Screening for target spot (*Corynespora cassiicola*) resistance in cotton (*Gossypium hirsutum* L.)

by

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Abstract

A critical problem for Alabama cotton growers is the presence of target spot (*Corynespora cassicola*), a foliar disease which is responsible for combined annual yield losses of >$70 million in AL, GA and the FL Panhandle. Currently, there are no resistant commercial cultivars and the only management defense are fungicide applications. Timing of application and implications for resistance to the fungicides are high. A resistant variety is the only long term solution, therefore this study focuses on the inoculation method and growing conditions of *C. cassiicola* in order to develop a greenhouse protocol to screen for the variety response to target spot. Preliminary experiments focused on inoculation techniques and environmental conditions favoring pathogen reproduction. It was determined that a spore suspension was preferred over a mycelium broth, the temperature required for growth was 24-32°C and that the plants had to be under consistent moisture for at least 12 hours. Future experiments will compare cultivars known to be different in a field setting to validate this protocol.

Key words; cotton, target spot, *Corynespora cassiicola*, inoculation

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List of Abbreviations

ANOVA	Analysis of variance
APDA	acid potato dextrose agar
BLAST	Basic Local Alignment Search Tool
bp	base pair
°C	Celsius
DI	deionized
DNA	deoxyribonucleic acid
g	gram
kg/ha	kilograms per hectare
lb/ac	pounds per acre
L	liter
ml	milliliter
µl	microliter
µm	micrometer
MS	mean square
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
PDA	potato dextrose agar
PVC	polyvinyl chloride
QPDA	quarter strength potato dextrose agar
RH	relative humidity

RCBD	randomized complete block design
TBE	tris-borate EDTA
UV	ultra violet
V	volts

LITERATURE REVIEW

Cotton is of major economic importance in the US, generating over 125,000 jobs in the private industry alone. On the production level, cotton is sold in 480lb bales, averaging 70 cents per pound, with 21 million bales harvested in 2017 (ERS, 2017). This crop is grown in the 13 most southern states which is referred to as the cotton belt, reaching from California to Virginia. Globally, the US ranks third in production, totaling over 21 billion dollars in goods and services (NCC, 2017) with over 12 million bales exported. Cotton is grown primarily for its lint which is purchased by the spinning industry to produce yarn. The cotton seed also has economic importance as animal feed and its oil has many applications (Lee, 1984). Cotton's vast uses make it an applicable commodity for stock exchange. Futures reached 78 cents in December 2018 (NASDAQ, 2018).

Gossypium hirsutum L., or upland cotton, is the most common of four domesticated cotton species (Wendel, 2009). All four species developed and diverged from wild ancestral cotton species found in many different geographical locations including Africa and Australia (Mauney, 1986). All cotton, including ancestral and wild cotton, is perennial, but cultivars developed for production are treated as annuals. Producers aim to grow cotton that is high yielding and has optimal fiber quality in the span of one growing season.

Cotton Growth and Development

Cotton development can be broken down into five stages: germination and emergence, seedling establishment, canopy development, flowering and boll

development, and maturation. These stages are not delineated by a specific physiological change. Not all stages are easily distinguishable to the naked eye (Oosterhuis, 1990).

Germination and emergence begins with the seed. The seed holds all of the organs and nutrients required for the beginning of growth. The embryo lies within the seed and consists of a radicle, hypocotyl, underdeveloped epicotyl, and cotyledons (Ooterhuis, 1990). These cotyledons will eventually be used for photosynthesis, but during germination, the cotyledons provide nutrients for growth (Oosterhuis and Bourland, 2001). High soil oxygen, temperatures above 60°F, and high soil moisture are required for optimal germination. Emergence occurs as the radical root exits the seed coat and begins to grow downward into the soil. Once the seed reaches the top soil barrier, the hypocotyl emerges, and the seed coat is shed (Oosterhuis, 2001). Seedling establishment is marked by the opening of the cotyledon leaves. During early stages of development, the primary focus of the plant is to produce a healthy root system, hence its ancestral perennial growth habit (Oosterhuis and Bourland, 2001).

Canopy development is a process that occurs through indeterminate growth of main stems and branches. Cotton has a main stem that continues to grow by means of apical dominance by the apical meristem or terminal bud (Ritchie *et al.,* 2007). The stem contains nodes throughout that allow branches to form off of the plant. Branches are separated into two categories, vegetative or reproductive. Vegetative branches function in the same way as the main stem. The branches are driven by apical dominance and grow in a straight line outward from the main stem. Carbohydrates from photosynthesis and nutrients taken up by the roots are primarily used for establishment of a canopy (Ritchie *et al.,* 2007). The formation of this canopy will allow for increased leaf area for

photosynthesis which will provide further nutrients for developing fruit. After approximately 75 days the canopy will close (Oosterhuis, 1990). This means that leaves from neighboring rows meet each other and the space between the rows are shaded by leaves. This canopy plays an integral part in weed control and control of evaporation by the soil (Oosterhuis, 1990).

Flower and boll development start approximately seven weeks after planting. Within five weeks cotton will begin to develop reproductive branches followed by reproductive structures only a few weeks later (Oosterhuis, 1990). Carbohydrates from photosynthesis and nutrients sourced from the roots are now used primarily for the development of reproductive branches and fruits. Vegetative growth does not cease once reproductive growth begins but is slowed because of repartitioning of nutrients. Cotton is indeterminate, which means it will continue to grow vegetatively as long as conditions are suitable.

The first reproductive fruiting structures to develop are squares which appear in three day intervals. Three weeks after formation, flowers will form (Oosterhuis and Bourland, 2001). During this three-week growth stage, the square will develop into a candle, which is the flowering structure. Once the flower is fully developed, it will open and be fertilized within hours of opening. Flowers are self-pollinated; however, bees and other insects may cause cross-pollination. The bracts still remain around the flower. Bracts account for about 10% of a boll's photosynthetic requirements once the flower is fertilized and boll development begins. Cotton flowers are white on the day of anthesis and will turn pink the following day and start to desiccate, leaving behind the fertilized fruit, or boll (Ritchie *et al.,* 2007).

Cotton will continue to flower and produce bolls (Oosterhuis, 1990) until cut-out. During cut-out the plant redistributes all of its energy into boll development and ceases flowering (Ritchie, 2007). Next, development of seed and lint within the boll takes place. It takes about 25 days after fertilization for fibers to reach their maximum length. Fibers are considered alive and will continue to thicken until the boll cracks and exposes the fibers to air. The air will dry the fibers causing them to curl and die. The end of the maturation phase is marked by boll opening and defoliation of the plant (Oosterhuis, 2001). After defoliation, harvesting may take place.

Common Foliar Diseases of Cotton

Foliar diseases are common in cotton grown in non—arid regions, partially due to the high temperature environments and high moisture content associated with canopy closure. These diseases are caused by bacteria or fungi and are not mutually exclusive, rather they are often synergistic.

Bacterial

Bacterial blight, also called angular leaf spot, is a common disease in cotton caused by *Xanthomonas campestris* pv. *malvacearum* (Nyvall, 1999). The bacterium can be found on seed lint, within the seed, in crop residue, and within the buds of asymptomatic plants. Wind, insects, water, and farm machinery pass it from plant to plant (Innes, 1983; Nyvall, 1999). This pathogen may also imbed itself within the seeds which will then serve as a source of inoculum when such seed is planted. *Xanthomonas campestris* is a cosmopolitan bacterium and when conditions are favorable, it will become pathogenic. It enters the plant through open stomata or wounds. Optimum environmental conditions include temperatures between 30°C and 36°C, abundant

moisture, and high relative humidity of 85% or more. Boll rot may accompany the leaf spot allowing the bacterium easy access to the seeds; if moisture is high, infection will occur (Nyvall, 1999).

Symptoms of bacterial blight are first seen on the lower cotyledon leaf surfaces within ten days after sowing. Spots will appear translucent green with a round or ovoid shape. After time, brown to black lesions will form on upper leaf surfaces (Nyvall, 1999). Lesions will appear angular or irregular and follow main leaf veins and petioles. As the disease progresses, leaf lesions will dry and become reddish-brown before eventual defoliation (Nyvall, 1999). The disease can also manifest on tissues other than leaves, this includes hypocotyls, stems, and bolls. Lesions on seedling hypocotyls appear as black cankers and may kill the plant. When the disease infects bolls, the bolls will form black sunken patches that will permit the entry of boll rot fungi (Nyvall, 1999; Thiessen *et al.*, 2017). Management practices to reduce inoculum load include growing resistant cultivars, using acid delinted seed, crop rotation, and sowing seeds from disease free plants. Plowing residues immediately after harvest may also help reduce the inoculum load within the field (Nyvall, 1999; Thiessen *et al.,* 2017).

Fungal

Areolate mildew, or false mildew, is a generally distributed foliar disease caused by *Mycophaerella areoli*, anamorph *Ramularia gossypii*. Nightly dew covering the leaves and daily drying, combined with temperatures ranging from 20°C to 30°C provide optimal growing conditions for this pathogen. Infection is commonly seen late in the season in highly humid environments (Nyvall, 1999). Symptoms include circular lesions on the leaves that appear water soaked and brown that begin at the bottom leaves and

quickly move up through the canopy (Nyvall, 1999; Theissen, 2018). These lesions may cover the leaf which eventually turns a dark reddish brown and withers. Premature defoliation can occur. Defoliated leaves are curly, dry, and fragile. White growth consisting of conidia may be visible to the naked eye on intact leaves as well as defoliated leaves, and seen on both the top and underside of the leaf (Nyvall, 1999).

