Two-Year Field Trial of Genetically Engineered American Chestnut Reveals Greater Fungal Blight Tolerance Compared to Wild-Type Full-Sibling Trees

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Abstract

The American chestnut (*Castanea dentata*) was a foundational forest canopy species in eastern North America until an imported fungal blight (caused by *Cryphonectria parasitica*) rendered it functionally extinct across its native range. Biotechnological approaches, such as the Darling 54 (D54) transgenic line, have potential for future restoration of American chestnut, but field-based evaluations of blight tolerance have been limited. Field-based evaluation is slowed by the many years it takes for seedlings to grow to saplings, then to full-fledged trees. Current regulatory restrictions also constrain the testing of transgenic chestnuts to just within permitted orchards. This research reports on a two-year field trial of four- to five-year-old, T3- and T4-generation transgenic D54(+) saplings and their non-transgenic full-siblings in a permitted orchard in southern Maine. The field trial deployed a randomized block design with wild-type American chestnut and Chinese chestnut (*Castanea mollissima*) controls. In the two years, 261 trees were branch-inoculated in three replicates with EP-155, a highly virulent strain of the fungal blight. D54(+) trees consistently outperformed their negative siblings and outperformed Chinese chestnut in a limited comparison. To our knowledge, this is the first multi-year field trial of fungal blight inoculations comparing advanced generation D54 families. This field-based evaluation of blight tolerance in D54 transgenic chestnuts contributes to the goal of restoring this iconic species to its eastern North American native range.

Key words: fungal blight · genetic engineering · field trials · inoculation · restoration · eastern US forests

Introduction: the American chestnut, fungal blight, genetic engineering, and inoculation

For millennia, the American chestnut (*Castanea dentata, Cd*) was a foundational species of eastern US forests stretching from Maine to Mississippi and west to Indiana (Powell 2014). In the late 1800s, a fungal blight (caused by *Chryphonectria parasitica, Cp;* Rigling and Prospero 2017) was accidentally introduced. By the 1950s, *Cp* had spread throughout the native range to kill the vast majority of the estimated four billion American chestnut trees (Powell et al. 2019). American chestnut survives today mainly as stump sprouts, as *Cp* does not kill the roots. However, trees rarely grow to maturity before succumbing to the blight.

Genetic engineering (GE) is one method being deployed to restore the American chestnut. Aiming to give American chestnuts fungal blight tolerance (FBT), a team headed by the late Dr. William Powell at the State University of New York Environmental Science and Forestry College (SUNY-ESF) inserted the oxalate oxidase (OxO) gene from wheat into Ellis 1, a pure American chestnut line, with the intention of detoxifying the oxalic acid that is excreted by the necrotrophic fungus, thus conferring blight tolerance (Steiner et al. 2017). The transformation of Ellis 1 using constitutively driven OxO generated two events, Darling 54 (D54) and Darling 58 (D58), and as reported in Klak et al. (2025), the D54 event is currently under consideration for deregulation (Jacobs et al. 2023; Newhouse et al. 2024). Since 2019, all genetically engineered materials (pollen, nuts, and seedlings) distributed from both SUNY-ESF and the University of New England (UNE) to permit holders across the native range have been determined to originate from D54 rather than D58 (Klak et al. 2025).

In this field trial, we utilized T3- and T4-generation D54 saplings, where D54(+) refers to hemizygous D54 lines that carry the 35S::OxO transgene while D54(-) refers to null segregants from the same families that are wild-type (WT). Previous field tests of D54's effect on FBT are few and based on small-scale field tests (e.g., only n=3 D54(+) and n=3 D54(-)) that were conducted on T1 or T2 saplings (Powell et al. 2020; ESF 2023; Newhouse et al. 2024, p.103). Additionally, a public comment from Purdue University's Hardwood Tree Improvement and Regeneration Center to APHIS concerning D54 deregulation cited an ongoing field inoculation trial from 16 D54 families and reported evidence of D54(+) saplings outperforming D54(-) (Kell 2025). The present report establishes a baseline for future transgenic fungal blight field trials.

To date, no definitive fungal blight assay has been established. There are only approximations of the impacts of natural fungal blight. These include the method deployed in this research, which we have termed the branch inoculation assay (BIA, also called AltSSA; Conn et al. 2023). Much trial and error over the years has revealed that chestnuts of different sizes and ages call for different inoculation test methods. For seedlings, a small stem assay (SSA) has often been used, although this method has been found to have a large "no take" (NT, failed

inoculations) rate, has outright killed many tested seedlings, and has tended to have a low correlation with later tests of larger trees (Conn et al. 2023; Newhouse et al. 2024, pp.102-3). Indeed, evaluating the blight tolerance of relatively young trees presents a challenge. Main stem inoculation (MSI) has been used extensively for trees with a 38mm (1.5") or larger diameter at breast height (DBH) and is not recommended for smaller trees (Georgi & Fitzsimmons 2015; Penn State 2025). In the present study, as of 2025, 69% of inoculated trees had stem diameters too small for MSI (measured at 15.2cm (6")) above the root collar). For trees under 38mm DBH, even highly resistant stems may be lethally girdled when inoculated at too small a diameter (Conn et al. 2023, p. 1576). Blight can aggressively penetrate through a small tree's living layers rather than surrounding them as is typical *Cp* behavior with larger trees (Hebard 2012).

A further complication is the American chestnut's natural lifespan of five hundred or more years in the absence of blight. Predictions about long-term survivability based on evidence from young trees must be taken with caution. Nonetheless, it is valuable for the sake of chestnut restoration progress to assess FBT early on. This is particularly true in the field where, instead of the relatively stable, consistent, mild and idealized lab/greenhouse conditions, trees are exposed to harsher weather, seasonality, herbivory, a wider range of environmental conditions, and vicissitudes such as late freeze and summer drought. As noted below, the young trees evaluated in this study have already experienced all of the above.

Methods

Tree Selection

Empirical research was conducted at a one-acre, APHIS-permitted orchard (permit #124-833JJQQ) in long-term partnership with a private landowner in Cape Elizabeth, Maine (Klak 2018; TACF 2025; **Supp. Figs. 1 and 2** for general location). The GE chestnuts and *Cp* fungus deployed in this research were under restricted use, governed by APHIS permits. Chestnuts in this orchard came from WT mother trees that had been bred the previous summer with GE pollen produced in speed breeding labs at UNE and ESF (Klak et al. 2025). The seedlings were grown from nuts assembled from crosses with WT trees in Maine and elsewhere provided by collaborators. Seedlings were outplanted at the orchard after growing indoors in UNE facilities for four months. First outplanted in 2021, the site remains the only orchard of transgenics and full-sibling WTs in New England. A full-sibling family is defined here as a group of OxO-positive and WT progeny from a cross between a single mother tree and a single father (pollen) in the same season. The study focused on comparing sibling saplings that differed only in whether they inherited the OxO gene.

