal bark treatment on stump sprouts from harvested trees. In February 2009, just prior to planting the chestnut seedlings, undesirable residual stems >2.5 cm diameter breast height (excluding the residual canopy trees) were cut and chemically treated with a 50 % mixture of triclopyr and water. In July 2011, a vegetation release using machetes was conducted to cut down sprouts that did not die from the herbicide site preparation treatment.

The chestnut seeds used in this study were produced at TACF's Research Farm in Meadowview, VA, with seed collection and seedling production described in Clark et al. (2012a). In February 2009, a 0.8 ha portion of the study site was planted with 1-0 nursery-grown seedlings of American chestnut, Chinese chestnut, and the BC_1F_3, BC_2F_3, and BC_3F_3 breeding generations, as part of a larger study designed to examine the field performance of the BC_3F_3 generation planted in several sites across the southeastern United States (Clark et al. 2012b). Seedlings were planted at 2.5×2.5 m spacing using KBC dibble bars modified to increase bar width to 30 cm. In total, there were 344 seedlings planted, including 66 chestnuts from two families of the American chestnut parental species, 61 chestnuts from one family of the Chinese chestnut parental species, 68 seedlings from two families of the BC_1F_3 generation, 63 seedlings from two families of the BC_2F_3 generation, and 86 seedlings from three families of the BC_3F_3 generation.

Data collection

All planted chestnuts were marked with numbered tags, and tree size [ground-line diameter (mm); height (cm)] and damage (evidence of browse, defoliation, chestnut blight, etc.) data were collected at the time of planting and at the end of each growing season after planting. At the beginning of the fourth growing season after planting (2012), we selected eight seedlings of each chestnut type (i.e., American chestnut, Chinese chestnut, BC_1F_3, BC_2F_3, and BC_3F_3) for photosynthetic gas exchange measurements ($n = 40$ total). Because we were interested in measuring the physiological characteristics of healthy individuals, we used previously collected data of survival, growth, and damage to reduce our potential sample population to living seedlings with negligible signs of mammal herbivory, chestnut blight, insect damage, or stem dieback. The seedlings sampled in this study were randomly selected from this 'healthy' population. This study was designed to determine effects of chestnut type on response variables but was not able to test effects of family within chestnut type due to the low sample sizes of different families in the sample population.

We measured gas exchange and photosynthesis of selected seedlings using a CIRAS-2 Portable Photosynthesis System (PP-Systems; Amesbury, MA) equipped with a PLC6 (U) Automatic Universal Leaf Cuvette to control the environment around each sampled leaf. For each seedling, we selected one fully expanded leaf that was growing in full sun from at or near the terminal branch. We conducted gas exchange measurements only on days with full sun to allow seedlings to equilibrate to a high light environment prior to measurement, and all measurements were done between 1000 and 1600 hours. We constructed light response curves by measuring the photosynthetic response following exposure to a sequence of light levels: 2,000, 1,800, 1,500, 1,200, 1,000, 800, 600, 400, 200, 100, 50, 25, and 0 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The conditions inside the cuvette were controlled to maintain 380 ($\pm$10) μmol CO_2, 50 % ($\pm$10 %) relative humidity, and 25° ($\pm$2°) C leaf temperature. We assumed that the seedlings were calibrated to ambient, full-sun light levels prior to measurement; as a result, we allowed seedlings 120 s to calibrate to the highest light level and 60 s for calibration at each subsequent light level. To account for changes in the ambient environment during the

sampling period and for spatial variability within the site, we grouped one of each of the chestnut types into blocks ($n = 5$ chestnut types per block; 8 blocks total) based on seedling proximity. Seedlings within each block were measured within a 3-h period, and the order of measurement for individuals within each block was randomized during each measurement period.

