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Oxalic acid leaf disk soak assay is a new possibility in screening for blight resistance in Castanea species

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Oxalic Acid Leaf Disk Soak Assay is A New Possibility in Screening for Blight Resistance in *Castanea* Species

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Abstract

Chestnut blight is a disease caused by the ascomycete fungus *Cryphonectria parasitica* in the *Castanea* species. The fungus uses oxalic acid (OA) to attack the tree's cells. *Castanea dentata*, the American chestnut, was wiped out by chestnut blight in the early to mid-20th century, but several East Asian *Castanea* species appear highly resistant to the fungus. To breed resistant American type trees, screening methods are used to enable selection of interspecific hybrids. The alternative small stem assay (aSSA) is a method of screening container-grown hybrid seedlings during their first growing season by directly infecting them with *C. parasitica*. Some plant species use oxalate oxidase enzymes to defend themselves from fungal OA attacks. Although *Castanea* species do not have endogenous oxalate oxidase genes (*OxO*), chestnut species vary in their responses to OA. OA tolerance (measured by the browning of tissues exposed to OA solutions) appears to correlate well with blight resistance (as measured by Small Stem Assays) in chestnut species and hybrids. This knowledge was used to characterize hybrid germplasm in *Castanea* breeding populations in Tennessee in 2022 and 2023. Three hundred twenty-five seedlings of eighteen half-sibling hybrid families and five species, were arranged for screening in a randomized complete block design at the UTC Fortwood Street Greenhouse. Results show that screening seedlings with an oxalic acid leaf disk soak yield results that correlate strongly with results of an alternate small stem assay of the same plants. The aSSA and oxalic acid leaf disk soak assay were thus shown to be complementary methods for distinguishing blight tolerance in hybrid chestnut seedlings. All trees from the 2022 trials will be planted in an experimental orchard in Middle Tennessee for long-term observations.

Introduction

There is a direct correlation between oxalic acid and virulence of *Cryphonectria parasitica* (Murr.) Barr (Bennett & Hindal, 1989). The role of oxalic acid in this patho-system led to the first attempt to use oxalate oxidase, or OxO, in genetically modified chestnut trees (Zhang et al., 2013). OxO allows for the breakdown of oxalic acid into carbon dioxide and hydrogen peroxide (Bolwell & Wojtaszek, 1997). As biologists study the transformed *Castanea* with OxO, characterization of trees using relative tolerance may be a new screening possibility. This is because the natural tolerance of oxalic acid vary greatly between *Castanea* species (Zhang et al., 2013). This knowledge was used to characterize our hybrid germplasm in seedling populations that we expected to vary. I hypothesized that an oxalic acid leaf disk assay can detect differences in oxalic acid (OA) response and the OA response can be used to select for blight resistance in container-grown chestnut seedlings. An oxalic acid leaf disk assay is where small 15 mm disks are soaked in 50 mM oxalic acid, and after this, the disks are measured based on the areas of browning tissue and green, healthy tissue. The current screening method for container-grown seedlings is the small stem assay (SSA) (Cipollini et al., 2021). Small stem assays are a screening method used in nursery settings on container-grown seedlings (Powell et al., 2007). This method is to be used to possibly screen trees before they are planted in an orchard setting, to save resources (Powell et al., 2007). For the present work, I compared SSA with a new method, titled "oxalic acid leaf disk soak assay." I predicted seedlings of *C. mollissima* would be most tolerant to oxalic acid, due to their resistance to *C. parasitica*, seedlings of *C. dentata* would be least tolerant to oxalic acid, due to their poor resistance to *C. parasitica*, and the hybrid families of seedlings would be intermediate in tolerance to oxalic acid because of their intermediate levels of blight resistance. There are still gaps in research with *C. pumila* and their resistance to

chestnut blight. Some scientists state that chinquapins are not resistant to blight (Barnard, 2000). However, Robert Morris considered it comparable to *C. mollissima* (Morris, 1914). I decided to include *C. pumila* on this leaf disk assay for this very reason, and I predicted their tolerance to be like that of the American chestnut.

Literature Review

Pre-Blight *Castanea*

The *Castanea* genus is held within the family Fagaceae. There are 10 species in total (Mellano et al., 2018; Mellano et al., 2012; Perkins et al., 2021). The genus is naturally found in Eastern North America, Europe, North Africa, and East Asia (Mellano et al., 2018). *Castanea dentata* used to be a major component of eastern American hardwood forests (Anagnostakis, 1987). Its tall slender shape made it a great use for lumber, and oftentimes the trunk grew to about six or seven feet in diameter (Brooks, 1937). The wood had a large amount of tannins, making it a great for outdoor use (Freinkel, 2009). The tree was also valued for its fruit. There is a rich history of chestnuts and those who lived in Appalachia (Freinkel, 2009). Many gathered, ate, sold, and fed their livestock the chestnuts (Freinkel, 2009). There are other species of *Castanea* within North America. Other than *Castanea dentata*, there is *Castanea pumila Mill. C. pumila*, also known as the chinquapin, found in the Southeastern United States (Mellano et al., 2012). Taxonomy and nomenclature of the chinquapins is in flux, with several species currently recognized at the level of botanical variety: *Castanea pumila* var. *alnifolia* Nutt., *Castanea pumila* var. *alabamensis* Ashe and *Castanea pumila* var. *ozarkensis* (Ashe) Tucker (Perkins et al., 2021).

In Europe, *Castanea sativa* Mill. is often considered the sweet chestnut, and is the only native species of Europe (Conedera et al., 2016; Lang et al., 2006). In Europe, chestnut forests are concentrated mainly in Italy, France, Spain, and the Iberian Peninsula (Conedera et al., 2016). The European chestnut is also valued for its timber and its desirable fruit.

Castanea is also found in East Asia, and is thought to be where the blight fungus originated from (Anagnostakis & Hillman, 1992). The Asian species include *Castanea mollissima* Blume, *Castanea crenata* Siebold & Zucc., *Castanea henryi* (Skan) Rehder & E.H.Wilson, and *Castanea seguinii* Dode (Mellano et al., 2012).

