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Physiological changes in advanced hybrid chestnuts do not alter blight resistance under co-occurring drought

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Complete List of Authors:	Fredericksen, Brett; Ohio University, Environmental and Plant Biology Kukor, Samuel; Ohio University, Environmental and Plant Biology Rosenthal, David; Ohio University, Environmental and Plant Biology
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7 Brett Fredericksen, Samuel Kukor, and David M. Rosenthal
8 Department of Environmental and Plant Biology Ohio University, Athens, OH, 45701, U.S.A.
9 Contact information: brettfredericksen@gmail.com

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Abstract

Chestnut breeding programs have been using a backcross breeding technique to produce trees with a predominantly American chestnut (*Castanea dentata*) genome and chestnut blight disease resistance from Asian species like the Chinese chestnut (*Castanea mollissima*). The potential for other physiological changes caused by breeding has yet to be widely studied. We quantify chestnut (American, Chinese, and BC₃F₃ hybrids) responses to water-stress and measure how co-occurring drought influences disease severity. The experiment was completed using 172 bare-root seedlings organized into a completely randomized factorial design in an outdoor rain-out shelter for one growing season. BC₃F₃ hybrid gas exchange (A_{sat} , g_s) rates were more similar to Chinese than American chestnuts over a 20-day dry-down period, and hybrid turgor loss point showed a more intermediate (between Chinese and American) response. The relationship of stomatal conductance to mid-day leaf water potential (Ψ_{md}) also exhibited both American and Chinese characteristics in the hybrid trees. There was no effect of drought on the disease severity for any of the chestnut groups. We find evidence that drought physiology has been altered in some BC₃F₃ hybrids, but do not find changes in disease severity when chestnuts are under co-occurring drought.

Keywords

American chestnut, hybrid chestnuts, chestnut blight, drought physiology, disease resistance

Introduction

The history of American chestnuts [*Castanea dentata* (Marsh.) Borkh.] in eastern North American forests has become a classic warning about the dangers of non-native pathogens. The fungus *Cryphonectria parasitica* (Murril) Barr., the causal agent of chestnut blight, was introduced to eastern hardwood forests in the early 20th century and reduced American chestnut to non-reproductive populations (Anagnostakis 1987, Stephenson et al. 1991). Soon after the extirpation of American chestnuts, the USDA and the Connecticut Agricultural Experiment Station began efforts to develop resistance through breeding with a goal to rescue the species (Anagnostakis 1982). Today, The American Chestnut Foundation (TACF) has inherited these efforts and has been working to develop blight resistant, hybrid chestnuts (third backcross generation or BC₃F₃) through introgressive hybridization using blight resistance genes from the Chinese chestnut (*Castanea mollissima*) BC₃F₃ (Burnham et al. 1986, Hebard. 2001). This goal was set based on the initial hypothesis that chestnut blight resistance was controlled by only a few loci in Chinese chestnuts making three generations of backcrossing sufficient for trait introgression of blight resistance (Clapper 1952; Burnham et al. 1986). Because the BC₃F₃ hybrid generation would still have 1/16th Chinese chestnut genetic material, the program had the additional goal of selecting for American chestnut characteristics. Achieving both of these goals would result in a hybrid chestnut that was disease resistant and could occupy the original American chestnut ecological niche (Hebard 2001).

The overarching goal of chestnut restoration efforts are the establishment of chestnut populations that are self-sustaining. To achieve this goal, the thousands of hybrid chestnut lines developed by TACF through the backcross breeding programs must be screened for disease

resistance and chestnut characteristics. So far, increased blight resistance has been demonstrated in hybrids when compared to pure American chestnuts in controlled and field settings (Clark et al. 2016; Steiner et al. 2017). However, recent evidence indicates that blight resistance in Chinese chestnut is a more polygenic trait than originally hypothesized (Kubisiak et al. 2013; Westbrook et al. 2020). Due to this deviation from the original breeding plan more Chinese genetic material has been inherited in some hybrids that express disease resistance (Westbrook, et al. 2020). Currently, little evidence exists if this increase in Chinese genetic material has caused changes to hybrid physiology and functional traits. Morphological similarity has been observed between hybrid and American chestnuts (Diskin et al. 2006) and some differences in growth have been observed between hybrid families (Sung et al. 2016) and hybrids compared to Americans (Clark et al. 2016), but physiological changes have been largely unstudied.

Quantifying physiological changes in hybrid genotypes is needed to ensure that the hybrid chestnuts will occupy a similar ecological niche as the American chestnut. One of the few physiological traits that has been studied in hybrids is light response (Knapp et al. 2014). In comparisons between hybrid generations (BC1, BC2, and BC3), pure American chestnuts, and pure Chinese chestnuts, Knapp et al. (2014) showed that advanced generations of hybrids perform more similarly to American chestnuts than Chinese chestnuts. In terms of restoration, we can assume from this similarity that hybrid chestnuts would perform well under intermediate shade as American chestnuts were found to be intermediate in shade tolerance (Joesting et al. 2007). More study is needed to determine if this similarity holds true for other

physiological processes relevant to restoration, like stress tolerance, soil preference, and plant hydraulics.