Following gas exchange measurements, each sampled leaf was collected and returned to the laboratory for determination of morphological characteristics and nitrogen concentration. For each leaf sample, we used an 8 mm diameter circular leaf punch to remove 10 tissue samples that were randomly distributed across the leaf surface. Each leaf and tissue sample was oven dried at 70 °C to a constant mass, and the mass was recorded. We determined the leaf mass per unit area (LMA; g m^{-2}), the specific leaf area (SLA; cm^2 g^{-1}), the total leaf mass per leaf (TLM; g) and the total leaf area per leaf (TLA; cm^2). The concentration of nitrogen (%) in each sampled leaf was analyzed by Kjeldahl digestion by the Agricultural Services Laboratory at Clemson University. To characterize the light environment at each seedling, photosynthetically active radiation (PAR) was measured using an Accupar ceptometer (Decagon Devices, Inc, Pullman, WA). Ceptometer readings taken at each seedling were compared to PAR measurements recorded every minute in open conditions to determine the percentage of full sunlight at each seedling. All light measurements were recorded between 1000 and 1400 hours in September 2009, 2010, and 2011, and the average of the three growing seasons was used to provide a general description of the relative light environment.

Data analysis

We constructed light response curves using the methods of Parsons and Ogsten (1999) to fit a non-linear regression function to the photosynthesis and light data collected for each leaf, with the following equation:

$$A_{net} = \frac{\varphi Q + A_{\max} - \sqrt{\left[(\varphi Q + A_{\max})^2 - 4\varphi Q k A_{\max}\right]}}{2k} - R_d \qquad (1)$$

where A_{net} is CO_2 assimilation (μmol m^{-2} s^{-1}), φ is the quantum efficiency (μmol CO_2 μmol^{-1} photon), Q is the photosynthetically active radiation (μmol m^{-2} s^{-1}), A_{max} is the maximum rate of photosynthesis (μmol m^{-2} s^{-1}), k is the curvature factor, and R_d is the dark respiration rate (μmol m^{-2} s^{-1}). From this data, we calculated the light compensation point (LCP; μmol m^{-2} s^{-1}) as:

$$LCP = \frac{R_d(R_d k - A_{\max})}{\varphi(R_d - A_{\max})} \qquad (2)$$

We fit light response curves to data from each sampled leaf, from which we derived A_{max}, φ, R_d, k, and LCP for each seedling and calculated the mean value of each variable by chestnut type.

We used analysis of variance (ANOVA) in a randomized complete block design to determine the effects of chestnut type on the photosynthesis response variables (A_{max}, φ, R_d, k, and LCP), on net carbon assimilation for each light level, and on leaf characteristics (LMA, SLA, TLM, TLA, and N) using Proc Mixed in SAS 9.3 (SAS Institute Inc., Cary, NC). We used the Satterthwaite approximation of degrees of freedom for each analysis, and we used pair-wise comparisons with Tukey's honestly significant difference test to

determine significant differences in pair-wise comparisons of chestnut types. To meet the assumptions of constant variance and normality of the residuals, we used a square root transformation of the LCP data and log-transformations of LMA and TLA; other variables did not need transformation to meet assumptions. For all analyses, statistical significance was determined when $p \leq 0.05$.

Results

The ambient light environment around the seedlings did not differ among the chestnut types ($F_{4, 35} = 0.20$; $p = 0.8900$), and PAR averaged 79.8 % full sunlight across the three growing seasons. The light response curves generated from the mean photosynthetic rates at each light level showed different patterns among the five chestnut types (Fig. 1). There were no significant differences in carbon assimilation (μmol m^{-2} s^{-1}) among the chestnut types for light levels of 600 μmol m^{-2} s^{-1} or below ($p \geq 0.0753$), but there were significant differences for light levels of 800 μmol m^{-2} s^{-1} and above ($p \leq 0.0278$). For each light level $\geq$800 μmol m^{-2} s^{-1}, American chestnut and the BC_3F_3 breeding generation had significantly higher carbon assimilation than Chinese chestnut (Fig. 1a). The BC_2F_3 breeding generation also had significantly higher carbon assimilation than Chinese chestnut at 1,500 μmol m^{-2} s^{-1}, but otherwise the BC_1F_3 and BC_2F_3 breeding generations were not significantly different from the other chestnut types.

