Conservation, collection, and characterization of *Castanea dentata* germplasm in the South: A Final Report to The American Chestnut Foundation, August 2020.

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Introduction

Breeding for resistance to introduced pathogens *Cryphonectria parasitica* and *Phytophthora cinnamomi*, whether through the TACF backcross breeding program or the proposed transgenic outcross program, requires access to a diverse population of flowering American chestnuts (*Castanea dentata*; Burnham, 1988; Westbrook, 2018; Westbrook et al., 2019). However, in most cases naturally occurring American chestnuts are confined to the understory due to chestnut blight and seldom receive enough light to flower (Paillet, 2002). As a result, breeding is limited to those rare flowering trees. Additionally, difficult terrain and distance from roads often preclude hard to reach individuals from traditional breeding methods (Alexander et al., 2004). Thus, there is a need to employ alternative methods to increase the number of accessible flowering American chestnuts to create a robust hybrid population for restoration (Fei et al., 2007; Westbrook, 2018).

Grafting is the primary vegetative propagation method in *Castanea* (Keys, 1978; McKay and Jaynes, 1969). It requires minimal material (scionwood) from the *in situ* plant from which multiple grafts attempts can be made (Garner, 2013). This is an important distinction from other methods, like transplanting, which risk the loss of the entire plant if unsuccessful (Rex Mann, pers comm, 2019). In reintroduction studies reviewed by Godefroid et al. (2011), transplanted individuals often fail to flower and set fruit, or do so rarely. Grafting, however, has been shown to promote flowing in as early as the first season (McKenna and Beheler, 2016; pers. observation, 2020).

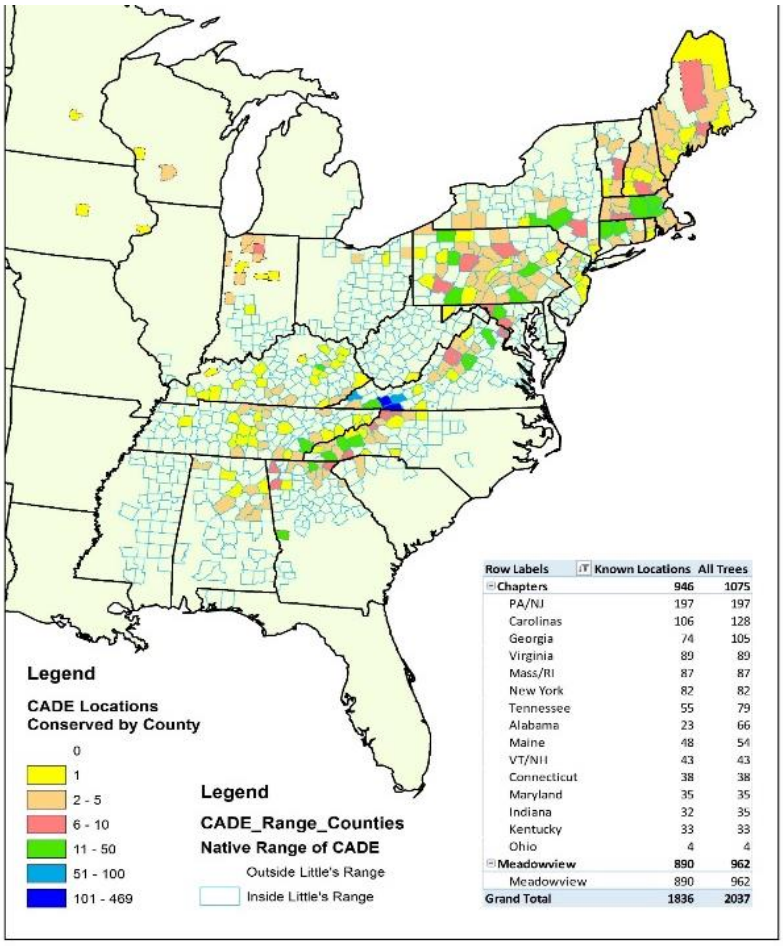
Accelerated growth under artificial conditions, known as speed breeding, has been used successfully in commercial crops (Ghosh et al., 2018; Sysoeva et al., 2016), but only recently been employed in American chestnut (Baier et al., 2012). Speed breeding can occur without respect to season and can allow the collection of pollen before wild and orchard-gown trees (Baier et al., 2012; Valverde et al., 2004). Obtaining pollen in advance can alleviate 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).

This multi-year study was designed to increase the number of flowering American chestnuts from highly diverse and under-represented populations in the South through grafting (Dane et al., 2003; Huang et al., 1998; Kubisiak and Roberds, 2006; Shaw et al., 2012). We also evaluated the use of speed breeding methods using increased photoperiod under artificial light on surviving grafts to accelerate flowering. Viable seeds produced from these light-treated grafts would represent the conservation of new and under-sampled genotypes into the TACF breeding program, likely with a reduced burden to breeders.

Materials and Methods

Study Area

This study focused on American chestnut populations throughout the southeast that are under-represented in the TACF breeding program. Guided by the county-by-county range map of conserved individuals (Figure 1; Westbrook, 2018), counties in Alabama, Georgia, Kentucky, and Tennessee with fewer than 10 trees were considered under-sampled and targeted for scionwood collection.



