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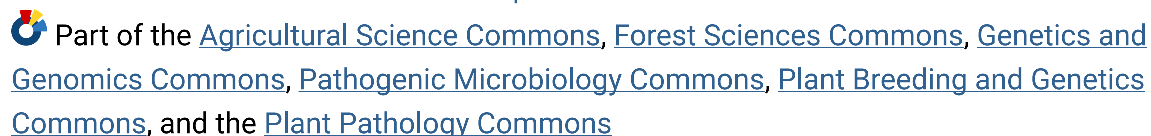
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PHENOTYPIC STUDY OF ANTHRACNOSE RESISTANCE IN BLACK WALNUT AND BUILDING A MAPPING POPULATION

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Plant Science

By

Sadie Land

December 2019

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PHENOTYPIC STUDY OF ANTHRACNOSE RESISTANCE IN BLACK WALNUT AND BUILDING A MAPPING POPULATION

Environmental Plant Science and Natural Resources

Missouri State University, December 2019

Master of Science

Sadie Land

ABSTRACT

Black walnut anthracnose, caused by *Gnomonia leptostyla*, is the most widespread and destructive disease affecting black walnut trees (*Juglans nigra*). Breeding cultivars for a higher resistance to anthracnose is a natural and efficient strategy for improving the health and production quality of black walnut trees. The two goals of this study were to reveal that the 'Sparrow' cultivar of black walnut contains a significantly higher resistance to anthracnose than the 'Football' cultivar when separated from environmental factors, and to expand the 'Football' × 'Sparrow' F_1 mapping population to evaluate how the trait of resistance is inherited in the progeny. A phenotypic assay was conducted under laboratory conditions using an ascospore inoculum to study the difference in symptom severity between two cultivars of black walnut named 'Football' and 'Sparrow.' The fungal suspension was used to artificially infect healthy 'Football' and 'Sparrow' leaflets to measure and compare the symptomatic responses over the course of thirteen days. The difference between the two cultivars was significant at eleven days post-inoculation with a p-value < 0.0001 as the leaflets from 'Football' averaged 15.1% diseased surface area while the 'Sparrow' leaflets had an average of only 1.2% diseased surface area. However, the most significant difference was seen at thirteen days post-inoculation as the 'Football' leaflets averaged 31.5% diseased surface area and the 'Sparrow' leaflets had an average of only 3.2%. The difference in susceptibility between the two cultivars confirmed a significant resistance to anthracnose in the 'Sparrow' cultivar compared to the 'Football' cultivar. To increase the F_1 mapping population, hybrid identification was performed utilizing DNA markers which resulted in a total of 52 hybrids added to the mapping population in 2019. A preliminary phenotyping procedure was applied to the existing 'Football' × 'Sparrow' F_1 mapping population which provided promising segregation for the trait of resistance to anthracnose.

KEYWORDS: black walnut, anthracnose, *Gnomonia leptostyla*, cultivar, resistance

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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INTRODUCTION

Background

The *Juglans* genus, more commonly known as walnut, is one of the world's most important nut crops as both its fruit and wood hold high commercial value. In total, over 800,000 tons of walnuts are produced annually (Tulecke et al. 1995). *Juglans* consists of 16 different species that are separated into four major generic sections—*Dioscaryon*, *Rhysocaryon, Cardiocaryon*, and *Trachycaryon*—by their leaf and flower morphologies. *Dioscaryon* (traditional *Juglans*) includes only *J. regia* also known as the English or Persian walnut. *Rhysocaryon* (black walnuts) includes *J. major*, J. *microcarpa*, *J. nigra*, *J. californica*, *J. hindsii*, *J. australis*, *J. boliviana*, *J. neotropica*, *J. guatemalensis*, and *J. olanchana*. *Cardiocaryon* (Asian butternuts) includes *J. ailantifolia*, *J. mandshurica*, and two varieties of *J. cathayensis*. The final section, *Trachycaryon* (American butternuts), includes only *J. cinerea.* (Stanford et al. 2000).

Of the *Juglans* genus, *J. nigra* (of section Rhysocaryon) is the most important walnut tree in the state of Missouri. The black walnut is the state tree nut of Missouri and is native to the central and eastern areas of the United States. This species is sensitive to soil conditions, but it grows best in the well-drained, deep, and fertile soils in northern Missouri (Nelson 2011). Depending on the location, it flowers and produces leaves around the same time from early to mid-April, and the nuts become ripe and drop soon after natural abscission of the leaves in the end of September to mid-October (Burns and Honkala 1990). Black walnuts are wind-pollinated and usually grow as scattered individual trees but can be found in small groups in the wild. They have highly valued timber and high-quality, edible nuts that are in high demand for baked goods and ice cream (Burns and Honkala 1990; Hilgedick 2017; Zhao et al. 2017). In comparison to the

English walnut (*J. regia*), black walnuts have a more intense and acquired taste. English walnuts also have a thinner shell and larger kernel, while black walnuts have a smaller kernel and are difficult to crack due to the thicker shell (Nelson 2011; Zhao et al. 2017). Black walnut can also have variable annual yield, which means that the production of the trees can vary year to year (Nelson 2011).

Economic Importance of Black Walnut

Missouri is the world's leading producer of black walnuts with approximately 32,000 tons harvested in the state each year comprising about 70 percent of the world's wild harvest (Nelson 2011; Hammons et al. 2015). Approximately 97 million wild black walnut trees exist in the state of Missouri, and the timber and nuts sell for high dollar starting at $700-$800 for a quality tree with at least a 60-inch circumference and around $9.00-$15.00 per one hundred pounds for nuts (Warmund 2009; Main 2013; Hammons 2019; Schneider et al. 2019). Most black walnuts are wild species, and wild walnuts vary in quality, yield, and moisture, which reduces the number of salable nuts produced from these wild varieties (Hammons et al. 2015; Schneider et al. 2019). Also, not many improved varieties currently exist, which means that there is a lot of room for improvement for black walnut commercial production (Schneider et al. 2019). There is great potential to improve the production quality and health of black walnut trees by using the strategy of selective breeding. As black walnut is an economically valuable dry fruit crop, growers are interested in improved varieties bred for specific favorable traits. These improved varieties are also known as cultivars or cultivated varieties (Hammons et al. 2015; Hassan et al. 2017). Some of the more favorable traits for black walnut include higher percent kernel yield, higher nut quality, thinner shells, later leafing date, and disease resistance.

Cultivated varieties of black walnut that contain more favorable traits make the prices for these varieties much higher. Walnuts from improved varieties are purchased at between $75.00-$92.00 per one hundred pounds, which averages about 7% profit increase compared to the profit from wild species (Hammons Products Company 2016; Schneider et al. 2019). This means that growers of black walnut can receive more for nuts that contain these desirable characteristics as high yield of good quality nuts is the key to profitable nut crops (Reid 1990; Hammons et al. 2015). However, one of the most important traits desired in improved cultivars is disease resistance. The expected yield of improved varieties can be inconsequential if the trees are consistently infected and weakened by disease. Therefore, breeding black walnuts with a high resistance to disease is an extremely important goal and highly desired trait.

The Problem

Anthracnose. Nearly all black walnut trees are infected with anthracnose disease—one of the most destructive diseases in the world affecting black walnut—every year (Matteoni and Neely 1979; Cline and Neely 1983). Of the *Juglans* genus, black walnut is the most susceptible to anthracnose disease, comparatively (Black and Neely 1978). Black walnut anthracnose is a disease caused by the ascomycetous fungus, *Gnomonia leptostyla,* which spreads its spores through water splash. The anamorph—asexual reproductive form—of *G. leptostyla* is known as *Neomarssoniella juglandis,* and it is recognized as the conidia of this fungus. When a black walnut tree is infected with anthracnose, black spots containing acervuli (fungal fruiting bodies) appear on the leaflets. These acervuli produce colorless, crescent-shaped conidia (*N. juglandis*) that can be separated from the leaflet and viewed underneath a microscope. The ascospores serve as the initial inoculum because the asci containing the spores are dislodged from the perithecia to

allow for spreading and growing. The ascospores cause the primary lesions (leaf spots) followed a few weeks later by a secondary cycle of infection spread by the conidia (Kessler 1984; Karov et al. 2014).

Black walnut anthracnose attacks the leaves, new shoots, and nuts after prolonged periods of leaf wetness (Kessler 1984; Reid 1990). The symptoms of black walnut anthracnose disease include brown to black slightly-sunken spots with irregular margins on the leaves and nuts followed by yellowing of the leaflets (Hassan et al. 2017). Upon early infection of a tree, the nuts can become deformed in development which can lead to poor kernel fill and unhealthy walnut production (Reid 1986; Karov et al. 2014). If a tree is highly susceptible to the disease, early defoliation is likely to follow the yellowing of the leaflets. Repeated defoliation due to anthracnose can have harmful effects on the growth rate of the plants (Woeste and Beineke 2001). Early defoliation can also move a tree toward the natural tendency to alternate bearing, which would decrease the production yield every other year (Reid et al. 2004). Although black walnut anthracnose is not lethal the first time a tree is infected with it, once a tree is infected with anthracnose, every year the tree will become significantly weaker allowing the tree to become susceptible to attack from other diseases, which can likely lead to the death of the tree, or cultivar altogether (Burns and Honkala 1990). In addition, most commercial plantations for black walnut are structured as monocultures, which can increase the probability of epidemic spread of foliar disease leading to a rapid decrease in tree growth, tree health, and commercial productivity (Cline and Neely 1983).

Strategy and Reasoning. Immunity to anthracnose has not yet been identified for black walnut, therefore, it is integral for natural defense strategies to be implemented as continued propagation of susceptible cultivars will only lead to a decrease in production yield and an

increase in production costs (Reid et al. 2004). However, there is much genetic potential for black walnut commercial production because anthracnose resistance heritability is high, which means that this trait can be passed down genetically and allow for resistant strains to this destructive disease to be generated (Beineke and Masters 1973; Reid 1990).

Previous phenotypic studies expressed that some cultivars of black walnut have shown greater resistance to anthracnose including 'Thomas,' 'Ohio,' and 'Sparrow' while cultivars including 'Surprise' and 'Football' have shown high susceptibility to the disease (Reid 1990; Reid et al. 2004). Also, varieties that are highly susceptible to anthracnose and have an alternate bearing pattern—like the cultivar 'Football'—would have no hope in developing a more steady annual bearing pattern unless the variety was bred with another variety that has a more steady annual production yield along with a higher resistance to anthracnose—like the 'Sparrow' cultivar (Reid et al. 2004). Therefore, incorporating breeding strategies is an important factor in producing improved cultivars of black walnut.

