ACCELERATED, GRAFT-BASED GERMPLASM CONSERVATION OF AMERICAN CHESTNUT (*CASTANEA DENTATA*) IN THE SOUTH

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ABSTRACT

Asexual propagation through grafting is a low-tech, noninvasive method for conservation of rare American chestnut germplasm. Particularly when *in situ* conditions prevent trees from reaching sexual maturity, graft-propagation allows release from shade conditions and disease pressure to promote flowering. Collection of pollen from containerized grafted trees allows conservation of genetic resources that were previously unavailable to breeders or difficult to access. Additionally, the use of high light environments may be able to reduce the generation time needed to develop a population of disease-resistant trees for restoration. As many new American chestnut individuals are required to advance both the current American Chestnut Foundation (TACF) backcross breeding program and the potential transgenic outcross program, the use of these methods provides an important proof of concept: accelerated conservation of novel genotypes from under-sampled southern populations is possible though graft propagation and the use of high light conditions.

DEDICATION

This project is dedicated to all those who have devoted their careers and volunteered their time to the restoration of American chestnut.

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LIST OF ABBREVIATIONS

ANOVA, analysis of variance

AL, Alabama

BB, bud break

CAES, Connecticut Agricultural Experiment Station

CD, catkin development or emergence

CM, catkin maturation or date of catkin collection

CO, CONSTANS gene

CP, *Cryphonectria parasitica*

cpDNA, chloroplast DNA

DNA, deoxyribonucleic acid

dsRNA, double stranded RNA

GA, Georgia

GCO, germplasm conservation orchard

GIS, Geographic Information System

KY, Kentucky

LD, long day

LSA, large surviving American chestnut

µmol $s^{-1}m^{-1}$, micromoles per second per meter

NY, New York

OTC, open top chamber

OxO, oxalate oxidase

PRR, Phytophthora root rot

RAPD, random amplified polymorphic DNA

SERNEC, Southeastern Regional Network of Expertise and Collection

spp., species

SUNY-ESF, State University of New York-Environmental Science and Forestry

TACF, The American Chestnut Foundation

TBSS, terminal bud scale scar

TN, Tennessee

UTC, University of Tennessee at Chattanooga

US, United States

USDA, United States Department of Agriculture

ybp, years before present

CHAPTER I

INTRODUCTION

Humans have been implicated in a number of environment impacts stemming from the intentional and unintentional movement of flora, fauna, and associated pests and pathogens around the globe (Lewis and Maslin, 2015; Steiner et al., 2017). Though unintentional, an infamous case of human-mediated ecological destruction is the near-obliteration of American chestnut (*Castanea dentata* [Marshall] Brokh.) by introduced pathogens that caused the phytophthora root rot (PRR; *Phytophthora cinnamomi* Rands) and chestnut blight (*Cryphonectria parasitica* [Murrill] Barr.; Anagnostakis, 1987; Crandall et al., 1945; Merkel, 1905). Formerly a dominant canopy tree common throughout much of the eastern hardwood forest, the American chestnut has little to no resistance to these pathogens (Griffin et al., 1985; Jeffers et al., 2009). As a result, it has been extirpated from much of its southeastern range by PRR (Crandall et al., 1945; Zentmyer, 1980) and reduced to an understory shrub throughout the remaining portion of its range by blight, seldom reaching the canopy before succumbing to lethal infection (Dalgleish et al., 2016; Paillet, 2002).

Evidence suggests that *P. cinnamomi* was introduced in the mid-1700s, with recorded fatalities by 1825 (Anagnostakis, 2001; Crandall et al., 1945). However, the demise of the American chestnut is attributed to the rapid spread of chestnut blight, first diagnosed in 1904 at the Bronx Zoological Park, New York, NY (Merkel, 1905). Within only decades, chestnut blight

spread throughout the entire range of American chestnut, causing the death of a culturally and economically important tree nearly everywhere it was found (Anagnostakis, 1987).

Through collaborative efforts from numerous organizations like the U.S. Department of Agriculture (USDA; Diller and Clapper, 1965), the Connecticut Agricultural Experiment Station (CAES; Graves, 1926), and more recently by The American Chestnut Foundation (TACF; Burnham, 1988), breeding for resistance to these pathogens has been underway dating back to the initial response to chestnut blight (Burnham, 1988; Diller and Clapper, 1965; Graves, 1926). Since 1983, TACF has focused on developing populations of advanced backcross hybrids of American chestnut and resistant Asian *Castanea* species, primarily *Castanea mollissima* Blume and *Castanea crenata* Siebold and Zucc. (Burnham, 1988). This method allows the introgression of genes for disease resistance from the Asian species while selecting for morphological characteristics of the American (Burnham, 1988; Diskin et al., 2006).

The natural breeding population of American chestnut is small because surviving trees are unable to reach sexual maturity due to pathogen pressure or light availability (Paillet, 2002). Additionally, flowering chestnut trees may be difficult to access due to distance from roads and/or terrain. Because traditional breeding methods require multiple visits to blooming trees with orchard ladders, bucket trucks, or professional tree climbers, the logistics alone make breeding a challenge (Alexander et al., 2004). Environmental and logistical obstacles have limited the genetic resources available to build the robust hybrid populations required for large scale restoration (Alexander et al., 2004; Fei et al., 2007).

To combat these challenges, I evaluated a graft-based germplasm conservation method targeting new and under-sampled populations of American chestnut in areas of high genetic diversity throughout the South (Dane et al., 2003; Huang et al., 1998; Kubisiak and Roberds,

2006; Shaw et al., 2012). Graft propagation is achieved by the collection and grafting of dormant scionwood onto rootstocks (Keys, 1978; McKay and Jaynes, 1969). Scionwood is a dormant twig of the previous season's growth, beyond the most apical terminal bud scale scar (TBSS), with unopened axillary buds (Garner, 2013). Each axillary bud has the genetic potential to become a new shoot, therefore each naturally-occurring wild-type tree can be cloned multiple times with minimal material removed from the *in situ* plant (Garner, 2013). Additionally, scionwood collection is independent of sexual maturity and surviving grafts can be grown under favorable conditions for flowering to occur *ex situ* (McKenna and Beheler, 2016).

Further, I assessed the effects of artificially increased photoperiod on surviving grafts with the intention of accelerating flowering and pollen collection. Plants respond to light as environmental cues to time certain physiological processes including flowering (Garner and Allard, 1920; Valverde et al., 2004). Therefore, artificial light can be used without respect to season in order to stimulate growth and flower induction (Valverde et al., 2004). Pollen can be collected from light-treated grafts and stored until the *in situ* population flowers, thus providing pollen to chestnut breeders in advance (Baier et al., 2012). *C. dentata* populations bloom nearly two weeks after *C. mollissima* in southeastern Tennessee, when *C. mollissima* female flowers are no longer receptive (J. Craddock, pers. comm., 2019). Thus, *C. dentata* pollen must be stored approximately 50 weeks prior to use in the following season. Light-treated grafts of *C. dentata* can be forced to flower in March or April, greatly reducing storage time and allowing crosses to be made in the year pollen was produced (J. Craddock, pers. comm., 2019).

Obtaining pollen in advance alleviates some of the logistical challenges required to collect pollen from wild trees, saving valuable time and resources during the breeding season. Importantly, as the grafts are containerized and maintained in a nursery, female flowers produced

from a light treatment can be pollinated with ease when receptive (Alexander et al., 2004; McKenna and Beheler, 2016). Viable seeds produced can then collected and stored for planting. Crosses from these graft-propagated clones from which seeds are produced would represent the conservation of new and/or under-sampled genotypes, and their introduction into the TACF breeding program, with a reduction in resources required to do so.

Collecting and conserving grafted American chestnut also benefits the greater research community beyond its utility in expanding the breeding program. Concentrating individuals from a wide geographic range into a single, easy to reach location allows collaborators access to cloned physical specimens *ex situ*. This project has contributed to a TACF-funded landscape genomics study and to the recognition of another *Castanea* species (Perkins et al., 2019). Future studies, chestnut breeding or otherwise, will benefit from this concentration of geographically and genetically diverse individuals.

Description of American chestnut and Range

Members of the genus *Castanea* Mill. (Fagaceae) are distributed throughout the Northern Hemisphere, with species in Asia (*C. mollissima, C. henryi*, *C. seguinii*, and *C. crenata*), Europe (*C. sativa*), and North America (*C. dentata, C. pumila*), though the North American taxonomy is currently under debate (Perkins, 2016; Perkins et al., 2019). The American chestnut differs from other North American *Castanea* species in a number of taxonomic characters, three of which are described here: leaf morphology—larger leaves (90-300 × 30-100 mm; Nixon 1997), absence of stellate trichomes on abaxial surface but with glandular trichomes on younger leaves (Weakley 2015), and occasional simple, appressed trichomes along abaxial veins (Nixon 1997); flower/fruit morphology—cupule with four-valve opening and three pistillate flowers/nuts per

bur (Nixon 1997); and habit—pre-blight records indicate that the tree commonly reached and exceeded heights of 30 m, making it the tallest *Castanea* species worldwide (Roane et al., 1987).

However, due to the impact of chestnut blight on morphology, flower and habit characteristics are generally not available or reliable (Shaw et al., 2012). Although large surviving American chestnut (LSA) do exist (Griffin et al., 1983), habit has been greatly altered by chestnut blight and what was once a large tree now commonly persists as small, multi-stemmed root sprouts typically 5-10 m tall (Nixon, 1997; Paillet, 1984). As a result, these surviving stems receive insufficient light to flower (Paillet, 2002), thus floral characters are generally not present.

The modern range of American chestnut—existing in a reduced, mostly vegetative form with the occasional LSA—extends along the Appalachian Mountains from central Alabama to Maine and southern Ontario, and from western Tennessee and Kentucky to central Virginia (Little, 1977; Westbrook, 2018). Prior to its demise, the American chestnut was an important economic and cultural figure in the eastern hardwood forest, particularly in Appalachia and possibly more than any other tree in this range (Ashe, 1911). Because of its large size (≥30 m) and timber quality, it was logged for a multitude of uses (Roane et al., 1987). For example, American chestnut has a high rot-resistance which made it a popular product for barns, fences, and telegraph poles (Brooks, 1937). Additionally, unlike other members of Fagaceae such as oak (*Quercus*), American chestnut produces a reliable annual mast (Diamond et al., 2000) of choice-edible nuts that were used as livestock feed and collected and sold for human consumption (Roane et al., 1987). The spread of chestnut blight (caused by *Cryphonectria parasitica*) severely impacted the culture and economy that depended on its quality wood and nut crop.

Biogeography of American chestnut

The range of American chestnut has, as the case for many eastern North American tree species (Delcourt and Delcourt, 1987), migrated over time in response to changes in climate (Davis, 1983). Throughout the Pleistocene epoch the eastern hardwood forest has been compressed repeatedly, some 18 to 20 times, as a result of glacial maxima and subsequently expanded following glacial retreat (Davis, 1983). During the latest glacial maximum, the Wisconsin glaciation between 18,000 and 20,000 ybp, the American chestnut range was compressed and survived glaciation in the southeastern U.S., specifically in the southern Appalachian Mountains and southern Alabama (Davis, 1983; Huang et al., 1998).

Davis (1983) and Delcourt (1979; Delcourt et al., 1980) reviewed the palynology of lake sediment in the eastern hardwood forest and were able to detect American chestnut pollen in Tennessee (Anderson Pond) as early as 15,000 ybp, while a Connecticut lake showed no evidence until 2,000 ybp. Palynology of American chestnut suggests it persisted in southern refugia during glaciation and migrated northward as the climate warmed (Davis, 1983). Along with other species, the rate of American chestnut migration was determined by variables such as fertilization requirements and seed dispersal methods (Delcourt and Delcourt, 1987). Davis (1983) notes that of the other deciduous species studied (*Acer* spp., *Carya* spp., and *Fagus grandiflora*), American chestnut was the slowest species to migrate northward at just 100 m per year.

Though the specific mechanism requires more study, the slow migration of American chestnut may be attributed to it being a monecious, obligate out-crosser. Although both male and female reproductive parts are formed on a single tree (monecious), the species is not self-fertile; thus, two trees are required for viable seed production (Hamrick and Godt, 1989; Huang et al.,

1998). Additionally, it is animal dispersed (Van der Pijl, 1969) and while long-distance dispersal by blue jay (*Cyanocitta cristata*) have been documented in related beech (*Fagus grandiflora*; Fenner, 1985; Johnson and Adkisson 1985), animal-vectored species are generally slow to migrate (Delcourt and Delcourt, 1987). However, Davis (1983) suggests that bird-vectored species are more efficient than wind dispersed as, in the case of the blue jay, seeds are more likely to be stored or distributed in areas amenable to germination rather than at random by wind.

Regardless of specific migration means, American chestnut has persisted in southern populations since that last glacial maximum, only migrating northward upon glacial retreat (Davis, 1983; Delcourt and Delcourt, 1987). The arrival of American chestnut in the northeastern U.S. some 2,000 ybp marks the extent of its range in this current interglacial period.

Genetic Diversity: Southern Hotspots

American chestnut, an obligate out-crosser, contains genetic diversity similar to other long-lived woody out-crossers (Hamrick and Godt, 1989; Huang et al., 1998). Although, American chestnut has lower diversity when compared to other members of Fagaceae and even congener species within the genus *Castanea* (Dane et al., 2003; Huang et al., 1998; Kubisiak and Roberds, 2006; Shaw et al., 2012). The extent of this diversity can be found in large part (95%) within-populations, though between-populations differences have been detected (Kubisiak and Roberds, 2006).

Huang et al. (1998) examined allozyme and random amplified polymorphic DNA (RAPD) from 12 populations of American chestnut and found the highest level of genetic diversity in a central-east Alabama population. Thus, Huang et al. (1998) suggests this region to be the center of diversity of the species. Diverse southern populations support biogeographical

evidence (Davis, 1983) that the species survived glaciation in these southern refugia, and expanded northward through successive founder events upon glacial retreat (Gailing and Nelson, 2017). It follows that Huang et al. (1998) noted a negative correlation between genetic diversity and geographic distance, whereby diversity decreases in northern populations.

Huang et al. (1998) adds that American chestnut segregates into four distinct populations: southernmost population, south-central Appalachian populations, north-central Appalachian populations, and northern Appalachian populations. However, Kubisiak and Roberds (2006) expanded genetic markers to include chloroplast DNA (cpDNA) and argue that results by Huang et al. (1998) were insufficiently quantified and not thoroughly tested statistically. They do agree that between-population variation exists, yet no distinct segregation of populations is warranted.