Row Labels	Known Locations	All Trees
Chapters	946	1075
PA/NJ	197	197
Carolinas	106	128
Georgia	74	105
Virginia	89	89
Mass/RI	87	87
New York	82	82
Tennessee	55	79
Alabama	23	66
Maine	48	54
VT/NH	43	43
Connecticut	38	38
Maryland	35	35
Indiana	32	35
Kentucky	33	33
Ohio	4	4
Meadowview	890	962
Meadowview	890	962
Grand Total	1836	2037

Figure 1 Conserved genotypes by county in the TACF breeding Program

Specific tree locations were sourced from TACF chapter members with local knowledge of trees in counties of interest. While locating previously unknown trees was also desired, this study sourced

scionwood primarily from known trees that had not been bred due to some limitation (i.e. sexual immaturity, difficult access, or other logistical obstacle).

Scionwood Collection

Depending on local dormancy conditions, scionwood collection occurred between December and March of 2017–2018 and 2018–2019. Over the two winter collecting seasons, scionwood was collected from 93 genotypes (Figure 2): 71 *C. dentata*, 19 *C. alabamensis*, and 2 unconfirmed *Castanea* spp. Of these sampled, 71 genotypes (78%) had not been bred previously.

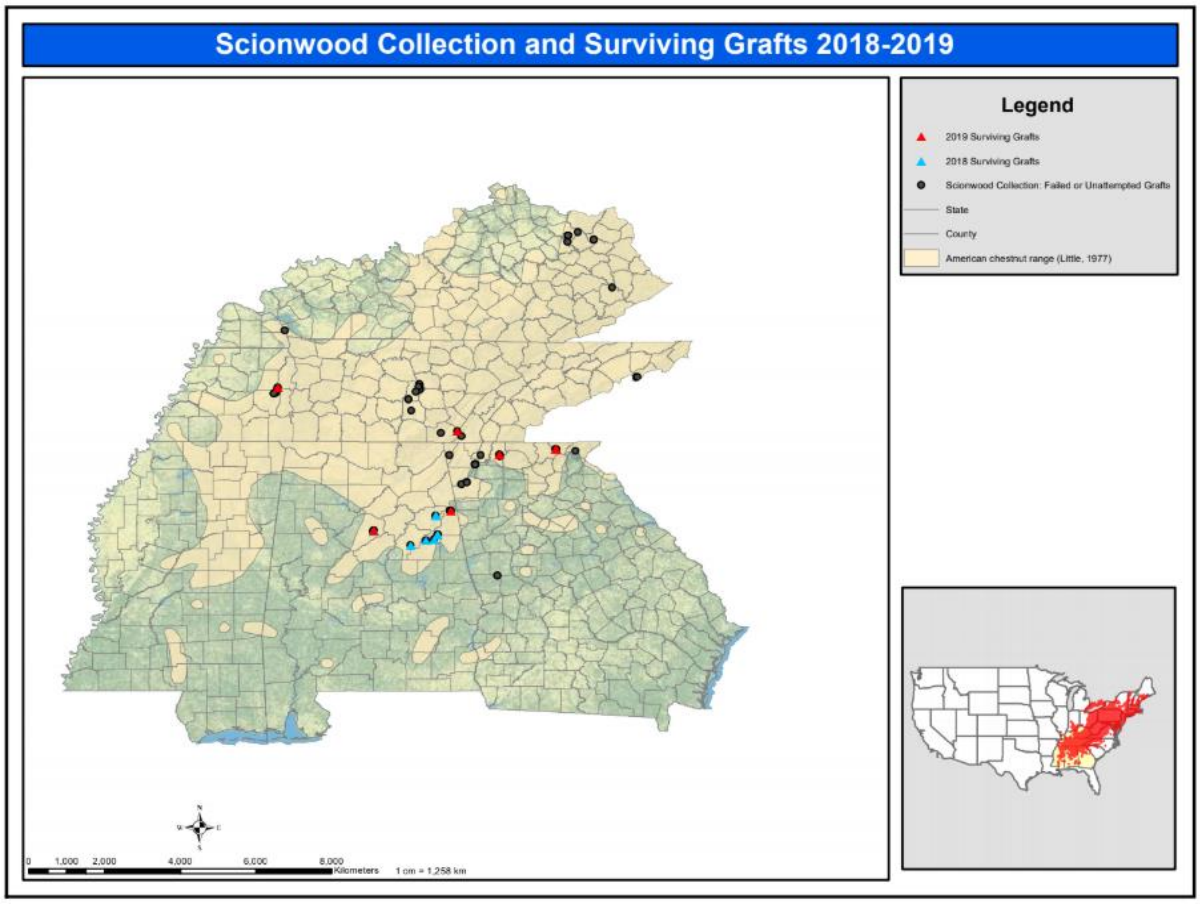


Figure 2 Scionwood Collection and Surviving Grafts 2018-2019

Grafting

Grafts were made between May and July of 2018 and 2019. In 2018, 14 genotypes from Alabama were grafted to 155 rootstocks and in 2019, 20 genotypes were grafted to 215 rootstocks for a two-year total of 34 genotypes and 370 attempts.

Light Chamber

Two open-top chambers (OTC) were constructed in the Fortwood Street Greenhouse on the UT Chattanooga campus, each enclosed by non-transparent plastic sheeting (Figure 3A). One chamber was

designated the artificial light treatment (16 hr photoperiod; Baier et al., 2012) using a 1000W high pressure sodium (HPS) bulb (Figure 3B). The other, no supplemental light chamber (No Supp), received only natural sunlight through the OTC. The light trials occurred over winter and the greenhouse was heated to 25 °C.