The two parent cultivars chosen for this project are named 'Football' and 'Sparrow,' and they show a visible difference in susceptibility to anthracnose disease based on past research and field observation. Figure 1 shows images of 'Football' and 'Sparrow' trees on the same day in the middle of August of 2018 after both cultivars have been heavily infected with anthracnose. In this figure, both cultivars have symptoms of the disease, however, 'Football' shows a significant amount of leaf drop while 'Sparrow' has maintained most of its leaves. These parents were chosen for a variety of complementary traits (Table 1) and for their high genetic dissimilarity (Schneider et al. 2019). The complementary traits and genetic diversity between 'Football' and 'Sparrow' suggest that the two cultivars would serve as excellent parents for the potential hybrid

progeny (Schneider et al. 2019). This specific project, however, focusses primarily on the high resistance to anthracnose that the ‘Sparrow’ cultivar displays in the field (Reid et al. 2004).

Based on the observations that some cultivars are more resilient to anthracnose than others, and that heritability of this phenotypic trait is high, it can be hypothesized that improved cultivars of black walnut can be bred for a high resistance to anthracnose in the hybrid progeny (Reid 1990; Woeste and Beineke 2001). If the leaflet surface area is observed and measured for percentage of disease after infection, then it would be expected that the leaflets of ‘Sparrow’ would reveal a higher resistance to the disease by showing a significantly lower percentage of diseased leaflet surface area compared to the ‘Football’ leaflets. It would also be expected that the resistance to anthracnose will be inherited by the ‘Football’ × ‘Sparrow’ progeny.

Purpose and Objective

There are two main objectives for this study: the first objective is to show that the ‘Sparrow’ cultivar of black walnut reveals a significantly higher resistance to anthracnose disease than the ‘Football’ cultivar without environmental factors involved, and the second objective is to expand the ‘Football’ × ‘Sparrow’ hybrid mapping population to study the inheritance of this trait of high resistance in the progeny.

Review of Previous Works

Details of Causal Pathogen. A phenotypic evaluation of anthracnose disease was performed on English walnut (*J. regia*) to identify the pathogen leading to the disease (Hassan et al. 2017). For this evaluation, four leaf-bearing branches from three individual walnut trees grown in natural conditions were tagged to be observed for the first appearance of symptoms of

anthracnose as well as shape and color of lesions. The diseased leaves and twigs were further evaluated in the laboratory to identify the pathogen associated with anthracnose. The leaf and twig samples were incubated at 21°C for 36 hours to allow for the acervuli to bulge so that they could be sampled from the leaves and twigs and viewed under the microscope with lactophenol cotton blue (LCB) stain. The acervuli and conidia were then examined and recorded for their color, shape, size, and septation. The morphological characteristics of the fungal pathogen causing anthracnose were studied on both the host and as a culture in the lab. The colony morphology of this causal organism on potato dextrose agar (PDA) media revealed that at 15 days old, the colony was white, circular in shape with whitish aerial tufts of mycelium in structure, and approximately 10-12.5 mm in diameter. At 30 days old, the colony grew to 23-24.4 mm in diameter, slightly changing color to a creamy white color. Finally, at 40 days old, the colony grew to a total of 44.5-45 mm in diameter and changed to a greyish-white color with concentric zones and well-defined lobate margins. This research also revealed information about the hyphae, conidia, and acervuli shape, structure, color, size, and septation (Hassan et al. 2017). From the leaf lesion samples, hyphae and conidia were both identified as hyaline in color as the acervuli were pitch dark. The hyphae were septate and had a smooth, branched structure. The conidia were crescent-shaped, bi-celled, with a singular septum sizing at 20-32µm (Hassan et al. 2017). These descriptions of the colony morphology and structure, color, and size of the acervuli and conidia will be used for identification of the *G. leptostyla* strain.

Hassan and Ahmad in 2017 recorded the asci structure to be 8-spored, straight to slightly-curved, and septate. The ascospores contained within the asci are the primary inoculum that performs the initial infection of the tree along with conidia produced from the acervuli. This initial infection is followed by a secondary cycle of spreading by only the conidia (Hassan and

Ahmad 2017). Hassan and Ahmad also mentioned previous studies which observed that the asci begin to form naturally in late February with ascospores developed in March. According to the observations from this study, the fungus then enters an incubation period that lasts up to 5 weeks. After the first symptoms appear on the leaflets, the conidia are developed within the acervuli and the secondary cycle of infection continues throughout the end of autumn (Hassan and Ahmad 2017). This information illustrates the life cycle of *G. leptostyla* and *N. juglandis*.

For growth of this strain of anthracnose in the lab, the best growing conditions must be provided for the fungus to keep it alive. A study was performed to determine the best nutrient media for growth and sporulation of *G. leptostyla* (Jamshidi et al. 2007). In this study, they tested the fungal growth of *G. leptostyla* on potato dextrose agar (PDA), nutrient agar (NA), malt agar (MA), corn-meal agar (CMA), water agar (WA), oat-meal agar (OMA), walnut leaf extract agar (WLEA), walnut leaf extract oat-meal agar (WLEOMA), and blood agar (BAB), and measured the maximum colony diameter of the *G. leptostyla* isolates every few days for up to 21 days. Incubation was conducted at 21°C with a 12-hour photoperiod and 30% relative humidity. After 48 hours, mycelial growth occurred on all nutrient media except for the BAB. *G. leptostyla* grew significantly faster on the OMA, WLEOMA, WLEA, and CMA. The OMA media demonstrated the fastest growth as the fungus completely covered half of the petri dish after 96 hours and the entire plate in two weeks (Jamshidi et al. 2007). Thus, OMA media was utilized in the process of growing and maintaining *G. leptostyla* in the lab for this project. PDA media was also used in the process of isolating *G. leptostyla* to see if its slower fungal growth (about half the growth rate of the OMA media) on this type of media was more favorable.

Differences in Susceptibility. Walnut anthracnose is the most widespread and important foliar disease in black walnut trees (Woeste and Beineke 2001). Although no specific genotype

has been discovered to be entirely resistant to this disease, it has been recognized that there are differences in susceptibility among different black walnut varieties. Woeste and Beineke gathered 42 black walnut clones that were selected from wild populations in the midwestern United States, and then grafted the clones onto seedling rootstock and planted them. For ten years, these 42 clones were evaluated and rated for symptoms of anthracnose infection, and there were visible differences in susceptibility to anthracnose among the clones (Woeste and Beineke 2001). This study was purely phenotypic based on a visual scale among trees maintained in a field, but it also initiated questions about how these phenotypic differences in susceptibility relay back to the genotypes of these individuals.

Figure 1: Comparison of the Two Parent Cultivars in the Field. The ‘Football’ cultivar is shown in the left image, and the ‘Sparrow’ cultivar is shown in the right image.

Table 1: Comparison of Phenotypic Traits in 'Football' and 'Sparrow' (Reid et al. 2004; Schneider et al. 2019).

Trait	Football	Sparrow
Leafing date	11.0 ± 2.1 DAD	16.8 ± 3.4 DAD*
Flowering type	protandrous	protogynous
Fruit-bearing	alternate	annual*
Spur-fruiting	yes*	no
Percent kernel	~30	>30*
Nut weight (g)	>20 g*	~20 g
Hull	thick	thin*
Wood strength	strong*	weak
Anthracnose susceptibility	high	low*

*agriculturally favorable trait

MATERIALS AND METHODS

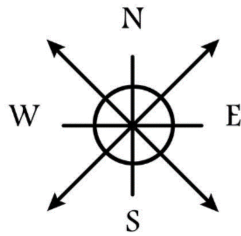
Study Area and Plant Material

Artificial Inoculation. The leaflets used for the experiments in this study were randomly sampled from all the ‘Football’ and ‘Sparrow’ parent trees located at the Missouri State Fruit Experiment Station (MSFES) in Mountain Grove, Missouri 65711. In the walnut orchard at the MSFES, there are four ‘Football’ trees and three ‘Sparrow’ trees spread throughout an orchard of 37 trees consisting of ten total cultivars of black walnut with fifty feet between-row and fifty feet within-row spacing, latitude 37.19749, -93.27925 longitude (Figure 2). The 37 trees were grown from cuttings that were provided by the University of Missouri’s Southwest Research Center (MUSRC) in Mt. Vernon, Missouri 65712.

Mapping Population. The walnut trees used for nut collection and hybrid identification of the ‘Football’ × ‘Sparrow’ F_1 progeny are located at the MUSRC (Figure 3). The black walnut orchard at the MUSRC contains a variety of cultivars; however, the walnuts used for hybrid identification purposes were collected from a row of three ‘Football’ trees. The three target ‘Football’ trees used for nut collection were positioned with four rows of ‘Sparrow’ trees (the intended pollinators) to the east and one row of ‘Sparrow’ to the west of the ‘Football’ row.

Although the plant materials used in this study were maintained at these two locations, most of the experiments took place in a controlled environment in the Molecular Breeding laboratory at Missouri State University in Springfield, Missouri 65897.

Figure 2. Map of Black Walnut Plot at the MSFES. The seven total parent cultivars (four 'Football' and three 'Sparrow') used in this study were located within the three rows of black walnut cultivars circled on the map.



ROW 7	ROW 8	ROW 9	ROW 10	ROW 11	ROW 12
			Shepard		
			Crosby		
			Cranz		
			Ohio		
		McGinnis	Pounds 2		
		South Fork	Ogden		
		Ridgeway	Abraham		
		Sparks 127	Hay #2		
	Thomas Meyer	Sauber 1	Sparks 147	Tomboy	Vander Sloot
	Thomas	Vander Sloot	Cutleaf - Shaw	Tomboy	Surprise
	Brown Nugget	Thomas	Lamb	Sparrow	Kwikkrop
Sauber 2	Mystery	Ohio	Sparks 147	Sparrow	Kwikkrop
DuBoise 8802	Ogden	Neel	Neel	Emma K	Football
Sparrow	Sparrow	Sparrow	Sparrow	Emma K	Football
Sparrow	Sparrow	Sparrow	Sparrow	Surprise	Tomboy
Sparrow	Sparrow	Sparrow	Sparrow	Surprise	Tomboy
Sparrow	Sparrow	Sparrow	Sparrow	Football	Sparrow
Sparrow	Sparrow	Sparrow	Sparrow	Football	Sparrow
Sparrow	Sparrow	Sparrow	Sparrow	Football	Sparrow

Figure 3. Map of Parent Black Walnut Cultivars at the MUSRC. Rows 7-10 of 'Sparrow' trees are positioned to the west of the 'Football' Row 11, and the final row of 'Sparrow' trees, Row 12, located to the east of the 'Football' row. Other black walnut cultivars were planted in the orchard, but the focus area remains within the first three trees of Rows 7-12 containing only 'Football' and 'Sparrow' trees. The three highlighted 'Football' trees were the individuals from which the walnuts were collected.