The decrease of genetic diversity in northern populations was also reported in works by Li and Dane (2013) and Shaw et al. (2012) through the study of haplotypes—distinct, maternally inherited patterns of cpDNA—across American chestnut populations. Both found that northern populations where fixed at more recently mutated haplotypes D1 (Li and Dane, 2013) and D2 (Shaw et al., 2012). In contrast, an Alabama population (Ruffner Mountain Nature Preserve, Birmingham, AL) exhibited rare “D-types” and other non-D-types found nowhere else across the sampled range. Shaw et al. (2012) add that predictable morphological variation occurs between haplotypes, thus morphology can be used to target these rare genotypes for additional study and conservation.

One deviation in the south-to-north decrease in diversity of chestnut is shown by Gailing and Nelson (2017), where they describe a longitudinal gradient in diversity. They investigated expressed sequence tag-simple sequence repeats (EST-SSRs) and cpDNA markers. While northern populations generally demonstrated lower levels of diversity, Gailing and Nelson

(2017) discovered that an east-to-west cline existed along the axis of the Appalachian Mountains. It may be that the decrease in American chestnut diversity can be more accurately described as a southwestern-to-northeastern cline rather than simply south-to-north.

Though population genetics studies of American chestnut vary in some respects as described above, one key factor remains constant: southern populations exhibit more rare alleles and in higher frequency (Kubisiak and Roberds, 2006; Li and Dane, 2013; Gailing and Nelson, 2017). These southern populations are of particular interest to TACF as restoration efforts rely on the conservation of rare alleles in their ongoing breeding program. However, individuals in southern populations are less dense and numerous than northern populations, thus conservation of these potentially rare genotypes is difficult. Special consideration for southern populations should be taken to ensure the conservation and incorporation of genetic resources into the TACF breeding program.

Chestnut Blight: *Cryphonectria parasitica*

Chestnut blight, caused by *Cryphonectria parasitica* (Murrill) Barr (CP), is an ascomycete fungal pathogen is characterized by necrotic lesions (cankers) in the bark of an infected plant host. Unlike other documented hosts (*Quercus* spp.; Davis et al. 1997; Phillips and Burdekin, 1992), American chestnut as little to no natural resistance to CP, thus infection almost always leads to rapid decline and mortality (Anagnostakis, 1987). This lethal fungus spreads primarily through wind-dispersed ascospores produced from bright orange pycnidial fruiting bodies, though formation of animal-vectored conidia is possible under certain conditions (Anderson, 1914; Gravatt, 1949). Chestnut blight attacks the cambium layer, impairing and eventually preventing the flow of nutrients and water across vascular tissue (Anagnostakis,

1987), thus killing the plant above the canker. Unable to spread to the soil, CP only attacks above ground tissue and blight-killed trees commonly coppice, or resprout, from the root collar of the original trunk (Graves, 1926). This then initiates a pattern of growth, infection, mortality, and coppicing which can persist for decades (Paillet, 2002).

The first evidence of chestnut blight was reported in 1904 at the Bronx Zoological Park, New York and mortality was recorded as soon as 1905 (Merkel, 1905; Roane et al., 1986). Blight spread rapidly by airborne spores and lack of resistance in American chestnut hosts (Anagnostakis, 1987, 2001; Gravatt, 1949; Gravatt and Marshall, 1926). Though efforts to slow its spread were undertaken as early as 1912 in Pennsylvania, where large fire-breaks were cut to create a buffer (Gravatt, 1949), they were unsuccessful, and blight was reported across the entire American chestnut range by 1926 (Gravatt and Marshall, 1926). In less than 30 years, chestnut blight killed nearly every mature American chestnut individual in the eastern hardwood forest (Anagnostakis, 2001). This ecological disaster, demonstrated by the rapid loss of billions of American chestnut individuals, has even been recorded in the pollen record (Russell et al., 1993).

Most populations of American chestnut have been reduced to small, vegetative resprouts that succumb to blight before reaching sexual maturity (Paillet, 2002), though in rare cases it is possible to find individuals 12 m to 18 m tall (Day et al., 1977; Diller and Clapper, 1965). These large surviving American chestnuts (LSA) can grow for many years despite infection by CP (Day et al., 1977). LSA trees were investigated further by Day et al. (1977) and they discovered that LSAs were infected by a hypovirulent strain of CP. Hypovirulence was first discovered in Italy, where chestnut blight also spread through European chestnut (*Castanea sativa*), and the cause attributed to a double stranded RNA (dsRNA) virus which attacked and weakened CP causing less severe and even reversal of symptoms (Grente and Sauret, 1969).

Isolates from LSAs were matched to known-hypovirulent strains found on *C. sativa* (Day et al., 1977), however hypovirulence in American chestnut has not produced the same results as the European at the landscape scale (Anagnostakis, 1977). Not long after its discovery, European scientists began inoculating blighted *C. sativa* with dsRNA-infected strains of CP which allowed the hypoviruses to spread throughout the CP population, leading to the recovery of European chestnut (Grente and Berthelay-Sauret, 1978). Anagnostakis (1977) found that spread of hypovirulence is restricted by a series of vegetative compatibility loci, were every allele must match for successful virus transmission. It is still unclear why hypovirulence spreads much slower in the United States than in Europe (Milgroom and Cortesi, 2004), but ongoing research (Zhang and Nuss, 2016) is investigating a genetically engineered knock-out strain to improve its transmission. It is possible that upon the approval of this strain, hypovirulence may prove to be an effective biocontrol against blight in North American as it has been in Europe.

Phytophthora Root Rot: *Phytophthora cinnamomi*

Considered one of the worst invasive plant pathogens worldwide (Cahill et al., 2008; Lowe et al., 2000; Weste and Marks, 1987; Zentmyer, 1980), Phytophthora root rot (PRR), otherwise known as ink disease, is caused by an introduced oomycete plant pathogen that, residing in soil, attacks and develops necrotic lesions on root tissue of susceptible hosts (Anagnostakis, 2001). As in the case of chestnut blight (caused by CP), American chestnut contains little to no natural resistance to PRR (Crandall et al., 1945; Jeffers et al., 2009). Pre-dating the introduction of chestnut blight, anecdotal evidence suggests that *P. cinnamomi* was introduced from Asia in the mid-1700s (Crandall et al., 1945). Reports of American chestnut mortality were recorded as early as 1825, where a landowner in Riceboro, GA described

symptoms now known to be caused by PRR (Anagnostakis, 2001). By the early 1900s, American chestnut trees in the Carolinas were showing signs of decline as a result of PRR (Crandall and Gravatt, 1967). However, Crandall and Gravatt (1967) suggest that this pathogen was likely overlooked due to the unprecedented destruction caused by chestnut blight around the same time.

PRR can be diagnosed most readily by inspection of roots, where black necrotic tissue forms along tap and/or feeder roots (Hein, 2018). Symptoms of PRR can also manifest in above ground tissue as leaf yellowing, wilt, branch die-back, and reduced vigor (Maurel et al., 2001). Because PRR kills the root systems of American chestnut trees, they are no longer capable of coppicing (Maurel et al., 2001). In this way, PRR requires more urgent attention than chestnut blight as individuals affected are completely killed, causing the permanent loss of valuable germplasm needed for future restoration efforts. Growth, survival, and pathogenicity of *Phytophthora cinnamomi* are apparently limited by soil temperature and moisture (Balci et al., 2007; Zentmyer, 1980). Presently, PRR is confined to southern populations (below 40º latitude) of American chestnut, where warm, moist soils offer protection to the pathogen from desiccation and sustained freezing temperatures (Balci et al., 2007). Climate change may increase habitat suitability, allowing migration of PRR northward (Thompson et al., 2014).

Relative to blight-resistance breeding, efforts to develop PRR-resistant American chestnut hybrids have begun only recently (Jeffers et al., 2009). Chinese chestnut contains genetic resistance to PRR and progeny from at least three breeding lines (*Castanea mollissima* ‘Clapper’, ‘Nanking’, and ‘Mahogany’) have retained loci for PRR-resistance despite only initially being selected for blight resistance (Westbrook et al., 2019a; Zhebentyayeva et al., 2014). These lines are critically important as breeding for resistance to both pathogens simultaneously is imperative if American chestnut restoration is to occur across its native range

(Zhebentyayeva et al., 2014). Additionally, breeding for PRR resistance will need to be carried out in northern populations as climate change threatens regions once thought too cold for *Phytophthora* to persist.

Restoration Efforts

Given the significant economic and cultural importance of American chestnut, coupled with the scale of its destruction, efforts to save and restore the species began almost immediately after the introduction of blight (van Fleet, 1914). Led by the USDA, a program was developed to (1) investigate genetic resistance in pure American chestnuts, (2) determine if an Asian *Castanea* spp. could function as a replacement, and (3) begin an interspecific breeding program to create resistant hybrids (Diller and Clapper, 1965).

The discovery of LSAs in decimated stands of blight-killed trees offered an early indication of resistance (Diller and Clapper, 1965). However, this phenomenon was later found to be caused by a hypovirulent strain of CP resulting from an infection by dsRNA (Anagnostakis, 1977). Researchers also investigated whether resistance was related to chestnuts ability to coppice from the base. Little hope remained as state and federal agencies, as well as chestnut hobbyists, reviled no practicable resistance to blight in either case (Diller and Clapper, 1965).

In 1927, Dr. R. Kent Beattie, authorized by the USDA, traveled through Asia in search of a blight-resistant *Castanea* spp. that might function as a suitable replacement for the American (Diller and Clapper, 1965). Replacement in this case was defined by the USDA for purposes of producing blight resistant forests for timber production, tannins, and mast for wildlife and orchard production (Clapper, 1954). Diller and Clapper (1965) describe that through 25 years of research and some 25 varieties tested, a Chinese chestnut from the Nanjing region—now

regarded as *Castanea mollissima* 'Nanking'—proved to be a potential replacement variety. Observations of 'Nanking' recorded a high degree of blight resistance and satisfactory growth that develops timber-quality form (Diller and Clapper, 1965). However, if ecological restoration is to be valued over simply replacing timber production, introducing *C. mollissima* 'Nanking' into the eastern hardwood forest does nothing to remedy the loss of the native *C. dentata* (Diskin et al., 2006).

The development of blight-resistant American chestnut hybrids dates back to successful interspecific crosses of *C. dentata* x *C. mollissima* by Gravatt and Clapper in 1925 (Beattie and Diller, 1954; Diller and Clapper, 1965). Decades of research by the USDA and the CAES investigated viability of Asian-American chestnut hybrids (Beattie and Diller, 1954; Berry, 1978; Diller and Clapper, 1965). Work by these institutions led to the 'Clapper' and 'Graves' trees: first backcross hybrids from initial crosses of *C. dentata* x *C. mollissima* M16 PI34517 and *C. dentata* x *C. mollissima* 'Mahogany', respectively (Burnham et al., 1986; Clapper, 1963).

Early successes by the USDA and CAES, such as the 'Clapper' and 'Graves' lines, were advanced by Burnham's backcross breeding program established in the early 1980s (Burnham, 1981; Burnham et al., 1986). Backcross breeding involves an initial cross of *C. dentata* x *C. mollissima* (or other resistant *Castanea* spp.), creating a first-generation (F_1) hybrid. Offspring containing sufficient levels of blight resistance are then crossed back to *C. dentata*, creating a first-backcross (BC_1) generation. Backcross breeding allows the introgression of blight resistance from the Asian species while recovering American characteristics required for ecological restoration (Burnham, 1988; Diskin et al., 2006).

When performed regionally, this approach can target and retain regional genetic diversity by backcrossing to a wide range of American individuals (Westbrook, 2018). Backcrosses are

advanced to the third generation (BC_3) and repeatedly intercrossed to create a second generation, third backcross (BC_3F_2). Relying on a three-gene model of blight-resistance inheritance (Hebard, 1994), Burnham et al. (1986) suggested that individuals at this generation (BC_3F_2) will be fully segregating for blight resistance. Those demonstrating high resistance will be selected for and propagated in restoration plantings. This model has recently been called into question as it appears that blight-resistance inheritance is more complex, and may be a polygenic trait existing on multiple loci (Steiner, 2017; Westbrook et al., 2019b).

Diskin et al. (2006) investigated morphological characteristics of BC_3F_2 hybrids and found them to be statistically identical to pure-American *C. dentata* in 16 of 24 (66.6%) characters analyzed. They suggest that recovery of American chestnut phenotype is possible through backcross breeding. It remains to be seen, however, whether these advanced hybrids contain similar ecological characteristics required for restoration (Diskin et al., 2006). Additional research is required in this regard to determine how BC_3F_2 hybrids perform in a forest setting once blight resistance is achieved, though results by Diskin et al. (2006) are promising.

Founded in 1983 upon the Burnham backcross breeding model, TACF has expanded its restoration efforts from backcross breeding and bio-control to include biotechnology. TACF has supported research by SUNY-ESF in the transformation of American chestnut to incorporate the wheat gene, oxalate oxidase (OxO; Steiner, et al., 2017). OxO detoxifies oxalate produced by CP, and is a common compound found in both monocots (cereals) and dicots (strawberry, beet, peanut, and apricot), but not *Castanea* (Steiner et al., 2017). Oxalate production by CP—and other fungal plant pathogens—causes cell death allowing the spread of advancing fungal hyphae (Hebard and Shain, 1988; Kim et al., 2008). OxO detoxifies oxalate, preventing or slowing the

spread of advancing hyphae, resulting in a non-lethal infection (Havir and Anagnostakis 1983; Chen et al. 2010).

SUNY-ESF has performed multiple transformation events on somatic embryonic clones of American chestnut for OxO gene insertion (Polin et al., 2006; Newhouse et al., 2014). Insertion and overexpression of OxO in genetically modified American chestnut has proven to enhance blight resistance to the level of blight-tolerance (Newhouse et al., 2014). Importantly, this trait is transmissible when outcrossed to wild-type (WT) genotypes. Newhouse et al. (2014) report that slightly less than the predicted 50% of the seeds harvested from controlled pollinations contained the OxO gene. Dr. Jared Westbrook, Director of Science for TACF, lays out a breeding plan designed build a robust population of blight-tolerant American chestnut, while conserving maximum genetic diversity (Westbrook et al., 2019c). They propose outcrossing a single transgenic founder tree to WT American chestnuts over five generations, selecting for offspring containing the OxO gene.

It should be noted that this transgenic founder is a clone of an American chestnut native to New York. As discussed earlier, northern populations are less diverse (Kubisiak and Roberds, 2006; Li and Dane, 2014; Shaw et al., 2012), thus in the same way that the backcross method required diluting Chinese genes, OxO breeding will require diluting the New York clone genes to restore regional diversity, as well as to reduce inbreeding potential (Westbrook et al., 2019c). Westbrook et al. (2019c) suggest that greater than 500 genetically distinct blight-tolerant individuals will be required to reduce genetic drift and the inbreeding coefficient. This population, containing sufficient genetic diversity and blight resistance, will be available for large-scale restoration in 20-35 years, pending federal approval (Westbrook et al., 2019c).