Figure 3 Light Chamber Interior and Exterior

Light trials ran 100 days and repeated twice: Trial 1 – December 10, 2018 to March 20, 2019; Trial 2 – November 13, 2019 to February 21, 2020. Each trial began with dormant surviving grafts randomly assigned to each treatment (16hr, No supp.). However, sole surviving ramets of a genotype were preferentially placed in the 16hr treatment to maximize breeding potential.

Trial 1 (2018-2019) consisted of 12 genotypes from two species (7 *C. dentata* and 5 *C. alabamensis*) totaling 39 ramets (28 *C. dentata* and 11 *C. alabamensis*); 16 hr: $n_1 = 23$; No supp.: $n_2 = 16$ (Figure 4). Trial 2 (2019-2020) consisted of 7 *C. dentata* genotypes, totaling 19 ramets; 16 hr = 11; No supp = 8 (Figure 5).

Light (photon density, µmol $s^{-1}m^{-1}$) and temperature (°C) measurements were taken in 9 designated locations (Figure 4 and 5) within each treatment. Four levels (Figure 6) were measured at each location in Trial 1 and 3 levels in Trial 2, totaling 36 and 27 points, respectively.

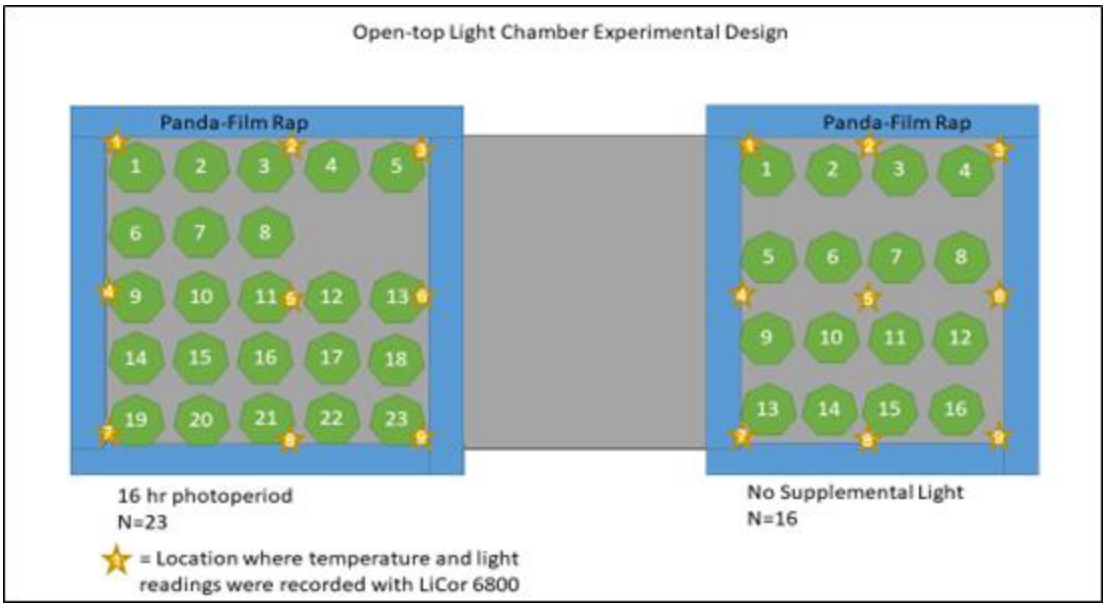


Figure 4 Light Trial 1 (2018-2019) Design with Light and Temperature Measurement Locations

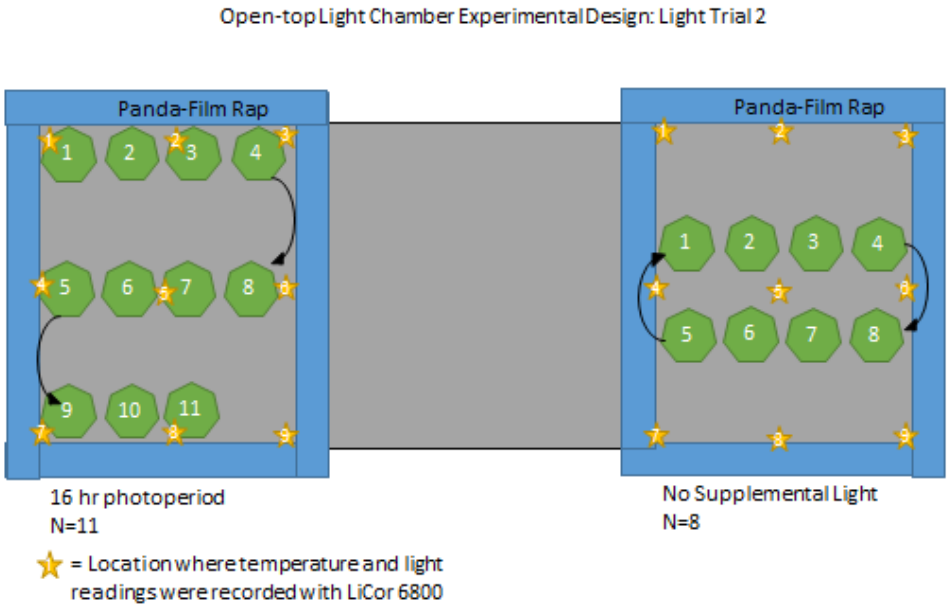


Figure 5 Light Trial 2 (2019-2020) Design with Light and Temperature Measurement Locations
Black arrows indicate direction of weekly rotation through the OTC

