Final report for the project **"Predicting growth and carbon uptake of American chestnut in current and future climates"**

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Abstract

This final report describes results from two growth chamber experiments on selected American chestnuts and blight resistant BC_3F_3 hybrids subjected to simulated climate change. The first experiment occurred in the spring and summer of 2016 and the second experiment (which was funded by TACF) in 2017 – 2018. These studies consider the response of hybrid and American chestnut genotypes to increases in temperature and atmospheric concentrations ($[CO_2]$) that are expected in the latter half of this century. In the first experiment chestnuts were grown from seed in controlled environment chambers at near ambient vs. elevated $[CO_2]$ of ca. 430 and 600 ppm, respectively, and at low vs. higher day/night temperatures of 21° C / ° C 15 and 25 °C / °C 19 in a full factorial experiment. In the first experiment, we did not detect the effect of elevated CO_2 on growth. Moreover, the modest increase in temperature did not have a detectable effect on growth and did not alter the response of seedlings to CO_2. Our power to detect differences was hindered by death of seedlings during the experiment. In the second experiment we opted to evaluate the effect of ambient vs. elevated CO_2 but not temperature, and we did detect a significant stimulation of growth by elevated CO_2 but this response was genotype dependent. Specifically, we found that genotypic differences in seed size underly seedling responses to elevated $[CO_2]$. Larger seeded American and Clapper hybrids (D families) had consistently greater stimulation of growth than any of the Graves hybrids (W families) in elevated CO_2. It appears that Clapper and Graves lineages are segregating for seed size with Graves families having smaller seeds on average than Clapper families. If Clapper and Graves lineages are segregating for seed size and seed size modulates both recruitment and the response to elevated CO_2, then how families are selected during restoration has important implication for chestnut recruitment and establishment in our forests.

Introduction

The American chestnut (*Castanea dentata* (Marsh.) Borkh.) was extirpated as a canopy tree by the introduced fungus, *Cryphonectria parasitica*, which causes Chestnut blight (Anagnostakis and Feb, 1987). The fungus was introduced on horticultural imports of Asian chestnut species brought to North America in the early 1900s. Restoration efforts aim to introduce millions of blight resistant chestnut trees into eastern deciduous forests (Jacobs et al., 2013). The reintroduction of American chestnut has the potential to alter forest carbon uptake and storage (Gustafson et al., 2018). Restoration of the chestnut is further complicated because it is occurring in a changing climate. The predicted magnitude of climate change varies, but consensus reports suggests that it will likely lead to increased summer droughts and temperature, both of which can also alter ecosystem structure and function (Romero-Lankao et al., 2014). Globally increasing atmospheric [CO_2] underlies our changing climate (IPCC, 2014). Thus, understanding how blight resistant hybrids will actually respond to elevated levels of atmospheric CO_2 and temperature predicted for this region is critical; however, data quantifying chestnut seedlings capacity to respond and acclimate to increasing CO_2 and temperatures are lacking. We addressed this knowledge gap by assessing the effect of future predicted increases in CO_2 and temperature on the growth of American chestnut and hybrids created for restoration. The specific aim was to assess the response of American chestnut and BC_3F_3 hybrid American x Chinese chestnuts to atmospheric carbon dioxide concentration ([CO_2]) and temperature predicted for the latter half of this century.

Experimental design and environmental parameters for pilot experiment

In January of 2016, the American Chestnut Foundation (TACF; Sara Fitzsimmons) provided BC_3F_3 seeds of two Clapper families (D2-10-3 & D1-26-19) and two Graves families (W6-32-143 & W7-15-8) and seeds of two American chestnut provenances (Eaton Center, NH; CC245xPryor NC). Our specific

