Report to TACF for the project:

"**Identifying genotypic variation in loss of blight resistance under drought in hybrid American chestnuts to inform restoration**"

Principal investigators and affiliations

Dr. David M. Rosenthal
Assistant professor
Ohio University
Department of Environmental and Plant Biology
Athens Ohio, 45701

Brett W. Fredericksen Jr.
Graduate Student – PhD – Advisor David Rosenthal
Ohio University
Department of Environmental and Plant Biology
Athens Ohio, 45701

Grant Duration

February 2019 – April 2020

Abstract

Environmental variation influences host-pathogen interactions, particularly in trees. Plant stresses like drought can increase the likelihood of a tree dying when infected by a pathogen due to additive or synergistic physiological effects. Some chestnut species, including American chestnuts, are hypothesized to have more severe blight infections when combined with an environmental stress. Here we assessed if and how a co-occurring, seasonal drought influences chestnut blight infection by imposing an artificial drought on infected and healthy Chinese, American, and disease resistant hybrid chestnuts from The American Chestnut Foundation (TACF). We measured carbohydrate concentrations, hydraulic conductivity, and carbon isotope fractionation (a measure of plant water use efficiency) to determine the effects of the drought and blight infections on the physiology of the tree seedlings. When applied alone, drought and pathogen treatments had similar effects on mortality; however, when combined drought and pathogen effects were additive – that is mortality was highest in the combined drought + pathogen (blight) treatment but was not greater than the sum of the individual treatment effects. The exact physiological mechanisms underlying the mortality (carbon starvation vs. hydraulic failure) between treatments have yet to be fully resolved.

*lab work and data analysis are delayed due the Covid pandemic and some results are preliminary. An addendum will be delivered to TACF when all analyses are completed.

Introduction

Understanding the genetics of chestnut blight resistance is only part of the information needed to determine the effective disease resistance in hybrids. The plant pathology paradigm of the "disease triangle" indicates that it is not only the host-pathogen relationship that dictates the occurrence and progression of a disease, but also the environment that both the host and pathogen experience. Environmental impacts on disease can have both positive and negative influences on the severity of a disease. Co-occurring or inciting stressors can weaken a plant's ability to respond to a pathogen attack, like chestnut blight, but the nature of the relationship between stresses can manifest in different ways. For instance, additive responses are simply the sum of what occurs in the plant when the stressors and disease happen alone. Antagonistic effects would result in the environmental stressor inhibiting the disease. Finally, a synergistic effect between stressor and disease would be more detrimental than either effect alone or an additive effect.

There is evidence for environmental stressors affecting chestnuts and chestnut blight in other *Castanea* species and in remnant populations of American chestnut. European sweet chestnut (*Castanea sativa*) has shown a synergistic effect of drought and chestnut blight on yearly growth, resulting in declines in growth that preceded mortality (Waldboth and Oberhuber, 2009). Climatic stresses like cold were postulated to increase blight severity in Chinese chestnut (*Castanea mollissima*) in Appalachian forests (Jones et al., 1980), and increased mortality to blight was observed in remnant American chestnut populations after drought (Parker et al., 1993). However, no studies have looked at the physiological mechanisms underpinning chestnut responses to blight when combined with other stressors, and no studies have looked at these effects in hybrid chestnuts.

We sought to determine if drought had additive, synergistic, or antagonistic effects on blight progression in disease resistant hybrid chestnut. Drought stress was selected as a co-occurring stressor as blight infection reportedly affects xylem hydraulics through cavitation (Bauerle et al., 2006), which we expected would be exacerbated by drought. How blight and drought stress interact to affect tree mortality has important implication for future reintroduction as climate models predict that precipitation regimes will changes during the growing season over most of the historic range of the American chestnut. Specifically, reintroduced chestnuts will face more extreme precipitation events and more seasonal droughts in the future (Romero-Lankao et al., 2014; Hubbart et al., 2016).

Experimental design

Seeds from both TACF (Meadowview Research Farm, VA) and the Pennsylvania chapter of the American Chestnut Foundation (State College, PA) were planted in late winter to early spring in the Ohio University Greenhouses. In total, we planted three disease resistant hybrid genotypes, one blight susceptible hybrid, one Chinese variety, one potentially disease resistant American chestnut and one highly susceptible American chestnut variety. Rot was observed on the Chinese, susceptible American, and half of the pure American chestnut seeds, which may have resulted in poor germination. To supplement the experiment, we obtained additional pure

American chestnut and Chinese chestnuts from University of Pennsylvania and Empire Chestnut, respectively.