Hybrid physiological changes in drought tolerance are of particular interest as drought tolerance in pure American chestnuts is poorly understood. Pure American chestnuts have been and continue to be found in upland, dry sites (Keever 1953; Abrams et al. 1990; Burke 2012). However, it is challenging to determine if this is a preference by the species or is an artifact of remnant chestnuts post-blight. Other studies have described the American chestnut as a more generalist species preferring well drained soil that still had ample water availability (Russell 1987; Stephenson et al. 1991). Historic records report chestnut as uniformly distributed along slopes supporting a generalist classification (Buttrick 1925). Across most of the historic chestnut range, precipitation changes are predicted under future climates with more winter precipitation and less summer precipitation expected (Hubbart et al. 2016). If the breeding program altered hybrid drought response, then their performance under these drier growing seasons may be diminished, limiting reintroduction efforts. Furthermore, water stress may prove to be an inciting stressor that could reduce blight tolerance in hybrid chestnuts (Manion 1991; Oliva et al. 2014). For remnant populations of American chestnuts, mortality was found to increase after a severe growing season drought (Parker et al. 1993). Parker et al. (1993) hypothesized that the combination of drought (inciting factor) and blight (contributing factor) caused mortality but failed to show causality as the drought occurred during an inter-census period. On a physiological level, blight and drought co-occurrence may provide synergistic effects on chestnuts as the blight is a vascular damaging disease, disrupting both xylem and phloem structure (Bramble 1938; McManus and Ewers 1990). *C. parasitica* conidial growth has been

observed to be associated with loss of water in excised, chestnut bark with the fungi able to continue growing at lower bark osmotic potentials than was observed in live chestnut wood (Gao and Shain 1995).

Hybrid chestnut blight tolerance has mostly been tested under controlled orchard conditions and well chosen field sites (Hebard 2005; Steiner et al. 2017; Clark et al. 2019). If these hybrid chestnuts are to be reintroduced on a range-wide basis, we will need to understand how robust the disease resistance of the hybrids is to environmental stressors such as drought. We also need to determine if and how drought tolerance has been affected by backcross breeding for blight resistance. Here, we address these knowledge gaps by comparing physiological responses of advanced generation hybrids (BC_3F_3) to pure American and pure Chinese chestnuts under an artificial drought similar to that which the trees may experience when reintroduced. Blight was administered and infection severity was quantified and compared among chestnut varieties to determine if severity of the disease was altered with drought. Given that conidial growth has been observed at low water potentials and the back cross breeding program is expected to yield hybrid physiological responses similar to American chestnuts or intermediate of American and Chinese chestnut, we initially hypothesized that blight infections should be more severe under co-occurring drought stress in all chestnut varieties.

Materials and Methods

Study Design

In 2018, a selection of pure American, Chinese, and BC_3F_3 hybrid chestnut seedlings were obtained from multiple orchards and nurseries as bare-root seedlings (1-0). Fifty pure

American and Chinese chestnut bare-root seedlings were procured from Clements State Tree Nursery in West Virginia. Seeds for the American chestnuts were procured from a seed source on site while the Chinese seeds were sourced from multiple locations before being germinated at the nursery. Fifty hybrid chestnuts were obtained from The American Chestnut Foundation facilities at Pennsylvania State University in State College, PA with original seed sources coming from TACF facilities in Meadowview, VA. Equal representation of the two major chestnut hybrid lineages, Clapper and Graves, were included in the hybrid group but were not replicated on a genotype level (S1). A total of 16 hybrid families were utilized within the hybrid group with anywhere from one to eight individuals per family. Another 25 pure American chestnut seedlings were obtained from The American Chestnut Foundation facilities in Meadowview, VA, with seeds sourced on site, to test for nursery source effects on the American physiological responses. Seedlings were kept bagged, refrigerated, and in the dark until all varieties were received (April 10, 2018).

Seedlings were transplanted on April 20th and 21st in 9.6 L tree pots (Stuewe and Sons TP818) before bud break at the Ohio University Student Farm. The potting mix contained pine bark mulch (60%), sand (30%), and vermiculite (10%) (Brown et al. 2014). Slow-release fertilizer was added to each pot according to the manufacturer's recommended quantity (Osmocote Plus 15-9-12). Seedlings were arranged in a completely randomized design across chestnut variety and treatment within a 2.6 m by 4 m covered shelter. Seedlings were re-randomized in June and July to mitigate confounding effects of placement within the shelter. The structure covered the chestnuts to prevent rainfall from reaching the pots but allowed plants to be exposed to ambient conditions otherwise.

Control, drought, pathogen, and drought-pathogen (co-occurring) treatments were implemented in groups of 5-12 individuals per treatment-variety combination (S2). The drought was implemented as a series of dry-downs on a per pot basis over the course of the growing season. Starting in late May, pots were dried until their volumetric water content (VWC, %) was < 5%, measured with a HydroSense II soil moisture probe (Campbell Scientific, Logan UT, U.S.A.), and then watered to field capacity. Volumetric water content measurements and necessary watering were completed once a week except during the measurement campaigns. Control and pathogen treatments were watered once a week regardless of volumetric water content to maintain soil moisture near field capacity.

Inoculation with blight was completed on the pathogen and drought-pathogen treatments in early July. The Ep155 strain of *Cryphonectria parasitica* used, which is a highly virulent strain of the fungus, was obtained from The American Chestnut Foundation. Infections were completed by making a one-centimeter incision along the stem of the seedlings approximately two centimeters above the soil. A small agar plug taken from a plate of actively growing *C. parasitica* (strain Ep155) was then placed onto the incision according to established protocols (Powell et al. 2007).

