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A small stem assay using hypovirulent Cryphonectria parasitica to screen Castanea dentata backcross F2 families may set the stage for long-term survival

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Abstract

A small stem assay using hypovirulent *Cryphonectria parasitica* to screen *Castanea dentata* backcross F2 families may set the stage for long-term survival

By combining the results of blight resistance breeding and the application of hypovirulence as a biocontrol, populations of resistant hybrid trees could be deployed together with a less pathogenic strain of *Cryphonectria parasitica* (Murr.) Barr. I used a small stem assay to screen seedlings in thirteen half-sibling backcross F2 families with an attenuated strain of *C. parasitica* containing the *Cryphonectria parasitica hypovirus-1* (CHV-1) Euro7 virus. The experiment was set up as a randomized complete block design in 2-gallon containers. Measurements of canker length and morphology were gathered at 90 days post-inoculation. Although statistically significant differences were seen between canker lengths in *C. dentata* and *C. mollissima* control groups, no statistically significant differences were seen between any of the hybrid families as shown by Duncan's multiple range test. Phenotypes elucidated using the SSA should not be used to make artificial selections within families, but the SSA can verify that parental selections were accurate. All surviving trees will be planted in an experimental orchard in middle Tennessee to create a potentially long-lasting population of disease resistant trees.

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Literature Review

Introduction

In efforts to conserve rare germplasm of the American chestnut C*astanea dentata* (Marshall) Borkhausen, biocontrol using a hypovirulent form of the ascomycete fungus *Cryphonectria parasitica* (Murrill) M.E.Barr infected with *Cryphonectria hypovirus-1* (CHV-1) has been long identified as a potential biocontrol against chestnut blight (Chen & Nuss, 1999). Albeit, the transmission of the hypovirus from one fungal individual to another is strongly hindered in the United States due to high levels of vegetative incompatibility (Chen & Nuss, 1999). *In vitro* studies often question the possibility of conversion of the fungal strain to hypovirulence, looking at the genomic level to identify hinderances and likelihood of conversion. *In vivo* studies regarding biocontrol creates a realistic image of the likelihood of conversion to hypovirulence and transmission of the hypovirus when the precise and repetitive methods of the laboratory are stripped away. As the three key characters, the interactions between *Castanea dentata, Cryphonectria parasitica,* and *Cryphonectria hypovirus-1* yield complexities surrounding vegtative incompatibility *vic* genotypes, conversion of fungal strains from virulent to hypovirulent both *in vitro* and *in vivo,* and the intended application of CHV-1 for mitigating infections.

Castanea dentata History

The American chestnut, *C. dentata,* was one of the most important trees in the Eastern hardwood forests because of its large nutrious chestnuts used by animals for food and by people living in the Appalachian forests as a cash crop (Roane et al., 1986). In some locations, up to 25% of the canopy was inhabited by *C. dentata,* providing shade for the forest floor (Roane et

al., 1986). *C. dentata* provided timber for lumber, furniture, and tannin for leather (Roane et al., 1986).

The chestnut blight was first observed on C. *dentata* in 1904 at the Bronx Zoological Park in New York City (Roane et al., 1986). Chestnut blight was accidentally introduced from Asia, and within 40 years the disease had spread all throughout the natural range of *C. dentata*. The native Asian species of *Castanea* have the most blight resistance: the Chinese chestnut, *C. mollissima,* and the Japanese chestnut, *C. crenata*. The European chestnut, *C. sativa,* is less susceptible to blight than the American chesnut but not as resistant as the Asian species.

The American Chestnut Foundation works to breed blight resistant *C. dentata* trees. The original idea to backcross *C. dentata* trees with *C. mollissima* was suggested by Burnham (1987). By backcrossing the *C. dentata* with *C. mollissima* and screening the trees at each generation for blight resistance, Burnham (1987) hoped to dilute the *C. mollissima* morphology while retaining the blight resistance genes in the *C. dentata* trees. However, backcrossing has not been as successful as hoped, because the blight resistance phenotype is coded by many genes at many loci rather than just one or a few (H. Craddock, personal communication).

Vegetative Incompatibility in *Cryphonectria parasitica*

Cryphonectria parasitica infects the tree via sexual and asexual spores through wounds on the tree. The fungus breaks down the vascular cambium, which prevents tree growth. Infected trees create bark lesions that have a reddish-brown discoloration at the site of infection. Exposures along the bark allow the ascospore to infect the tree, and points of entry are often caused by growth cracks in the bark or moribund tissue caused by cuts, fires, or drought (Rigling & Prospero, 2018).

Like other filamentous ascomycetes, *C. parasitica* uses vegetative incompatibility to prevent the spread of infections such as CHV-1 (Zhang & Nuss, 2016). The hypovirus *Cryphonectria hypovirus-1* (CHV-1) hinders the ability of the fungus to sporulate and harm the tree. CHV-1 is a double-stranded RNA virus that spreads mostly through hyphal anastomoses. Hyphal anastomosis is the fusion of two mating fungi using its branching filaments. Transmission of the hypovirus is prevented through vegetative incompatibility controlled genetically by *vic* alleles. Vegetative incompatibility is a self/non-self recognition system to prevent the spread of infection in fungi in which programmed cell death (PCD) occurs if the two anastomosing hyphae do not have compatible *vic* alleles. *vic* is controlled by two alleles at six loci. For mating hyphae to be compatible, all six loci must have identical alleles, resulting in 64 vegetative compatibility types (Cortesi & Milgroom, 1998). In other words, two hyphae can mate if they share the same vegetative compatibility *vc* type, characterized by matching alleles at all six *vic* loci. In the instance that two vegetatively incompatible hyphae attempt to fuse, programmed cell death occurs and prevents the transmission of CHV-1. The virulence of each strain of CHV-1 is based on how well the hypovirus can attenuate the ability of the fungus to sporulate and on the transmissibility of the hypovirus between fungal hosts.

Furthermore, the hypovirus aids in American chestnut conservation by allowing the infected tree to heal through the formation of cankers at the site of infection and limiting the spread of ascospores to other trees (Chen & Nuss, 1999). The strain CHV-1 spreads mostly widely and is most often seen in research surrounding hypovirulence. The heightened virulence of CHV-1 is due to the balance in both debilitating the host and inhibiting PCD after hyphal fusion. Though a hypovirulent fungus has a reduced ability to spread asexual spores, the hypovirus counteracts the reduced sporulation with increased infectivity of each spore. Furthermore, CHV-1 spreads

vertically to ascospore progeny if the hypovirus DNA has already been integrated into the host DNA, resulting in a transgenic strain of *C. parasitica* (Chen & Nuss, 1999).

vic Genotypes

The ability to undergo anastomosis is defined by allelic interactions. In *C. parasitica*, the six loci used in the *vic* system are *vic 1, vic 2, vic 3, vic 4, vic 6, vic 7*, and each locus is inhabited by one of two idiomorphic alleles (Cortesi & Milgroom, 1998). Milgroom excluded the locus *vic 5* from the set that controls vegetative incompatibility, because the effects of heteroallelic interactions at that locus could not be seen on the agar medium in his study (1998). From the six *vic* loci arise 64 vegetative compatibility *vc* types, a method of categorizing the sequences of alleles that can mate. A difference in an allele at one locus would change the *vc* type of the fungus, therefore changing the individuals that the fungus could fuse with. In individuals with the same *vc* type, anastomoses—and transmission of CHV-1—is almost guaranteed (Bryner & Rigling, 2012).

