Germinability, Viability, and Transgene Inheritance of Blight Resistant American Chestnuts

Sara Klopf[1] and Jason Holliday[2]

[1]School of Plant and Environmental Sciences

[2]Department of Forest Resources and Environmental Conservation

Virginia Tech, Blacksburg, VA 24061

Introduction:

The American Chestnut was formerly the dominant canopy species in many hardwood forests of the eastern United States, representing between 20-25% of stems in the Appalachians and serving as an important resource for people and wildlife. In the early 1900s, a fungal blight (*Cryphonectria parasitica*) was introduced from imported Japanese nursey stock that caused topkill of American chestnuts. The disease spread rapidly through Appalachia, and by the 1950s, only a few seemingly blight resistant trees remained, as well as resprouts from the dead trees. The resprouts repeatedly die back such that they never reach mature height, rendering the American chestnut canopy tree, functionally extinct.

Coal surface mining has also greatly impacted the forests of Appalachia. At least 1 million acres have been disturbed to date in Appalachia, much of which is still in need of reclamation. The forestry reclamation approach (FRA) under the Surface Mining Control and Reclamation Act (SMCRA) has improved the success of coal mine reforestation in Appalachia (Burger et al. 2005) through better substrate selection and site preparation methods. The extent of coal surface mining in the Appalachians, and thus land in need of reclamation, overlaps significantly with the historic range of the American chestnut (Figure 1). This overlap presents the opportunity to field-test improved American chestnuts for blight resistance, growth, survival, and blight resistance on land already in need of reforestation.

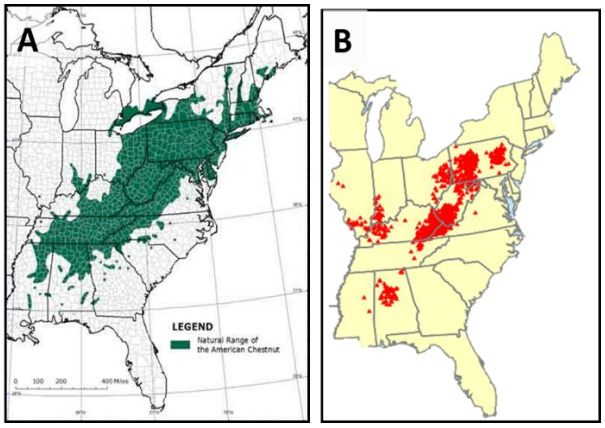

Figure 1. A) Map of the historic range of American chestnut in the United States (TACF 2014), B) Map of active coal surface mines in the eastern United States as of 2012 (CDC 2012).

Since 1983, the American Chestnut Foundation (ACF) has been conducting a backcross breeding program to produce a tree resistant to the blight. While American chestnuts are extremely vulnerable to the blight pathogen, Chinese chestnuts have some blight resistance and typically show less severe symptoms of blight infection. The ACF breeding program aims to boost American chestnut resistance to blight by crossing American chestnuts that ostensibly have some blight resistance with Chinese chestnuts. Genetic diversity is achieved by subsequently doing a series of backcrosses (hybrid x American) and intercrosses (hybrid x hybrid) to produce offspring with varying levels of resistance. All offspring are inoculated with blight to assess their resistance to blight. Trees with the highest blight resistance are selected to produce the next generation.

Unsurprisingly, blight resistance of offspring produced from backcross breeding is variable and the process overall is time-consuming. Additional research has focused on developing blight resistance trees through the use of genetic engineering. Genes that are suspected to provide resistance to blight infection are inserted into the genome of American chestnut somatic cell embryo clones using the plant pathogen *Agrobacteria parasitica*. Somatic embryo clones are produced using the methods described in Andrade and Merkle (2005) and Carraway and Merkle (1997). Embryos are exposed to a solution containing the modified *A. parasitica*; the bacterium then infects the embryos and transfers a DNA plasmid, carrying the resistance gene, into the genome of the American chestnut. While researchers are able to select specific resistance genes to incorporate, where they end up in the genome has thus far been imprecise, so while they may

be present somewhere in the genome, they may not be expressed (i.e. provide blight resistance). Therefore, it is important to test resulting seedlings on a variety of sites to assess their viability as restoration tools.

For the last six years, our research group has been field-testing traditionally cross-bred and transgenic American chestnuts on orchard sites as well as reclaimed coal mines. Our goal has been to determine which genetic constructs (background genotype + inserted transgene) produce trees that survive and grow well, have American chestnut morphology, and show resistance to blight infection after inoculation. One genetic construct that was planted on our orchard site in November 2013 has performed well in all of these aspects: CBS1. This construct has a background genotype of American chestnut hybridized with Chinese and Japanese chestnuts, and the cystathionine-beta-synthase (CBS) domain-containing gene from Chinese chestnut that expresses a CBS domain-containing protein. The CBS domain-containing protein appears to be expressed in response to stress, such as from pathogen infection. As of January 2019, survival, height, basal diameter, and canker response after inoculation of CBS1 trees were similar to the pure Chinese chestnuts (Qing), which would be expected to have the highest blight resistance in the study (Figures 2-6).

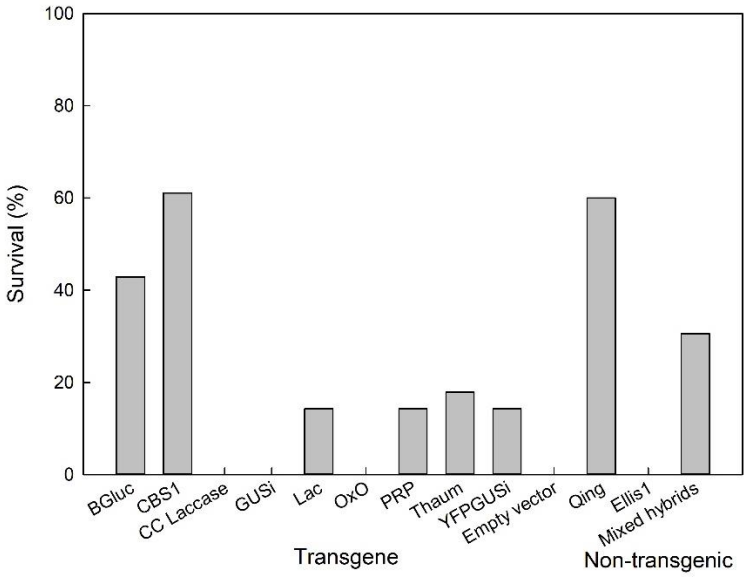

Figure 2. Survival of transgenic and non-transgenic American chestnuts planted at Kentland Farm orchard on November 2013. Data from January 2019.

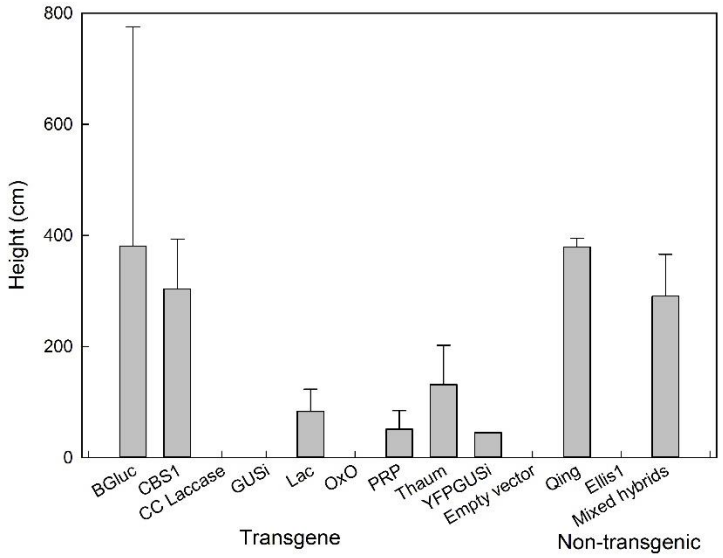

Figure 3. Height of transgenic and non-transgenic American chestnuts planted at Kentland Farm orchard on November 2013. Data from January 2019.