Chestnut Blight

The pathogen *Cryphonectria parasitica* (Murrill) M.E.Barr is suspected to have been carried from Japan to the United States on nursery stock of *Castanea crenata*, the Japanese chestnut, in the early twentieth century (Anagnostakis & Hillman, 1992). The ascomycete fungus was first noted by Murrill in 1905 at the New York Botanical Garden (Murrill, 1906). It is characterized by necrotic lesions on the limbs and trunk and orange sporulation, and this develops into fatal cankers (Murrill, 1906; Rankin, 1912).

When the fungus decimated the tree, an estimated four-billion American chestnut trees were lost on the eastern hardwood forest (Roane et al., 1986). Environmentally, there were organisms that depended on this tree, like *Synanthedon castaneae* (Sesiidae), a moth that feeds on the trunk (Opler et al., 1978). The *S. castaneae* (Sesiidae), among various moths (seven in total) may have gone extinct due to the loss of *Castanea dentata* (Opler et al., 1978). Ecologically, *Castanea dentata* was an important factor in the Eastern hardwood forest

(Anagnostakis & Hillman, 1992). Major conservation efforts have started since the discovery of the fungus in 1905 (Anagnostakis & Hillman, 1992; Murrill, 1906).

Hypovirulence

C. sativa, was also affected by *Cryphonectria parasitica* (Rigling & Prospero, 2018). In Europe, the fungal pathogen was successfully defeated through a virus. The use of hypoviruses to attack the fungus as a biocontrol has helped to attenuate chestnut blight disease in Europe (Anagnostakis & Hillman, 1992). The viruses attack *Cryphonectria parasitica* directly, which weakens the fungus and makes it less harmful to chestnut trees. They were discovered in Italy when some of the fungal strains had less of a distinct orange color, and the trees seemed to survive with these strains (Anagnostakis & Hillman, 1992). These hypovirulent strains produce less spores, meaning they pass less often between trees (Anagnostakis & Hillman, 1992).

Oxalic Acid

Oxalic acid is used by many pathogenic fungi to weaken the cell wall. Oxalic acid acidifies host tissues and sequestrates calcium from host cell walls (Dutton & Evans, 1996). There is a direct correlation between virulence factors and oxalic acid production (Dutton & Evans, 1996). It was discovered many years ago the relationship between oxalic acid and *Cryphonectria parasitica* (Bennett & Hindal, 1989). A higher virulence indicated a higher production of oxalic acid (Bennett & Hindal, 1989).

Cryphonectria parasitica effectively uses oxalic acid to attack vulnerable trees (Zhang et al., 2013). To infect the tree, *C. parasitica* excretes oxalic acid as oxalate in the chestnut tree's stem to decrease pH (Zhang et al., 2013). Some research indicates that oxalic acid may lead to programmed cell death, which enables this necrotrophic fungus (Errakhi et al., 2008; Rigling &

Prospero, 2018). When the stem is vulnerable, the fungal hyphae enter through cambium, which causes the distinct canker formation (Griffin, 2011). Studies have revealed that scientists may be able to inhibit this pathway. When removing the ability to create oxalacetate acetylhydrolase, or OAH, in *C. parasitica*, the fungus's ability to create cankers greatly reduces (Chen et al., 2010). In the metabolic pathway, OAH catalyzes the hydrolysis between oxalacetate to oxalic acid and acetate (Chen et al., 2010).

Chestnut Breeding

American chestnut tree breeding efforts began after blight first began by Arthur Graves and R. Clapper (Clapper, 1954; Graves, 1950). Because of this, legendary B1 trees have been named after these individuals (the Graves Tree and the Clapper Tree) and have been used in The American Chestnut Foundation breeding program (Hebard et al., 2012).

Backcross breeding was proposed by Burnham and Rutter (Burnham, 1988). The purpose is for introgression of alleles from *C. mollissima* (resistant species) into *C. dentata* (susceptible species) while conserving adaptive traits in the recurrent species (Burnham, 1988). Burnham believed that blight resistance was partially dominant (Hebard et al., 2012). For example, the first offspring, F1 generation (half-mollissima, half-dentata at the genomic level), are intermediate in blight resistance. When F1s are bred again with American trees, the Chinese traits are lost by one-half in the resulting first-backcross generation (An F1 crossed with *C. dentata* produces a BC1 or B1 generation) (Hebard et al., 2012). An B1 crossed with *C. dentata* produces a BC2 generation (B2). After three such backcrosses to the recurrent species *C. dentata*, only one-sixteenth of the genetic material from *C. mollissima* will remain (Burnham, 1988). However, after generations of breeding hybrid trees, genetic studies reveal that Burnham's hypothesis cannot be supported (Miller, 2020). A preponderance of recent evidence suggests that blight

resistance in *C. mollissima* is controlled by genes at several loci (possibly dozens of genes) distributed among the twelve chromosomes. Thus, backcross breeding as envisioned by Burnham and Rutter may not be tenable. Nonetheless, there is great conservation value in the populations of surviving backcross hybrids. All of this is a major conservation effort held up by The American Chestnut Foundation (TACF). The main purpose is to retain as much as possible of the adaptive variation in the extant populations of *C. dentata* and to maintain those characteristics that are the defining characters of *C. dentata* (Westbrook et al., 2019). These hybrid trees, accumulated over more than 30 years of breeding effort, harbor vast stores of genetic diversity, and will lend themselves to advances in plant breeding that take advantage of new technologies.

Transgenic Chestnut Trees

An example of an emerging new technology is genetic engineering. No *Castanea* trees have a natural genetic factor to metabolize oxalic acid. Plants like *Triticum,* wheat, contain the OxO gene, which does metabolize oxalic acid. (Carlson et al., 2022; Zhang et al., 2013). Scientists at SUNY-ESF have used agrobacterium-mediated transformation to insert the gene into *C. dentata* (Westbrook et al., 2020). By using the OxO gene from wheat plants, *Castanea* species can breakdown oxalic acid into carbon dioxide and hydrogen peroxide (Bolwell & Wojtaszek, 1997). The use of the OxO gene elevates *C. dentata's* ability to fight *C. parasitica* (Carlson et al., 2022; Powell et al., 2019). The trees studied in early trials showed heightened resistance to blight (Steiner et al., 2017).

