FUNGICIDE SENSITIVITY OF *CORYNESPORA CASSIICOLA* AND ASSESSMENT OF MANAGEMENT OF TARGET SPOT OF COTTON IN GEORGIA

by:

MA. KATRINA SHIELA E. LAUREL

(Under the Direction of Robert C. Kemerait, Jr.)

ABSTRACT

Target spot, caused by *Corynespora cassiicola,* is a serious foliar disease of cotton in the southeastern United States. Baseline (current) isolates of *C. cassiicola* were tested for sensitivity to metconazole (DMI), fluxapyroxad (SDHI) and pyraclostrobin (QoI). Further work compared fungicide sensitivity of *C. cassiicola* isolates from cotton to isolates from other hosts. Field experiments were conducted to establish a relationship between fungicide sensitivity in laboratory experiments and fungicide efficacy in managing target spot on cotton. Based on the sensitivity distribution, all isolates tested were considered sensitive to fungicides. However, these sensitivities varied among isolates offering an early indication that resistance can happen in the future. Additionally, all fungicides reduced disease severity and premature defoliation; however, Priaxor (pyraclostrobin + fluxapyroxad [QoI + SDHI]) proved to be most effective. Results from this study can help optimize fungicide sensitivity monitoring practices in an effort to improve fungicide use patterns for optimum disease management.

INDEX WORDS: *Corynespora cassiicola*, Cotton, DMIs, Fluxapyroxad, Fungicide Resistance, Metconazole, Pyraclostrobin, QoIs, SDHIs, Target spot

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# DEDICATION

To my loving mother, Marsha, who single-handedly raised us with so much love and wisdom, to whom I owe so dearly for all that I am and all that I aspire to become. To my siblings, Kiefer and Krystal, your encouragements kept me going and has meant a lot to me. To my grandmother, Mama Clemen, for all the love and support.

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# CHAPTER 1

## INTRODUCTION AND LITERATURE REVIEW

Cotton (*Gossypium* sp.) is affected by a number of important diseases that limit production in all locations where cotton is grown. As a subtropical to tropical crop that is grown over a wide range of latitudes, as well as a perennial plant grown as an annual crop, cotton is often under stress that may exacerbate specific disease problems (Rothrock et al., 2015). In Georgia, this crop is grown typically from May to October and can be affected by a number of fungal and bacterial diseases that can impact yield and lint quality. Use of fungicides to control foliar fungal diseases of cotton in the United States is a recent development. More research is needed to better understand the best strategies to increase profitability with these applications.

There are four domesticated species of cotton, to include *G. arboreum* L. and *G. herbaceum* L. which are both diploid and native to the Old World, and *G. barbadense* L. and *G. hirsutum* L., both allotetraploid, which evolved in the New World (Lee et al., 2015). *Gossypium arboreum* remains an important crop in India, while *G. herbaceum* is grown today for local use in the drier areas of Africa and Asia. *Gossypium barbadense*, also known as extra-long-staple, Egyptian and Pima cotton, supplies about 3 to 5% of the current world production of fiber. This type of cotton is mostly for the production of luxury fabrics and sewing thread (Lee et al., 2015). It is favored for some purposes due to its long, strong, and fine fibers; however its relatively low yield has limited its importance in the total world production. *Gossypium hirsutum*, commonly known as upland cotton, contributes about 95% to the current world production of 118 million bales of fiber where there are about 225 kg lint/bale of cotton (Lee et al., 2015). Upland cotton

fibers are used in the manufacture of variety of textile products, cordage and other non-woven products. Modern upland cultivars are high-yielding, day-length neutral, early-cropping plants, with easily ginned, abundant fiber (Wendel et al., 1992).

The United States ranks third in the world for cotton production, following China and India, respectively (USDA-ERS, 2016). Georgia remains the second largest cotton producing state, trailing behind Texas in acres and production. According to the National Agricultural Statistics Service (NASS-USDA), the area planted to cotton in the U.S. (2016) was estimated at 10.0 million acres, with upland cotton planted to an estimated 9.82 million acres. In Georgia, 1.18 million acres of upland cotton was planted in 2016 and was valued at $749 million USD. Production of cotton occurs in 17 states in the southern half of the United States, to include Alabama, Arkansas, Arizona, California, Florida, Georgia, Kansas, Louisiana, Mississippi, Missouri, New Mexico, North Carolina, Oklahoma, South Carolina, Tennessee, Texas and Virginia. Major concentrations of cotton production include areas of the Texas High and Rolling Plains, the Mississippi, Arkansas and Louisiana Deltas, southern Georgia and California's San Joaquin Valley (USDA-ERS, 2016).

Cotton is a typical woody perennial shrub. The plant simultaneously develops its vegetative and flowering, or reproductive, structures (Mauney, 2015). To fully understand the productive capacity of cotton, it is important to understand its structures. The vegetative axis is composed of a tap root and vertical stem with alternate leaves which continues to produce nodes as long as temperature and moisture allow. The leaves on the vegetative branch arrange themselves with a 3/8 phyllotaxy. Fruiting branches result from the transformation of these initially vegetative meristems into flower primordia. Flowering branches are sympodial and arise from the first, sometimes second, axillary branch position at leaves above about the fifth node on

the stem (Mauney, 2015). The differentiation of the flower begins with formation of a whorl of three bracts after the first true leaf has developed. The floral differentiation consumes the meristem of the axis, subsequently; the axis terminates with the flower. Afterwards, the primordium develops a prophyll, an internode, a true leaf and a terminal flower. A thorough understanding of how a cotton plant grows can aid in choosing better management practices to produce maximum yields (Deterling & El-Zik, 1982).

The importance of leaves in crop productivity should not be overlooked. Sustaining healthy leaves and boll retention are crucial in cotton production. The quality of cotton and yield benefit when healthy, young leaves are maintained on the plant (Oosterheis et al., 1990). There are three categories of cotton leaves; the cotyledons or seed leaves, the main-stem leaves and the subtending leaves. Cotyledons serve as storage tissues that feed the developing shoot tip and root system. Cotton maturity can be severely delayed if both cotyledons are lost within the first week after emergence (Oosterheis et al., 1990). Main-stem leaves feed the developing terminal, branches and bolls of the cotton plant. The subtending leaves, attached to the fruiting branches, are critical for boll set and filling. Like the nearest main-stem leaf, the subtending leaf provides most nutrition to the young boll. If the subtending leaf is damaged or shaded, that young boll is more likely to be lost as it is most vulnerable to shedding during the early stages of growth (Oosterheis et al., 1990).