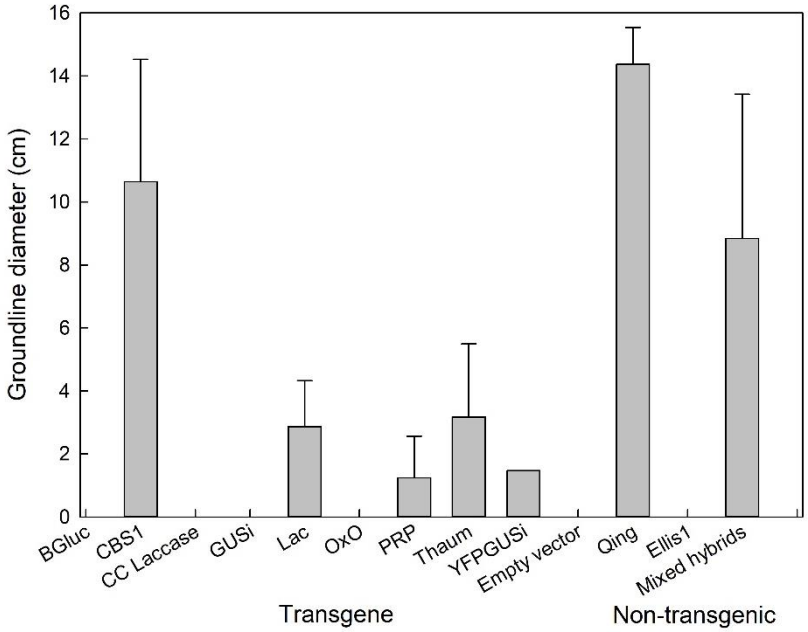

Figure 4. Groundline diameter of transgenic and non-transgenic American chestnuts planted at Kentland Farm orchard on November 2013. Data from January 2019.

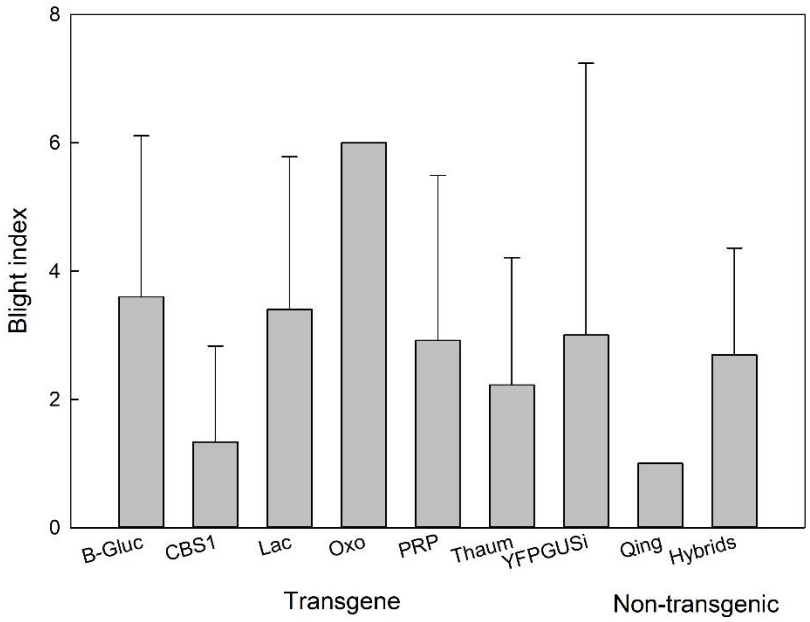

Figure 5. Blight incidence of transgenic and non-transgenic American chestnuts planted at Kentland Farm orchard on November 2013. Data from November 2018, after inoculation in May 2018. Higher numbers indicate more severe blight symptoms.

Figure 6. Stem canker at inoculation site on pure Chinese chestnut (left) and CBS1 transgenic tree (right). Note that larger, more swollen and split wound on the Chinese chestnut, as well as the orange discoloration on the tape, indicating the growth of *C. parasitica* fruiting bodies.

In addition, the CBS1 construct has flowered for several years and produced thousands of open-pollinated nuts that, per our permit guidelines, we have thus far been destroying and discarding (chestnut fruits must be either caged or destroyed to prevent wildlife tampering). Due to the promising performance of the CBS1 trees on our orchard site, we proposed to investigate these constructs further through the following questions:

1) What is the germination rate of nuts produced from open-pollinated CBS1 trees?
2) How well do seedlings grown from open-pollinated CBS1 nuts survive and grow on a reclaimed coal mine site and the orchard site the nuts were collected from?
3) What proportion of seedlings grown from open-pollinated CBS1 nuts inherit the CBS transgene?

Objectives:

To assess germination of open-pollinated transgenic trees with observed blight resistance, assess transgene inheritance of offspring, and quantify survival and growth on our existing field sites (Kentland Farm orchard site and the Powell River Project).

Research Site History:

The two research sites involved are located at the Kentland Research Farm in Blacksburg, VA (37°12'20.65"N 80°35'27.74"W) and a location on the Powell River Project (PRP) (36°59'9.75"N 82°42'5.47"W).

Soils at the Kentland Farm planting location are in the Unison and Braddock series, which are well-drained and characterized by a loamy texture with common cobbles. The Kentland site was formerly a peach orchard, converted to upland pasture, and was planted with a mix of transgenic and non-transgenic chestnuts in November 2013, including the CBS1 trees.

The Powell River Project (PRP) site was seeded after regrading in 2012 with a tree compatible seeding mix. At planting, the substrate was very rocky and comprised primarily of weathered sandstones and shales. Fine materials collected from the site before planting ranged in pH from 4.83 to 5.83 (mean 5.19), and organic matter was overall fairly low ranging from 0.4 to 1.1%

(mean 0.7 %). The site was first planted in June 2013 with a mix of hybrid chestnuts, then planted with additional hybrid chestnuts as well as transgenic chestnuts in May 2014 and May 2015.

Methods:

Tree selection and nut germination

We selected five CBS1 trees representing three different gene insertion events that have shown particularly strong resistance to blight inoculation (Table 1). Aluminum window screening material was used to cage approximately 50-100 burs per tree to prevent tampering by squirrels or other wildlife while they mature (Figure 7). The remainder of nuts were removed and destroyed to comply with our APHIS permit requirements. Chestnut burs were harvested from cages when they were mature, noted by the burs browning and opening up to release the nuts. Nuts were removed from burs and cold-stratified in moist peat moss at 0° C from 4 October 2019 to 16 December 2019 to break dormancy (Hebard and Rutter, unknown date).

Table 1. Number of nuts planted from each of the five mother trees.

Mother #	Event #	Nuts planted (#)
166	2	185
174	3	70
175	7	196
177	7	136
179	7	86

Figure 7. Tree with caged chestnut burs (29 August 2019, Sara Klopf).

To allow us to assess germination rates of the collected nuts, we planted the stratified nuts in individual cone-tainers at the BIOL/BI Plant Growth Service greenhouse facility which provides daily watering and pest control. In addition, planting in a greenhouse allowed us to minimize winter seed predation and obtain more accurate germination data. Planting media was mixed, following recommended chestnut planting methods (Hebard and Rutter, unknown publication date) (Table 2). Some trees were more prolific nut producers than others, and some seeds were lost to rot during stratification, so the number of nuts planted varied (Table 1). After planting, germination was monitored and recorded weekly through 18 February 2020.

Table 2. Planting media recipe used for chestnut germination.

Mix component	**Volume (qt)**
Perlite	12
Vermiculite	12
Ground peat moss	12
Lime	0.06

Quantifying transgene inheritance

Prior to planting seedlings on our field site, tissue samples were collected from all offspring for PCR analysis to quantify whether the CBS1 transgene had been passed on to offspring. DNA extraction was completed using the DNeasy Plant Mini Kit (Qiagen). PCR analysis was performed by Qian Zhang, in the Department of Forest Resources and Environmental Conservation. DNA extract concentration and quality were analyzed with NanoDrop and Qubit. For PCR analysis, the forward primer (FP_CmCBS (tm=54C)) and reverse primer (RP_CmCBS (Tm=55.6)) were used. A positive control (plasmid with the inserted transgene) was included in every PCR run, as well as tissue samples from the mother trees. The preliminary results indicated than none of the offspring carried the transgene and that none of the mother trees carried the transgene, suggesting that the PCR methods needed to be modified to ascertain whether the trees were transgenic.