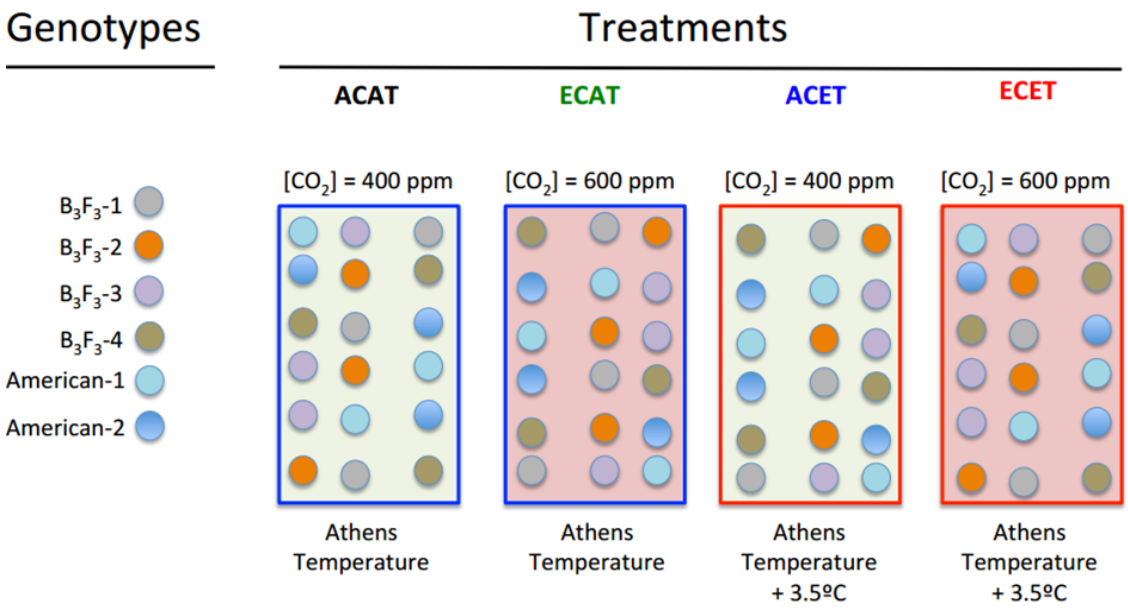

Figure 1. Experimental design illustrating treatments and genotype replicates in experiment 1.

goal was to assess if the response of BC_3F_3 genotypes to predicted higher temperature and CO_2 was similar to pure American chestnuts (Fig. 1).

All seeds were weighed, planted and placed in environmental growth chambers on February 3rd 2016. Canopy light intensity was maintained at photosynthetic photon flux density (PPFD) of ca. 350 µmol m^{-2} s^{-1} at the top of seedling canopy. Light levels exceed 350 µmol m^{-2} s^{-1} prior to canopy closure but are substantially lower than 350 µmol m^{-2} s^{-1} after canopy closure in our native forests. Therefore, light levels in the growth chamber provided adequate seasonal light availability to encourage seedling growth. To mimic a natural photoperiod, the day length was increased from 11 hours at planting to 14.5 hours in June and was decreased thereafter until leaf senescence. Growth temperature at planting was 21 °C (day) /15 °C (night) for ambient temperature (AT) and 25/19 for elevated temperature (ET). After seedling emergence, day and night temperature for all treatments was increased by 1.5 °C. Three replicates (n=3) of each genotype (n=6) were randomly placed in one of four growth treatments (Fig. 1). Chamber environmental conditions were maintained to one of four preset conditions: ambient CO_2 (ca. 430 ppm, Fig. 2) and ambient temperature (ACAT); elevated CO_2 (ca. 600 ppm, Fig. 2) and ambient temperature (ECAT); ambient CO_2 and elevated temperature (ACET); and elevated CO_2 and elevated temperature (ECET). Plants were uniformly fertilized and watered as needed. To avoid confounding chamber and treatment effects, seedlings were rotated within chambers, and treatments were rotated among chambers weekly.

Experimental set up for CO_2 control

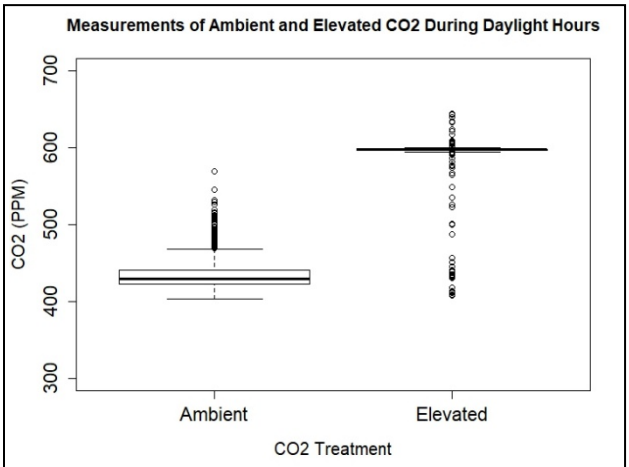

Figure 2. Box and whisker plot of hourly [CO_2] over 4 weeks in ambient and elevated CO_2 treatments during daylight hours in mid-summer. Points are outliers that typically occur when chambers are disturbed during data collection campaigns.