Field studies in the United States and in Europe indicate a negative correlation in the transmissibility of CHV-1 and the number of *vc* types. In Europe, hypovirulence has been established in the population of *C. parasitica* and is an effective biocontrol in favor of the European chestnut tree *C. sativa*. European *C. parasitica* strains only contain 31 *vc* types, nearly half of the 64 *vc* types present in North America (Cortesi & Milgroom, 1998).

Conversion of Virulent Strains to Hypovirulent

When conversion is attempted between incompatible strains, the two hyphae undergo PCD and a barrage zone forms where the two strains meet (Rigling & Prospero, 2018). The two fungal strains used in the failed conversion do not change in morphology (Hwang, 2001). When conversion is attempted in compatible strains, virus-free strains of *C. parasitica* change from

their original orange to white, an indicator that the fungus is hypovirulent. Successful conversions could also yield a third sector of white concentric circles *in vitro* (Hwang, 2001).

The process of conversion requires tester strains of known *vc* types which are used to attempt conversions with the unknown strain (Rigling & Prospero, 2018). Hwang (2001) shows that conversion between a virus-containing strain with brown background and a local orange virulent strain is possible. However, rates of conversion are low, because *vc* types of the two strains are typically unknown and most likely do not match (Hwang, 2001). Hwang suggests conversion of a local virulent strain yields more success when the donor is an isogenic hypovirulent strain (2001). Isogenic V and H strains are guaranteed to have the same *vc* type, therefore the transmission of the hypovirus is much more likely to occur (Hwang, 2001).

Conversion of Blighted Cankers *in vivo*

Furthermore, the specific fungal strain influences the transmissibility of the virus (Bryner & Rigling, 2012). Transmissibility is how well the virus is transferred from one hypha to the other, which is controlled by the virus strains. Whereas *vc* types control whether transmission can occur at all. Virus-infected strains of *C. parasitica* EP155 and Euro-7 grew as fast or faster than the virus-free version of each strain (Chen & Nuss, 1999). Chen showed the growth of EP155 was more aggressive than that of Euro-7 (1999). The cankers of the stronger EP155 grew to be around twice the size of the canker of Euro-7 infected tree.

Phenotypes, such as canker morphology, expansion, and asexual sporulation levels, of the lesion upon infection with a virus-free strain and of the canker upon infection with the virus-containing strain vary based on the virulence of CHV-1 (Chen & Nuss, 1999). When *C. parasitica* infects the tree, a bark lesion forms at the site of infection. The lesions are sunken and reddish-brown in appearance (Rigling & Prospero, 2018). Nuss and Chen showed that more

virulent forms of *C. parasitica* produce cankers with stromal pustules and viable conidia (1999). Cankers healed due to hypovirulence are superficial, are calloused, and have a swollen appearance (Rigling & Prospero, 2018), but a closer look at the strain of the hypovirus transferred to the canker displays different healing characteristics (Chen & Nuss, 1999). Cankers healed using weaker transgenic strains such as CHV1-EP713 produce smaller superficial cankers with fewer stromal pustules. Whereas, stronger transgenic strains such as CHV1-Euro7 form healing cankers with distinct margins where callous tissue forms and significantly more stromal pustules with more viable asexual spores (Chen & Nuss, 1999). Orchards in Indiana have also attested to this phenomenon with the presence of a variety in hypovirus strains such as CHV-1 subtype-1, subtype-F1, and subtype-F2 (Lawson et al., 2021). In Indiana, CHV-1 subtype-1 showed potential to establish in the population, but the cankers develop slowly in comparison to CHV-1 subtype-F1 and subtype-F2, whose canker showed reduce virulence of the fungal host.

Methods of Application of Hypovirulent Inoculum

Methods of applying the hypovirulent fungal strain range from painting a slurry of hypovirulent slush, inserting the hypovirulent inoculum, and spraying the hypovirulent slush to the blighted cankers (Double et al., 2017).

Metheny (2019) used the painting method for application of the hypovirus to the canker. Using a leather punch, she created a wound in the bark along the margin of the canker and a wound at the center of the canker. Then, Metheny painted a slurry made from blending the hypovirulent fungus with water onto the canker using a paintbrush and covered the open would with laboratory pads and masking tape (2021).

Double et al. (2017) also used the painting method and inserted the hypovirulent inoculum onto the canker, referred to as the Punch-Initiated method. Double first removed 7 mm diameter

discs of the canker near the center and covered the hole with hypovirulent mycelium grown on potato dextrose agar cultures. Then, the open wound was bandaged (2017).

The method of spraying diluted hypovirulent water onto trees has been used but is often cited as ineffective (Root et al., 2005); (Metheny, 2021).

Limitations of Biocontrol Attempts

Double et al. (2017) noted that conversion *in vivo*, and subsequently transmission of the hypovirus to surrounding individuals, is unlikely to occur if the strain that initially infects the tree varies significantly in *vic* genotypes from the strain applied by researchers. Double et al. (2017) showed that the virulent fungus continues to grow its lesion around the stem of the tree despite the presence of a hypovirulent fungus on the infected region. Therefore, effective biocontrol would use a hypovirus that can disseminate to the rest of the fungal population. Chen and Nuss (1999) showed that effective biocontrol requires a continuous supply of hypovirulent inoculum due to the inability of the hypovirus to colonize and sporulate on chestnut bark.

In a field study over a seven-year period, bigger *C. dentata* have a greater potential to survive and could aid in the establishment and dissemination of a hypovirulent population (Double et al., 2017). Albeit, at the end of the seven-year study, nearly 75% of the trees treated with the hypovirulent fungus died or was of declining health (Double et al., 2017). The condition of the trees in this study were not ideal for hypovirulent establishment anyway, noting the generally small tree diameter and high *vic* diversity that often inhibits establishment in North America (Double et al., 2017). *C. dentata* is also known for its increased susceptibility to *C. parasitica* in comparison to the *C. sativa*, which does not aid in the process of establishing biocontrol.