Additional PCR analyses were run with the CmCBS F/R primer, as well as three pairs of NptII F1/R1 primers [**F1**:(94-113): GATGGATTGCACGCAGGTTC, **R1**:(670-651): TGATATTCGGCAAGCAGGCA; **F2**:(138-157): ATTCGGCTATGACTGGGCAC, **R2**:(503-484): ATGCGATGTTTCGCTTGGTG; **F3**: (329-348): TTGTCACTGAAGCGGGAAGG, **R3**:(764-745): ATATCACGGGTAGCCAACGC]. The NptII gene is often used in the development of transgenic plants, as it confers neomycin and kanamycin antibiotic resistance and provides a method for screening resulting embryos to determine whether the modified plasmid is incorporated in their genome. The presence of this gene would indicate that the tree was transgenic, whether or not it carried the CBS1 transgene. For these analyses, we focused on the mother trees and offspring with the best quality DNA since previous analyses suggested that either the mother trees were not transgenic or there was an issue with the methodology. The absence of the NptII gene among the mothers would indicate that somehow the selective media selected non-transgenic embryos instead of transgenic embryos, or that there were still issues with the methodology. The results of these PCR runs indicated that the issues detecting the transgene were likely due to lower quality DNA from older tissue samples and programming issues.

Field testing CBS1 offspring

The original intent of the project was to plant the resulting seedlings at both our Kentland Farm study site and the Powell River Project study site in spring 2020, under our existing USDA-APHIS permit, however the Covid-19 pandemic affected our ability to travel and we were forced to modify our research plans. As of summer 2021, we have only planted and monitored seedlings at the Kentland Farm study site in 2020. Remaining seedlings have been cared for in the BIOL greenhouse on the Virginia Tech campus and were moved to Reynolds Homestead in Critz, VA to overwinter. We are hoping to plant some seedlings at the Powell River Project, but due to our current APHIS permit expiring soon, we may be unable to do so and would instead plant remaining seedlings at Kentland Farm.

Seedlings were planted at Kentland Farm on April 11 and were watered as needed through May. After planting, we measured initial height, groundline diameter (GLD), and diameter at 10 cm. Diameter at 10 cm is considered to be a more accurate comparison among chestnut seedlings, as the seedling stem can be swollen near the base. Preliminary seedling survival was recorded on July 29, 2020. Survival was recorded, and height and diameters were measured on October 21, 2020 at the end of the growing season after buds had set. Height and diameter growth were calculated from the differences between initial and end of growing season measurements.

Statistical analysis:

All data were analyzed using Sigmaplot 12.5 software (Systat Software, San Jose, CA). Germination data were analyzed with one-way ANOVA with repeated measures for the multiple recording dates to look for differences among the mother trees and the genetic construct events. Preliminary survival, initial height, and initial diameters (GLD and diameter at 10 cm) were analyzed using one-way ANOVA to detect differences among blocks, mother trees, and genetic construct events.

Results:

Seedling germination

Germination was 63.6% overall and differed among construct event (p=0.008) (Figure 8). Events 2 (74%) and 7 (64.4%) had the highest germination, and Event 3 (25.5%) had the lowest germination. Germination did not differ among mother trees, although low tray replication among trees 174 and 179 may have limited our ability to detect differences among groups (p = 0.245) (Table 3).

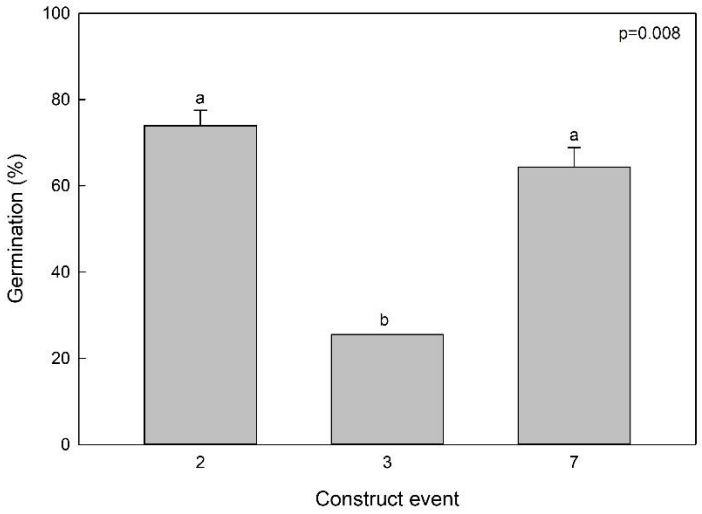

Figure 8. Mean percent germination among the three construct events. Error bars represent mean standard error and different letters signify differences among groups at the α=0.05 level.

Table 3. Summary statistics for percent germination among mother trees.

Mother	Event	N	Median	25% percentile	75% percentile	Mean germination (%)	Standard deviation	Standard error
166	2	2	74.0	70.4	77.6	74.0	5.0	3.6
174	3	1	25.5	25.5	25.5	25.5	0.0	0.0
175	7	2	54.6	50.0	59.1	54.6	6.4	4.6
177	7	3	67.4	56.0	76.9	66.8	10.5	6.0
179	7	1	76.7	76.7	76.7	76.7	0.0	0.0

PCR analysis of transgene inheritance

When this project was proposed, the assumption was that the genotypes of all the mother trees that we received from our collaborators at the University of Georgia (UGA) included the CBS1 gene. Prior to transplanting, PCR analysis was conducted by UGA on the embryogenic culture material for the three events within our genetic construct of interest, which determined that the CBS1 transgene was present in all three events. Embryos were then separated and grown on selective media which specifically selects embryos with the NptII gene, a transgene present in each construct. The assumption was that the CBS1 transgene was present in all resulting seedlings, including the five mother trees in this study. However, as PCR analysis of offspring tissue began, we found that none of the seedlings grown in the greenhouse had inherited the transgene, though we were able to obtain a strong band when we ran the positive control. It is very unlikely that none of the offspring would have inherited the transgene if at least one parent tree is carrying the transgene, which suggested either that the transgene was not present in any of the mother tree genomes or there was an issue with the PCR methods used. Subsequent PCR analysis of the mother tree tissues failed to obtain a positive band from any of the mother trees for the CBS1 transgene, and it was assumed that there was an error with the methodology.

In our final attempts with PCR analysis in which we used primers for both CBS1 and NPTII we observed weak to strong bands for several of the trees, though it still appears that there may still be some issues with the methodology we are using to detect the two transgenes which we have been unable to fully explore and address. The data presented below represent the final PCR runs in December 2020. Initial DNA concentration was quantified by both NanoDrop and Qubit; after sample dilution, DNA concentration was quantified by NanoDrop on December 1 and 2, and by Qubit on December 7.

On December 1, 2020, the control and mothers 174, 175, 177, and 179 showed strong bands for both CBA and NptII, while mother 166 produced a weak band for NptII only (Table 4). Of the offspring, only seedlings 30 and 36 produced weak bands for NptII and none of the offspring produced weak bands for CBS. The 280/260 and 230/260 ratios indicate that the DNA quality of the mothers was better than the offspring, so there may have been an issue with the collection or DNA extraction of these tissues.

Table 4. Results of PCR run from December 1, 2020.

ID	Mother	ng/ul Nanodrop	DNA (ul)	H2O (ul)	CmCBS*	NptII*	ng/ul Nanodrop after dilution	280/260[1]	230/260[2]
Plasmid (+ control)	**N/A**	1ng/ul			+++	+++			
Mothers									
166	N/A	135.8	9.2	40.8	-	-+?	21.5	1.71	1.36
174	N/A	229.4	5.4	44.6	+++	+++	17.4	1.93	2.56
175	N/A	125.7	9.9	40.1	+++	+++	21.2	1.87	1.83
177	N/A	196.9	6.3	43.7	+++	++++	20.6	1.94	2
179	N/A	47.6	26.3	23.7	++	++	17.7	1.73	0.73
Offspring									
21	166	47	26.6	23.4	-	-	17.6	1.29	0.47
22	166	104.3	12	38	-	-	17.4	1.43	0.85
23	166	132.9	9.4	40.6	-	-	7	1.12	0.51
24	166	172.9	7.2	42.8	-	-	22.1	1.68	1.47
25	166	151.4	8.3	41.7	-	-	11.6	1.56	1.15
26	166	131.3	9.5	40.5	-	-	14.1	1.64	1.14
27	166	102.3	12.2	37.8	-	-	21.8	1.68	0.98
28	166	81.9	15.3	34.7	-	-	13.7	1.41	0.69
29	166	95.2	13.1	36.9	-	-	15	1.55	0.97
30	166	97.7	12.8	37.2	-	-+?	18.3	1.53	0.92
33	166	109.6	11.4	38.6	-	-	19.4	1.71	1.37
36	166	90.3	13.8	36.2	-	-+?	15.5	1.42	0.63
37	166	103.3	12.1	37.9	-	-	13.8	1.55	0.83
40	166	110.5	11.3	38.7	-	-	21.9	1.63	1.21
43	174	105.7	11.8	38.2	-	-	16	1.54	0.89
57	175	94.9	13.2	36.8	-	-	17.8	1.61	0.98
58	175	88	14.2	35.8	-	-	22.7	1.78	1.51
60	175	91.2	13.7	36.3	-	-	15	1.52	0.85

*Run 1 Dec 2020. DNA diluted to 25 ng/ul, 1 ul used per reaction. Program: 94C 3 min; 94C 30 sec, 53C 1 min, 68C 1 min, 35 cycles; 68C 5 min. [1]Absorbance ratios indicating purity of DNA. 280/260 ratio of ~1.80 is considered pure DNA. [2]Absorbance ratios indicating the presence of contaminants. 230/260 ratio of ~2-2.2 considered acceptable.