Cotton fibers grow inside of a cotton boll. Cotton fiber is a highly elongated and thickened single cell of the seed epidermis and is considered the most important agricultural textile commodity in the world (Haigler et al., 2012; Wakelyn et al., 2010). Upland cotton includes a number of varieties and cultivars with different fiber length and tolerances to different growing conditions. Upland cotton has fibers that range in length from about 7/8 to 15/16 inches

(2.22 to 2.38 cm). The short fibers removed from seeds before crushing are known as linters, and they are an important source of industrial cellulose (Lee et al., 2015). Although cotton is grown mostly for fiber, the seeds are also important. Seeds are pressed to make oil used for culinary purposes and its residue, being protein-rich, serves as feed for ruminant livestock (Lee et al., 2015).

Production potential of cotton may be limited by a number of important foliar diseases. Most are caused by fungal pathogens, but a bacterial pathogen *Xanthomonas citri* subsp. *malvacearum* is also important (Rothrock et al., 2015; Hillocks, 1992). In the United States, an estimated 8 to 17% of yield was lost to diseases and nematodes in 2015. (Rothrock et al., 2015). Foliar diseases caused by fungal pathogens are frequently detected in cotton; however, the impact of these diseases varies by geographical location (Rothrock et al., 2015). Among the foliar diseases, bacterial blight caused by *Xanthomonas citri* subsp. *malvacearum* (Schaad et al., 2006), Stemphylium leaf spot caused by *Stemphylium solani* (G.F. Weber) and target spot caused by *Corynespora cassiicola* (Berk. & M.A. Curtis), can cause significant yield loss and are considered important diseases of cotton. Correct diagnosis of foliar diseases of cotton is necessary to effectively manage the disease.

***Corynespora cassiicola***

*Corynespora cassiicola* (Berk & M.A. Curtis) C. T. Wei, the cause of target spot, an emerging disease on cotton in the southeastern United States, is a ubiquitous plant pathogen causing major problems on many crops with high economic importance. The fungus is commonly found in tropical and subtropical regions. It is widely diverse in the utilization of substrates and has a very broad host range (Dixon et al., 2009). The most studied plant disease caused by *C. cassiicola* is Corynespora leaf fall on rubber (Silva et al., 2003).

Taxonomically, *C. cassiicola* belongs to the kingdom Fungi, phylum Ascomycota, class Dothideomycetes and order Pleosporales which contains other known plant pathogens like *Alternaria*, *Pyrenophora* and *Cochliobolus*. On the basis of morphological characteristics, the fungus was initially classified as *Helminthosporium*, as both genera have similar conidial structure. However, subsequent phylogenetic analyses revealed that the genus *Helminthosporium* belongs to the family *Massarinaceae* while the genus *Corynespora* is revealed to be polyphyletic (Voglmayr and Jacklitsch, 2017). *Corynespora cassiicola* is closely related to and in the same clade as *C. smithii* but does not show a clear relationship to any other currently established families (Schoch et al., 2009). *Corynespora cassiicola* is known to produce asexually via conidia. Unlike other fungal pathogens, its sexual structure has not been documented and its life cycle is poorly understood.

Species belonging to the genus *Corynespora* are characterized by conidia produced either solitarily or acropetally in chains and are variable in shape, obclavate to cylindrical, straight to curved. The conidia can be subhyaline to pale olivaceous-brown or brown, several-celled or pseudoseptate. Conidial measurements range from 40 – 220 µm long (up to 520 µm in culture), 9 – 22 µm thick in the broadest part, 4 – 8 µm wide at the truncate base. The spore's hilum is often dark with a slight rim, having a thick, colorless exospore with dark prominent basal scar (Schoch et al., 2009; Barnett and Hunter, 1998). Its conidiophores are erect, simple or occasionally branched, straight or slightly flexuous, smooth, septate, and 10 – 85 µm long, and 4 – 11 µm thick (Barnett and Hunter, 1998). Characteristics specific to *C. cassiicola* in culture include effused colonies of either grey or brown color, thinly hairy, and when viewed under a binocular dissecting microscope, the conidiophores appear iridescent (Ellis et al., 1971).

**Diseases Caused by *Corynespora cassiicola***

Symptoms of diseases caused by *C. cassiicola* are typically found on the foliage of the plant. *Corynespora cassiicola* has a broad host range which is able to infect at least 530 different plant species (Dixon et al, 2009). *Corynespora cassiicola*, was first reported as a pathogen on cotton in Mississippi in 1961 (Jones, 1961). *Corynespora cassiicola*, causative agent of target spot, affects numerous hosts worldwide including fruits, vegetable crops and ornamental plants. Specific hosts include papaya (*Carica papaya* L.), tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*), cowpea (*Vigna unguiculata*), soybean (*Glycine max),* patchouli (*Pogostemon cablin* Benth) and *Hydrangea* spp. (Bala, 1993; Koenning et al., 2006; Blazquez, 1972; Blasquez, 1967; Boosalis et al., 1957; Chase, 1993; Chase et al., 1986; Chase, 1984; Hansen et al., 1994; McGovern, 1994; McMillan et al., 1995; Miller, 1974; Chen, 2010; Raffel et al., 1999).

Previous studies have also shown tremendous variations in the host range for individual isolates of *C. cassiicola*. Some isolates were shown to have a wide host range beyond their host of origin. In a study conducted by Dixon et al. (2009), a pair of related isolates from cucumber could infect 2-5 other host species. In contrast, some isolates were specific to their host of origin. Host specificity among lineages and/or isolates has also been observed on *C. cassiicola* isolates from other hosts such as papaya (Dixon et al., 2009). In pathogenicity tests conducted by Sumabat et al. (2015), 32 *C. cassiicola* isolates collected from different hosts were tested for pathogenicity on cotton, soybean, tomato and cucumber resulting in significant differences in virulence. Isolates originally from cotton were more aggressive on cotton than were isolates from other hosts. Based on the study, *C. cassiicola* was shown to be more aggressive when inoculated to the same host of origin. All isolates from cotton were most aggressive on cotton, but were less

aggressive on other inoculated hosts. This was also seen on tomato and soybean. This suggests that the *C. cassiicola* populations causing emerging diseases in the southeastern U.S. are likely to be host-specialized.