The Darling 58 tree is the transformed *C. dentata* tree (Westbrook et al., 2020). Darling 58 tree is currently undergoing FDA deregulation for distribution and breeding in unrestricted areas. Before this, they had to be sure it had little ecological impact as a transformed tree. Studies

had to be done with the Darling 58 tree to ensure that it would be safe to introduce throughout the east coast. They found no ecological impacts with the transformed chestnut tree, such as little impacts on the photosynthesis physiology in transformed plants (Onwumelu et al., 2023). The transformed trees do not affect pollinators, such as the honeybee (Newhouse et al., 2021).

If the Darling 58 tree is bred with American chestnut trees, then half of the progeny will receive the oxo-gene (Westbrook et al., 2020). This allows for a completely "American" tree, with all *C. dentata* traits, while maintaining blight resistance. This implies that the American chestnut tree could be fully restored and remain virtually the same with the insertion of the *oxo* gene (Powell et al., 2019; Westbrook et al., 2020).

Screening Methods for Blight

Before the modern application of the small stem assay, the previous screening method used trees already planted in orchards. They typically grew for four to five years before their trunk diameter was 2.5-5 cm to be adequately assessed (Griffin et al., 1983). A hole of 5mm was made for pathogen infected agar (cork-borer method) to be inserted into the tree (Griffin et al., 1983). Those screening the trees then allow the fungus to infect the tree. The advantage of the traditional screening method is that it can detect intermediate or partial resistance to *C. parasitica* within hybrid trees (Anagnostakis & Hillman, 1992) and due to its high resolution, can be used to make selections within backcross families. However, this means that all trees are purposely infected with the fungal pathogen. Most of them will die. Many resources will be spent for the four or five years of growing these seedlings in their respective orchards before they are infected and eventually succumb to blight. Attempts to reduce the costs, time required and resources necessary to properly screen trees for blight resistance led to an alternative method for screening, the small stem assay (Cipollini et al., 2021).

Small Stem Assays

Small stem assays are a screening method used in nursery settings on container-grown seedlings (Powell et al., 2007). This method is to be used to possibly screen trees before they are planted in an orchard setting, to save resources (Powell et al., 2007). The small stem assay was first used in 1989 (Hebard, 1989). However, this first attempt did not display significant differences between low, moderate, or high resistance between the control species, *C. dentata* and *C. mollissima*. Then, in 2017 TACF worked with US Forest Service Resistance Screening Center (RSC) to develop a better small stem assay (Westbrook, 2018). Cipollini et al. (2021) proposed an alternative SSA, in which a cut stem is inoculated at its tip, rather than through an incision along the side of the stem. The cut stem method alleviates some persistent problems of the earlier SSA methods: the inoculum (fungus) is applied more consistently, resulting in fewer "no takes" (inoculation failures). Because clipping the shoot tips stimulates the growth of lateral buds and basal shoots, seedlings screened using cut stem method appear to have better out planting and survival rates than trees inoculated on the side of the stems (Cipollini et al., 2021).

Materials and Methods

Hypothesis

I hypothesized that the two controls, *C. dentata* (American, blight-susceptible), and *C. mollissima* (Chinese, blight-resistant) would show statistically significant differences in their oxalic acid tolerance, and that the hybrid families used in the small stem assay would show intermediate levels of oxalic acid tolerance. Relative oxalic acid tolerance is expressed as measurable differences in leaf disk necrosis following an oxalic acid soak of leaves of *C. alabamensis, C. dentata, C. mollissima, C. ozarkensis, C. henryi, C. alnifolia, C. pumila*, and

open-pollinated seedlings of selected backcross hybrids. The resistance of *C. pumila* species to *C. parasitica* is understudied, and I thought their endogenous OA tolerance was going to be somewhere between *C. dentata* and *C. mollissima*.

Preliminary Tests

The oxalic acid leaf disk assay soak being a new idea, many preliminary trials were completed to determine the right configuration for testing. First, the age of the leaf was determined to affect the soak. One leaf was collected that was fresh (first fully expanded), one from the middle of the same tree, and one closest in proximity to the root collar. Both the old and new leaves reacted better than the middle-aged ones. Reacting better in this case means a more distinct brown/green area on the actual disk. This determined that the fully expanded fresh leaves would be used for the leaf soak.

Another preliminary test was for time duration. This is because it was suggested by Andy Newhouse (personal communication) that I conduct the assays for 12 hours. However, when looking at *C. dentata* controls, they turned completely brown within 12 hours. A trial was conducted where tubes of *C. dentata* (both hybrid families and pure *C. dentata*) disks were observed every hour for 12 hours, and ten hours seemed be the threshold before most hybrids and American turned completely brown. Thus, all oxalic acid leaf soaks were conducted for ten hours.

The final trial was conducted to see if different orientations of the acid solution tube on the orbital shaker affected the browning areas on leaves due to clumping of leaves within liquid. It was concluded that horizontal placement of the tubes on the orbital shaker was most effective to prevent disks from clumping.

Oxalic Acid Leaf Disk Soak

Eighteen half-sib families of open-pollinated seedlings were used in my oxalic acid leaf disk assay. They were originally intended for TACF TN chapter SSA of 2022. The control *C. dentata* family came from Connecticut. The control *C. mollissima* family came from an orchard located at Auburn university. The trees with "RC" in the name come from the Ruth Cochran orchard in middle TN (Moore county), where the Tennessee chapter breeds trees. Families with "TTU" in the name come from the Tennessee Tech University backcross orchard in Putnam Co., and come from the Dave Cantrell backcross orchard in Knox County, TN. All hybrid families used in this study are from Tennessee.

Trish Nguyen used these same trees for an aSSA in 2022 (Nguyen (2023). She inoculated every tree with a hypovirulent strain of *C. parasitica*. Because they were used for the small stem assay, their arrangement in the nursery was a randomized complete block design, in four nonadjacent blocks. I collected the leaves after the small stem assay inoculation, when they were in the randomized block design. My *C. pumila* collections were also at the same location. However, they were not included in these randomized blocks.

The eighteen half-sib families used in the Oxalic Acid Leaf Disk Soak Assay are listed in Table 1 along with seedling type and the quantity of plants in each family. Only one leaf per tree was used in this study. Three-hundred and twenty-five container grown seedlings were used in this study. I used one leaf per individual tree to make ten disks. This means each tree had ten disks each. The experiment had a total of 3250 disks in the assay.