A second PCR run was performed on more of the offspring on December 2, 2020 and no bands were obtained for either CBS or NptII, even for samples with high quality DNA (Table 5). A control was not run on this day and the PCR program was slightly different from the run on December 1, so it is unclear where the error originated from.

Table 5. Results of PCR run from December 2, 2020.

ID	Mother	ng/ul Nanodrop	DNA (uL)	H2O (uL)	CmCBS*	NptII*	ng/ul Nanodrop after dilution	280/260[1]	230/260[2]
Offspring									
111	175	101.3	12.3	37.7	-	-		1.68	1.49
112	175	61.3	20.4	29.6	-	-		1.36	0.71
113	175	48.3	25.9	24.1	-	-		1.45	0.89
114	177	95.6	13.1	36.9	-	-		1.62	1.2
115	177	89.3	14	36	-	-		1.51	0.95
116	177	67.2	18.6	31.4	-	-		1.7	1.58
117	177	138.6	9	41	-	-		1.62	1.11
118	179	73.3	17.1	32.9	-	-		1.47	0.9
119	179	153.8	8.1	41.9	-	-		1.61	1.18
120	179	270.6	4.6	45.4	-	-		1.84	2.11

*Run 2 Dec 2020. DNA diluted to 25 ng/ul, 1 ul used per reaction. Program: 94C 3 min; 94C 30 sec, 53C 45 seconds, 68C 1 min, 35 cycles; 68C 5 min. [1]Absorbance ratios indicating purity of DNA. 280/260 ratio of ~1.80 is considered pure DNA. [2]Absorbance ratios indicating the presence of contaminants. 230/260 ratio of ~2-2.2 considered acceptable.

A final PCR was run on two of the mothers and a subset of offspring that still had enough remaining sample for analysis. This run resulted in positive bands for CBS (somewhat weak) and NptII (strong) for mothers 174 and 179 (Table 6). Seedling 51 from mother 174 also produced bands for both CBS (moderate) and NptII (somewhat weak).

Table 6. Results of PCR run from December 7, 2020.

ID	Mother	Nano (ng/ul)	Original Qubit (ng/ul)	DNA (ul)	H2O (ul)	Re-checked Qubit ng/ul	CmCBS*	NptII*	280/260[1]	230/260[2]
Offspring										
30	166	97.7	88.8	20.4	39.6	14.7	-	-	1.6	0.84
36	166	90.3	80.8	25.2	34.8	11.9	-	-	1.64	0.83
51	174	267	224.3	4.3	55.7	69.4	++	+	1.88	2.27
55	175	213.7	200.6	4.8	55.2	62.6	-	-	1.85	1.83
102	166	145.6	151.4	4.2	55.8	71	-	-	1.87	1.76
109	166	72.3	62.9	9.3	50.7	32.2	-	-	1.9	2.14
111	175	80.4	80	8.7	51.3	34.6	-	-	1.79	1.51
120	179	191.2	129.6	3.3	56.7	91.4	-	-	1.88	2.05
Mother										
174	N/A	229.6		4.1	55.9	73	+	+++	1.78	1.77
179	N/A	47.6		46	14	6.52	+	++	1.74	1

*Run 7 Dec 2020. DNA extracts diluted to 5 ng/ul, 1 ul used per reaction. Program: 94C 3 min; 94C 30sec, 53C 45 seconds, 68C 1 min, 35 cycles; 68C 5 min. [1]Absorbance ratios indicating purity of DNA. 280/260 ratio of ~1.80 is considered pure DNA. [2]Absorbance ratios indicating the presence of contaminants. 230/260 ratio of ~2-2.2 considered acceptable.

Initial seedling size metrics

Mean initial seedling height at Kentland was 22.6 ± 5.1 cm and did not differ among blocks (p=0.452), mother trees (p=0.549), or events (p=0.343). Mean initial groundline diameter (GLD) was 0.60 ± 0.17 cm and differed among mother trees (p=0.032) (Figure 9) and construct events (p=0.011) (Figure 10). Seedlings grown from Mother 177 had the largest GLD, while seedlings from Mother 166 were the smallest. Among the mother tree construct events, seedlings grown from Event 7 had the largest initial GLD, while trees from Event 2 had the smallest diameter. Initial GLD did not differ among blocks (p=0.918).

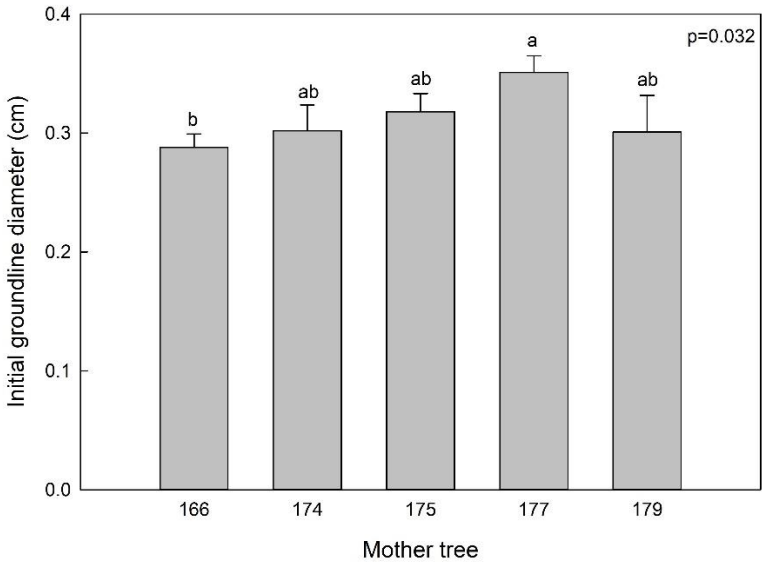

Figure 9. Initial groundline diameters of seedlings from the five mother trees. Error bars represent mean standard error, and different letters signify differences among groups at the α=0.05 level.

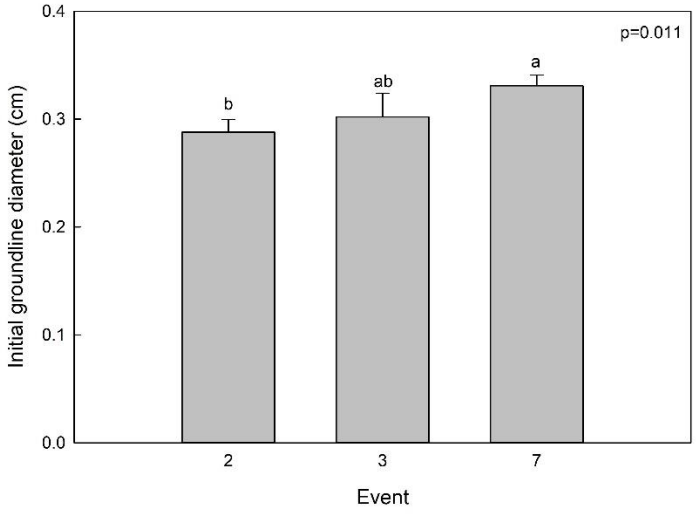

Figure 10. Initial groundline diameters of seedlings from the three mother tree construct events. Error bars represent mean standard error, and different letters signify differences among groups at the α=0.05 level.

Initial diameter at 10 cm on the stem differed among mother trees (p=0.017) (Figure 11) as well as mother tree construct event (p=0.019) (Figure 12). At this time, the relationships among mother trees and events for the diameter at 10 cm mirror those observed for GLD, though this may change over time. No differences in initial diameter at 10 cm height were detected among blocks (p=0.660).