Experimental Design for Artificial Inoculation

The overall design for artificial inoculation began with obtaining anthracnose-infected black walnut leaf tissue, followed by the growth and maintenance of *G. leptostyla* in the lab, identification and isolation of the fungal strain, detached-leaflet artificial inoculation of the parent black walnut trees, and phenotypic analysis.

Identification and Isolation of *G. leptostyla*. To begin, fresh leaf tissue from anthracnose-infected leaflets was obtained from the parent plot at the MSFES in the middle of June in 2018. The black walnut trees in the orchard were evaluated for signs of infection: brown-black spots on the leaflets along with yellowing around the spots. Five infected leaflets were gathered for the isolation process for *G. leptostyla* in the lab. Two types of media were poured into sterile petri dishes and prepared in the lab for the fungal isolation process: PDA (39g/L; Cole-Parmer, Vernon Hills, IL) and OMA (72.5g/L; Difco, Sparks, MD). The infected leaflet tissue was cut with a sterile scalpel into small 1 cm size pieces containing acervuli and plated onto both types of media plates followed by incubation (Heratherm incubator, Thermo Scientific) at 22°C for 72 hours or until significant growth of various fungi and bacteria was visible (Figure 4). The OMA media plates showed more growth at a much faster rate than the growth on the PDA plates—approximately double the growth and rate of growth of the PDA media plates—which was more favorable for the purpose of separating the different strains from each other. Thus, only the OMA was the media type selected for moving forward in this project.

After the growth of various fungi and bacteria appeared on the OMA media plates, the visibly different individual strains of fungi were separated from each other and placed onto small, ½ inch squares of OMA media located on clean microscope slides and then covered with a 20 X 20 mm cover slip (Figure 5). This technique was demonstrated by Yuri (2011); it conserves

the amount of media used while maintaining a pure culture environment and makes it easier to view the different fungi on a microscope slide. These microscope slides prepared with the various unidentified fungal strains were incubated at 22°C for 24-72 hours which allowed for the individual colonies to grow onto the media as well as the coverslip (Yuri 2011). Once a significant amount of growth appeared on the individual pure culture OMA squares, each coverslip was removed and transferred onto a clean microscope slide with LCB stain (Yuri 2011), a common stain used to observe fungi under microscopic view. The phenol in the LCB stain kills any live organisms, the lactic acid preserves the fungus, and the cotton blue stains the chitin within the fungal cell walls (Leck 1999).

To identify *G. leptostyla*, each individual colony pure culture was observed under a stereoscopic light microscope (Leica DME; North Central Instruments) up to 400X magnification and compared to structural and morphological descriptions in previous research (Jamshidi et al. 2007; Hassan and Ahmad 2017; Hassan et al. 2017). *G. leptostyla* grown on an OMA media plate under the conditions chosen for this experiment reveals LCB-stained ascospores at 400X magnification. When *G. leptostyla* was identified from an OMA square, an isolate from the square was transferred onto a new OMA media plate to grow a pure culture of only *G. leptostyla*. After the pure culture of *G. leptostyla* was achieved, the strain was kept alive in the incubator for use in the artificial inoculation procedure. To ensure that the pure culture fungus grown in the lab was the same fungus producing symptoms of anthracnose in black walnut, artificial inoculation of healthy leaflets was performed with the pure culture obtained in the lab to confirm that this strain would lead to the same symptoms of anthracnose seen in the field.

Figure 4: Growth of Various Fungi and Bacteria on OMA Media.

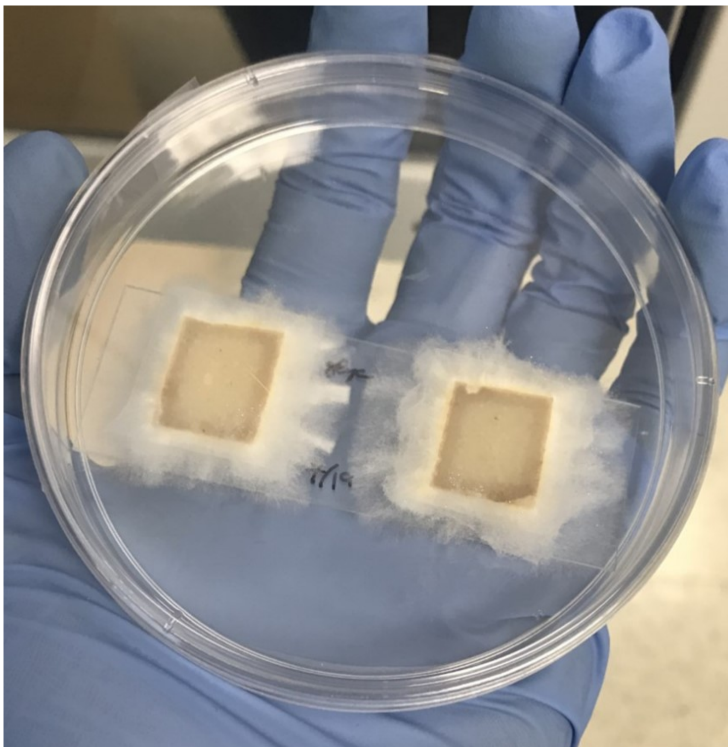


Figure 5: Yuri's Technique Used for the Isolation and Identification of *G. leptostyla*.

Preservation Methods for *G. leptostyla.* To preserve the pure culture of *G. leptostyla* for future use, two different methods of preservation for long-term storage were utilized. The first preservation method was performed by drying the pure culture onto fast flow rate filter papers (11.0 cm diameter; Fisher Scientific) (Morales 2008). To achieve this, autoclaved filter papers cut into 2 cm squares were placed on OMA media plates. Samples of mycelia from the pure culture of *G. leptostyla* were placed in the center of each filter paper on the OMA media. The plates were then sealed with parafilm and incubated at 22°C for 48-72 hours. After incubation, *G. leptosyla* grew onto each filter paper square, usually covering it entirely with the fungus (Figure 6). If the filter papers were not completely covered, they were allowed one more day in incubation. When the filter papers were covered with the fungus, they were removed from the OMA media plates and transferred into clean petri dishes on autoclaved wax paper. These petri dishes containing the fungus-covered filter papers were incubated slightly ajar at 22°C for nine to twelve hours to allow the fungus to dry. The drying step is crucial for proper preservation of the fungus because if it is dried too quickly, the fungus will die, and if it is dried too slowly, contamination can occur. After the fungus-covered filter papers were completely dried, they were wrapped in autoclaved wax paper and then wrapped in foil to store at 4°C or -20°C for five to ten years (Morales 2008). The second preservation method used was fungal preservation in 1mL of 4M glycerol in sterile Eppendorf tubes. For each 1mL of glycerol, sterilized tweezers were used to submerge mycelia from the lab-grown fungus into the glycerol followed by vortexing and storage at -80°C.

The first samples of fungus dried on filter papers or suspended in glycerol were stored in early July of 2018. As live growth of *G. leptostyla* was maintained in the lab throughout the duration this project, many samples were dried on filter papers or stored in glycerol for future

use. The samples of the dried fungus stored in July of 2018 at 4°C and -20°C along with the fungus stored in glycerol at -80°C were plated on fresh OMA media one year after storage to observe if and how the fungus grew after this prolonged period of storage using the various preservation methods.

Figure 6. *G. leptostyla* Grown on Filter Papers for Storage. Autoclaved filter papers cut into small, 2 cm squares positioned on freshly poured OMA media for samples of *G. leptostyla* to be grown. After 72 hours, all filter papers should be completely covered with the pure *G. leptostyla* isolates.

Artificial Inoculation of Healthy Leaflets. One hundred and twenty fresh leaflets showing no signs of infection were randomly sampled on July 8th, 2019 from the parent cultivars including samples from four 'Football' trees and three 'Sparrow' trees located at the MSFES to begin the artificial inoculation process using the isolated *G. leptostyla* strain grown in the lab. To produce the ascospores for use in artificial inoculation, the culture of *G. leptostyla* was grown on OMA media at 22°C in an incubator for up to 96 hours. The fungal solution used as the inoculum was generated by submerging four-day-old mycelia in a 1.5 mL Eppendorf tube filled with sterilized diH2O followed by vortexing. A hemocytometer was used underneath a microscope lens to measure the density of the fungal suspension and adjust the concentration to 700 ascospores/µL.

Artificial inoculation of sixty healthy 'Football' leaflets and sixty healthy 'Sparrow' leaflets was performed using the fungal solution generated in the lab. The one hundred and twenty leaflets plus controls from each parent were surface-sterilized by rinsing with diH2O then placed for ten seconds in 10% bleach followed by a final thorough rinsing in diH2O. After the leaflets dried, they were placed individually in sterilized petri dishes, abaxial surface facing up on a damp, autoclaved paper towel. Each leaflet was injured with a small, 1 cm incision on the midrib of the abaxial surface starting near the petiole moving toward the tip of the leaflet. According to previous research, inoculations from the abaxial surface develop ten-fold the number of lesions compared to inoculations from the adaxial surface of the leaflet (Matteoni 1977). Therefore, the leaflets were inoculated with 1 µL of the 700 ascospores/µL fungal solution into the midrib injury on the abaxial surface for maximum symptom development. The controls for each parent were treated under the same conditions only with diH2O replacing the fungal solution. Finally, the artificially inoculated leaflets along with the controls were sealed

and incubated at 22°C, and then evaluated every day for up to thirteen days after inoculation to measure the difference in susceptibility to anthracnose between both parent cultivars.

Phenotypic Analysis. For evaluation of the leaflets post-artificial inoculation, the total leaflet surface area and amount of infection was measured using WinRHIZO scanning technology (LA2400 Scanner; Regent Instruments Canada Inc. 2017) and color analysis. WinRHIZO is a software program that utilizes a scanner to perform various types of measurements including total leaflet surface area and percentage of leaflet surface area covered in disease. These measurements were obtained via color analysis using color classes defined by the user to perform precise measurements daily for each leaflet. The leaflets were first scanned as an image while remaining sealed inside their petri dishes positioned face-down on the scanner (Figure 7). Then, each day—from day zero to day thirteen—the leaflets were measured for total surface area in cm^2 and analyzed based on the different color classes on the leaflet surface area. The color classes included every shade of green or brown/black defined by the user to recognize and distinguish between the areas of healthy leaflet tissue and diseased leaflet tissue. The color classes were then designated into one of two color groups in the color analysis program: ‘Healthy’ and ‘Diseased.’ The ‘Healthy’ and ‘Diseased’ color groups were used to measure the amount of total leaflet surface area covered by the ‘Healthy’ color group along with the amount of total leaflet surface area covered by the ‘Diseased’ color group. From these measurements, the percentages of diseased leaflet surface area and healthy leaflet surface area were calculated. The WinRHIZO color analysis allowed for multiple perspectives of the color classes and color groups each time an analysis was run (Figure 8). The two perspectives that were most important for this study were the ‘Color Classes’ view and the ‘Color Groups’ view. The ‘Color Classes’ view shows all of the defined color classes from the color analysis, and the ‘Color Groups’ view

shows the two groups showing the healthy leaflet surface area against the diseased surface area. A statistical comparison between the two cultivars using the WinRHIZO measurements of diseased surface area percentage was conducted.