There are two primary management strategies for the control of Areolate mildew. One, grow resistant cultivars. Resistant cultivars may show minimal symptoms of tiny reddish-brown spots surrounded by a chlorotic halo (Nyvall, 1999). Second, apply foliar fungicides that are labeled to treat Areolate mildew. If climatic conditions are favorable and disease is already present in the field within the sixth week of bloom, a fungicide should be sprayed. However, fields that are within four weeks of defoliation may not need to be sprayed as the disease will produce little to no yield loss (Theissen, 2018).

Alternaria leaf spot is common foliar fungal disease caused by *Alternaria gossypina*, *A*. *alternata* and *A. macrospora*. The pathogen can survive as mycelium on infested crop residues before proper conditions for sporulation occur. Sporulation is triggered by moist conditions, and the spores are carried by the wind to surrounding leaf tissue. It is common for *A. gossypina* to infect a host plant previously compromised by other pathogens, making them predisposed to new infections. Nutrient deficiency, such as limited potash due to poor soil quality, may also aid in the development of disease. A pre-plant potash application has been shown to reduce the incidence of Alternaria leaf spot. Symptoms can occur at all stages of cotton growth and development. Leaf spots are brown and circular with a papery feel. Lesions form concentric rings similar to that of target spot, another fungal foliar disease. Severely diseased plants in moist environments

will exhibit premature defoliation, however, use of fungicides provide no significant yield gains (Hagan, 2013).

Management practices for Alternaria leaf spot include five main practices. Seeds should be treated with fungicidal seed protectant. Proper soil fertility should be maintained to reduce the incidence of disease aided by poor soil quality. Since Alternaria leaf spot is commonly a secondary disease due to other issues that compromise the plant, proper fertilization will aid in prevention. Injury due to insects and machinery should be avoided. Wounding increases the likelihood of infection by allowing an entry for the pathogen into plant tissues. Lastly, plowing under residues and growing later maturing varieties will provide some protection for this disease.

Ascochyta blight caused by *Ascochyta gossypii* is commonly referred to as wet weather blight because of its preference to wet or extremely humid conditions. This disease is more likely to occur in the Northern perimeter of the eastern U.S. cotton growing region. Spores and mycelia overwinter in the soil and on crop residue providing an inoculum source for the following year (Nyvall, 1999). Wet conditions permit spore dispersal through splashing, but wind will also distribute spores through a field. The disease will manifest as preemergence or postemergence seedling blight, while in later growth stages causes brown foliar spots with reddish borders (Nyvall, 1999). As the disease progresses, the spots will shift from brown to grey before the necrotic centers fall out (Nyvall, 1999; Wade *et al.,* 2015). Symptoms are not limited to leaves. Bolls, stems, and branches may also present sunken dark brown spots. Lesions may eventually encircle stems, suffocating and killing the plant above that point.

Since the disease overwinters on crop residue it is a good idea to plow under residues if possible (Wade *et al*., 2015). Secondly, treating seeds with a fungicidal seed treatment is the best way to minimize pre/post emergence seedling blight along with proper soil fertility (Nyvall, 1999; Wade *et al.,* 2015). Ascochyta blight is more severe when cotton is not rotated, so rotating with a resistant crop is a positive management strategy. However, yield losses are rarely reported.

Cercospora leaf spot, caused by *Cercospora gossypina* (*Mycosphaerella gossypina*), causes reddish lesions during the onset of disease. Over time the center of the lesions will shift from the reddish color to a light brown or white. Lesions will present concentric rings and may be mistaken for another foliar disease, target spot. However, the red margins that persist throughout lesion development differentiate Cercospora leaf spot from target spot. Other misdiagnoses may include Stemphylium leaf spot and Alternaria leaf spot. Reliable identification may be made by viewing the septate spores microscopically to confirm the disease.

All areas of cotton production are prone to Cercospora leaf spot. Premature defoliation has been documented as caused by *C. gossypina* infection and reduced yield and fiber quality occur as a result. The best management practices typically do not include chemical application, but rather maintaining proper nutrient levels within the soil, encouraging plant vigor, and eliminating drought stress to reduce primary infection.

Target spot is a common foliar disease caused by *Corynespora cassiicola.* The fungus is ubiquitous in nature and may grow saprophytically on residues in the soil. Residues allow the fungus to overwinter. Common in the southeast, target spot prefers hot conditions with relative humidity at or exceeding 80% (Nyvall, 1999). Symptoms

include brown lesions that form concentric rings growing until they eventually coalesce (Fulmer et al., 2012; Nyvall, 1999). Symptoms have also been documented on bracts, stems and bolls. *Corynespora cassiicola* infected bolls may eventually lead to boll rot (Fulmer *et al.,* 2012; Lakshmanan *et al.,* 1990) Target spot, if untreated, can lead to premature defoliation and yield losses (Hagan, 2017; Fulmer *et al.,* 2012).

Currently, the only management strategy for target spot is the use of fungicide sprays as no resistant varieties are commercially available at this time. The spraying regime will depend on the type of fungicide being used. For example, Headline® (BASF, Ludwigshafen Germany), and Quadris® (Syngenta, Basel, Switzerland) should be applied at early stages of disease development and repeated every 14 days, while Twinline® (BASF, Ludwigshafen Germany) is more effective as a preventative measure being applied before disease development occurs (Hagan, 2013).

Target Spot

Target Spot as a Threat

In cotton, target spot was identified in Mississippi as early as 1961 (Jones, 1961). Reports of the disease have been uncommon until the last decade. Symptoms of the disease were more recently identified in Alabama in 2011 on dryland and irrigated cotton. It is most commonly seen after canopy closure since it provides a conducive growth environment for *C. cassiicola;* such as longer periods of wetness, shade, and warmth (A. Hagan, personal communication). The disease will continue to move upward through the plant and through the canopy as time progresses. If left untreated, target spot can lead to premature defoliation, loss of bolls, and death of the plant. Because of its tendency to appear underneath the canopy, the occurrence is often missed by producers. In 2012, the

estimated yield loss in select cultivars ('Deltapine 1050 B2RF' and 'Phytogen 499 WRF') surpassed 336 kg/ha seed cotton (Hagan, 2017). Due to increased yield losses in cotton, it is important to develop a screening protocol to identify varieties that exhibit promise for resistance.

Target spot was previously considered only a minor threat to all crops. However, research suggests that yield loss due to *C. cassiicola* infection in at least rubber tree, soybean, cotton, and tomato are significant. Target spot occurrence has become increasingly common since the late 1980's. In 1987, target spot had devastating effects on the rubber tree industry in Sri Lanka, leading to the uprooting and burning of more than 46000 ha of trees to reduce disease incidence (Silva *et al*. 2003). Research on tomato in 2002 concluded that the use of fungicides as compared to no treatment doubled marketable yields. Similarly, a study found that target spot was responsible for a 20-40% yield suppression in soybean in 2004 (Koenning *et al.,* 2006).

Corynespora cassiicola

Corynespora cassiicola is a fungus that infects 530 plant species from 380 genera, including cotton and soybean. It has a large geographical distribution from Japan, the tropics of Brazil, to North America and can be found on leaves, stems, roots, and within nematode cysts, among monocots, dicots, and on one species of cycad. The fungus is ubiquitous in nature and can act as an endotroph or saprotroph, meaning it can act as a pathogen or be present on plant material with no pathogenic effects (Seaman *et al,.* 1965). Morphology is similar among isolates. The pathogen produces conidiophores which are solitary or in clusters that generate a single conidium at the broad apical pore. This single conidium is the spore of the fungus. The conidium can adopt a variety of

different shapes and shades from hyaline and straight, to brown and slightly curved, further proving genetic diversity in the fungus. Pathogenic infection of susceptible species' roots will lead to root rot, while pathogenic infection of susceptible species' leaves produces a distinguishable necrotic target-shaped spot or spots. Bordering the target spot is a light yellow to light green halo. These lesions, if not controlled by fungicides or unfavorable environment, will lead to premature defoliation, otherwise known as leaf fall.

Corynespora cassiicola causes disease on a limited number of host species per geographic location which serves as an indicator for host specificity among isolates. However, some isolates have been recorded to infect multiple hosts, while others will not. Isolates, like those from rubber trees, will infect papaya. However, papaya isolates have no effect on rubber. Adaptation of isolates to alternative hosts may occur over time. Spore producing ability, virulence, and pathogenicity are variable among isolates.

Isolation and Culture Methods

Culture methods of *C. cassiicola* isolates are similar regardless of host source. Koch's postulates can be applied with *C. cassiicola* and can be isolated from infected plant material and reapplied to cause disease. During a soybean study in Canada, the pathogen was isolated from primary and secondary roots. Conidia from these samples were plated on V8 agar, PDA, and Alphacel agar. Within 4-5 days the cultures became dark with conidia and conidiophores with a yellow pigment arising 2-5 days later in cultures. Soybean isolates may also be taken from the leaves. This is a common practice when dealing with the foliar leaf disease caused by *C. cassiicola*. Temperature requirements for growth depend on the host source. Isolates of tomato have an optimal

temperature range of 20°C - 28°C, while soybean isolates from Canada prefer 15°C to 20°C (Jones, 1984; Seaman *et al.,* 1965). This is not the only culture method, however. When leaf lesions occur, fungal cultures can be initiated from collected leaves.