Ex situ Conservation: Graft Propagation

Restoration of the American chestnut, whether accomplished through backcross or transgenic breeding, will require access to a numerous and diverse population of sexually mature trees (Westbrook, 2018; Westbrook et al., 2019c). As many individuals currently persist in the understory where flowering is rare (Paillet, 2002), intervention is required to bolster the breeding population to capture genetic diversity in the restored population (Westbrook, 2018). There are multiple methods available to promote flowering, both *in* and *ex situ*. An *in situ* solution may be to clear the trees around an American chestnut to free it from shade conditions where it can then receive enough light to flower (Paillet, 2002; Wang et al., 2006). However, access to flowers may still be a challenge if travel over difficult terrain with ladders or bucket trucks is required. Additionally, clearing may be difficult to authorize depending on landowner wishes, especially if land is privately owned. *Ex situ* conservation can be achieved through transplanting, collection/sowing of seeds, somatic embryogenesis, rooting, and grafting (Alexander et al., 2004; Carraway and Merkle, 1997; Craddock and Bassi, 1999; Keys, 1979; McKenna and Beheler, 2016). Though each offers a unique set of challenges, these methods allow the tree to be propagated and maintained in an area (i.e. nursery or orchard) that offers increased light availability, reduction in disease pressure (PRR), and minimizing in logistical difficulties.

With exception of the European (*C. sativa*), species in the genus *Castanea* have been found to be difficult to root, proving less responsive to traditional rooting techniques (Galic et al., 2014; Wright, 1976). Transplanting is of course a viable option; however, caution should be applied with small populations and rare genotypes (Rex Mann, pers comm, 2019). Transplanting removes the plant entirely from its natural habitat and as survival rates are highly variable, large numbers of individuals may be lost (Rex Mann, pers comm, 2019). Considering the high genetic

diversity in the south (Gailing and Nelson, 2017; Li and Dane, 2014; Shaw et al., 2012), transplanting individuals should only be attempted if other propagation means have failed and if rescue is required (Alexander et al., 2004).

As methods and rates of success have improved, somatic embryogenesis has become a more reliable propagation method (Carraway and Merkle, 1997; Gonzales et al., 1985; Merkle et al., 1991) and is of significance in the OxO transformation of American chestnut by *Agrobacterium* (Polin et al., 2006). However, this method requires tissue from developing embryos (seeds), which are not typically available in naturally occurring American chestnut populations. Thus, somatic embryogenesis by itself would not increase the number of new genotypes added to the TACF breeding program.

Considering the other methods described, grafting is the primary technique for propagation of American chestnut (Keys, 1978; McKay and Jaynes, 1969). Grafting creates a duel-organism by combining tissue from two organisms into one (Hartman et al., 2010). Dormant buds of the desired genotype or species—scionwood—are spliced onto a rootstock of the same or related species (Garner, 2013). Proper alignment of vascular tissue during grafting allows the transfer of water and nutrients to and from the rootstock and scion (Garner, 2013; Huang et al., 1994). Additionally, grafting requires no complex equipment and can done relatively quickly (Craddock and Bassi, 1999). Minimal material is required from the *in situ* plant, or ortet, leaving it relatively undisturbed and is independent of sexual maturity (Garner, 2013). Consequently, successful grafts (ramets) from non-flowering ortets can be allowed to flower in as early as the first growing season (McKenna and Beheler, 2016). Additionally, over a five year study, McKenna and Beheler (2016) found that 38% of grafts survived and, importantly, produced more seeds than seedlings over the same span.

Graft Incompatibility

Studying grafting success is a difficult task given the number of variables associated with it. Jaynes (1979) describes four factors that commonly influence graft success or failure in chestnut: (1) winter hardiness, (2) CP infection at graft union, (3) improper grafting technique (i.e. human error), and (4) scion-rootstock incompatibility. Graft incompatibility has been studied in *Castanea*, and though limited, some evidence has emerged to suggest it can occur. Santamour et al. (1986) examined 10 *Castanea* species and found three variable phenotypic patterns of anodal isoperoxidase bands in the cambial zones. They report that graft incompatibility exists when bands do not match, even when rootstock and scion are sourced from the same species. Although isoperoxidase bands were not examined, McKenna and Beheler (2016) do support that graft success can vary between genotypes of the same species.

Huang et al. (1994) also investigated chestnut grafting and disputed incompatibility by isoperoxidase mismatch. However, they did discover that graft failure is influenced by improper alignment of vascular bundles, age of rootstock selected, and development of non-vascular calluses or masses at the graft union. Vascular bundles in chestnut are condensed at younger ages causing grooved or fluted stem morphology (Huang et al., 1994). In these instances, misalignment of these bundles at the graft union prevents proper connectivity between scion and rootstock, leading to graft failure. As suggested by Huang et al. (1994), this can be mitigated by using older rootstocks (2-3 years old) where bundles are more defined, and vascular tissue alignment is less difficult.

Still, graft failure has been known to occur after initial success some months or even years later, known as delayed graft failure or incompatibility. Perhaps the most notable investigation of delayed graft incompatibility is that of grafted walnut (*Juglans*; Mircetich et al.,

1980; Schuster and Miller, 1933). Commercial orchards of Persian walnut (*Juglans regia*) scions grafted onto eastern black walnut (*J. nigra*) rootstocks have been in decline since the 1920s, where mature grafts began to fail (Schuster and Miller, 1933). Initially thought to be caused by delayed graft incompatibility, researchers discovered a black line of necrotic tissue at the graft union. Eventually described as a walnut isolate of the cherry leafroll virus, graft failure is caused by differential susceptibility to the disease, where *J. regia* responds asymptomatically and *J. nigra* is hypersensitive (Mircetich et al., 1980). When *J. nigra* encounters the disease, it shuts down cellular activity, thus cutting off connectivity with grafted *J. regia* at the graft union (Mircetich et al., 1980). Failure among mature grafted-chestnut has been observed, though more study is required to determine whether the cause can be attributed to an infection similar to black line disease.

Though it is common to use scion-rootstock combinations of the same species to minimize potential graft incompatibility (Weber and MacDaniels, 1969), consideration of rootstock survival is important in areas impacted by *P. cinnamomi*. While limited evidence suggests incompatibility occurs with interspecific combinations (Huang et al., 1994; Santamour et al., 1986), grafting *C. dentata* scionwood to *C. dentata* rootstock would not be advised in orchards or nurseries known to contain *P. cinnamomi*. PRR would likely cause rootstock failure, leading to the loss of the scion. In these cases, selecting an Asian *Castanea* rootstock resistant to PRR may be more advantageous**.**

Photoperiod Manipulation

Plants respond to light as an environmental signal to synchronize major physiological processes such as dormancy (Hemberg, 1949; Rohde and Bhalerao, 2007) and flowering

(Valverde et al., 2004). Responses to light are influenced by three factors: (1) light intensity, (2) quality, or wavelength, and (3) duration, or photoperiod (Garner and Allard, 1920). Photosynthetically-active radiation (PAR) defines the wavelength of light (400-700 nm) at which plants respond to perform photosynthesis (Alados et al. 1996). Plant growth can be stimulated by saturating leaves with PAR in growth chambers (Baier et al., 2012). Light intensity, a measurement of photon density reported as μmol $s^{-1}m^{-1}$, corresponds to the ability to saturate the laminar surface with PAR (Powell, 1984; Ruban, 2009). The genes and regulatory pathways that control these processes have primarily been studied in herbaceous model species and those of commercial value. In *Arabidopsis*, a heavily studied genus of model species, flower development has been associated with the *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) genes (Valverde et al., 2004). These genes are regulated by exposure to long-days (LD) sensed by photoreceptors phytochromes and cryptochromes (Eriksson and Millar, 2003; Kobayashi and Shimizu, 2013; Valverde et al., 2004). Similar genetic control of flowering has been found in the woody tree genus *Populus* (Bohlenius et al., 2006).

Subjecting plants to variable photoperiods and growing conditions allows researchers to better understand molecular processes and, importantly, how those conditions influence phenotype. Along with genomics and molecular biology, plant ecologists are experimenting with photoperiod and growing condition manipulation to predict adaptability to projected future climate conditions (Sanz-Perez et al., 2007; Way and Montgomery, 2015). Although few studies exist on American chestnut, understanding how temperature and photoperiod influence the species is key to ensuring TACF proceeds with guided restoration efforts (Wang et al., 2006).

Wang et al. (2006) studied how different light environments impacted growth and form of American chestnut to establish ideal conditions for restoration plantings. They measured

photosynthetic rate, biomass allocation (above and below-ground), and growth of containerized American chestnut seedlings grown under increasingly darkened shade cloth at four levels of irradiance (4%, 12%, 32%, 100%). Wang et al. (2006) found that American chestnut is shade-tolerant, able to accumulate below-ground biomass until released from light competition. Although American chestnut responses to light were similar to other eastern deciduous species (Groninger et al., 1996; Kubiske and Pregitzer, 1996), when release occurs, American chestnut is capable of rapid growth, often out growing neighboring species (Wang et al., 2006). Wang et al. (2006) determined the light saturation point American chestnut to be 203 µmol $s^{-1}m^{-1}$.

In contrast to Wang et al. (2006), Baier et al. (2012) examined the acceleration of growth and flower induction using a growth chamber. While Wang et al. (2006) utilized shade cloths to reduce light in natural conditions, Baier et al. (2012) subjected containerized plants to artificial light. Initially designed to promote rapid vegetative growth of transgenic seedlings ('Hinchee 1'), Baier et al. (2012) discovered that it was possible to expedite sexual maturity. Under high light conditions (16-hour photoperiod of 700-900 µmol $s^{-1}m^{-1}$) viable catkins were produced on 14 (43%) of 'Hinchee 1' seedlings between 9 and 11 months after planting. Although flowering can occur on year-old seedlings (personal observation, Fortwood Street Greenhouse, UTC), it is rare and most individuals take between 8 and 10 years to become sexually mature (Zon, 1904).

Baier et al. (2012) then subjected WT American and Chinese chestnuts to the same conditions as the previous test, and similar results were found. Although sample size was small—just 6 seedlings per species (12 total)—viable pollen was collected from 4 (67%) Chinese and 1 (17%) American seedling as early as 6 months after planting. Perhaps most importantly, a single Chinese seedling formed female flowers, demonstrating the ability to perform controlled pollinations on containerized, light induced plants. This method of accelerating sexual maturity

to reduce generation time—speed breeding—has been employed in commercial crops for years (Ghosh et al., 2018; Sysoeva et al., 2016). Baier et al. (2012) provide evidence that the same approach can be taken in chestnut.

Research Objectives

This study was divided into three main objectives. First, I asked whether it was possible to conserve new and under-sampled American chestnut individuals through graft propagation. If successful, containerized grafted plants could be grown in favorable conditions to promote flowering. Second, I tested the effects of high light conditions on grafted plants, where my hypothesis was that accelerated growth and flowering would occur under high light with extended photoperiod. Finally, I intended to contribute to the broader American chestnut research community by creating a collection of genetically and geographically diverse genotypes and support the creation of long-term germplasm conservation orchards.

CHAPTER II

MATERIALS AND METHODS

Study Area and Tree Location

The study area was divided into four regions: (1) southeast Tennessee/northwest Georgia, (2) south-central Tennessee/northern Alabama, (3) north-central Tennessee/southwestern Kentucky, and (4) western Tennessee/northern Mississippi. Specific areas for scionwood collection were informed by a county-by-county range map of American chestnut individuals conserved in the TACF breeding program (Figure 1; Westbrook, 2018). Counties containing between 0 and 10 conserved individuals were considered under-sampled and targeted for scion collection.

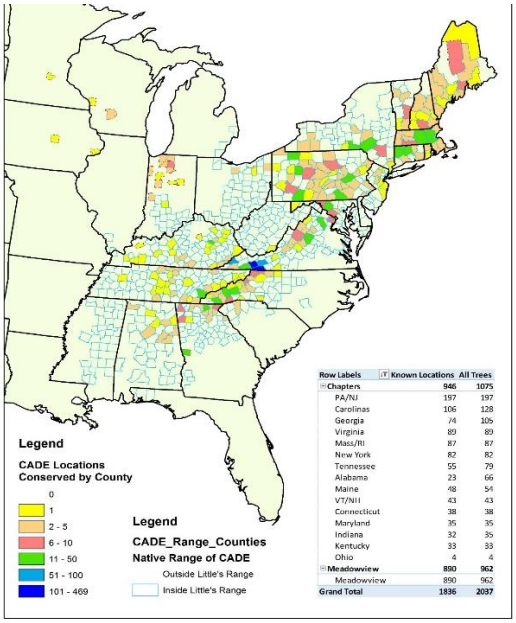


Figure 1 Conserved genotypes by county in the TACF breeding Program

Assistance in tree location and scionwood collection was solicited from TACF members in Alabama, Georgia, Kentucky, and Tennessee chapters through a member-wide announcement. Additionally, tree locations were sourced from herbarium records of the Southeastern Regional Network of Expertise and Collection (SERNEC). Though locating previously unknown individuals was desired, this study relied heavily upon the location of known individuals that had not been bred due to some limitation (i.e. sexual immaturity, difficult access, or other logistical obstacle). When possible, locations of American chestnuts were to be visited during the growing season to confirm location, species identification, and to collect a voucher specimen. To maximize the number of genotypes collected in the second season (winter 2018-2019), I drafted a scionwood collection protocol (Appendix A) to guide TACF volunteers willing to collect and ship scionwood in support of this project.

Scion collection in 2018-2019 was improved by re-visiting sites from 2017-2018 and confirming species identity during the growing season (Chester, 2015; Perkins et al., 2019). Finally, instructed by the scionwood collection protocol (Appendix A) in 2018-2019, TACF volunteers submitted scionwood samples via mail. In these cases, species identification was limited to winter twig and bud characters (Petrides et al., 1988).

Scion Collection and Storage

Ideal dormant scionwood consists of twigs, beyond the most distal TBSS, with multiple unopened buds (Garner, 2013). Diameter of scions is an important factor which can influence the grafting method used (Garner, 1947). To perform techniques such as the whip-and-tongue, the diameter of the scion and rootstock must match (Craddock and Bassi, 1993). However, shade dominated American chestnut trees seldom receive sufficient light for vigorous growth. The

result of which produces small diameter scions that are generally too small to match the diameter of the rootstock. In these cases, the bark-flap graft is a viable method, as it is used with smaller diameter scionwood (Garner, 1947).

Dormancy requirements of scionwood collection limited collecting trips to December through March for most southern populations (Garner, 2013). When tree height permitted, scionwood was collected by hand pruners, though taller trees required the use of pole pruners (3-10 m). Scionwood cut from dormant trees was trimmed to the length of a standard gallon freezer bag, then placed inside (Figure 2). The bag was labeled with the date and local tree name (i.e. Bradford Trail 2) or TACF tree code (TNHEN02), if known. Additionally, a note card containing more detailed information (see Appendix A) was placed in the bag as well. Each sample was then double-bagged and rolled to evacuate excess air to prevent desiccation. While in the field, scionwood was stored in an iced cooler (0°- 4 °C) for the duration of the collecting trip. Upon return from the field, samples were stored in a walk-in cooler (0°- 4 °C) at the Fortwood Street Greenhouse on the campus of the University of Tennessee at Chattanooga (UTC).