Gas-exchange and water potentials measurements

Before gas exchange measurements began, the drought treatment was allowed to dry until the volumetric water content was significantly lower than the control across all pots ($t = 6.51$, $df = 171.48$, $p < 0.001$). Water was withheld starting in late May and the drought treatment was significantly lower by early June. After this initial dry-down, all seedlings were

watered to field capacity and the drought treatments were then allowed to dry-down for 20 days (June 10th to July 1st). Using a LI-6400XT (Li-Cor Biosciences, Lincoln NE, U.S.A), gas exchange measurements were collected every 2-3 days, weather permitting, on a random selection of each chestnut group in each treatment ($n = 6$). Specifically, photosynthetic rates (A_{sat} $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) and stomatal conductance (g_s $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) were measured at saturating light ($1500 \mu\text{mol m}^{-2} \text{ s}^{-1}$ photosynthetic photon flux density) and otherwise ambient environmental conditions. Measurements occurred from 0900 to 1300 on days without rain. Water use efficiency was then calculated from A_{sat} and g_s . The relative chlorophyll content of leaves was estimated with a SPAD meter during the dry-down period (SPAD-502Plus, Konica Minolta, Tokyo, Japan). SPAD is a unitless measure that is useful in indicating relative amounts of foliar leaf nitrogen which is most concentrated in chlorophyll content (Chang and Robison 2003). After the 20-day dry-down, the drought was maintained using the 5% VWC cut off.

Another campaign of gas exchange measurements was conducted in conjunction with measurements of mid-day leaf water potential (Ψ_{md}) to determine how the different chestnut varieties were responding in terms of stomatal conductance and leaf water potential. Over the course of five days in late July, gas exchange was measured on a set of randomly selected seedlings in the drought and control treatments from 1200 to 1500 until all seedlings in each group were measured. This time was selected to capture the hottest parts of the day where leaf water potential would likely be lowest. Individuals in the drought treatment that were watered in the last week were excluded from the selection pool. The Li-6400XT measurement chambers were set to control leaf temperature at 25 °C at a constant PPFD of $1000 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Mid-day water potentials were determined using the same leaves used for gas exchange and were

measured using a pressure chamber (PMS Instrument Company, Albany OR, U.S.A) in the field less than two minutes after gas exchange measurements (Scholander 1965).

Pre-dawn water potential and turgor loss point

Measurements of pre-dawn leaf water potential (Ψ_{pd}) and osmotic potential (Ψ_{π}) were used to track changes in turgor loss point over a range of leaf water statuses. Between 7-15 individuals from each chestnut variety – treatment combination were measured over the course of four days in mid-August, totaling to 122 measurements. Branches were selected after sundown and wrapped in plastic to mitigate the chance of nighttime transpiration (Caird et al. 2007). Leaves measured for pre-dawn potential were excised from the plants and transported back to the laboratory (~15 minutes) double wrapped in plastic with a damp paper towel. In addition, stem segments were excised from the top third of the plant with the cut ends kept in water during transit to the lab. Upon reaching the lab, stem segments were recut underwater using a fresh razor blade and rehydrated. After rehydration, one leaf was selected from each stem and measured again for water potential. Leaves below 0.2 MPa were considered rehydrated and used for solute measurement. Solute concentration was determined using a Vapro Vapor Pressure Osmometer 5520 (Wescor Inc. Elitech Group, Logan UT, U.S.A) and turgor loss point was estimated using protocols detailed in Bartlett et al. (2012).

Quantification of infection severity

Two methods were used to determine the severity of blight infection in the infected treatments at the end of the experiment. A qualitative based scoring system of one through five similar to that used by The American Chestnut Foundation and other chestnut studies

(Table 1). This scoring system involves assigning a number based on how visually infected the stem cankers appear (Hebard 2012), with low numbers indicating mild infections (small cankers) and high numbers indicating severe infection (larger cankers). Because qualitative metrics are inherently subjective, we developed an objective and quantifiable metric of infection severity called the canker severity ratio:

(Table 1: Qualitative infection scoring for chestnut blight)

$$\text{Canker Severity Ratio} = \frac{\text{Surface Area of canker (cm}^2\text{)}}{\text{Basal diameter of infected stem (mm)}} \quad (\text{eq. 1})$$

where blight severity is the ratio of the size of the infected area to the basal diameter of the infected stem. The logic of this metric was to take advantage of the highly visible fungal canker on infected stems but also accounting for differences in stem size. After 16 weeks of infection, we photographed each stem canker and then quantified the surface area via image analysis software (ImageJ). Basal diameters were obtained at the point of harvest. Individuals that showed no signs of a successful infection were removed from this data set.

Statistical Analysis

All statistical analyses were completed using R statistical software (version 3.6.2). The two sources of American chestnut were not significantly different in any physiological parameter measured and were thus grouped into a single American variety. Analysis of photosynthesis (A_{sat}), stomatal conductance (g_s), and water use efficiency (WUE) during initial dry-down utilized a repeated measures, mixed-effects model with individuals as a random factor. Fixed effects (chestnut variety, treatment, and day of measurement) and covariates

(SPAD, basal diameter, volumetric water content, and leaf counts) were included in the full model prior to model selection. Interactions of all fixed effects were also included. From the full model, all possible model parameter combinations were fit with maximum likelihood and resulting models were ranked according to lowest AICc and highest Akaike weights to determine which covariates and interactions to keep before refitting with restricted maximum likelihood. Type III repeated measures ANOVA was used to determine differences in the final model and a post hoc comparisons of least – square means with Tukey adjustment was used for pairwise comparisons.