Families

Table 1: Eighteen half-sib families used in the Oxalic Acid Leaf Disk Soak Assay. Thirteen of these families were from the UTC 2022 SSA (Nguyen 2023).

Family	Seedling Type	n
CT-EL007 (*C. dentata*)	American	17
AU-1-26 (*C. mollissima*)	Chinese	13
TN-DC12-2-8	B4	23
TN-DC12-4-6	B4	22
TN-RC09-2-22	B4	24
TN-RC09-2-35	B4	21
TN-RC09-3-62	B4	26
TN-RC09-3-9	B4	25
TN-RC09-5-15	B3	24
TN-RC09-5-30	B1	23
TN-RC09-6-46	B3	28
TN-RC09-7-33	F2 (*dentata* x *crenata*)	24
TN-TTU-A34	F2 (*dentata* x *mollissima*)	24
C. pumila	Chinquapin	4
C. pumila var. alabamensis	Alabama Chinquapin	2
C. pumila var. ozarkensis	Ozark Chinquapin	1
C. pumila var. alnifolia	Trailing Chinquapin	6

C. henryi	Henryi	4

Data Collection and Measurements Using Image J

ImageJ is an open-source Java-based image editing program (Rasband, 1997-2018). Used by many in biological sciences, it can be used to measure objects in images (Abràmoff et al., 2004). In this case, I used it to measure individual leaf disks, and then to measure the remaining area of green after selecting the brown areas out. Following the ten-hour soak, they were rinsed with distilled water and photographed and processed this way in ImageJ. All ten disks for one tree were photographed at once, so only one photograph was taken for each tree.

Statistical Analysis in R

RStudio is an open-source software for statistical analysis and developing graphics (RStudio, 2019). Ggplot2 was used specifically for developing visually appealing graphics (Wickham, 2016). Other packages used were easyanova and dyplr (ARNHOLD, 2013; Wickham, 2023).

Results

The first two disks in the top row in Figure 1 are *C. mollissima* (AU-1-26, Chinese, blight resistant) and *C. dentata* (CT-EL-007, American, blight-susceptible), respectively. The main measurement taken in the assay is the brown area vs total area. As seen in Figure 1, *C. pumila* and its hybrids had distinctly different reactions to the same amount of oxalic acid when compared to the SSA families. These images correspond with the families mean value of necrosis. That means that the image of *C. mollissima* (AU-1-26) is representative of a disk that is approximately 63% brown.

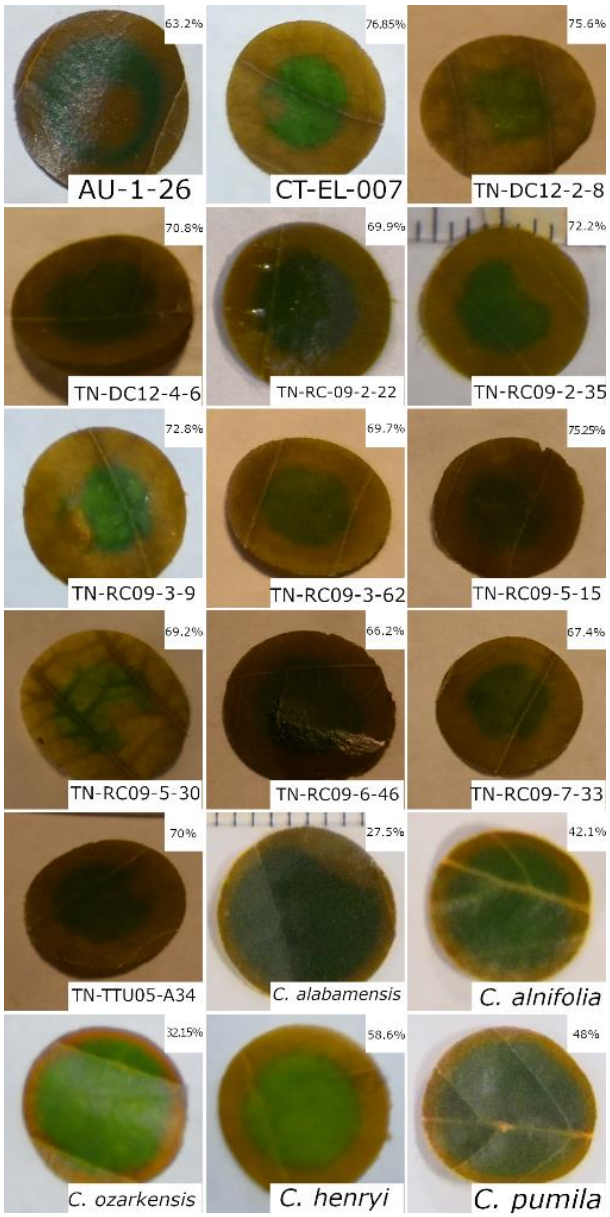

Figure 1. Photographs of individual 1.5 cm diameter disks after oxalic acid solution soak. Each image is representative of the median of the families. (Image created by Micheal Harden)

In Figure 2 are the plotted the family means for percent disk necrosis by half-sib family, *C. dentata* was least tolerant of OA with a family mean of 79.2% browning, and *C. mollissima* was most tolerant of OA with a family mean of 63% browning. All hybrid families had means for percent disk necrosis intermediate between the two controls, which was expected. However,

there is large variation in percent disk necrosis within the hybrid families. The whiskers on the plots sometimes take up the entire y-axis.