Furthermore, blind conversion of virulent to hypovirulent strains *in vitro* yields little success.

Hwang (2001) converted only 30% of the brown hypovirulent strains with a known *vc* type to the local virulent strains. Despite sterile laboratory practices, contamination often taints the cultures (Hwang, 2001).

Small Stem Assay

Orchard assay methods to screen for blight resistance require five-year old trees, which is taxing for labor and land needs. The small stem assay (SSA) method requires one- to two-year old seedlings and can be conducted in a blight-free greenhouse setting.

Hebard and Shain (1989) first tested for blight resistance by inoculating five- to eighteen-month-old seedlings. The trees screened were F1 and backcross hybrids using *C. parasitica* strains EP155, EP408, and EP905. Inoculations were made using a miniature cork-borer on opposite sides of the stem. Though Hebard and Shain (1989) were able to distinguish between *C. dentata* and *C. mollissima* controls, they failed to find significant differences in blight resistance among the hybrid trees.

Westbrook and Jarret (2018) proved that the small stem assay method was an effective method for differentiating levels of blight resistance. Seedlings from 68 families of BC3F2 families were planted, inoculated, and measured for canker length at 7-, 15- and 24- weeks post-inoculation. Differences in blight resistance were distinguished at the seedling stage and results were most defined when inoculated with highly pathogenic strains of *C. parasitica.*

Cipollini et al (2021) cut the stem using sanitized pruning shears at 5 mm in diameter. The open cut was immediately inoculated with a disc of mycelium grown on potato dextrose agar for one week. The inoculation site was covered with sealed end plastic straw sleeves. The straw sleeves were removed after one week. Only two out of over 200 stems did not produce a canker,

testifying to the efficacy of this method.

Hypothesis

This experiment seeks to answer two questions:

1. Can the small stem assay using a hypovirulent fungus separate levels of blight resistance in progeny of intermediately resistant hybrid *C. dentata* trees?
2. Can the levels of blight resistance in these hybrid trees be ranked using the small stem assay with a hypovirulent fungus?

I hypothesize that the small stem assay using a hypovirulent strain of *Cryphonectria parasitica* will show a statistically significant difference in chestnut blight resistance between susceptible *Castanea dentata* and resistant *Castanea mollissima* controls. Because previous small stem assays had enough resolution to distinguish between *C. dentata* and *C. mollissima* controls, the two controls may be separated here using the hypovirulent strain too. Furthermore, the hypovirulent strain, because of its attenuated pathogenicity, may be sensitive enough to rank each family of backcross hybrids by its level of blight resistance.

Based on the negative correlation between mother *C. dentata* ancestry and average family blight resistance, I predict that the *C. mollissima* controls will show higher levels of blight resistance than the *C. dentata* controls. I predict that all hybrid families will show levels of blight resistance intermediate to the controls. I predict that the BC1F2 hybrids will show the highest levels of blight resistance among all backcross families, because it has the most *C. dentata* lineage. I predict that BC3F2 hybrids will show the second highest level of blight resistance among backcross families and that BC4F2 will show the third highest level of blight resistance.

Lastly, I predict that the F2 hybrids will show the highest level of blight resistance among all hybrid families. If the results support the predictions, then the small stem assay using a hypovirulent fungus can be used to determine levels of blight resistance among other families of *Castanea dentata* easily without the time and financial constraints of orchard screening.

Materials and Methods

Planting

Seeds stored in the Fortwood Greenhouse freezer were used in the small stem assay. The seeds were given from various institutions and were progeny of Chinese-American hybrids. *C. dentata* and *C. mollissima* controls were also planted. The seeds were planted on February 17th, 2022 in D40 pots at the Fortwood Greenhouse. The D40 pots were changed to larger CP512 pots when the seedlings started to outgrow the smaller size on May 5th, 2022. The seeds were planted in Pro-Mix BK55 potting medium and were top-dressed with a tablespoon of Osmocote Plus with micronutrients after its first and second potting. During the second potting, the trees were also staked to encourage upward growth. Bamboo poles were placed vertically and taped at points of leverage along the tree. The trees were treated with a fungicide once during the summer to control *Phytophthora* root rot. The fungicide is a mix of the Plantex 21-7-7 Acid with an Alude brand systemic fungicide and applied using a 3-gallon SOLO backpack sprayer.

Table 1.

Total Number of Trees Planted for Each Family with Phylogeny Noted for the Small Stem Assay

Name	Generation	Phylogeny	Total *n*
AU-1-26	Chinese control	Ch x opCH	18
TN-RC09-3-9	BC4F2	B4 x opB1-4	30
TN-RC09-3-62	BC4F2	B4 x opB1-4	30
TN-RC09-6-46	BC4F2	B4 x opB1-4	30
TN-RC09-7-33	F2	F1 x op, *dentata* x *crenata*	28
TN-RC09-2-22	BC4F2	B4 x opB1-4	29
TN-RC09-5-15	BC3F2	B3 x opB1-4	30
TN-RC09-5-30	BC1F2	B1 x opB1-4	30
TN-RC09-2-35	BC4F2	B4 x opB1-4	28
TN-DC12-2-8	BC4F2	B4 x opB4	27
TN-DC12-4-6	BC4F2	B4 x opB4	30
CT-EL007	American control	Am x opAm	25
TN-TTU05-A34	F2	F1 x opF1	30

Greenhouse Management

The trees were grown in full sun and were watered by hand and using the drip irrigation line. The trees were watered to saturation and re-staked as needed throughout the growing period.

The trees were set up in a randomized complete block design with four non-adjacent blocks. Each block was separated by one guard row of trees that were not a part of the experiment, so each experimental block does not affect each other.

Figure 1.

Randomized complete block design showing the empty experimental rows and the adjacent guard rows.

Inoculum

Hypovirulent (virus-infected) and virulent (virus-free) cultures of the Weekly strains of *Cryphonectria parasitica* were shipped from West Virginia University. The Weekly isolate was infected with the Euro7 strain of *Cryphonectria parasitica hypovirus-1*. Twenty-four PDA plates of both the hypovirulent and the virulent Weekly strain were grown from excisions of the original plates from WVU. The cultures were set near a window on a lab bench at room temperature to promote growth for one week before the inoculation date.

Inoculation

By July 16th, 2022, the trees had ample time to grow to a 5mm diameter. Of the 365 seeds planted, only 320 trees were large enough to be inoculated. The trees that did not reach the 5 mm diameter requirement were logged and excluded from the study. Also, trees disfigured due to virus infection were excluded from the experiment. Using the uniform 5.5 mm cutout, the highest point on the tree that has a 5mm diameter was snipped off using pliers flame-sterilized with 70% ethanol (Cipollini et al., 2021). Mycelium-containing agar was excised from the petri dish and placed onto the top of the tree with the mycelium side facing the wound. A flame-sterilized spatula was also used to place the mycelium on the tree. Then, the tree was covered using a closed straw tip. The straw tips used to cover the tree wounds were clean by soaking them in bleach and dried in the herbarium drier.