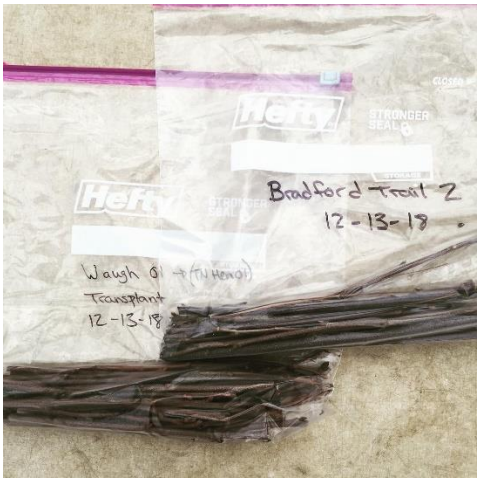


Figure 2 Scionwood Collection

Over two scionwood collection seasons—December through March of 2017-2018 and 2018-2019—scionwood was collected from 93 genotypes (Figure 3): 71 *C. dentata*, 19 *C. alabamensis*, and 2 unconfirmed *Castanea* spp. (1). 38 genotypes were collected in 2017-2018 and 69 genotypes in 2018-2019, including 16 genotypes collected in both seasons. Scions were collected from all four designated regions: (1) southeast Tennessee/northwest Georgia, (2) south-central Tennessee/northern Alabama, (3) north-central Tennessee/southwestern Kentucky, and (4) western Tennessee/northern Mississippi. Importantly, 71 genotypes (78%) from which scionwood was collected came from individuals that had not been bred previously. This was determined by searching through breeding records of the TACF data base (DentataBase).

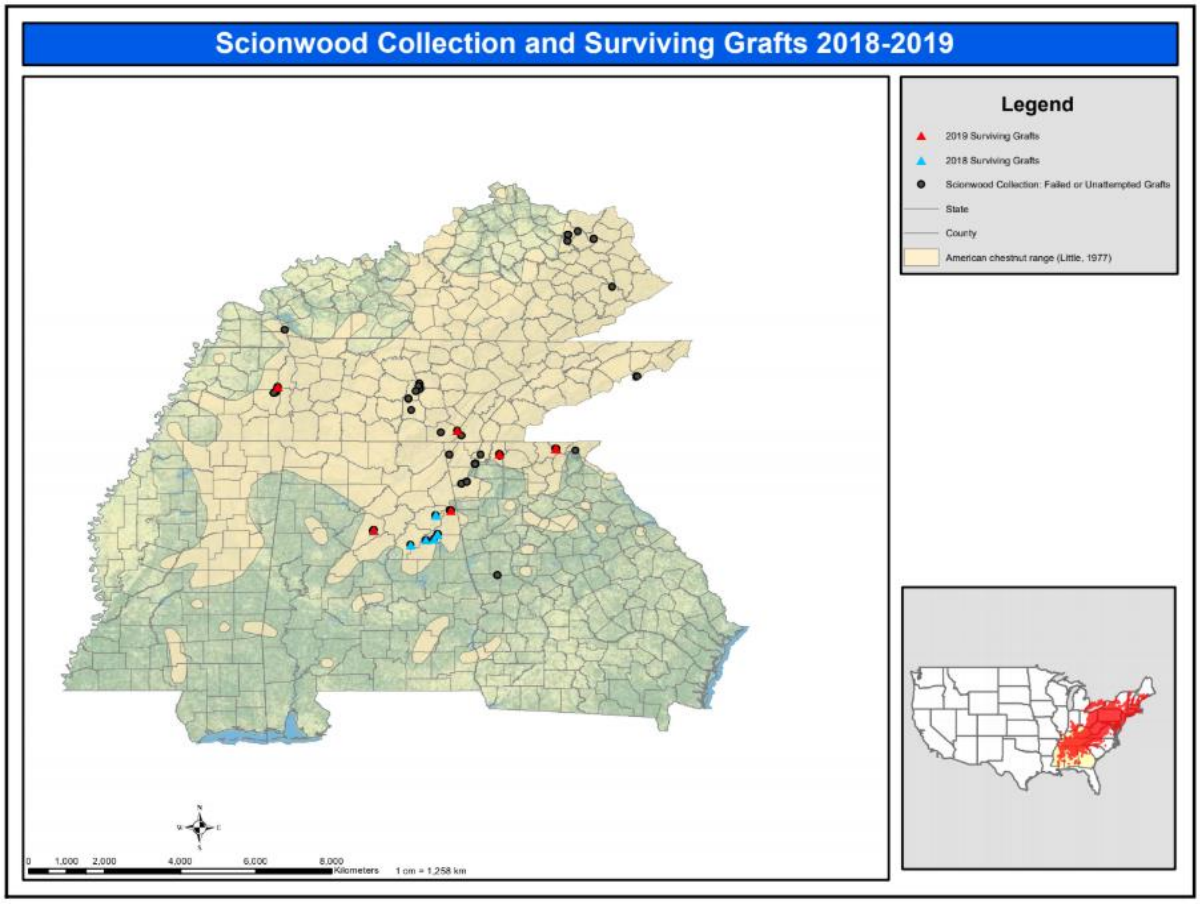


Figure 3 Scionwood Collection and Surviving Grafts 2018-2019

Table 1 Scionwood Collection Data 2017-2018 and 2018-2019

[1]Scions collected in winter (1) 2017-2018 and/or (2) 2018-2019 between December and March. 91 total ortets sampled, 14 and 20 genotypes grafted in 2018 and 2019, respectively. As not all scionwood collected was of desired condition or diameter, grafting attempts were prioritized based on scionwood quality or rarity of ortet. NC denotes "not confirmed" genotypes through which winter characteristics were inconclusive and grafts failed before leaf characters could be used.

Name	Species	State	County	Location	Collecting Season[1]	Grafting Year	Surviving Graft	Previously Bred (Y/N)	Volunteer Sample
5	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1	2018	Y	Y	N
10101A	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1,2	2018, 2019	Y	N	N
10101B	C. dentata	AL	Jefferson	Ruffner Mountain Nature Preserve	1		N	N	N
1CN	C. dentata	AL	Jefferson	Ruffner Mountain Nature Preserve	1		N	N	N
3CN	C. dentata	AL	Jefferson	Ruffner Mountain Nature Preserve	2	2019	N	N	N
4CN	C. dentata	AL	Jefferson	Ruffner Mountain Nature Preserve	2		N	N	N
6CN	C. dentata	AL	Jefferson	Ruffner Mountain Nature Preserve	2		N	N	N
7CN	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1,2	2018, 2019	Y	Y	N
9CN	C. dentata	AL	Jefferson	Ruffner Mountain Nature Preserve	1		N	N	N
Cheaha07	NC	AL	Talladega	Adams Gap	1		N	N	N
Cheaha08	*C. dentata*	AL	Talladega	Adams Gap	1	2018	Y	N	N
Cheaha17	NC	AL	Talladega	Adams Gap	1		N	N	N
Choco01	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	Y	N	N
Choco02	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	Y	N	N
Choco22	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	Y	N	N

Table 1 Continued

Name	Species	State	County	Location	Collecting Season	Grafting Year	Surviving Graft	Previously Bred (Y/N)	Volunteer Sample
Choco27	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	Y	N	N
Choco28	*C. dentata*	AL	Calhoun	Choccolocco Mountain	1	2018	Y	N	N
Clarke01	C. dentata	AL	Clay	Sonny Clarke Property	1	2018	Y	Y	N
Frames03	*C. dentata*	AL	Cleburne	Frames Property	1	2018	Y	Y	N
Frames05	*C. dentata*	AL	Cleburne	Frames Property	1	2018	Y	Y	N
Hutch11	*C. alabamensis*	AL	Cleburne	Hutchinson Property	2		N	N	N
Hutch04	*C. dentata*	AL	Cleburne	Hutchinson Property	2	2019	Y	N	N
MS31	*C. alabamensis*	AL	Jefferson	Ruffner Mountain Nature Preserve	1		N	N	N
MS38	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1,2	2019	Y	N	N
MS41	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1		N	N	N
MS42	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1	2018	Y	Y	N
MS63	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	2	2019	N	N	N
T3	*C. dentata*	AL	Talladega	Talladega National Forest	1	2018	Y	Y	N
Unmarked01	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	2		N	N	N
Unmarked04	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	2		N	N	N
GAFL14	*C. dentata*	GA	Floyd	Berry College	2		N	Y	Y
GAFL3	*C. dentata*	GA	Floyd	Berry College	2		N	Y	Y
GAFL4	*C. dentata*	GA	Floyd	Berry College	2		N	Y	Y
GAMU8-A	*C. alabamensis*	GA	Murray	Fort Mountain	2		N	Y	Y

Table 1 Continued

Name	Species	State	County	Location	Collecting Season	Grafting Year	Surviving Graft	Previously Bred (Y/N)	Volunteer Sample
GAMU8-B	*C. dentata*	GA	Murray	Fort Mountain	2		N	Y	Y
GAMU9-A	*C. dentata*	GA	Murray	Fort Mountain	2	2019	Y	Y	Y
GAMU9-B	*C. dentata*	GA	Murray	Fort Mountain	2	2019	Y	Y	Y
GARA5	*C. dentata*	GA	Rabun	Glassy Mountain	2	2019	N	Y	Y
GAUN5	*C. dentata*	GA	Union	Brasstown Bald	2	2019	Y	Y	Y
GAUN8	*C. dentata*	GA	Union	Brasstown Bald	2		N	Y	Y
GAUN3XGAWA7	*C. dentata*	GA	Union	Brasstown Bald	2	2019	Y	Y	Y
GAWH687	*C. dentata*	GA	White	-	2	2019	N	N	Y
GAWA17	*C. alabamensis*	GA	Walker	John's Mountain	2		N	Y	Y
Johns Mt02	*C. alabamensis*	GA	Walker	John's Mountain	2		N	N	N
Johns Mtn03	*C. alabamensis*	GA	Walker	John's Mountain	2		N	N	N
Johns Mtn04	*C. alabamensis*	GA	Walker	John's Mountain	2		N	N	N
Johns Mtn05	*C. alabamensis*	GA	Walker	John's Mountain	2		N	N	N
Johns Mtn06	*C. alabamensis*	GA	Walker	John's Mountain	2		N	N	N
Johns Mtn07	*C. alabamensis*	GA	Walker	John's Mountain	2		N	N	N
Shiloh FDR 01	*C. dentata*	GA	Harris	FDR State Park	2		N	N	Y
Alcorn01	*C. dentata*	KY	Rowan	Alcorn Property	2		N	N	Y
Conley08	*C. dentata*	KY	Knott	Conley Property	2		N	N	Y
Fleming03	*C. dentata*	KY	Fleming	Gossett Property	2	2019	N	N	Y
Frazier	*C. dentata*	KY	Fleming	Gossett Property	2		N	N	Y
Galloway03	*C. dentata*	KY	Fleming	Gossett Property	2		N	N	Y
Gossett02	*C. dentata*	KY	Fleming	Gossett Property	2		N	N	Y

Table 1 Continued

Name	Species	State	County	Location	Collecting Season	Grafting Year	Surviving Graft	Previously Bred (Y/N)	Volunteer Sample
LBL Big Tree	*C. dentata*	KY	Marshall	Land Between the Lakes SP	2	2019	N	Y	N
Stevie07	*C. dentata*	KY	Carter	Stevie Property	2		N	N	Y
Willis06	*C. dentata*	KY	Carter	Stevie Property	2		N	N	Y
Woods05	*C. dentata*	KY	Rowan	Lewman Property	2		N	N	Y
Bill Hill 01	*C. dentata*	TN	Cannon	Middle TN	2		N	N	Y
Bradford Trail 01	*C. dentata*	TN	Henderson	Natchez Trace SP	2	2019	N	N	N
Bradford Trail 02	*C. dentata*	TN	Henderson	Natchez Trace SP	2	2019	Y	N	N
Bradford Trail 03	*C. dentata*	TN	Henderson	Natchez Trace SP	2		N	N	N
Clear Fork 01	*C. dentata*	TN	Cannon	Middle TN	1,2	2019	N	N	N
Headwaters01	*C. dentata*	TN	Cannon	Middle TN	1,2	2019	N	N	N
Old Stone Fort 01	*C. dentata*	TN	Coffee	Old Stone Fort Park	1,2	2019	N	N	N
Sample1	*C. dentata*	TN	Unicoi	Private Land, Conservation Easement	2		N	N	Y
Sample2 L1	*C. dentata*	TN	Unicoi	Private Land, Conservation Easement	2		N	N	Y
Sample3 L1/L2	*C. dentata*	TN	Unicoi	Private Land, Conservation Easement	2		N	N	Y
Signal Mtn01	*C. dentata*	TN	Hamilton	Signal Mountain	2	2019	Y	Y	N
Stringers Ridge 01	*C. dentata*	TN	Hamilton	Stringer's Ridge Park	2		N	N	N
TNCAN01	*C. dentata*	TN	Cannon	Todd Jr Property	1		N	N	N
TNCAN02	*C. dentata*	TN	Cannon	Todd Jr Property	1		N	N	N
TNHAM02	*C. dentata*	TN	Hamilton	Signal Mountain	1		N	N	N

Table 1 Continued

Name	Species	State	County	Location	Collecting Season	Grafting Year	Surviving Graft	Previously Bred (Y/N)	Volunteer Sample
TNHEN01	*C. dentata*	TN	Henderson	Natchez Trace SP	1,2		N	N	N
TNHEN02	*C. dentata*	TN	Henderson	Natchez Trace SP	1,2		N	N	N
TNHEN03	*C. dentata*	TN	Henderson	Natchez Trace SP	1,2		N	N	N
TNHEN04	*C. dentata*	TN	Henderson	Natchez Trace SP	1,2		N	N	N
TNHEN05	*C. dentata*	TN	Henderson	Natchez Trace SP	1,2		N	N	N
TNHEN06	*C. dentata*	TN	Henderson	Natchez Trace SP	1,2		N	N	N
Waugh01 Transplant	*C. dentata*	TN	Henderson	Natchez Trace SP	1,2		N	N	N
Willmouth01	*C. dentata*	TN	Cannon	Middle TN	1,2		N	N	N
Young Hollow 01	*C. dentata*	TN	Cannon	Middle TN	1,2		N	N	N

Rootstock Selection

To account for potential graft incompatibility (Huang et al., 1994; Santamour et al., 1986), a variety of rootstocks were chosen for the first grafting season (May-July 2018). However, as both chestnut blight and PRR are known to occur in the greenhouse and nursery (Fortwood Street Greenhouse, UTC, Chattanooga, TN), rootstock survival was a major concern. In 2018, scions were grafted to *C. mollissima*, *C.* dentata, and F_1 and BC_3F_2 hybrids of *C. mollissima* x *C. dentata*. In 2019, scions were grafted primarily to *C. mollissima* to improve statistical analysis of subsequent light chamber experiment, and to obtain rootstock resistance to chestnut blight and PRR more uniformly. Rootstocks were sourced from researchers at TACF (Sara Fitzsimons, Penn State University), U.S. Forest Service (James McKenna, Hardwood Improvement), commercial nurseries (Route 9 Cooperative and Forrest Keeling Nursery), and stock grown on site at the Fortwood Greenhouse.