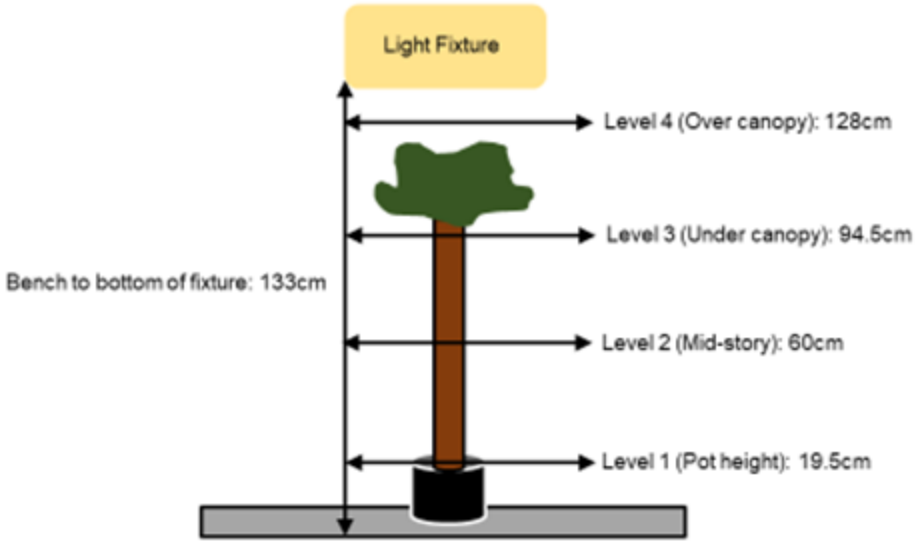


Figure 6 Illustration of Light and Temperature Measurement Levels

Data Collection and Analysis

Each treatment was monitored twice weekly for 3 phenological events: bud break (BB), catkin emergence/development (CD), and catkin maturation (day of catkin collection; CM). 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.

Pollen and Seed Collection and Storage

Catkins produced from flowering grafts were collected and laid out onto clean panes of glass (Figure 7A). After 24 hours, anthers dehisced onto glass (Figure 7B). Pollen was scraped by a razor blade and transferred into glass vials (Figure 7C). 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 February 2020.

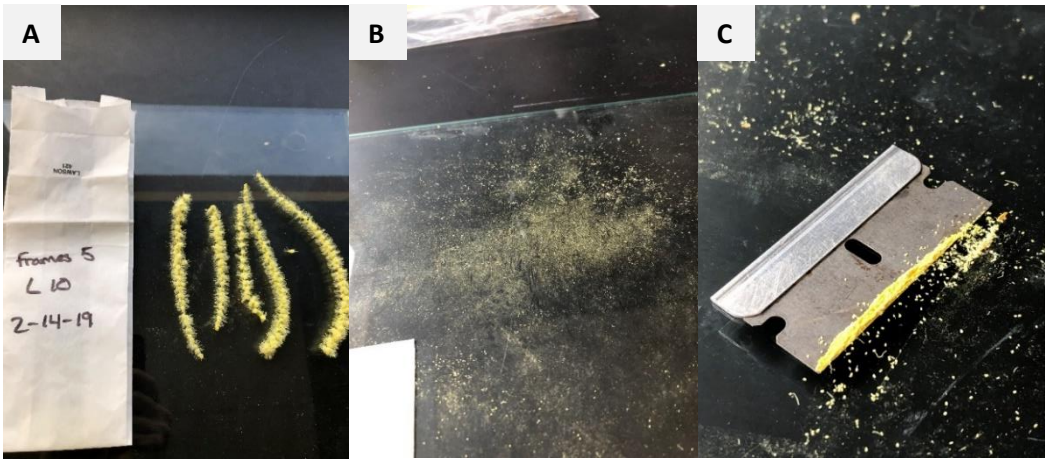


Figure 7 Pollen Processing

Results

Grafting

2018: May—July: 14 of 38 genotypes were grafted to 155 rootstocks. Twelve (12) genotypes survived, totaling 39 ramets (25.2% survival; Table 1). Surviving genotypes represent 7 *C. dentata* and 5 *C. alabamensis* individuals from Alabama. Grafts performed by Dr. J. Hill Craddock.

2019: May–July: 20 of 69 genotypes (6 AL, 6 GA, 2 KY, and 6 TN) were grafted to 215 rootstocks. Nine (9) survived, totaling 19 ramets (11.9% survival; Table 1 and 2). Survival percentage calculated from 159 graft attempts as 56 grafts were attempted with freeze damaged scionwood caused by cooler malfunction. Grafts performed by Trent Deason.

