

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

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 will be gathered at four, eight, 12-, and 16- weeks post-inoculation. A secondary experiment was conducted to further understand the in vivo effects of hypovirulence. Trees from five of the thirteen half-sibling families were inoculated with isogenic virulent (virus-free) and hypovirulent (virus-containing) strains of the fungus. Virulence of the virus-free (V) and virus-containing (H) strains was measured using the Granny Smith apple assay. The possibility of in vivo canker conversion from V to H will be tested later (after eight) weeks by inoculating half of the V-infected trees with the H strain. Measurements of canker length and morphology will be gathered weekly for another eight weeks post-inoculation. Only preliminary results are presented here as the SSA portion of this project is expected to continue through November 2022. All surviving trees will be planted in an experimental orchard in Middle Tennessee to create a potentially long-lasting population of disease resistant trees together with an effective, long-term biocontrol for chestnut blight.

Experiments

Small Stem Assay using a Hypovirulent Strain: We plan to continue measuring the canker growth of hypovirulent *C. parasitica* on the progeny of *C. mollissima*, *C. dentata* and selected backcross hybrids expected to have varied levels of blight resistance.



Image 1. (left) Block 3, placed on Row 8 at the greenhouse, is shown empty before the experimental trees were placed at random and attached to the automatic irrigation line. Image 2. (right) Greenhouse volunteer Jalen precisely administers 1 tbsp of Osmocote to every tree using the fertilizer applicator.

Small Stem Conversion from V to H: Measuring the Canker Growth of converted *C. parasitica* from virulent to hypovirulent on the progeny of hybrids with intermediate blight resistance.



Image 3. (left) Tree identified as TN-RC09-3-9V14 was inoculated with virulent *C. parasitica*. The full canker length was measured using a caliper on 6 Sept. 2022. Image 4. (right): Tree identified as TN-RC09-6-46 H8 was inoculated with hypovirulent *C. parasitica* and its full canker length was measured using a caliper on 21 Sept. 2022.

Granny Smith Apple Assay: Measuring the lesion growth of hypovirulent and virulent *C. parasitica* on Granny Smith apples.



Image 5. The 15-day lesions shown here were caused by inoculation with the virulent strain of *C. parasitica*.

Materials and Methods

Small Stem Assay using a Hypovirulent Strain

- Inoculation and Data Collection: 365 open-pollinated progeny of selected backcross hybrid trees with intermediate levels of blight resistance were planted in four completely randomized blocks. Only 321 trees were inoculated with a hypovirulent Weekly strain of *C. parasitica*. Infected with the CHV-1 Euro7 hypovirus. At four, eight, twelve, and sixteen weeks, the cankers will be measured for canker length and morphology (Cipollini et al. 2021).



Image 6. (left) Dr. Craddock, Paola, Evelyn, and Kathryn each completing their task in the assembly line on inoculation day 16 July 2022. Image 7. (right) Trish and Kathryn completing the first measurements on 13 August 2022. Trish uses the calipers to measure the full length, the orange zone, and the sunken tip of the canker and calls out each measurement for Kathryn to record on the chart.

Small Stem Conversion from V to H

- Inoculation and Data Collection: A total of 109 trees were inoculated in the secondary experiment. For each family, eight out of 24 trees were inoculated with hypovirulent *C. parasitica* and sixteen out of 24 were inoculated with virulent *C. parasitica*. After four weeks, the cankers were measured weekly using a caliper for canker length and morphology. After eight weeks post-inoculation, eight of the sixteen trees originally inoculated with the virulent fungus were re-inoculated with the hypovirulent fungus to convert the canker in vivo. The cankers will be measured weekly for canker length and morphology for the next four weeks.



Image 8. (left) Tree identified as TN-RC09-2-22 H1 was measured on 30 August 2022 using the caliper. Image 9. (center) Tree identified as TN-RC09-3-36 V11 was re-inoculated with the hypovirulent strain of isogenic *C. parasitica* on 13 Sept. 2022. The sunken tip of the tree was snipped off and a plug of fungus-containing PDA was placed on the open wound. The entire tip was then wrapped with a small square of parafilm. Image 10. (right) All four trees in the same tray were randomly selected to be re-inoculated.