Not all seedlings survived the first or even the second year of outplanting. Rodents, drought, late frost, and naturally-introduced blight were among the causes. Of these, predation by rodents (voles and mice), resulting in excessive root loss and girdling of young stems, was especially significant. Replacement plantings of D54(+) and D54(–) full-siblings were made in the following summers. In 2023, OxO(+) seedlings from a WX162 event that included a wound-induced promoter from poplar (*Populus deltoides*; Carlson et al. 2022) were added to the orchard and as of 2025, nine were alive. This provided an opportunity for a limited comparison with four D54(+) plants of the same age. By 2025, the chestnuts used for this field trial were between three- and five-years-old and sapling-sized or nearly so.

Inoculation Method

More than one hundred strains of *Cp* fungus are known. EP-155 is the most virulent documented strain and in recent years has been widely used in chestnut challenge assays (Hebard 2012; Conn et al. 2023). For this research, *Cp* was cultured at West Virginia University's Forest Pathology and Environmental Microbiology lab. After culturing, it was dried on filter paper and then stably frozen at -20ºC until used in 2024 or 2025. *Cp* was reconstituted in potato dextrose agar in petri dishes, following standard chestnut inoculation protocols. To plate the spores onto the agar in an aseptic environment, the Midwest Grow Kit's oven-plating procedure was used (Midwest Grow Kits 2019). In the field, inoculum discs were cut to fit within the diameter of crimped straws and positioned so that *Cp* was in direct contact with each newly cut branch (following May 2025; **Supp. Fig. 3**).

The branch inoculum assay (BIA) was developed and previously tested in Georgia, near the southern edge of the American chestnut's range (Cipollini et al. 2021). The present study is the first to deploy BIA outside of the south, indeed near the northern edge of the chestnut's native range. In addition, BIA is appropriate for plants of sapling size, and has another advantage discovered in prior studies. It has yielded canker production of over 90%

(i.e. few "no takes" (NT)). This rate is higher in many cases than both the SSA and MSI inoculation methods (Conn et al. 2023).

Note that previous publications on the BIA describe it as "intended primarily for forward selection" (Conn et al., p. 2023). This means that very young chestnuts can be screened with BIA and only those with small cankers can be outplanted, thus saving orchard space and effort. At the same time, BIA deployed in previous work correlated with other measures of blight tolerance, including a multifactorial method applied to the mother tree (Conn et al. 2023). Previous research utilized BIA to differentiate susceptibility between trees ranging from *Cd* to *Castanea mollissima* (*Cm)* and hybrids in between. To our knowledge, the present study is the first application of BIA to compare GE chestnuts to full-sibling WTs. BIA is deployed in the present research as the best nondestructive tool available to assess trees beyond seedling-size but not yet large enough for MSI.

Branches at different heights on the chestnut plant play different metabolic roles and will react differently to a *Cp* infection. Therefore, in both years of this study, three branches were selected for inoculation on each tree rather than the one inoculation in previous studies. This improvement was recommended to better account for "experimental or environmental variation" and to capture a more generalized response (Cipollini et al. 2021, p. 583). To further standardize, selected branches were, whenever possible, south-oriented and mid-level on the tree. Branch diameters needed to be in the 4.5–5.5mm range to firmly secure the crimped straw containing the *Cp* plug against the fresh wound (**Supp. Fig. 3**). A few smaller trees did not have three branches of ≥4.5mm, in which case only one or two branches were inoculated. In both years, trees were inoculated on June 5, the crimped straws containing the inoculum were removed one week later, and the cankers were removed and measured on September 3. Previous research determined that measurements gathered 90 days post-inoculation are most useful (Conn et al. 2023).

At 90 days, when cankered branches were removed for measurement, the sites from where they were cut were disinfected with 70% isopropyl alcohol to reduce the likelihood of further infection to the tree. Following protocol, two measurements were taken. One was the orange or darkened zone where *Cp* had advanced from the inoculation site up the branch. According to Conn et al. (2023, p. 1580), this discoloration "may be indicative of a relatively deep penetration of the fungal mycelium in the host tissue". A second measure called full canker length (FCL) was taken of a longer canker if there was evidence of additional, patchy discoloration up the branch. This measure in both 2024 and 2025 parallelled previous studies. Where a difference was observed was in what previous studies called the "orange canker" (May 2025). In 2025, that zone was darker in color and more shriveled than in 2024 and so is referred to as "darkened canker" for 2025.

Statistical Methods

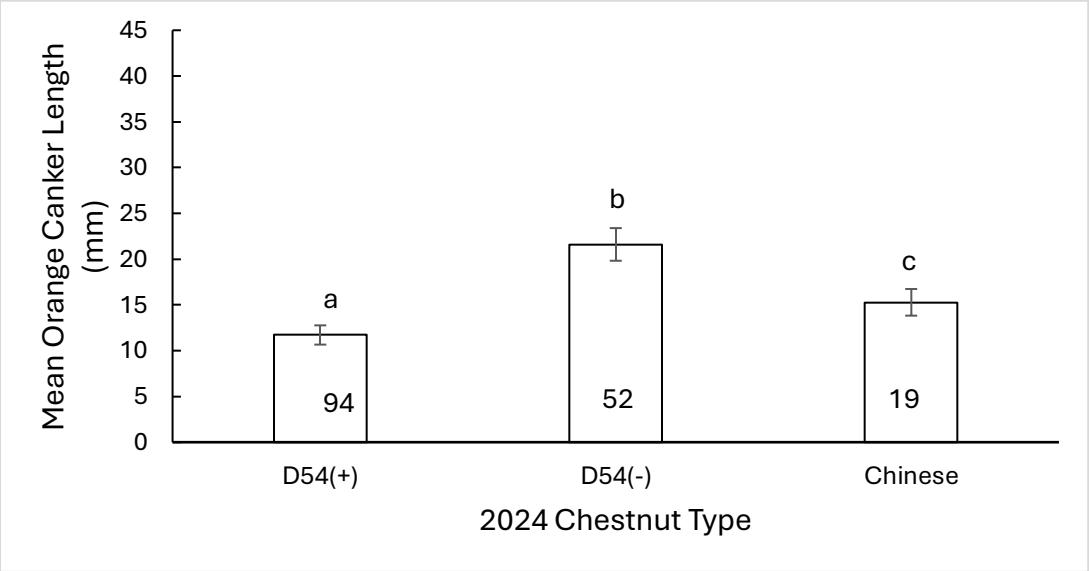
Data were analyzed following a standard analysis of covariance (ANCOVA) approach, which treated chestnut type (i.e., D54(+), D54(-), *Cm* alleles) as the principal factor of interest. Tree height was treated as a covariate of both orange/darkened canker and full canker length responses to *Cp* infection. To fully meet model assumptions, each blight response, as well as the height covariate, were log- or square-root-transformed prior to analysis. Initially, split-plot ANCOVAs were run using just the D54(+) and D54(-) families from the larger data set collected in 2025, which allowed for a comparison of chestnut types nested within their full-sibling families and year of planting. Although the data set collected in 2024 did not include sufficient full-sibling families to allow for the same approach, it was still possible to compare combined full- and half-sibling families separately by mother tree and by pollen source. This comparison was calculated for 2025 as well to assure consistency of results. Neither year nor family was a significant variable in either the single 2025 full-sibling model nor any of the 2024 full- and half-sibling models. These two factors were therefore omitted from subsequent analyses, which allowed us to broaden our comparisons to include a third chestnut type, trees with *Cm* alleles. These tests evaluated the effect of type on both orange/darkened canker and full canker length, holding height constant. Significant overall effects of chestnut type were followed by multiple pairwise comparisons of type, which were evaluated by applying a Bonferroni correction to test-wise alpha levels. We sought to strike a balance between minimizing the probability of committing Type I and Type II errors and thus set our test-wise α-levels at 0.033 ($\alpha = 0.10/3$).