Statistical Analysis. An ANOVA and Tukey's pairwise comparison was run using the SAS software (SAS Institute 2013) to determine whether there was a significant difference in the symptom intensity between the two parent cultivars based on the percentages of healthy and diseased leaflet surface area. 98 leaflets were able to be statistically tested as some leaflets died in the process not due to the disease. The two comparable groups consisted of 49 'Football' leaflets and 49 'Sparrow' leaflets. An ANOVA was also performed to measure the variance between the two cultivars over the course of the thirteen days after inoculation. A Tukey's pairwise comparison was run along with the ANOVA to locate the day in which the difference in symptom severity was significant between the two parent cultivars.

Preliminary Run of Artificial Inoculation with Hybrid Progeny. Before the 'Football' × 'Sparrow' hybrids from 2019 were added to the mapping population, a preliminary run of the detached-leaflet artificial inoculation assay was performed with the thirty hybrids existing in the field at the MSFES. The healthy leaflet samples from the thirty hybrid individuals endured the same inoculation process as the parents, and they were observed up to nine days post-inoculation. The same phenotypic evaluation using WinRHIZO scanning technology and color analysis was applied to the thirty existing hybrids to measure the percent diseased leaflet surface area. Based on the WinRHIZO measurements and visual evaluations of the leaflets, each was given a score of one, two, or three to categorize the severity of symptoms among the population. A score of one represents high resistance to anthracnose or 0-12% diseased surface area, a score

of two represents average susceptibility to anthracnose or 12.1-60% diseased surface area, and a score of three represents high susceptibility to anthracnose or 60.1-100% diseased surface area.

Experimental Design for Increasing the Mapping Population

The overall design for expanding the mapping population began with obtaining walnuts from under ‘Football’ trees at the MUSRC, followed by growing the walnuts in a greenhouse at the MSFES, and hybrid identification.

Growing the Walnuts. In October of 2018, approximately 1,860 walnuts were collected from underneath the three ‘Football’ trees located at the MUSRC. Immediately after gathering, the walnuts were hulled on-site and then allowed to dry on a greenhouse bench for 48 hours. On October 23rd, after the walnuts were dry, they were buried underground for approximately 100 days for cold stratification. This process allows the walnuts to enter a period of dormancy so that they will be ready to germinate in the spring. In February of 2019—after the 100-day period—the walnuts were dug up and transferred to the MSFES where the walnuts were planted in 44-ounce Styrofoam cups and stored in a greenhouse.

The walnuts began to germinate only two weeks after planting and continued to germinate for the following three months. Five weeks after planting, approximately 35% of the 1,860 walnuts germinated. Seven weeks after planting, the germination rate increased to 54%. After approximately three months, germination had come to a halt and a total of 1,163 walnuts germinated from the 1,860 walnuts planted in the greenhouse, equaling a final germination rate of 62.5%. When the plants had five to six true leaflets, healthy young leaflets were sampled from every germinated individual for DNA isolation.

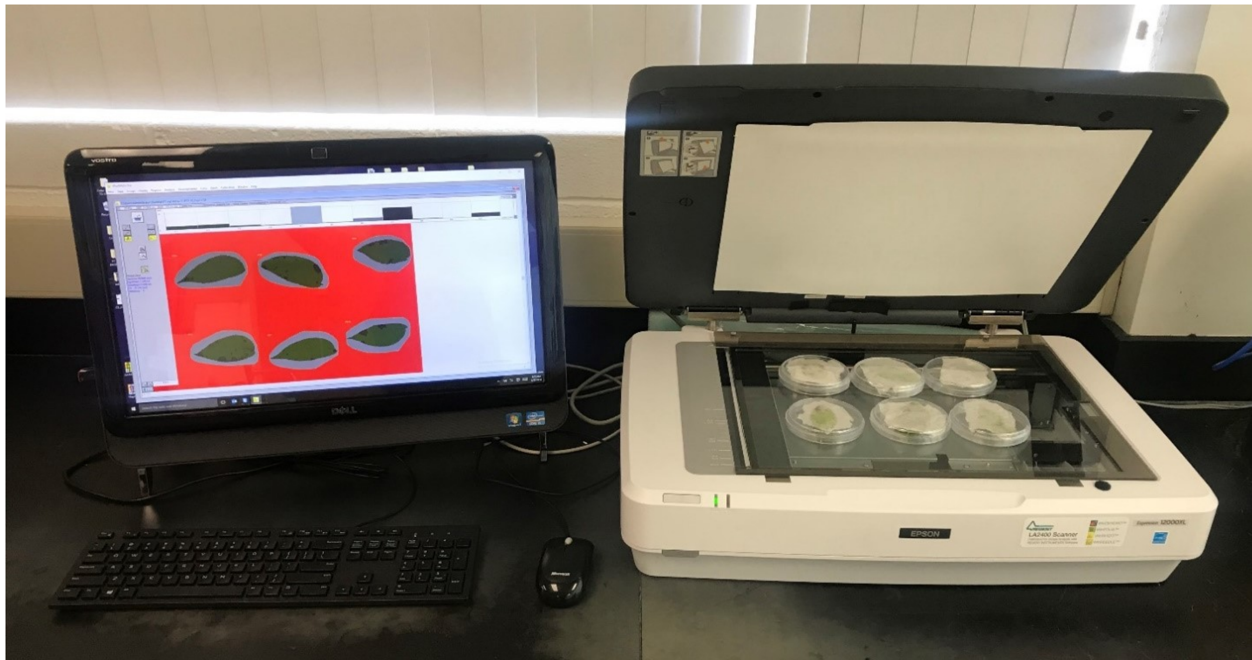


Figure 7. WinRHIZO Scanner Set Up. The WinRHIZO scanner connected to a computer shows the resulting view of the color analysis of six sealed petri dishes containing six different artificially inoculated leaflets.

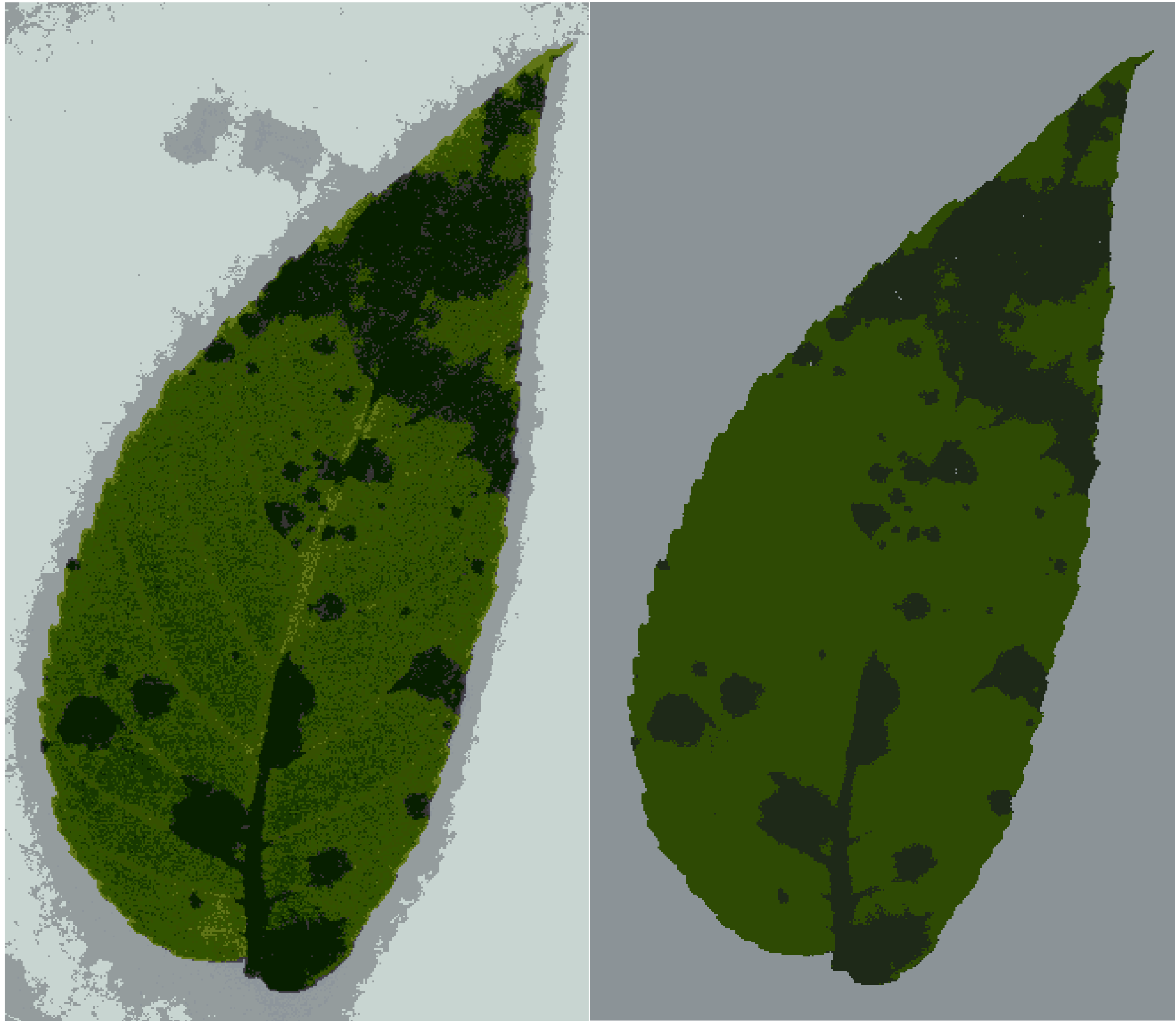


Figure 8. WinRHIZO Color Analysis Views. The image on the left shows the ‘Color Classes’ view of a scanned leaflet and the image on the right shows the ‘Color Groups’ view of the same scanned leaflet. The color analysis feature also allows for a third color group labelled ‘Background’ which can contain any shade of the background color or shadow.