Rootstocks in 2018 were potted in 7.19 L or 14.76 L Rootmaker pots (Stuewe and Sons, Inc.), depending on size, though in 2019 all rootstocks were potted in 14.76 L containers for standardization. Sun Gro Metro-Mix 852 was used as the potting medium and fertilized with Osomocote Plus 15-9-12 slow release (8-9 months) and Peters Professional water soluble 21-7-7 Acid Special fertilizer. Rootstocks were treated with systemic fungicide (Allude) to prevent infection by *P. cinnamomi*.

Grafting

Scionwood was grafted between May and July of 2018 and 2019. During the 2018 season, 14 genotypes from Alabama (9 *C. dentata* and 5 *C. alabamensis*) were grafted to a variety of rootstocks, totaling 155 graft attempts. Grafting technique was determined by

scionwood diameter. The whip-and-tongue was used when scion-rootstock diameter matched, and the bark-flap used when they did not. During the 2019 season, 20 genotypes (19 *C. dentata* and 1 *C. alabamensis*) from Alabama, Georgia, Kentucky, and Tennessee were primarily grafted to *C. mollissima* rootstocks, though F_1 and BC_3F_2 hybrids were used sparingly, totaling 215 attempts. Grafting technique was limited to the bark-flap as required by small scion diameter. Graft survival was only reported in overall totals, not by graft type or rootstock.

Each graft union was wrapped in Parafilm nursery grafting tape to prevent desiccation and secure scion to rootstock. Additionally, the exposed cut surface of the scion tip was sealed with grafting wax. Prior to the increased heat of summer, grafted stock was allowed to acclimate in the greenhouse for two weeks before being moved to the nursery, where they were placed on irrigation lines in part-sun. Late season grafts were immediately placed in the nursery due to excessive heat in the greenhouse. Grafts were monitored for survival and watered daily, as needed.

Light Chamber Design and Experiment

Following the day-night regime established by Baier et al. (2012; 16 hr photoperiod), an experiment was designed to test the effect of an artificial, high light environment on the reduction in time to floral initiation and development of grafted American chestnut. This experiment was performed twice: December 2018 to March 2019 and November 2019 to February 2020, 100 days per trial. The 2019-2020 trial attempted to address replication issues of the previous year by standardizing pot size, graft type, and rootstock, though poor graft survival restricted the study group significantly (n = 20). Additionally, plant stress and photoinhibition (Powell, 1984; Ruban, 2009) were considered in the 2019-2020 trial. Plants in both treatments

were rotated in a serpentine pattern each week to avoid overexposure to high intensity light at the same angle of incidence for prolong periods.

Two 1.52 m x 1.22 m open-top chambers (OTC) were constructed by enclosing sections of a greenhouse bench with non-transparent plastic sheeting (PandaFilm; Figure 4A). In each chamber, a single fixture (PhotonMax 1000W DE) with a high pressure sodium bulb (HPS; 400-700 nm; Figure 5A) was hung in the center overhead at 133 cm from the bench top (Figure 4B). One OTC was assigned as the no-supplemental light treatment, where the light fixture would remain off, illuminated only by solar radiation through the greenhouse. The other OTC was assigned to function as the 16 hr photoperiod treatment, with the fixture illuminated. The HPS bulb and fixture were rated for 2,050 µmol $s^{-1}m^{-1}$ at 1000W output (Figure 5B). To account for radiant heat given off from the light fixture, two fans were arranged (one outside overhead and one inside) to circulate air and minimize temperature difference between chambers. The fixture in the 16 hr photoperiod treatment was controlled by an automatic programmable timer set to run from 6:00am to 10:00pm, seven days a week. A bench-top irrigation line was installed, where each container was watered by individual micro-sprayers, twice a week as needed. As these experiments took place over winter, the greenhouse was heated to 25 °C.

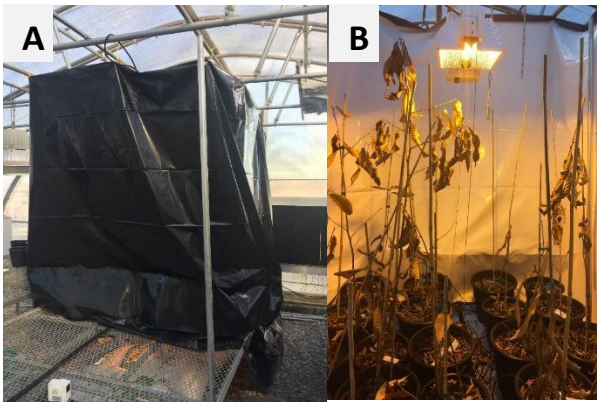
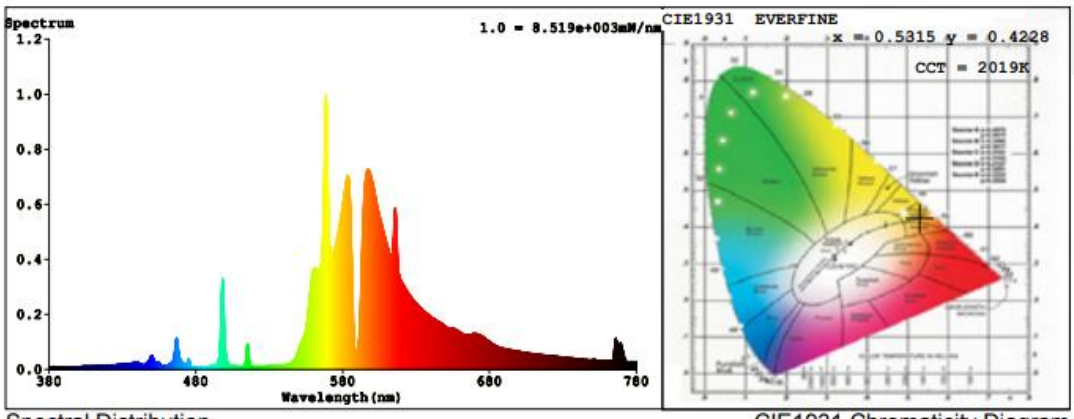


Figure 4 Light Chamber Interior and Exterior

SPECTRAL ANALYSIS:



A

Spectral Distribution — CIE1931 Chromaticity Diagram

SPECIFICATIONS:

ITEM	SPECIFICATION	PH-1000HPS-F-DE	PH-1000HPS2-F-DE	PH-1000HPS3-F-DE
		DETAILS		
GENERAL PERFORMANCE	Wattage	1000W		
	PPF	2,050 umol/s		
	Ballast Efficiency	93%		
	Lifetime (Lamp)	10,000 Hours		
	BTU Output	3,412 BTU's/Hour		
ELECTRICAL	Frequency	50/60Hz		
	Input Voltage	120V	277V	347V
	THD	≤10%		
	Power Factor	≥.98		
PHYSICAL	Start/Warm up	5 Mins.		
CERTIFICATION	Certifications	ETL, CETL		

B

Figure 5 PhotonMax 1000W DE HPS Spectral Distribution and Specifications

Dormant surviving grafts were randomly assigned to one of two light treatments (16 hr photoperiod or no-supplemental light). However, as the study group depended upon graft survival and not all genotypes survived evenly or at all, not all genotypes were represented in the two treatments. Additionally, sole surviving ramets or those of potentially rare genotypes were disproportionately assigned to the 16 hr treatment to maximize breeding potential.

The 2018-2019 study group consisted of 12 surviving Alabama genotypes from two species (7 *C. dentata* and 5 *C. alabamensis*) totaling 39 ramets (28 *C. dentata* and 11 *C.*

alabamensis). Ramets were divided into each treatment (Figure 6): 16 hr photoperiod ($n_1 = 23$) and no-supplemental light ($n_2 = 16$). The treatment ran for 100 days (December 10, 2018 to March 20, 2019) and grafts in each chamber were monitored 2 days a week. Observations recorded phenological events (1) bud break (BB), (2) catkin emergence (CD; earliest signs of developing catkins), (3) mature catkin collection (CM). Four ramets (two from each chamber) failed during the treatment and were removed. In both treatments, light intensity (µmol $s^{-1}m^{-1}$) and temperature (°C) measurements were taken (LiCor 6800) at 9 designated locations (Figure 6) at 4 levels each: 128 cm, 94.5 cm, 60 cm, and 19.5 cm from the bench top during both cloudy and clear weather conditions (Figure 7). Differences between light intensity and temperature between treatments were tested by a two-sample *t*-test using SAS (TTEST function; SAS Institute © 2018).

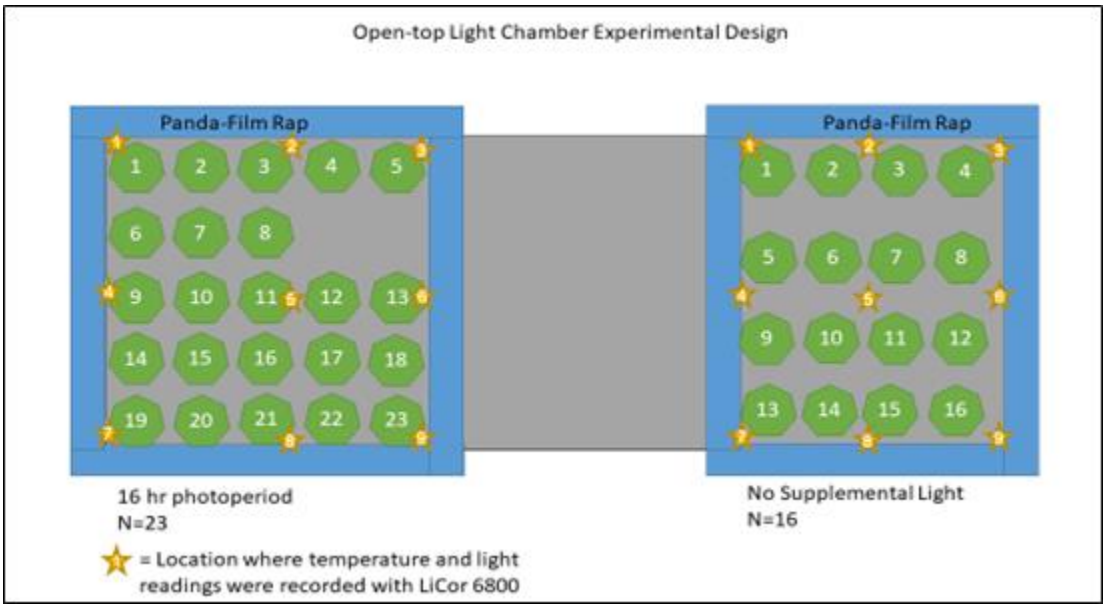


Figure 6 Light Trial 1 (2018-2019) Design with Light and Temperature Measurement Locations

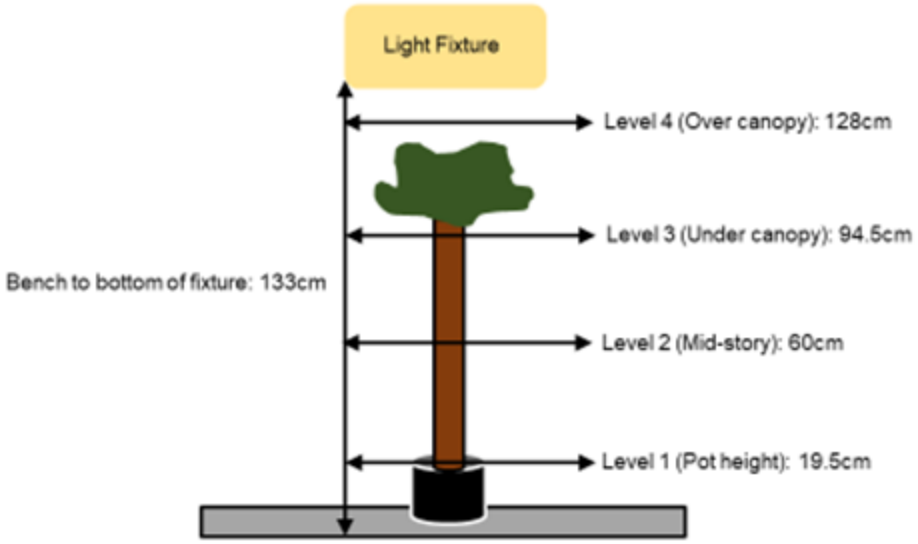


Figure 7 Illustration of Light and Temperature Measurement Levels

The number of days to each event (BB, CD, CM) were analyzed through multiple two-way analysis of variance (ANOVA) to test for effects of (1) photoperiod and genotype, and (2) photoperiod and species on the reduction in time to each event. These analyses were performed in SAS (SAS Institute © 2018) using the PROC GLM function.

To induce dormancy to expedite the second light experiment start date, surviving grafts from 2019 were placed in a darkened walk-in cooler and chilled at 4 °C for 12 days (October 31—November 11, 2019). The 12 day chilling period was chosen arbitrarily and ended when grafted stock appeared to be dormant. Dormancy is a difficult event to define and observe precisely; literature suggests a combination of two definitions: (1) cessation of cell elongation and/or (2) cessation of cell division (Lang, 1987; Rohde and Bhalerao, 2007). At the end of the 12 day chilling period, it was determined that surviving grafts had ceased apical growth (cell elongation) and many grafts had ceased photosynthesis (abscission of brown leaves).

Surviving dormant grafts from the 2019 season were randomly divided into the same 16 hr photoperiod and no-supplemental light treatments, though randomization was adjusted to avoid over representation of a genotype into a single treatment. Again, special consideration was given to sole surviving ramets of a particular genotype, which were placed in the 16 hr photoperiod treatment to maximize breeding potential. The 2019-2020 trial began on November 13, 2019 and ran 100 days, ending February 21, 2020. Treatments consisted of 7 *C. dentata* genotypes, totaling 19 ramets: 16 hr photoperiod ($n_1 = 11$), no-supplemental light ($n_2 = 8$; Figure 8). During Light Trial 2 four grafts failed and were removed from the trial. Light intensity and temperature measurements were taken in the 2019-2020 trial by the LiCor 6800 at the same 9 positions of the previous year, though were only at three levels (128 cm, 94.5 cm, and 19.5 cm; Figure 7. The number of days to the same three phenological events (BB, CD, and CM) were recorded and analyzed in the same manner as the first trial.