Results

Year 1 Results

As in previous studies, two canker lengths were measured along the branch from the inoculation site: orange/darkened canker and full canker (**Supp. Fig. 4**). The most significant contrast was between the D54(+) and D54(-) types which differed statistically for both measures of canker length (orange canker length: $F_{2,143}=39.55$,

p<0.0001; full canker length: $F_{2,143}$=32.50, p<0.0001; **Fig. 1** and **Supp. Tables**). D54(+) also had shorter orange canker lengths than trees with Chinese alleles ($F_{2,110}$=6.58, p=0.0117; **Fig. 1** and **Supp. Tables**). However, saplings with *Cm* alleles were less genetically diverse and from limited families.



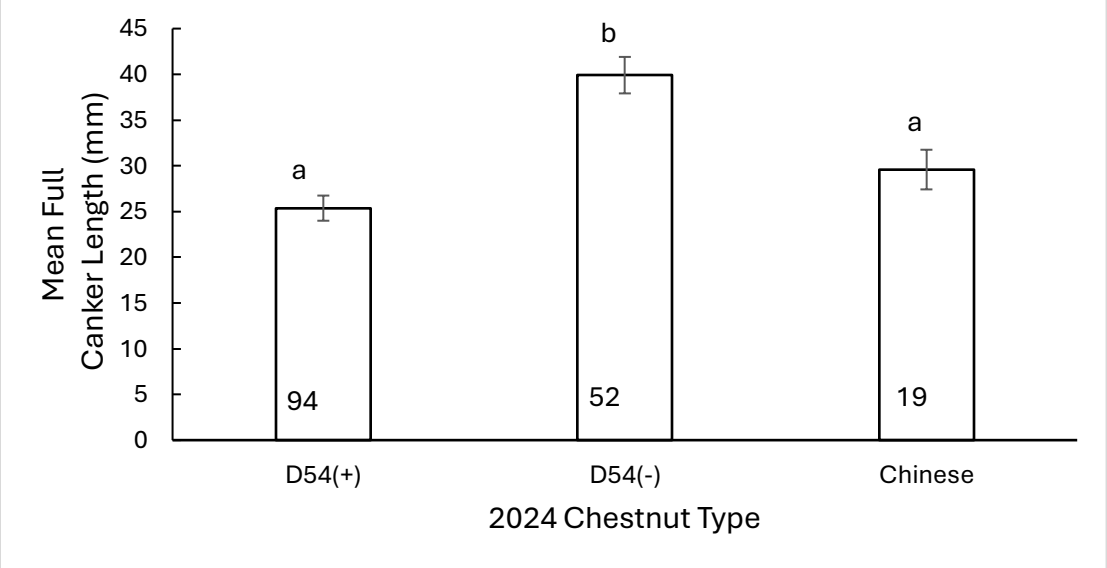


Fig. 1 90-day mean canker lengths for the 3 chestnut types in year 1. The number inside each bar graph is the sample size. D54(+) refers to hemizygous D54 lines that carry the 35S::OxO transgene, D54(-) refers to null segregants from the same families that are WT, and Chinese refers to plants with *Cm* alleles. Error bars represent standard errors. Shared letters above the bars indicate non-statistically-significant differences in means.

At 90 days, the presence of sporulation on cankers was also recorded. Sporulation occurs when the *Cp* infecting the inoculation starts reproducing. Sporulation was recorded when orange clumps of spores, forming over the branches' normally white lenticels, were visible (**Supp. Fig. 4**). If the orange spores were present, they could easily transfer onto fingers or equipment. Both were sanitized between saplings. If spores were present on one of a plant's inoculations, the entire individual was recorded for the presence of sporulation. A $\chi 2$ test was run on the presence or absence of sporulation between types, then a series of odds ratio tests was run as planned contrasts to determine if D54(+) saplings differed in sporulation presence from the D54(-) type or the trees with *Cm* alleles. The only significant result of the three planned contrasts was between D54(+) and D54(-) saplings. There was

significantly less sporulation observed on D54(+) saplings compared to D54(-) saplings (odds ratio 0.403, confidence interval 95% [0.181, 0.901], p-value=0.013).

Year 2 Results

Unfavorable weather conditions significantly impacted younger chestnut trees across Maine in 2025. Whereas 2024 was a relatively normal summer for temperatures and rainfall by historical standards, 2025 was exceptionally difficult for younger chestnuts. Compared to 2024, 2025 had greater swings from a wet early summer followed by prolonged drought. Commenting on the impacts on agriculture in 2025, which would also apply to young chestnut trees, Maine's Drought Task Force explained that "Crops typically require about 1" of water per week and this has not happened during June through August" (Maine 2025; see also **Supp Figs. 1** and **2**). The timing of the drought corresponded with the field trial.

Phenotypic impacts of the 2025 extreme weather were visible during the 90-day period. Wilted leaves, leaf drop, seedling death, and, for mature trees outside the orchard, bur drop and the aborting of flowers were observed. Indeed, across five monitored WT orchards that host 5- to 8-year-old trees in different Maine locations, the production of burs was severely diminished and even zero in some places. In prior, more normal years, there was an increasing crop of flowers followed by burs and fertile nuts on trees as they matured, as expected with chestnut maturation. However, 2025 saw a substantial drop from the trajectory of the previous years. These observations, along with what we observed in terms of disease development on these trees in 2025 compared to 2024, are potentially associated with drought, resulting in an atypical growing season in 2025.[1]

One methodological improvement for 2025 was that additional surviving 4- to 5-year-old D54(+) and D54(-) orchard trees with the same family ancestry were identified and added to the study. This allowed for more intra-family comparisons of D54(+) and D54(–) (i.e., same mother tree and pollen source). At the same time, some D54 saplings that were challenged in 2024 but did not have a full-sibling counterpart were excluded in 2025. Lastly, all surviving saplings with *Cm* alleles in the orchard were inoculated in 2025. This increased the number of *Cm* plants with canker measurements from 19 to 30. However, the differences among the *Cm* saplings in population size and percentage *Cm*, and the limited parental variety (only 5 families) render statistical comparisons less definitive.