Infection severity ratios were analyzed using Kruskal-Wallis ranked sum tests and Dunn's multiple comparisons post hoc test for pairwise comparisons of the ratio data. The relationship of stomatal conductance and mid-day leaf water potential was analyzed by first logarithmically transforming stomatal conductance to make the relationship linear with mid-day water potential. Droughted individuals that were recently watered were removed from the analysis. A linear regression was then used to determine different chestnut variety responses in water potential for each group. Values were predicted from the model and back-transformed before plotting. Analysis of turgor loss point data utilized linear regression models to compare response to pre-dawn water potentials among chestnut varieties.

Results

Evidence of drought effects

We used a threshold of 5% soil volumetric water content (VWC) for re-watering plants to full hydration in the drought treatments. This 5% threshold of soil VWC proved sufficient to

cause declines in mid-day leaf water potential across all chestnut varieties (Figure 1). Allowing each pot to dry individually to this threshold also allowed a spectrum of drought severity to be present for each set of physiological measurements.

(Figure 1)

Blight infection severity

Quantitative assessment of blight infection using the canker severity ratio (Eq1) showed similar patterns of blight severity when compared to the qualitative score's assessment (Figure 2, top). As the ratio metric accounted for the relative size of the plants and had less potential for observer bias, the ratio metric was used for analysis. Chestnut variety was significant using a Kruskal-Wallis rank sum test ($\chi^2 = 6.84$, $df = 2$, $p = 0.033$). American chestnuts had more severe infections than Chinese chestnuts, which was expected ($z = 2.50$, $p = 0.019$). Hybrid chestnuts showed an intermediate response not being significantly different than Americans ($z = 1.64$, $p = 0.15$) or Chinese chestnuts ($z = -0.66$, $p = 0.76$). Drought treatments did not affect the severity of blight infection (Figure 2) for any of the chestnut varieties ($\chi^2 = 0.07$, $df = 2$, $p = 0.80$). Main stem mortality was observed during the experiment ($n=10$, $< 7\%$ of total sample size), but no patterns were observed across treatments.

(Figure 2)

Gas exchange over dry-down period varied by variety

Mixed-effects models were determined for light saturated photosynthetic rate (A_{sat}), stomatal conductance (g_s), and water use efficiency (WUE) over the course of the dry-down. Photosynthetic rate was significantly different across varieties and days measured (Table 2).

Treatment was not significant, but the interaction of treatment and day of measurement was significant with the drought treatment becoming lower on day 9 on average (t -ratio = 2.88, df = 359, p = 0.004, Figure 3, top row) and remaining lower until the end of the 20-day period. SPAD proved to be a significant covariate for photosynthetic rate, while volumetric water content and leaf counts were not significant but were kept in the model for biological relevancy and based on model selection by AICc (Table 2). Averaged over the entire measurement period and across treatments, pairwise comparisons show that American chestnuts exhibited the highest rates of photosynthesis and was higher than both Chinese and hybrid chestnuts (Table 3). Photosynthetic rates of the hybrid chestnuts were not significantly different from those of Chinese chestnuts.

Mixed-effects models for stomatal conductance also showed significant differences in variety and over time, and like photosynthesis showed a significant interaction between treatment and day (Table 2) with the drought treatment becoming significantly lower on day 9 across all varieties (t -ratio = 1.64, df = 270, p = 0.005, Figure 3 middle row). Days in which the drought treatment was significantly lower than the control were not as consistent as the photosynthetic model, with no treatment difference on day 13 (p = 0.11) before treatment difference reemerged on days 15 (p = 0.002) and 20 (p < 0.001). Also, unlike the model for photosynthesis, there was a significant interaction of chestnut variety and treatment for stomatal conductance (Table 2). Covariates of leaf count, and volumetric water content were kept as covariates in the model but were not significant. SPAD was not found to be an important covariate for stomatal conductance and was removed from the model. Averaged over the measurement period, stomatal conductance of American chestnuts was significantly

286 higher than Chinese and hybrid chestnuts (Figure 3, middle row) in the control group (Table 3).
287 In the droughted group, the same pattern is present with Americans showing higher rates of
288 conductance than both the Chinese and hybrids (S4). In neither control nor drought treatments
289 did pairwise comparisons show the Chinese and hybrid groups were statistically different.
290 Chinese chestnuts were the only variety not to show a significant decline in stomatal
291 conductance for the measurement period between the control and drought group.
292 (Figure 3)
293 (Table 2)
294 (Table 3)

295 Main effects in the WUE model showed no significance; however, interactions of variety
296 and treatment, treatment and day, and day and variety all were significant (Table 2). Covariates
297 of leaf counts, SPAD, and volumetric water content were all included in the model. The drought
298 treatment was significantly higher than the control group on day 9 (t -ratio = -2.14, df = 349, p =
299 0.033) and on day 15 (t -ratio = -4.63, df = 349, p < 0.001) across all varieties. The spike in values
300 observed on day 15 drove the variety day interaction and may be due to increased
301 measurement temperatures (S3) Averaged across days, American chestnuts were significantly
302 lower in the control group compared to Chinese and not different than hybrid chestnuts (Figure
303 3, bottom row). However, in the drought treatment, hybrid chestnuts showed significantly
304 higher WUE compared to both Chinese and American chestnuts (Table 3). Within varieties,
305 Chinese chestnuts showed no difference between drought and control groups when averaged

over the measurement period while both American and hybrid WUE rates showed increases in the drought treatment.