Granny Smith Apple Assay

- Ten Granny Smith apples were inoculated with a control plug of PDA, virulent *C. parasitica*, and hypovirulent *C. parasitica* (Kuhlman, 1983). Measurements were recorded with a caliper at five, ten, and fifteen days. Each lesion was recorded for three different diameters from which the mean area was calculated. Photos were also taken for each lesion on each measurement date and the surface area was determined using ImageJ.



Image 11 (left): The fume hood where the apples were inoculated, and supplies used to inoculate the apples. Image 12 (center): Apple lesion identified as GSA6V was inoculated with the virulent form of the isogenic *C. parasitica*. A ruler is included in the photo for ImageJ analysis, but the mean area of the lesion was also measured using a caliper. Image 13 (right): The binary image is of apple lesion GSA6V, which was used to calculate the area in ImageJ.

Preliminary Results

SSA and Conversion Experiments: Data collection and analysis is ongoing. I expect to complete all data collection in late November and complete all analyses before the end of 2022.

Granny Smith Apple Assay: For each measurement day, an unpaired Student's T-test was conducted to test for significant differences between the mean areas (gathered using a caliper and using ImageJ) of the virulent and hypovirulent lesions. The mean area of the lesions caused by V was larger than the mean area of the lesions caused by H for 5-, 10-, and 15-days for both caliper-measured and ImageJ-measured lesions, but the differences were not statistically significant.

x	y	T-value	df	p-value	Accept or Reject H ₀ ?	Mean of x (mm ²)	Mean of y (mm ²)
Day 5 V Canker Mean Area from Caliper	Day 5 H Canker Mean Area from Caliper	0.56374	13.88600	0.58190	accept	329.5	307.6
Day 10 V Canker Mean Area from Caliper	Day 10 H Canker Mean Area from Caliper	1.07010	15.03800	0.30140	accept	1327.8	1207.9
Day 15 V Canker Mean Area from Caliper	Day 15 H Canker Mean Area from Caliper	1.52580	17.99500	0.14440	accept	2228.9	1977.6
Day 5 V Canker Mean Area from ImageJ	Day 5 H Canker Mean Area from ImageJ	0.39782	16.34400	0.69590	accept	331.6	314.9
Day 10 V Canker Mean Area from ImageJ	Day 10 H Canker Mean Area from ImageJ	0.92776	15.46300	0.36780	accept	1434.8	1320.1
Day 15 H Canker Mean Area from ImageJ	Day 15 H Canker Mean Area from ImageJ	1.09300	17.72400	0.28900	accept	2546.4	2289.3

Table 1. Results from R of Unpaired Student's T-test on the Granny Smith Apple Mean Areas
All data sets resulted in a p-value greater than 0.05, meaning that observed differences between H and V were not statistically significant.

Conclusion

Small Stem Assay using a Hypovirulent Strain

- The data collection and analysis of the main experiment is still ongoing. I plan to record two more canker measurements at 12- and 16-weeks post-inoculation. Only two measurements have been recorded for the main experiment so far. Because analysis of already gathered data is still underway, no conclusion can be drawn yet.
- The results of the Small Stem Assay will help to develop screening methods for progeny testing. Surviving trees will be grown in an orchard setting with the prospect of establishing a population of hypovirulent *C. parasitica* along with blight resistant trees.

Small Stem Conversion from V to H

- I have already completed the first set of measurements prior to re-inoculation, the re-inoculation of half the V-infected trees, and two out of four post-inoculation measurements. I am currently analyzing the data already obtained but I cannot yet draw any conclusions.

Granny Smith Apple Assay

- The results of the Granny Smith Apple assay were statistically insignificant, which does not support my prediction that the virulent lesion would grow faster than the hypovirulent lesion. The two strains—virulent and hypovirulent—of the isogenic fungus may have similar levels of pathogenicity, resulting in no distinction in terms of growth rates. An excised stem assay may provide more distinctive growth differences in comparison to the Granny Smith Apples.

References

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