Plastic straw tips were used to create a waterproof seal over the inoculum. The diameter of the straw is approximately 5 mm which fits over the snipped tree. The straws were cut into 50 mm lengths, and one end was sealed at the 40 mm mark with a FoodSaver food vacuum sealer.

The uniform 5.5 mm cutout was intended to be made as a 5 mm cutout. The spot where the tree was snipped needed to be 5 mm so the straw tip can fit securely over the open wound. When using the improperly sized cutout, the person snipping the tree approximated the 5mm mark based on how the cutout fit the diameter of the tree. A straw tip was occasionally placed over the top of the stem to check that it would fit. After the inoculation step, a straw tip was placed over the open wound. If the straw tip was too small for the top of the stem, then two slits were cut into the open end of the straw tip it was placed over the tree. The loose end of the straw tip was then secured to the stem using Parafilm. If the straw tip was too loose after placing it on the stem, then the loose end of the straw was also secured to the stem using Parafilm.

After the individual was inoculated, it was returned to the same block in random order. The extra hypovirulent plates were returned to the lab fridge for storage.

Screening

The trees were monitored, and any abnormalities were noted. At 90-days post-inoculation, the orange zone of the canker was measured on each tree. The orange zone was measured as the furthest point of orange seen on the tree.

Cutting and Sanitizing

On all trees of the SSA, the blighted canker was cut at least one node down from the end of the canker using clippers sanitized with 70% ethanol. The blades were recleaned after clipping every few trees to prevent the spread of *C. parasitica*. The open wounds were also sprayed with 70% ethanol.

Analysis

During analysis, only the October orange zone lengths were considered, and data from all other months were excluded. The August, September, and November measurements were excluded due to systematic error while measuring. 90-day canker measurements have been used in previous studies (Cipollini et al., 2021), so only the October measurements, taken 90 days post-inoculation, were considered in this study.

Data were analyzed using the RStudio (Team, 2023) and were subjected to a two-way ANOVA to determine the effect of the hypovirulent inoculum and the block effect. ANOVA was performed on means for each family and each generation. Duncan's multiple-range test was used for post hoc analysis to determine differences between canker ratings and scaled orange zones. Canker rating variables are assigned to each orange zone canker length based on where the

measurement fell in the four quartiles of the orange zone length distribution. Scaled orange zones are an inflated version of the orange zone length in which the *C. mollissima* means are inflated to 100 and the *C. dentata* means are shrunk to 0. The scaled orange zone enhances the differences in each hybrid family. Differences were considered statistically significant at $p \leq 0.05$.

Results

Average Canker Lengths

Table 2.

Average Canker Lengths and Standard Deviations for Each Generation

Generation	mean (mm)	sd (mm)
BC1F2	18.6	13.2
BC3F2	20.4	19.3
BC4F2	17.0	11.4
C. dentata	25.9	16.8
C. mollissima	9.6	8.7
F2	15.9	13.3

Table 3.

Average Canker Lengths and Standard Deviations for Each Family

Family	mean (mm)	sd (mm)
AU-1-26	9.6	8.7
CT-EL007	25.9	16.8
TN-DC12-2-8	16.7	9.7
TN-DC12-4-6	14.2	8.2
TN-RC09-2-22	19.6	12.9
TN-RC09-2-35	16.6	11.3
TN-RC09-3-62	21.0	14.2
TN-RC09-3-9	17.0	10.9
TN-RC09-5-15	20.4	19.3
TN-RC09-5-30	18.6	13.2
TN-RC09-6-46	14.0	10.2
TN-RC09-7-33	17.1	12.3
TN-TTU05-A34	14.8	14.2

Table 4.

Average Canker Lengths and Standard Deviations for Each Block

Block	mean (mm)	sd (mm)
1	17.7	12.0
2	14.2	11.7
3	17.5	14.9
4	20.4	13.1

Table 5.

Average Canker Lengths and Standard Deviations for Each Generation in Each Block

Generation	Block 1		Block 2		Block 3		Block 4	
	mean (mm)	sd (mm)	mean (mm)	sd (mm)	mean (mm)	sd (mm)	mean (mm)	sd (mm)
BC1F2	14.1	7.4	23.6	19.2	11.6	11.4	22.6	10.1
BC3F2	19.0	12.5	15.3	16.0	25.7	25.2	16.2	15.0
BC4F2	17.5	10.8	13.9	10.4	16.5	11.2	20.5	12.4
C. dentata	28.9	19.5	20.0	10.4	17.0	19.0	36.8	17.8
C. mollissima	14.4	18.6	4.8	3.5	9.5	3.0	10.8	6.7
F2	15.2	11.0	9.7	7.2	18.7	17.2	18.	13.6

Table 6.

Average Canker Lengths and Standard Deviations for Each Generation in Each Block

Family	Block 1		Block 2		Block 3		Block 4	
	mean (mm)	sd (mm)	mean (mm)	sd (mm)	mean (mm)	sd (mm)	mean (mm)	sd (mm)
AU-1-26	14.4	18.6	4.8	3.5	9.5	3.0	10.8	6.7
CT-EL007	28.9	19.5	20.0	10.4	17.0	19.0	36.8	17.8
TN-DC12-2-8	17.2	10.6	17.0	11.0	15.8	9.0	17.0	11.8
TN-DC12-4-6	12.5	6.4	11.5	7.3	14.1	7.8	22.5	10.0
TN-RC09-2-22	24.3	13.8	17.3	11.3	15.4	10.9	21.3	16.3
TN-RC09-2-35	14.1	11.4	17.4	15.3	11.5	2.7	23.0	11.5
TN-RC09-3-62	18.0	12.2	18.7	19.5	18.1	9.5	28.3	15.6
TN-RC09-3-9	22.0	9.6	10.8	3.0	22.5	15.0	15.1	9.9
TN-RC09-5-15	19.0	12.5	15.3	16.0	25.7	25.2	16.2	15.0
TN-RC09-5-30	14.1	7.3	23.6	19.2	11.6	11.4	22.6	10.1
TN-RC09-6-46	11.9	2.0	9.8	2.4	14.8	16.9	16.5	9.6
TN-RC09-7-33	17.2	6.8	11.6	13.4	20.8	16.5	17.0	12.2
TN-TTU05-A34	13.5	14.1	8.8	1.7	17.3	18.4	21.6	17.0

ANOVA

The data collected passed the Shapiro-Wilk Normality Test ($p=5.97x10^{-16}$). The data passed the Levene's Test for equal variances for each family (p=0.037). The data did not pass the Levene's Test for equal variances for each block (p=0.34). The data passed the Levene's Test for equal variances for each generation (p=0.016). The data collected did not pass Grubb's test for outliers. The outlier tree TN-RN09-5-30 268 from Block 3 was removed from the data and its orange zone length was 331 mm.