As mentioned earlier, the orange zone was darker in color and more shriveled in 2025 than in 2024. It is possible that, if measurements had been taken before the standard 90 days, cankers would have looked more orange. We speculate that the more shriveled and darker-colored branches were impacted by the drought. Consistent with this interpretation is the fact that whereas fungal sporulation was observed on most (i.e., 69%) orange zone branches in 2024, it was entirely absent at 90 days in 2025. Like the chestnut hosts, *Cp* appeared phenotypically drier during the 2025 drought. Whether the canker zone was orange as in 2024 or darkened as in 2025, there is considerable consistency in the inter-year findings for D54 (**Figs. 1** and **2**). To accurately capture the inter-year differences, we refer to the orange canker for the 2024 observations and the darkened canker for the 2025 observations. In 2025, the darkened cankers on D54(+) trees were shorter than those on D54(-) full-siblings ($F_{1,63}$=5.61, p=0.0209) and also shorter than those of trees with *Cm* alleles ($F_{1,61}$=16.80, p=0.0001; see also **Supp. Tables**).

[1] In one orchard in Saco, Maine, representative of the wider trend, in 2024 a total of 9 burs containing 17 fertile nuts were harvested from 7-year-old trees. In 2025, following an identical transgenic pollination method as in 2024, the yield from the then 8-year-old trees was 2 burs containing a total of 5 fertile nuts, which represents a 71% decline in nut production. Those burs/nuts that did mature in several orchards were noticeably smaller than previous years. So, reduced fertile nut development was one impact of drought even on trees mostly older than the 4–5-year-old inoculated ones.

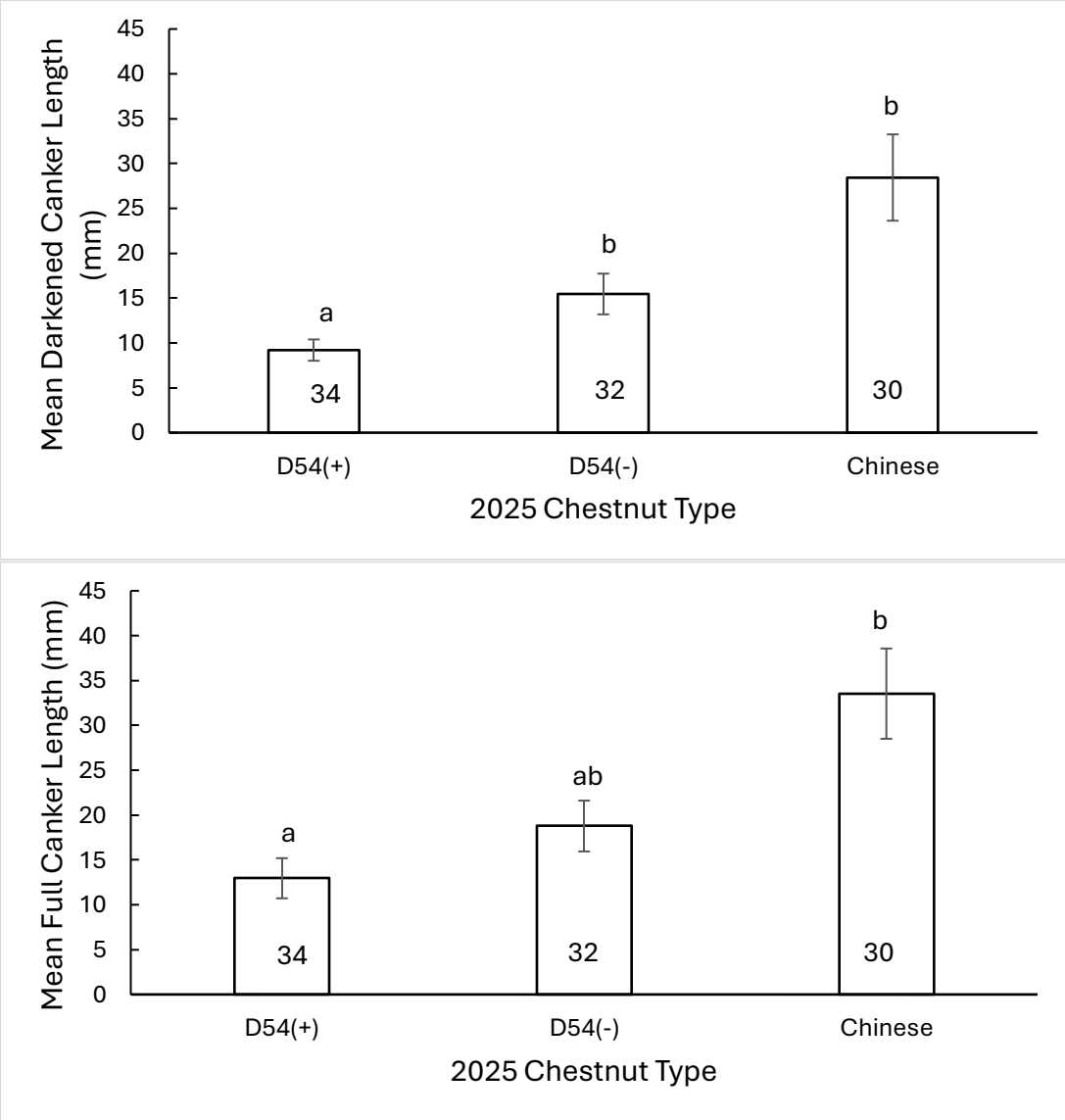


Fig. 2 90-day mean canker lengths for the 3 chestnut types in year 2. The number inside each bar graph is the sample size. D54(+) refers to hemizygous D54 lines that carry the 35S::OxO transgene, D54(-) refers to null segregants from the same families that are WT, and Chinese means plants with *Cm* alleles. Error bars represent standard errors. Shared letters above the bars indicate non-statistically-significant differences in means.

Another question was whether cankers differed between trees that were 4- versus 5-years-old in 2025. A well-replicated test of outplanting year and family via comparison of D54(+) and D54(-) showed no effect of either factor on blight canker metrics (e.g., for darkened canker length, Year: $F_{1,10}$=0.06, p=0.8064; Family: $F_{10,12}$=0.31, p=0.9646). After these alternative explanations were discounted, the conclusion was that both canker measurements showed that D54(+) trees outperformed their WT full siblings ($F_{12,40}$=2.69, p=0.0095).

Another addition for 2025 was the comparison of a limited number of very young (3-year-old) trees that differ in terms of OxO promoter. Whereas the D54(+) trees have a constitutive promotor (CaMV35S; n=4), the WX162(+) trees have a wound-induced promoter (WX162; n=9; Guilley et al. 1982; Carlson et al. 2022). There was no statistical difference in canker length between these two transgenic types in the limited number of 3-year-old saplings tested (**Supp. Tables**).