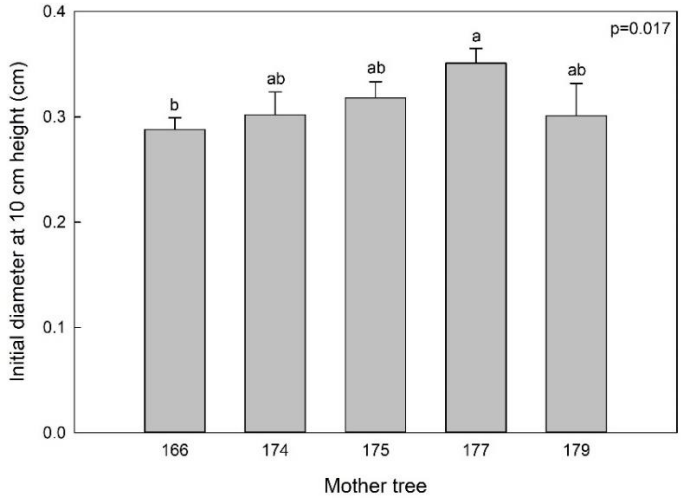

Figure 11. Initial diameters at 10 cm of seedlings from the five mother trees. Error bars represent mean standard error, and different letters signify differences among groups at the α=0.05 level.

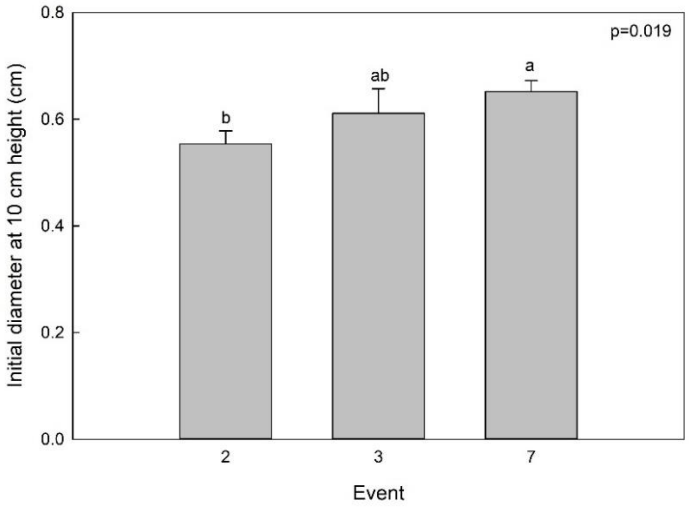

Figure 12. Initial diameters at 10 cm of seedlings from the from the three mother tree construct events. Error bars represent mean standard error, and different letters signify differences among groups at the α=0.05 level.

Seedling survival

Survival, as of July 29, 2020, was 65.8 % overall and differed among blocks (p=0.035) (Figure 13). Survival was highest in Block 2 (79.2 %) and lowest in Block 4 (37.5%), though neither differed from any of the other blocks. By October 21, 2020, survival was 55.8 % overall and continued to differ among blocks (p=0.015) (Figure 14).

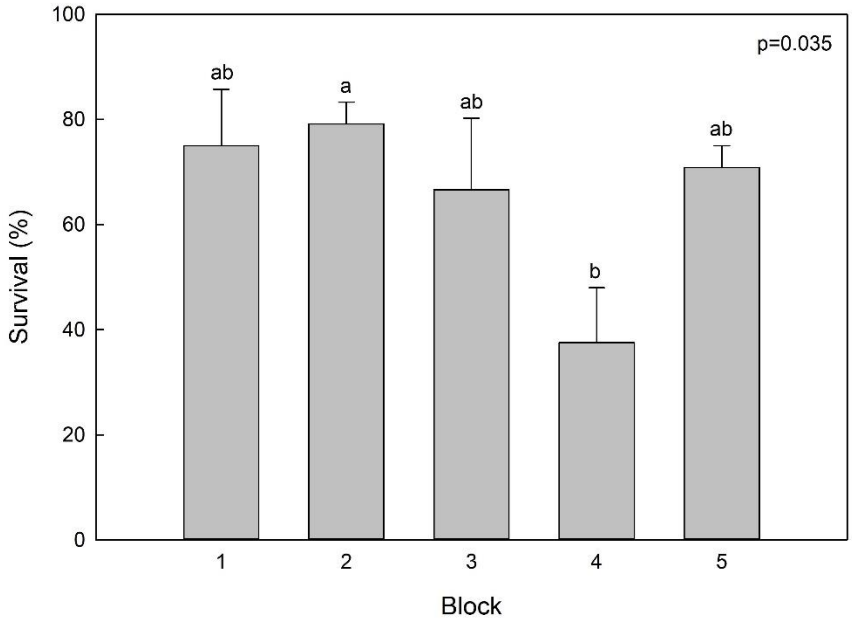

Figure 13. Survival of all CBS trees among planting blocks at Kentland Farm in July 2020. Error bars represent mean standard error, and different letters signify differences among groups at the α=0.05 level.

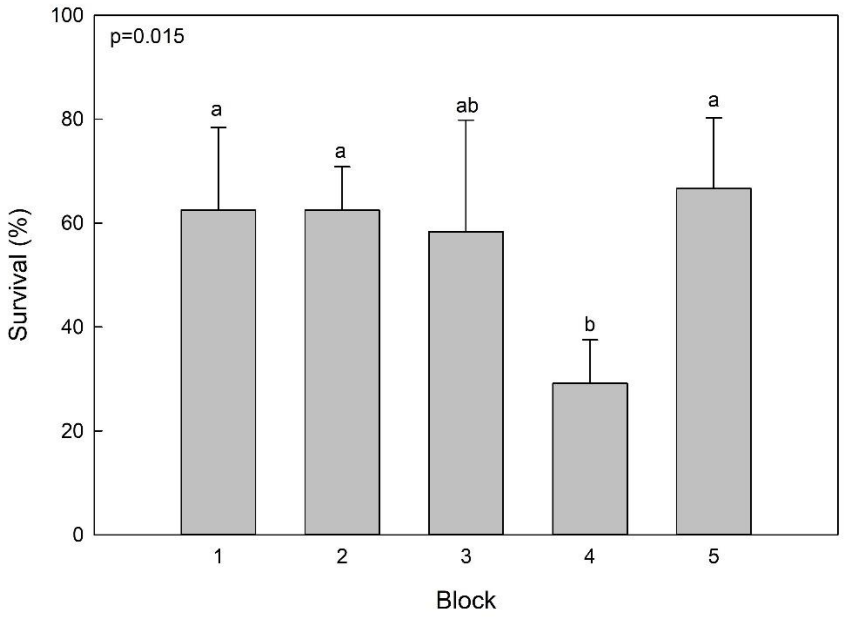

Figure 14. Survival of all CBS trees among planting blocks at Kentland Farm in October 2020. Error bars represent mean standard error, and different letters signify differences among groups at the α=0.05 level.

There were no statistically significant differences in July seedling survival among mother trees (p=0.645) (Figure 15), though survival was nominally higher among seedlings from Mothers 174 and 175. Similarly, July survival did not statistically differ by construct event (p=0.619) (Figure 16), but was nominally higher in Event 3, which includes only trees from Mother 174. Survival in October 2020 did not differ among mother trees (p=0.702) (Table 4). Survival in October 2020 also did not differ among construct events (p=0.659) (Table 5).

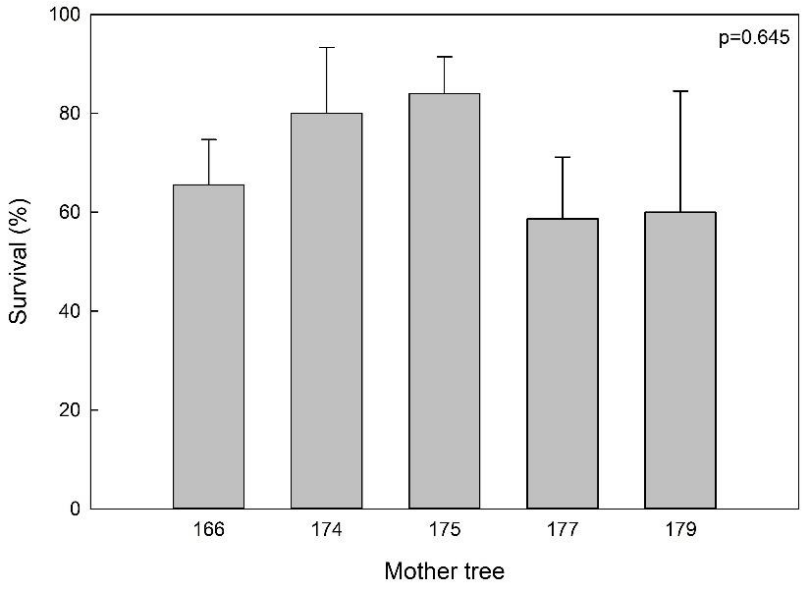

Figure 15. July 2020 survival of all CBS trees among mother trees at Kentland Farm. Error bars represent mean standard error, and different letters signify differences among groups at the α=0.05 level.