Table 7.

ANOVA Table for Response of Each Family to the Hypovirulent Inoculum with the Block Effect

Predictor	df	Sum Sq	Mean Sq	f	P
Family	12	3872	322.6	1.951	0.0283
Block	1	711	711.3	4.302	0.0389
Residuals	307	50763	165.4		

Table 8.

ANOVA Table for the Interaction between Families and Blocks

Predictor	df	Sum Sq	Mean Sq	f	p
Family	12	3872	322.6	1.904	0.0336
Block	1	711	711.3	4.197	0.0414
Family:Block	12	766	63.8	0.376	0.9711
Residuals	295	49998	169.5		

Table 9.

ANOVA Table for Response of Each Generation to the Hypovirulent Inoculum with the Block Effect

Predictor	df	Sum Sq	Mean Sq	f	p
Generation	5	2695	539	3.257	0.00698
Block	1	686	685.9	4.145	0.04261
Residuals	314	51965	165.5		

Table 10.

ANOVA Table for the Interaction between Generations and Blocks

Predictor	df	Sum Sq	Mean Sq	f	p
generation	5	2695	539	3.21	0.00767
Block	1	686	685.9	4.086	0.04411
Generation:Block	5	89	17.8	0.106	0.99091
Residuals	309	51876	167.9		

Duncan's Multiple Range Test (DMRT)

Table 11.

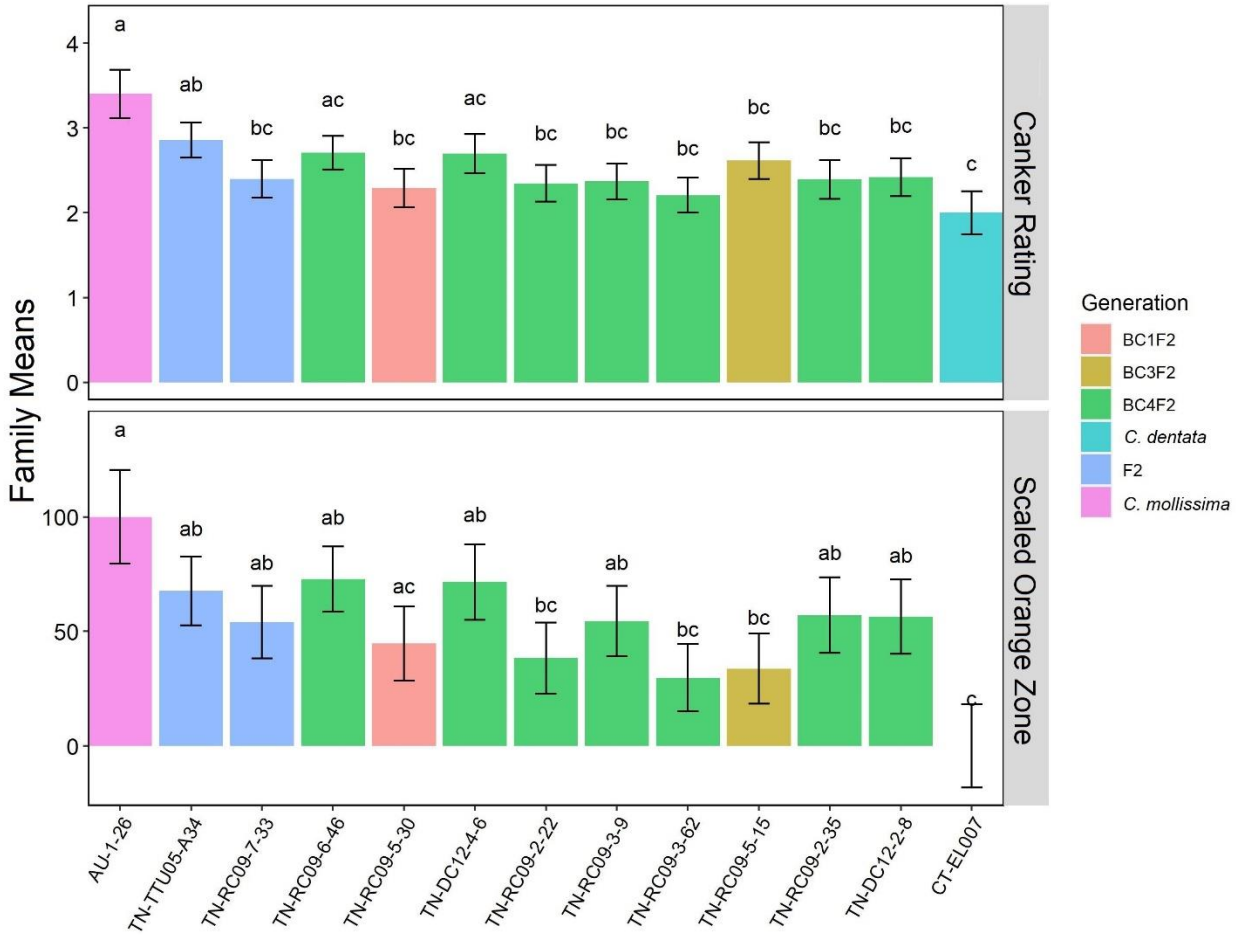
Outline of Each Family's Average Canker Rating with Standard Deviations and Scaled Orange Zone with Standard Deviation

Family	Generation	Canker Rating		Scaled Orange Zone	
		mean	se	mean	se
AU-1-26	*C. mollissima*	3.4^{a}	0.3	100^{a}	20.5
CT-EL007	*C. dentata*	2.0^{c}	0.3	0^{c}	18.2
TN-DC12-2-8	BC4F2	2.4^{bc}	0.2	56.5^{ab}	16.2
TN-DC12-4-6	BC4F2	2.7^{ac}	0.2	71.6^{ab}	16.5
TN-RC09-2-22	BC4F2	2.3^{bc}	0.2	38.4^{bc}	15.6
TN-RC09-2-35	BC4F2	2.4^{bc}	0.2	57.1^{ab}	16.5
TN-RC09-3-62	BC4F2	2.2^{bc}	0.2	29.8^{bc}	14.7
TN-RC09-3-9	BC4F2	2.4^{bc}	0.2	54.5^{ab}	15.3
TN-RC09-5-15	BC3F2	2.6^{bc}	0.2	33.7^{bc}	15.3
TN-RC09-5-30	BC1F2	2.3^{bc}	0.2	44.8^{ac}	16.2
TN-RC09-6-46	BC4F2	2.7^{ac}	0.2	72.9^{ab}	14.3
TN-RC09-7-33	F2	2.4^{bc}	0.2	54.0^{ab}	15.9
TN-TTU05-A34	F2	2.9^{ab}	0.2	67.7^{ab}	15.0

Note. Values having a common letter are not statistically significantly different ($p<0.05$) as determined by Duncan's multiple range test.

