FINAL REPORT NARRATIVE

Comparing the shade tolerance of American chestnut, Chinese chestnut, and their hybrids

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1. Objectives

- To investigate and compare the shade tolerance of American chestnut (*Castanea dentata*), Chinese chestnut (*C. mollissima*), and F_1 and BC3-F3 hybrid chestnuts
- To aid chestnut breeders in understanding how shade tolerance of hybrid chestnuts could be influenced by parent species
- To assist land managers in planning reintroduction trials of hybrid chestnuts by elucidating how light environment could affect their establishment and early growth

Background

Prior to the 20th century, American chestnut (*Castanea dentata*) was one of the most important tree species in the deciduous forests of eastern North America. At its peak, an estimated 3.5 billion individuals of this species comprised a population that ranged from New England to the Gulf Coast and from the Atlantic Coast to the Ohio River Valley. During the past century, the magnitude and extent of American chestnut in eastern North American forests have been devastated by chestnut blight, a disease caused by the accidental introduction of the Asian fungal pathogen *Cryphonectria parasitica* around the turn of the twentieth century. Spreading at a rate of up to 55 km yr^{-1}, chestnut blight virtually eliminated all native stands of American chestnut by the 1950s. Currently, the remaining American chestnuts in eastern North American include widely dispersed stump spouts of pre-blight trees limited in growth and confined to the forest understory by the continued presence of chestnut blight and a small number of isolated mature specimens planted in blight-free areas away from its historical range.

In addition to its inherent effect on forest composition, the decimation of American chestnut has been associated with other ecological and economic impacts. These impacts range from altered productivity of forest ecosystems to the loss of an important food source for associated wildlife to a decline in the historical subsistence culture of Appalachia. To help remediate these and other negative impacts, restoration efforts are currently underway that include an active backcross breeding program for production of hybrid trees that retain the morphological characteristics and adaptive variation of American chestnut with the blight resistance of Chinese chestnut (*C. mollissima*). These efforts are supported by The American Chestnut Foundation (TACF), a non-profit organization that aims to restore the American chestnut to eastern North American forests (TACF website). The TACF breeding program involves a series of backcrosses between hybrids of American and Chinese chestnuts and full American chestnuts. Individuals are selected prior to each backcrossing for Chinese chestnut blight resistance but against Chinese chestnut morphological characteristics. Resultantly, Chinese chestnut genes are decreased by one half, on average, per backcross generation. Initially, it was estimated that three backcrosses to full American chestnuts (producing BC_3s) would be sufficient to recover blight resistant trees that appear and function like historical American chestnuts . However, because blight resistance is only partially dominant, third backcross trees have been further intercrossed with one another (producing BC_3F_2s), to allow the offspring to inherit blight resistance genes from both parents and thus theoretically be homozygous for those genes. Most recently, these putative homozygous blight-resistant offspring have been intercrossed among themselves producing BC_3F_3 hybrids that are 94% American chestnut and breed true for the blight resistance conferred by their Chinese chestnut ancestry.

Although Chinese chestnut provides a potential solution to the blight susceptibility of American chestnut, Chinese chestnut does differ in some potentially significant ways from its American congener. Perhaps most obviously, the widely cultivated Chinese chestnut is relatively short in stature with a broad crown, as is typical of common orchard trees, while American chestnut can grow to more than 30 m tall as a component of the deciduous forest canopy. As for habitat, Chinese chestnut is associated with sunny open exposures, while American chestnut must contend with the low light environment of the forest understory to grow and eventually become successfully established as a component of the well lit forest canopy. Historically, successful growth from the forest understory, through the mid-canopy, and into the canopy clearly was possible and common for American chestnut in the absence of blight. However, their associated habitats suggest that American chestnut and Chinese chestnut could be adapted to different light environments, and thus could differ in their abilities to tolerate sun and shade. We also suggest that by conducting its hybrid breeding program primarily in greenhouse and orchard-like settings, TACF may be artificially and inadvertently selecting its hybrid chestnuts for adaptation to high-light environments. Given these concerns, the ability of hybrid chestnuts to tolerate sun and shade could be an important characteristic to consider regarding their use in any future restoration efforts.