Table 1 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/Code	Short Code	Species	State	County	Location	Collecting Season [1]	Grafting Year [2]	Previously Bred [3]	# Ramets	Flowers Induced
5	ALJEFF78	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1	2018	Y	2	Y
10101A	ALJEFF80	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1,2	2018, 2019	N	5	Y
7CN	ALJEFF74	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1,2	2018, 2019	Y	1	
Cheaha08	ALCLAY08	*C. dentata*	AL	Talladega	Adams Gap	1	2018	N	3	Y
Choco01	ALCALH01	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	N	3	Y
Choco02	ALCALH02	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	N	1	Y
Choco22	ALCALH22	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	N	1	
Choco27	ALCALH27	*C. alabamensis*	AL	Calhoun	Choccolocco Mountain	1	2018	N	1	
Choco28	ALCALH28	*C. dentata*	AL	Calhoun	Choccolocco Mountain	1	2018	N	1	
Frames03	ALCLEB04	*C. dentata*	AL	Cleburne	Frames Property	1	2018	Y	1	
Frames05	ALCLEB06	*C. dentata*	AL	Cleburne	Frames Property	1	2018	Y	2	Y
Hutch04	ALCLEB14	*C. dentata*	AL	Cleburne	Hutchinson Property	2	2019	N	2	
MS38	ALJEFF19	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1,2	2019	N	1	Y
MS42	ALJEFF42	*C. dentata*	AL	Jefferson	Ruffner Mountain Nature Preserve	1	2018	N	2	Y
T3	ALTALL03	*C. dentata*	AL	Talladega	Talladega National Forest	1	2018	Y	5	Y
GAMU9-A	GAMU9-A	*C. dentata*	GA	Murray	Fort Mountain	2	2019	Y	5	
GAUN5	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	TNHEN02	*C. dentata*	TN	Henderson	Natchez Trace State Park	2	2019	N	1	
Signal Mtn01	TNHAM01	*C. dentata*	TN	Hamilton	Signal Mountain	2	2019	Y	1	Y

Table 2 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

16 hr Treatment: BB observed at 10 days and for all ramets (n = 23) at 30 days (Table 3). Average days to BB = 18.72 days (*SD* = 5.79; Figure 8). CD observed (Figure 9) between 27 and 39 days (n = 9). Average days to CD = 34.11 days (*SD* = 5.40; Figure 10). CM recorded between 43 and 88 days (n = 8). Average days to CM = 70.5 days (*SD* = 13.41; Figure 11).

No Supp Treatment: BB observed at 28 days and for all ramets (n = 15) at 62 days (Table 3). Average days to BB = 44.33 days (*SD* = 9.07; Figure 8). CD observed between 43 and 70 days (n = 7; Table 3). Average days to CD = 59.00 days (*SD* = 9.47; Figure 10). CM recorded at 97 days (n = 1; Figure 11). The other 6 ramets aborted developing catkins prior to maturation.

Table 3 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**	**Short Code**	**BB**	**CD**	**CM**
16 hr	L1	Choco01	ALCALH01	18	27	43
16 hr	L2	Frames5	ALCLEB04	10	-	-
16 hr	L3	Frames5	ALCLEB04	27	39	-
16 hr	L4	MS42	ALJEFF42	15	27	72
16 hr	L5	Choco02	ALCALH02	20	-	-
16 hr	L7	Cheaha08	ALCLAY08	10	-	-
16 hr	L8	Choco22	ALCALH22	18	-	-
16 hr	L9	5	ALJEFF78	18	37	76
16 hr	L10	Frames 5	ALCLEB04	15	27	65

Table 3 Continued

Treatment	Plant Code	Genotype	Short Code	BB	CD	CM
16 hr	L11	T3	ALTALL03	15	-	-
16 hr	L12	Frames3	ALCLEB02	20	-	-
16 hr	L13	Choco27	ALCALH27	27	-	-
16 hr	L14	Cheaha08	ALCLAY08	18	37	76
16 hr	L15	Choco01	ALCALH01	27	37	65
16 hr	L16	10101A	ALJEFF80	10	-	-
16 hr	L17	10101A	ALJEFF80	30	37	88
16 hr	L18	Choco02	ALCALH02	23	-	-
16 hr	L19	Cheaha08	ALCLAY08	15	-	-
16 hr	L20	10101A	ALJEFF80	23	-	-
16 hr	L21	Choco28	ALCALH28	15	-	-
16 hr	L22	T3	ALTALL03	15	-	-
16 hr	L23	T3	ALTALL03	23	39	79
No Supp	C1	T3	ALTALL03	48	53	-
No Supp	C2	Choco01	ALCALH01	43	65	-
No Supp	C3	Unknown01	-	48	-	-
No Supp	C4	Choco28	ALCALH28	43	-	-
No Supp	C5	MS42	ALJEFF42	37	-	-
No Supp	C6	Frames5	ALCLEB04	30	43	-
No Supp	C7	Frames5	ALCLEB04	43	-	-
No Supp	C9	T3	ALTALL03	48	-	-
No Supp	C10	Frames5	ALCLEB04	57	70	-
No Supp	C11	Choco02	ALCALH02	62	68	97
No Supp	C12	Cheaha08	ALCLAY08	43	-	-
No Supp	C13	T3	ALTALL03	43	57	-
No Supp	C14	10101A	ALJEFF80	53	-	-
No Supp	C15	5	ALJEFF78	28	57	-
No Supp	C16	10101A	ALJEFF80	39	-	-