Figure 2.

Histogram of 90-day Canker Rating and Scaled Orange Zones Separated by Families



Note. Values having a common letter are not statistically significantly different ($p<0.05$) as determined by Duncan's multiple range test.

Mother Ancestry Correlations

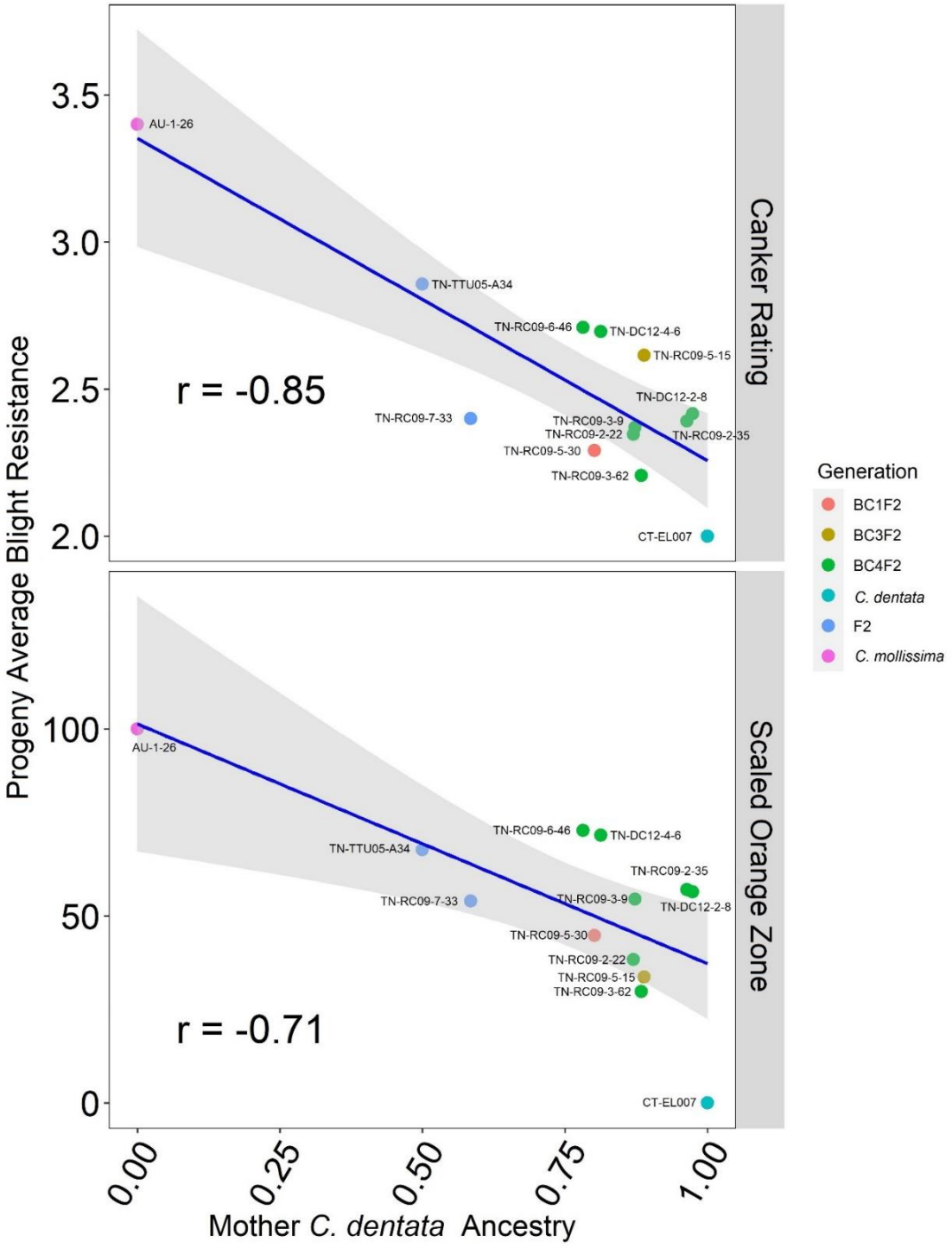
When the *C. dentata* ancestry is correlated against the progeny average blight resistance in terms of canker rating, the r-value resulted as -0.85. The negative correlation is most tightly packed around 0.75 mother *C. dentata* ancestry. Most families lie above the 0.5 mother *C. dentata* ancestry and cluster around the canker rating of 2.4. The Chinese control lies at the leftmost end of the trendline. The rightmost family is the American controls with a canker rating

of 2. The F2 families fall at the center of the x-axis with about 0.5 *C. dentata* ancestry.

When the *C. dentata* ancestry is correlated against the progeny average blight resistance in terms of the scaled orange zone, the r-value resulted as -0.71. The negative correlation is most tightly packed around 0.75 mother *C. dentata* ancestry. Most families lie above the 0.5 mother *C. dentata* ancestry and cluster around the scaled orange zone of 50. The Chinese control lies at the leftmost end of the trend with a scaled orange zone of 100 mm. The American control lies at the rightmost end with a scaled orange zone of 0 mm.

Figure 3.

Correlation Between the Amount of C. dentata *Ancestry in Each Family and Progeny Average Blight Resistance*



When the blight resistance of the mother is correlated against the progeny average blight resistance in terms of canker rating, the r-value resulted as 0.75. The positive correlation is tightest around 0.25 mother blight resistance and have a canker rating less than 2.5. Most families lie below 0.5 mother blight resistance. The Chinese controls have a canker rating of 3.4, and the American trees have a canker rating of 2.0.

When the blight resistance of the mother is correlated against the progeny average blight resistance in terms of scaled orange zone, the r-value is 0.69. The positive correlation is tightest around the 0.30 mother blight resistance. The American trees have a scaled orange zone of 0 and the Chinese have a scaled orange zone of 100.

Figure 4.