Figure 1: Experimental setup. The experiment took place at the Ohio Student Farm. Seedlings were grown in 2-gallon tree pots, outdoors in two 3 x 6 meter shelters covered in 4 mm greenhouse plastic to keep out rain. The sides of the shelters were open to enhance airflow across seedlings. Pots arrayed in crates away from shelter edges. Treatments were randomly assigned within and across crates.

All healthy seedlings were transplanted to a rainout shelter (Figure 1.) at the Ohio University Student Farm. A completely randomized design was used for planting and treatment assignment. Treatments consisted of drought, chestnut blight infection, drought+blight infection, and well-watered control treatment. The drought was maintained as a series of dry-downs on a per pot basis where seedlings were dried to the point of wilting before being rehydrated to field capacity. This drought was used to mimic future precipitation conditions expected over the chestnut range with long dry periods interspersed with heavy rainfall events (Hubbart et al., 2016). Non-droughted plants were watered twice weekly to maintain the soil at field capacity. Blight infection was accomplished by inoculating stems with the Ep155 strain of the fungus. Control and non-blighted drought groups also went through the same inoculation procedure but with sterile agar plugs used instead of agar with blight. Inoculation procedures were separated temporally to prevent cross-contamination, with the non-blighted groups (July 16th) undergoing the procedure one day before the blighted groups (July 17th). We measured carbon isotope fractionation, hydraulic conductivity, and carbohydrate concentrations to determine the physiological effects of the treatments and mortality for each genotype at the end of the study. We determined the drought's effectiveness by measuring tissue carbon isotope ratios ($\delta^{13}C$) which is a seasonally integrated estimate of water-use-efficiency (Farquhar et al., 1989). Larger values of $\delta^{13}C$ are interpreted as higher integrated WUE as long as all plants experience similar vapor pressure deficits during their growth (Farquhar et al., 1989).

Results

The drought was effective as $\delta^{13}C$ was higher in all chestnut varieties in drought treatments compared to their counterparts in the control and pathogen treatments (Figure 2). The significantly higher $\delta^{13}C$ ratios found in the drought and drought + pathogen groups were from woody tissue and integrate both treatment and pretreatment wood. Therefore, these are conservative estimates of the drought's effect on leaf physiology following drought. Pathogen treatment showed intermediate values between droughted and control groups.

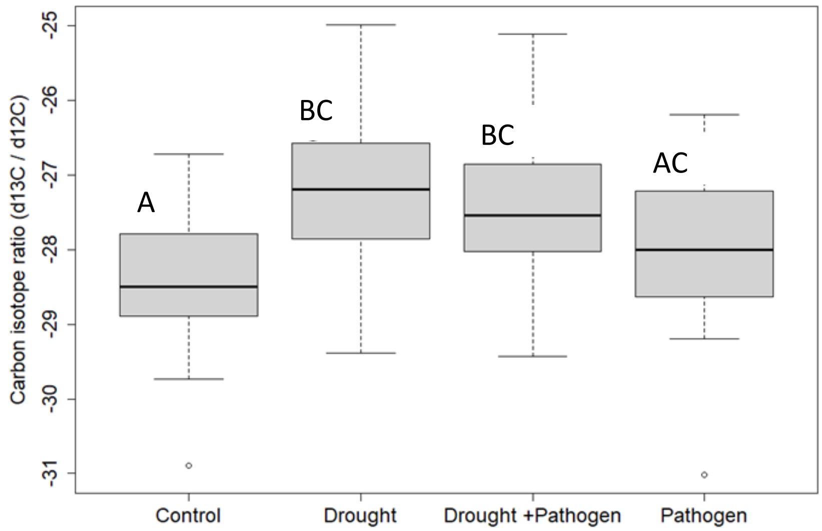
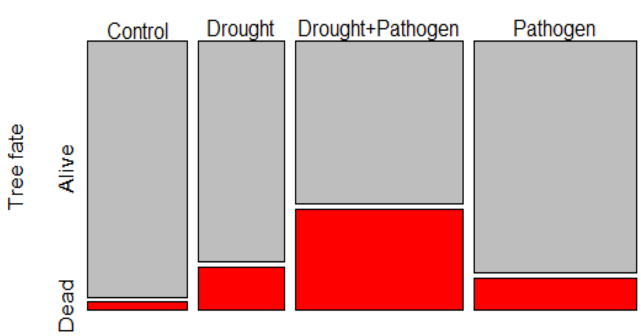


Figure 2: The box and whisker plots of carbon isotope ratios for each treatment. Boxes represents the 25th to 75th percentile, whiskers the 95th percentile with open circles being extreme observations. Carbon isotope ratios ($\delta^{13}C$) are an indicator of integrated water use efficiency and stomatal closure over the lifetime of the plant. Higher ratios are indicative of increased water use efficiency and drought stress. Stem tissue isotope ratios were significantly higher in the drought groups compared to the control and pathogen groups (p-value < 0.001). Groups that share letters are not significantly different following Tukey post hoc test (p-value < 0.05).