DNA Isolation and Amplification. For the DNA isolation process, the Synergy 2.0 Plant DNA Extraction Kit (OPS Diagnostics) was used to isolate the DNA of all the germinated individuals. This process began with approximately 50 mg of fresh, young leaflet tissue that was then homogenized with buffer and small, metal beads followed by centrifugation to separate the supernatant containing the DNA from the pellet containing the other contaminants and debris. After the pellet was formed, the supernatant was transferred to a tube with RNase solution and incubated at 37°C to ensure that the resulting product contains only DNA. Isopropanol was then added to the solution and placed in -20°C to purify the DNA. Then, the DNA went through a process of binding to a silica filter, washing with ethanol, and eluting with diH2O which resulted in a pure DNA product. The concentration of the final DNA product was measured using a Nanodrop spectrophotometer (Nanodrop 2000; Thermo Scientific) along with the quality of the DNA for each sampled individual.

Each DNA sample isolated from the 1,163 germinated individuals was used as a template for Polymerase Chain Reaction (PCR) to replicate specific regions of the DNA—using seven DNA markers (Table 2)—for amplification. In the PCR process, an M13-tailed 5'-TGTAAAACGACGGCCAGT-3' forward primer was used along with a reverse primer. In addition, a third primer—an M13 sequence containing a WellRED fluorescent label (Sigma, St. Louis, MO)—was added to allow the PCR product to be used in capillary electrophoresis. Each reaction had a total volume of 8.4 µL which included 0.8 µL diH2O, 0.2 µL $MgCl_2$ (25 mM), 0.8 µL DNA (15 ng/µL), 0.8 µL forward primer (0.1 µM), 0.8 µL reverse primer (2 µM), 0.8 µL WellRED primer (2 µM), and 4.2 µL AmpliTAQ Gold 360 master mix polymerase (ThermoFisher Scientific, St. Louis, MO). The amplification conditions were run for 1 cycle of 10 minutes at 95°C, 40 cycles of 30 seconds at 95°C, 1 minute at 60°C, and 1 minute at 72°C,

and a final cycle of 7 minutes at 72°C followed by a holding temperature of 4°C. The PCR products were confirmed with a 1.5% agarose gel and then prepared for capillary electrophoresis using a Beckman Coulter CEQ 8000 (Beckman Coulter, Inc., Brea CA) to begin the process of hybrid identification using DNA markers for the increasing of the F_1 mapping population.

Hybrid Identification. The DNA markers used for hybrid identification are known as simple sequence repeats (SSR), which are short base-pair patterns that repeat in tandem several to many times at a specific length in the DNA sequence of an individual. These fragment lengths can differ between individuals which allows for the differentiation between cultivars using the SSR marker regions. Black walnuts have two fragment lengths for each SSR marker as they are diploid—having two sets of chromosomes. After the SSR regions were replicated and by polymerase chain reaction, capillary electrophoresis was performed to identify the exact fragment lengths of the amplified SSR marker regions of the PCR products. The amplified fragment lengths of every DNA sample were compared to the fragment lengths of the two parent cultivars to identify which individuals contained fragment lengths pertaining to the parents at that specific SSR region. A 'Football' × 'Sparrow' hybrid would show a fragment length that matches a 'Football' fragment length at that specific marker region and one that matches a 'Sparrow' fragment length. After each sample was screened with the DNA markers, the 'Football' × 'Sparrow' hybrids were separated from the total and added to the mapping population. All the sampled individuals were screened with the seven SSR markers to ensure the 'Football' × 'Sparrow' hybrids were as accurate as possible.

Table 2: Seven SSR Markers used in Hybrid Identification Procedure for 2019.

Marker	Sequence	Football	Sparrow
		Allele size	
WLLC497-J_N_32364_f	TGTAAAACGACGGCCAGTTGCTTTCTCAGAATCCAAACGC	281/283	277/279
WLLC498-J_N_32364_r	TGTGAAGGCTTGTGGATGGG		
WLLC605-J_N_29438_f	TGTAAAACGACGGCCAGTTTGTGTTTACGCAGCAAGGC	147/149	151
WLLC606-J_N_29438_r	CGTCTTCCACTCTGCATTTGC		
WLLC609-J_N_106830_f	TGTAAAACGACGGCCAGTAGAATCCAACGGTGTGCGC	158/162	148/150
WLLC610-J_N_106830_r	GCGATGGAAGCTTGGAAAGG		
WLLC697-J_N_26530_f	TGTAAAACGACGGCCAGTACATGGATGAGGGCAAGAGC	184	181
WLLC698-J_N_26530_r	AAACCCAAGACCCTCAGTGC		
WLLC831-J_N_221533_f	TGTAAAACGACGGCCAGTTCAGAGACCCAATTAGCACC	232	244/246
WLLC832-J_N_221533_r	TCGACCACGTCCTCTCTAGG		
WLLC933-J_N_31676_f	TGTAAAACGACGGCCAGTAATTTGCCTTTGGATGGCGC	262/267	264
WLLC934-J_N_31676_r	ATGCCACATGAGCCAAATCC		
WLLC1305-J_N_13427_f	TGTAAAACGACGGCCAGTACTGGAACGTCGTTTCCTGC	345	351
WLLC1306-J_N_13427_r	ATTCGTTCTTGTTGCTGGCG		

RESULTS

Identifying *G. leptostyla*

Based on previous research, the fungus isolated and cultured in the lab on the OMA media contained the ascospores of *G. leptostyla.* These ascospores contained 6-8 spores within asci that were straight to slightly-curved with tapered ends (Figure 9) (Hassan et al. 2017; Hassan and Ahmad 2017). The artificial inoculation performed with the healthy leaflets revealed that the inoculum using the pure strain generated in the lab induced symptoms of anthracnose—brown-black leaf spots. The leaf spots produced by the artificial inoculation were sampled for microscopic evaluation, and the structure and morphology of the conidia from the inoculated leaflets were identical to the source image of *N. juglandis* from bioimages.org (2007), which further confirmed that the fungal strain isolated in the lab is *G. leptostyla* (Figure 10). It was observed that the OMA media only produced growth of the ascospores of *G. leptostyla*, while the conidia (*N. juglandis*) and acervuli were only produced on the leaflet tissue.

Preservation Methods and Re-growth

In July of 2019, the samples that were set aside in July of 2018 for storage were retrieved and re-plated on freshly poured OMA media plates and observed for up to 120 hours. At 48 hours, the isolates from the dried filter papers—those stored at both 4°C and -20°C—grew healthy, uncontaminated fungal colonies that had a diameter of approximately 18 mm. However, the isolates from the glycerol did not show much growth until 72 hours after being plated. After 72 hours, every one of the isolates showed fungal growth with a diameter of approximately 25 mm (Figure 11). Ultimately, all the fungal preservation methods have proven to be successful for

at least up to one year. In following years, the same process will be repeated to observe how long these strains could be stored for and still be viable when re-cultured.

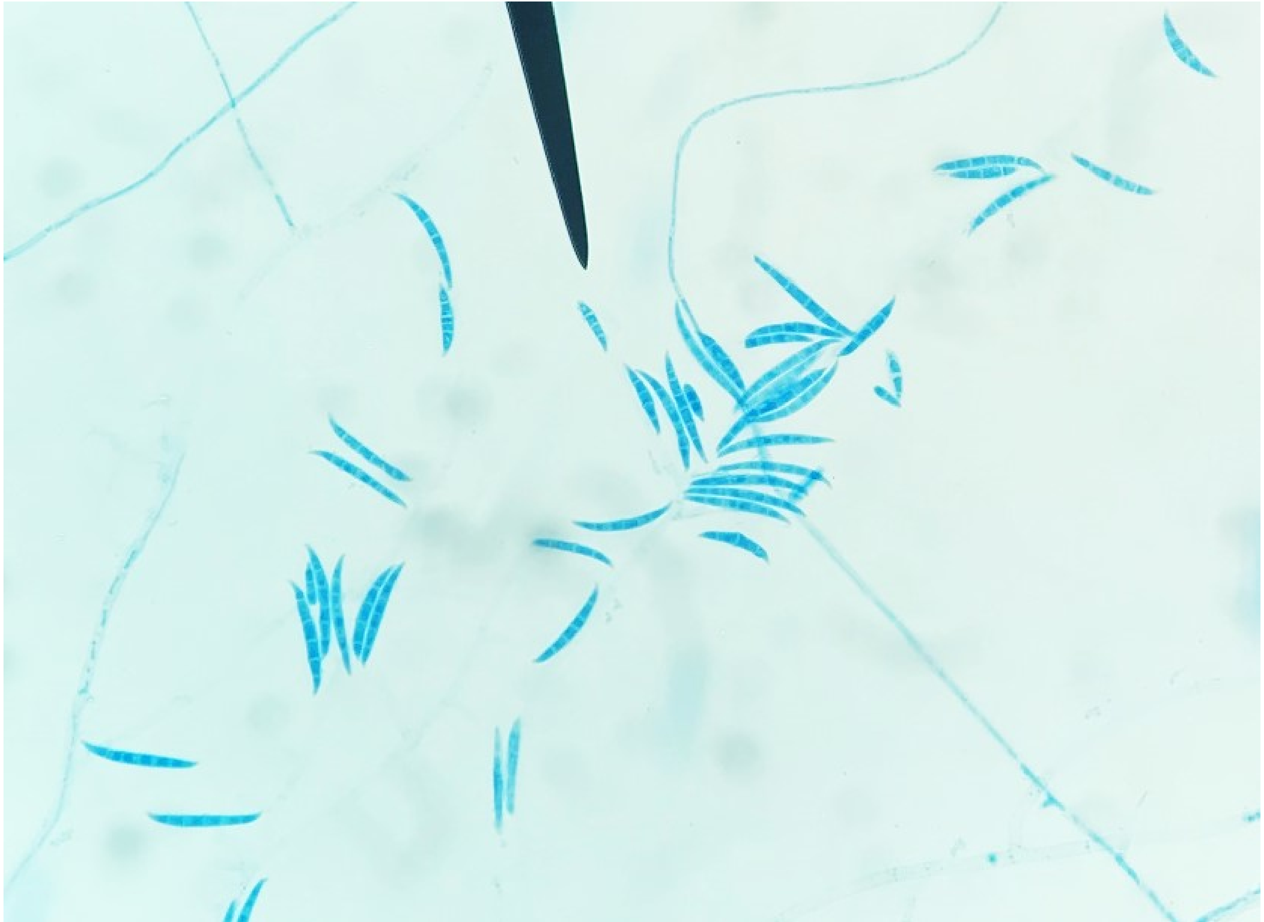
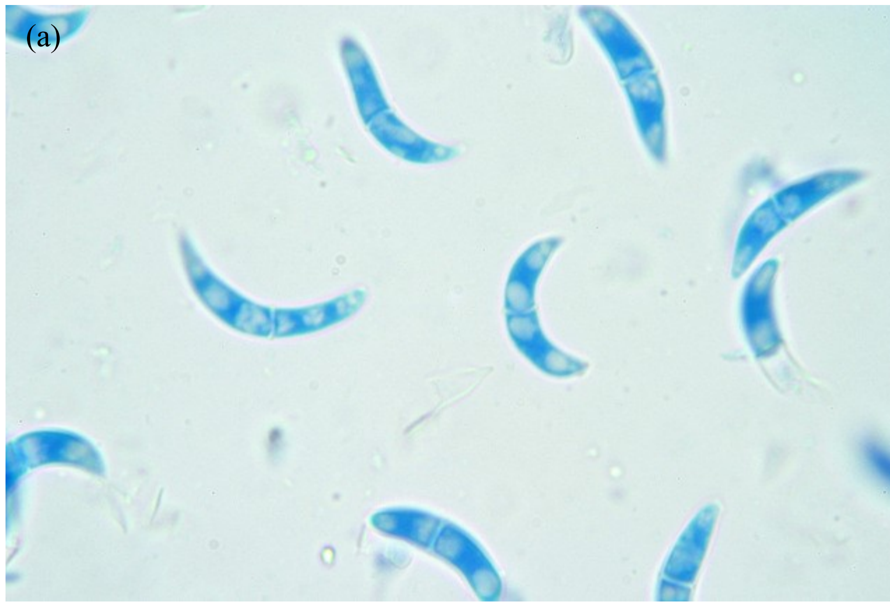