Stomatal conductance and water potential

We compared stomatal conductance to declining mid-day leaf water potential for each chestnut variety to determine at what leaf water status each variety began to close stomata and limit gas exchange under drought (Figure 4). Response of stomatal conductance (g_s) to mid-day leaf water potential (Ψ_{md}) showed a significant logarithmic relationship in hybrid ($t = 4.65$, $p < 0.001$, $R^2 = 0.61$) and Chinese ($t = 3.86$, $p < 0.001$, $R^2 = 0.39$) chestnuts but showed no significant relationship in American chestnuts ($t = 1.38$, $p = 0.18$, $R^2 = 0.08$) within the measured range of water potentials (Figure 4). Chinese and hybrid models were not significantly different in slope or intercept. The range of mid-day water potentials was smallest in the Chinese group compared to the American and hybrid ranges of water potentials.

(Figure 4)

Turgor loss point responses to pre-dawn leaf water potential

Linear regression showed that turgor loss point had a negative relationship with pre-dawn leaf water potential in all variety ($t = 4.16$, $p < 0.001$) (Figure 5). The American chestnut's regression has a significantly more negative intercept than the Chinese chestnut regression ($t = 2.23$, $p = 0.027$) but did not have a different slope ($p = 0.42$; Figure 5). This is further confirmed by variety comparisons in the control treatment which showed that estimated turgor loss point in the American chestnuts is significantly lower than Chinese chestnuts (Figure 6). The hybrid chestnut regression was not statistically different from the American chestnut's, but it was

trending towards significance at $\alpha = 0.1$ for both slope and intercept. Across both the drought and control groups, Chinese chestnuts showed a smaller range of pre-dawn water potentials when compared to the hybrid and American water potential ranges. Hybrid chestnuts were found to have the best regression fit at an $R^2 = 0.45$ compared to $R^2 = 0.24$ for Chinese and $R^2 = 0.245$ for American chestnuts. The blight infection treatment alone had no influence on turgor loss point (S5).

(Figure 5)

(Figure 6)

Discussion

The central goals of the chestnut hybrid breeding program run by The American Chestnut Foundation are to impart chestnut blight resistance from Chinese chestnuts while retaining as many American chestnut traits as possible in the hybrids. The ideal BC₃F₃ hybrid chestnuts should on average display over 90% genetic similarity to American chestnuts with the remaining genetic information containing the Chinese loci for chestnut blight resistance (Burnham et al. 1986). However, recent genotyping of BC₃F₂ hybrids show that the average percent genetic material inherited from *C. dentata* is 83% (Westbrook et al. 2020). A potential explanation for this deviation is that Chinese chestnut blight resistance has now been found to be controlled by more loci than originally thought, and this may have resulted in a general selection for Chinese genetic material rather than specific loci. This increased amount of Chinese genetic material may also imply a trade-off in that blight resistance would be acquired at the cost of American traits. We find evidence to support that some hybrid chestnuts do

show altered physiological behavior when compared to American chestnuts under both droughted and control conditions. However, this altered physiology did not result in notable losses of blight resistance under co-occurring drought.

Blight infection severity

We measured blight infection severity using two metrics, one qualitative and one quantitative with similar results in both methods (Figure 2). As expected, American chestnuts showed more severe infections compared to the Chinese (Figure 2). While, the hybrid chestnuts showed no statistical differences between American or Chinese chestnuts, average canker severity was significantly different between the American and Chinese groups. This lack of hybrid differences is explained by the greater variation seen in hybrid group infections. Drought treatments did not show evidence of exacerbating blight infection severity in any of the chestnut varieties contrary to our initial prediction. While previous studies have postulated increased mortality due to blight under drought stress (Parker et al. 1993), we found no evidence that our simulated drought directly increased blight severity on stems (Figure 2). This is likely due to the relatively short-term and mild drought used in our study compared to more severe drought observed in Parker et al. 1993. It is also possible that water stress does not directly increase the size of the canker but contributes to increased mortality.

Physiological differences in hybrid chestnuts

We would predict that hybrid chestnuts should perform more similarly to American chestnuts in physiological aspects not related to blight tolerance based on the selection preferences of the breeding program. However, we see that hybrid rates of stomatal

conductance deviate from Americans and are more similar to Chinese chestnuts in both the control and drought group over the dry-down (Figure 3). While both the American and hybrid groups showed similar patterns of decline in their conductance rates from control to drought treatment, hybrids still averaged lower rates in both treatments. Unlike both American and hybrid groups, the Chinese chestnuts showed no effect of drought on their gas exchange rates. We found that hybrids, in fact, showed the greatest declines in conductance rates (but not photosynthetic rates) in the drought treatment compared to their control group, which resulted in higher WUE rates over the dry-down – as intrinsic WUE is the ratio of photosynthetic assimilation (A_{sat}) and stomatal conductance (g_s). These significantly higher WUE rates compared to both American and Chinese groups can be interpreted as an emergent response not equal or intermediate to either parental lineages. While this difference between varieties is in part due to a sharp increase of WUE on day 15 of the dry-down shown in all chestnut groups but exacerbated in the hybrid group, WUE was still high on average on most other days. While hybrid phenotypes that are different than both parental lineages are not uncommon in breeding (Dickinson et al. 2003), no studies of transgressive segregation in hybrid chestnuts have occurred to our knowledge.