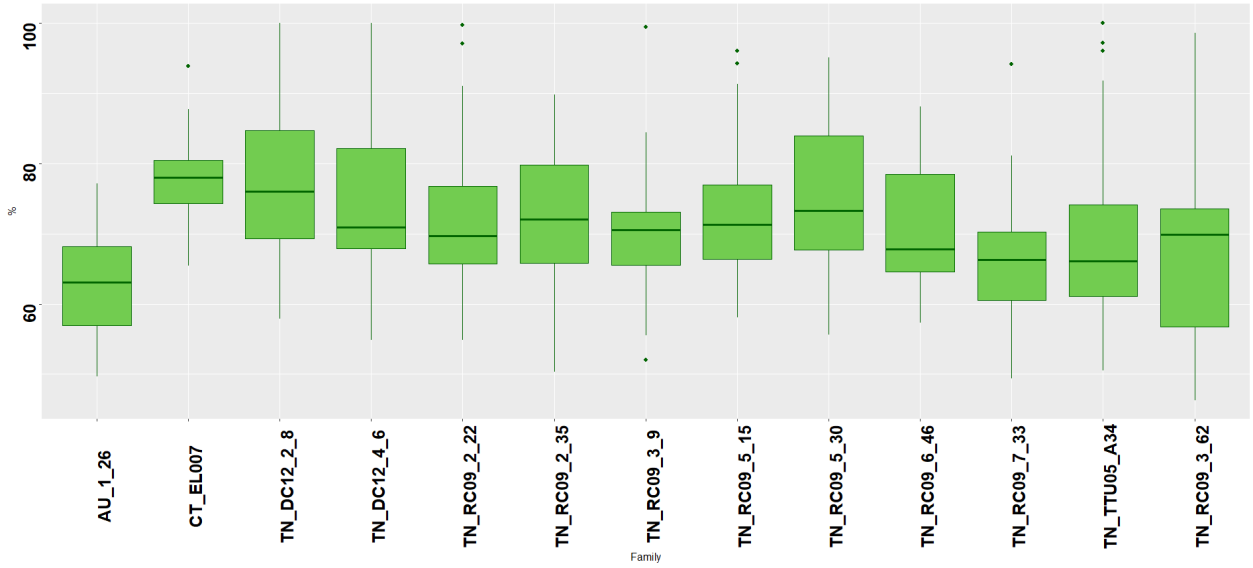

Figure 2. Box plots for all SSA families. X-axis is the family (treatment). The Y-axis is the percentage of brown seen on the disks. The first boxplot is *C. mollissima*, and second is *C. dentata*. The following are hybrid families. Generated in RStudio with ggplot2 (Wickham, 2016).

The results of *C. pumila* in Figure 3 are unexpected. How are chinquapins supposedly tolerant of oxalic acid? All their medians fall below both *C. mollissima* (AU-1-26) and *C. dentata* (CT-EL007). To have a median browning percentage of under 50% is remarkable. The results are also clear with the sample images seen in Figure 1.

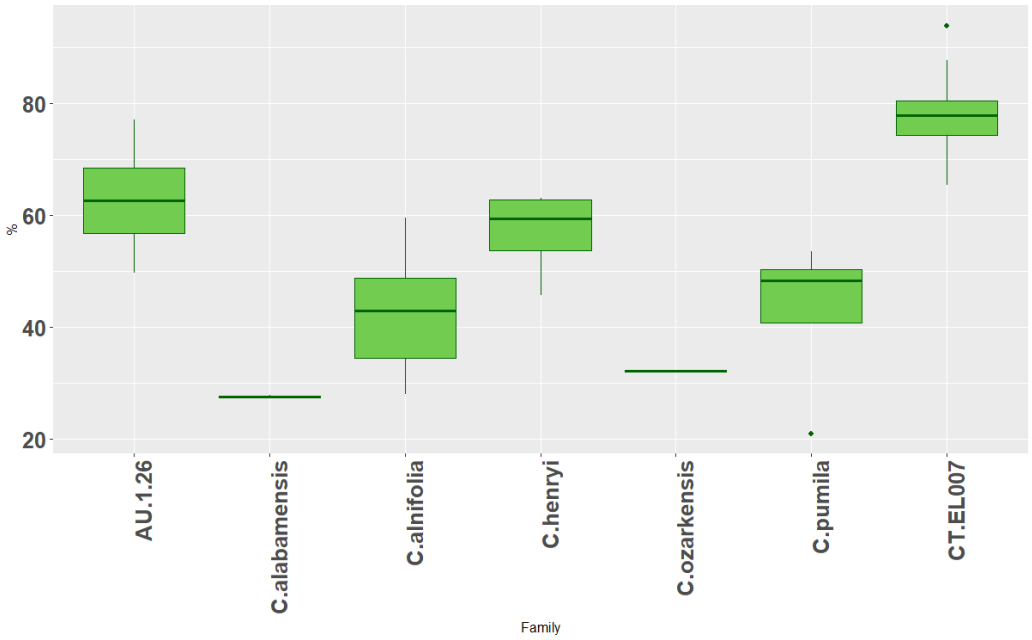

Figure 3. Box plots for all *Castanea* species (non-hybrids). X-axis is the family (treatment). The Y-axis is the percentage of brown seen on the disks. The first boxplot is *C. mollissima*, and second is *C. dentata.* This graph includes chinquapins despite the small sample size. Generated in RStudio with ggplot2 (Wickham, 2016).

One-Way ANOVA

Table 2. One-way ANOVA results produced by RStudio.

	Df	Sum	Mean sq	F value	Pr (>F)
group	12	3881	323.4	3.198	0.000252
Residuals	294	29732	101.1		
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1					

One-way ANOVA computed in R confirmed statistically significant results within the means of the thirteen families in UTC’s aSSA. I used only the small stem assay families due to the *C. pumila*’s small sample sizes. This meant I had 12 degrees of freedom, from the 13 families used in the small stem assay. The F-value was 3.198, and the p-value was 0.00025. Since the p-

value is smaller than 0.05, the mean browning area values of the oxalic acid leaf soak are significant when compared between family treatments. Based on Figure 2, *C. mollissima* (AU-1-26) and *C. dentata* are statistically different (based on their box plots), but it is hard to tell with the remaining families (hybrid half sib families). This means I then had to compare the family means and find out which ones were statistically different using Tukey's HSD.

Tukey's HSD and Duncan's Range Test

I used Tukey's range test to determine which means were significantly different from one another. The easyanova software provided me with Tukey's HSD and Duncan's range test, so I included them both in Table 3 (ARNHOLD, 2013). According to Tukey's HSD, the pairwise significant comparisons for this data was seen in Table 4. As is seen in Tables 3 and 4, the controls were significantly different from one another. Most comparisons were statistically different from AU-1-26, *C. mollissima*. However, TN-RC09-7-33, an F2 hybrid family, showed statistically significant differences from CT-EL007, *C. dentata*. It also differed from TN-DC12-2-8, which is a B4 seedling type. The B4 would be most closely related to the *C. dentata.* The rest of the means were not statistically different. This means the hybrid families are not easily differentiated between each other. This method, the oxalic acid leaf disk assay cannot differentiate between intermediate levels of OA tolerance.