Target spot of cotton was first reported in Mississippi in 1961 (Jones, 1961). The fungus *C. cassiicola*, was identified as the pathogen causing the disease (Jones, 1961). The disease was not reported again in the United States until it was observed in southwest Georgia in 2005 (Fulmer et al., 2012). The disease produces leaf spot lesions that first appear as brick red dots which may expand to form larger concentric rings of alternating dark and light tan bands. Symptoms initially occur on mature mainstem and subtending leaves found in the lower canopy. In severe infestations, lesions may be noted in the upper canopy as well. Symptoms may also be found on the bracts and bolls (Raper & Young-Kelly, 2016). Premature defoliation starting in the lower canopy can rapidly occur given the right environmental conditions conducive for target spot (Kelly, 2016). Target spot can cause premature defoliation of up to 70% and a 200lb/A lint loss (Fulmer et al., 2012). Premature defoliation from other causes has been found to reduce yield and alter fiber quality (Burmester, et al.,2009).

*Corynespora cassiicola* has been found to cause diseases of cotton elsewhere in the world. In 2013, cotton plants infected with irregular to circular concentric rings of alternating light and dark brown bands were observed in Sanya, Hainan Province, China (Wei et al., 2014). Symptoms as well as fungal isolates obtained from symptomatic leaves were consistent with the description of *C. cassiicola*. Target spot and other fungal diseases on cotton, are major production limiting factors in China (Wei et al., 2014). In Brazil, target spot was first reported on cotton in the state of Mato Grosso, in 1995 (Mehta et al., 2005). In recent years, target spot has spread over the cotton growing regions in Brazil, causing heavy yield losses (Galbieri, et al.,

2014). In India, *C. cassiicola* pathogenic to cotton bolls was observed as early as 1988 (Lakshmanan et. al, 1990). Target spot continues to receive widespread attention in the United States as outbreaks have been documented in other cotton producing states such as Alabama (Conner et al., 2013), Louisiana (Price et al., 2015b), and Tennessee (Butler et al., 2016). Target spot has now been observed in almost every cotton producing state in the southeastern and the mid-south region of the USA.

Effect of cotton foliar diseases on fiber quality has been poorly documented; however, research on cotton leaf curl virus concluded that the disease had overall adverse effect on plant growth, yield and fiber quality, with a 4.5% decrease in fiber length (Ahmad et al., 2002). Foliage injury or complete leaf removal indirectly affected yield by reducing leaf area that provides photosynthates to mature bolls. In a similar research on cotton leaf curl virus, Mahmood et al. (1996) reported that in cotton cultivars, the average reduction in fiber length and fiber strength were 3.4% and 0.7% respectively.

An understanding of the development of the disease affecting leaves of the cotton plant is essential in order to develop best management practices. The disease cycle for *C. cassiicola* begins with the source of primary inoculum, which could be conidia in the soil, infested crop residue, or other host species. Primary infection typically occurs on the older leaves low in the canopy where leaf spots first appear. The disease then spreads upward through the canopy towards the shoot tips (Rothrock et al., 2015; Hagan and Sikora, 2012). Initial infection is likely the result of spores splashed from debris on the soil or spread from other diseased plants. The disease progress as conidia produced from target-like lesions serve as a source for secondary inoculum of *C. cassiicola*. Prolonged periods of leaf wetness are critical for the development and spread of target spot (Rothrock et al., 2015). Frequent showers or irrigation will contribute to an

increase in disease whereas prolonged periods of dry weather patterns should slow disease spread (Hagan and Sikora, 2012). Conditions of extended periods of leaf wetness and high humidity are typical of the cotton growing season in Georgia. Coupled with a dense canopy, these factors can lead to development and spread of foliar diseases such as target spot (Whitaker et al., 2018).

Estimated yield losses in select cultivars exceeded 336kg/ha seed cotton (Conner et al., 2013). Predicted losses, estimated at 5%, would cost $70 million in Alabama and Georgia and losses of 40% in a grower's field would be devastating to the economies of these cotton producing states (Conner et al., 2013; Hagan, 2014).

**Management of target spot**

**Resistance:** While no varietal host resistance has been identified, target spot seems to be more severe on some cotton cultivars than on others. There are no known cotton cultivars that are resistant to target spot, although previous studies show that some cotton varieties like PHY 499 WRF were more susceptible to target spot than was variety DP 1050 B2RF (Hagan, et al., 2013). In addition, multistate trials conducted over a three year period (FL, GA, LA and VA in 2014; AL, FL, GA, LA, MS, TN and VA in 2015; AL, FL, GA, LA, MS and TN in 2016) revealed disease incidence and defoliation were greater on PHY 499 WRF than DPL 1137 B2RF (Mehl et al., 2017). Still, more studies are needed to compare susceptibility among other cotton varieties grown in the southeastern US.

**Cultural practices:** *Corynespora cassiicola* can survive in crop debris (Rothrock et al., 2015). For this reason, growers can take steps to manage the diseases by cultural and production practices such as rotating fields away from cotton production, destroying crop residue, by burying the debris, and by managing "volunteer" cotton plants that may grow between seasons

(Rothrock et al., 2015). Such steps should help to reduce the amount of primary inoculum for the next growing season.

Cotton is an indeterminate crop that grows vegetatively and reproductively at the same time (Mao et al., 2014). Excessive vegetative growth often results negatively on the yield of the crop (Eaton, 1955). Plant growth regulators are used extensively in cotton production to inhibit the negative effects of excessive vegetative growth on the indeterminate crop (Mao et al., 2014). This allows for cotton to produce sufficient vegetative growth to support fruiting bodies without allowing the plant to become rank. Various chemistries can be used but the most commonly utilized chemical worldwide is mepiquat chloride (Dodds et al., 2010). Mepiquat chloride is a gibberellin biosynthesis inhibitor that is known to control morphological growth by reducing leaf area, controlling plant height, and reducing internodes (Mao et al., 2014). The result is a more compact plant that allows adequate penetration of light into the canopy (Reddy et al., 1990). Preliminary reports suggest that reduction in humidity and extended periods of leaf wetness are useful in the management of target spot (Hagan, 2014). Growers can use growth regulators to manage excessive growth in the cotton field. This will allow for increased airflow in the canopy and reduction of humidity and periods of leaf wetness (Kelly, 2016).