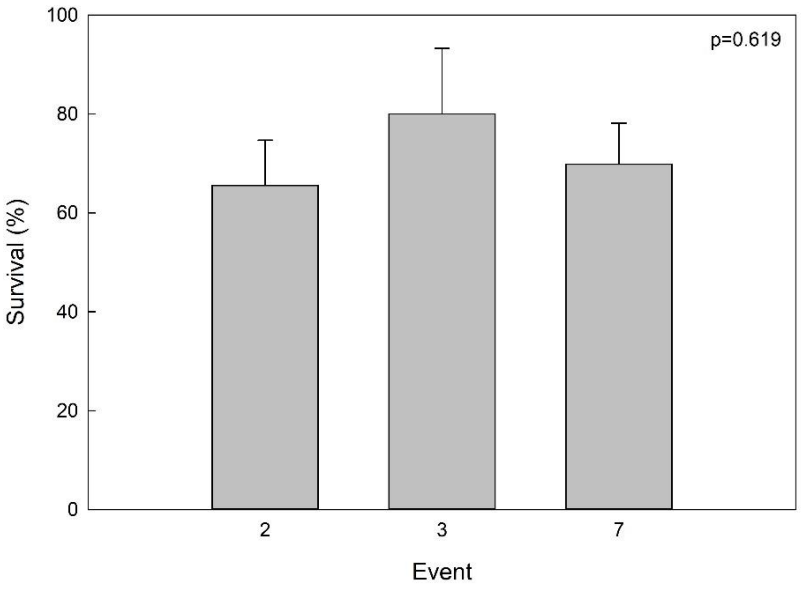

Figure 16. July 2020 survival of all CBS trees among mother tree construct events at Kentland Farm. Error bars represent mean standard error, and different letters signify differences among groups at the α=0.05 level.

Table 4. Mean ± SD October 2020 survival among offspring from five mother trees.

Mother tree #	Event	Survival
		%
166	**2**	48.9 ± 21.7
174	**3**	56.7 ± 27.9
175	**7**	64.0 ± 16.7
177	**7**	63.3 ± 13.9
179	**7**	40.0 ± 54.8

Table 5. Mean ± SD October 2020 survival among offspring from three construct events.

	Survival
Event	%
2	48.9 ± 21.7
3	56.7 ± 27.9
7	61.7 ± 13.8

October 2020 height and diameter

Mean height in October 2020 was 24.1 ± 5.9 cm and did not differ among blocks (p=0.089) (Table 6), mother trees (p=0.906) (Table 7), or Events (p=0.739) (Table 8). Mean groundline diameters in October 2020 was 0.70 ± 0.23 cm and did not differ among mothers (p=0.765) (Table 7) or events (p=0.612) (Table 8). Mean diameter at 10 cm in October 2020 was 0.30 ± 0.07 cm and did not differ among mothers (p=0.130) (Table 7). A significant difference for diameter at 10 cm was detected among events (p=0.044) (Table 8), but pairwise comparisons did not detect any differences between groups.

Table 6. Mean ± SD height for seedlings within all blocks.

Block	Height
	cm
1	26.0 ± 5.8
2	24.1 ± 7.0
3	25.2 ± 5.4
4	18.7 ± 2.9
5	23.9 ± 5.4

Table 7. Mean height, groundline diameter, and diameter at 10 cm for seedlings from all mothers.

Mother tree #	Event	Height	Groundline diameter	Diameter at 10 cm
			cm ± SD	
166	**2**	23.5 ± 6.2	0.62 ± 0.14	0.29 ± 0.05
174	**3**	25.7 ± 6.3	0.75 ± 0.42	0.29 ± 0.09
175	**7**	24.0 ± 6.0	0.66 ± 0.17	0.32 ± 0.07
177	**7**	24.4 ± 6.0	0.72 ± 0.29	0.33 ± 0.07
179	**7**	24.9 ± 4.5	0.58 ± 0.13	0.30 ± 0.06

Table 8. Mean height, groundline diameter, and diameter at 10 cm for seedlings within all construct events.

	Height	Groundline diameter	Diameter at 10 cm
Event		cm ± SD	
2	23.5 ± 6.2	0.62 ± 0.14	0.29 ± 0.05
3	25.7 ± 6.3	0.75 ± 0.42	0.29 ± 0.09
7	24.3 ± 5.8	0.68 ± 0.23	0.33 ± 0.06

October 2020 groundline diameters ($p=0.008$) (Figure 17) and diameter at 10 cm ($p=0.019$) (Figure 18) differed among blocks. For both measurements, diameters were largest in Block 1 and smallest in Block 4, while the remaining blocks did not differ from any other block.

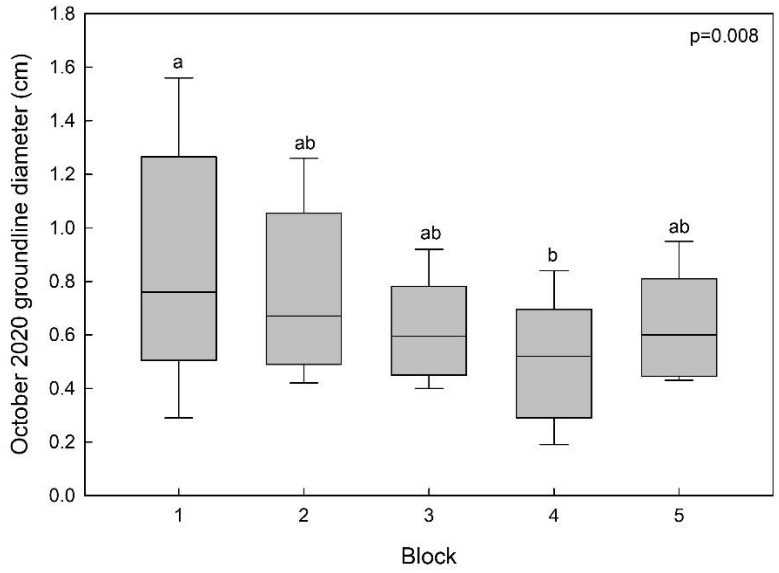

Figure 17. October 2020 groundline diameters within blocks. Box plots show median diameters, 25th and 75th percentiles, and error bars represent the 5th and 95th percentiles. Different letters indicate significant differences among groups at the α=0.05 level.

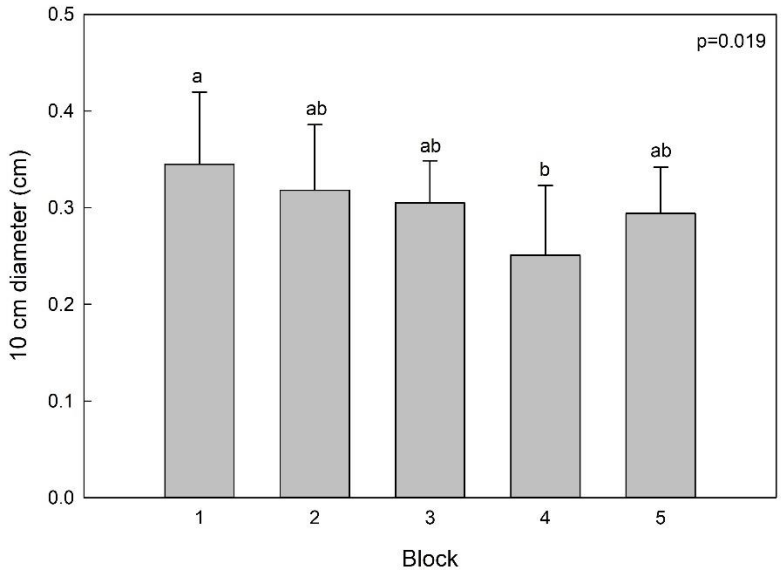

Figure 18. October 2020 mean diameter at 10 cm height on stem within blocks. Error bars represent standard deviations. Different letters indicate significant differences among groups at the α=0.05 level.

Year one growing season growth

Mean height growth in the first growing season was 0.2 ± 4.6 cm and differed among blocks (p=0.047) (Figure 19). Height growth was greatest in Block 1 and lowest in Block 4. Mean height growth did not differ among mothers (p=0.251) (Table 9) or events (p=0.074) (Table 10).