A_{max} was significantly higher for American chestnut and the BC_3F_3 breeding generation than for Chinese chestnut, and the BC_1F_3 and BC_2F_3 breeding generations were not different from any of the other chestnut types (Table 1). The quantum efficiency (φ) was significantly higher for BC_3F_3 seedlings than for Chinese chestnut and BC_2F_3 seedlings (Table 1). We found no differences in R_d or LCP among the five chestnut types ($p = 0.0520$ and $p = 0.1420$, respectively). We found few differences in leaf morphology or chemistry among the five chestnut types. TLA was significantly lower for Chinese chestnut than for any of the other chestnut types, with no differences among American chestnut and/or any of the breeding generations (Table 2). There were no effects of chestnut type on TLM, LMA, SLA, or N.

Discussion

The most notable difference among the five chestnut types was in the maximum rate of photosynthesis, with the American chestnut and the BC_3F_3 breeding generation values nearly double that of Chinese chestnut. As expected, the BC_1F_3 and BC_2F_3 breeding generations had maximum rates of photosynthesis that were between American chestnut and Chinese chestnut, although the differences were not statistically significant from either parent. Although our study design and sample size did not account for variability from the breeding families within each chestnut type, our results suggest that this variability was low relative to that among chestnut types for carbon assimilation and A_{max}. Interestingly, the BC_3F_3 breeding generation also had higher quantum efficiency than Chinese chestnut, suggesting that the BC_3F_3 seedlings have the capacity for higher carbon assimilation at low light levels when compared to Chinese chestnut. However, we did not find significantly different levels of carbon assimilation, R_d, or LCP among the chestnut types, indicating that the primary difference among the chestnut types is the capacity for carbon assimilation in high light environments.
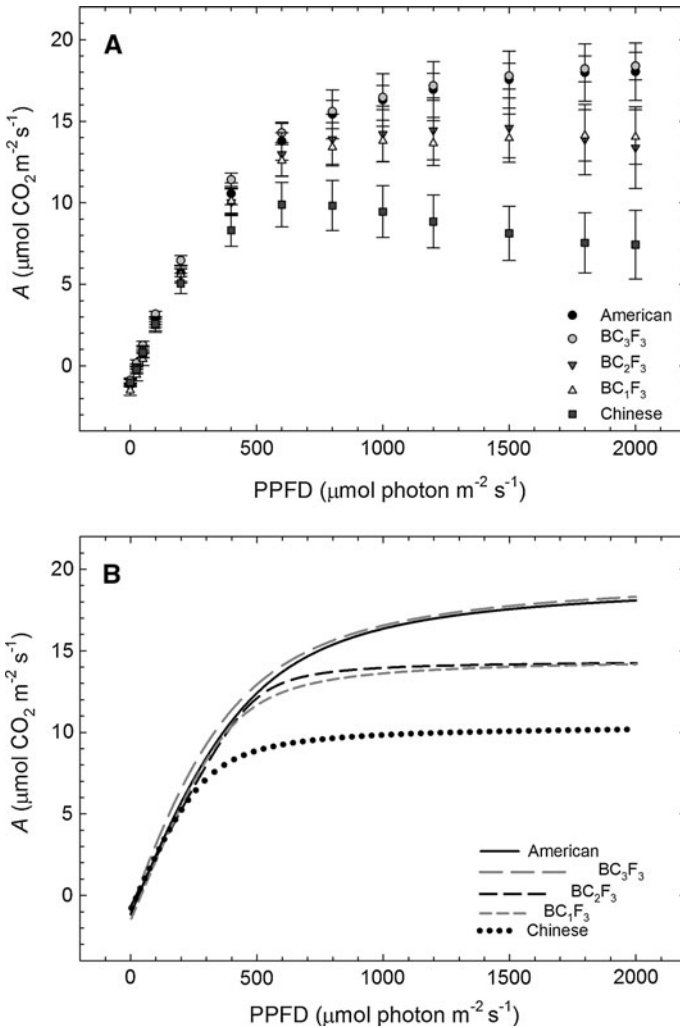
Fig. 1 Light response curves plotted by **a** field data and **b** best-fit curves from nonlinear regression using Eq. (1) for American chestnut, the BC₃F₃, BC₂F₃, BC₁F₃ breeding generations, and Chinese chestnut. Each point in (**a**) represents the mean ± 1 SE for eight seedlings measured in the field