Mortality Quantification

Mortality assessments focused on mainstems (used for infection) with resprouted stems excluded. At the end of the study, stems that exhibited complete dieback and no green leaves were considered dead. Chi-squared test showed the most mortality occurred in the drought + pathogen treatment at 38% across all chestnut types (Figure 3). The drought treatment also showed significantly more mortality than the control at 16% vs. 3%, respectively.



TREATMENT	MORTALITY (%)	SIGNIFICANCE GROUPS
CONTROL	3%	A
DROUGHT	16%	B
DROUGHT+ PATHOGEN	38%	C
PATHOGEN	12%	AB

Figure 3: Mortality at the end of the experiment. Lower portion of bars are numbers of dead for each category at the end of the experiment. Treatments were associated with increased mortality compared to control as determined by a Chi-squared contingency analysis ($p < 0.001$). The drought-pathogen group showed the highest proportion of mortality across all varieties of chestnut at 38% ($p<0.001$). Treatments with different letters are significantly different following pairwise Chi-squared comparisons with a false discovery rate correction.

Specific treatment by genotype mortality rates are reported below (Table 1). All chestnut varieties showed the highest rates of mortality in the drought + pathogen treatment except for the American 1 variety, which exhibited complications during the transplanting making its mortality results questionable. The American 2 variety showed greater mortality in the pathogen treatment as is expected.

Table 1. Mortality for each chestnut variety as a percentage of sample size (N) for each treatment combination.

	Genotype or Orchard	**Drought**		**Pathogen**		**Drought + Pathogen**		**Control**	
		N	**Dead**	**N**	**Dead**	**N**	**Dead**	**N**	**Dead**
Chinese	Empire Chestnut	13	15%	17	17%	22	32%	10	0%
American 1	PA-Haun-m	6	50%	7	14%	8	0%	6	14%
American 2	TACF State College	6	0%	12	50%	14	71%	6	0%
Resistant American	PA-KxA	7	14%	13	8%	13	30%	6	0%
Resistant Hybrid 1	PA-AR3-2-5-135	8	12%	21	0%	17	24%	7	0%
Resistant Hybrid 2	D2-29-122	11	9%	22	10%	20	32%	10	10%
Resistant Hybrid 3	D4-11-52	10	20%	18	0%	18	50%	9	0%

Infection Severity Quantification

Canker severity ratio (canker area / stem basal diameter) differed among groups but there was no difference overall between drought and non-drought infected groups (Figure 4). The American group from State college showed the most severe infection severity followed by the Chinese group (Figure 4). The hybrid groups and resistant American group had either significantly less severe infections or similar infections to the Chinese group across both treatments. The American 1 group was also more similar to Chinese and hybrids in infection severity, but this result reflects small samples sizes and difficulty in transplanting as previously mentioned.