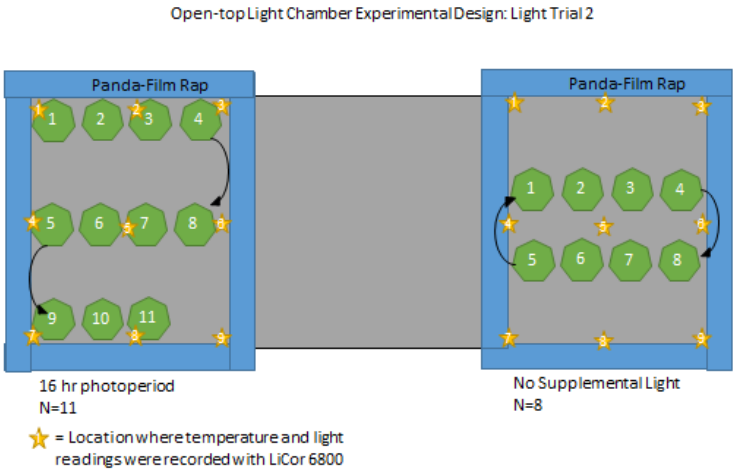


Figure 8 Light Trial 2 (2019-2020) Design with Light and Temperature Measurement Locations

Pollen and Seed Collection and Storage

Catkins produced from flowering grafts were collected and laid out onto clean panes of glass (Figure 9A). After 24 hours, anthers dehisced onto glass (Figure 9B). Pollen was scraped by a razor blade and transferred into glass vials (Figure 9C). Pollen vials were cold-stored at -10 °C in a sealed desiccator until orchard-grown American chestnuts/hybrids were receptive. Pollination was performed by shaking vial to collect pollen on vial top, then the top was spread over the stigmas of receptive female flowers. Seed produced from controlled crosses were shucked from the burrs and stored in plastic freezer bags filled with slightly moistened peat. Seeds were stored at 4 °C for three to four months to allow stratification prior to planting in 2020.

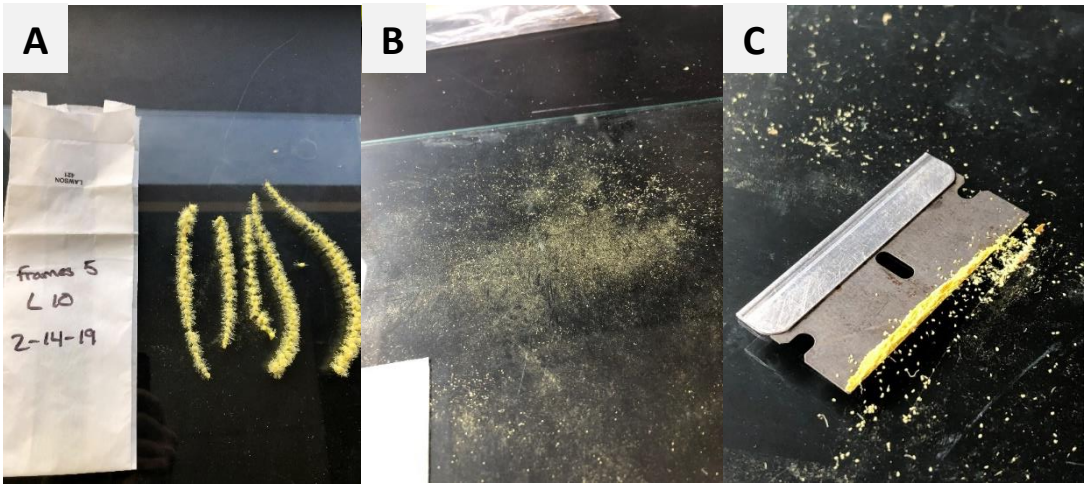


Figure 9 Pollen Processing

CHAPTER III

RESULTS

Grafting Success

2018: May—July

Fourteen of the 38 genotypes collected, all from Alabama (Figure 3; Tennessee scions were excluded inadvertently), were grafted to a variety of rootstocks. Graft type depended on scion-rootstock diameter, but the bark-flap method was used primarily (Figure 10). In total, 155 grafts were attempted across the 14 genotypes, where 12 genotypes—39 ramets (25.2% survival)—survived (Table 2) to be included in the first light experiment (December 10, 2018). The 12 surviving grafted genotypes represent 7 *C. dentata* and 5 *C. alabamensis* individuals. Note that grafts in the first season were performed by an experienced grafter, Dr. J Hill Craddock.

Figure 10 Bark-Flap Grafting Technique

Observations of graft survival noted high initial graft-failure, where many scions broke bud but did not survive longer than a few days. Of the initial survivors, about half failed after a few weeks, and halved again after a few months. Survival rates were relatively stable after a few months. Few grafts failed through fall of 2018, although additional grafts failed throughout the duration of the light experiment.

Additionally, one *C. dentata* ramet (T3) produced male catkins just three months after grafting under natural conditions in the nursery (Figure 11). Pollen was collected and cold-stored for breeding the following breeding season (June—July 2019).

Figure 11 Flowering Ramet in Natural Light Conditions

2019: May—July

Twenty of the 69 genotypes (6 AL, 6 GA, 2 KY, and 6 TN; Figure 3) were grafted primarily to *C. mollissima* using the bark-flap technique (Figure 10), although *C. dentata* and F_1, BC_1F_1, BC_3F_2 hybrids were used as *C. mollissima* rootstock was depleted. In total, 215 grafts were attempted, though survival percentage was calculated from 159. The walk-in cooler in which the scions were stored malfunctioned late in the grafting season. Based on 159 attempts with viable scionwood, 9 *C. dentata* genotypes (Figure 3)—19 ramets (11.9% survival)—survived to begin second light experiment (November 13, 2019; Tables 2 and 3). Note that grafts in the second season were performed by the author and a reduction in survival percentage may be attributable to less experience.

Graft survival in the second season largely paralleled those of the first, where graft failure was highest initially, but became less frequent the longer a graft survived. Similar to the first year, three grafts failed during the course of the second light experiment, which reduced the graft survival to 10.1% (Table 3).

Table 2 Graft Survivorship for 2018 and 2019 seasons by genotype.
[1] Collecting season reflects the winter season in which scionwood was collected: 1 = 2017-2018; 2 = 2018-2019; 1,2 = collected in both seasons. [2] Graft survivorship by year: 2018 = 25.02%; 2019 = 10.1%. [3] Previously bred indicates whether genotype has been conserved in the TACF breeding program, based on TACF database records (*DentataBase*).

Name	Species	State	County	Location	Collecting Season [1]	Grafting Year [2]	Previously Bred [3]	# Ramets	Flowers Induced
5	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1	2018	Y	2	Y
10101A	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1,2	2018, 2019	N	5	Y
7CN	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1,2	2018, 2019	Y	1	
Cheaha08	*C. dentata*	AL	Talladega	Adams Gap	1	2018	N	3	Y
Choco01	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	N	3	Y
Choco02	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	N	1	Y
Choco22	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	N	1	
Choco27	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	N	1	
Choco28	*C. dentata*	AL	Calhoun	Choccolocco Mountain	1	2018	N	1	
Frames03	*C. dentata*	AL	Cleburne	Frames Property	1	2018	Y	1	
Frames05	*C. dentata*	AL	Cleburne	Frames Property	1	2018	Y	2	Y
Hutch04	*C. dentata*	AL	Cleburne	Hutchinson Property	2	2019	N	2	
MS38	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1,2	2019	N	1	Y
MS42	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1	2018	N	2	Y
T3	*C. dentata*	AL	Talladega	Talladega National Forest	1	2018	Y	5	Y
GAMU9-A	*C. dentata*	GA	Murray	Fort Mountain	2	2019	Y	5	
GAUN5	*C. dentata*	GA	Union	Brasstown Bald	2	2019	Y	3	
GAUN3XGAWA7	*C. dentata*	GA	Union	Brasstown Bald	2	2019	Y	1	
Bradford Trail 02	*C. dentata*	TN	Henderson	Natchez Trace State Park	2	2019	N	1	
Signal Mtn01	*C. dentata*	TN	Hamilton	Signal Mountain	2	2019	Y	1	Y

Table 3 2019 Graft Survivorship by State

[1] Attempted grafts reflect total number of grafts attempted, however, Percent Survival was calculated without consideration of 56 attempts with freeze-damaged scionwood. Survival at time of Light Trial 2 = 19 (11.9%). Three graft failures during treatment reduced survival percentage to 10.1%, shown below.

2019 Graft Survivorship By State:	**AL**	**GA**	**KY**	**TN**	**TOTAL**
Attempted Genotypes:	6	6	2	6	**20**
Attempted Grafts [1]	79	60	18	58	**215**
Surviving Genotypes	3	3	0	2	**9**
Surviving Grafts	5	8	0	3	**16**
Percent Survival: Genotypes	50.00	50.00	0.00	33.33	**45.00**
Percent Survival [1]	6.33	13.33	0.00	5.17	**10.1**

Light Chamber

Light Trial 1: 2018-2019

Dormant surviving grafts from 12 of 33 genotypes—7 *C. dentata* (27 ramets) and 5 *C. alabamensis* (10 ramets)—were placed in assigned chambers (16 hr photoperiod supplemental light, n = 23; no supplemental light, n = 16; Figure 6) on 10 December 2018 and terminated on 22 March 2019.

16 hr photoperiod chamber: Bud break (BB) was first observed at 10 days, and for all ramets (n_{bb1} = 23) at 30 days (Table 4). Average days to BB = 18.72 days (*SD* = 5.79; Figure 12). Catkin Development (CD) was observed between 27 and 39 days on 9 ramets (n_{cd1} = 9; Table 4; Figure 13). Average days to CD = 34.11 days (*SD* = 5.40; Figure 14). Eight individuals (n_{cm1} = 8; 2 Alabama chinquapin, 6 American chestnut) produced mature male catkins (Figure 15), where observations recorded Catkin Maturation (CM) date of collection. The first CM occurred at 43 days and the last at 88 days (Table 4). Average days to CM = 70.5 days (*SD* = 13.41).

No supplemental light chamber: BB was first observed at 28 days, and for all ramets (n_{bb2} = 15) at 62 days (Table 4). Average days to BB = 44.33 days (*SD* = 9.07; Figure 12). CD was observed on 7 ramets (n_{cd2} = 7), between 43 and 70 days (Table 4). Average days to CD = 59.00 days (*SD* = 9.47; Figure 14). Only one ramet maintained catkins to maturity (n_{cm2} = 1), which occurred at 97 days (Table 4; Figure 16). The other 6 ramets aborted developing catkins prior to maturation.

Table 4 Light Trial 1 Days to BB, CD, CM by Treatment
Trial began December 10, 2018 and was terminated on March 20, 2019: total of 100 days. All grafts were dormant at time trial began. All values represent number of days to observed phenological event: bud break (BB), earliest indication of catkin development or emergence (CD), and catkin maturation (day catkin was collected; CM). Not all ramets demonstrated CD or CM, indicated by "-".

Treatment	Plant Code	Genotype	BB	CD	CM
16 hr	L1	Choco01	18	27	43
16 hr	L2	Frames5	10	-	-
16 hr	L3	Frames5	27	39	-
16 hr	L4	MS42	15	27	72
16 hr	L5	Choco02	20	-	-
16 hr	L7	Cheaha08	10	-	-
16 hr	L8	Choco22	18	-	-
16 hr	L9	5	18	37	76
16 hr	L10	Frames 5	15	27	65
16 hr	L11	T3	15	-	-
16 hr	L12	Frames3	20	-	-
16 hr	L13	Choco27	27	-	-
16 hr	L14	Cheaha08	18	37	76
16 hr	L15	Choco01	27	37	65
16 hr	L16	10101A	10	-	-
16 hr	L17	10101A	30	37	88
16 hr	L18	Choco02	23	-	-
16 hr	L19	Cheaha08	15	-	-
16 hr	L20	10101A	23	-	-
16 hr	L21	Choco28	15	-	-
16 hr	L22	T3	15	-	-
16 hr	L23	T3	23	39	79
No Supp	C1	T3	48	53	-
No Supp	C2	Choco01	43	65	-
No Supp	C3	Unknown01	48	-	-
No Supp	C4	Choco28	43	-	-
No Supp	C5	MS42	37	-	-
No Supp	C6	Frames5	30	43	-
No Supp	C7	Frames5	43	-	-
No Supp	C9	T3	48	-	-
No Supp	C10	Frames5	57	70	-

Table 4 Continued

Treatment	Plant Code	Genotype	BB	CD	CM
No Supp	C11	Choco02	62	68	97
No Supp	C12	Cheaha08	43	-	-
No Supp	C13	T3	43	57	-
No Supp	C14	10101A	53	-	-
No Supp	C15	5	28	57	-
No Supp	C16	10101A	39	-	-