Currently at Auburn University, infected cotton leaves are collected, surface sterilized, and divided into cuttings before being plated. Single conidia or a hyphal tip are later selected from that plate and re-plated on V8 or PDA agar. The same is being done with soybeans and tomato. Plates are kept in an incubator at 28°C. Within 11-13 days, the culture will begin to produce conidia. Spores may be used to make a spore suspension which can be used as inoculum. Within 14 days the plate will be covered with mycelium. Variability of color will occur among isolates. This is another indicator of genetic diversity within the species.

Inoculum

Common Laboratory Inoculum Sources

Use of a spore suspension is a common form of inoculation in target spot research. After the first report of target spot in Alabama, Dr. Conner produced a $2x10^4$ spore suspension for application to ten cotton seedlings. Within six days the seedlings showed symptoms of disease. After re-isolation, Koch's postulates were verified, and genetic verification was completed (Conner et al., 2013). A spore suspension provides an exact concentration of principal inoculum. Conidia as inoculum is important as it mimics how the disease would spread naturally in a field which involves spore dispersal and dissemination, not relocation of mycelium (Bowen, personal communication).

V8 broth, comprised of 3g calcium carbonate, 160mL of V8 juice, and 840mL of water, culture of the fungus is another form of inoculum that consists of mycelial

colonies that form within the solution. After colony formation, the solution is homogenized in a blender and is ready for use. This form of inoculum can be made in large quantities as long as shaker table space is readily available; however, it is not a principal type of inoculum because no spores are present within the suspension. Environmental variability, such as room temperature fluctuations, will impact how/if colonies form within the broth. Some batches may form only a few large round colonies, others may form hundreds of tiny colonies, and in some cases no colonies form at all. Counting mycelial fragments using a hemocytometer is not a common form of determining concentration. Colony-forming units can be enumerated, but this requires additional time and resources. There is no general consensus on how long the V8 broth inoculum may be stored before use although it is commonly stored for up to one week (K. Lawrence, personal communication).

Corynespora cassiicola is known to produce a toxin, cassicolin, that induces disease symptoms (Onesirosan, 1974). Research done in tomatoes shows that when applied directly to the plant, the toxin produces lesions within 18 hours and can kill plants within 72 hours (Onesirosan, 1974). Resistant tomato varieties do not show symptoms. Cassicolin can be extracted from *C. cassiicola* cultures through a lengthy process broken down into four filtrate fractions with a final evaporation step leaving a dry cassicolin residue (Onesirosan, 1974). The advantages to use of the toxin for inducing symptoms are that it can be made in large batches and transported internationally without the risk of introduction of a new isolate to an untouched region. However, it does not discriminate between moderately and highly resistant individuals (Onesirosan, 1974)

Resistance in Soybean

Cultivars of soybean that are resistant to *C. cassicola* have been identified, but the mechanism of resistance is still unknown. Fortunato *et al* (2016) suggests that resistance is due to an increase in at least 6 defense related enzymes coupled with an increase in lignin and flavonoid concentration at the infection site (Fortunato, 2010, 2016). Flavonoids are naturally occurring phenolic substances found within plants that are known for their beneficial health properties and ability to control some enzymatic functions (Panche, 2016), making sense of Fortunato's discovery. Target spot resistance in soybean is likely due the plant's capability to regulate the phenylpropanoid pathway (Fortunato et al., 2016). No research has been conducted on whether differing phenylpropanoid levels occur among cotton cultivars.

Fungicide Control

Corynespora cassiicola is classified as a high-risk fungus (FRAC). The likelihood of the fungus developing resistance to fungicides is high due to genetic variability and the fungus's tendency to adapt to its surroundings. Fungicide resistance has been documented in *C. cassiicola* isolates from tomatoes and cucumber for some commonly used SDHI (e.g., boscalid)and QoI fungicides (Miyamoto, 2009). Timing of application of fungicides according to proper protocols can allow for some protection against yield loss (Hagan et al 2017). However, constant use of fungicides over time can lead to fungicide resistance.

Corynespora cassiicola resistance to fungicides has yet to be documented in cotton. One study, documenting the efficacy of a variety of different fungicides applied to tomato, found that plots treated with Endura® (boscalid; BASF, Ludwigshafen Germany), an SDHI, and Quadris®(azoxystrobin; Syngenta, Basel, Switzerland), a quinone outside inhibitor (QoI), showed increased symptoms compared to the nontreated

control. The isolates used were known to be resistant only to boscalid. QoI fungicides have proven most effective thus far, but an increase in resistance has recently been seen. This resistance is not entirely understood. QoI resistance in *C. cassiicola* is unique because there is a lack of cross resistance among chemistries in this group. Some QoI fungicides have proven to be and still are effective while others are losing efficacy (Ishii, 2010). QoI fungicides act by effectively deactivating cellular respiration through the cytochrome b6 complex in the mitochondria. The mode of action (MOA) of QoI fungicides have very specific target binding sites allowing them to inhibit respiration of the fungus, but not of the plant (Wyenandt, 2015). The question is, why is cross resistance not seen among these fungicides and is it safe to continue depending on them for a treatment of such a high-risk pathogen?

Objectives

Because of the tendency for *C. cassiicola* to become resistant to fungicides, Alabama and the rest of the southeastern U.S. needs an alternative option for target spot management. Therefore, the primary objective is to discover varying degrees of resistance to target spot. If genotypic differences are observed, varieties that are identified as tolerant or resistant can serve as a starting point for future experiments similar to the work of Fortunato et al. (2016) work in soybean. By utilizing what is known about target spot in other species, a protocol must be developed to evaluate cotton varieties for resistance. A protocol implemented in a greenhouse setting and at an early growth stage will save time, money, and space. It must also be validated by what is occurring under normal field conditions.

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CHAPTER 1

INTRODUCTION

Target spot caused by the casual fungus, *Corynespora cassiicola,* was first identified in Mississippi cotton in 1961 (Jones, 1961). Until the past decade, the disease has not been seen, either not being present or gone unreported. In 2011, the disease resurfaced in Alabama on dryland irrigated cotton with yield loss estimates exceeding 336kg/ha by the next year (Hagan, 2017). Although previously considered a minor threat, increased yield losses has given growers cause for concern. The best way to combat these losses is by planting resistant varieties. To date, no known target spot resistant cotton variety is on the market for growers to utilize.

Closure of the cotton's canopy provides a conducive environment for the causal fungus, allowing longer periods of wetness, shade, and higher temperatures (A. Hagan, personal communication). Target spot generally starts on the lower leaves and will continue to move up the plant and through the canopy as time progresses. If left untreated, target spot can lead to premature defoliation, loss of bolls, and death of the plant. Because of target spot's tendency to appear within the canopy, its occurrence is often missed by producers.

Target spot is recognized by its distinct concentric circles, reaching ½ inch diameter with bands of light and dark, hence the name. Symptoms first appear after canopy closure, which creates an environment conducive for disease development. Once established, target spot spreads quickly and defoliation levels can reach 70-90%. This reduces the available carbohydrates (energy resources) for developing reproductive fruit (bolls) in cotton. The boll receives the most carbohydrates from the closest leaf (Ashley,

1972). If the leaf is absent, boll shed will occur in order to support existing lower bolls, which results in reduced boll load and yield loss (Johnson and Addicott, 1967). It is advantageous to have the bolls already set and starting to open when the disease attacks, this minimizes yield loss.

The current method of control of target spot is by using scheduled fungicide treatments (Hagan, 2017). It is unwise to depend solely upon fungicides for target spot management within a field because *C. cassiicola* has been classified as a high risk fungus (FRAC). This classification indicates that *C. cassiicola* is extremely likely to develop fungicide resistance (FRAC) because it asexually reproduces and has already been documented in other crops such as tomato (Ishii, 2010).

Boscalid resistance is of primary importance. While Fluopyram has proven to be effective, penthiopyrad does not suppress the disease. This indicates a lack of cross resistance within fungicides (Ishii, 2011). Timing of application is important. Hagan (2017) determined that fungicide application methods, other than those currently available specifically for target spot, are equally to more effective. However, the possibility for *C. cassiicola* to develop fungicide resistance cannot be eliminated. If the causal fungus develops resistance to available fungicides, there are no effective management strategies to protect the crop.

Recent research conducted at Auburn University found that temperature, incubation time, and leaf wetness duration played a major role in spore germination and lesion development in tomato (Sharma, 2017). Spores on water agar germinated best when exposed to temperatures between 24-32°C with an incubation period of at least twelve hours. When spores were applied to plants, the minimum leaf wetness duration

required for germination was also at least twelve hours. However, an increase in leaf wetness duration lead to a steady increase in germination percentage. The highest germination rate occurred at 48 hours, the maximum number of hours tested in this experiment. Lesions appeared at roughly one week post inoculation with 48 hours of leaf wetness (Sharma, 2017).

In order to efficiently screen numerous lines of cotton for resistance to *C. cassiicola* a protocol must be established. By doing so, growers can minimize yield losses, and prevent the development of fungicide resistance. With field studies being a season long and requiring extensive amounts of space, a greenhouse protocol would be quicker and more suitable for screening. The protocol should be rapid, easy to duplicate, and allow for the evaluation of a large number of genotypes.

First, analyzing plants at the seedling stage was deemed to be the best available option. Second, by using a large mist chamber many different genotypes could be screened with relative ease and at low cost. Lastly, using a spore suspension with a known concentration of inoculum would allow for the test to be duplicated. Therefore, the objective of the current study is to determine the optimum growing conditions for the pathogen for successful inoculation, in terms of temperature, moisture and light. This information is necessary in order to provide a consistent protocol. In addition, the amount of replications, planting media and inoculum type will be investigated.