Combined 2024 and 2025 Results

The two previous BIA studies had very low NT rates and ≥98% inoculation success (Cipollini et al. 2021; Conn et al. 2023). In our study, the NT rates were slightly higher, with a 91% inoculation success rate in 2024 and 95% in 2025. In comparison, the BIA NT rate was much lower than the ~40% NT rate sometimes produced by traditional SSAs (Gentner 2018).

For both years, height and diameter were significant positive covariates with canker length. We determined which covariate had a more significant relationship with each response variable using a simple linear regression (response ~ covariate). Height was more highly correlated with canker length so it was included in the model.

All family effects and influence from the height covariate were factored out for all types, so the only difference between these two types was their presence or absence of the transgene. When height was held constant and included in the model, D54(+) trees had statistically shorter dead-zone cankers than full-sibling D54(-) trees. The WT D54- siblings without the transgene produced orange/darkened cankers that were on average ≥10mm (2024) and ≥6mm (2025) longer than their OxO positive counterparts.

In both 2024 and 2025, combined full- and half-sibling family comparisons by mother tree and pollen source were run to rule out family effects and to justify treating all trees as independent indicators of blight tolerance. In both years, as in previous applications of BIA, orange/darkened zone cankers provided clearer statistical results compared to full canker length. In addition, for 2025 we were able to run a larger full-sibling comparison to further rule out family effects.

Genomic analysis earlier revealed that three of the mothers of the orchard trees in the study had hybridity with European chestnut (*Castanea sativa* or *Cs*). Thirty-seven of the 65 (57%) trees tested in the D54 full-sibling analysis for 2025 had some *Cs* genes, with the *Cs* percentage ranging from 10.5 to 20%. *Cs* trees are also susceptible to blight although somewhat less so than *Cd*. The question arose as to whether the percentage of *Cs* genes could explain canker length. To determine if European-American hybrids had a differing response to the blight than the 100% *Cd* trees, a series of ANCOVAs was run, once again treating height as a covariate. In both years, the *Cs* influence was not statistically significant (p-values ranged from 0.30–0.47). Canker length was not explained by whether a tree had a minority share of *Cs* genes.

Discussion

Methodologically, this study provides further support for the efficacy of Cipollini et al.'s (2021) branch inoculation assay (BIA), specifically as a tool for assessing chestnut saplings in an orchard setting. We demonstrate the ability of the BIA to yield meaningful results in a different outdoor environment at the northern edge of the species' distribution. Furthermore, we were able to improve on previous studies by inoculating each sapling three times, with each being treated as a subsample. The subsampled canker measurements were averaged together to reduce variation and more inclusively detect blight response. Lastly, whereas the BIA has previously been used to compare blight resistance among *Cd*, *Cm*, and their hybrids, this study was the first to establish a baseline response for D54(+) blight tolerance in the field compared to full-sibling WT *Cd*.

This study contributes field evidence regarding the genetic engineering approach to American chestnut and its fungal blight tolerance. This is the first multi-year field trial of fungal blight inoculations comparing T3- and T4-generation D54(+) and D54(-) trees by family. The two trial years in this study differed markedly in precipitation levels and this likely impacted canker observations. Marked sporulation differences between the two years showed that the fungus-chestnut interactions differed. In 2024, when there was more moisture available for both fungus and trees, significantly less sporulation was observed on D54(+) saplings compared to D54(-) saplings. In 2025, with much less precipitation, there was no observed sporulation on the inoculated branches. The canker color also differed noticeably between the two seasons. Nonetheless, despite the differences in environmental conditions between the two summers, results from both years showed that D54(+) trees had statistically shorter cankers than their full-sibling D54(-) counterparts.

We also provide some preliminary evidence that D54(+) trees outperformed trees with Chinese alleles using the BIA, although this finding requires particular caution because of the small number of trees with Chinese alleles used in this comparison. In three of the four comparisons in the two years, D54(+) significantly outperformed trees with *Cm* alleles (**Figs. 1** and **2**). This might be viewed as an unexpected finding, considering that *Cm* coevolved with *Cp* and therefore should have natural blight tolerance. However, some previous studies using the SSA found a similar pattern, including the ESF submission to APHIS for nonregulated status for D54 (Newhouse et al. 2024, pp. 102-5). The American Chestnut Foundation also reported that D54(+) (erroneously referred to then as D58(+)) had shorter cankers than full *Cm* trees, although the difference was not significant (TACF 2023). In conclusion, the comparison of D54(+) and those with *Cm* alleles is a subject especially in need of further testing.

One might be tempted to discount the 2025 results owing to the marked impacts of drought, but note that climate change science predicts more weather extremes such as those observed. It may be more accurate to think of 2025's conditions as "new normal" weather rather than as an outlier (WMO 2023). Indeed, two months after the field trial, the precipitation deficit at the orchard site had worsened from "Moderate Drought" to "Extreme Drought" (US Drought Monitor 2025). This raises questions about additional drought impacts on young chestnut trees going into the 2026 season. Nonetheless, for 2025, the results for D54 was consistent with that of 2024, when the weather was more in line with historical trends.

As a side note, concern has been raised about D54 because the D54 event resulted in the insertion of the 35S::OxO T-DNA within Caden.04G062600, which encodes the putative *SAL1* gene in American chestnut (Westbrook and Levine 2025). This concern is not relevant to this study because only hemizygous D54(+) lines were included. D54(+) harbors a wild-type allele that presumably maintains *SAL1* function.

Importantly, it is not yet possible to know what the better canker scores for the D54(+) trees compared to WT siblings mean for American chestnut restoration. For example, we do not know if the D54(+) trees are sufficiently blight-tolerant to mature and reproduce like their pre-blight ancestors. Additional field-based trials involving long-term studies of canker growth and tree survival are needed, under differing habitat and environmental conditions, in order to fully address the restoration potential of D54(+) trees. Ideally, future experiments should incorporate natural blight exposure to local strains of *Cp*. In nature, blight strains find their own ways to penetrate bark and overcome a tree's protective layers. Inoculation by humans, on the other hand, enables the fungus to artificially bypass the protective bark and attack the living layers of a tree. The impacts of natural blight in field plots, however, are likely to take decades to reveal themselves. In the meantime, we suggest that experimentally challenging trees with highly virulent fungal strains, such as EP-155, should continue in order to provide insights into whether the inserted OxO gene offers sufficient protection to D54(+) American chestnuts.

Reference List

Carlson E, Stewart K, Baier K, McGuigan L, Culpepper T 2nd, Powell W. (2022) Pathogen-induced expression of a blight tolerance transgene in American chestnut. Mol Plant Pathol. 23(3):370–382.