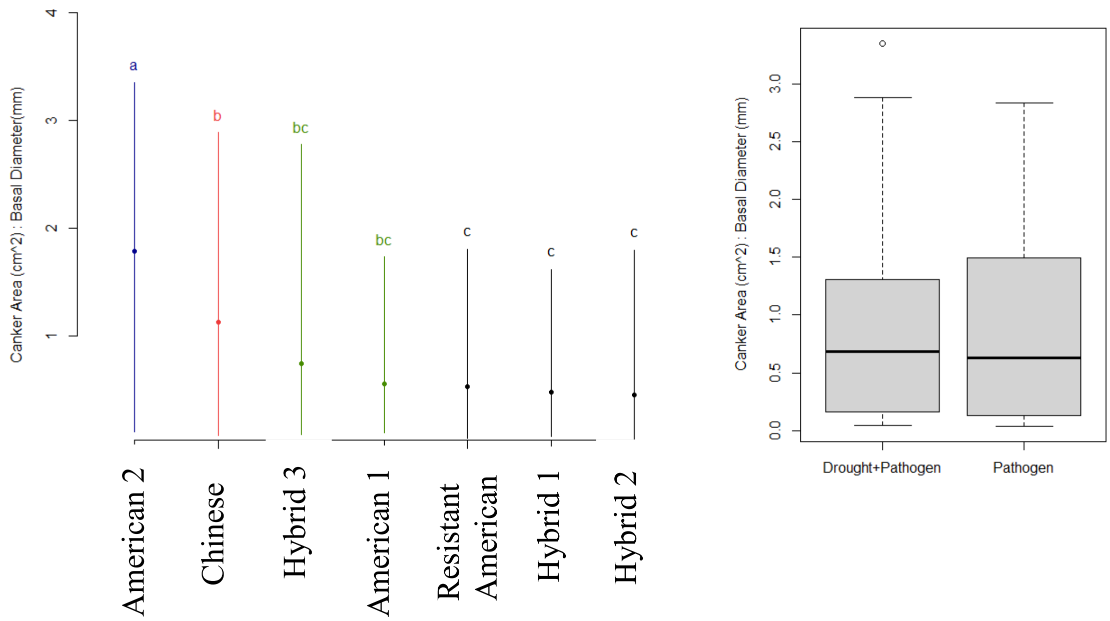


Figure 4: Left panel: infection severity measured as a ratio of the canker area (cm^2) to stem diameter (mm). Symbols are means and range of values (min and max). Groups with different colors/letters are significantly different (p-value < 0.05). Right panel: box and whisker plots show no effect of cooccurring drought on the severity of infection across all chestnut varieties (p > 0.05).

<u>Carbohydrate Analysis – *partial dataset</u>

Nonstructural carbohydrates in the forms of soluble sugars and starch were quantified in three tissue types (distal stems, canker margins, and roots) across all varieties and treatments. Due to delays caused by the COVID-19 pandemic, the carbohydrate data represents half of the final dataset that will be collected. Therefore, these results are preliminary.

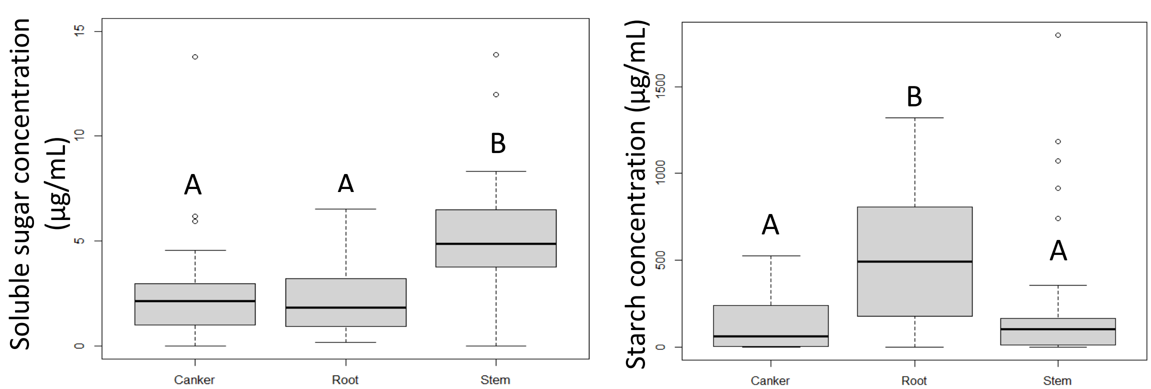


Figure 5: Box and whisker plots of soluble sugars and starch among tissue types. See figure 2 for boxplot details. Boxes with different letters are significantly different (p< 0.05). Starch concentrations were generally orders of magnitude higher than soluble sugar concentrations.

The distal stem tissue showed the highest concentrations of soluble sugars while the roots showed the greatest concentrations of starch across all treatments (Figure 5). Soluble sugars were the highest at branch tips and significantly higher than cankers ($p=0.03$) and root tissue ($p < 0.001$). Root tissue showed a higher starch concentration than both cankers and stem tissue ($p < 0.001$). Statistical differences determined using a type 2 ANOVA and Tukey post hoc test.

Preliminary data also suggest that starch may be more responsive to treatments compared to the soluble sugars (Figure 6).