MATERIALS AND METHODS

Inoculum

Two types of inocula were tested: a mycelium fragment V8 broth culture and a distilled water spore suspension, used in previous research (May, 2017; Sharma, 2017). Instructions for inoculum production are as follows.

Isolate Collection for V8 Broth Inoculum

Infected leaves collected from plants in Brewton and Fairhope, AL were placed in labeled plastic bags and put in a cooler for transportation back to the lab. Single spores from the leaves were isolated onto PDA plates, incubated, and transferred a cutting from the outer perimeter of growth to a PDA slant tube. The slant tubes were stored in a refrigerator. Ten to 15 V8 agar plates were inoculated using the isolates preserved in tubes. These plates were kept in an incubator at 29°C for two weeks before using them as a source for the fungal plugs needed to make V8 *C. cassiicola* broth.

Mycelium fragment V8 broth

An 8oz (~237mL) can of V8 was poured in a one liter glass Pyrex bottle, containing a magnetic stir rod and 3 grams of calcium carbonate. The bottle was then filled to 1L with deionized water and allowed to stir for one minute. The contents are equally divided into 2- 500ml bottles and autoclaved for 30 minutes. The preserved *C. cassicola* plates were recultured once a month and kept in an incubator at 28°C. Single spores or single mycelia directly from the isolate were placed onto a V8 agar plate. They are grown in the incubator for at least 14 days before being used for inoculum. Autoclaved V8 broth was cooled at room temperature for at least one day. If the broth is too hot, the fungus will not grow. When cooled, the bottles of broth were aseptically

inoculated with *C. cassiicola* plugs taken from 2-week-old plates described above. Once this task was completed, the bottles were placed on a shaker table for 10 days at room temperature. During shaking, fungal colonies formed inside the broth. The bottles were emptied into a blender, homogenized, and poured back. The inoculum is now ready for use.

Spore suspension

Fungal isolates began with a single spore isolation onto a PDA agar plate. These plates were incubated at 29°C for one week. Using aseptic technique, a small square from the outer perimeter of the fungal growth is cut, removed from the plate, and placed fungal side down inside a PDA slant tube. The tubes were labeled and stored in a refrigerator. These tubes served as the source for all plating in these trials.

Ten to fifteen QPDA plates were inoculated using the BRW1 isolate (discussed in detail below). QPDA is quarter the strength of regular PDA and is made by mixing 11g granular PDA and 7g agar powder with 500mL deionized water. Using aseptic technique, a small sample of the isolate was transferred from the tube and onto the plate. After each plate had been inoculated, they were labeled and wrapped with parafilm before being placed in an incubator at 29°C. The plates grew for ten days. At day ten, the plates were checked for spores. If no spores are visible, the plates will be checked each day following. Spores usually form within 10-14 days.

To confirm the presence of spores, a small sample of mycelium was taken from the inoculated plate and transferred to a microscope slide. The sample was stained with cotton blue dye. It was then evaluated under a compound light microscope. If spores were present, the plates were washed.

To wash fungal cultures to make spore suspension, DI water was added to each plate until the fungal growth was covered. Using a glass stir rod or loop the growth was carefully agitated. This removes the spores without uplifting mycelium. Using a mesh strainer, the spore suspension from the plate was poured into a glass bottle and rinsed with DI water using a wash bottle to remove any remaining spores. The strainer will catch any large pieces of mycelium. Once all plates had been washed, the concentration was evaluated using a hemocytometer. If the concentration was too high, the spore suspension was diluted. Immediately before inoculation, Tween20 was added to a concentration of 0.05%.

Genetic Confirmation

DNA extraction was done by using an E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek, Norcross, Georgia, USA) according to the manufacturer's protocol with one major deviation. Fresh tissue was used instead of dried tissue. The main goal was to extract the DNA, amplify a specific region, and send the amplicon for sequencing and confirmation that the BRW1 isolate is *C. cassiicola*

The BRW1 isolate used in the mist chamber experiments was plated on four QPDA dishes incubated at 29°C until the plates were covered with fungal growth. The growth was scraped off the plate using a scalpel and 50mg sample of fresh tissue was weighed, placed in a powerbead tube, and placed in a bead beater for 2 minutes. From this point forward, the manufacturer's protocol was followed. DNA concentration and quality were measured using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and its integrity was checked on agarose gel.

PCR was done using the extracted DNA as a template and primer pair ITS1 and ITS4 (Raja et al. 2017). PCR was performed in a 96 well plate with 25 µl volume of reaction mixtures. The reaction mixture contained 2.5 µl of standard buffer, 0.5 µl of dNTPs (10 mM), 30 ng of DNA in 1.5 µl, 0.5 µl of each ITS1 and ITS4 primer, 0.125 µl of Taq DNA polymerase, and 19.375 µl of water to make the final volume of 25 µl. The PCR conditions consist of: 1) 95 °C for 30 seconds, 2) 40 cycles of 20 seconds at 95°C (denaturation), 1 minute at 54 °C (annealing), and 1 min at 72°C (extension), 3) final extension step of 10 minutes at 72°C, and 4) forever at 4°C.

Gel electrophoresis

Gel electrophoresis separates DNA by fragment size. A 1.5% agarose gel was prepared using 1X TBE (Trisborate EDTA) solution and was heated using a microwave to dissolve the agarose. After cooling for 5 minutes, ethidium bromide was added. Ethidium bromide detects and stains the DNA fragments after they are run through the gel. The agarose solution was poured in a gel tray and allowed to solidify. A total of 5 µl of loading dye (6X) was added in 25 µl of PCR product and was mixed before loading it into the well. A DNA ladder (4 µl) of 100 bp was used to estimate the size of PCR product. The gel was run at 90 V for one hour and then examined under UV light. The band size of about 550 bp was cut from the gel and was purified using E.Z.N.A.® Gel Extraction Kit (Omega Bio-tek, Norcross, Georgia USA). The purified product was sent for sequencing.

BLAST

A program called 4PEAKS was used to view the sequence to determine the quality of the sequence. The sequence was analyzed through BLAST (basic local alignment search tool) on NCBI database to identify the fungal species.

After submitting the BRW1 DNA fragment sequence selected by the primer pair IST1 and IST4, the NBCI database concluded that it was a 99% match to known *C. cassiicola* sequence. This confirmed beyond visual identification that the BRW1 isolate is *C. cassiicola.*

Preliminary Inoculation

Cotyledons were inoculated 3, 5, and 7 days post emergence. For preliminary studies, 50 count Jiffy pellet trays were used for planting. Two seeds of 'Phytogen 499WRF' (susceptible check), and Bayer breeding lines 'BCT1', 'BCT2', and 'BCT3' were placed in their respective pellets. Before inoculation, if both seeds had emerged, the smaller seedling was removed. No quantitative data were recorded, only visual observations were made. Seedlings were first sprayed with water and immediately after with V8 *C. cassiicola* inoculum until runoff. After inoculation, the plants were placed inside 14x20x12 PVC enclosures with no additional water. The enclosures were covered with white translucent trash bags to preserve moisture. Lesions were observed and confirmed by Austin Hagan to be target spot.

V8 *C. cassiicola* broth contains no spores, therefore the concentration of inoculum was not quantified. The suggested mode of quantifying this inoculum would be through colony forming unit analysis, but this would add extra resources and time. Going

forward, it was decided that use of a spore suspension would be more appropriate for the research goals. Secondly, the small PVC enclosures did not provide ample space.

A 8'x4'x4' mist chamber was constructed from 1'' PVC pipe and covered with clear poly sheeting. Corners were folded under and duct taped. A main flap was cut to serve as a door. When not in use the flap was secured by two clips to ensure that heat and humidity was retained within the chamber. Sprayer heads were suspended using 1" tube 2 ¼ feet from the top of the chamber at a spacing of 16'' from the outer edge and 32'' in between.

Experiment – Trial one:

In October 2018, nine lines were evaluated in a RCBD with four replications. Thirty-six 4 1/2 inch pots were filled with potting mix, packed down, and filled again to the top. Three seeds were sown in each pot to ensure emergence of at least one seedling. The seeds were planted at a depth less than one inch, covered, watered in thoroughly, and placed on the benchtop outside of the mist chamber. Once the seedlings emerged, the seedlings were reduced to one plant per pot. A soil drench of Admire Pro (Bayer Crop Science, Thane, India) was applied at 20µL per plant to reduce insect pressure.

On the same day as planting, 5-10 cultures of *C. cassiicola* were started in 100mm diameter plates. During the 10-14 days for the fungus to sporulate, the seedlings continued to grow. Once spores were confirmed, a 1.5x10^4spores/ml spore suspension was established. This suspension was sprayed on all plants except for the non-treated Phytogen499WRF control, using a regular household bottle sprayer. The Phytogen499WRF non-treated control was sprayed with water. Each plant was sprayed two times for a total of about 1.5 mL per plant and allowed to sit on the benchtop for five

minutes. After this time, the plants were organized according to the RCBD within the mist chamber. The Mist Timer II (Drips Incorporated, Concho, Arizona, USA) was set to mist for 1 second every ten minutes for three days; to keep the leaves wet but without runoff. After three days, the mist was turned off and the plants remained in the mist chamber for 11 more days before evaluation. The plants were watered as needed, watering at the base of the stem so as to not wet the foliage.

Plants were evaluated for disease 14 days after inoculation. Each plant was given a severity rating based on the percent of the leaf area damaged for both cotyledon leaves. First true leaves were not considered. Each half of a cotyledon leaf was considered to be 25%, with ¼ of a cotyledon leaf being 12.5%. Each plant was visually assessed and the rating was recorded.