Cipollini, ML, Moss, JP, Walker, W, Bailey N, Foster C, Reece H, Jennings C. (2021) Evaluation of an alternative small stem assay for blight resistance in American, Chinese, and hybrid chestnuts (*Castanea* spp.). Plant Disease https://doi.org/10.1094/PDIS-06-20-1272-RE

Conn, CE, Howie, N, Lynch, M, M, Lee S, Young, E, Westbrook J, Holliday, J, Zhang Q, Cipollini ML. (2023) Validation of an alternative small stem assay for blight resistance in chestnut seedlings and recommendations for broader use. Plant Disease https://doi.org/10.1094/PDIS-06-22-1489-RE

ESF. (2023) ESF Darling chestnut science summary. State University of New York College of Environmental Science and Forestry. [accessed 2025 Mar 25]. https://www.esf.edu/chestnut/science-update/index.php#garden

Georgi L and Fitzsimmons S. (2015) Inoculation best practices. Chestnut Magazine (Summer). https://tacf.org/wp-content/uploads/2017/03/Chestnut_Summer2015_FINAL.pdf

Guilley H, Dudley RK, Jonard G, Balàzs E, Richards KE. (1982) Transcription of Cauliflower mosaic virus DNA: detection of promoter sequences, and characterization of transcripts. Cell. 30(3):763–773.

Hebard FV. (2012) The American Chestnut Foundation Breeding Program. Proceedings of the 4th International Workshop on Genetics of Host-Parasite Interactions in Forestry. https://www.fs.usda.gov/psw/publications/documents/psw_gtr240/psw_gtr240_221.pdf

Jacobs, DF et al. (2023) Reintroduction of at-risk forest tree species using biotechnology depends on regulatory policy, informed by science and with public support. New For. 54:587–604 https://doi.org/10.1007/s11056-023-09980-y

Kell, CE.(2025) Comment from Purdue University Hardwood Tree Improvement and Regeneration Center.

https://www.regulations.gov/comment/APHIS-2020-0030-19913

Klak T. (2018) TACF-Landowner Partnerships: An Unfolding Story From Maine. Chestnut: The Journal of the American Chestnut Foundation, 32(3):10-12.

Klak T, Pilkey H, May VG, Matthews D, Oakes AD, Tan EH, Newhouse AE (2025) Speed breeding transgenic American chestnut trees towards restoration. bioRxiv 2025.05.19.654928
doi: https://doi.org/10.1101/2025.05.19.654928

Maine, State of. (2025) Drought Task Force Report on Current Drought Conditions September 4, 2025 https://www.maine.gov/mema/sites/maine.gov.mema/files/inline-files/Drought%20Task%20Force%20Report%2020250904%20%281%29.pdf

May, VG. (2025) Speed Breeding and Fungal Blight Testing of The Darling 54 American Chestnut (*Castanea dentata*). Master's Thesis, School of Marine & Environmental Programs, UNE. https://dune.une.edu/theses/449

Midwest Grow Kits. (2019) Midwest Grow Kit's Full Audio Video Grow Guide with Tips and Extras. https://vimeo.com/374391334/b9da11d504

Newhouse, AE et al. (2024) Petition For Determination Of Nonregulated Status For Blight-Tolerant Darling 54 American Chestnut (*Castanea dentata*). downloadable at: https://www.regulations.gov/document/APHIS-2020-0030-17585

Penn State. (2025) Inoculation. Penn State College of Agricultural Sciences. [accessed 2025 Mar 14]. https://ecosystems.psu.edu/research/chestnut/breeding/inoculation

Powell WA, Newhouse AE, Coffey V. (2019) Developing blight-tolerant American chestnut trees. Cold Spring Harb Perspect Biol. 11(7): a034587.

Powell WA. et al. (2020) Petition for determination of nonregulated status for blight-tolerant Darling 58 American chestnut. https://www.aphis.usda.gov/sites/default/files/19-309-01p.pdf

Rigling D, Prospero S. (2017) *Cryphonectria parasitica*, the causal agent of chestnut blight: invasion history, population biology and disease control. Mol Plant Pathol. 19(1):7-20.

Steiner KC, Westbrook JW, Hebard FV, Georgi LL, Powell WA, Fitzsimmons SF. (2017) Rescue of American chestnut with extraspecific genes following its destruction by a naturalised pathogen. New For. 48(2):317-336.

TACF. (2023) 2022 Annual Report.
https://tacf.org/wp-content/uploads/2021/05/2022_TACF_AnnualReport.pdf

TACF. (2025) Plot: Cape Elizabeth - D58 Common Garden 2021. *dentata*Base. [accessed 2025 Mar 13]. https://dentatabase-staging.herokuapp.com/orchards/1101/plots/3360

Westbrook, J and B Levine. 2025. Comments of the American Chestnut Foundation on Draft Environmental Impact Statement and Draft Plant Pest Risk Assessment for Determination of Nonregulated Status for Blight-Tolerant Darling 54 American Chestnut; Docket No. APHIS-2020-0030 https://www.regulations.gov/comment/APHIS-2020-0030-19911

WMO (World Meteorological Organization, the United Nations). (2023) Extreme weather is the "new norm". https://wmo.int/media/news/extreme-weather-new-norm

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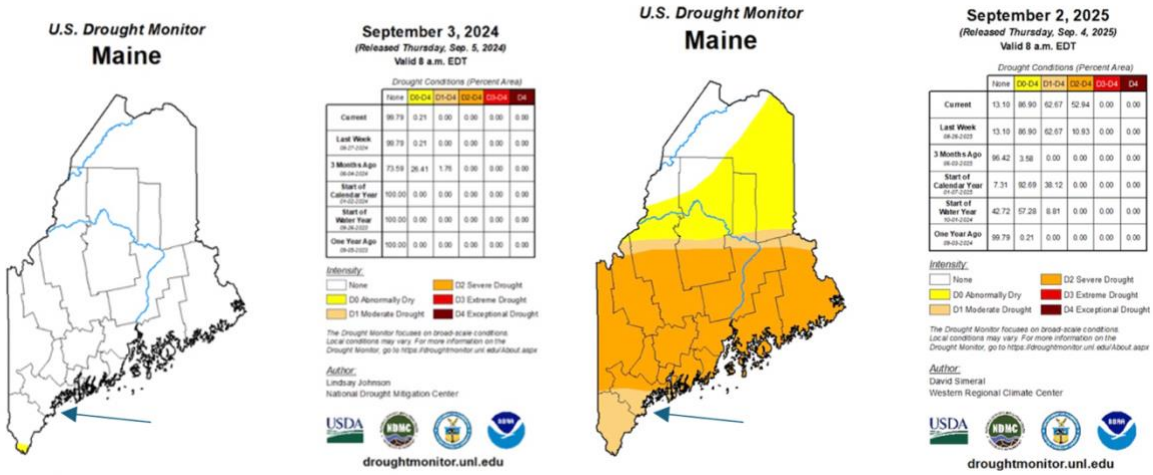
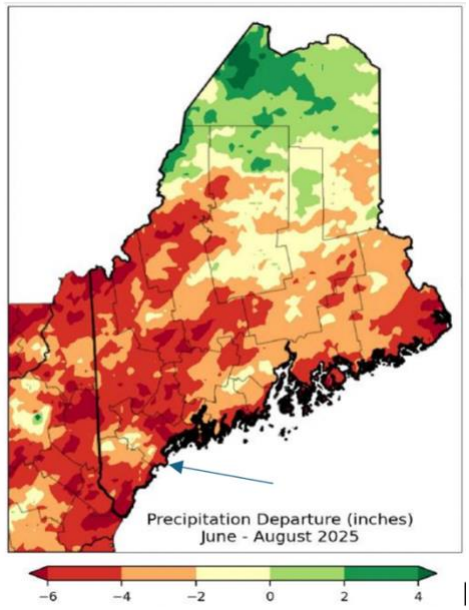
Supplemental Material