Four infrared gas analyzers (IRGAs; PP systems WMA-5) and associated electronics measured and recorded the CO_2 concentration in environmentally controlled growth chambers located in the Environmental and Plant Biology Department at Ohio University (Fig. 2). The IRGA's are connected to proportional integrative derivative (PID) controllers that maintain [CO_2] near a set point. The CO_2 within chambers was continuously monitored throughout our experiments to ensure controllers were functioning properly and that seedlings experienced the appropriate growth conditions. The [CO_2] in ambient CO_2 treatments (ACAT and ACET) remained significantly lower than our elevated CO_2 treatment (ECAT and ECET) target of 600 ppm throughout the experiment. Hourly averaged ambient CO_2 concentration (ACAT and ACET chambers) varied around a mean of 433.9 $\pm$ 19.14 sd (Fig. 2) and was significantly lower than in the elevated CO_2 (ECAT and ECET) treatments 594.5 $\pm$ 22.2 sd (Fig. 2).

Results and discussion experiment 1

In experiment 1, the effects of simulated climate change (elevated temperature and elevated CO_2) were assessed on four hybrid (D2-10-3; D1-26-19; W6-32-143; W7-15-8) and two American chestnut provenances (Eaton Center, NH; CC245xPryor NC) by comparing seedling biomass at the end of the experiment. We were surprised to find that simulated climate change, as either increased temperature or elevated [CO_2] did not have a detectable impact on final biomass (Figure 3). The early death of several plants prior to the biomass harvest coupled with substantial variation among the remaining individuals reduced our power to detect treatment differences. Prior work has shown that growth stimulation by elevated CO_2 can be lower than expected if roots become restricted (Thomas and Strain, 1991). We confirmed that none of the seedlings in our experiment experienced root restrictions that might have diminished the expected stimulation of seedlings by elevated CO_2.

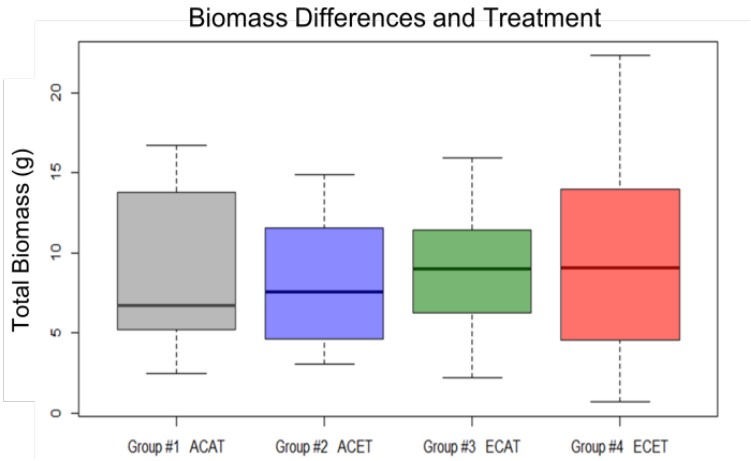

Figure 3. Total biomass across treatment groups in experiment 1. Box and whisker plots from left to right are ACAT (grey) ACET (purple), ECAT (green), ECET (red).

It is well established that seed size is a good predictor of initial seedling growth and biomass in herbs and trees (Stanton, 1984; Gómez, 2004). Nevertheless, it was surprising that seed mass was a stronger predictor of final biomass differences across provenances than elevated CO_2 or the combined effect of increasing CO and temperature (compare Fig. 3 and Fig. 4).