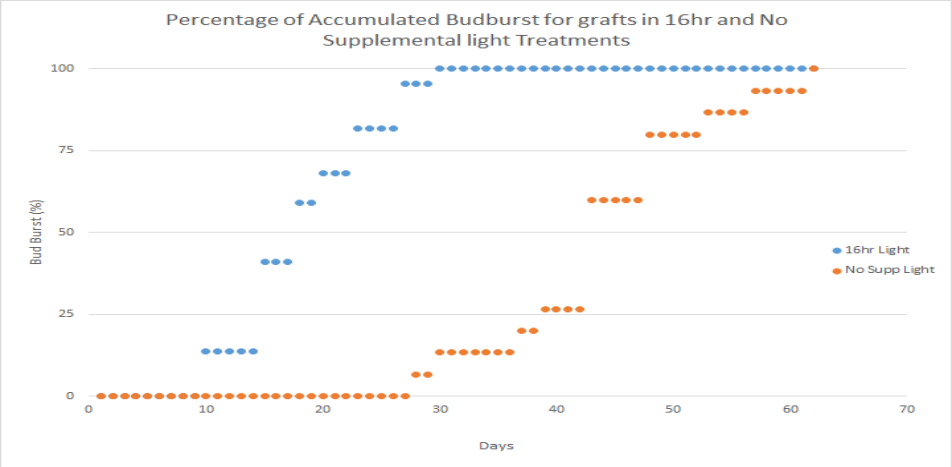


Figure 8 Percent of Accumulated Days to Phenological Event BB

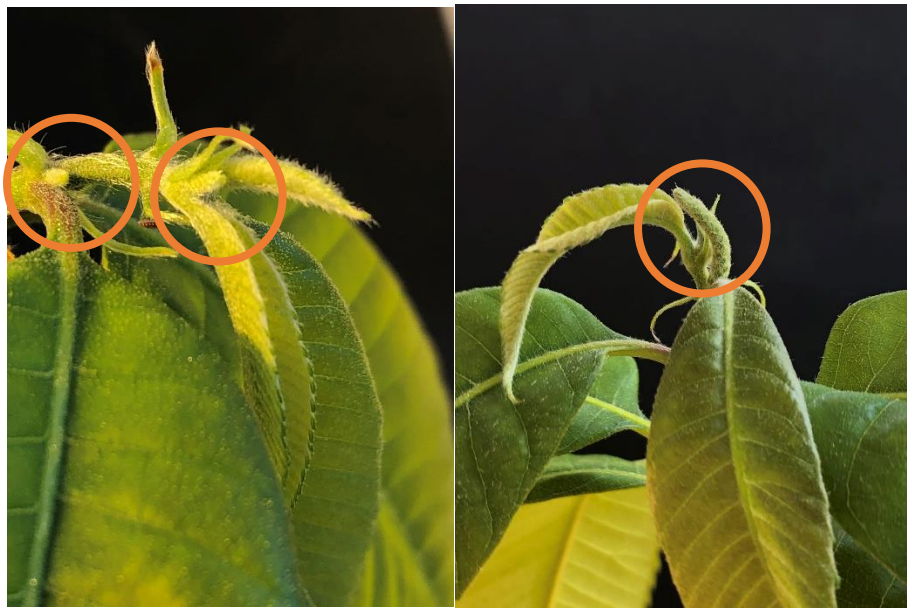


Figure 9 Developing Catkins on Light-Treated Grafts

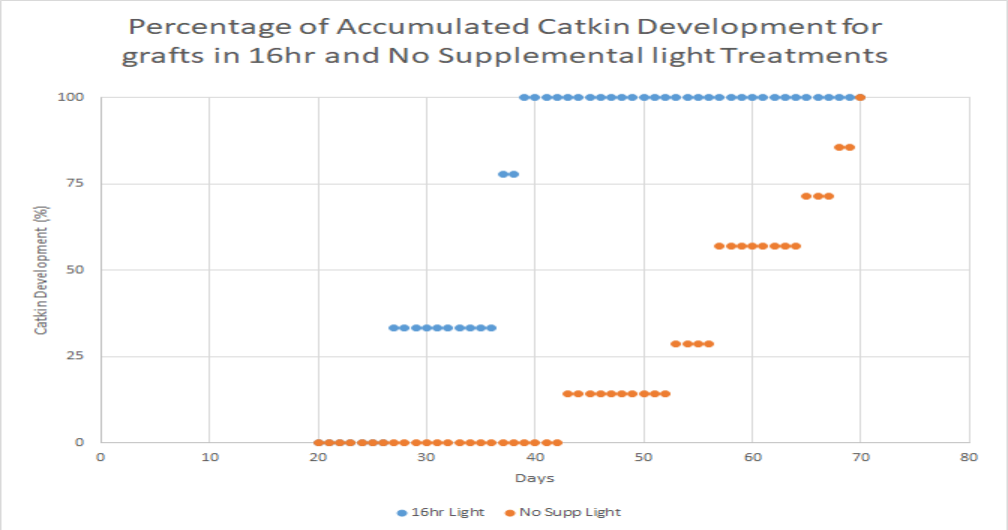


Figure 10 Percent of Accumulated Days to Phenological Event CD

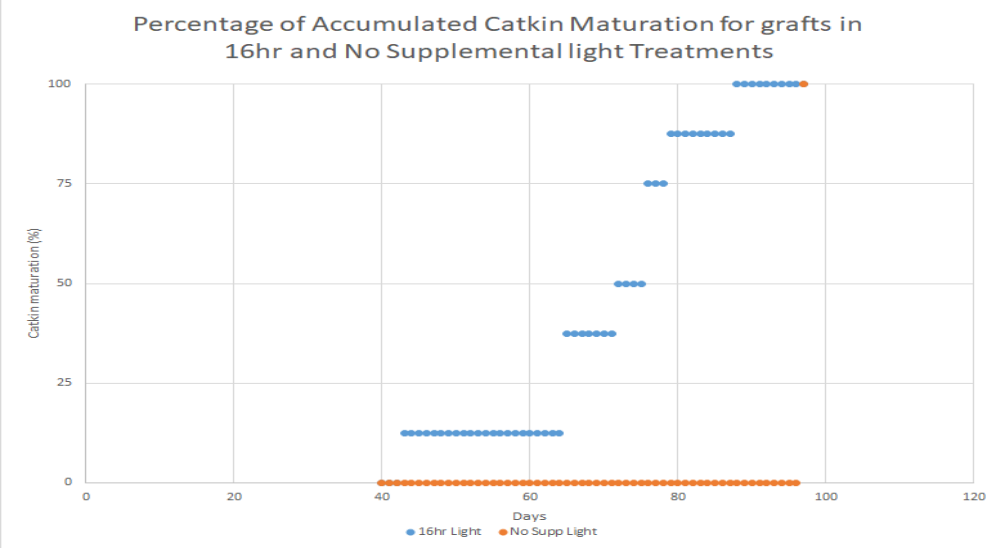


Figure 11 Percent of Accumulated Days to Phenological Event CM

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 with respect to photoperiod 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 12). 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	