Supplemental Fig. 1 Moisture Conditions in 2024 and 2025 by the end of the 90-day inoculation period. Arrow points to the field trial orchard's location
Source: US Drought Monitor



Supplemental Fig. 2 Precipitation deficit during the 2025 90-day inoculation test. Arrow points to the field trial orchard's location
Base Map Source: Maine, 2025

Supplemental Fig. 3 Flagged branch immediately after inoculation, with crimped, clear plastic straw sealing the inoculum disc snugly to the cut end of branch

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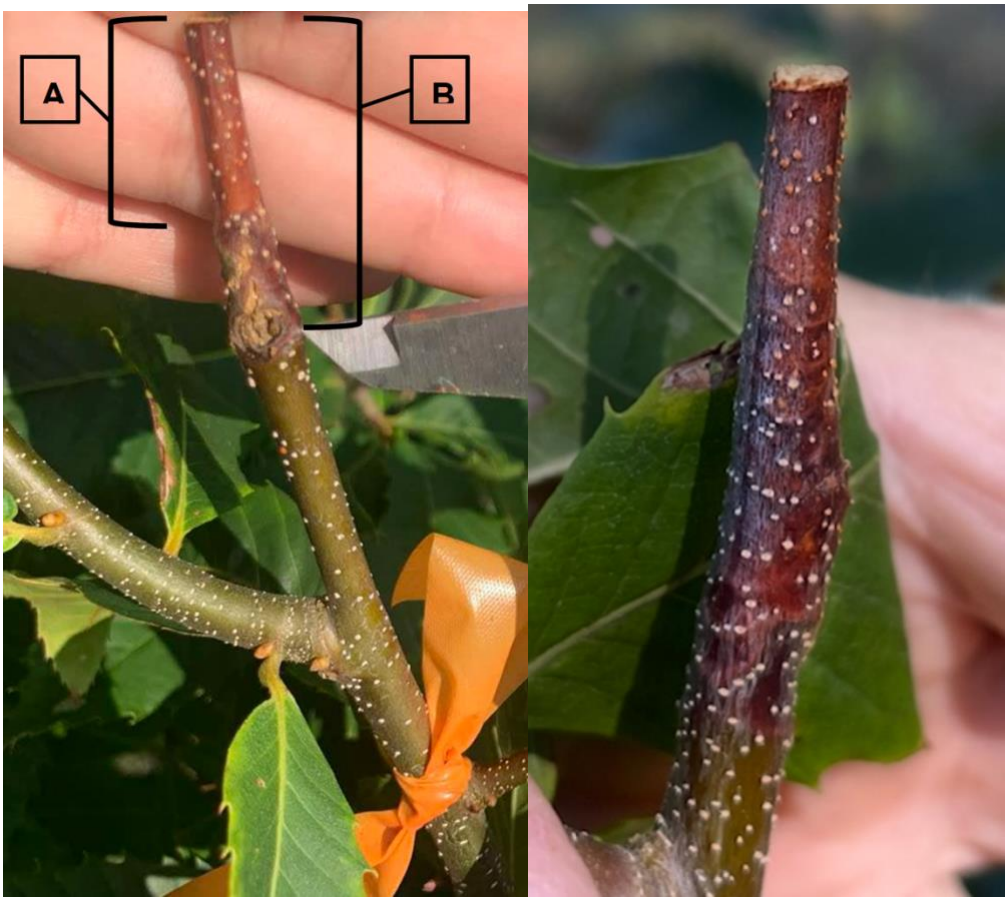

Supplemental Fig. 4 Example cankers from 2024 (top; A=orange canker length including normally-white lenticels turned orange indicating sporulation; B=full canker length); and 2025 (bottom) showing darkened, more shriveled canker where in this case both canker lengths are nearly the same

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Supplemental Tables

2024 Data

Statistical Models using Height as a covariate

FullCanker sqrt-transforme OrangeCanker log-transfor height sqrt-transformed

Check to see if canker varies by Mom for D54+ and D54- including Height as a covariate

D54+

OrangeCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Mom	8,84	1.2157	0.2999	NS	Mom	8,84	1.0627	0.397	NS
Height	1,84	2.4752	0.1194	NS	Height	1,84	5.9457	0.0169	*

D54-

OrangeCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Mom	8,42	0.902	0.5238	NS	Mom	8,42	1.6437	0.1415	NS
Height	1,42	1.4253	0.2392	NS	Height	1,42	2.9296	0.095	NS

Check to see if canker length varies by Dad for D54+ and D54- including Height as a covariate

D54+

OrangeCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Dad	7,85	1.9706	0.0685	NS	Dad	7,85	3.1691	0.005	**
Height	1,85	5.0005	0.028	*	Height	1,85	7.9466	0.006	**

D54-

OrangeCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Dad	6,44	0.682	0.6649	NS	Dad	6,44	1.8249	0.1162	NS
Height	1,44	1.9391	0.1708	NS	Height	1,44	4.8625	0.0327	*

Conclusion: Combined full- and half-sib families show no impact of family on canker metrics in all but one instance (Full canker length varies among paternal families in the D54+ line)

Pairwise comparisons of Types from 2020/2021 outplanting, with all families of Chinese descent combined

Overall ANCOVA with all types

OrangeCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Type	2,161	17.579	<0.0001	***	Type	2,161	22.096	<0.0001	***
Height	1,161	5.9865	0.0155	*	Height	1,161	8.9757	0.0032	**

D54+ vs. D54-

OrangeCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Type	1,143	32.899	<0.0001	***	Type	1,143	39.55	<0.0001	***
Height	1,143	8.3857	0.0044	**	Height	1,143	11.69	0.0008	***

D54+ vs. CH/F1

OrangeCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Type	1,110	2.1243	0.1478	NS	Type	1,110	6.5779	0.0117	*
Height	1,110	4.0244	0.0473	*	Height	1,110	6.0566	0.0154	*

D54- vs. CH/F1

OrangeCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Type	1,68	8.3383	0.0052	**	Type	1,68	5.1344	0.0266	*
Height	1,68	0.3475	0.5575	NS	Height	1,68	0.7285	0.3964	NS