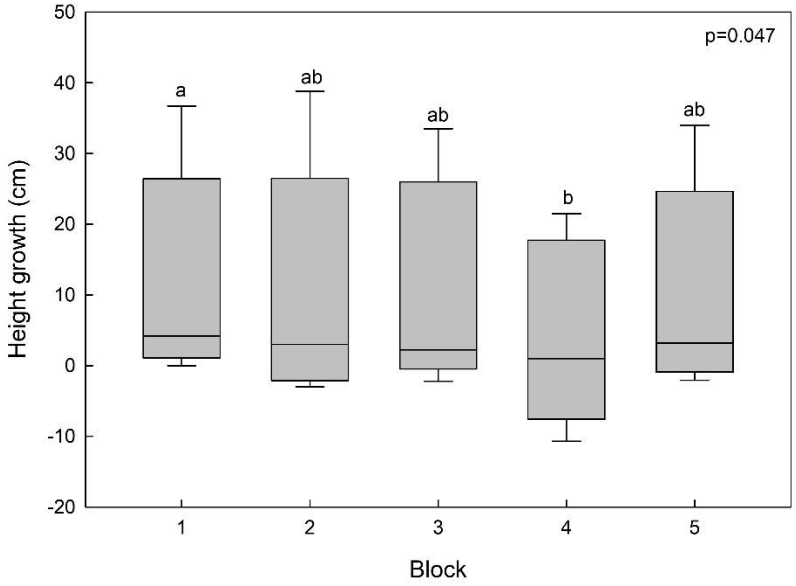

Figure 19. Year one height growth within blocks. Box plots show median diameters, 25th and 75th percentiles, and error bars represent the 5th and 95th percentiles. Different letters indicate significant differences among groups at the α=0.05 level.

Table 9. Mean year one height growth, groundline diameter growth, and diameter at 10 cm growth for seedlings from all mothers.

Mother tree #	Event	Height growth	Groundline diameter growth	Diameter at 10 cm growth
			cm ± SD	
166	**2**	0.0 ± 4.3	0.03 ± 0.14	-0.03 ± 0.05
174	**3**	2.8 ± 2.2	0.10 ± 0.24	-0.05 ± 0.04
175	**7**	0.4 ± 3.5	0.02 ± 0.11	-0.01 ± 0.06
177	**7**	-0.7 ± 6.3	-0.01 ± 0.26	-0.04 ± 0.10
179	**7**	0.6 ± 3.5	-0.07 ± 0.10	-0.03 ± 0.07

Table 10. Mean year one height growth, groundline diameter growth, and diameter at 10 cm growth for seedlings within all construct events.

	Height growth	Groundline diameter growth	Diameter at 10 cm growth
Event		cm ± SD	
2	0.0 ± 4.3	0.03 ± 0.14	-0.03 ± 0.05
3	2.8 ± 2.2	0.10 ± 0.24	-0.05 ± 0.04
7	-0.2 ± 5.0	-0.00 ± 0.20	-0.03 ± 0.08

Mean year one groundline diameter (GLD) growth differed among blocks (p=0.001) (Figure 20), but did not differ among mothers (p=0.699) (Table 9) or events (p=0.588) (Table 10). Among blocks, Blocks 1 and 2 had the most GLD growth, and were the only blocks with an overall increase in GLD. Blocks 3 and 5 showed very little change in GLD and Block 4 had a considerable decrease in GLD.

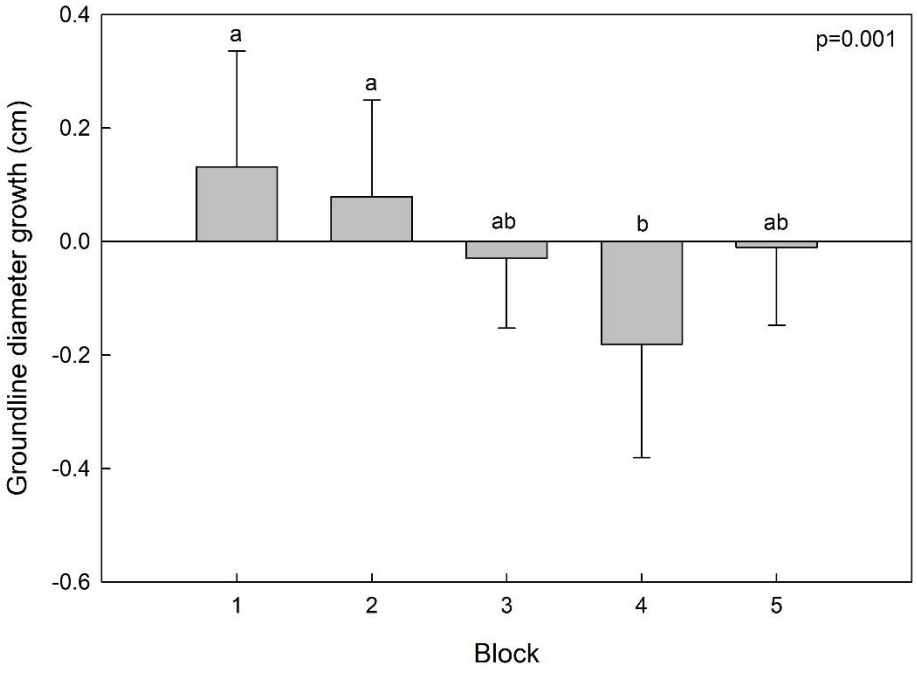

Figure 20. Mean year one GLD growth within blocks. Error bars represent standard deviations. Different letters indicate significant differences among groups at the α=0.05 level.

Mean year one diameter at 10 cm growth differed among blocks (p=0.004) (Figure 21), but did not differ among mothers (p=0.878) (Table 9) or events (p=0.732) (Table 10). Among blocks, Blocks 1, 2, and 3 had the greatest 10 cm diameter growth, but Block 1 was the only block with an overall increase in 10 cm diameter. Block 4 had the largest decrease in diameter at 10 cm. Decreases in diameter are associated with the death of the original stem and formation of new shoots from the rootstock, which is a typical response of chestnut trees to blight infection. However, no obvious blight symptoms were observed, so stem death was likely a response to other environmental stressors.

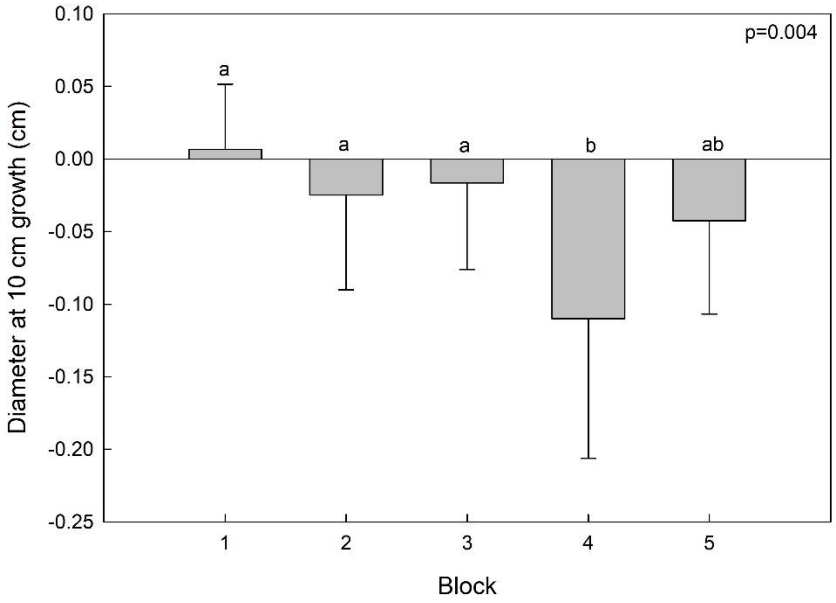

Figure 21. Mean year one diameter at 10 cm within blocks. Error bars represent standard deviations. Different letters indicate significant differences among groups at the α=0.05 level.