Correlation Between Mother Blight Resistance in Each Family and Progeny Average Blight Resistance

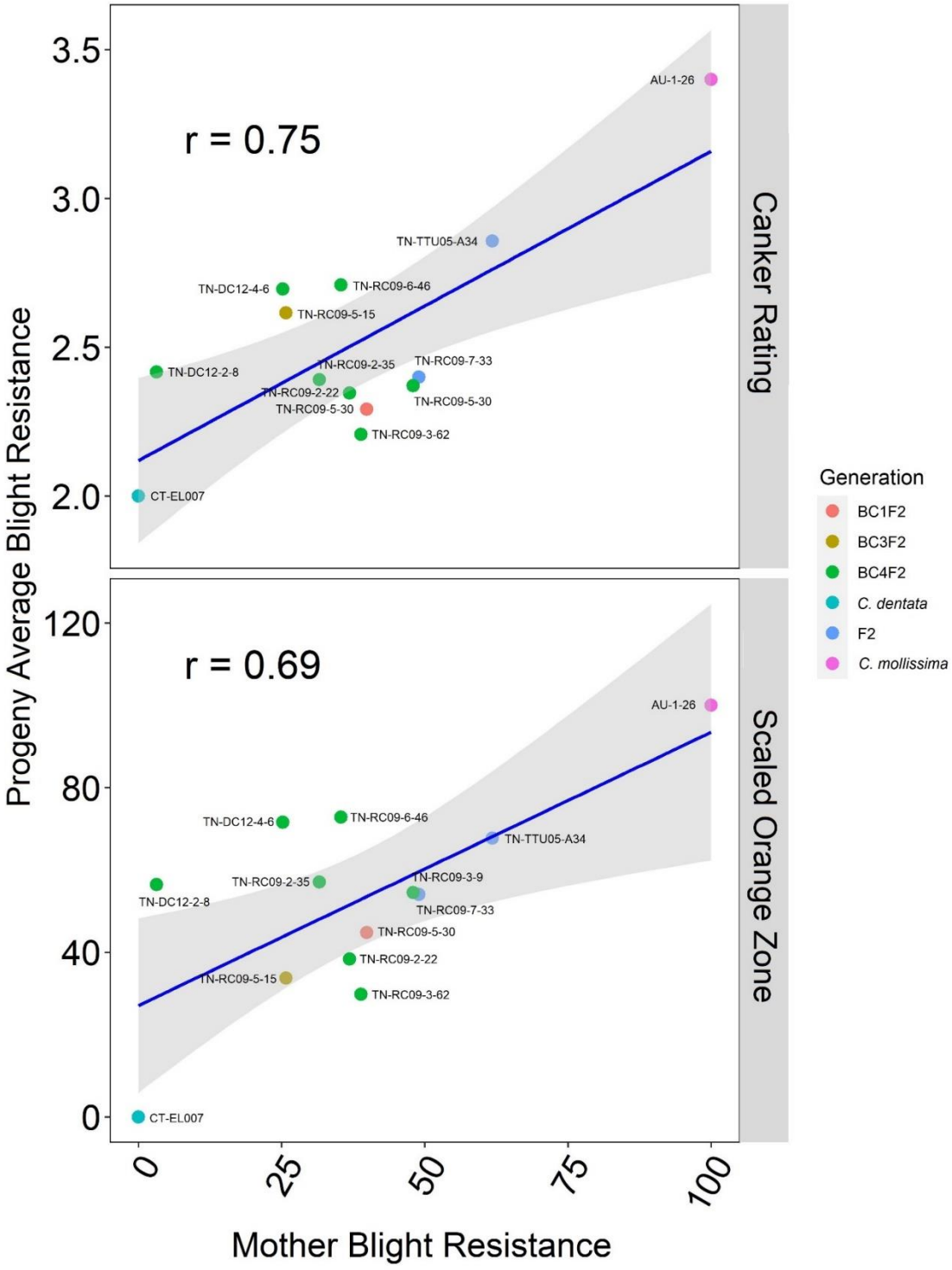


Table 12.

SSA Heritability H2 Estimated for Canker Rating and for Scaled Orange Zone Lengths for Both Families and Individual Trees

	H^2 individual ± SE	H^2 family ± SE (N = 28)
Canker Rating	0.15±0.14	0.53±0.22
Orange Zone Length	0.08±0.10	0.36±0.31

Discussion

Average Canker Length

Average Canker Lengths by Generation

The *C. dentata* generation had the highest mean of all other generations. *C. mollissima* had the lowest mean than all other generations. All hybrid generations had means intermediate to the two controls. ANOVA analyzed differences in means for each generation while factoring in the block effect and resulted in a statistically significant difference for both the generation and the block factor. This corroborated the DMRT results for canker ratings from Figure 1, because *C. dentata* and *C. mollissima* generations were statistically different. However, the differences in the hybrids were largely indiscernible based on DMRT results. For instance, the F2 generation contained the TN-TTU05-A34 family, which resembled the *C. mollissima* family and had a mean that was different enough to distinguish it from the *C. dentata*. The F2 generation also contained TN-RC09-7-33, which had a canker rating that resembled *C. dentata* but was not statistically significantly different from *C. mollissima.* Though both mother trees had about generally the same amount of *C. mollissima* lineage as they had *C. dentata* lineage, TN-TTU-A34 had slightly more *C. mollissima* lineage, which may explain why that family resembled *C. mollissima* more in terms of canker rating and why TN-RC09-7-33 resembled *C. dentata* more in canker ratings. Albeit, when looking at scaled orange zones, both trees resembled *C. mollissima* and were

statistically significantly different from *C. dentata.* This is one example why the SSA is not a good indicator for making artificial selection within generations or families. The SSA is not an accurate enough indicator for blight resistance.

Furthermore, of the backcross generations, BC4F2 generation had the smallest mean canker lengths, followed by BC1F2, then the BC3F2 with the largest canker lengths. BC4F2 was expected to have a higher canker length than other backcross generations, because it has been crossed with C. dentata more. From Figure 2, higher *C. dentata* ancestry correlated with a worse canker as defined by a lower canker rating. Therefore, BC3F2 should have an average canker length between the BC1F2 generation and the BC4F2 generation. Instead, BC3F2 has the highest average canker lengths of all three generations. A possible explanation for this incongruity was that the random error in measurement was significantly worse while measuring the BC3F2 generation than other generations. When comparing the standard deviations to the means of all three backcross generations, we can see that the standard deviation for BC3F2 is nearly the size of the average canker length itself. Meanwhile, the other backcross generations had lower standard deviations in comparison to their means. The high standard error points to a limitation in the methods of the experiment.

Another possible reason for the incongruity seen between the backcross generations is that the sample size for the BC1F2 and BC3F2 generations are small in comparison to the BC4F2 generation. The BC4F2 generation contains seven families and 204 seedings total. The BC1F2 and BC3F2 generations both contain one family each. The BC1F2 generation contains 29 seedlings with one outlier seedling removed during data analysis, and the BC3F2 generation contains 30 seedings total. Because of the small sample size for BC1F2 and BC3F2 generation, the results may not provide a clear picture of the level of blight resistance at each generation.