Experiment – Trial 2

In November 2018, a second trial was performed using the same method as above except the number of plates inoculated with *C. cassiicola* was increased from 5-10 to 10-15 plates. The inoculum concentration was increased from 1.5x10^4spores/mL to 4.0x10^4spores/mL.

Experiment – Trial 3 and 4

In December 2018 and January 2019, trials three and four were conducted. They consisted of 6 lines and the replications were increased to eight. Forty-eight pots total were planted. Breeding lines BCT1, BCT2, and BCT3 were eliminated from the trial. The inoculum concentration was maintained at 4.0x10^4spores/mL.

Data Analysis

Statistical analysis for the four trials were analyzed using a mixed model ANOVA with Tukey's mean separation in SAS 9.4.

RESULTS AND DISCUSSION

Mist Chamber Analysis

Trial 1, using 1.5x10^4 spores/mL water inoculum, indicated no significant differences between the eight genotypes (pvalue= 0.341; Table 2). However using 4.0x10^4 spores/ml, a significant pvalue at <0.001, was observed. The inoculated Phytogen499WRF treatment had the highest severity (93.8%), while the control had 0% (Table 3). These tests had four replications which were able to detect large differences among genotypes in severity/tolerance (i.e. greater than 10%).

Based on the positive results with 4.0x10^4 spores/mL, two additional trials using this concentration were performed with increased replications in order to determine if smaller differences between cultivars could be identified. The ANOVA table indicated that there was no significant differences between the trials of testing (Table 4). The genotypes were significantly different in their response at pvalue <0.0001. The test by genotype interaction (p=0.36) indicated that the ranking of the genotypes stayed the same across trials, suggesting that this concentration produced consistent results when duplicated.

Among the 6 genotypes tested, the controls performed as expected with Phytogen499WRF being the most susceptible; however, when nontreated, this cultivar had 0%, as expected (Table 5). LA and UA preformed similarly at 18 and 29.7%, respectively. NM and TAM lines were not significantly different, averaging ~36%.

V8 C. cassiicola Broth

Variation was seen among Phytogen499WRF individuals inoculated with a V8 *C. cassiicola* broth with near death of the plants being the most common. Although no

quantitative data were collected, a field experiment further corroborates the idea that V8 *C. cassiicola* broth is not a useful tool when evaluating plants at either the seedling or mature plant stage. The study concluded that when using V8 *C. cassiicola* broth as an inoculum source, correlation between field and greenhouse experiments were poor and the inoculum had a tended to be fatal to mature plants (May, 2018). In order to evaluate inherent genetic resistance, resistance should not be overcome with a lethal dose of inoculum. In order to quantify V8 *C. cassiicola* broth, colony forming units must be evaluated, which takes extra time and resources. With no general consensus on how long the broth is viable, using a spore suspension that can be quickly and precisely quantified is an easier and more reliable option. The concentration can be determined and then sprayed onto plants within minutes of each other.

Leaf Wetness Duration, Temperature, and Environment

To further support previous findings (Sharma, 2017), leaf wetness duration exceeding 12 hours, in this case 72 hours, allowed for the development of lesions on seedlings in at least 7 days. Increasing the leaf wetness duration to 3 days was suggested to keep the chamber at a high relative humidity for an extended period of time. With plants being small and having no canopy to trap moisture, the leaves dry within 10 to 15 minutes after being exposed to mist. Temperatures could not be controlled within the mist chamber but remained naturally at or above the 28°C required for *C. cassiicola* growth. Temperature fluctuations between ambient seasons did occur, but did not impact whether or not lesions developed.

Concentration and Replication Adjustments

After an initial experiment of 1.5x10^4 spores/mL inoculum significant differences among genotypes were not observed (Table 1). It was hypothesized that increasing the concentration to 4.0x10^4 spores/mL and adding four additional replications would produce statistically significant differences among genotypes. After increasing the inoculum load, separation among genotypes was evident. This confirmed that the problem was not with the method or protocol itself, but with the precision of the experiment. When evaluating the performance of a 1.5x10^4 spores/ml suspension, numerical differences were observed when rating for percent severity (Table 2). The treated control performed the worst and the nontreated control showed no symptoms of disease. However, these differences were found to be statistically insignificant.

Severity amplified with an increase in inoculum concentration. As expected, the treated control performed the worst, with the untreated control presenting no signs or symptoms of disease. Significant differences were observed among the genotypes. This confirmed the suspicion that an increase in inoculum concentration would be more fitting for observing genotypic differences. Increasing the replications from four to eight provided double the amount of data points per line which increased the precision of the trial. An inoculum concentration of 4.0x10^4 is sufficient enough to see genotypic differences under mist chamber conditions and at the seedling stage (Table 3).

CONCLUSION

The findings of this study determine that the isolate being used is genetically identical to known *C. cassiicola*. This is important to confirm when evaluating disease. Furthermore, while a spore suspension is the preferred form of inoculation, a concentration at 1.5x10^4 spores/ml is not sufficient for observing differences among cotton genotypes tested under our conditions. It was hypothesized that increasing the inoculum concentration could provide better discrimination. The hypothesis was not immediately confirmed. However, by increasing the concentration to 4.0x10^4 spores/ml along with increasing the number of replications of the study from four to eight, statistically significant differences among genotypes were observed. The Phytogen499WRF nontreated control performed better than all other genotypes with the LA14063001 variety ranking second. Phytogen499WRF performed the worst as expected.

Based on these findings, it is clear that this mist system screening method has the potential to be used for screening cotton lines for resistance to *C. cassiicola*. Additional varieties from more diverse genetic backgrounds should be tested in the near future. Digital imagery analysis could be considered to remove the bias of visual ratings and provide greater precision. Also, isolates of C. *cassiicola* from different geographic locations should be evaluated to determine if resistance to the disease is consistent.

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CHAPTER 2

INTRODUCTION

Cotton is a high value row crop grown in the southern US. In 2016, the Mid-South and Southeast regions planted a combined 3.79 million acres. Alabama and Georgia had a combined planting of 1.4 million cotton acres with revenues of $371 and $947 million, respectively (NASS, 2016). Each year diseases cause approximately 12% in cotton yield losses (Kirkpatrick and Rothrock, 2001).

In recent years a foliar disease, target spot, caused by *Corynespora cassiicola*, has become a problem in the Southeast. The disease appears on cotton, soybean, sesame, and various vegetable crops. Jones (1961) first reported a *C. cassiicola* incited leaf spot disease on cotton in Mississippi; however, it was not until 2005 that this disease was responsible for an estimated 200 lbs/acre yield loss in southwest Georgia. While the reason for the resurgence of the disease is not known, it has become a high priority in cotton. Symptoms of the disease were more recently identified in Alabama in 2011 on dryland and irrigated cotton (Conner, 2011).

In 2012, the estimated yield loss in select cultivars (Deltapine 1050 and Phytogen 499) surpassed 336 kg/ha seed cotton (Hagan, 2014a). By 2013, target spot appeared in cotton fields in Alabama, Tennessee, Louisiana, the Florida panhandle, South Carolina, North Carolina and Virginia (Hollis, 2013). That year, Alabama and Georgia reported an estimated $70 million in yield and seed losses (Hagan, 2014a). Since the disease only recently resurfaced, there are major gaps in the knowledge surrounding the source, spread and survival of the causal fungus (Kemerait, 2016). While the reservoir is not known, *C. cassiicola* can survive on plant residue over the winter (Kemerait, 2016) and initial

infection requires humidity >80% or free moisture from rainfall or irrigation (Faske et al, 2014).

Yield loss by target spot can be devastating and fungicide application is the only proposed management strategy in cotton (Hagan, 2014b). These applications can increase gross income by $120 per acre if applied early, otherwise they may be ineffective and the expense is wasted. One issue is that the infection starts at the bottom of the canopy and moves up and outward; making early diagnosis difficult unless a producer is walking his field (Price and Fromme, 2014). Though fungicides are effective at suppressing disease, they are costly at approximately $30/acre (Hagan, personal communication) with the caveat that no more than two concurrent applications of the same fungicide are applied. This is due to the ability of *C. cassiicola* to develop resistance to certain fungicides, which is a problem in the vegetable industry (Miyamoto et al., 2009). The fungus has been classified as high-risk regarding fungicide resistance, which has been identified in cucumber and lettuce (FRAC, Miyamoto et al., 2009).

Objectives

The objective of this study is to rate the 2017 RBTN field to determine if genotypic differences to target spot are observed at the field level. If differences are observed, the mist chamber protocol will be used to evaluate the highest and lowest performing varieties. The severity ratings from the mist chamber and the ratings from the field will be analyzed to see if they correlate and, if so, how well.

MATERIALS AND METHODS

2017 Field Experiments

In 2017, the Regional Breeders Testing Network (RBTN) experiment was grown as a RCBD with single row plots. It consisted of 34 varieties in eight replications and was planted at the Gulf Coast Research and Experiment Station in Fairhope, Alabama. The planting rate was 4 seeds per foot and plots were ten feet in length. The trial was planted on May 15th, 2017.