In terms of photosynthetic rates for the chestnuts, we find only that Americans showed the highest on average rates of photosynthesis when averaged across both days and treatments. American chestnuts having higher rates of photosynthesis compared to Chinese chestnuts is consistent with findings in Knapp, et al 2014. However, the finding that averaged over treatment and measurement days photosynthetic rates are different between American and hybrids, contradicts findings in Knapp et al 2014, who also reported that advanced

generation hybrids showed similar photosynthetic light response to Americans. This difference in findings could be due to the differences in experimental set up as the Knapp study used older saplings in a field planting setting or could be due to the specific hybrid families used. Few hybrid families were used in both the Knapp study and this study and all families in the Knapp study came from the Clapper lineage of hybrids (Clark et al. 2012; Knapp et al. 2014).

To further explore this difference in gas exchange, we measured the response of stomatal conductance to mid-day leaf water potential. The relationship between leaf water potential and conductance can indicate the point at which stomata begin to close under declining water availability and illustrate if a plant has a conservative or risky drought strategy (Brodribb et al. 2003; Brodribb and Holbrook 2003). The results showed that the range of water potentials that the hybrids occupied was more similar to American chestnuts and was almost a full megapascal greater (more negative) than Chinese chestnuts. However, the logarithmic shape of the hybrid relationship was more similar to Chinese chestnuts (Figure 5). Surprisingly, American chestnuts showed no overall relationship of water potential and stomatal conductance. This lack of a relationship is likely due to water potentials not declining enough in the American groups to elicit a stomatal response and helps explain why stomatal conductance was able to be maintained in the dry-down experiment. We interpret these findings as showing that Americans have a riskier relationship of gas exchange and water potential, allowing water potentials to decline further than hybrids or Chinese while stomata stay open. Such a relationship is often seen in more drought tolerant species and is consistent with the idea of American chestnut having a degree of drought tolerance (Sade et al. 2012). Conversely, Chinese chestnuts showed a more conservative strategy as they began limiting conductance under a

tighter range of water potentials. This conservative strategy is consistent with the relatively low gas exchange rates observed in the dry-down and little change between treatments. Considering the generally lower rates of stomatal conductance observed in both the control and drought treatment, the hybrids seem to be doing a bit of both and are showing range of water potentials of Americans (risky) but the overall relationship of Chinese (more conservative).

Previous studies have speculated that American chestnuts utilize osmotic adjustment (Brown et al. 2014) as a drought tolerance mechanism but those that have tested for the trait have been inconclusive (Abrams et al. 1990). Here we are able to show that all chestnut groups (American, Chinese, and hybrids) showed a capacity to buffer their turgor loss points in response to declining pre-dawn leaf water potential (Figure 5). These changes to turgor loss point were measured using vapor pressure osmometry at full turgor (Bartlett et al. 2012), so we can infer that this buffering of turgor loss point is due to osmotic adjustment. While other drought tolerance traits like modification of elastic modulus of the cell wall are not excluded, many woody plants use osmotic adjustment to maintain turgor under drought stress conditions (Kozlowski and Pallardy 2002). We show that even at water potentials close to zero American chestnuts have lower turgor loss points than Chinese chestnuts again supporting the idea that American chestnuts are drought tolerant (Figure 5), a point further supported by their lower turgor loss point in the control treatments (Figure 6). Hybrids again were intermediate to American and Chinese chestnuts in turgor loss points and not more similar to American chestnut values as would be expected by the breeding program.

Implications for chestnut restoration

While the physiological data indicate that not all hybrids perform identically to American chestnuts, the altered response to drought does not alter severity of chestnut blight infection in the chestnuts (Figure 2). American chestnuts did show higher canker infection severity than Chinese chestnuts as expected with the average hybrid severity in the middle of the two groups, not significantly different than either. This lack of difference in the hybrids is likely due to the large amount of variability observed across hybrid chestnut families. Contradicting our expectations, the drought treatment showed no effect on canker severity in any of the chestnut varieties (Figure 2). Thus, there is reason to believe that moderate droughts within a single growing season will not result in changes to blight resistance. Prolonged or more severe drought stress would certainly cause increased damage and mortality in infected trees. Studies on mature American chestnut in Wisconsin did report increases in blight progression in years directly following drought (Gilland et al. 2012) and studies on the remnant American populations in eastern North American forests postulated increased mortality observed between census periods could have been driven by a combination of blight and drought (Parker et al. 1993). While our study shows no effect of drought on disease progression in a single year, this finding needs to be tested across, genotypes, age classes, and years before the extent of its relevance to chestnut reintroduction can be fully understood. Age class and years are of particular interest as longer field trials looking at hybrid resistance seem to correlate better with the resistance of hybrid parents (J. Westbrook. Personal communication, Feb. 2021)

The changes to drought response in the hybrid chestnuts did not result in a loss of blight tolerance, but the changes in drought tolerance should still be considered in chestnut reintroduction and breeding efforts. This is one of the first studies to show evidence of

physiological changes in the hybrid chestnuts that result in significant differences from pure American chestnut. While we do not extend our findings to all hybrid chestnut genotypes, it is notable that these findings are a result of aggregating across multiple genotypes with representation from the two major lineages of hybrids, Clapper and Graves lines (S1). With recent genetic work showing that there is a greater range of Chinese genetic material among BC₃F₃ chestnut genotypes than expected, our results indicate that physiological changes follow this genetic pattern (Westbrook et al. 2020). Because we did not do any genetic work in this study, it may be that we simply used hybrid genotypes that were more Chinese, and that other genotypes may be better suited to fulfilling the goals of blight tolerance and American similarity. Increased screening, either genetic or physiological, will likely be needed to identify genotypes that meet both the blight resistance goals and American similarity goals.