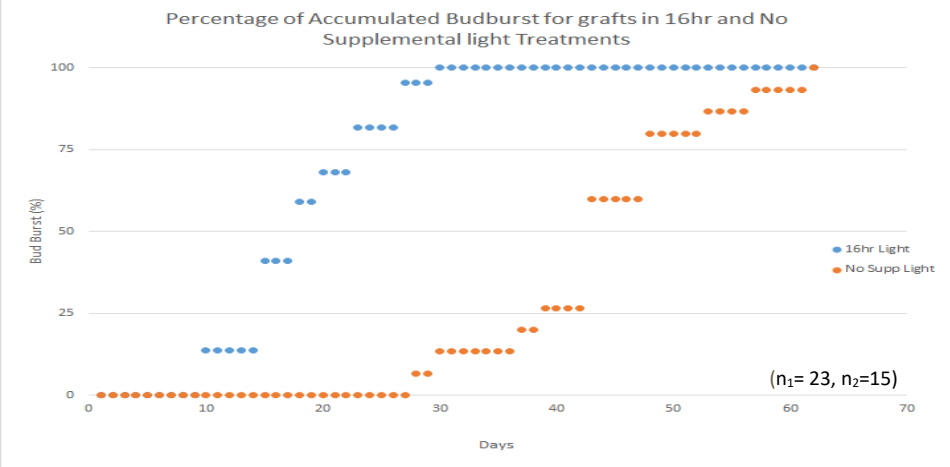


Figure 12 Percent of Accumulated Days to Phenological Event BB

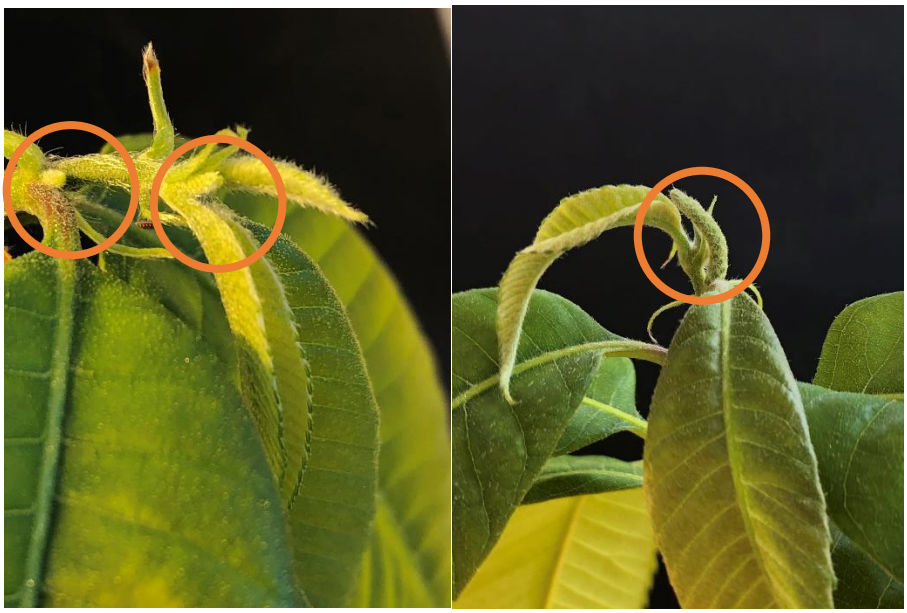


Figure 13 Developing Catkins on Light-Treated Grafts

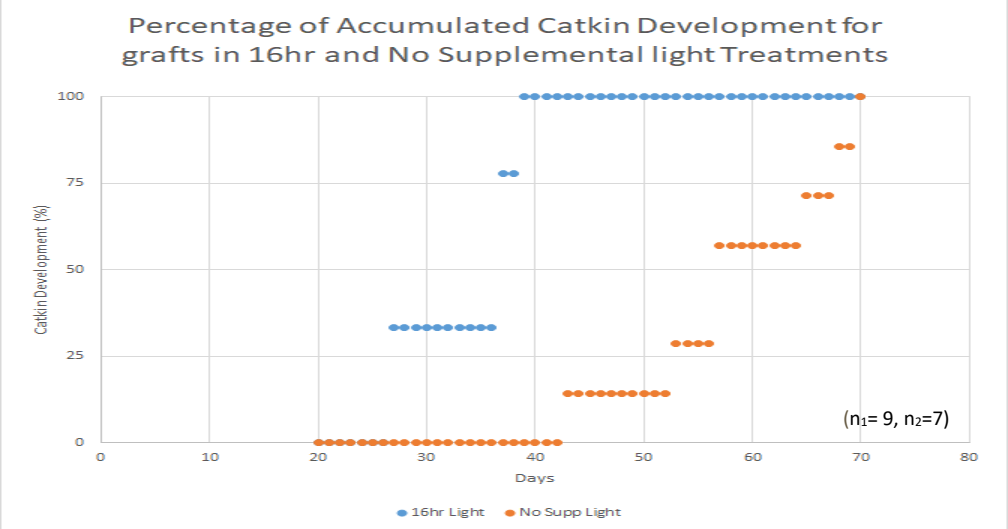


Figure 14 Percent of Accumulated Days to Phenological Event CD

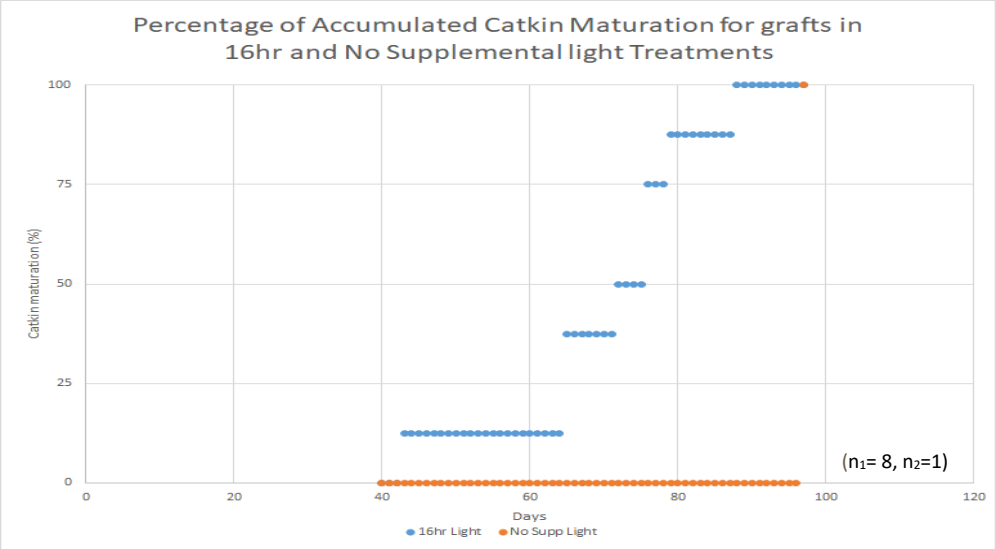


Figure 15 Percent of Accumulated Days to Phenological Event CM

Data Analysis: Average days to each phenological event were reduced in the 16 hr photoperiod treatment: BB reduced by 25.61 days, CE reduced by 24.89 days, and CM reduced by 26.50 days (Table 5). A two-factor ANOVA (genotype and photoperiod) found significant differences between BB ($n_1 = 23$, $n_2 = 15$, $F = 65.86$, $p = <0.0001$) and CE ($n_1 = 9$, $n_2 = 7$, $F = 10.63$, $p = 0.0311$) but not in CM ($n_1 = 8$, $n_2 = 1$; Figure 13). However, 8 individuals in the supplemental light treatment produced male flowers, while the no-supplemental light treatment only produced one. All tests found no significance between genotypes, species, or interaction between factors.

Table 5 Light Trial 1 Average Days to BB, CD, CM by Treatment.
Values reported in mean days to phenological event. Only one ramet in the No Supplemental light treatment produced a mature catkin, therefore, no standard deviation (*SD*) can be calculated. Two-way ANOVA and subsequent Tukey's test indicates days to BB and CD are significantly different (*).

	Phenological Event					
Treatment	Bud Break	*SD*	Catkin Emergence	*SD*	Catkin Maturation	*SD*
16hr	18.72*	5.79	34.11*	9.07	70.50	13.41
No Supp	44.33	5.40	59.00	9.47	97.00	-
Reduction	25.61		24.89		26.5	

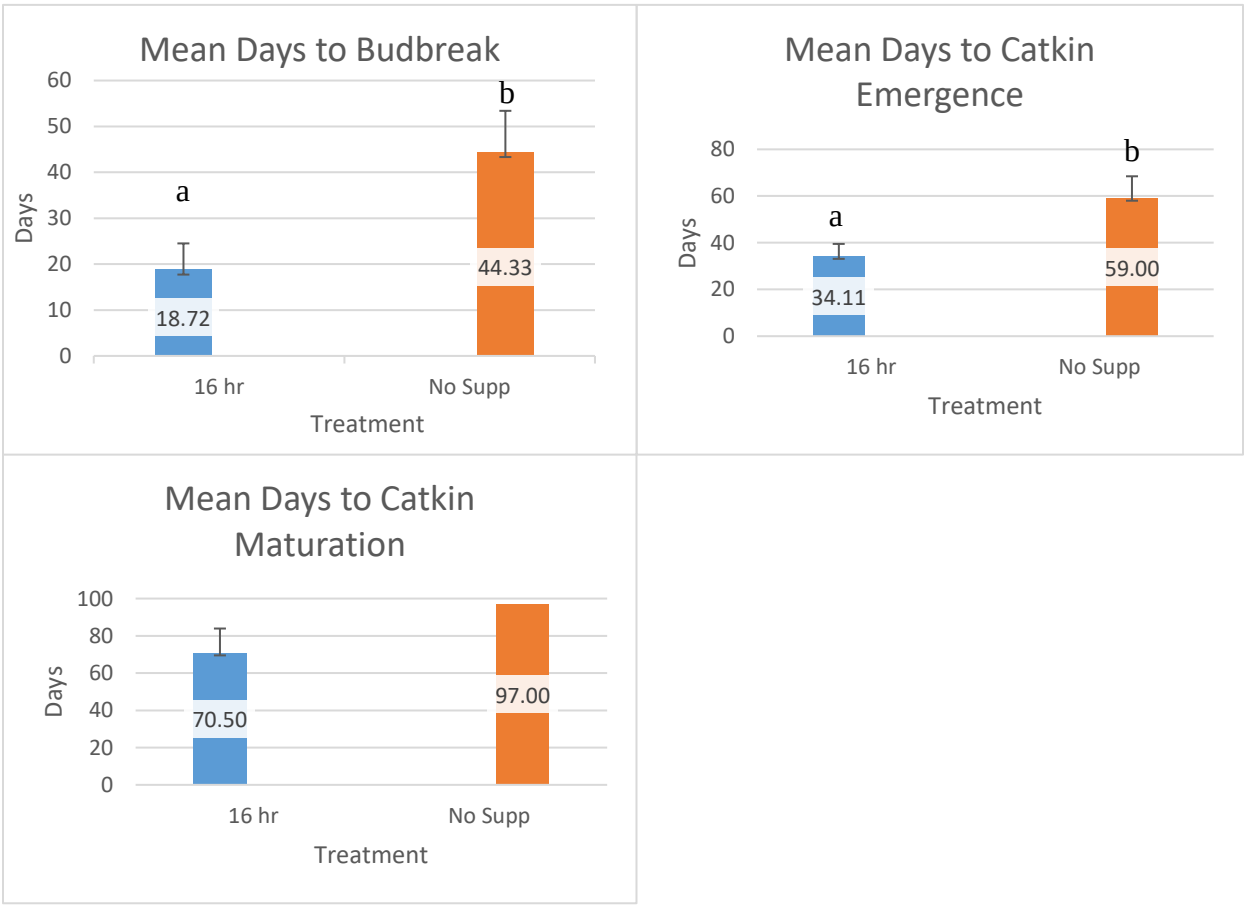


Figure 16 Mean Days to Phenological Event per Treatment of Grafted American chestnut and Alabama chinquapin

Light and Temperature: The 16 hr photoperiod chamber averaged 348.05 µmols $s^{-1}m^{-1}$ and 29.78 ℃ and the no-supplemental chamber averaged 72.11 µmols $s^{-1}m^{-1}$ and 29.94 ℃ (Table 6). Temperatures recorded in each chamber were not significantly different ($t(203) = 1.74$, $p = 0.0832$).

Table 6 Trial 1 Light (µmol s^{-1} m^{-1}) and Temperature (℃) Averages per Position.
Light Trial 1: Light and temperature measurements were taken by LiCor6800 at 9 positions at 4 levels: 128 cm, 94.5 cm, 60 cm, and 19.5 cm from the bench top during both cloudy and clear weather conditions. Two-sample t-test found no significant temperature differences between chambers ($t(203) = 1.74$, $p = 0.0832$).

	16 hr Light Chamber		No-Supplemental Light Chamber	
Position	**Light (µmol $s^{-1}m^{-1}$)**	**Temp (℃)**	**Light µmol $s^{-1}m^{-1}$)**	**Temp (℃)**
1	173.42	29.55	93.40	29.86
2	271.34	29.75	57.92	29.86
3	181.44	29.98	46.10	29.94
4	193.94	29.43	101.04	29.96
5	1415.23	30.07	63.81	29.94
6	239.15	29.80	56.05	29.93
7	146.44	29.65	100.06	30.01
8	311.97	29.80	71.96	29.98
9	199.49	29.97	58.63	29.96
Average	**348.05**	**29.78**	**72.11**	**29.94**

Plant Stress: Because plants were not rotated during the first trial, many grafts showed signs of stress and photoinhibition through yellow and brown leaves. After the light trial ended, plants were moved into natural light conditions of the nursery, yet stress symptoms persisted for three months, at which point new healthy leaves/shoots emerged.

Light Trial 2: 2019-2020

This trial ran 100 days: November 13, 2019 to February 21, 2020. The trial consisted of 8 genotypes of *C. dentata*—19 ramets— divided between the two light treatments: 16 hr photoperiod ($n_1 = 11$) and no-supplemental light ($n_2 = 8$; Figure 8).

16 hr Photoperiod Treatment: BB was first observed at 28 days and was observed in all ramets ($n_{bb1} = 8$) by 49 days (Table 7). Note that 3 grafts failed prior to BB. Average days to BB = 35.13 days (*SD* = 7.1). CD was only observed by 49 days ($n_{cd1} = 2$; Table 7). CM was recorded on 1 graft at 90 days (Table 7). The other graft produced stunted catkins with few stamens and were not collected due to small size.

No-Supplemental Light Treatment: BB was first recorded at day 63, and in all ramets by 91 days ($n_{bb2} = 6$). Average BB = 80.33 days (*SD* = 9.83; Table 7). CD and CM were not observed.

Table 7 Light Trial 2 Days to BB, CD, CM by Treatment
Trial ran 100 day: Nov 13, 2019 to Feb 21, 2020. All grafts were dormant at time trial began. All values represent number of days to observed phenological event: bud break (BB), earliest indication of catkin development or emergence (CD), and catkin maturation (day catkin was collected; CM). Not all ramets survived or demonstrated BB, CD, or CM, indicated by "-". Average days to BB = 33.14 days (SD = 4.7).

Treatment	Plant Code	Genotype	BB	CD	CM
16 hr	3	SIGNAL MTN 01	31	49	90
16 hr	1	BRADFORD TR 2	28	49	-
16 hr	2	BRADFORD TR 2	-	-	-
16 hr	7	HUTCH04	-	-	-
16 hr	8	HUTCH04	31	-	-
16 hr	10	GAUN5	49	-	-
16 hr	12	MS38	28	-	-
16 hr	13	GAMU9-A	38	-	-
16 hr	15	GAMU9-A	38	-	-

Table 7 Continued

Treatment	Plant Code	Genotype	BB	CD	CM
16 hr	16	GAMU9-A	38	-	-
16 hr	18	GAMU9-B	-	-	-
No Supp	4	SIGNAL MTN 01	-	-	-
No Supp	6	HUTCH04	80	-	-
No Supp	9	GAUN5	91	-	-
No Supp	11	GAUN5	84	-	-
No Supp	14	GAMU9-A	63	-	-
No Supp	17	GAMU9-A	77	-	-
No Supp	19	GAMU9-B	-	-	-
No Supp	20	GAUM3XGAWA7	87	-	-

Data Analysis: Days to BB were reduced between treatments by 45.21 days on average. A two-way ANOVA indicate significant differences between photoperiod with respect to BB (F = 107.61; p = 0.0005). No observations of CD or CM occurred in the No-supplemental light treatment.

Light and Temperature: The 16 hr photoperiod chamber averaged 474.65 µmols $s^{-1}m^{-1}$ and 26.01 °C and the no-supplemental chamber averaged 65.48 µmols $s^{-1}m^{-1}$ and 25.84 °C (Table 8). Temperatures between treatments were found to be significantly different ($t(52) = 2.25$, p = 0.0288).