On August 29th, 3 liters of V8 broth *C. cassiicola* inoculum was diluted, and sprayed on the RBTN just prior to canopy closure. This was done to ensure disease presence within the field. A disease rating was done on just prior to inoculation on August 28th and again on September 8th, 2017, using a 1-10 ordinal rating scale by 0.5 intervals developed by Chiteka et. al. (1988), 1 to 10 leaf spot scoring system:

Rating Scale

1 = no disease,

2 = very few lesions in canopy,

3 = few lesions noticed in lower and upper canopy,

4 = some lesions seen and < 10% defoliation

5 = lesions noticeable and < 25% defoliation

6 = lesions numerous and < 50% defoliation,

7 = lesions very numerous and < 75% defoliation

8 = numerous lesions on few remaining leaves and < 90% defoliation,

9 = very few remaining leaves covered with lesions and < 95% defoliation, and

10 = plants defoliated

Three plants, one at the beginning, middle, and end of each row were singled out and visually assessed from the ground up. Lesions on only one of the three selected plants would result in a score of 1.5. If any defoliation was seen at all, the rating would automatically result in a rating of 4. The rating for the row was averaged from these three individuals.

The more tolerant and susceptible lines from this field trial will be selected to be used in the mist chamber protocol in order to investigate the differences in the results and to help validate it.

2018 Field Experiments

In 2018, the same 2017 RBTN trial was planted again at the Gulf Coast Research and Experiment Station in Fairhope, Al. It was planted on May 17th, 2018 and rated once which gave no indication of incidence. The plants were laid over by tropical storm Gordon on September 2nd, which resulted in abandoning the test.

The 2018 RBTN trial consisting of 25 varieties was planted at the Bretwon Agriculture Research and Experiment Station in Brewton, Al. While the entries differed from the 2017 trial, the design was the same – one row plots, ten foot rows, four seeds per foot, and eight replications. This field was not inoculated with *C. cassiicola* as in 2017, due to heavy disease presence early in the season. The field was rated four times using the same method and scale as 2017 – August 15th, September 9th, September 19th, and October 3rd.

Data analysis

Statistical analysis for the field trials were analyzed using a mixed model ANOVA with Tukey's mean separation in SAS 9.4. Comparison of greenhouse trials with field was analyzed with the PROC CORR command.

RESULTS AND DISCUSSION

For the 2017 RBTN field trial, large genotypic differences were observed post inoculation. Across the 34 varieties, the range in the severity was 3.99 -5.63%, with a mean of 4.81(Table 6). Mean separation was able to indicate large genotypic differences between the lines but not small differences. However, large genotypic differences were observed under natural infestation of the disease. Therefore 4 lines, the more tolerant LA 14063001 and NM 16-13P1088B were selected along with more susceptible Phytogen 499WRF and TAM LBB130218, for greenhouse trials.

The visual rating results of two trials at 4.0 x10^4 spores/ml in the mist chamber are presented in Table 7. Ratings, not comparing with the untreated check, ranged from 23.8 -91.9% severity. It was hypothesized that the field results would be mirrored in the mist chamber protocol, and it did trend in that direction. The regression analysis shows that based on the averaged ratings for two trial 3 and trial 4 mist chamber ratings, the values have a correlation coefficient of 0.99. This proves that given the four data points, the greenhouse and field are highly correlated. More data points will be needed to determine a more reliable consensus. The R^2 of 0.75 indicates that this regression can predict a rating with ~75% accuracy using the trend line.

The 2018 RBTN test was not inoculated using a V8 *C. cassiicola* broth but did show that Phytogen 499WRF as the more susceptible line. The rating scale was able to show large genotypic differences however it was unable to separate small differences. This is probably due to the fact that the RBTN genotypes are more advanced breeding lines, which possibly contain common parents, and therefore a lack of genetic diversity is

present within these lines. It would be more advantageous to screen a large group of very diverse material from the germplasm collection.

Genotypic variation can be consistently documented at the field level. However, with the potential for more than one disease to be present in the field, the Chiteka et. al. (1988) rating system may not be appropriate. Regrowth near the base of the plant is also not considered when using this scale. It would be interesting to evaluate the 2017 RBTN again under inoculated and noninoculated conditions to compare the results.

CONCLUSION

The ranking of genotypes evaluated in the mist chamber correlated with the rankings seen in the field, R=0.99. This suggests that the mist chamber screening method is performing as expected and can be used in conjunction with or in place of field screening. However, increasing the amount of genotypes in the mist chamber trial would further validate the protocol.

Future work to further refine the protocol, would be to determine the natural concentration of spores within a field. Also, a rating system that accounts for regrowth and disease complexes should be considered, as ratings could be falsely decreased due to green regrowth at the base of the plant giving the appearance of declining defoliation or falsely elevated when more than one disease is present within the field.

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Appendix 1

Table 1. Anova table for mean target spot ratings comparing 1.5x10^4 spores/ml with 4.0x10^4 spores/ml.

Source of Variance	df	F Value	Pvalue
Trial	1	79.07	<.0001
Genotype	8	5.14	0.0002
Trial*Genotype	8	4.65	0.0004

Table 2. Mean values for target spot ratings for 1.5x10^4 spores/ml.

Genotype	Rating
Phytogen 499WRF (control) untreated	0.00
LA 14063001	4.08
BCT2	5.25
Tam LBB130218	8.00
UA48	10.7
BCT3	14.3
Phytogen499WRF (control) Treated	17.1
BCT1	19.8
NM 16-13P1088B	25.0
	ns
Pvalue	0.341

Table 3. Mean values for target spot ratings for 4.0x10^4 spores/ml.

Genotype	Rating %	
Phytogen 499WRF (control) Nontreated	0.0	e*
UA48	27.5	d
BCT1	38.8	bcd
NM 16-13P1088B	45.0	bcd
LA 14063001	46.3	bcd
BCT2	51.3	bc
BCT3	61.3	b
Tam LBB130218	87.5	a
Phytogen 499WRF (control) Treated	93.8	a
LSD	20.5	
Pvalue	<.0001	

*Means followed by the same letter are not significantly different at P=0.05.

Table 4. Anova of two rounds of 4.0x10^4spores/ml with 8 replications in a greenhouse mist chamber.

Source of Variance	df	Fvalue	Pvalue
Trial	1	1.57	0.2143
Genotype	5	14.54	<.0001
Trial*Genotype	5	1.12	0.358

Table 5. Phenotypic differences for 4.0x10^4spores/ml in greenhouse mist chamber.

Genotype	Rating %	
Phytogen499WRF (control) Nontreated	0.00	d*
LA 14063001	18.24	c
UA48	29.69	bc
NM 16-13P1088B	34.69	b
Tam LBB130218	37.81	b
Phytogen499WRF (control) Treated	63.44	a
LSD	11.00	
Pvalue	<.0001	

*Means followed by the same letter do not differ at p=0.05.

Table 6. Target Spot ratings for 2017 inoculated RBTN trial conducted in Fairhope, AL.

Genotype	Rating %	
LA14063001	3.99	f[†]
LA14063038	4.06	ef
NM 16-13P1088B	4.13	ef
NM 13R1015	4.25	def
PD 08028	4.44	c-f
GA 2015090	4.46	c-f
LA14063046	4.50	c-f
Ark 0908-60	4.50	c-f
AU 90098	4.50	c-f
Tamcot G11	4.63	b-f
PD 09084	4.65	b-f
Ark 0912-18	4.67	b-f
TAM 13Q-51	4.69	b-f
Ark 0921-31ne	4.71	b-f
LA14063083	4.75	a-f
TAM 13Q-18	4.75	a-f
PD 07040	4.75	a-f
TAM WK-11L	4.81	a-f
TAM 13S-03	4.88	a-f
GA 2012141	4.88	a-f
GA 2015073	4.88	a-f
TAM LBB131001	4.94	a-e
DP 493 CK	4.94	a-e
Ark 0921-27ne	5.00	a-e
Acala 1517-08	5.00	a-e
Ark 0911-13	5.06	a-d
GA 2015032	5.06	a-d
LA14063101	5.17	abc
PD 2013016	5.31	abc
FM 958 CK	5.31	abc
PD 09046	5.44	ab
DP 393 CK	5.46	ab
TAM LBB130218	5.50	ab
PHYTOGEN499WRF (control)	5.63	a
Mean	4.81	
LSD (0.05)	0.66	

[*]Means followed by the same letter do not differ at p=0.05.

Table 7. Mean visual ratings for target spot damage to cotyledons across 2 rounds of inoculation at 4.0x10^4 spores/ml.

Genotype	Visual Rating %	
PHYTOGEN499 WRF (control) Nontreated	0	e*
UA 48	23.8	de
LA 14063001	35.6	cd
BCT 7701	37.5	cd
NM 16-13P1088B	41.3	bcd
BCT 7702	41.9	bcd
BCT 7703	46.3	bc
TAM LBB 130218	58.1	b
PHYTOGEN 499 WRF (control) Treated	91.9	a
LSD	18.9	

*Means followed by the same letter do not differ at p=0.05.

Table 8. Target spot ratings for 2018 RBTN trial under natural infestation in Brewton, AL.