Discussion:

Germination

American chestnuts and chestnut hybrids germinate well in various settings, but germination of chestnuts can vary considerably with genetic background, and predation can significantly decrease germination and seedling survival. In two studies that took place in nursery settings, germination rates differed among genotypes, with consistently moderate germination of Chinese

chestnuts (< 70%) and variable germination rates among hybrid genotypes (23% to 97%) (Clark et al. 2012, Pinchot et al. 2015). Conversely, Skousen et al. observed fairly similar germination rates of 75% to 85% among pure Chinese, pure American, and hybrid chestnuts sown on a reclaimed coal mine, with the highest germination among hybrids, suggesting that hybrid chestnuts may germinate better in a field setting or in different soil types (2013). The background genotype of all our mother trees is a clone of a Virginia Department of Forestry hybrid of Chinese, American, and Japanese chestnuts. Mother trees may have been pollinated by hybrids (transgenic or non-transgenic), pure American chestnuts, or pure Chinese chestnuts. The variability in offspring genotypes likely contributed to our wide range in germination rates, while germinating them in a greenhouse setting may have resulted in decreased germination rates.

Germination rates are typically lower when nuts become damaged, such as from weevil predation during nut maturation, fungal rot, or rodent predation after planting (Dalgleish et al. 2012, Fields-Johnson 2012, Skousen et al. 2013). In this study, seed germination rates varied by Event, ranging from 25.5% to 74% and averaging 63.6% overall. Event 3, which included only Mother 174, had the lowest germination rates. Anecdotally, Mother 174 is the most isolated from other flowering chestnuts on the site and nuts seemed smaller, possibly suggesting incomplete pollination, which may have resulted in reduced germinability. In addition, although we observed differences among events, we think that the relatively lower germination rates observed in this study were predominantly related to nut damage from insects and fungal damage after planting. Though we did not quantify insect damage in this study, we assume that at least some insect damage occurred in the field while nuts matured, given the documented frequency at which it occurs (Dalgleish et al. 2012). In terms of fairly low germination overall, an infestation of fungus gnats due to overwatering in the BIOL greenhouse likely reduced germination; both by direct damage to cotyledons by the insects before seedling emergence as well indirectly by the spread of root pathogens (Braun et al. 2012).

Survival and growth

Chestnut seedling survival is strongly affected by a number of site variables, including soil texture, pH, and moisture content (Braun 1935, McEwan et al. 2005, Rhoades et al. 2009, Gilland and McCarthy 2012, Gilland and McCarthy 2014). While we did not quantify

differences across the site, we can anecdotally say that at least texture, moisture content, and soil compaction vary noticeably across the planting area. Overall, the seedlings survived fairly well on the orchard site and differed only among blocks. Survival did not differ by mother tree or event, but was significantly lower in Block 4 suggesting that survival was more strongly influenced by spatial differences at the site, though it is unclear what is driving those spatial differences. Initial measurements of height and diameters showed differences among mother trees and events, but in July and October, only block differences were detected. The region experienced a record-setting heat wave in July 2020. As seedling survival decreased considerably between July and October, it seems likely that this weather pattern strongly affected survival and growth. We suspect that any relatively small differences in soil texture, organic matter content, bulk density, or other soil characteristics that may affect water availability and root growth could have become more influential during the heat wave in terms of both seedling survival and growth.

Transgene inheritance

All mother trees, except for Mother 166, eventually produced bands for the CBS and NptII genes. Of the offspring, only sample 51 (Mother 174) produced bands for both transgenes, while samples 30 and 36 (Mother 166) produced weak bands for NptII. As it is impossible that all or most of the offspring did not inherit the transgene, we continue to suspect that there is an issue with the methodology and/or the quality of offspring DNA. Unfortunately, we were unable to fully investigate these issues with the funding and time available, so our ability to fully discuss these results is limited. Many of the 280/260 and 230/260 wavelength ratios suggest though, that DNA quality was poor. Poor DNA quality was likely due to collected leaves being too old at the time of collection, but there may have been errors during DNA extraction such as cross-contamination.

Conclusions

Overall, while some environmental issues and human error contributed to decreased germination and survival, the nuts produced by the CBS mother trees germinated well and have performed

well at the Kentland Farm field site. Ideally, we would have been able to investigate some of the spatial differences we observed across the site, but this was well outside the scope and funding for this project. We were unfortunately unable to quantify transgene inheritance in the offspring due to myriad issues with the methodology and sample quality, but we will apply what we have learned to future projects.

Future plans

Unfortunately, because of travel restrictions associated with the pandemic, we were unable to plant any seedlings in order to assess the growth or survival of the CBS seedlings on the mine site at the appropriate time of year. We were hoping to be able to plant them on the mine in 2021, but may be unable to due to our permits for that site expiring soon. We do plan to replace any dead seedlings at Kentland in the fall, although we may not be able to represent all of the mother trees or events due to lack of availability. Currently, the remaining seedlings are being cared for at Reynold's Homestead in Critz, VA.

References:

American Chestnut Foundation (ACF) (2014) Retrieved 21 Sep 2014 from <http://www.acf.org/history.php>

Andrade, G.M. & S.A. Merkle (2005) Enhancement of American chestnut somatic seedling production. *Plant Cell Reports*, **24**: 326-334.

Beyhan, N. & U. Serdar. (2009) Pollen viability and germination rates of some hybrid and European chestnut pollens. *Acta Horticultura*, **815**: 107-114.

Braun, E.L. 1935. The vegetation of Pine Mountain, Kentucky: an analysis of the influence of soils and slope exposure as determined by geologic structure. *The American Midland Naturalist*, **16**: 517-565.

Braun, S.E., J.P. Sanderson, & S.P. Wright. (2012). Larval *Bradysia impatiens* (Diptera: Sciaridae) potential for vectoring Pythium root rot pathogens. *Phytopathology*, **102 (3)**: 283-289.

Burger, J., D. Graves, P. Angel, V. Davis, and C. Zipper. (2005) The Forestry Reclamation Approach. The Appalachian Regional Reforestation Initiative. Forest Reclamation Advisory No. 2.

Carraway, D.T. and S.A. Merkle. (1997) Plantlet regeneration from somatic embryos of American chestnut. *Canadian Journal of Forest Research*, **27**: 1805-1812.

Centers for Disease Control and Prevention: Office of Mine Safety and Health Research (CDC) (2012) Locations of active surface coal mining operations. Retrieved 20 Sep 2014 from www.cdc.gov

Clark, S.L.; Schlarbaum, S.E.; Saxton, A.M.; Hebard, F.V. (2012). Nursery performance of American and Chinese chestnuts and backcross generations in commercial tree nurseries. *Forestry*, **85**: 589–600.

Dalgleish, H.J., J.T. Shukle, & R.K. Swihart. (2012). Weevil seed damage reduces germination and seedling growth of hybrid American chestnut. *Canadian Journal of Forest Research*, **42**: 1107-1114.

Fields-Johnson, C.W. (2011). Appalachian surface mine reforestation techniques: effects of grading, cultural treatments and species selection. Master's Thesis, Virginia Tech, Blacksburg.

Gilland, K.E. & B.C. McCarthy. 2012. Reintroduction of American chestnut (*Castanea dentata*) on reclaimed mine sites in Ohio: microsite factors controlling establishment success. *Northern Journal of Applied Forestry*, **29**: 197-205.

Gilland, K.E. & B.C. McCarthy. 2014. Performance and phenotypic variation of American chestnut (*Castanea dentata*) hybrids on newly reclaimed mine sites in eastern Ohio, USA. *Ecological Restoration*, **32**: 379-387.

Hebard, F.V. and P.A. Rutter (unknown publication date) Growing chestnut trees from seed. Retrieved 15 August, 2019 from https://www.acf.org/ma-ri/the-project/quick-growing-guide/

McEwan, R.W., C.C. Rhoades, S. Beiting. 2005. *Castanea dentata* ([Marsh] Borkh.) in the pre-settlement vegetation of Mammoth Cave National Park, central Kentucky. *Natural Areas Journal*, **25**: 275-281.

Pinchot, C.C., S.L. Clark, S.E. Schlarbaum, A.M. Saxton, S.S.Sung, and F.V. Hebard. (2015) Effects of temporal dynamics, nut weight, and nut size on growth of American chestnut, Chinese chestnut, and backcross generations in a commercial nursery. *Forests*, **6**: 1537-1556. doi: 10.3390/f6051537

Rhoades, C., D. Loftis, J. Lewis, & S. Clark. 2009. The influence of silvicultural treatments and site conditions on American chestnut (*Castanea dentata*) seedling establishment in eastern Kentucky, USA. *Forest Ecology and Management*, **258**: 1211-1218.

Sigmaplot 12.5. Systat Software, San Jose, CA.

Skousen, J., T. Cook, L. Wilson-Kokes, & E. Pena-Yewtukhiw. (2013). Survival and growth of chestnut backcross seeds and seedlings on surface mines. *Journal of Environmental Quality*, **42**: 690-695. doi:10.2134/jeq2012.0368