**Chemical foliar treatments:** The use of fungicide applications is relatively new in the management of foliar diseases of cotton in the United States. Until recently, use of fungicides had been primarily for the management of southwestern cotton rust; however use of foliar-applied fungicides could become more widespread in the United States as research efforts continue on the management of target spot and as additional fungicides become available (Hagan, 2014).

Fungicides from different chemical classes are approved for use on cotton in the U.S. and include demethylation inhibitors (DMI), quinone outside inhibitors (QoI), and succinate dehydrogenase inhibitors (SDHI) (Fungicide Resistance Action committee [FRAC] Code 3, 11 and 7 respectively).

The demethylation inhibitors (DMI) group of fungicides (FRAC Code 3) target sterol 14α-demethylase CYP51, an important enzyme in the ergosterol biosynthetic pathway. These fungicides render the CYP51 catalytically inactive which prevents the demethylation of lanosterol and eburicol, thereby inhibiting the production of ergosterol. Ergosterol is a sterol, necessary to maintain fungal membrane fluidity and permeability (Köller 1992; Price et al., 2015a). Use of DMI fungicides leads to the disruption of membrane structure and prevents active membrane transport. This is due to a combination of two factors: the depletion of ergosterol in the cell and the accumulation of 14α-demethylated sterols, resulting in fungistasis (Price et al. 2015a). DMI fungicides are used elsewhere to control diseases caused by *C.* cassiicola. In tomato, the mixture of DMI difenoconazole (FRAC Code 3) + amino acid and protein synthesis inhibitor cyprodinil (FRAC Code 9) (Inspire Super; Syngenta Crop Protection, LLC) is registered to control target spot (Paret et al., 2015). In Brazil, the mixture of DMI prothioconazole (FRAC Code 3) + quinone outside inhibitor trifloxystrobin (FRAC Code 11) (Stratego YLD; Bayer Crop Science) is registered to control target spot in soybean (Xavier et al., 2013).

The quinone-outside inhibitor (QoI) fungicides (FRAC Code 11) are important in the management of target spot. They inhibit mitochondrial respiration by binding to the quinol oxidation site of the cytochrome $bc_1$ enzyme complex, blocking electron transfer between the cytochrome *b* (cyt *b*) and cytochrome $c_1$; a process that halts the production of ATP resulting in

an energy deficiency in fungal cells (Bartlett et al., 2002; Fernández-Ortuño et al., 2008; Gisi et al., 2002). However, they are considered to be at high risk for resistance development. Azoxytrobin (Quadris; Syngenta Crop Protection, LLC) as well as premixed azoxystrobin (FRAC Code 11) + tetraconazole (FRAC Code 3) (Quadris Top; Syngenta Crop Protection, LLC) are both registered for control of target spot on tomato (Paret et al., 2015). Quadris Top is also registered for control of target spot in soybean (Allen and Irby, 2017).

The succinate dehydrogenase inhibitor (SDHI) fungicides (FRAC Code 7) inhibit fungal respiration through the inhibition of the enzyme succinate dehydrogenase (SDH, also known as complex II) in the mitochondrial electron transport chain (FRAC 2016). SDH consists of four subunits, the hydrophilic flavoprotein (SdhA), the iron-sulfur protein (SdhB), and two lipophilic transmembrane C- and D-subunits (SdhC and SdhD). Studies on the molecular mechanisms responsible for the resistance to SDHI fungicides have shown that mutations which lead to amino acid substitutions in the SdhB, SdhC or SdhD subunits of SDH confer laboratory resistance (Matsson et al., 1998; Matsson & Hederstedt, 2001). Simply put, fungicides in the SDHI group bind to the ubiquinone binding site (Q-site) of the mitochondrial complex II and thus inhibit fungal respiration. SDHI fungicides are also registered to control target spot on other hosts. Boscalid (FRAC Code 7) (Endura; BASF Corporatio, Research Triangle Park, NC) is used on tomato (Paret et al., 2015) and cucumber (Miyamoto et al., 2009).

Field trials conducted in 2012 at multiple locations in southwestern Georgia and Virginia confirmed that disease suppression and yield increases can be obtained with single or multiple applications of labeled fungicides (Walls et al., 2012). Results from small plot trials in Georgia demonstrated that two applications of a DMI QoI premix of metconazole and pyraclostrobin (Twinline; BASF Corporation, Research Triangle Park, NC) or pyraclostrobin alone (Headline;

BASF Corporation, Research Triangle Park, NC) during the first and third week of bloom provided the most consistent suppression of disease intensity (Walls et al., 2012). Based on a multi-year regional evaluation of one and two applications of registered and experimental fungicides, it appeared that fungicides (Headline SC, Priaxor, Quadris and Topguard) delayed disease progress and reduced overall defoliation. However, yield increases with fungicide applications were infrequent (Mehl et al., 2017). More studies are needed to evaluate the efficacy of fungicides and to determine the best timing applications to protect cotton from target spot triggered yield loss.

**Fungicide Resistance**

The repeated large scale use of fungicides having a similar mode of action places selection pressure on the pathogen population that can lead to fungicide resistance (Brent and Hollomon, 2007a). Fungicide resistance is the acquired and heritable reduction in sensitivity of a fungus to a fungicide that occurs as a result of the selection of insensitive members within a population (FRAC 2016). Practical resistance occurs when a pathogen population has shifted to one that is predominantly resistant, leading to disease control failures after application of the recommended dose of a fungicide. This process may develop in either a quantitative or qualitative manner (Brent and Hollomon, 2007a; FRAC 2016). Quantitative, or multi-step resistance, is a process where multiple mutations occurring in the target site result in a gradual shift from sensitivity to insensitivity in the pathogen population over several years (Brent and Hollomon, 2007a; FRAC 2016). Qualitative or single-step resistance on the other hand occurs as the result of a single mutation in the target site which leads the sudden loss of product efficacy as two sub-populations develop with vastly different sensitivities to a given fungicide (Brent and Hollomon, 2007a; FRAC 2016).