In general, tree species can be classified as either shade-tolerant or shade-intolerant depending on their ability to become established and grow in various light environments. Shade-tolerant species are able to grow in low-light environments, while shade-intolerant species require high light availability. Examining leaf-level gas-exchange properties and related leaf morphology can provide a means to assess the degree of shade tolerance and intolerance of a species. Specifically, low photosynthetic rates, respiration rates, and light compensation points (LCP), a measure of the minimal light required for photosynthetic carbon gains to offset respiration carbon losses, are characteristic of shade tolerance. Similarly, low leaf LMA has been considered to be indicative of shade-tolerant tree species. Shade tolerance also has been associated with relatively high light-induced plasticity of leaf-level morphology. In contrast, a high maximum rate of photosynthesis in response to increasing light availability ($A_{\max}$), which may be indicative of increased incorporation of photosynthetic pigments and enzymes in leaves, often is characteristic of shade-intolerant species. Inclusion of such photosynthetic materials may be associated with relatively high leaf mass per unit surface area (LMA) in shade-intolerant species as well. In addition, shade-intolerant tree species have been associated with relatively high light-induced plasticity of physiological characteristics.

Given the contrasting light availability of their associated habitats, Chinese chestnut would not need to tolerate shade to successfully grow and reproduce, while American chestnut would require the ability to both contend with low light availability in the understory and take advantage of high light availability in the forest canopy. But because American chestnut has been functionally extinct during the past century, quantifiable assessments of its ability to tolerant shade and sun have been limited in comparison with information about co-occurring tree species. Early scientific observations of American chestnut responses to light availability were conflicting, with some suggesting that this species should be regarded as moderately or highly shade tolerant and others suggesting that it should be considered shade intolerant. The conclusions of more recent research have been similarly inconsistent. A study of American chestnut seedlings grown in a range of light treatments characterized them as shade tolerant due to their relatively low LCP compared to other co-occurring deciduous forest tree species, light-induced leaf morphological plasticity, and a lack of mortality due to light limitation. However, the recent research on chestnut seedlings, saplings, and mature trees growth in naturally available light gradients in forest settings concluded that American chestnut possesses an intermediate level of shade tolerance given its combination of characteristics associated with shade-tolerance and shade-intolerance. Specifically, it has been reported that American chestnut has both a low LCP and LMA in combination with a high A_{max}, suggesting that it can both persist in the understory like shade-tolerant species and take advantage of periodic high light provided by canopy openings like more shade-intolerant species. Consequently, these researchers recommended that hybrid chestnut seeds be planted in understory or shaded environments, and techniques such as thinning or burning be used to form periodic canopy gaps once saplings are established to maximize restoration success. However, the findings of other recent research suggested that American chestnut plantings would benefit from high-light availability during initial seedling growth.

Although assessments of the shade-tolerance or intolerance of American chestnut have differed, the applicability of these studies on American chestnut restoration efforts hinge on a similar presumption that hybrid chestnuts bred for restoration will function like their American ancestors. Yet, to date, no quantifiable research has examined the response of BC_3F_3 chestnuts to light availability or compared these responses to those of their American and Chinese ancestors. In recent field trials, hybrid chestnuts planted in a shaded southern Appalachian forest understory with mid-story clearing at the Lula Lake Land Trust (Walker County, Georgia, USA) observably exhibited much lower survival in comparison with those planted in a sunny open agricultural setting at nearby Bendabout Farms (Bradley County, Tennessee, USA), suggesting that hybrids may have a relatively low degree of shade tolerance. However, other non-studied differences between these field sites such as elevation, temperature, and water-availability could have influenced these results.

Certainly, the success of any efforts to remediate the impacts of chestnut blight via the introduction of hybrid chestnuts to eastern North American forests would be partially dependent on the selection and management of reintroduction sites based on optimal environmental conditions. To test the assumption that BC_3F_3 hybrid chestnuts will function like their presumably shade-tolerant American ancestors rather than their presumably shade-intolerant Chinese ancestors, we quantifiably assessed and compared the shade tolerance of American chestnut, Chinese chestnut, and BC_3F_3 hybrids chestnut saplings. Specifically, we investigated the shade tolerance of saplings of these chestnut types when grown in either ambient light or a half-ambient shade treatment as determined by survival, growth, leaf-level morphological characteristics, and leaf-level photosynthetic light responses. Although American and Chinese chestnuts are associated with contrasting light environments, we hypothesized that BC_3F_3 hybrid chestnuts would exhibit characteristics indicative of shade tolerance similar to those of American chestnuts given their closer genetic relationship.