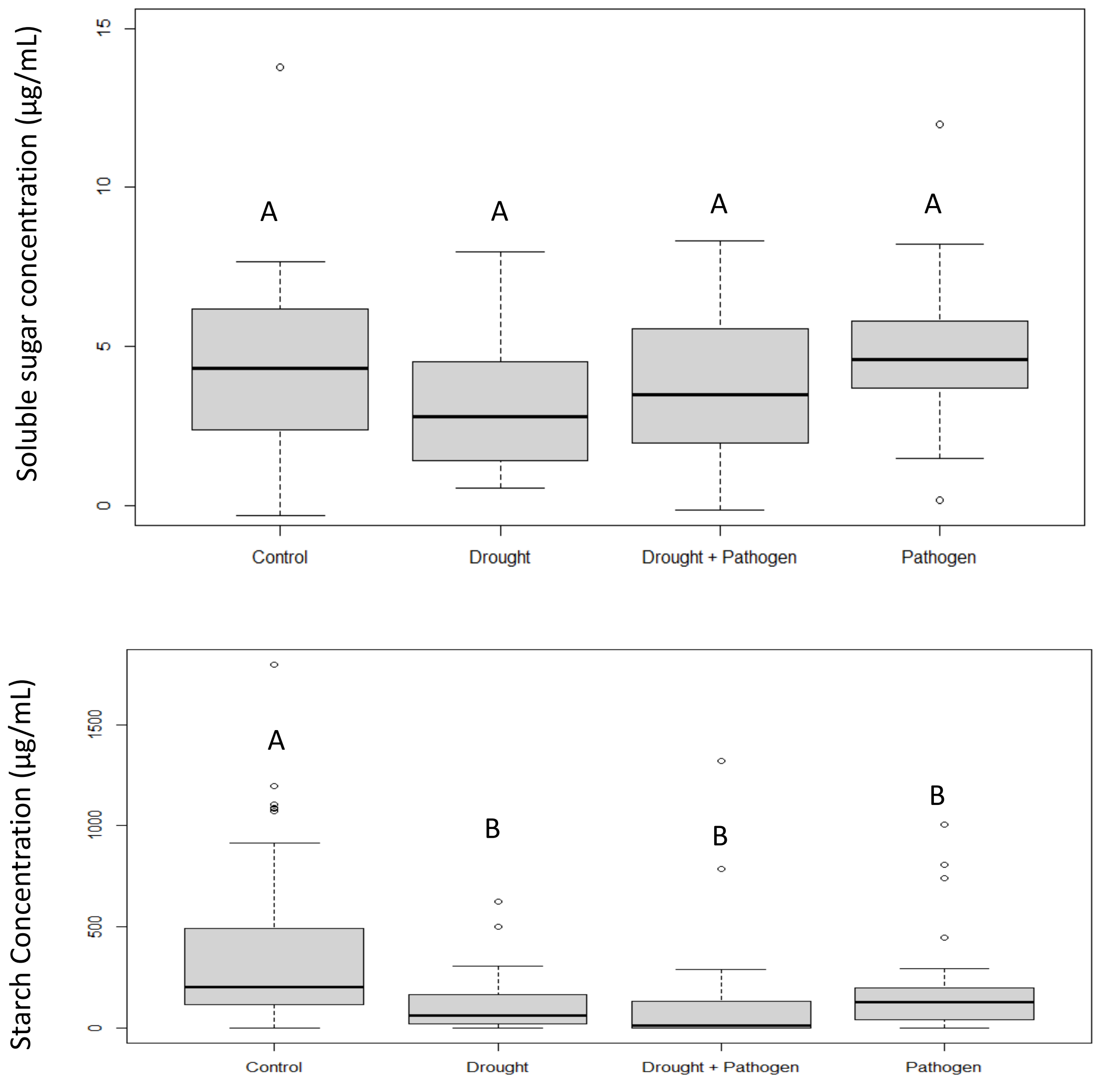


Figure 6: Box and whisker plot of soluble sugars and starch concentrations across treatments. Treatments with different letters are significantly different ($p<0.05$). Drought+pathogen groups had individuals with starch concentrations near zero/too low to quantify.

Across all chestnut varieties (Chinese, hybrids, and Americans) and treatments, the relative differences in soluble sugar and starch pools among tissue types remained the same. Concentrations of NSCs (soluble sugars and starch) were compared by treatment across tissue types and varieties. No differences were found in soluble sugar pools ($p=0.12$) among treatment groups (Figure 6 top panel). Starch pools did show a difference with both drought ($p < 0.01$) and drought pathogen treatments ($p < 0.001$) being lower than the control group (Figure 6 lower panel). The pathogen group was trending toward a significant difference with the control ($p=0.054$).

<u>Hydraulic conductivity</u>

Using a hydraulic conductivity apparatus (Figure 7), stem specific hydraulic conductivity (K_s) was quantified across all treatments and chestnut varieties near the end of the growing season.