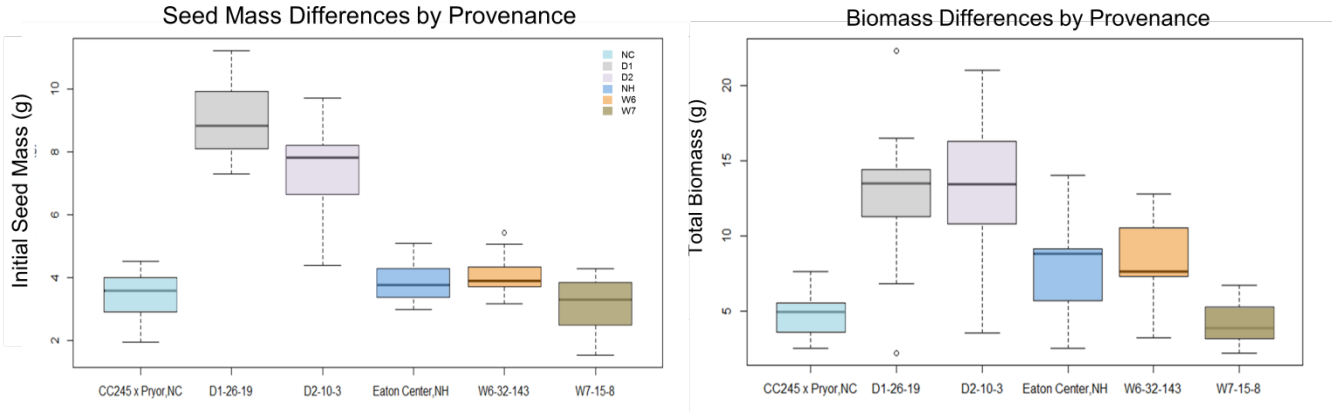

Figure 4. Initial seed mass and final plant biomass of American (light blue, NC; dark blue, NH) and hybrid chestnut provenances (D1, D2, W6, W7) in experiment 1. Note that box and whisker plots are seed and biomass data for a given genotype in all treatment combinations.

Indeed combined increases in CO_2 and temperature are expected to synergistically enhance photosynthesis (Long, 1991; McMurtrie and Wang, 1993). Of all the genotypes in experiment 1, two

hybrid “Clapper” genotypes, D1-26-19 and D2-10-3, both had significantly greater seed sizes and final biomass when compared to the “Graves” hybrids and either of the pure Americans. Thus, while we should expect larger seeds to produce larger seedlings, we were surprised that simulated climate change had no detectable effect on growth of these seedlings. Because these results were contrary to expectations and it appeared that W and D genotypes were segregating for seed size, we proposed to replicate this experiment as above with a different set of genotypes. We initiated the TACF funded experiment in February of 2017.

Experiment 2

In experiment 2 we eliminated the season long high temperature treatment used in experiment 1 in favor of increasing sample size and power to detect a response to elevated [CO_2]. The experimental set up for CO_2 controls were identical for the second experiment therefore we had two “ACAT” and two “ECAT” chambers (c.f. Fig. 1) set to the same day/night temperature. The American Chestnut Foundation provided four different BC_3F_3 genotypes, which included two Clapper “D” and two Graves “W” lineages (D4-9-46; D7-13-131;W4-24-52; W1-31-4-7) and two Americans, one from Fryeburg, Maine, and the other from Huckleberry Knob, Virginia. Seeds were weighed and planted in Ray Leach cone-tainers (SC10, Stewe and Son), and germinated in the Ohio University greenhouse in mid-April 2017. Seedlings were then transplanted into 2 gallon tree pots and placed in environmental growth chambers the second week in June 2017. As in experiment 1, growth chamber light intensity at the top of the seedlings was maintained at photosynthetic photon flux density of ca. 350 µmol. Following a two week acclimation to growth in the chambers seedling were randomly assigned to Ambient [CO_2] (ca. 430 ppm) or Elevated [CO_2] (600 ppm) treatments and at day and night temperature of 25 °C / 19 °C. After leaf senescence seedlings were removed from chambers and placed outside and left to overwinter on October 30th 2017. The goal was to re-initiate the experiment on seedlings for a second year. In midwinter, Athens experienced extremely cold temperatures during a so called “polar” vortex. Unfortunately, the seedlings and soil had frozen solid, in spite of being buried and surrounded by mulch. Once thawed, we harvested, dried, and weighed all plants to estimate seedling biomass (Fig. 5).