2. Methods

Forty-eight seeds each of American chestnut, Chinese chestnut, and B_3-F_3 hybrid chestnuts collected from populations at TACF's Meadowview Research Farms in Meadowview, Virginia, USA and at experimental orchards throughout Tennessee, USA, were propagated in a greenhouse on the campus of the University of Tennessee at Chattanooga in March 2010 according to methods developed and regularly used by J. Hill Craddock. Each seed was planted in a separate 3-gallon nursery pot filled with a commercially available southern perennial soil mixture containing pine bark, peat moss, and pearlite. Twenty-four pots of each chestnut type were be placed on a bench with full sunlight exposure inside the greenhouse, while the remaining 24 pots of each chestnut type were placed on a bench shaded with aluminum shade cloth designed to filter 50% of sunlight. All pots were treated with a commercially available slow-release encapsulated fertilizer and watered biweekly to saturation. In addition, all pots received an application of liquid fertilizer once every two weeks.

To eliminate the impacts of insect and mite infestation inside the greenhouse, which become common in late spring, all pots were moved outdoors to a location adjacent to the greenhouse in mid-May 2010. The 24 individuals of each chestnut type propagated with full sunlight exposure inside the greenhouse continued to be grown with full sunlight exposure outside of the greenhouse, while the 24 individuals of each chestnut type propagated in the shade treatment inside the greenhouse were placed under a shade house structure covered with shade cloth designed to filter 50% sunlight. All potted individuals continued to be watered and fertilized according to the methods used inside the greenhouse. In early spring 2011, all individuals were transplanted to separate 5-gallon nursery pots to allow adequate room for root growth for the duration of the 1.5-year growth period of our research.

Photosynthetic characteristics were measured *in situ* on one fully expanded top canopy leaf from all chestnut individuals that survived to summer 2011 with a portable gas-exchange system (Li-Cor 6400XT, Li-Cor, Lincoln, Nebraska, USA) equipped with a CO_2-control module. Measurements on the ~1.5-year-old saplings were made throughout July and August 2011 on warm, clear afternoons. Specifically, the photosynthetic response to light level (an *A*/Q curve) was assessed for each selected leaf by measuring the steady-state responses of photosynthesis to externally supplied photosynthetically active radiation (PAR) provided in 16 steps from 2000 μmol photons m-2 s-1 (maximum sunlight) to 0 (complete darkness). At each PAR setpoint, a photosynthesis measurement was made after gas exchange had equilibrated as was determined when the coefficient of variation for the CO_2 partial pressure differential between the sample and reference analyzers was below 1%. During these photosynthesis measurements, leaf temperature was kept at 28ºC by using thermoelectric coolers and the water vapor pressure deficit was maintained between 1.0 and 1.5 kPa to replicate average ambient conditions. A constant external CO_2 partial pressure of 40 Pa was provided by the CO_2-control module.

Photosynthetic light-response curves were analyzed to estimate the maximum rate of photosynthesis at saturating photon flux density ($A_{max;}$ μmol CO_2 m^{-2} s^{-1}), light compensation point (LCP; μmol photons m^{-2} s^{-1}), quantum yield (Φ; mol CO_2 mol photons^{-1}), and rate of leaf respiration in dark (R_d; μmol CO_2 m^{-2} s^{-1}) of all measured individuals. These estimations were made with Photosynthesis Assistant (Dundee Scientific, Scotland, UK), a software program that uses a standard biochemical model describing photosynthesis (Farquhar et al., 1980).

All leaves selected for physiological measurements were harvested directly following those measurements for leaf structural 3000A, Li-Cor, Lincoln, Nebraska, USA) and then dried in a 60°C laboratory oven for 72 h to determine leaf mass per unit area (LMA; g m^{-2}) of each chestnut individual. Concurrent with leaf harvests, we measured the stem height and stem diameter at 20 cm above the soil surface of all studied individuals.