Table 3. First family is CT-EL007, *C. dentata* control, and last family is AU-1-26, *C. mollissima* control. Table is composed of Tukey's HSD rankings, Duncan's Range Test rankings, means, and standard errors for each treatment. Table was created in RStudio using easyanova (ARNHOLD, 2013).

Treatment (Family)	Tukey's HSD	Duncan's Range Test	Mean	Standard error
CT_EL007	a	a	79.2	2.37
TN_DC12_2_8	a	ab	77.7	2.05
TN_RC09_5_15	ab	ac	76.4	2.01
TN_RC09_3_9	ab	ad	74.6	1.97
TN_DC12_4_6	ac	ad	74.4	2.1
TN_RC09_2_22	ac	ad	72.8	2.01
TN_RC09_2_35	ac	ad	72.6	2.14
TN_RC09_5_30	ac	ad	72	2.05
TN_RC09_3_62	ac	bcd	71.8	1.94
TN_TTU05_A34	ac	bcd	71.7	2.01
TN_RC09_7_33	ac	cd	70.5	2.01
TN_RC09_6_46	bc	de	68.4	1.87
AU_1_26	c	e	63	2.69

Table 4. Significantly different pair-wise comparisons from Tukey's HSD, shows the two-family comparison and its adjusted p-value from the test. Results from RStudio.

Pairwise Comparison	Adjusted p-value
CT_EL007-AU_1_26	0.000674
TN_DC12_2_8-AU_1_26	0.00129
TN_RC09_5_15-AU_1_26	0.031509
TN_RC09_5_30-AU_1_26	0.005335
TN_RC09_7_33-CT_EL007	0.02561
TN_RC09_7_33-TN_DC12_2_8	0.048879

Discussion

Variation in Hybrid Families

In Figure 2, there is large variation in percent disk necrosis within the hybrid families. The whiskers on the plots sometimes take up the entire y-axis. The variation is further evidence

that blight tolerance (and in this case, OA tolerance) is a polygenic trait, making breeding very difficult (Westbrook et al., 2019).

Comparisons between Small Stem Assay and Oxalic Acid Leaf Soak

Figure 4 shows a high negative correlation value for oxalic acid necrosis, progeny means, and mother blight resistance. As the mother's blight resistance increases, the generation's progeny means for necrosis in the leaf soak decrease. More resistance to blight indicates more resistance to oxalic acid. SSA correlation values are lower than the OA leaf necrosis, so there may be outside interferences on the alternative small stem assay experiment, or the OA necrosis assay may be a better predictor for mother blight resistance.

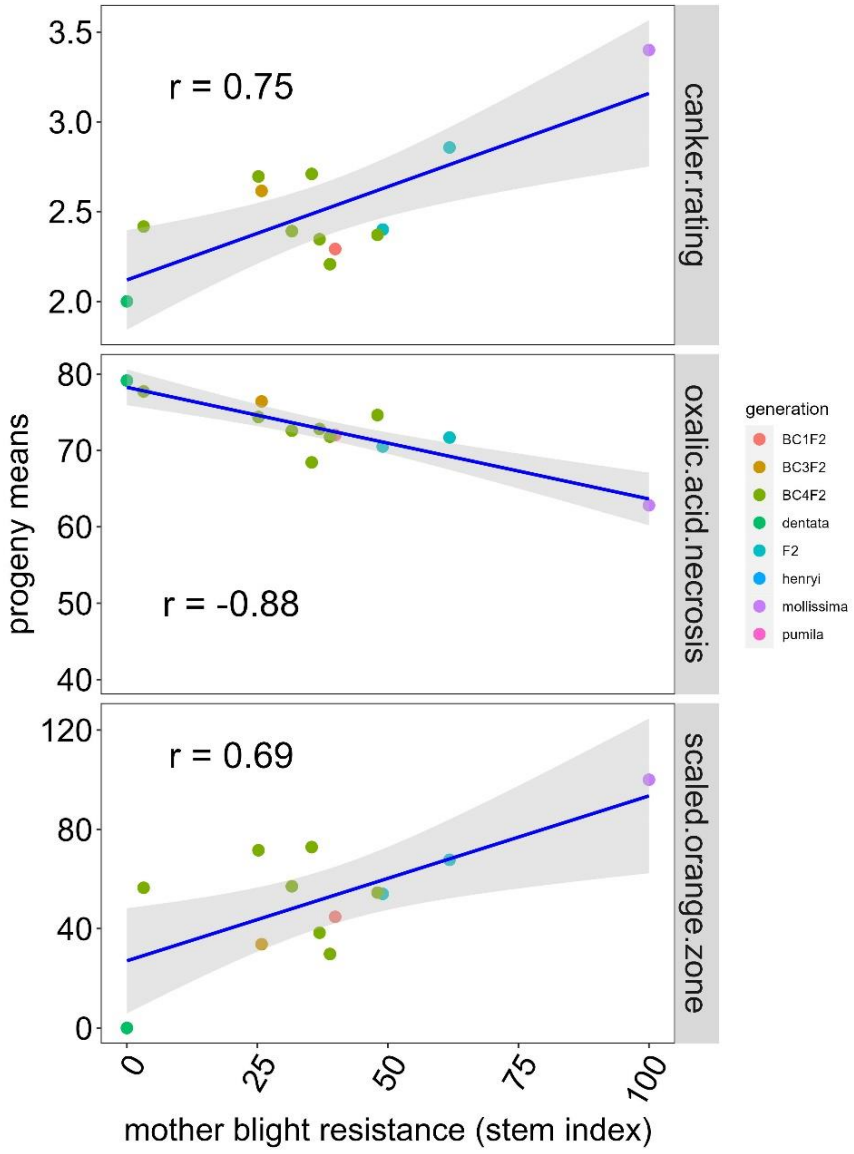

Figure 4. Graph for Mother Blight Resistance vs. Progeny means and their correlation with SSA (canker.rating and scaled.orange.zone) and OA leaf disk assay (oxalic.acid.necrosis).