Genotype	Rating %	
TAM LBB150107	2.13	i
16-13P1115	2.44	hi
PD2011081	2.56	ghi
TAM LBB150824	2.94	f-i
TAM 13S-03	3.13	f-i
UA 222	3.19	f-i
Ark 1005-35	3.25	e-i
PD2011026	3.25	e-i
Ark 1015-42	3.50	d-i
Ark 1005-41	3.50	d-i
PD2011021	3.63	c-i
TAM LBB150921	3.81	c-h
GA 2015024	3.88	c-h
TAM 12J-39	4.00	b-h
LA11309040	4.13	b-g
DP 493	4.31	b-f
LA14063075	4.44	b-f
MS 2010-87-5	4.50	a-f
LA14063083	4.81	a-e
Ark 1004-38	4.94	a-d
FM 958	4.94	a-d
GA 2012141	5.13	abc
PHYTOGEN499WRF	5.50	ab
Ark 1007-15	5.56	ab
DP 393	6.06	a
Mean	3.98	
LSD (0.05)	1.59	

Appendix 2

Figure 1. Regression of Field and Greenhouse ratings for 4 genotypes

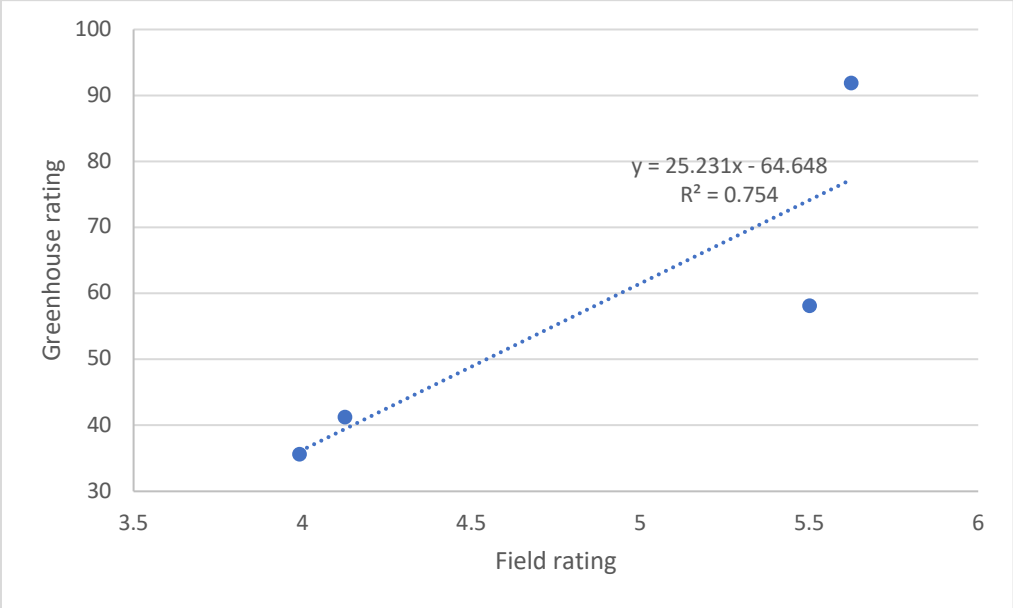


Figure 2. Mist Chamber

Figure 3. *Corynespora cassiicola* BRW1 isolate spores

Figure 4. Temperature ranges for mist chamber on a November day in 2018

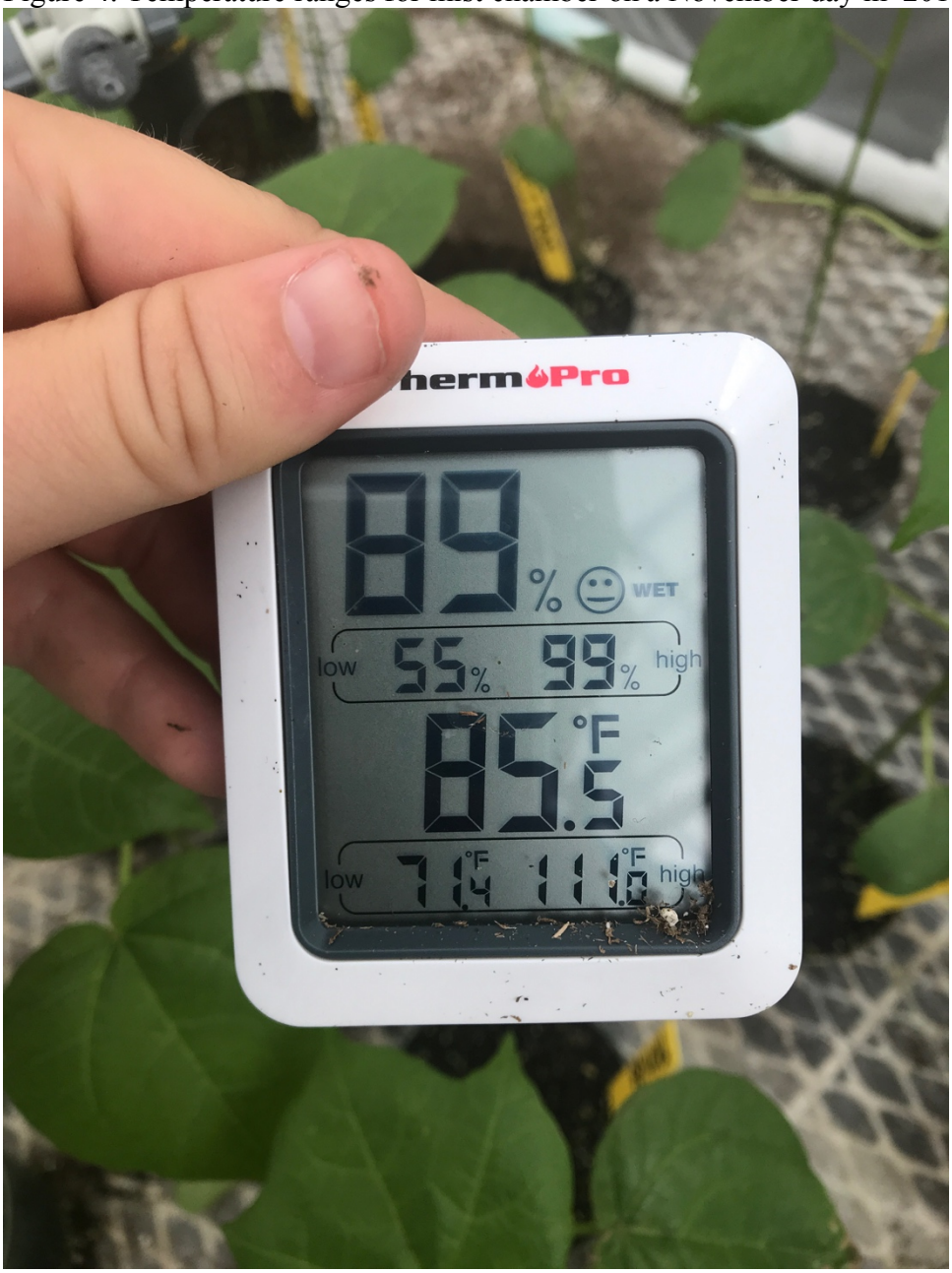


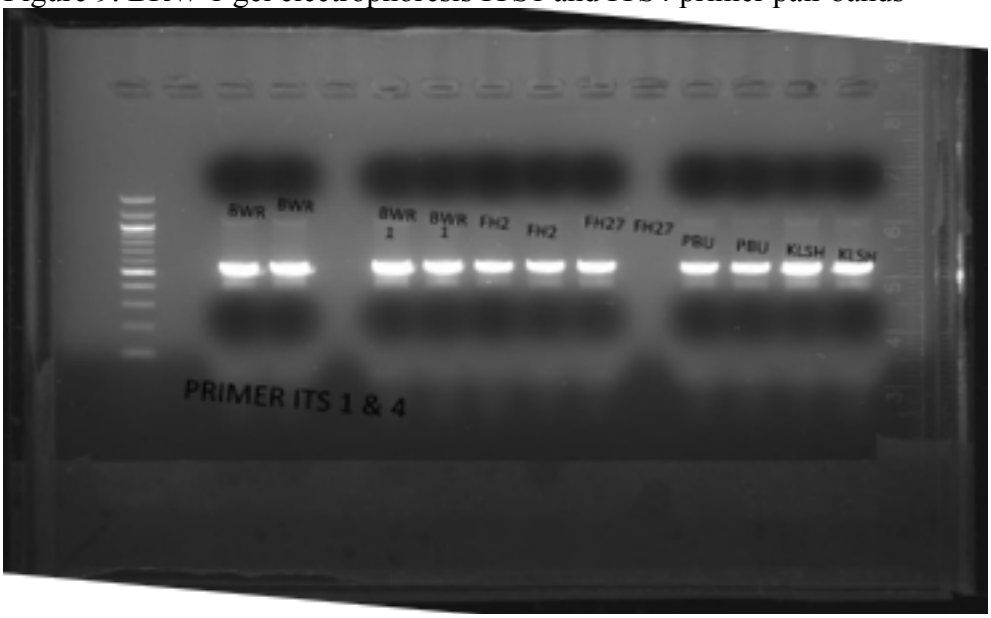
Figure 5. Phytogen499WRF plant ~2 weeks post exposure to 1.5x10^4 spores/ml inoculum

Figure 6. Target spot infested cotyledon leaf

Figure 7. V8 *Corynespora cassiicola* broth before blending – small colonies

Figure 8. V8 *Corynespora cassiicola* broth inoculum before blending – large colonies

Figure 9. BRW 1 gel electrophoresis ITS1 and ITS4 primer pair bands



Appendix 3

Protocol Development Troubleshooting

Which enclosure to use?

Growth chamber vs. PVC enclosure

There were three available enclosure options for this project; small PVC enclosures, a growth chamber, or a mist chamber. Initially, the growth chamber was chosen because it was readily available. However, it would not maintain a high enough RH to be used for target spot evaluation. Attempts to fix the growth chamber were made, but regardless of following the manufacturers troubleshooting protocols and replacing parts, the RH issue was never resolved. Secondly, enclosures are more cost efficient than buying a growth chamber. They are cheaper and can be quickly assembled. One of goals of this research is to produce a protocol that is applicable to a wide audience. As a result, 4 PVC enclosures were built and covered with translucent trash bags to maintain 80%+ RH.