As growers turn to fungicides for control and management of foliar diseases on cotton, sensitivity monitoring programs can be useful for detecting changes in the frequency of less sensitive or resistant isolates before control failure occurs. Establishment of baseline sensitivity is the first step of fungicide sensitivity monitoring program and fungicide resistance management strategies (Russell, 2004). Baseline is defined as a profile of the sensitivity of the target fungus to the fungicide constructed by using biological or molecular biological techniques to assess the response of previously unexposed fungal individuals or populations to the fungicide (Russell, 2004). For some fungicides, sensitivity monitoring is done by germination tests and, for QoIs, by PCR tests for the G143A mutation (Brent and Hollomon, 2007a). Common techniques include mycelial growth inhibition, spore germination assays and germ tube elongation assays (Russell, 2004). To obtain the fungicide sensitivity data, in vitro assays are conducted to determine the 50% effective fungicide concentration ($EC_{50}$) for each isolate. The $EC_{50}$ value corresponds to the dose that reduces the growth of mycelium or spore germination to a value of 50% for each isolate (Russell, 2004). Monitoring allows for detection of impending fungicide resistance situation. This is a vital area of research; all of knowledge of the distribution, evolution and impact of resistance in the field relies heavily on monitoring (Brent and Hollomon, 2007a).

Although not yet reported in *C. cassiicola* from cotton, shifts in sensitivities to fungicides from DMI, QoI and SDHI groups have been reported in isolates from other hosts for target spot as well as in other closely related fungal species. Demethylation inhibitor fungicides are thought to have a medium risk for resistance developing (FRAC 2016). Practical resistance to the DMIs has been observed in 17 fungal species and tends to develop as a result of target site mutations in the *cyp51* gene, overexpression of *cyp51*, or reduced intracellular fungicide accumulation due the activity of efflux transporters (Price et al., 2015a; Ziogas and Malandrakis, 2015; FRAC 2016).

Similar to other fungicides, point mutations in the *cyp51* gene that lead to amino acid substitutions are the most frequently observed cause of resistance (Price et al., 2015a). Although DMI resistance on cotton has not yet been reported, DMI resistance has been documented for over 30 diseases including powdery mildew, apple scab and brown rot of stone fruit. In one study, resistance of *C. cassiicola* isolates from soybean to carbendazim (MBC, FRAC Code 1), was observed in samples collected from Mato Grosso, Brazil (Xavier et al., 2013). In this same study, resistance of these isolates was not concluded for prothioconazole (DMI, FRAC Code 3); however, the $EC_{50}$ values for this DMI fungicide ranged from 0.47 μg/mL to 26.44 μg/mL (mean 5.02 μg/mL). This is noteworthy because an $EC_{50}$ of 1.0 to 50 μg/mL is considered as moderately resistant (Xavier et al., 2013) and some authors considered 1.0 μg/mL ($EC_{50}$) as a cut off for possible resistance to triazoles (Teramoto et al., 2011; Edgington and Klew, 1971). In peanut, the development of late leaf spot (*Cercosporidium personatum* Berk. and Curt.) resistance to the DMI fungicide tebuconazole (FRAC Code 3) was demonstrated since 2003 (Gremillion et al., 2011).

The respiratory inhibitor fungicides, including the QoI and SDHI fungicides, are prone to resistance development due to their site-specific mode of action (Amiri, 2010). Both groups of fungicides are classified as "high-risk" for resistance to develop in fungal populations. Practical resistance to the QoI fungicides has been documented in over 30 fungal species representing 20 genera (FRAC 2013) and primarily occurs as a result of nucleotide point mutations in the cyt *b* gene (Fernández-Ortuño et al., 2008). Moreover, pathogens with a short generation time and which produce abundant spores that are widely dispersed as with wind-borne pathogens, are generally associated with greater risk of resistance (Brent and Hollomon, 2007a). Rapid selection for resistance to QoI fungicides in populations in the field has been documented for several

pathogens including *Pseudoperonospora cubensis* (Ishii et.al, 2001), *Blumeria graminis* (Chin et. al., 2001), and *Didymella bryoniae* (Stevenson et.al, 2004). In 2012, field resistance to azoxystrobin (Quadris, FRAC Code 11) by *C. cassiicola*, causative agent of target spot on tomato, was reported in Qunicy, Florida (Paret et al., 2015). Moreover, *C. cassiicola* isolates from tomato collected in Florida from 2015 to 2017 were evaluated for fungicide sensitivity to seven respiration inhibitor fungicides including QoIs and SDHIs. The results revealed that nearly 90% of the isolates were resistant to QoI fungicides azoxystrobin and fenamidone (MacKenzie et al., 2017). Approximately 75% of these isolates exhibited reduced sensitivity to one or more of the tested SDHI fungicides. In this same study, 76 out of 79 isolates that had reduced sensitivity to penthiopyrad were also cross resistant with boscalid. Moreover, in 48 out of 49 assessments, when an isolate had reduced sensitivity to fluxapyroxad, the isolate was cross resistant with benzovindiflupyr (MacKenzie et al., 2017). Also, *Alternaria solani*, causal agent of early blight of potato, developed in-vitro resistance to the SDHI boscalid (FRAC Code 7) (Gudmestad et al., 2013). Resistance to boscalid was also observed in *C. cassiicola* on cucumber in Japan (Miyamoto et al., 2009). These monitoring studies showed that frequencies of resistance could increase rapidly from one year to the next in response to selection.

In Japan, benzimidazole (MBC, FRAC Code 1) and QoI (FRAC Code 11) resistance has been reported for *C. cassiicola* from cucumber (Hasama 1991; Hasama and Sato 1996; Date et al., 2004; Takeuchi et al., 2006). Resistance was also documented in Brazil where Teramoto et al. (2011) evaluated the sensitivity of *C. cassiicola* isolates from cucumber to five different fungicides and found $EC_{50}$ values greater than 50 μg/mL for the QoI fungicide azoxystrobin (FRAC Code 11) and MBC fungicides carbendazim and thiophanate-methyl (FRAC Code 1).

With the presence of resistant pathogens in different pathosystems, monitoring programs should be initiated to detect potential shifts in pathogen sensitivity.