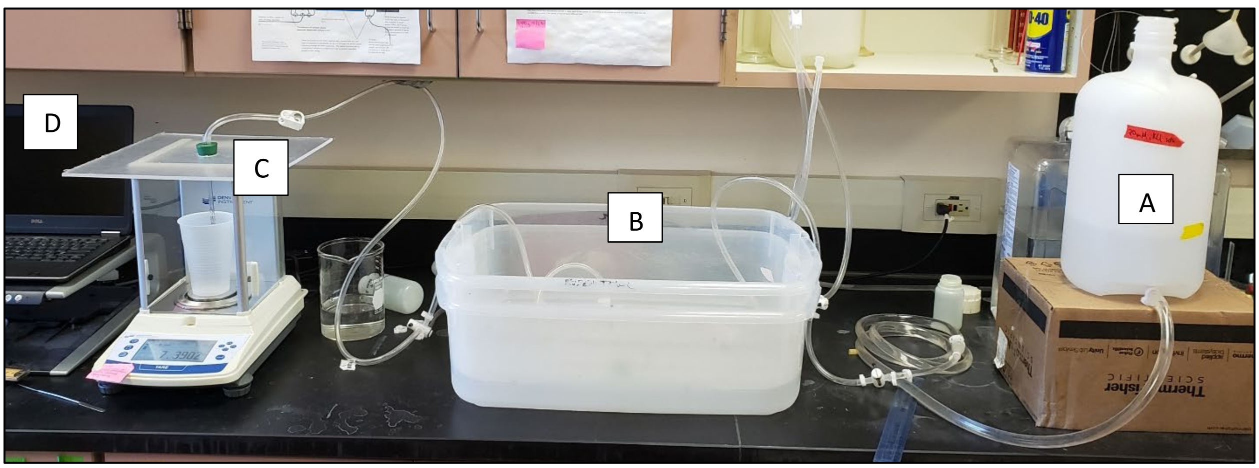
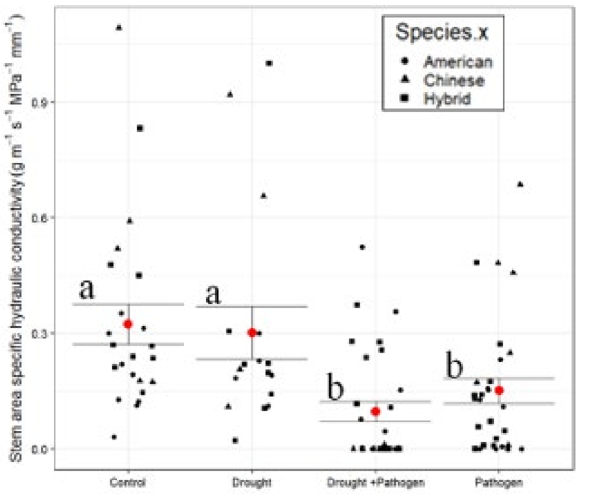


Figure 7. Hydraulic conductivity apparatus. Degassed water in A moves through tubing under low pressure. Tubing is connected to stem in water bath B. Water that passes through stem is collected and weighed on scale C. Flow rate is collected in D and used to calculate hydraulic conductivity Ks.

The pathogen and drought + pathogen treatments showed significant declines compared to the control and drought treatments (Figure 8 A). Because infected stems could not have emboli flushed as is normally done to calculate percent loss of conductivity (PLC), PLC was calculated on a treatment wide basis for each chestnut group (American, Chinese, and Hybrid). Chestnut genotypes were combined into species groups for this analysis due to a lack of notable differences between genotypes. Across all species hydraulic conductivity decreased sequentially from drought to pathogen with the greatest percent loss of conductivity in the drought + pathogen group, which was driven by the large (> 80%) decrease in the Chinese group (Figure 8 B). American and hybrid groups had similar PLC values in the pathogen group but hybrids had higher PLC than Americans in the drought + pathogen group.

A.



B.

$$PLC_{proxy} = 1 - \left(\frac{K_S \ of \ treatment \ average \ per \ variety}{K_S \ of \ control \ treatment \ for \ variety} \right)$$