All measured variables were averaged across chestnut type and across light treatment. Two-way analysis of variance (ANOVA) was conducted to evaluate the main effects and interactive effect of chestnut type and light treatment on all measured variables. Main and interactive effects of treatments were considered significant if $P \leq 0.05$. Treatment means were compared to determine if means of the dependent variable were significant at the 0.05 probability level with least significant difference (LSD) post-hoc analysis. These statistical analyses were performed with IBM SPSS Statistics 19 (SPSS, Inc., 2010, Somer, NY). Means of select measured variables were used to model representative *A*/Q curves for each chestnut type in each light treatment.

3. Results

Survival and Growth

All planted seeds germinated successfully by April 2010, but survival to the time that measurements were made in summer 2012 differed among chestnuts types. Both Chinese chestnut and hybrids chestnut saplings exhibited > 85% survival whether propagated and grown in full ambient sunlight or half ambient sunlight provided by shade cloth, while American chestnut saplings exhibited ≤ 75% survival in both light treatments. Specifically, 18 American chestnuts, 22 Chinese chestnuts, and 23 BC_3F_3 hybrid chestnuts remained in the full light treatment, while 24 Chinese chestnuts, 21 BC_3F_3 hybrid chestnuts, and 16 American chestnuts remained in the shade treatment in August 2011.

Across chestnut types, stem height and stem diameter both were significantly greater for surviving individuals grown in full ambient sunlight compared with individuals grown in shade ($P = 0.48$ and $P = 0.003$, respectively). Specifically, chestnuts grown in full ambient sunlight grew 5.7% taller and had 15.9% larger stem diameter than chestnuts grown in half ambient sunlight. Stem height also was influenced by the main effect of chestnut type ($P = 0.11$), with Chinese chestnuts growing significantly taller than either American or hybrid chestnuts across light treatments. In contrast, stem diameter did not differ significantly between chestnut types when averaged across light treatments ($P = 0.780$). In addition, the interaction of chestnut type and light treatment did not influence significantly either stem height ($P = 0.589$) or stem diameter ($P = 0.916$).

Photosynthetic Light Response

Representative curves modeled using mean gas-exchange characteristics illustrated differences among chestnut types and light treatments in photosynthetic responses to PAR. Both within the full ambient sunlight treatment and the shade treatment, American chestnut saplings exhibited the greatest modeled photosynthetic response to increasing PAR at relatively high light intensity (>1000 µmol photons m^{-2} s^{-1}), while Chinese chestnuts exhibited the lowest response. The photosynthetic light response of BC_3F_3 hybrid chestnuts was intermediate to that of the pure chestnut types at relatively high light intensity. However, at relatively low light intensity, the photosynthetic rate of Chinese chestnuts appeared to exceed that of both American chestnuts and hybrid chestnuts, which exhibited similar responses. When grown in full sunlight, both American and hybrid chestnuts exhibited greater modeled photosynthetic response to increasing PAR than when grown in half-ambient sunlight; however, the photosynthetic light response of Chinese chestnuts appeared to be similar whether grown in full sun or shade. Photosynthetic response curves also suggested that Chinese chestnuts experience significant down-regulation of photosynthetic processes at relatively high light levels whether grown in full sunlight or half-ambient sunlight. It should be noted that this down- regulation was exhibited by the majority of individual *A*/Q curves, as well as representative curves for Chinese chestnuts. It also appears that sun-grown BC_3F_3 hybrid chestnuts may experience a more minor degree of photosynthetic down-regulation relative to those grown in shade. In contrast, down-regulation was not evidenced by the modeled *A*/Q curves of American chestnuts grown in either light treatment or by hybrid chestnuts grown in half-ambient sunlight.

Gas-exchange Characteristics and Related Measures

There was a significant main effect of chestnut type on most measured variables of gas-exchange activity, including A_{max} ($P < 0.001$), R_d ($P = 0.018$), and Φ ($P < 0.001$). Across light treatments, A_{max} and R_d were significantly greater in both American chestnuts and BC_3F_3 hybrid chestnuts than in Chinese chestnuts. However, neither A_{max} nor R_d, differed significantly between American chestnuts and hybrid chestnuts when averaged across light treatments. Mean Φ also was significantly greater in American chestnuts and hybrid chestnuts than in Chinese chestnuts, but Φ of American chestnuts was significantly greater than that of hybrids as well. In contrast to other measured gas-exchange variables, mean LCP was not significantly influenced by chestnut type ($P = 0.249$).