**Justification for research and objectives**

The risk of resistance to fungicides and the mode of resistance in plant pathogens against fungicides should be concluded separately for each fungicide/pathogen combination (Gisi et al., 2002). The primary objectives for this research were to refine management strategies for target spot and to develop information helpful in fighting fungicide resistance in cotton through fungicide efficacy and sensitivity studies. Focus was on isolate sensitivity of the causative fungal pathogen, *C. cassiicola,* to three different classes of fungicides: sterol demethylation inhibitors (DMI), quinone-outside inhibitors (QoI) and succinate-dehydrogenase inhibitors (SDHI). Sensitivities of *C. cassiicola* isolates from cotton as well as from other economically important hosts were assessed. Monitoring fungicide sensitivities helps to identify developing resistance problems and can provide important information on how to effectively manage the disease. Results of in vitro sensitivity assays will help determine the risk of resistance development in pathogen populations treated with these three different classes of fungicides. These chemical classes were chosen because they encompass currently registered fungicides that can be used in the field for control of target spot. Metconazole (DMI) and pyraclostrobin (QoI) were chosen for the test as these fungicides are present in the mixture that is registered for control of target spot (Twinline). Pyraclostrobin is also available as a stand-alone product (Headline) or as a QoI and SDHI premix combination with fluxapyroxad (Priaxor). Fluxapyroxad, as previously mentioned, was chosen for the test as this fungicide is present in the mixture with the seemingly highest field efficiency, alongside Headline, among the products registered for control of target spot (Mehl et al., 2017; Kelly 2016).

At present, the timing of a fungicide application for management of target spot is problematic. When a fungicide is applied too early, it may not be as effective when the disease begins to develop later. However, a delay in the application of fungicide may result in missing the critical period when disease is initiated and management is most appropriate. Additionally, growers are concerned about the impact of the disease and are generally willing to use fungicides in the management of target spot although they are cautious in adding expense to production without assurance that applications will increase profit (Rothrock et.al, 2015). This research will compare one and two application programs of selected registered and candidate fungicides for the control of target spot on two different cotton cultivars in a field setting as well as yield response and lint quality. As research on target spot progresses, impact of fungicides on yield, the optimal number of applications for control of the disease and the best timing for those applications, will be determined. Results of this research can help optimize fungicide sensitivity monitoring practices in an effort to improve fungicide use patterns for optimum disease management.

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# CHAPTER 2

# FUNGICIDE SENSITIVITY OF *CORYNESPORA CASSIICOLA* (TARGET SPOT) ISOLATES FROM COTTON

[1] Laurel, M. K. S., Kemerait Jr., R. C., Brewer, M. T., Stevenson, K. L., & Newsom, L. J. To be submitted to *Cotton Science*.

**Abstract**

*Corynespora cassiicola*, causal agent of target spot of cotton, is a serious foliar disease in the southeastern United States. Fungicides are used to control target spot. However, *C. cassiicola* has a remarkable ability to adapt and become resistant to effective fungicides. To facilitate fungicide resistance monitoring, baseline (current) sensitivity distributions were established for metconazole (DMI), fluxapyroxad (SDHI) and pyraclostrobin (QoI). Forty isolates were tested using a mycelial growth assay (DMI and SDHI) or spore germination assay (QoI) to determine the effective fungicide concentration at which mycelial growth or spore germination was inhibited by 50% ($EC_{50}$). Mean $EC_{50}$ values of metconazole, fluxapyroxad and pyraclostrobin were 0.07, 0.03 and 0.06 µg/mL respectively. Additionally, there was no evidence of cross-resistance between the sensitivity to metconazole and fluxapyroxad, between metconazole and pyraclostrobin and between fluxapyroxad and pyraclostrobin, as correlation analyses found no relationships of statistical significance. Based on the sensitivity distribution, all isolates tested were considered sensitive to the fungicides. However, these sensitivities varied among isolates offering an early indication that resistance can happen in the future. Since *C. cassiicola* is sensitive to the three fungicides evaluated in this study, with each fungicide having a different mode of action, a resistance management strategy can be utilized. This can involve rotational use of these fungicides, providing timely applications of each chemical.

**Introduction**

Cotton is an important crop in the southeastern United States. In 2016 it was estimated that cotton was planted on 10.0 million acres, with upland cotton area planted on an estimated 9.82 million acres (National Agricultural Statistics Service- United States Department of Agriculture). The bulk of cotton production included areas of Texas High and Rolling Plains, the

Mississippi, Arkansas and Louisiana Delta, southern Georgia and California's San Joaquin Valley (USDA-ERS, 2016). In the state of Georgia, 1.18 million acres of upland cotton were planted in 2016, with yields valued at $749 million.

Target spot is an economically important disease of various ornamental, vegetable and field crops and is caused by the ascomycete *Corynespora cassiicola* (Berk. & M.A. Curtis). Under environmental conditions of high relative humidity and extended periods of leaf wetness, the fungus causes target-like spots on leaves and fruits as well as premature defoliation on various hosts including tomato (*Solanum lycopersicum*), rubber (*Hevea brasiliensis*), cucumber (*Cucumis sativus*) and cotton (*Gossypium hirsutum*). In the United States, target spot was first reported on cotton in Mississippi in 1961 (Jones, 1961). The disease was not noted again until 2005 when crop consultants in southwestern Georgia reported an unusual occurrence of leaf spot on cotton. When these symptomatic leaves were submitted to the University of Georgia Tifton Plant Disease Clinic for identification in 2008, the causal agent was identified as *C. cassiicola* (Fulmer et al., 2012). In recent years, target spot incidence has increased in cotton growing regions to include Alabama (Campbell et al., 2012), North Carolina (Edmisten, 2012), Louisiana (Price et al. 2015b), and Tennessee (Butler et al., 2016). For the last six years, target spot has spread to other southern cotton producing states, with estimated losses of $70 million during its peak in 2013 (Hagan et al., 2016). Currently, the disease is best managed by cotton growers using fungicide options that include azoxystrobin, pyraclostrobin, pyraclostrobin + metconazole, pyraclostrobin + fluxapyroxad, and azoxystrobin + benzovindiflupyr (solatenol), flutriafol, and prothioconazole + trifloxystrobin. Tebuconazole is labeled, but specifically for the control of southwestern rust (Hagan and Sikora, 2012). There are no known resistant varieties of cotton at present.