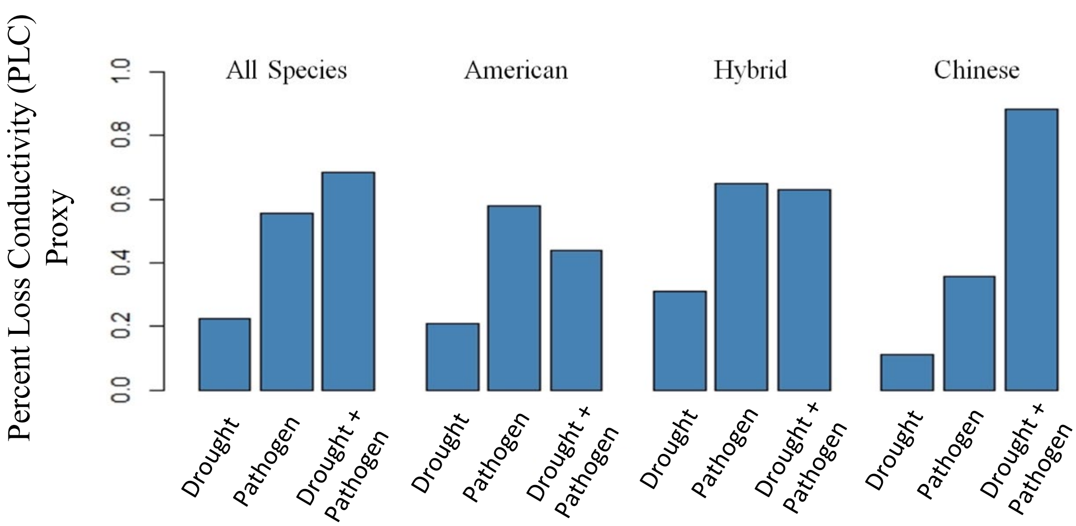


Figure 8: Hydraulic conductivity apparatus measured stem area specific hydraulic conductivity (K_s) on stems in each treatment. Kruskal-Wallis rank sum tests shows K_S showed significant differences between treatments (panel A, p-value <0.001). We calculated our PLC_{proxy} value as described in (B) and values plotted in the lower panel illustrate the relative effect of the treatment. Overall treatment differences are on the left with subgroups listed to the right.

Discussion

As expected, the greatest rates of mortality were in the drought + pathogen group (Figure 3). This increase in mortality is consistent across all chestnut varieties except for the American 1 group. As stated, this group had low samples sizes and initial issues with transplanting at the beginning of the study making an interpretation of these results tenuous. The American 2 variety performed more consistently, and within expectations, and therefore assume it is representative of the American chestnut response. We found no evidence for a strong synergistic effect of blight and drought in any chestnut variety, so we conclude that this combination of stressors is likely to be an additive response (Table 1). The lack of synergistic effects is further supported by the canker size quantification which showed no differences between the drought + pathogen and pathogen groups (Figure 4). While mortality increased in the drought+pathogen group, the lack of a difference in canker severity between these groups implies that the cooccurring drought does not directly increase the severity in blight. The ratio of canker size and basal diameter did follow expectations for the chestnut varieties with the American 2 variety being the highest and all resistant hybrids showing significantly less severe infections compared to the American 2 group. The Chinese group showed the second highest canker severity ratios, which was unexpected, but is explained by the increased swelling and callusing the Chinese group exhibited in response to the infection. This response gave the Chinese group an artificially inflated canker area even though their response indicated the active resistance to blight. This finding is supported in the limited mortality seen in the Chinese groups. In both mortality and canker size, the resistant American group did show evidence of being similar to the hybrid groups in both pathogen and drought + pathogen treatments (Table 1).

Of the three hybrid genotypes tested, the range of morality rates in the drought + pathogen group was 24% to 50%. This range of mortality is not due to variance in general blight resistance as almost no hybrids died in the pathogen treatment – moreover, there were no differences between hybrids in canker size ratios. However, these differences were not significant and more study at larger samples sizes may reveal variation among hybrid genotypes.

We measured hydraulic conductivity and carbohydrate concentrations to determine the underlying physiological effects of these coocurring stresses. The trade-off between tree hydraulics and carbohydrate usage is a central area of study in tree responses to drought (McDowell, 2011; Adams et al., 2017). Because chestnut blight affects xylem and disease responses requires carbohydrate allocation, we expected these parameters to be altered by combined drought and blight stress (Oliva et al., 2014; Stenlid and Oliva, 2016). Carbohydrate data collection is still underway but initial results suggest starch depletion will play a major role in all treatment groups (Figure 6). The drought + pathogen group specifically showed almost complete depletions of starch reserves even in the root tissue, which is typically the largest starch pool (Figure 5). While soluble sugars appear to be unaffected by treatments, analyses of remaining samples may reveal subtle differences among treatments and genotypes. Indeed, as starch is broken down into soluble sugars for usage by the plant across tissues, the overall treatment effect on starch levels will manifest before we see changes in soluble sugars. Analyses of genotype by treatment interaction effects on carbohydrate cannot yet be completed with the partial dataset but the depletion of starch reserves seems consistent across treatment groups.

The effects of treatment on stem hydraulics is a bit more interesting. Only the drought + pathogen and pathogen group showed significantly lower conductivity compared to the controls (Figure 8). It is possible that the repeated dry-downs of the drought did not reach the necessary prolonged soil dehydration that would induce xylem cavitation and lower hydraulic conductivity. Alternatively, the rehydration after wilting allowed the trees to recover some amount of the conductivity in the drought treatment. Notable in the drought + pathogen treatment is the number of zero values in conductivity. Even in some seedlings that did not show cankers fully girdling the stem, water movement could not be detected. This may be due to the fungal mycelium extending past the visible canker margin on the outside of the stem or the cavitation of the remaining xylem not infected with blight. The percent loss of conductivity on a treatment level shows that this strong reduction of conductivity in the drought + pathogen treatment is actually driven by the Chinese group which shows over ~80% losses in conductivity, even though the drought and pathogen treatments alone did not have a strong effects. This pattern did not hold for the American or hybrid groups, which showed the largest losses of conductivity in the pathogen groups but no large increases in the drought + pathogen groups.