Similar to the main effect of chestnut type, the main effect of light treatment significantly influenced most measured gas-exchange variables. Specifically, saplings grown in full sunlight exhibited 45.8% greater R_d than those grown in half-ambient sunlight ($P = 0.018$; -0.70 ± 0.06 vs. -0.48 ± 0.07 µmol CO_2 m^{-2} s^{-1}, respectively). Similarly, the mean LCP was 104.9% greater for chestnuts grown in full sunlight than those grown in shade ($P = 0.004$; 29.5 ± 3.0 vs. 14.4 ± 4.1 µmol photons m^{-2} s^{-1}, respectively). Conversely, chestnuts grown in full sunlight exhibited 37.8% lower Φ than chestnuts grown in the half-ambient sunlight treatment ($P < 0.001$; 0.023 ± 0.002 vs. 0.037 ± 0.002 mol CO_2 mol $photons^{-1}$, respectively). In contrast to other measured gas-exchange variables, mean A_{max} was not significantly influenced by the main effect of light treatment ($P = 0.564$).

Mean Φ was influenced significantly by the interaction of chestnut type and light treatment in the studied saplings ($P < 0.001$). However, this interaction did not significantly influence mean A_{max} ($P = 0.572$), $R_{d,}$ ($P = 0.186$), or LCP ($P = 0.096$). In both American chestnuts and BC_3F_3 hybrid chestnuts, Φ was significantly greater when saplings were grown in shade than in full sunlight than in half-ambient shade; however, light did not significantly influence Φ in Chinese chestnuts.

Like gas-exchange measures, mean LMA also was influenced significantly by the main effect of light treatment ($P = 0.05$). Specifically, LMA was 21.1% greater in for chestnuts grown in full sunlight than those grown in shade (91.23 ± 3.16 vs. 75.34 ± 3.44 g m^{-2}). In contrast, LMA did not differ significantly among chestnut types when considered across light treatments. However, mean LMA was influenced significantly by the interaction between chestnut types and light treatment ($P = 0.001$). Specifically, both Chinese chestnuts and BC_3F_3 hybrid chestnuts exhibited greater LMA when grown in full sunlight than when grown in our shade treatment, while the LMA of American chestnuts did not differ between saplings grown in sun and those grown in shade.

Synthesis of Findings

Collectively, the survival, growth, and leaf-level physiological and morphological characteristics of young American chestnuts reported in our study suggest that this species possess a combination of some factors that typically are associated with shade tolerance and others that generally are not associated with shade tolerance. Specifically, our saplings survived and grew in a half-ambient sunlight shade treatment, but both survival and growth were enhanced by increased light availability, which suggests an intermediate degree of shade tolerance. The leaf-level A_{max} and R_d values we report correspond with those associated with shade-intolerant tree species, while our LCP and Φ values are similar to those associated with shade-tolerant tree species. Similarly, the A/Q curves and the lack of plasticity of A_{max} that we observed are suggestive of shade tolerance, although the lack of plasticity of LMA that we report is characteristic of shade intolerance. Given our combined results, we agree with previous researchers who characterized the American chestnut as a species with intermediate shade tolerance and disagree with assessments that this species should be regarded as purely shade tolerant.

Similarities in the mean A_{max} and R_d of American and BC_3F_3 hybrids chestnut saplings across full sunlight and half-ambient shade treatments indicate that these chestnut types have similar overall gas-exchange rates given their close genetic relationship in accordance with our hypothesis. In contrast, the intermediate Φ of hybrid chestnuts relative to American and Chinese chestnuts suggests that hybrids may retain some leaf-level physiological similarities to their Chinese ancestors. Collectively, the greater values of A_{max}, R_d, and Φ of American and hybrids chestnut saplings compared with those exhibited by our Chinese chestnut saplings suggest that both the American and hybrid chestnut are less tolerant of shade than the Chinese chestnut, a finding which we did not expect given the contrasting associated habitats of the American and Chinese chestnut. We suggest that photoinhibition at very high light levels (>1000 µmol photons m^{-2} s^{-1}) by Chinese chestnut saplings during our mid-summer midday measurement times could have negatively impacted instantaneous gas-exchange activity in this species, but that such down-regulation could provide photoprotection, which could have a positive impact over time.