Figure 9: Ascospores of Lab-Grown Strain of *G. leptostyla*. These ascospores were sampled from fresh mycelia and then stained using LCB fungal stain to be visualized under the microscope at 400X magnification.



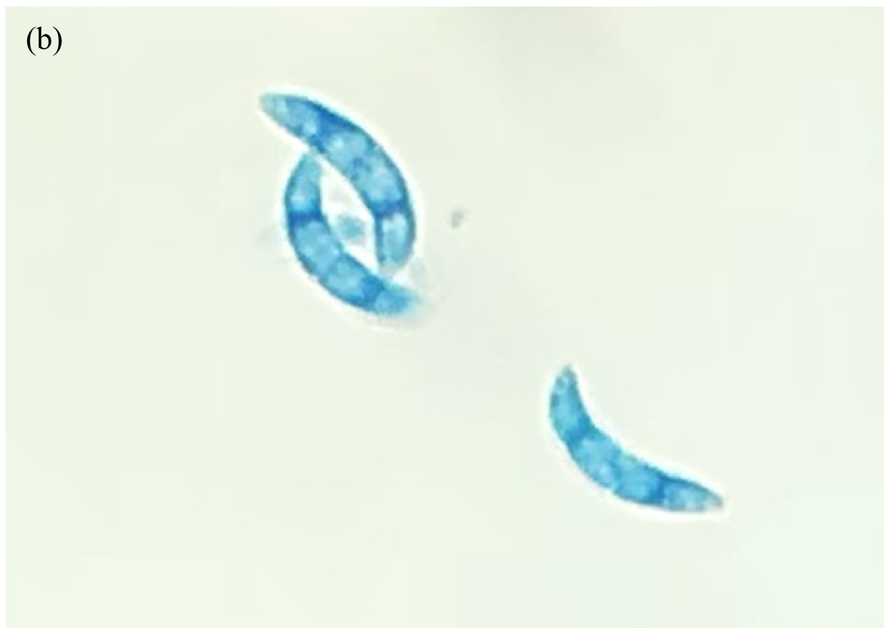


Figure 10: *N. juglandis* Sampled from Leaf Spot. The known structure of *N. juglandis* sampled from a leaf spot of a *J. regia* leaf from previous research (8a; bioimages.org 2007) is compared to the structure of the conidia sampled from a leaf spot from an artificially inoculated *J. nigra* leaflet produced in the lab (8b).

Figure 11. Three Days of Re-growth of Preserved *G. leptostyla* Isolates after One Year of Storage. The image on the left shows re-growth of *G. leptostyla* that was dried on filter paper and stored at 4°C, the center image shows re-growth of *G. leptostyla* after it was dried and stored at -20°C, and the image on the right shows *G. leptostyla* that was re-grown after being stored in 4M glycerol at -80°C.

Artificial Inoculation of Healthy ‘Football’ and ‘Sparrow’ Leaflets

Color Analysis. At thirteen days post-inoculation, the visual observations revealed a difference in susceptibility to anthracnose between the two cultivars (Figure 12). Out of the one hundred and twenty total leaflets artificially inoculated with anthracnose, the sixty leaflets from ‘Football’ showed visible symptoms earlier and spread faster than the ‘Sparrow’ leaflets. The sixty artificially infected leaflets from ‘Sparrow’ showed little to no symptoms of anthracnose at day thirteen, and the most affected area was localized to the midrib at the point of inoculation. From the color analysis, both the ‘Football’ leaflets and the ‘Sparrow’ leaflets started at ~0% diseased surface area (Table 3). The controls for both ‘Football’ and ‘Sparrow’ leaflets remained at ~0% diseased surface area throughout the duration of the scanning, which confirms that there was no contamination. The difference in symptom severity between the two cultivars was seen at day seven where the average percentage of diseased surface area for ‘Football’ was 2.5% while ‘Sparrow’ had shown nearly no increase in diseased surface area and remained close to 0% (0.7%). At day nine, the average diseased surface area for ‘Football’ leaflets was 6.1% while ‘Sparrow’ only raised to 0.8%. Day eleven showed a spike in diseased surface area for ‘Football,’ showing an average of 15.1% while ‘Sparrow’ was still only at an average of 1.2% diseased surface area. At thirteen days after inoculation, the average percentage of diseased surface area for the ‘Football’ leaflets was 31.5% while the average percentage of diseased surface area for the ‘Sparrow’ leaflets was 3.2% (Figure 13).

Figure 12: Difference in Symptom Severity between 'Football' and 'Sparrow' Leaflets. The 'Football' (right) and 'Sparrow' (left) leaflets showed the most significant difference in symptom severity at thirteen days post-inoculation as the 'Football' leaflets showed significantly more leaf spots than the 'Sparrow' leaflets.

Table 3: Daily Average Disease Percentage for 'Football' and 'Sparrow' Leaflets. The average diseased surface area percentage was calculated using the measurement of the surface area in the 'Diseased' color class for each individual leaflet. The individual percentages were used to formulate an average percentage of diseased surface area per day for each parent cultivar. The color analyses for every odd day were used to formulate a graph to show the trend of symptom growth.

Day	Football		Sparrow	
	Total SA	% Diseased SA	Total SA	% Diseased SA
0	21.2	0.3	14.9	0.1
3	21.1	0.7	15.0	0.5
5	21.3	1.1	15.0	0.6
7	21.3	2.5	15.0	0.7
9	21.2	6.1	15.1	0.8
11	21.2	15.1	15.0	1.2
13	21.1	31.5	15.0	3.2

***significant at $p < 0.0001$

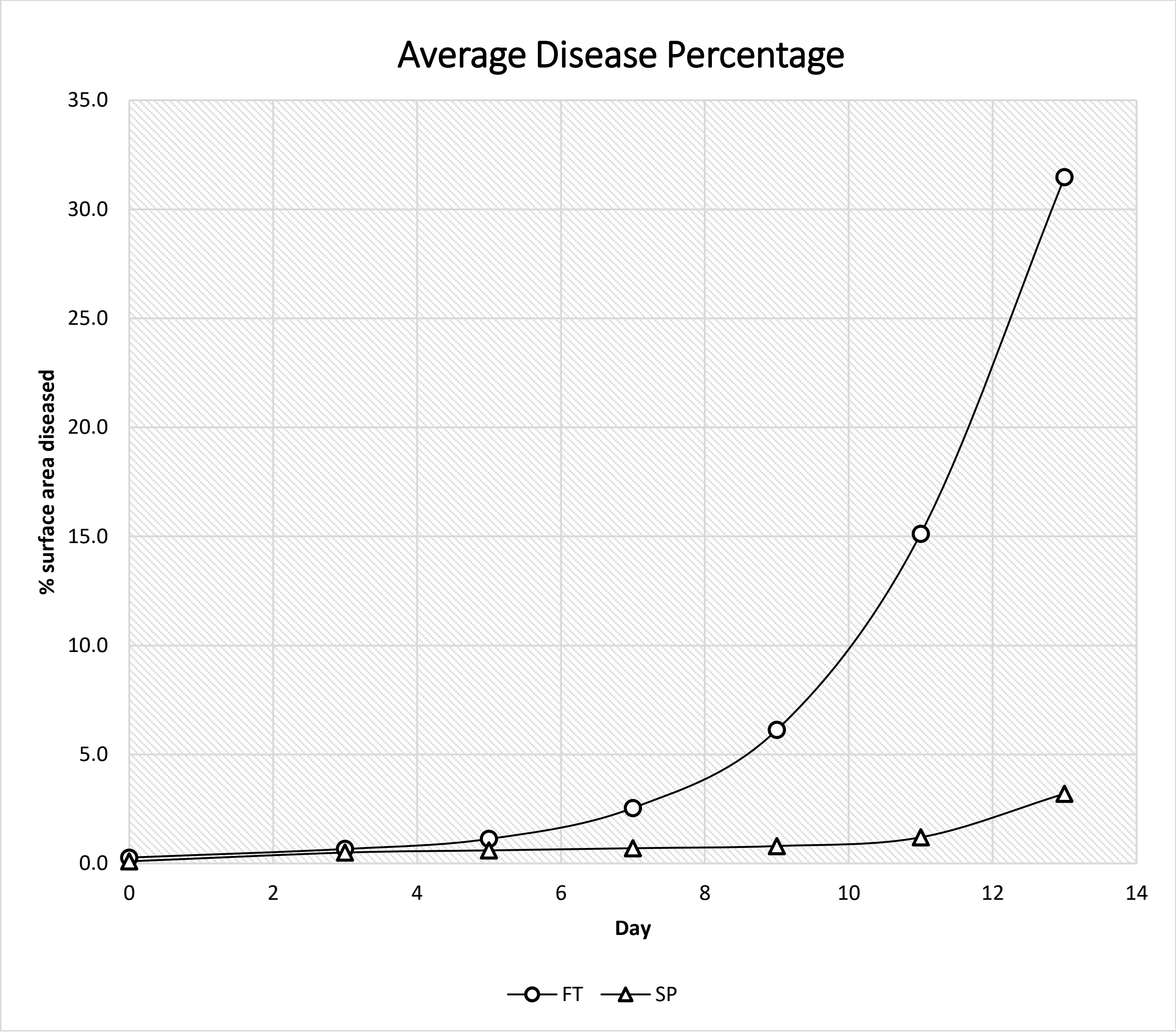


Figure 13: Average Disease Percentage for 'Football' and 'Sparrow' Based on Surface Area. The points plotted in this figure are based on the average disease percentages for 'Football' and 'Sparrow' from Table 3.