Outside of disease resistance, changes in the hybrid water use strategies and drought tolerance have potential implications for the competitive abilities of the hybrid chestnuts when reintroduced. In previous physiological studies, American chestnut has been shown to have higher rates of WUE compared to other native eastern forest species when under water stress (Abrams et al. 1990; Bauerle et al. 2006). As hybrid WUE was observed to be even higher than the American chestnuts in this study, hybrid chestnut WUE may be an important competitive trait when drought stress occurs. Additionally, water use efficiency is known to have interactive effects with rising atmospheric CO₂ which generally results in increases to water use efficiency (Huang et al. 2007). Thus, our finding of increased WUE for hybrid chestnuts under drought stress could be further enhanced under future climates of increased CO₂.

Finally, we must not assume traits observed in pure American chestnut are present in hybrid chestnuts without validation. While previous studies have identified American chestnuts as being most competitive in well drained, dry soil, this might not be the case for all hybrid genotypes (Keever 1953; Abrams et al. 1990). As climate and precipitation regimes are expected to change over most of the historic range of the American chestnut, identifying the extent of the physiological changes in each hybrid genotype will be needed to determine competitive viability at different sites.

Conclusions

The goal of The American Chestnut Foundation breeding program is to develop blight resistant hybrid chestnuts that perform similarly to American chestnuts in all other aspects. While we report that hybrid physiological performance is not always identical to pure Americans under water stress in terms of stomatal conductance and response to leaf water potential, we do find that these changes do not alter blight resistance under cooccurring drought stress.

Supplementary Data

Conflict of Interest

Authors have no conflicts of interest to report.

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Authors' Contributions

Brett Fredericksen designed the experiment, collected data, and wrote the manuscript. Samuel Kukor collected data and provided edits to written materials. David Rosenthal provided guidance throughout the experimental design and provided edits to written manuscript.

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652 List of Tables

653 Table 1: Qualitative infection scoring for chestnut blight

654 A qualitative scoring system was used to quickly evaluate the progression of the disease
655 throughout the experiment. The scores were based off the size of the canker in relation to the
656 initial infection markings of 1 cm.

Score	Description
1	Infection localized to the immediate perimeter of infection site. No detrimental influence on plant health observed. Canker < 1 cm in length
2	Minor infection expanding past the margins of the infection site. No detrimental influence on plant health observed. Canker < 1 cm in length
3	Infection expanding past initial infection site (either vertically or horizontally). Overall plant health is maintained. Canker ≥ 1 cm in length
4	Infection severe with canker expanding past infection site. Other symptoms like swelling or sinking of the stem observed. Fruiting bodies may be present. Noticeably declining health of plant. Canker > 1 cm in length
5	Most severe infection. Stem may be entirely girdled, and plant is either dead or dying. Stem may be splitting open around infection area. Canker margins difficult to determine due to damage.

657

Table 2: ANOVA results from mixed effects model

Fixed effects and interactions determined by model selection using AICc and biological relevance of parameters. Fixed effects included Chestnut Variety (American, Chinese, or hybrids), day of gas exchange measurement, and treatment. Interactions of Variety*Treatment and Treatment*Day were found to be significant. The interaction of variety and day was only found to be significant in the WUE model. The three-way interaction of variety*treatment*day and variety*day were not found important to model fitting and were removed during model selection. Columns are broken up by gas exchange rate (A_{sat} , g_s , WUE). Leaf count, SPAD, and VWC are used as covariates. NA represent models where the effect was not included.

Fixed effects	A_{sat}			g_s			WUE		
	Chisq	df	P-value	Chisq	df	P-value	Chisq	df	P-value
Chestnut Variety	16.64	2	< 0.001	22.99	2	< 0.001	5.72	2	0.06
Day	38.24	5	< 0.001	15.33	5	0.009	5.78	5	0.33
Treatment	0.6	1	0.269	0.9	1	0.344	0.85	1	0.36
Variety*Treatment	3.7	2	0.157	6.29	2	0.043	6.62	2	0.033
Treatment*Day	32.08	5	< 0.001	21.87	5	< 0.001	13.54	5	0.016
Variety*Day	NA	NA	NA	NA	NA	NA	22.54	10	0.013
Leaf count	2.51	1	0.113	2.17	1	0.14	3.52	1	0.061
SPAD	6.75	1	0.009	NA	NA	NA	5.74	1	0.017
VWC (%)	3.54	1	0.222	2.27	1	0.132	0.95	1	0.328

Table 3: Pairwise comparisons of gas exchange rates

Planned pairwise comparisons are listed based on the results of the repeated measures ANOVA. Test statistics (t-ratio), degrees of freedom (df), and p-values are listed for each comparison of photosynthetic rate, stomatal conductance (g_s), and water use efficiency (WUE). Planned pairwise comparisons within variety and within treatments are shown for these variables. Photosynthetic rate and stomatal conductance showed difference in variety across both day and treatment, but water use efficiency did not. Planned pairwise comparisons were used to limit excessive comparisons and focus on treatment and varietal differences. P-values adjusted using a Tukey post hoc adjustment with an alpha of 0.05. NAs represent sections where model effects were not significant.