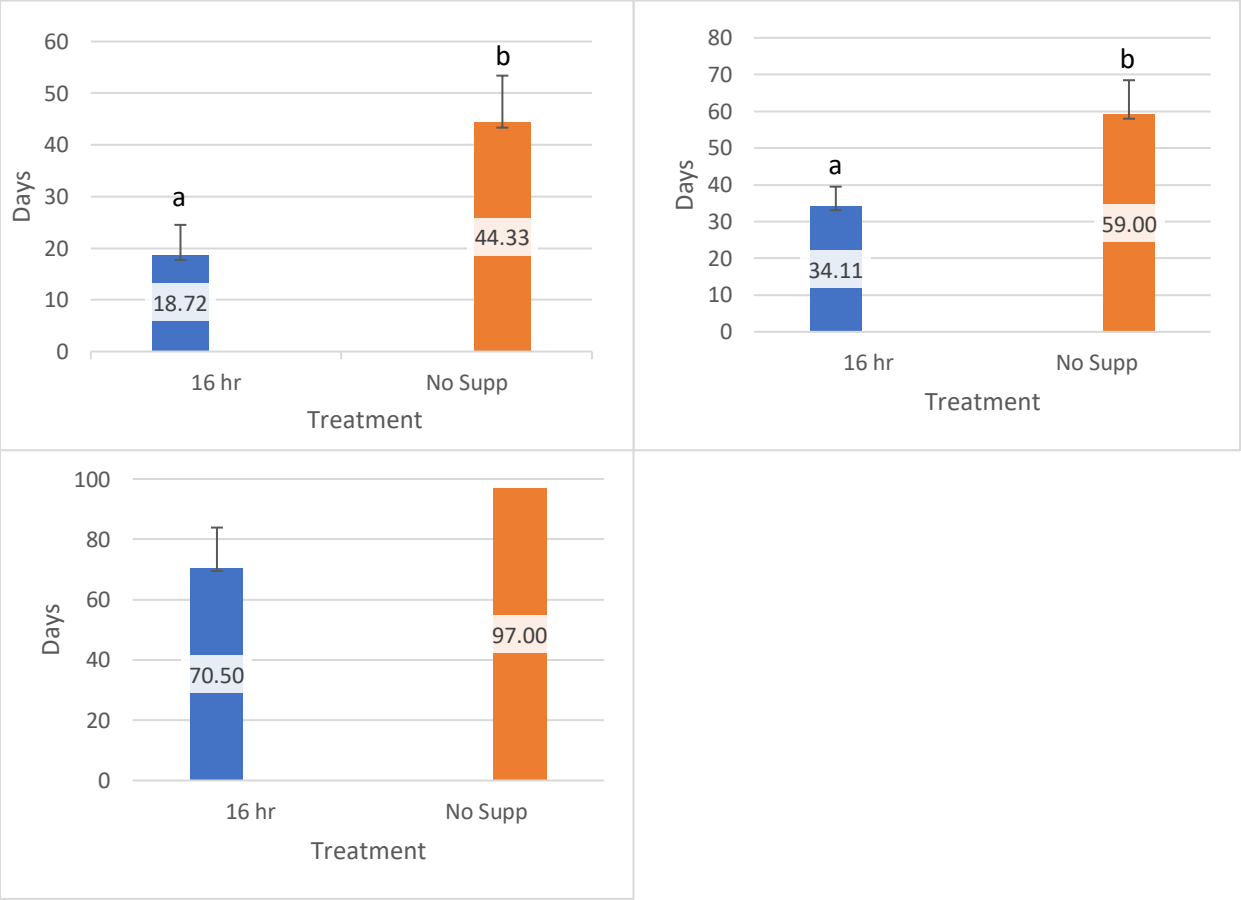


Figure 12 Mean Days to Phenological Event per Treatment of Grafted American chestnut and Alabama chinquapin

Light and Temperature: The 16 hr photoperiod chamber averaged 348.05 µmols $s^{-1}m^{-1}$ and 29.78 ℃. The No supp 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 Light (µmol s^{-1} m^{-1}) and Temperature (℃) Average 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. Only then were new healthy emergent leaves/shoots observed.