Figure 5 is looking at the mother's *C. dentata* ancestry and progeny means. As the mother's *C. dentata* ancestry increases, families lose tolerance to oxalic acid, further supporting the idea that the resistance is polygenic.

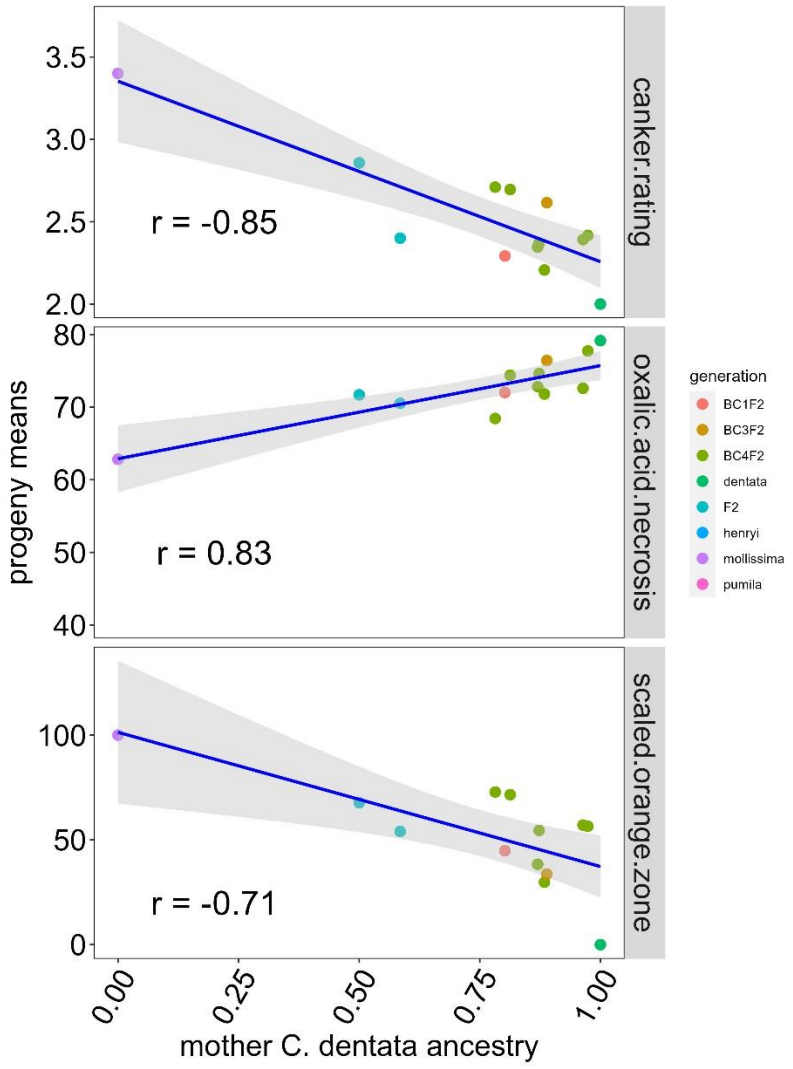

Figure 5. Mother *C. dentata* ancestry vs. Progeny Means and their correlations with the SSA (canker.rating and scaled.orange.zone) and OA leaf disk assay (oxalic.acid.necrosis).

Duncan's range test was used in Figure 6 for all families used in the SSA and in the OA leaf soak. Interestingly, *C. henryi* is not statistically different from *C. mollissima*, but *C. pumila* is. Each generation is color-coded, which is not included in my original statistical analysis. The statistical differences between SSA and the OA leaf soak are different, which is interesting. It was concluded by the SSA that the scaled orange zone was an ineffective measurement in screening for blight resistance.