Comparisons			A_{sat}			g_s			WUE		
			t-ratio	df	p-value	t-ratio	df	p-value	t-ratio	df	p-value
Variety											
American	vs	Chinese	3.79	157	< 0.001	5.1	158	< 0.001			NA
American	vs	Hybrid	4.49	157	<0.001	5.15	158	< 0.001			NA
Chinese	vs	Hybrid	0.575	157	0.834	0.18	158	0.983			NA
Within Variety											
American											
Control	vs	Drought			NA	2.78	359	0.006	-2.28	349	0.02
Chinese											
Control	vs	Drought			NA	0.71	359	0.477	-0.36	349	0.721
Hybrid											
Control	vs	Drought			NA	4.3	359	< 0.001	-3.83	349	< 0.001
Within Treatment											
Control											
American	vs	Chinese			NA	4.67	158	< 0.001	-2.72	157	0.02
American	vs	Hybrid			NA	3.04	158	0.008	-1.91	157	0.138
Chinese	vs	Hybrid			NA	-1.58	158	0.256	0.78	157	0.714
Drought											
American	vs	Chinese			NA	2.89	158	0.012	-1.07	157	0.537
American	vs	Hybrid			NA	4.8	158	< 0.001	-4.03	157	< 0.001
Chinese	vs	Hybrid			NA	1.72	158	0.201	-2.62	157	0.026

8192

Figure captions

Figure 1: Through concurrent measurements of midday leaf water potential (MPa) and volumetric water content (%), it was determined that the 5% volumetric soil water content (black dashed line) was sufficient to influence leaf water status. Water potentials began to decline at soil volumetric water contents below 10% with the most drastic declines in the 0%-5% range of volumetric water content. Chestnut varieties are represented by shape with circles (American), triangles (Chinese), and squares (hybrids).

Figure 2: Average canker severity ratios are shown to increase with increasing quantitative infection severity scores across all chestnut types (top). Analyzed by Kruskal-Wallis rank sum tests, chestnut variety was shown to be significant ($\chi^2=6.84$, $df = 2$, $p = 0.033$). Pairwise comparisons showed American chestnuts to have higher canker severity ratios than Chinese chestnuts ($p = 0.019$) but are not statistically higher than hybrid chestnuts ($p = 0.15$). Drought treatments showed no indication of increased canker severity ratio and was not significantly different than the well-watered blight infection treatment for any chestnut group ($\chi^2=0.065$, $df = 2$, $p = 0.80$) (bottom). Larger points represent means of each group with standard error bars while smaller points represent individual observations from that group. Inoculations that failed to take were removed from the analysis.

Figure 3: Gas exchange (A_{sat} , g_s , and WUE) was measured over a 20-day period. Control plants were watered regularly while the droughted plants were saturated on the first day of the dry-down and allowed to dry. Days are reported in relation to time passed since saturation. Each graph shows the comparative dry-down between treatments across each variety. Model adjusted means determined from models reported in Table 2. Solid lines represent American chestnuts, dashed lines represent Chinese chestnuts, and dotted lines represent hybrid chestnuts. Photosynthetic rate showed no differences by variety and treatment, but stomatal conductance and water use efficiency did. Hybrid chestnuts showed more similarity to Chinese chestnuts in their rates of stomatal conductance under both the control and drought treatments when averaged over the dry-down (Table 3).

Figure 4: The response of stomatal conductance (g_s) to midday leaf water potential measured directly after measurements of stomatal conductance on the same leaf. To determine how conductance responds to midday water potential, a logarithmic transformation was used to linearize data and develop regressions. The predicted values of this regression were back transformed and plotted with the raw data points of each variety. Both Chinese ($p < 0.001$, $R^2 = 0.39$) chestnuts and hybrid chestnuts ($p < 0.001$, $R^2 = 0.61$) had a significant response of midday water potential (MPa) on stomatal conductance. American chestnuts ($p = 0.18$, $R^2 = 0.08$)

719 showed no effect of midday water potential on conductance over this range of water
720 potentials.

721 Figure 5: Response of estimated turgor loss point to predawn water potentials measured on
722 leaves of the same branches of those used to estimate turgor lost point using osmotic data at
723 full leaf turgor. All varieties showed linear declines in turgor loss point (p -value < 0.001) as
724 predawn water potentials became more negative. Chinese chestnuts had significantly different
725 intercepts compared to American chestnuts ($t = 2.23$, $p = 0.027$). The hybrid chestnut's
726 response was not significantly different to American chestnuts in terms of intercept ($t = 1.74$, p
727 $= 0.085$) or slope ($t = 1.93$, $p = 0.057$) but was trending towards significance ($p < 0.1$). Infected
728 treatments were also measured but showed no effect on turgor loss point across variety ($p >$
729 0.05).

730 Figure 6: Box and whisker plot of estimated turgor loss point (MPa). Within the well – watered
731 control group TLP was found to vary by variety ($F = 7.83$, $df = 2$, $p = 0.002$). Tukey post hoc
732 analysis showed that Chinese chestnuts had higher turgor loss points compared to American
733 chestnuts ($p = 0.002$). Hybrid chestnuts showed the largest interquartile range and were not
734 different than either American ($p = 0.08$) or Chinese groups ($p = 0.325$).

Draft

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