Rate of Increase. The rate of increase for average percentage of diseased leaflet surface area was measured for both parents and compared between the parents at day three, day five, day seven, day nine, day eleven, and day thirteen after inoculation. The comparison showed that the 'Sparrow' leaflets had almost no increase in percentage of diseased surface area until a small rise on the final day, while the 'Football' leaflets had a spike in percentage of diseased surface area at day seven and continued to drastically increase every day following (Figure 14). From day zero to day three post-inoculation, both the 'Football' and 'Sparrow' leaflets showed the same average percentage increase of around 0.40%. From day three to day five post-inoculation, the 'Sparrow' leaflets only increased 0.10% while the 'Football' leaflets showed an increase of 0.46% diseased surface area. From day five to day seven, the 'Sparrow' leaflets had the same average increase as the days prior, while the 'Football' leaflets had a three-fold increase of the days before at 1.42%. From day seven to day nine, the 'Football' leaflets showed an average increase of 3.58% diseased surface area while the 'Sparrow' leaflets remained at an average increase of only 0.10%. From day three to day nine, the average rate of increase of diseased surface area for the 'Sparrow' leaflets remained the same—approximately 0.10% average increase every two days for a total of only 0.30% increase over six days. From day nine to day eleven, the 'Sparrow' leaflets showed a slightly raised average increase of 0.40% diseased surface area while the 'Football' leaflets showed an average increase of 9.00%. On the final day, the average increase of diseased leaflet surface area from day eleven to day thirteen for 'Sparrow' reached 2.00% while 'Football' had an average increase of 16.35%. The rate of increase of diseased surface area for 'Sparrow' was significantly slower than 'Football' as the rate of increase for 'Sparrow' plateaued for six days but consistently continued to rise for 'Football.'

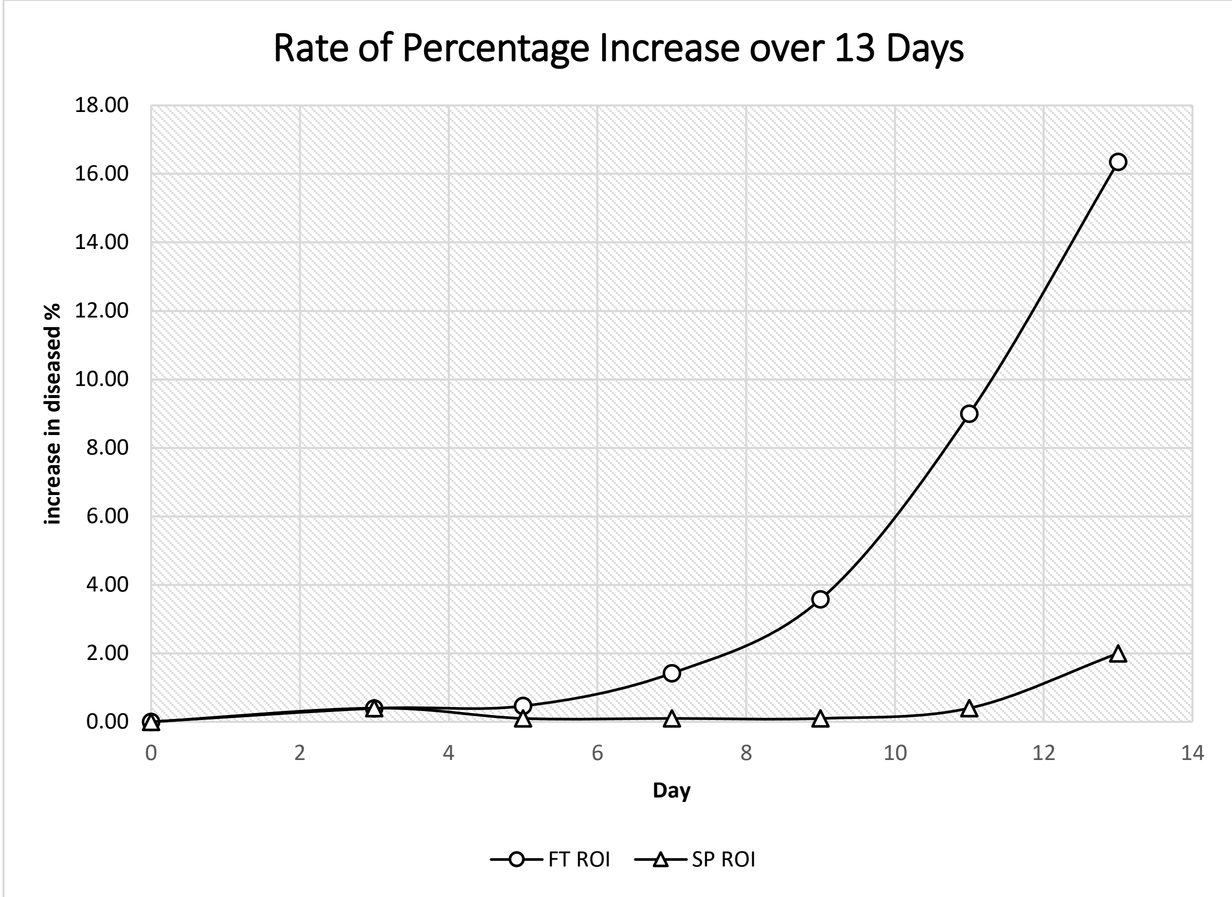


Figure 14: Rate of Increase for Percentage of Diseased Surface Area between 'Football' and 'Sparrow' Leaflets. The 'Football' leaflets continued to increase at levels much higher than the 'Sparrow' leaflets. From day eleven to the final day (day thirteen), there was a small spike in average increase of 2.00% diseased surface area for 'Sparrow;' however, 'Football' showed a more drastic spike of 16.35% average increase of diseased surface area from day eleven to day thirteen.

Statistical Analysis. The results of the ANOVA and Tukey's test presented a significant difference between the two groups at day eleven with a p-value < 0.0001 as the average percent of diseased leaflet surface area for the 'Sparrow' leaflets was 1.2% and the average for the 'Football' leaflets was 15.1%. Therefore, in future studies, the length of the detached-leaflet artificial inoculation assay can be shortened to eleven days. However, the difference in symptom intensity between the two parents reached the greatest significance at day thirteen according to the ANOVA and Tukey's test. Overall, the results of the statistical tests performed in this study confirmed that the 'Sparrow' cultivar's resistance to anthracnose in comparison to the 'Football' cultivar is significant at day eleven post-inoculation, with the greatest significance shown at day thirteen post-inoculation.

Artificial Inoculation with F_1 Progeny

Nine days post-artificial inoculation revealed that the hybrid population showed visible segregation of the trait of resistance to anthracnose also confirmed by the measurements obtained using WinRHIZO color analysis that revealed a large range of symptom severity across the entire population (Figure 15). Of the thirty hybrids measured, fifteen individuals (50%) received a score of one, nine individuals (30%) received a score of two, and six individuals (20%) received a score of three (Table 4). This data already shows how this trait segregates phenotypically in the hybrids, but it will need to be replicated when there is a larger mapping population available.

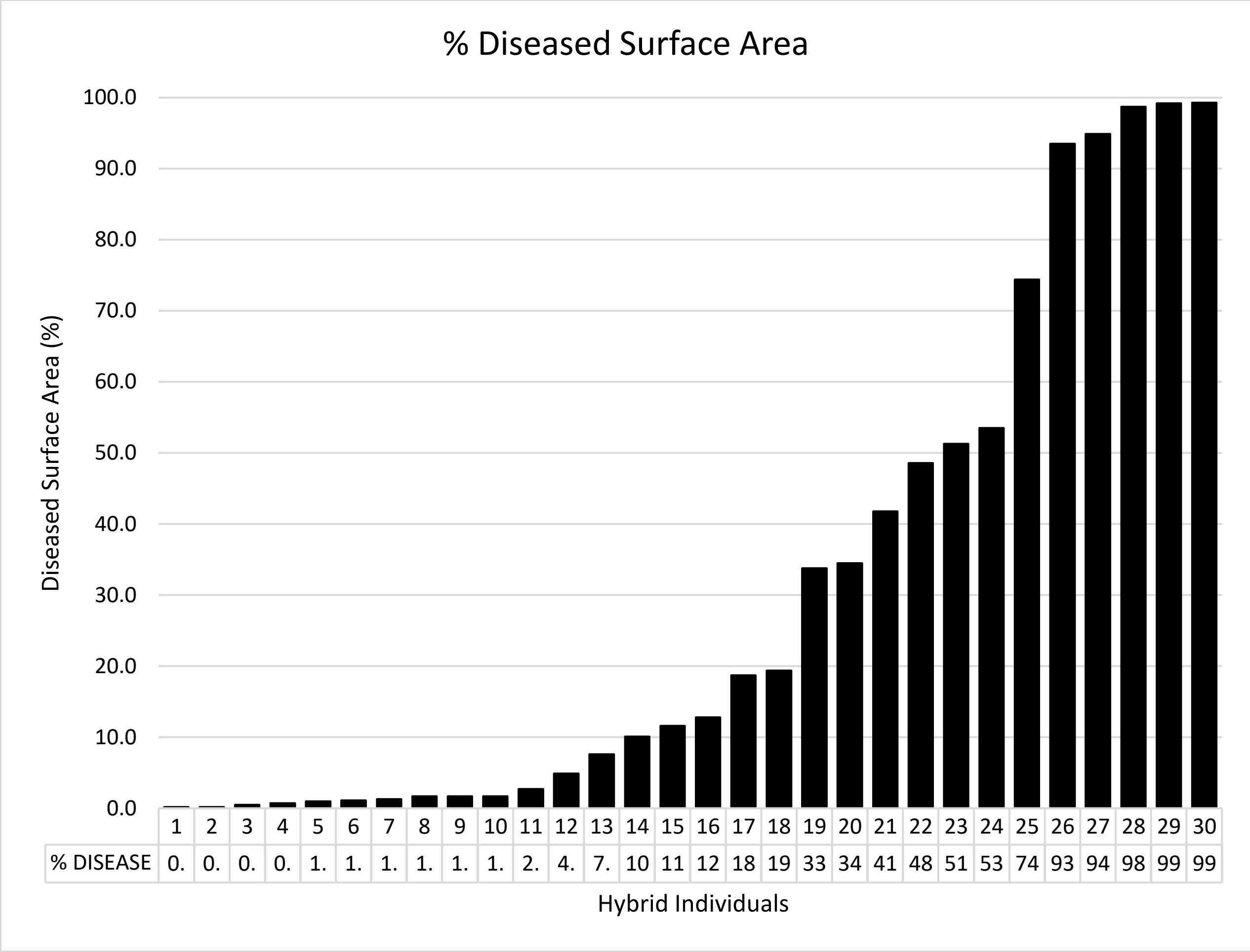


Figure 15: Preliminary Artificial Inoculation Data for the 'Football' × 'Sparrow' F_1 Hybrid Population for 2019. At nine days after inoculation, the leaflets revealed segregation of the high resistance to anthracnose.