Overall, our research findings should aid chestnut restoration efforts by elucidating how shade tolerance (as well as blight resistance) could be influenced by parent species, as well as aid land managers in planning reintroduction trials of BC_3F_3 hybrid chestnuts and other backcross hybrid chestnuts by determining how light environment could influence their successful establishment and growth. Specifically, we determined that the responses of BC_3F_3 hybrid chestnuts to light availability were largely similar to that of their closely related American ancestry, suggesting that BC_3F_3 hybrid chestnuts should contend well with light environment of the historical range of the American chestnut within the temperate deciduous forest of eastern North America.

Differences from Objectives

Due to issues with obtaining an adequate supply of F_1 seeds prior to the time that seeds were propagated for this project, we had to proceed with the research project without the inclusion of these intermediate hybrids. This prevented us from determining if F_1 hybrid chestnuts were characterized by shade tolerance that was intermediate between that of their American and Chinese parents, which was part of our original objectives. However, this omission still allowed us to meet the most relevant of our objectives to chestnut restoration efforts. Specifically, our findings should still be useful to the TACF and land managers in planning reintroduction trials of hybrid chestnuts by elucidating how light environment could affect their establishment and early growth since these trials are proposed to concentrate on BC_3F_3 hybrid chestnuts.

4. Published Works and Presentations

Manuscript in Progress

Boyd JN, Craddock JH. Comparing the tolerance of American chestnut (*Castanea dentata*), Chinese chestnut (*C. mollissima*), and their hybrids to sun and shade. In preparation for *Forest Ecology and Management*. Expected submission by August 31, 2012. Copy of this manuscript will be provided to TACF upon its completion.

Presentations

Lyon A*. Investigating the shade tolerance of American chestnut, Chinese chestnut, and their hybrids. Beta Beta Beta Undergraduate Research Symposium. University of Tennessee at Chattanooga. April 2012.

Lyon A*, Craddock JH, Boyd J. Using leaf-level gas-exchange characteristics to investigate the shade tolerance of *Castanea dentata*, *C. mollissima*, and hybrid chestnuts. Association of Southeastern Biologists. Athens, GA. April 2012.

Lyon A*. Investigating the shade tolerance of American chestnut, Chinese chestnut, and their hybrids. Posters on the Capitol. Nashville, TN. February 2012.

Boyd J, Lyon A*, Craddock JH. Using leaf-level gas-exchange characteristics to investigate the shade tolerance of *Castanea dentata*, *C. mollissima*, and their hybrids. Tennessee Academy of Science. Jackson, TN. October 2011.

Lyon A*, Craddock JH, Boyd J. Investigating the shade tolerance of *Castanea dentata*, *C. mollissima*, and their hybrids. Tennessee Academy of Science. Jackson, TN. October 2011. NE-1033 Technical Committee Meeting Biological Improvement of Chestnut through Technologies that Address Management of the Species, its Pathogens and Pests. Ivoryton, CT. October 2011.

Hughes K*, Craddock JH, Boyd J. Comparing shade tolerance of *Castanea dentata*, *C. mollissima*, and their hybrids. NE-1033: Biological Improvement of Chestnut through Technologies that Address Management of the Species, its Pathogens and Pests. Maggie Valley, NC. September 2010.

Boyd JN. Not made for the shade? An ecophysiological approach to American chestnut restoration efforts. Biology Seminar Series, UTC, October 2010.

Boyd J, Craddock JH. Not made for the shade? Comparing the shade tolerance of *Castanea dentata*, *Castanea mollissima* and their hybrids. Association of Southeastern Biologists. Asheville, NC. April 2010.

Boyd JN. Not made for the shade? An ecophysiological approach to American chestnut restoration efforts. E.O. Grundset Lecture Series, Southern Adventist University, Collegedale, TN, September 2009.

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5. Press Coverage

N/A