Average Canker Lengths by Family

All hybrid families showed average canker lengths intermediate to those of the *C. mollissima* and *C. dentata* families as expected. Of the BC4F2 generation, the family with the highest average canker length is TN-RC09-3-62, which correlated with its high mother *C. dentata* ancestry and relatively low *C. mollissima* ancestry.

For the average family canker lengths, the ANOVA revealed that the differences in each family were statistically significantly different from one another, so the SSA using hypovirulent inoculum was able to separate each hybrid family based on its blight resistance. However, the standard deviations were also high in comparison to its mean, leading to the conclusion that the random error in measurement greatly disturbed the results of the SSA. The DMRT was not able to separate and rank the hybrid families from one another, but all families were intermediate to the two controls, as expected. Based on the DMRT, the SSA showed that backcross selections for blight resistance are making a positive trend towards the desired phenotype, but the SSA method itself may not be a good method to make those selections.

Heritability (H^2) of Blight Resistance

Gains in blight resistance are proportional to the heritability (H^2) of the trait used to measure blight resistance and selection intensity, defined in the SSA as the percentage of individuals selected prior to planting. For the SSA, heritability can be estimated for the family and for individual trees using canker ratings and scaled orange zones. The heritability for individual trees (H^2 individual in Table 12) aids in the effectiveness of the SSA by selecting trees prior to planting in the field. The H^2 for individual trees is a ratio of how strongly the trees' phenotypes are correlated with their underlying genetic blight resistance. For half-sibling or backcross families, the following equation can be used to find the H^2 value, where VAR stands for

estimated variance components for the subscript "mom" and "error," respectively estimated in a mixed-model equation:

$$H^2 individual = 4 \times \frac{VAR_{mom}}{(VAR_{mom} + VAR_{error})}$$

"Mom" refers to the known tree in an open-pollenated cross. Mom's variance is estimated via pedigree relationships among trees (J. Westbrook, personal communication).

SSA can also backwardly select the most resistant mother based on the average canker severity of their progeny by finding the average family heritability:

$$H^2 individual = \frac{VAR_{mom}}{VAR_{mom} + \frac{VAR_{error}}{average \text{ n } individuals\ per\ family}}$$

The average family heritability is how accurately the phenotypic canker rating or scaled orange zone reflects the genetic resistance of the parent.

Unfortunately, the H^2 estimates for individual tree phenotypes were very low as seen in Table 12, so phenotypes elucidated using the SSA should not be used to make selections within families. The selection gains in blight resistance will be very small, if any. Fortunately, with large family sizes of at least 100 progeny, the average family heritability in the SSA were > 0.9 (Odle, 2022). The SSA can verify that parental selections were accurate and that gains result from the expected crosses of the most resistant trees. While this SSA only had about a family size of about 28 trees, the H^2 resulted follow the expected pattern of previous experiments.

Future Small Stem Assays

Based on this SSA, the two families with a scaled orange zone of at least 40, on the scale of *C. dentata* as 0 and *C. mollissima* as 100, should be crossed. Each cross should generate 150 seeds, which will be enough to produce clearer resolution in the SSA. Also, open-pollinated

seeds from the same mothers should be collected to compare any additional gains in resistance from the controlled pollinations to the open pollinations (J. Westbrook, personal communication). Future SSA should cross two of the most resistant trees and better BC1 progeny. The SSA should include larger sample sizes per family (>100), which will verify the expected gains in resistance from crossing the best families. After confirming that the progeny of the two best families meet minimum resistance expectations using the SSA, the progeny can then be grown in a greenhouse or nursery for genotyping to predict resistance and to assess the *C. dentata* ancestry before planting the best 10% of individuals in an orchard setting.

The horticulture and selection practices would also apply for the Darling58 (D58) transgenic chestnuts. D58 can be crossed with diverse backcross trees and the progeny can be grown in the greenhouse for a year. The progeny would then be genotyped to select individuals with minimal *C. mollissima* ancestry and selected individuals would be planted in orchards.

The small stem assay method for chestnut blight is an example of early screening methods, which also has broad reaching effects to screening for other diseases of American chestnut and for other plants as well. The American Chestnut Foundation has used young one-year-old American chestnut seedlings to screen for *Phytophthora* root rot caused by *Phytophthora cinnamomi* Rands. (Hein 2018). The small stem assay method can also be used to screen other tree species for prevalent diseases such as Dutch elm disease caused by *Ophiostoma ulmi.*

Random Error

Most trees in this experiment seemed to have shrunk in canker lengths when viewing the progression of measured canker lengths each month, which is biologically impossible. Possible random error while measuring the cankers may have occurred, because the point where the orange zone ended was not consistently defined. Discoloration in the canker ranged from pale

white to light brown to orange. The ill-defined orange zone length caused high standard error in the measurements as seen in Tables 2, 3, 4, 5, and 6. To avoid this issue in future SSA experiments, I suggest creating a color scale for what is considered the orange zone. I also suggest having another trained eye to double-check the measurements. In my SSA, I was the only person to measure the cankers. I made this decision to avoid measurement error in case multiple volunteers measure cankers, and each volunteer identifies the orange zone length using different characteristics. Yet, I still encounter random error, so I suggest more than one person check the length of each canker to ensure accuracy.

I also suggest taking breaks in between each block or section of trees. The measurements took about 6 hours to complete. Taking one or two small breaks leads to fatigue and lack of clarity while measuring. I measured from Block 1 to Block 4. In hindsight, fatigue can be at its worst by the time I reach Block 4. While I do not suggest rearranging the order in which the trees are measured, rushing through measurements can lead to sloppy work. The measurement period can be split into 3-hour sessions over the course of two days rather than 6-hour sessions all in one day.

The small stem assay method was used to screen seedlings in thirteen half-sibling backcross F2 families with a hypovirulent strain of *C. parasitica* containing the *Cryphonectria parasitica hypovirus-1* (CHV-1) Euro7 virus. Similar to a previous SSA experiment conducted by Odle (2022), the SSA method differentiated between orange zone lengths of *C. dentata* and *C. mollissima* control groups and all hybrid families using the visualization methods of canker ratings and scaled orange zones. However, Duncan's multiple range test was unable to rank each hybrid family by its level of blight resistance. The SSA should not be used to make artificial selections for blight resistance within families due to its low resolution, but it can verify that the

parental selections for each family were accurate. All surviving trees from the experiment will be planted in an experimental orchard in Middle Tennessee and monitored for survivability.

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