Table 8 Trial 2 Light (µmol s^{-1} m^{-1}) and Temperature (°C) Averages per Position.
Light Trial 2: Light and temperature measurements were taken by LiCor6800 at 9 positions at 3 levels: 128 cm, 94.5 cm, and 19.5 cm from the bench top during both cloudy and clear weather conditions. Two-sample t-test found significant temperature differences between chambers ($t(52) = 2.25$, $p = 0.0288$).

	16 hr Light Chamber		No-Supplemental Light Chamber	
Position	**Light (µmol $s^{-1}m^{-1}$)**	**Temp (°C)**	**Light µmol $s^{-1}m^{-1}$)**	**Temp (°C)**
1	160.00	25.77	62.00	25.83
2	301.50	25.77	54.67	25.83
3	239.17	25.80	60.00	25.83
4	185.50	25.80	70.00	25.83
5	2538.00	26.07	74.33	25.80
6	225.67	26.20	61.33	25.90
7	167.33	26.17	83.33	25.80
8	274.17	26.30	72.33	25.80
9	180.50	26.23	51.33	25.93
Average	474.65	26.01	65.48	25.84

Pollen Collection, Controlled Pollinations, and Seed Collection

Nine ramets produced catkins, only 3 of 8 genotypes (Cheaha08, T3, and Frames5) produced enough pollen to be used effectively in controlled pollinations. Controlled pollinations took place at experimental orchards at Tennessee Tech University (TTU; Cookeville, Tennessee) and the TACF Meadowview Research Orchard (Meadowview, VA). One cross was made at TTU onto a BC_3F_1 hybrid: TTU-M13 x Cheaha08; and two crosses at Meadowview onto *C. dentata*: AN-65 x T3 and AN-86 x Frames5 (Table 9). While genotypes T3 and Frames5 were already represented in the TACF breeding program, Cheaha08 had not been bred prior to this study. A total of 80 seeds were harvested in October 2019 (Table 9). The no-pollen-control bags on TTU-M13 x Cheaha08 contained 4 nuts, when none should be expected.

Table 9 Pollination and Seed Collection

Pollen collected from grafts in Light Trial 1 over winter 2018-2019 was cold stored until July 2019. No-pollen control bags from TTU-M13 x Cheaha08 contained seeds, indicating potential contamination from undesired adjacent males prior to hand-pollinations. [1] BC_3F_1 hybrid, *C. dentata* x *C. mollissima*; Tennessee Tech Backcross Orchard, TTU, Cookeville, TN; [2] *C. dentata*; TACF Research Farms, Wagner Orchard, Meadowview, VA. [3] Burr data not available from AN-65 and AN-86 crosses.

Mother	Father	Cross Year	Pollen Bags (#)	Control Bags (#)	Burrs (#)[3]	Total Seeds (#)	Seeds in Control Bags
TTU-M13 [1]	Cheaha08	2019	21	2	42	21	4
AN-65 [2]	T3	2019	15	3	-	11	0
AN-86 [2]	Frames5	2019	25	3	-	52	0

CHAPTER IV

DISCUSSION

This study sought to conserve new and under-sampled genotypes of American chestnut from southern populations by graft propagation. Then, to accelerate their introduction into the TACF breeding program by growing survivors under a high light environment to induce flowering. Species identification errors were made during the first scionwood collecting season, winter of 2017-2018. As only one location was visited during the growing season, the remaining trees were identified through winter characters (Petrides et al., 1988), which resulted in the collection of non-target species. In the Ruffner Mountain (Birmingham, AL) population where multiple *Castanea* spp. co-occur, winter identification led to the collection of a confounding member of *Castanea* currently under debate: *C. alabamensis* (Perkins et al., 2019). Although this study collected scionwood from 93 genotypes, graft success—2018: 25.01%, and 2019: 10.1%—was less than expected (38% achieved by McKenna and Beheler, 2016). Different from the present study, McKenna and Beheler (2016) grafted all *C. dentata* scions to *C. dentata* rootstocks. This reduction in graft survival is most likely due to grafting experience, particularly in 2019, but the use of interspecific scion-rootstock combinations may also be a factor (Huang, 1996).

Additionally, McKenna and Beheler (2016) reported significant differences in graft survival between genotypes, which was also observed in the present study. Though differences in genotype survival in this study may be due to variations in quality of scionwood collected from

the ortet, as not all samples collected were of desired diameter. Nonetheless, surviving grafts consist of 20 genotypes (16 *C. dentata* and 4 *C. alabamensis*) which represent conserved individuals from southern counties with fewer than 10 conserved individuals. This demonstrates that graft propagation is a viable method of *ex situ* conservation of under-sampled populations.

Containerized grafts also contributed to the greater research community, where leaf tissue was collected from each genotype and supplied to an on-going TACF landscape genomics study. Had these grafts not been available, researchers would have had to expend significantly more time and resources sampling leaves from their *in situ* ortets. Also, the collection and grafting of the non-target species *C. alabamensis* contributed to another study (Perkins et al., 2019), and represents the first *ex situ* conservation of this species. Pollen collected from *C. alabamensis* can be used to investigate potential hybridization between *C. dentata* through controlled crosses.

This study is the first attempt at speed breeding grafted American chestnut. Although the power of statistical analysis of this study was limited by small sample sizes, results indicate that extended photoperiod (16 hr) under high light conditions can reduce the time to both vegetative growth (BB reduced by 25.61) and male flower production (CD and CM reduced by 24.89 and 26.5 days, respectively), compared with the no-supplemental light treatment. Light Trial 2 (2019-2020) sought to improve statistical power. The 12-day cooler treatment used to induce dormancy for the second trial was effective at achieving earlier dormancy. However, release from dormancy was delayed compared to the previous trial. Observations of BB, CD, and CM were delayed by 16.41, 14.89, and 18.50 days, respectively. Although these events were delayed in the second light trial, similar differences between treatments were observed. BB was reduced by 45.21 days between treatments; an additional reduction of 19.60 days compared to the first trial. During the second trial, results indicate that temperature did vary between treatments, though not

in the first trial. This is likely the result of an inadvertent change in experimental design between Light Trial 1 and 2. A fan placed over the 16 hr treatment during Light Trail 1 was moved in error, no long circulating air directly over the 16 hr chamber during Light Trail 2. With less overhead circulation, the 16 hr treatment was warmer than the no-supplemental light chamber.

Like the seedling study by Baier et al. (2012), accelerated growth under high light conditions produced viable pollen more rapidly than would be available under natural and reference chamber conditions (i.e. no-supplemental light). However, different from Baier et al. (2012) no female flowers were produced in artificial light portion of this study. Interestingly, during the first trial 7 ramets in the no-supplemental light treatment initiated male catkin development, all but one aborted development. Further investigation is required to determine the exact cause, but floral development may have been aborted due to low light conditions (Van Tuyl et al., 1985).

As speed breeding occurs under artificial growth conditions, it can take place over winter where pollen can be produced and stored well in advance of the *in situ* population. This provides a real advantage to chestnut breeders as, demonstrated in this study, pollen was produced between January and March, three months sooner than under natural conditions. Allowing it to be processed in advance to permit timely pollination of desired crosses. Three controlled crosses made during this study: TTU-M13 x Cheaha08, AN-65 x T3 and AN-86 x Frames5, which produced viable seeds. Importantly, seeds produced by TTU-M13 x Cheaha08 represent the conservation of a new genotype, as Cheaha08 had not been bred previously. This provides an important proof of concept: accelerated conservation of novel genotypes from under-sampled southern populations is possible though graft propagation and the use of high light growth chambers.

Though it should be noted that no-pollen control bags placed during the TTU-M13 x Chaha08 cross contained four viable seeds. This result represents potential pollen contamination, probably because the female flowers were bagged too late and thus may have been already pollinated prior to the controlled crosses. The other crosses made in this study (AN-65 x T3 and AN-86 x Frames5) contained no seeds in the no-pollen control bags, indicating that seeds collected were in fact the result of controlled pollination of their respective male parents. However, seeds collected from controlled pollinations have been planted and germination occurred. These seedlings will be grown and maintained in the Fortwood Street Greenhouse and nursery. Surviving seedlings will be planted out in research orchards corresponding to the TACF state chapters from which the parent trees originated.

CHAPTER V

CONCLUSION

Despite the occurrence of rare and unique alleles (Gailing and Nelson, 2017; Li and Dane, 2014; Shaw et al., 2012), southern populations of American chestnut are underrepresented in the TACF breeding program. Given the shade-dominated conditions under which most American chestnuts persist (Paillet, 2002), sexual immaturity will continue to prevent introduction of these genotypes into the program without intervention (Westbrook, 2018). When other methods of propagation rely on sexually mature individuals (somatic embryogenesis), high-risk methods (transplanting), or those unlikely to be successful (rooted cuttings), graft propagation seems to be the most viable alternative.

Asexual propagation through grafting is a low-tech, noninvasive method for conservation of potentially rare American chestnut germplasm. Particularly when *in situ* conditions prevent trees from reaching sexual maturity, graft-propagation allows release from shaded conditions and disease pressure to promote flowering. Collection of pollen from containerized grafted trees allows conservation of genetic resources that were previously unavailable to breeders or difficult to access. Further, the use of high light environments may be able to reduce the generation time to develop a restoration population (Sysoeva et al., 2016). As many new American chestnut individuals are required to advance both the current backcross breeding and the potential transgenic outcross programs (Westbrook, 2019c), the use of these methods may accelerate the 20-30 year timeline suggested.

Finally, a collection of grafted American chestnuts can be conserved in a nursery or germplasm conservation orchard (GCO) to facilitate long-term conservation. Additionally, concentrating genetically diverse individuals from a wide geographic region will reduce logistical challenges for future research. This study has demonstrated the importance of such concentration to the greater research community.

Future Direction

This study relied heavily upon local knowledge of chestnut occurrence by TACF state chapter members. The use of GIS habitat suitability modeling should be investigated to locate new individuals in locations not previously sampled (Fei, 2007). Additionally, to better evaluate the ability of high light environments to reduce flowering time, a more robust study is required. This study was restricted to few surviving grafted trees, thus future studies may require a more robust experiment to include higher numbers of grafted trees and additional light chambers to create replication and investigate multiple photoperiods (i.e. 12 hr). Efforts are ongoing at the state chapter levels of TACF to graft American chestnut on a larger scale. Professional grafters from the commercial nursery industry may be able to provide the number of ramets required for future light experimentation. More importantly, these increased efforts in graft propagation should lead to the incorporation of still more genotypes into the TACF breeding program and maximize conservation of genetic diversity in the restored population.

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APPENDIX A

SCIONWOOD COLLECTION PROTOCOL

Scionwood collection for American chestnut conservation project

The American Chestnut Foundation is seeking scionwood from 100 wild American chestnut trees throughout the South. The objectives of this study are:

1. Conserve genetic diversity of southern populations of American chestnut through grafting.
2. Grow these grafted plants in favorable conditions (including growth chambers) to promote flowering and ease of pollen collection.

The results of this study will provide new and under-sampled sources of pollen to the backcross breeding program to increase its genetic diversity. Grafted plants maintained in a nursery setting that represent a wide geographic span can support other research with reduced logistical obstacles.

Protocol

- Scionwood collection should be targeted to those counties that do not have a representative tree conserved through the breeding program and/or counties with few conserved trees (fewer than 10; Figure 1). Collection from counties with more than 10 conserved trees will be accepted, but not preferred.
- Scionwood collection should be done during winter dormancy only. For most southern states, this occurs between December and March.
- It may be necessary to visit sites twice: once, prior to winter dormancy to ensure species can be identified to American chestnut, particularly in areas where they co-occur with chinquapin. Second, when trees have gone dormant to collect scionwood.

- Scionwood should be approximately the diameter of a pencil and of the previous season's growth (beyond the last bud scale scar). It should be at least 3 inches long and contain 1 or more unopened buds.
    - Ideal scionwood has 2-3 inches between buds, though not common in shade conditions.
    - When the previous season growth was not robust, common with shade dominated trees, previous-season growth may be less than the diameter of a pencil and may be shorter than 3 inches.
        - In these instances, collecting second-year growth is acceptable but should be limited to a case-by-case basis.
- Collect 5-10 pieces of scionwood per tree, when possible. However, use judgement when collecting from small trees as to not jeopardize the survival of the tree by over collecting.
- Take GPS coordinates from each tree using the TreeSnap application for smartphones (https://treesnap.org/) or with a GPS unit or smartphone.
- Place scionwood from a single tree in a one-gallon sealable bag.
- For each tree, place a note card in the bag containing:
    - Date, State, and County
    - Latitude and Longitude
    - Burs present or absent (on ground or tree)
    - Previously used for breeding or not: Yes, No, or Unknown
    - TreeSnap ID (if applicable) or wild tree code from Regional Science Coordinator
    - Public or Private land
- Before sealing, place card in bag, then roll bag from bottom to top remove excess air.

- Write on bag with permanent marker only for personal organization. Do not rely on it for all collection information as it may fade or be scratched off during handling and storage. Temporary writing on bag is fine, but do not rely on them for storage.
- Do not place a damp paper towel in the bag. Moisture from the towel commonly causes mold during storage.
- While in the field, store scionwood in a cooler with cold packs and place in the crisper drawer of the refrigerator until shipping. Do not place in freezer.

Shipping:

- Please ship scionwood no later than 2-3 weeks after collection.
- Contact your Chapter representative for shipping address.
- Ship on Monday (excluding holidays) through Wednesday via 2 day shipping. Do not ship Thursday through Saturday.
- Ship samples on cold packs and in small foam cooler, if possible.

Supplies:

- Sealable gallon freezer bags
- GPS unit or smartphone with TreeSnap (https://treesnap.org/)
- Permanent marker for making notes on cards
- Note cards or paper
- Pruners hand and/or pole, depending on tree size
- Cooler and cold packs

Questions: Email Trent Deason at hvj617@mocs.utc.edu

VITA

Trent Deason was born to parents Tony and Amy Deason and raised Elkton, Kentucky, a small rural community in the southwestern region of the state, where he grew up helping his father tend to his large produce garden. After high school, Trent joined the U.S. Air Force and served six years in radio communication and maintenance. It was his assignment to Ramstein, Air Base, Germany where he was exposed to a more environmentally conscience community, then to central North Carolina where he witnessed the large, intact longleaf pine ecosystem. These experiences led him to pursue an education in environmental science after the end of his enlistment. Trent settled in Chattanooga and attended the University of Tennessee at Chattanooga for his undergraduate degree, earning a B.S. in Environmental Science in 2018, then continuing to pursue a M.S. in Environmental Science. Trent plans to start a career in landscape level environmental stewardship, in environmental consulting or regulation, with a focus on minimizing human impacts to the environment while working to restore degraded landscapes. He also plans to continue his involvement with The American Chestnut Foundation (TACF) as he has developed a passion for species restoration projects, especially as those with committed professionals and volunteers such as TACF.