PVC enclosure vs. mist chamber.

After multiple inoculations of Phytogen499WRF, it was determined that the enclosures were not large enough for the study. Target spot symptoms were not showing up on cotyledons before the first true leaf stage. This means that the plants were becoming too big for the enclosure at the time of final evaluation. Some leaves from plants around the borders were coming into contact with and sticking to the trash bag. Leaves touching the bag quickly became water logged, yellowed, and eventually senesced.

Enclosure can only be as big as the trash bag using this method. Furthermore, multiple separate PVC enclosures were used, meaning we could not guarantee RH and temp were identical across enclosures. Although no hard data was ever taken, it was obvious that some enclosures were maintaining moisture better than others. Trash bags were folded underneath enclosure to seal. An air tight seal was not guaranteed. Through opening and closing to access plants inside enclosure inadvertent tearing of the bag occurred in some cases. Many times, it was unknown that holes had formed. All in all, the process was cumbersome and annoying.

Mist chamber

In a field, a period of leaf wetness is very important alongside temperatures at or above 20 ℃ for the development of target spot lesions. This could be replicated using a mist chamber. A chamber was built, enclosed with plastic sheeting, and three mist heads were spaced evenly along the length of the chamber. The benefits of using this method as opposed to others are: All plants are being exposed to relatively the same environmental conditions because they are all in the same space. Leaf wetness can be controlled by mist set to a timer. The chamber could be almost completely sealed with no risk of tearing or unexpected holes. A door flap was created but could be sealed with clips.

However, some problems were evident. Temperature fluctuations occurred seasonally, with the chamber becoming incredibly hot during the summer months and cooler during the winter changing the time to onset of disease. Too much mist caused inoculum to run off and too little will not keep leaves wet enough. Both problems were manageable, so it was determined that the mist chamber should be used for the remainder of the studies.

Inoculum selection

V8 Broth - Inoculation of cotyledons

In preliminary studies, cotyledons were inoculated until runoff at 3,5 and 7 days post emergence. While no data was officially recorded, visually no apparent differences were noted among these intervals. Lesions did not form until first true leaf. After inoculation, the plants were placed in the enclosures with no additional water. The enclosure maintained such a high RH that watering was not necessary. Lesions occurred within a week. Visually, varying degrees of severity were observed among inoculum batches. Some Phytogen499WRF subjects were entirely necrotic, while others showed visible lesions with green tissue still remaining.

Inoculation of Field using V8 Broth.

10 250mL batches were combined into 2L bottles and sent to Fairhope AL. V8 broth was diluted by the research station to a volume that would cover the entire field. The concentration of the final spray is unknown. Since target spot requires specific environmental conditions for natural infection, it was decided that the field should be inoculated to ensure disease presence within the field. Field ratings were taken before and after inoculation.

The V8 broth has many pros. It can be produced in large batches; enough to cover a field with the limiting factor in broth production being shaker table space. It adheres to leaves. There is no real risk of the inoculum running off of the plant. With leaf wetness being of critical importance, an inoculum that remains on the leaf without the risk of running off with mist is of extreme importance. Lastly, *C. cassiicola* broth can be stored and will continue to produce disease for weeks. In this study, plants continued to show

signs of disease even after the broth was stores in a refrigerator for four weeks. However, whether stored inoculum maintained its original virulence over time is unknown. The recommended storage time is one week (K. Lawrence, personal communication).

However, the cons of using this form of inoculum outweigh the benefits. Because this is a blended inoculum made of only mycelium and V8, the concentration is unknown. Comparisons cannot be made between tests. The only suggested way to determine concentration is to assess colony forming units (CFU) which adds extra time to an already lengthy production schedule. The broth contains no spores and is not considered a primary source inoculum. Primary inoculum is considered to be the original source of disease while secondary inoculum is the inoculum produced from the primary symptom.

Isolate Preservation for Spore Suspension

Filter paper preservation

A single spore isolate from a leaf is plated on potato dextrose agar (PDA) and allowed to grow for 10-14 days. If no contamination is present, a sample of this isolate is cut from the PDA plate and placed onto a new plate. The cutting is then surrounded by sterile pieces of filter paper. After about one to two weeks the fungus has grown over the filter paper. The papers are removed and placed in a sterile petri dish to dry. These papers can be a source for future plating.

Slant tube preservation

How much PDA is made for this task depends on how many tubes are needed. The manufacturers label is followed when creating the mix. The mixture is then microwaved at 15 second intervals until the PDA is dissolved completely in the DI water. Fifteen mL of PDA solution is poured into test tubes until no more PDA solution

remains. The tubes are then autoclaved for 30 minutes. Once removed from the autoclave the racked tubes placed at an angle, about 45°, until solidified.

A section from the single spore isolation PDA plate, as described above, is cut and placed fungal side down onto the middle of the slant. The tubes are incubated at 29°C for five days before being removed from the incubator and placed in the refrigerator for storage.

Spore suspension production

The benefits of using this method of production are that a very accurate concentration may be determined. By making a 1.5x10^4 spores/ml solution and a 4.0x10^4 spores/ml solution differences can be observed. Using this method an optimal concentration may be discovered that does not kill the plants while still producing disease. Each plant will be exposed to the same level of inoculum ensuring that comparisons can be made.

However, this method is laborious. Even with tween being used as a surfactant, special care must be taken to make sure the mist does not cause the inoculum to drip off the plants. A drying time of five minutes before being exposed to mist was used to combat this issue. Secondly, it is hard to make the suspension in large batches, so field analysis using a spore suspension is unlikely.

After careful consideration, it was determined that the spore suspension was the only reliable form of inoculum. After successfully producing disease at both 15k spores/ml and 40k spores/ml, the V8 broth was no longer used. Disease was present without damaging the entire plant. A visual severity rating was used for evaluation.

Mist Chamber Settings

The mist chamber consisted of a basic 1" PVC frame with three even spaced misting heads hanging 18 inches from the top of the PVC frame. A door flap was created for easy access into the chamber. The flap was kept sealed using three extra-large binder clips.

Mist is sprayed for one second every ten minutes. This keeps the leaf wet but with minimal water droplet runoff from leaf. The leaves will be nearly dry by the time the next round of mist is sprayed. This is done for the first 3 days post inoculation.

Alternative timing that was been attempted:

10 seconds every 30 minutes = runoff and complete drying of leaves

5 seconds every 15 minutes = runoff and complete drying of leaves

2 second every 10 minutes = runoff

1 second every 8.5 minutes = runoff

Planting method

Jiffy pellets were initially used during the PVC enclosure portion of the preliminary experiments. Jiffy pellet trays can hold a large number of plants. Little to no labor is required. The pods are sprayed with warm water, slowed to swell, and then seeds are inserted into the pod. Cleanup is easy, as the entire tray may be thrown away upon completion of the experiment.

However, the pods are oriented very close together. As the plants grow, the leaves begin to overlap and touch each other. This is detrimental to the experiment as the leaves that begin to overlap trap water between them causing disease to manifest at a rapid rate. This does not give a reliable representation of target spot severity.

A similar situation can be observed with using 3X4 inserts. While many can be filled at one time the plants grow too close together and begin to overlap and stick. Inserts would be a good option for evaluation on very small plants at the cotyledon stage, but since disease does not seem to present itself until the first true leaf stage, it was determined that both Jiffy Pods and inserts would be unacceptable for this test.

4" pots proved to be the most reliable planting container. Each plant has its own pot separate from other plants. Subjects may be oriented within the mist chamber at a distance suitable for disease evaluation. There is no worry of overlapping leaves. However, it takes much more time to fill individual pots than it does to fill inserts or use Jiffy Pods. Also, fewer individuals can fit within the mist chamber. It was determined that given the experimental design for this test, 4" pots would fit, but the test could not become any larger.

Selecting an isolate for testing

Initially, FH27, an isolate out of Fairhope, Alabama, was chosen for the study. The isolate produced spores at ten days and contained more spores per plate than other isolates that were available. In theory, less plates would need to be inoculated since FH27 performed so well, saving time and resources. However, halfway through mist chamber experiments this isolate "crashed" and failed to produce spores time and time again. This isolate was preserved on filter papers only, not on slant tubes. The isolate would produce limited growth with very few spores (sometimes none) on QPDA, grew on PDA but failed to produce spores, and would produce no growth on APDA.

It was suggested by Dr. Bowen through personal communication that reisolating FH27 off of plant tissue may work as a last effort to save the isolate. This would require

producing only a small amount of spores and exposing a plant to them. This was achieved, a Phytogen499WRF seedling was inoculated, placed in the mist chamber, and within 11 days lesions were present. Spores were isolated from the lesions and transferred back to QPDA. The isolate continued to perform poorly even after this. New QPDA plates were made to eliminate the potential that the plates were unfit for growth. Even with new plates, FH27 failed. It was decided that a new isolate would be selected.

After attempting and failing to obtain enough spores from isolates KSL4 and PBU1805 it was discovered that isolates preserved on filter paper were the problem. All isolates preserved on paper failed to grow, while older isolates maintained on slant tubes continued to spore on QPDA. This indicates that filter papers need to be recultured more often than slant tubes, at least for these isolates. However, it was determined that all filter paper isolates would be thrown away. At this point, BRW1, another isolate that consistently produced many spores, was selected. It was never preserved on filter paper. Instead, more slant tubes were made of BRW1 to ensure enough of the isolate was available to complete the experiments. BRW1 was confirmed visually to be C. *cassiicola,* and DNA analysis was also completed. The result was a 99% match to known *C. cassiicola* DNA.