The most important fungicides currently labeled for use on cotton belong to the demethylation inhibitor (DMI, FRAC Code 3), succinate dehydrogenase inhibitor (SDHI, FRAC Code 7) and quinone outside inhibitor (QoI, FRAC Code 11) classes. The DMI fungicides interfere with the C-14 demethylase of the ergosterol biosynthesis pathway resulting in membrane leakage (Brent and Hollomon, 2007b). Currently, DMIs are registered for use on many crops, providing a broad spectrum activity against numerous fungal pathogens. Metconazole, tebuconazole and prothioconazole are DMI fungicides registered for use on cotton. Metconazole is marketed as a product premixed with a QoI fungicide pyraclostrobin (Twinline; BASF Corporation, Research Triangle Park, NC). Prothioconazole is also marketed as a premixed product with a QoI fungicide trifloxystrobin (Stratego YLD; Bayer Crop Science).

Quinone outside inhibitor (QoI) and succinate dehydrogenase inhibitor (SDHI) fungicides are respiration inhibitors (RI). Fungicides within these classes are used in the management of target spot. Registered QoI fungicides for use on cotton include pyraclostrobin and azoxystrobin, which are marketed as both individual and as premixed products. Fluxapyroxad and solatenol are SDHI fungicides currently marketed as premixed products with QoI fungicides.

QoI fungicides such as azoxystrobin, pyraclostrobin and trifloxystrobin inhibit mitochondrial respiration. These fungicides bind to the $Q_o$ site of cytochrome b, blocking electron transfer along the mitochondrial respiratory chain of the complex bc1, also known as complex III. This disrupts the energy cycle by stopping ATP production. SDHI fungicides such as fluxapyroxad and solatenol specifically bind to ubiquinone-binding site (Q-site) of the mitochondrial complex II consequently halting fungal respiration (Bartlett et al., 2002; Avenot and Michailides, 2010).

**Fungicide resistance**

"Fungicide resistance is the acquired and heritable reduction in sensitivity of a fungus to a fungicide that occurs as a result of the selection of insensitive members within a population" (FRAC 2016). The likelihood of resistance occurring depends on characteristics of both the pathogen and fungicide, respectively (Brent and Hollomon, 2007b).

Practical resistance occurs when a pathogen population has shifted to one that is prevalently resistant, prompting disease control failures after utilization of the recommended dose of a fungicide. This process may develop in either a quantitative or qualitative manner (Brent and Hollomon 2007a; FRAC 2016). Quantitative, or multi-step resistance, is a process where multiple mutations occurring in the target site result in a gradual shift from sensitivity to insensitivity in the pathogen population over several years (Brent and Hollomon 2007a; FRAC 2016). Qualitative or single-step resistance occurs as the result of a single mutation in the target site. This leads to abrupt loss of product efficacy producing two sub-populations having vastly different sensitivities to a given fungicide (Brent and Hollomon 2007a; FRAC 2016).

As growers turn to fungicides for control and management of foliar diseases on cotton, sensitivity monitoring programs can be useful for detecting changes in the frequency of less sensitive or resistant isolates before control failure occurs. Establishment of baseline sensitivity is the first step of fungicide sensitivity monitoring program and fungicide resistance management strategies (Russell, 2004). Baseline is defined as a profile of the sensitivity of the target fungus to the fungicide constructed by using biological or molecular biological techniques to assess the response of previously unexposed fungal individuals or populations to the fungicide (Russell, 2004). Sensitivity monitoring for QoI fungicides is mainly done by spore germination tests with additional PCR tests for the G143A mutation (Brent and Hollomon, 2007a). For other classes,

various techniques are possible including mycelial growth inhibition, spore germination assays and germ tube elongation assays (Russell, 2004). To obtain the fungicide sensitivity data, in vitro assays such as mycelial growth inhibition and inhibition of spore germination are conducted to determine the 50% effective fungicide concentration ($EC_{50}$) for each isolate. The $EC_{50}$ value corresponds to the dose that reduces the growth of mycelium or spore germination to a value of 50% for each isolate (Russell, 2004). Monitoring allows for detection of impending resistance. This is a vital area of resistance research; all of the knowledge of the distribution, evolution and impact of resistance in the field relies heavily on monitoring (Brent and Hollomon, 2007a).

Demethylation inhibitor fungicides are thought to have a medium risk for the development of resistance (FRAC 2016). Practical resistance to the DMIs has been observed in 17 fungal species and tends to develop as a result of target site mutations in the *cyp51* gene, overexpression of *cyp51*, or reduced intracellular fungicide accumulation due the activity of efflux transporters (Price et al., 2015a; Ziogas and Malandrakis, 2015; FRAC 2016). Similar to other fungicides, point mutations in the *cyp51* gene that lead to amino acid substitutions are the most frequently observed cause of resistance (Price et al., 2015a). Although DMI resistance to *C. cassiicola* from cotton has not yet been reported, DMI resistance has been documented in pathogens that cause over 30 diseases including powdery mildew, apple scab and brown rot of stone fruit. In one study, resistance of *C. cassiicola* isolates from soybean to carbendazim (MBC, FRAC Code 1), was observed in samples collected from Mato Grosso, Brazil (Xavier et al., 2013). In this same study, resistance of these isolates was not concluded for prothioconazole (DMI, FRAC Code 3); however, the $EC_{50}$ values for this DMI fungicide ranged from 0.47 µg/mL to 26.44 µg/mL (mean 5.02 µg/mL). This is noteworthy because an $EC_{50}$ of 1.0 to 50 µg/mL is considered as moderately resistant (Xavier et al., 2013) and some authors considered 1.0 µg/mL

($EC_{50}$) as a cut off for possible resistance to triazoles (Teramoto et al., 2011; Edgington and Klew, 1971).