Conclusion: D54+ showed shorter cankers than D54- for full canker length, and shorter than both D54- and Chinese trees for orange zone canker length

2025 Data

Statistical Models using Height as a covariate

DarkenedCanker log-transformed FullCanker log-transformed Height sqrt-transformed

Test of year and family via comparison of 2020/2021 D54+ and D54-
(run on residuals of DeadCanker or DiscolorationCanker as a function of Height)

DarkenedCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Year	1,10	0.0634	0.8064	NS	Year	1,10	0.1469	0.7096	NS
Family	10,12	0.3073	0.9646	NS	Family	10,12	0.6258	0.7672	NS
Type	12,40	2.6877	0.0095	**	Type	12,40	1.6679	0.1116	NS

Conclusion: Well-replicated full-sib families show no effect of year or family on canker metrics

Check to see if canker varies by Mom for D54+ and D54- including Height as a covariate

D54+

DarkenedCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Mom	6,23	0.2495	0.9545	NS	Mom	6,23	0.876	0.5276	NS
Height	1,23	3.4304	0.0769	NS	Height	1,23	1.7926	0.1937	NS

D54-

DarkenedCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Mom	7,23	1.3866	0.258	NS	Mom	7,23	1.0981	0.3969	NS
Height	1,23	0.4604	0.5042	NS	Height	1,23	0.0398	0.8436	NS

Check to see if canker length varies by Dad for D54+ and D54- including Height as a covariate

D54+

DarkenedCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Dad	5,23	1.0471	0.4144	NS	Dad	5,23	0.6247	0.6825	NS
Height	1,23	1.3407	0.2588	NS	Height	1,23	1.5418	0.2269	NS

D54-

DarkenedCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Dad	7,23	2.1206	0.0822	NS	Dad	7,23	2.0609	0.0902	NS
Height	1,23	7.0222	0.0143	*	Height	1,23	2.8081	0.1073	NS

Conclusion: Combined full- and half-sib families show no impact of family on canker metrics

Pairwise comparisons of Types from 2020/2021 outplanting, with all families of Chinese descent combined

Overall ANCOVA with all types

DarkenedCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Type	2,92	8.8545	0.0003	***	Type	2,92	7.1873	0.0013	**
Height	1,92	8.0651	0.0056	**	Height	1,92	6.6028	0.0118	*

D54+ vs. D54-

DarkenedCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Type	1,63	5.6117	0.0209	*	Type	1,63	3.307	0.0737	NS
Height	1,63	10.861	0.0016	**	Height	1,63	6.4594	0.0135	*

D54+ vs. CH/F1/B3F3

DarkenedCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Type	1,61	16.797	0.0001	***	Type	1,61	13.808	0.0004	***
Height	1,61	4.2188	0.0443	*	Height	1,61	6.0499	0.0167	*

D54- vs. CH/F1/B3F3

DarkenedCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Type	1,59	3.8364	0.0549	NS	Type	1,59	3.9685	0.051	NS
Height	1,59	3.1443	0.0814	NS	Height	1,59	1.6739	0.2008	NS

Conclusion: D54+ showed shorter cankers than both D54- and Chinese trees for full canker length, but only greater than Chinese trees for full canker length

Supplemental Tables (cont.)

Comparison of 2020/2021 D54+ vs. D54- with %Sativa included as an additional factor

OrangeCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Type	1,140	33.512	<0.0001	***	Type	1,140	42.962	<0.0001	***
%Sativa	3,140	1.233	0.3001	BS	%Sativa	3,140	1.2336	0.2999	NS
Height	1,140	7.3208	0.0077	**	Height	1,140	11.938	0.0007	***

Conclusion: %Sativa had no additional affect on canker lengths

Comparison of 2020/2021 D54+ vs. D54- with %Sativa included as an additional factor

DarkenedCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Type	1,59	5.0378	0.0286	*	Type	1,59	2.8819	0.0948	NS
%Sativa	2,59	0.7581	0.473	NS	%Sativa	2,59	0.921	0.4038	NS
Height	1,59	10.397	0.0021	**	Height	1,59	4.8667	0.0313	*

Conclusion: %Sativa had no additional affect on canker lengths

Comparison of Types from 2022 outplanting

DarkenedCanker					FullCanker				
Source	DF	F	p		Source	DF	F	p	
Type	1,10	1.0784	0.3235	NS	Type	1,10	1.261	0.2877	NS
Height	1,10	0.7694	0.401	NS	Height	1,10	0.8477	0.3789	NS

Conclusion: D54+ and DRW+ did not differ in canker lengths

Full and Half Sibling Analysis

2024

Moms

OrangeCanker					FullCanker			
Source	DF	F	p		Source	DF	F	p
Year	1,7	3.2316	0.1153		Year	1,7	3.3251	0.111
Mom	7,9	0.3022	0.9356		Mom	7,9	0.3329	0.9192
Type	9,128	3.9339	0.0002		Type	9,128	4.6295	<0.0001

Dads

OrangeCanker					FullCanker			
Source	DF	F	p		Source	DF	F	p
Year	1,6	2.5207	0.1634		Year	1,6	1.539	0.2611
Dad	6,6	0.3843	0.8653		Dad	6,6	0.6863	0.6704
Type	6,122	5.3146	0.0001		Type	6,122	6.645	<0.0001

Full and Half Sibling Analysis

2025

Moms

DeadCanker					FullCanker			
Source	DF	F	p		Source	DF	F	p
Year	1,8	0.1818	0.6811		Year	1,8	0.0379	0.8506
Mom	8,10	0.4114	0.8894		Mom	8,10	0.6308	0.7371
Type	10,44	3.037	0.0052		Type	10,44	2.1735	0.038

Dads

DeadCanker					FullCanker			
Source	DF	F	p		Source	DF	F	p
Year	1,6	0.388	0.5563		Year	1,6	0.066	0.8059
Dad	6,8	0.1781	0.9752		Dad	6,8	0.3009	0.9197
Type	8,48	2.8938	0.0103		Type	8,48	2.4592	0.0256

2024 Summary Statistics

Variable	OrangeCanker			
	mean	sd	se(mean	n
Chinese	15.28	6.3464	1.456	19
D54-	21.605	12.856	1.7828	52
D54+	11.714	10.191	1.0511	94

Variable	FullCanker			
	mean	sd	se(mean	n
Chinese	29.586	9.453	2.1687	19
D54-	39.909	14.391	1.9957	52
D54+	25.367	13.325	1.3744	94

2025 Summary Statistics

Variable	DarkenedCanker			
	mean	sd	se(mean	n
Chinese	28.442	26.349	4.8106	30
D54-	15.459	12.842	2.2701	32
D54+	9.2053	6.9113	1.1853	34

Variable	FullCanker			
	mean	sd	se(mean	n
Chinese	33.535	27.562	5.0321	30
D54-	18.778	16.018	2.8316	32
D54+	12.956	13.043	2.2368	34