Light Trial 2: 2019-2020

16 hr Treatment: BB observed at 28 days and in all ramets (n = 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 observed by 49 days (n = 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-Supp Treatment: BB observed at day 63 and in all ramets by 91 days (n = 6). Average days to 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	Short Code	BB	CD	CM
16 hr	3	SIGNAL MTN 01	TNHAM01	31	49	90
16 hr	1	BRADFORD TR 2	TNHEN02	28	49	-
16 hr	2	BRADFORD TR 2	TNHEN02	-	-	-
16 hr	7	HUTCH04	ALCLEB14	-	-	-
16 hr	8	HUTCH04	ALCLEB14	31	-	-
16 hr	10	GAUN5	GAUN5	49	-	-
16 hr	12	MS38	ALJEFF19	28	-	-
16 hr	13	GAMU9-A	GAMU9-A	38	-	-
16 hr	15	GAMU9-A	GAMU9-A	38	-	-
16 hr	16	GAMU9-A	GAMU9-A	38	-	-
16 hr	18	GAMU9-B	GAMU9-B	-	-	-
No Supp	4	SIGNAL MTN 01	TNHAM01	-	-	-
No Supp	6	HUTCH04	ALCLEB14	80	-	-
No Supp	9	GAUN5	GAUN5	91	-	-
No Supp	11	GAUN5	GAUN5	84	-	-
No Supp	14	GAMU9-A	GAMU9-A	63	-	-
No Supp	17	GAMU9-A	GAMU9-A	77	-	-
No Supp	19	GAMU9-B	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-supp treatment.

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

Table 8 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

Eleven ramets produced catkins (Trial 1: 9; Trial 2: 2), however only 4 genotypes (Trial 1: ALCLAY08, ALTALL03, and ALCLEB04; Trial 2: TNHAM01) produced enough pollen to be used effectively in controlled pollinations. Pollen collected during Light Trial 1 was used in controlled crosses 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 ALCLAY08; and two crosses at Meadowview onto *C. dentata*: AN-65 x ALTALL03 (T3) and AN-86 x ALCLEB04 (Frames5; Table 9; Figure 13). While genotypes ALTALLO3 and ALCLEB04 were already represented in the TACF breeding program, ALCLAY08 (Cheaha08) had not been bred prior to this study. A total of 80 seeds were harvested in October 2019 (Table 9) and planted in February 2020. The no-pollen-control bags on TTU-M13 x ALCLAY08 contained 4 nuts, when none should be expected. Additionally, although female flowers were not observed during either Light Trial 1 or 2, female flowers were observed in June 2020 on a single ramet of ALCLAY08 (Figure 14). This genotype/ramet was used during Light Trial 1 (2018-2019), thus production of pistilate flowers occurred 15 months from the end of the trial.

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 ALCLAY08 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**	**Year**	**Pollen Bags (#)**	**Control Bags (#)**	**Burrs (#)[3]**	**Total Seeds (#)**	**Seeds in Control Bags**
TTU-M13 [1]	ALCLAY08	2019	21	2	42	21	4
AN-65 [2]	ALTALL03	2019	15	3	-	11	0
AN-86 [2]	ALCLEB04	2019	25	3	-	52	0

Figure 13 Controlled pollination at Wagner Orchard (TACF Research Farms, Meadowview, VA) of AN-65 x ALTALL03 (T3) and AN-86 x ALCLEB04 (Frames5; not pictured) in June 2019.

Discussion

Though our survival rate was lower than expected, 10% - 25% rather than 38% (McKenna and Beheler, 2016), graft propagation is a viable, minimally invasive method of vegetative propagation. As 78% of the scionwood collected came from non-flowering individuals and those not yet conserved in the TACF breeding program, grafting can quickly expand the genetic base of the breeding program. Twenty genotypes (16 *C. dentata* and 4 *C. alabamensis*) were successfully grafted, all from counties with fewer than 10 individuals in the breeding program. Thus, this collection may offer access to potentially novel genetic resources.

This study was the first attempt at speed breeding grafted American chestnut. Light chamber results demonstrate that both vegetative growth and floral development (male) of grafted germplasm can be accelerated under high light conditions. Mature male catkins were collected between February and March during each light trial, allowing breeders access to pollen in advance of *in situ* populations. This is a step towards reducing the generation time required to develop a viable hybrid and/or transgenic restoration population (Sysoeva et al., 2016; Westbrook, 2019). While no pistilate flowers were produced during either light chamber experiment, they have been observed subsequently on ALCLAY08 (Cheaha08). Pollinations were made and thus allows the conservation of maternal germplasm in viable seed. This represents a valuable proof of concept, where if not for graft propagation of this difficult to reach novel genotype, its genetic resources may not have been introduced into the breeding program.

Finally, surviving grafts are maintained in a nursery, which allows researchers access to germplasm and reducing the need for field visits. As such, grafts conserved in this study offer continued access to pollen which has been used in additional crosses not reported here. Additionally, they have been used to support the TACF landscape genomics project, where leaf samples were collected in minutes, yet represented some 500 miles of travel. This highlights the value in conserving genetic and geographic diversity in easily accessible areas. These surviving grafts will be out-planted in germplasm conservation orchards (GCOs) for long-term management, ideally in the states from which they represent, to benefit regional breeding and research efforts.

Figure 14 Ripening burr on a graft of ALCLAY08, pollinated in July 2020. Photo by Dr. J. Hill Craddock

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Public Presentations:

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Presentation. Deason, T. Accelerated, graft-based conservation of under-sampled American chestnut populations in the South. Master's Thesis Defense. Chattanooga, TN. February 14, 2020.