Results and discussion experiment 2

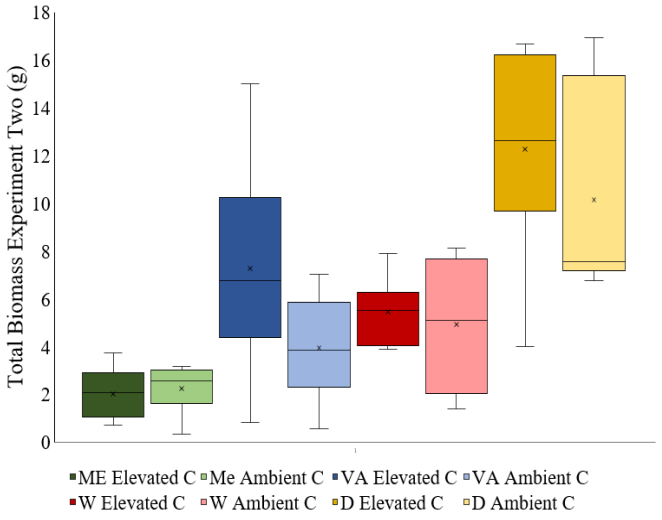

Figure 5. Variation in total biomass by genotype and treatment in experiment 2. The dark and light boxes of a given color are data for a given genotype in elevated CO_2 and ambient CO_2, respectively. The x inside each box is the mean and the line the median for each genotype. We detected an elevated CO_2 effect, but only when seed mass and genotype were accounted for in the model ($p = 0.036$). Thus, genotype and seed mass and both modulate the seedling response to elevated CO_2.

While we were not able to continue the experiment for a second year, we did detect greater biomass overall in elevated CO_2 (Fig. 5, compare darker and lighter boxes of a given color). Note that we only detected the effect of elevating [CO_2] on biomass if we accounted for seed size and genotype identity in the statistical model. That is, seedling in the elevated CO_2 treatment were on average larger but this varied according to the genotypes and seed size. This effect was driven by the American chestnuts from Virginia ($p = 0.023$; compare the dark and light blue boxes in Fig. 5) and the Clapper (D) genotypes (compare the orange to the manilla colored boxes on the far right of Fig. 5).

Overall, and across both experiments, seed size varied, and was larger on average in the hydrid lineages than in the American populations, though with some exceptions (Fig. 6). Moreover, and consistent with the first experiment, Clapper genotypes have larger seeds and gained more biomass in one year and Graves seed sizes more comparable to pure American genotypes (Fig. 6).

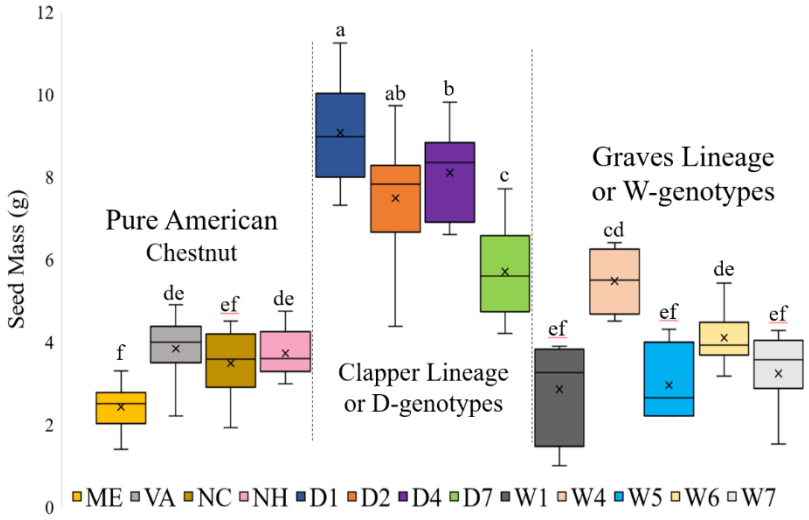

Figure 6. Seed mass variation among genotypes in both experiment 1 and experiment 2. The first four boxplots represent pure American chestnuts denoted by the state of origin. The Clapper tree seeds (D genotype) are consistently larger than Graves (W genotypes) and pure American chestnuts ($p<0.0001$). Genotypes that have the same letter above the box plots are not statistically different, determined by two-way ANOVA and Tukey HSD ($\alpha=0.05$).

We confirmed that seed size differences in our experiment were not an artifact of the genotypes we received from TACF by analyzing data from the fall 2017 seed harvest provided by Jared Westbrook. While there is considerable overlap in the mass of Clapper and Graves genotypes, Clapper seeds collected in 2017 were larger than Graves seeds (Fig. 7).