The levels of maximum photosynthesis for American chestnut determined in this study exceed those previously published for many species (e.g., Ellsworth and Reich 1993; Wayne and Bazzaz 1993; Herrick and Thomas 1999; Henderson and Jose 2005). For example, Reich et al. (1999) reported that photosynthetic capacity of 25 species in Wisconsin ranged from 5.8 $\mu mol\ m^{-2}\ s^{-1}$ for black walnut (*Juglans nigra* L.) to 14.8 $\mu mol\ m^{-2}\ s^{-1}$ for eastern cottonwood (*Populus deltoids* W. Bartram ex Marshall). In the southern Appalachians, Sullivan et al. (1996) found that maximum photosynthesis rates for twelve common species ranged from 1.63 $\mu mol\ m^{-2}\ s^{-1}$ for striped maple (*Acer pensylvanicum* L.) to 27.53 $\mu mol\ m^{-2}\ s^{-1}$ for scarlet oak. Our results support the findings of previous studies that report relatively high rates of photosynthesis for American

Table 1 Means and one standard error of photosynthesis response variables by chestnut type

Breeding generation	R_d (µmol m^{-2} s^{-1})		φ (mol mol^{-1})		LCP (µmol m^{-2} s^{-1})		A_{max} (µmol m^{-2} s^{-1})	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
American	1.17	(0.15)	0.039[AB]	(0.002)	30.64	(3.62)	20.73[A]	(2.11)
BC$_3$F$_3$	0.84	(0.17)	0.042[A]	(0.001)	20.70	(4.31)	20.90[A]	(1.10)
BC$_2$F$_3$	0.77	(0.12)	0.034[B]	(0.002)	24.07	(4.78)	15.80[AB]	(1.93)
BC$_1$F$_3$	1.47	(0.32)	0.038[AB]	(0.002)	40.53	(10.65)	16.43[AB]	(1.68)
Chinese	0.79	(0.21)	0.035[B]	(0.002)	22.19	(6.36)	11.22[B]	(1.25)
p value	0.0520		0.0162		0.1420		0.0013	

R_d dark respiration, φ quantum efficiency, *LCP* leaf compensation point, A_{max} maximum photosynthesis

The same superscript letter within a response variable indicates no significant difference in pair-wise comparisons of chestnut types at $\alpha = 0.05$

Table 2 Means and one standard error of per-leaf leaf morphology and chemistry variables by chestnut type

Breeding generation	LMA (g m^{-2})		SLA (cm^2 g^{-1})		TLM (g)		TLA (cm^2)		N (%)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
American	83.50	(1.05)	120.20	(1.49)	0.78	(0.08)	94.14[A]	(8.84)	1.70	(0.11)
BC$_3$F$_3$	78.75	(5.38)	131.33	(9.90)	0.84	(0.12)	106.12[A]	(12.29)	1.81	(0.11)
BC$_2$F$_3$	86.25	(4.37)	117.83	(6.08)	0.80	(0.12)	92.44[A]	(12.54)	1.65	(0.11)
BC$_1$F$_3$	87.50	(5.05)	117.37	(7.55)	0.84	(0.06)	95.80[A]	(4.84)	1.73	(0.07)
Chinese	97.88	(8.21)	106.91	(8.08)	0.58	(0.09)	57.22[B]	(6.16)	1.60	(0.10)
p value	0.1933		0.2349		0.1101		0.0011		0.6161	