Table 4: Scoring of 'Football' × 'Sparrow' F_1 Population. Fifteen of the total 30 individuals (50%) received a score of 1 as their percentage of diseased surface area was between 0% and 12%. Nine of the total 30 individuals (30%) received a score of 2 as their percentage of diseased surface area was between 12% and 60%. Six of the total 30 individuals (20%) received a score of 3 as their percentage of diseased surface area was between 60% and 100%.

Score	1	2	3
	0-12% diseased surface area	12-60% diseased surface area	60-100% diseased surface area
	0.2 %	12.8 %	74.4 %
	0.2 %	18.7 %	93.5 %
	0.5 %	19.4 %	94.9 %
	0.7 %	33.8 %	98.7 %
	1.0 %	34.5 %	99.2 %
	1.1 %	41.8 %	99.3 %
	1.3 %	48.6 %	
	1.7 %	51.3 %	
	1.7 %	53.5 %	
	1.7 %		
	2.7 %		
	4.9 %		
	7.6 %		
	10.1 %		
	11.6 %		

Hybrid Identification

Based on the parent data for the markers used in the hybrid identification process, the total number of ’Football’ × ‘Sparrow’ hybrids identified with seven SSR markers was 52 out of the 1,163 individuals germinated in 2019. These individuals were separated from the other germinated walnuts and prepared to be planted in the field for future replications using the ‘Football’ × ‘Sparrow’ hybrid mapping population.

DISCUSSION

Artificial Inoculation

There were some difficulties in the culturing process of the causal pathogen for anthracnose. *G. leptostyla* is of the order Diaporthales (sac fungi) and has a similar ascospore structure to a variety of genera and species within that order. Therefore, when the ascospores were identified in the laboratory as *G. leptostyla* based on the structure and morphology from previous studies, the presumed strain of *G. leptostyla* was further tested to ensure accurate identification (Hassan et al. 2017; Hassan and Ahmad 2017). The identified ascospores were finally confirmed as *G. leptostyla* as the symptoms of anthracnose disease (caused by *G. leptostyla*) appeared on healthy black walnut leaflets after the ascospores were used for artificial infection. The symptoms included the brown/black spots appearing on the leaflet surface with the production of conidia (*N. juglandis*) from the leaf spots. It has also been stated that the conidia can be cultured on media (Jamshidi and Zare 2012); however, the attempts in culturing the conidia on media in the laboratory were unsuccessful in this study.

Another factor that should be noted includes the window of time for sampling leaflets for the detached-leaflet artificial inoculation assay. According to what was seen in the field in 2018, there is a short window of time after budbreak where the leaves have not yet been affected by disease. For the purposes of this study, mature leaflets were desired for the artificial inoculation process to accurately reflect the infections of anthracnose in the stage of which the tree is naturally affected. Therefore, the window of time was narrowed to about one to two months for the collection of mature leaflets that were healthy and showing no sign of anthracnose. Thus, the collection process needed to be done quickly to have the most accurate results with mature,

healthy leaflets. However, in 2019, symptoms of anthracnose seemed to arise earlier than in 2018, which caused the process of leaflet sampling to be hurried, and the sampling size to be smaller than expected.

Additionally, in the artificial inoculation process for evaluating anthracnose symptoms in the two parent cultivars, the phenotypic observation of natural disease incidence—number of leaves per plant displaying symptoms—of ‘Football’ and ‘Sparrow’ trees over time could have provided more information on the rate of increase for this disease. In this study, the rate of symptom increase was measured from the controlled detached-leaflet artificial inoculation assay; however, looking at the entirety of the individual trees could have offered a more accurate interpretation of how quickly the disease naturally spreads throughout the entire organism. All the listed limiting factors should be considered as this study continues to move forward.

Increasing the Mapping Population

Although this study yielded promising results, there were some limiting aspects. In order to identify hybrids with the desired parents, many walnuts must be collected, germinated, and screened. However, the walnuts collected for this project were from beneath ‘Football’ trees, which means that some years the walnut yield is high, and some years are very low as ‘Football’ has an alternate bearing pattern. This inconsistency can potentially slow down the process of expanding the hybrid mapping population.

In the wind-pollination process, the number of ‘Football’ × ‘Sparrow’ hybrids was very low in 2019 compared to the overall total of collected walnuts. While the germination rate of the wind-pollinated walnuts was 62.5% in 2019, only 4.6% of the germinated individuals were ‘Football’ × ‘Sparrow’ hybrids. In previous years, controlled pollination was attempted, but the

germination success rate (only ~2.0%) along with number of desired hybrids produced was very low. One reason for the low ‘Football’ × ‘Sparrow’ hybrid percentage was because the ‘Football’ walnuts used for this project were collected from an orchard containing other varieties and cultivars of black walnut, which means that there were many other cultivars competing with the ‘Sparrow’ trees to pollinate the ‘Football’ trees. Another reason for low ‘Football’ × ‘Sparrow’ hybrid numbers was due to the flowering date and flower type of the two parent cultivars. Leafing and flowering date is based on the length of time in days it takes for a tree to produce leaves and flowers after the first cultivar produces leaves and flowers—'Davidson’—which is why the leafing and flowering date is referred to as number of days after ‘Davidson’ (DAD). ‘Football’ is one of the earliest black walnut cultivars to flower (11.0 ± 2.1 DAD), and ‘Sparrow’ is known to flower later than ‘Football’ at 16.8 ± 3.4 DAD (Reid et al. 2004). The flower type of the ‘Football’ cultivar is protogynous which means that the female reproductive organs mature before the male reproductive organs. ‘Sparrow’ on the other hand has a protandrous flower type, which means that the male reproductive organs mature before the female reproductive organs (Reid et al. 2004). Because of the different flowering dates and flower development of these two cultivars, it can be challenging for the ‘Sparrow’ trees to pollinate ‘Football’ trees in an orchard as the window of opportunity is small. Although the current process of growing the F_1 mapping population from wind-pollination is labor-intensive, it has had the highest success rates of all the processes that have been attempted thus far.

SSR Marker Screening and Future Objectives

A total of 629 SSR markers were screened for the purpose of constructing a genetic linkage map for ‘Football’ and ‘Sparrow.’ So far, only 175 of the 629 SSR markers were

polymorphic and could be useful for genetic linkage map construction. These markers were also screened with the hybrid F_1 mapping population for the purpose of shortening the length of time of the hybrid identification process. The reason it can be challenging to locate markers that show polymorphism for both parents is because the SSR markers for black walnut are intraspecific—existing within the same species. Therefore, the majority of the SSR regions in one cultivar are the same in another cultivar. However, SSR markers will continue to be screened for linkage map construction and hybrid identification purposes until new marker strategies arise to make these processes easier and more efficient.

In future steps, the detached-leaflet artificial inoculation procedure will be replicated with a larger 'Football' × 'Sparrow' F_1 mapping population to gain a better understanding of the segregation of the trait linked to anthracnose resistance. Once this is performed, a genetic linkage map will be generated for the two parent cultivars as well as the 'Football' × 'Sparrow' hybrid progeny using DNA markers. After a genetic linkage map is constructed, the phenotypic data from this study will allow for the identification of a QTL (quantitative trait locus) in the black walnut genome that links to high resistance to anthracnose. A quantitative trait locus is a location on a chromosome that contains the gene for a specific favorable phenotypic trait—in this case, high resistance to anthracnose. As soon as a QTL is identified on the genetic linkage map, the hybrid progeny can be screened at three to four weeks old (or after true leaves are produced) with DNA markers to determine which individuals contain the QTL linked to high anthracnose resistance. The two types of DNA markers that will be used are SSRs and single-nucleotide polymorphisms (SNPs), or single nucleotide differences. This process is important because using DNA markers to compile a genetic linkage map and locate a QTL expedites the typical process of selective breeding to shorten the length of time it would take to see positive traits; rather than

waiting up to 20 years to see questionable results, clear and efficient results can be seen at three to four weeks old.

The overall goal for the genetic linkage map construction and QTL identification is to eventually release a new cultivar that consists of phenotypic and agronomically favorable traits. Although the hybrids produced from this project may carry the gene for high resistance to anthracnose, it is possible that they may also include some unfavorable traits from the two parent cultivars. For example, the hybrids could likely inherit the weak branching structure of 'Sparrow,' the weaker rootstock from 'Sparrow,' and the less-favorable walnut traits from 'Football.' The hybrids that inherit high resistance to anthracnose may not be weakened by disease, however, these hybrids could have different susceptibilities due to other traits inherited from 'Football' and 'Sparrow.' Therefore, every individual in the 'Football' × 'Sparrow' hybrid mapping population will undergo a cultivar evaluation process where they will be tested to see if the individual would survive and serve as a productive cultivar in the future.

CONCLUSION

The results from the artificial inoculation assay support the hypothesis that the ‘Sparrow’ cultivar contains a higher resistance to anthracnose than the ‘Football’ cultivar as the difference in the average percentage of diseased leaflet surface area between ‘Sparrow’ and ‘Football’ was significant at day eleven according to the ANOVA and Tukey’s tests. However, the difference in percentage of diseased leaflet surface area was most significant at thirteen days after inoculation where the ‘Sparrow’ leaflets had an average of only 3.2% compared to 31.5% for the ‘Football’ leaflets. Therefore, ‘Sparrow’ revealed a higher resistance to anthracnose as the leaflets observed and measured showed nearly no symptoms compared to the leaflets from ‘Football.’ Seeing that the parents have shown significant difference in susceptibility to anthracnose and that the F_1 progeny preliminary data has shown promising results, more replications of artificial inoculation for the ‘Football’ × ‘Sparrow’ F_1 hybrid progeny can be performed and measured in the future for the phenotypic range of resistance to anthracnose.

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