Conclusions and implications

Ample evidence confirms that sapling and tree growth in eastern deciduous forests will be stimulated by increasing CO_2 (Bazzaz et al., 1990). Early seedling growth in chestnuts will be affected by changes in atmospheric carbon dioxide but this depends on the genotype and seed size. Specifically, in our second experiment, the American chestnut from Virginia responded more than the one from Maine, and similarly the Clapper (D genotypes) responded more than Graves (W genotypes). One other study has specifically assessed native tree seedling responses to elevated CO_2 while considering seed size as an

explanatory variable (Bazzaz and Miao, 1993). The authors demonstrated that larger seeded species such as red oak showed a significantly larger increase in biomass than smaller seeded plants (birch) in response to elevated CO_2 (Bazzaz and Miao, 1993). Consistent with that observation both VA and D genotypes which have larger seeds showed greater responses to elevated CO_2 than the smaller seeded genotypes in the second experiment (Fig. 5).

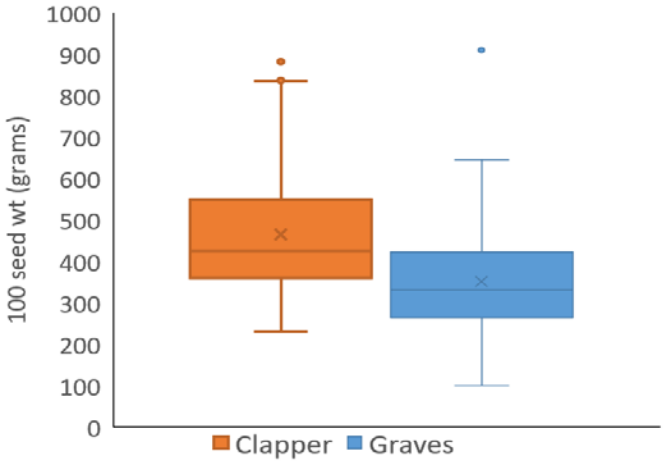

Figure 7. Mass per 100 seeds for 65 Clapper genotypes and 57 Graves genotypes. Seeds were harvested and weighed in fall of 2017 at Meadow View Farm (Jared Westbook, pers. comm.). A t-test shows significant differences between the two lineages (p-value < 0.01) with average for the Clapper lineage at 464.6 $\pm$ g and the average for the Graves lineage being 351.3 $\pm$ 20.2 (se) grams.

Variation in seed size within and among hybrids should be considered with respect to seedling recruitment, as well as growth, during reestablishment (c.f. fig. 7). For instance, rapid seedling establishment and early growth may enhance recruitment and therefore benefit reintroduction; however, while seed mass is a good predictor of early seedling growth and biomass, and response to CO_2, there is also a direct negative effect of seed size on survival in the face of predation from granivores (Gómez, 2004). So larger seeded Clapper genotypes may grow faster but may not actually recruit more than smaller seeded Graves genotypes. Even if not predated upon, rodents cache and disperse BC_3F_3 and American chestnut seeds differentially (Blythe et al., 2015) indicating that hybrids may not be functionally equivalent to American chestnuts when it comes to dispersal (Blythe et al., 2015) or recruitment. Yet, Blythe (2015) did not directly test if or how seed size differences in different hybrid lineages impacted seed preference by rodents. Larger seeded hybrid genotypes may represent remnant Chinese chestnuts traits, as Chinese chestnuts are known for their larger seeds. If such a polymorphic trait as seed size can be conserved through the breeding program it is likely that other physiological and functional aspects of the chestnut hybrid genotypes have been altered during breeding as well. If these changes are identified, they could be correlated with blight resistance, growth, or resistance to phytopthera root rot, all of which will help inform genotype selection for restoration.

Broader impacts of the study

During the spring of 2016, 22 students in Plant Ecophysiology Class PBIO 3260-5260 learned a about the American chestnut in the classroom and in the context of our work. Students were engaged in the experiment and data collection of experiment 1 during our laboratory research assignments.

During the summer of 2016, two additional undergraduate students also participated in various aspects of the project and presented results at the Ohio University research EXPO. In the summer of 2017, I presented our work at the Ecological Society of American Meeting in Portland, OR. Brett Fredericksen, a graduate student in the lab, also presented the second year experiment in the fall of 2017 at the annual meeting of the chestnut foundation in Maine where he received 2nd place in the poster contest. In 2018, I organized a special session about American chestnut restoration at the 2018 Ecological Society of American Meeting in New Orleans. Locally, Ohio University has also recognized our work funded by TACF through the Ohio University Forum, a university wide online publication.

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Supplemental Figure 1. Layout of seedling in growth chambers in experiment 1 and 2.