LMA leaf mass per unit area, *SLA* specific leaf area, *TLM* total leaf mass, *TLA* total leaf area, *N* foliar nitrogen concentration

The same superscript letter within a response variable indicates no significant difference in pair-wise comparisons of chestnut types at $\alpha = 0.05$

chestnut. However, the rates of maximum photosynthesis for American chestnut have varied among the published studies and throughout chestnut ontogeny. For example, Wang et al. (2006) reported a maximum photosynthetic rate of 13.40 µmol m^{-2} s^{-1} for 1 year-old American chestnut seedlings grown in rainout shelters, and Joesting et al. (2009) determined maximum photosynthesis rates of 9.03, 7.97, and 13.88 µmol m^{-2} s^{-1} for seedling, sapling, and mature trees, respectively, measured in a field study in Wisconsin. Previous studies have shown the photosynthetic potential and growth of American chestnut to increase following silvicultural treatments in which light is increased (McCament and McCarthy 2005; Joesting et al. 2007; Rhoades et al. 2009; Clark et al. 2012c), supporting our findings of high rates of photosynthesis of chestnut seedlings in high light environments (80 % of full sun) created after a shelterwood harvest.

The high photosynthetic potential of American chestnut provides an explanation for the comparatively rapid rates of growth previously reported for the species (Latham 1992; Jacobs and Severeid 2004; Griscom and Griscom 2012). For example, Jacobs et al. (2009) found that American chestnut size (basal diameter, diameter at breast height, height, and/or volume) exceeded that of interplanted black walnut and northern red oak through 19 years

of growth. Our results indicate that differences in photosynthetic rates among the chestnut types only occurred at light levels ≥ 800 μmol m^{-2} s^{-1} PPFD. Given that the PAR levels averaged 80 % of full sunlight, and the maximum PAR recorded in open conditions was 1,620 μmol m^{-2} s^{-1} PPFD, the total carbon accumulation of American chestnut and the BC$_3$F$_3$ breeding generation probably exceeded that of the other chestnut types during periods of full sunlight. Seedlings of American chestnut and the BC$_3$F$_3$ breeding generation would likely have faster rates of growth than the other generations and the Chinese chestnut due to their efficiency in converting carbon; however, complete analyses of the growth and survival of the chestnut types in this study are beyond the scope of this publication but are currently in preparation (S. Clark, personal communication, 2013).

Conclusion

Our study is the first to compare the photosynthetic response of hybrid chestnut breeding generations (i.e., BC$_1$F$_3$, BC$_2$F$_3$, and BC$_3$F$_3$) and their parental species (i.e., American and Chinese chestnuts). Our results provide evidence of the similarities between American chestnut and the BC$_3$F$_3$ breeding generation produced by the TACF backcross breeding program. Given the wide ecological amplitude of American chestnut, the inferences from our results may be limited due to the fact that data were collected during only one growing season, for one stage of ontogeny, and on only one study site. Similar to previous research, we found physiological traits of American chestnut that are typically associated with shade-tolerant species (e.g., low dark respiration rates, low light compensation points, and high quantum efficiency rates), as well as physiological traits typically associated with shade-intolerant species (e.g., high maximum photosynthesis rates) (Wang et al. 2006; Joesting et al. 2007, 2009). Taken together, these results suggest that American chestnut and the BC$_3$F$_3$ breeding generation have growth strategies that allow for persistence in the forest understory and the potential for rapid growth following canopy disturbances that increase light availability (Paillet 2002; McEwan et al. 2006; Wang et al. 2006; Joesting et al. 2009; Jacobs et al. 2013). Although our study provides insight into the physiological response of chestnuts planted for restoration, additional research is required to determine if these findings will be common to other breeding lines, across other site types throughout the American chestnut range, or throughout the tree's ontogeny.

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