When combined, the results of the study indicate that the American chestnuts are more robust to losses of hydraulic conductivity through the combined drought + pathogen treatments; however, this robustness does not prevent mortality due to blight. Conversely, the Chinese chestnuts are less likely to die compared to the Americans in either pathogen alone or drought + pathogen treatment but are more susceptible to strong losses in hydraulic conductivity. It is encouraging that the hybrids respond with less mortality and less loss of conductivity compared to American and Chinese groups, respectively. It is yet unknown how carbohydrates may buffer this hybrid loss of hydraulic conductivity.

Broader Impacts of Study

As expected, the drought + pathogen treatment did increase mortality across chestnut varieties. However, there is no evidence that there were strong, synergistic increases in mortality, particularly in the hybrid chestnuts. The hybrid chestnuts showed mortality rates similar to the Chinese chestnuts in the combined drought + pathogen treatment and even showed less percent loss of conductivity compared to the Chinese. This bodes well for chestnut reintroduction efforts as large rates of mortality due to blight and drought may not occur under seasonal droughts. More mortality is to be expected in years of water stress in infected trees, but the blight resistance of hybrid chestnut should be robust to this moderate drought stress. This study is an important step in understanding how co-occurring stresses may affect seedling establishment, which is the most susceptible stages of a tree's life cycle. However, the response of saplings and mature hybrid trees to these co-occurring stresses is not resolved. Nevertheless, the fact that mortality rates for the hybrids was at or below 50% in all treatment groups implies that large scale mortality is not likely due to droughts. Thus, we conclude that hybrid genotypes blight resistance is robust under co-occurring drought, and while mortality is expected to increase in infected trees during drought, it does not insure mortality.

References

Adams, H. D., M. J. B. Zeppel, W. R. L. Anderegg, H. Hartmann, S. M. Landhäusser, D. T. Tissue, T. E. Huxman, et al. 2017. A multi-species synthesis of physiological mechanisms in drought-induced tree mortality. *Nature Ecology & Evolution* 1: 1285–1291.

Bauerle, W. L., G. Geoff Wang, J. D. Bowden, and C. M. Hong. 2006. An analysis of ecophysiological responses to drought in American Chestnut. *Annals of Forest Science* 63: 833–842.

Farquhar, G. D., J. R. Ehleringer, and K. T. Hubick. 1989. Carbon Isotope Discrimination and Photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* 40: 503–537.

Hubbart, J. A., R. Guyette, and R.-M. Muzika. 2016. More than Drought: Precipitation Variance, Excessive Wetness, Pathogens and the Future of the Western Edge of the Eastern Deciduous Forest. *Science of The Total Environment* 566–567: 463–467.

Jones, C., G. J. Griffin, and J. R. Elkins. 1980. Association of Climatic Stress with Blight on Chinese Chestnut in the Eastern United States. *Plant Disease* 64: 1001–1004.

McDowell, N. G. 2011. Mechanisms Linking Drought, Hydraulics, Carbon Metabolism, and Vegetation Mortality. *Plant Physiology* 155: 1051–1059.

Oliva, J., J. Stenlid, and J. Martínez-Vilalta. 2014. The effect of fungal pathogens on the water and carbon economy of trees: implications for drought-induced mortality. *New Phytologist* 203: 1028–1035.

Parker, G. G., S. M. Hill, and L. A. Kuehnel. 1993. Decline of understory American chestnut (Castaneadentata) in a southern Appalachian forest. *Canadian Journal of Forest Research* 23: 259–265.

Romero-Lankao, P., N. S. Smith, D. J. Davidson, N. S. Diffenbaugh, P. L. Kinney, P. Kirshen, P. Kovacs, and L. Villers Ruiz. 2014. North America. *In* K. L. E. Barros, V.R., C.B. Field, D.J. Dokken, M.D. Mastrandrea, K.J. Mach, T.E. Bilir, M. Chatterjee, and and L. L. W. Y.O. Estrada, R.C. Genova, B. Girma, E.S. Kissel, A.N. Levy, S. MacCracken, P.R. Mastrandrea [eds.], Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part B: Regional Aspects. Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change,.

Stenlid, J., and J. Oliva. 2016. Phenotypic interactions between tree hosts and invasive forest pathogens in the light of globalization and climate change. *Phil. Trans. R. Soc. B* 371: 20150455.

Waldboth, M., and W. Oberhuber. 2009. Synergistic effect of drought and chestnut blight (Cryphonectria parasitica) on growth decline of European chestnut (Castanea sativa). *Forest Pathology* 39: 43–55.