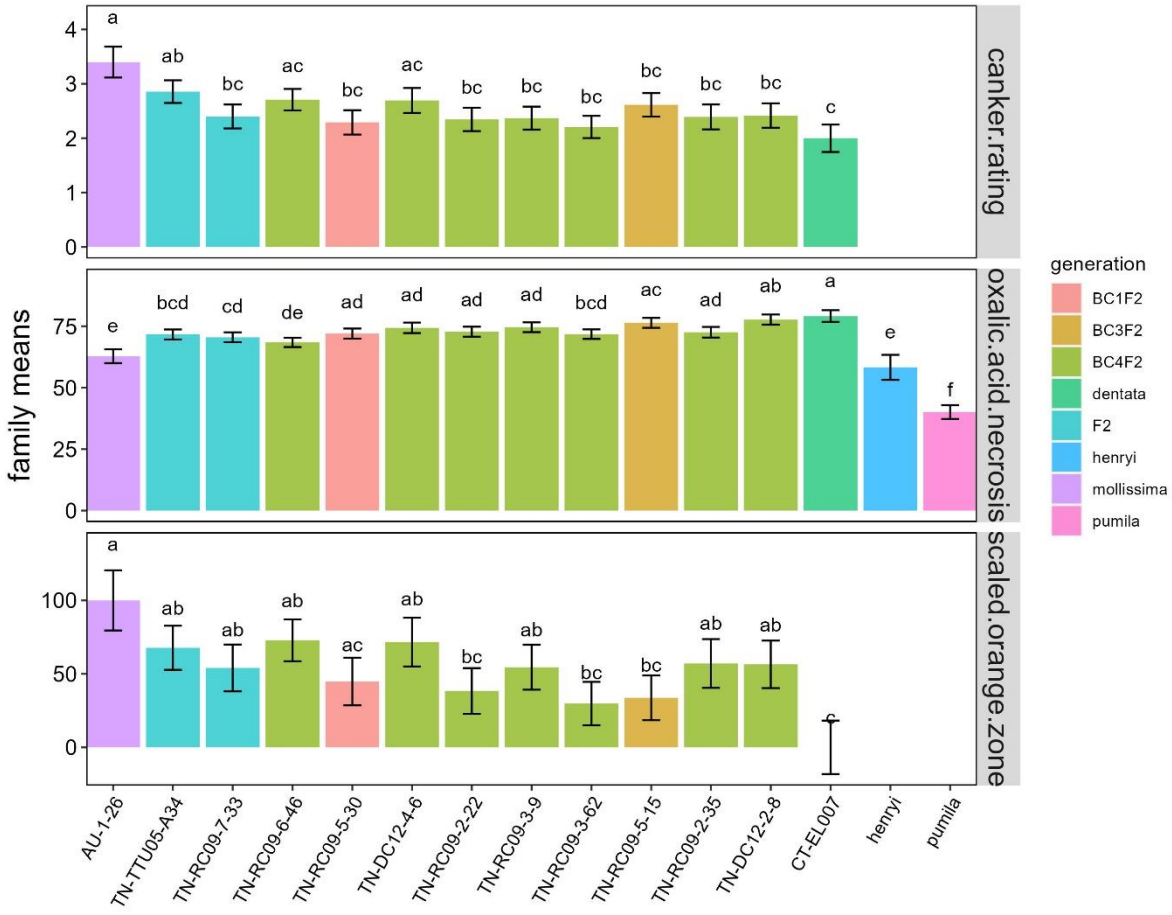

Figure 6. Duncan's Range Test with SSA (canker.rating and scaled.orange.zone) and OA leaf disk assay (oxalic.acid.necrosis).

Due to the strong correlation between the small stem assay and oxalic acid leaf disk assay, they both might be comparable screening methods for blight. This is seen in Figure 7. A high mean canker rating would indicate most resistance to blight according to the graph. So, as mean canker rating increases (blight resistance increases), then OA necrosis decreases.

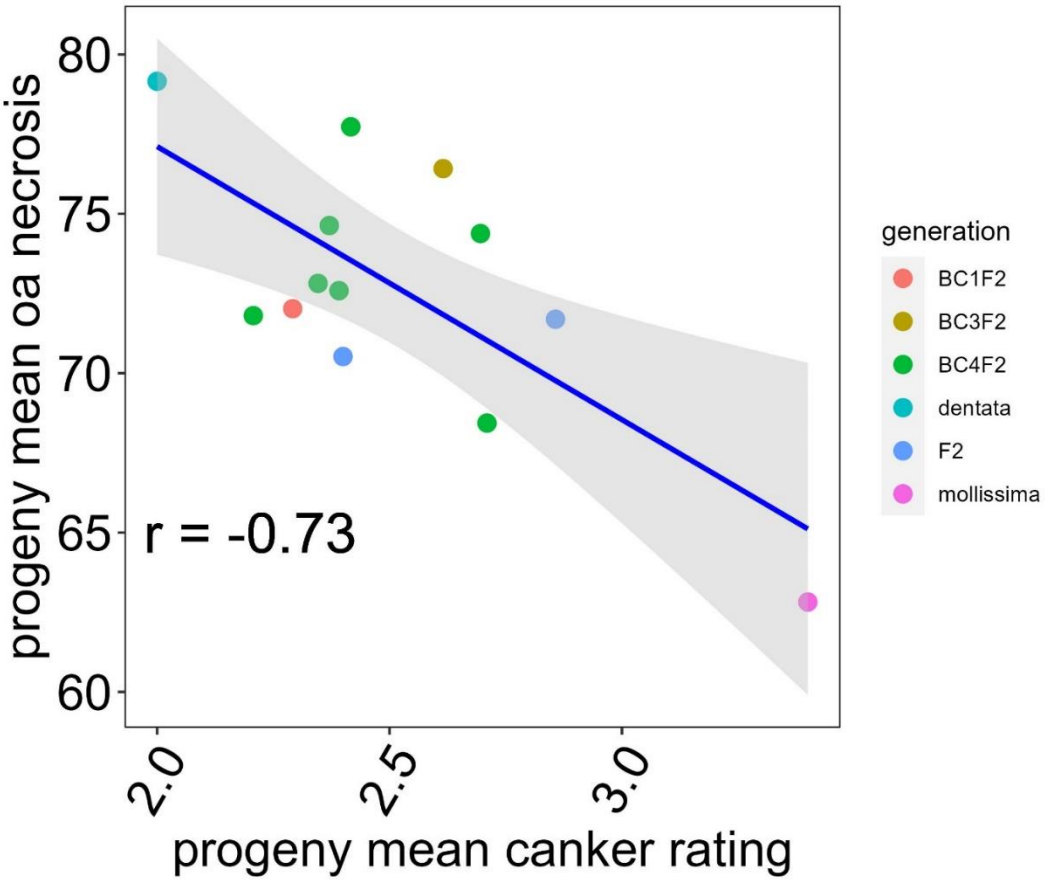

Figure 7. Correlation between progeny mean canker rating and progeny mean oa necrosis in TN 2022 SSA. Created with ggplot2 and dplyr (Wickham, 2016; Wickham, 2023).

Conclusion

Based on Table 4, the best hybrid families would be TN-RC09-6-46 (BC4F2), mean of 68.4% browning, and TN-RC09-7-33 (F2), with a mean of 70.5% browning. To reiterate, the *C. mollissima* control had an average of 63.0, and *C. dentata* had a mean of 79.2% browning. The family that performed the worst, or most like *C. dentata* was TN-DC12-2-8 with a mean browning area of 77.7% browning.

In 2023, another student will continue both the small stem assay and OA leaf disk assay on an even larger sample of trees. Further studies about correlations between the two methods of

screening will be made. Because of my unusual results with *C. pumila*, the 2023 trial will also include chinquapins. So hopefully, further conclusions about the chinquapins will be made in the next year regarding both their oxalic acid tolerance and blight resistance.

Results may be more significant if sample sizes are increased. Changing the number of disks used might be another issue. Another consideration could be using different parts of the plant. However, this method is still new and should be considered alongside the small stem assay for comparison.

The small stem assay and oxalic acid leaf disk assay appear similar in their statistical validity in distinguishing blight tolerance. It could be used to detect the absolute best and worst family within the hybrids, but not much else. These trees will be planted in the orchard in Moore County for long-term observations. In 4-5 years, they will be reinfected with *C. parasitica* to check their blight resistance. Then, these tests can be further compared to see if they are true early screening methods for blight resistance.

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