The respiratory inhibitor fungicides, including the QoI and SDHI fungicides, are prone to resistance development due to their site-specific mode of action (Amiri et al., 2010). Both groups of fungicides are classified as high-risk for resistance to develop in fungal populations. Practical resistance to the QoI fungicides has been documented in over 30 fungal species representing 20 genera (FRAC 2012) and primarily occurs as a result of nucleotide point mutations in the cyt *b* gene (Fernández-Ortuño et al., 2008). Moreover, pathogens with a short generation time and which produce abundant spores that are widely dispersed as with wind-borne pathogens, are generally associated with greater risk of resistance (Brent and Hollomon, 2007a). Rapid selection to resistance to QoI fungicides in populations in the field has been documented in several pathogens including *Pseudoperonospora cubensis* (Ishii et al., 2001), *Blumeria graminis* (Chin et al., 2001), and *Stagonosporopsis spp.* (Stevenson et al., 2004). These monitoring studies showed that frequencies of resistance could increase rapidly from one year to the next in response to selection.

In other countries such as Japan, MBC (FRAC Code 1) and QoI (FRAC Code 11) resistance has been reported for *C. cassiicola* from cucumber (Hasama, 1991; Hasama and Sato, 1996; Date et al., 2004; Takeuchi et al., 2006). Resistance was also documented in Brazil where Teramoto et al. (2011) evaluated the sensitivity of *C. cassiicola* isolates from cucumber to five different fungicides and found $EC_{50}$ values greater than 50 μg/mL for the QoI fungicide azoxystrobin (FRAC Code 11) and MBC fungicides carbendazim and thiophanate-methyl (FRAC Code 1). In the United States, field trials conducted at the University of Florida in 2012 to evaluate effective fungicides for management of target spot of tomato caused by *C. cassiicola*

demonstrated the impact of fungal resistance to azoxystrobin (Quadris; Syngenta Crop Protection, Greensboro, NC) (Paret et.al., 2015). Resistance to this fungicide primarily occurs as a result of a single nucleotide point mutation in the cytochrome *b* gene *CYTB*, such as the substitution of glycine by alanine at position 143 (G143A) that occurs in several phytopathogenic fungi (Fernández-Ortuño et al., 2008). In addition, *C. cassiicola* isolates from tomato collected in Florida from 2015 to 2017 were evaluated for fungicide sensitivity to seven respiration inhibitor fungicides including QoIs and SDHIs. The results revealed that nearly 90% of the isolates were resistant to QoI fungicides azoxystrobin and fenamidone (MacKenzie et al., 2017). Approximately 75% of these isolates exhibited reduced sensitivity to one or more of the tested SDHI fungicides. In this same study, 76 out of 79 isolates that had reduced sensitivity to penthiopyrad were also cross resistant with boscalid. Moreover, in 48 out of 49 assessments, when an isolate had reduced sensitivity to fluxapyroxad, the isolate was cross resistant with benzovindiflupyr (MacKenzie et al., 2017). In Japan, isolates with reduced sensitivity to SDHI were identified in a field population of *C. cassiicola* on cucurbits (Miyamoto et al., 2009; Ishii et al., 2011). Boscalid-resistant isolates were detected in 17 out of 19 greenhouses with a history of use of this fungicide. Mechanisms of resistance to this fungicide typically involve mutations which lead to amino acid substitutions in the SdhB, SdhC or SdhD subunits of succinate dehydrogenase.

With limited fungicides available to the growers, lack of resistant cotton cultivars and the potential for *C. cassiicola* isolates to rapidly develop resistance to single-site-mode-of-action fungicides, fungicide sensitivity monitoring is crucial. Characterizing fungicide sensitivity profiles for *C. cassiicola* will help improve target spot management. Although fungicide sensitivity monitoring is helpful for detecting shifts in sensitivity, it is difficult to predict the

relative efficacy of a fungicide for control of target spot based solely on in vitro sensitivity assay results. The objective of this research is to provide some level of insight about the baseline sensitivities of effective fungicides and the potential for mutliple-resistance among them. Results from this research can be used to monitor fungicide sensitivity and detect significant shifts in fungicide sensitivity. Hence, the objectives of this study were to determine the sensitivity of *C. cassiicola* isolated from cotton to metconazole (DMI, FRAC Code 3), fluxapyroxad (SDHI, FRAC Code 7), and pyraclostrobin (QoI, FRAC Code 11). These chemicals were chosen because they are currently registered for management of target spot on cotton.

**Materials and Methods**

**Sampling, Isolation, Incubation and Isolate Maintenance.** A total of 40 isolates of *C. cassiicola* was obtained from cotton symptomatic for target spot from Georgia and the southern U.S. (Table 2.1). Isolates were obtained from cotton leaves with typical target spot lesions and isolated on quarter-strength potato dextrose agar (qPDA). Tissue was incubated at 25°C for 3 days in the dark to allow for mycelial growth and sporulation. Plugs were taken from the edges of the mycelial growth from each isolate and transferred to qPDA as subcultures. After 7 days these active cultures were used in fungicide sensitivity assays. For long term conservations, all isolates were maintained on filter paper discs with dried mycelial growth and stored at -20°C until further use. Stored isolates were recovered by placing a piece of filter paper with fungal mycelium on a fresh plate of qPDA and incubating at 25°C for 7 days in preparation for fungicide sensitivity assays.

**Fungicide-amended media preparation.** Technical grade metconazole, fluxapyroxad and pyraclostrobin (BASF Corporation) were dissolved in acetone to obtain stock solutions of 30,000 µg/mL for metconazole and fluxapyroxad and 100,000 µg/mL for pyraclostrobin. Serial

dilutions of the stock solution for metconazole and fluxapyroxad were made in acetone and added to autoclaved and cooled (55°C) potato dextrose agar (PDA) to obtain desired concentrations of 0, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0 and 3.0 µg of a.i./mL. To test the sensitivity of *C. cassiicola* to pyraclostrobin, water agar (WA) media was amended with 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 10 µg of a.i./mL of the fungicide. In addition, salicylhydroxamic acid (SHAM) at 100 µg/mL dissolved in methanol was either added or not added to the WA. SHAM is used in QoI fungicide in vitro assays to prevent fungi from using an alternative respiration pathway (Ziogas et al., 1997).

**Fungicide sensitivity assays.** Sensitivity of each isolate to metconazole, and fluxapyroxad was determined by using an in vitro mycelial growth assay on fungicide-amended and non-amended PDA plates. Sensitivity of each isolate to pyraclostrobin was determined through a spore germination assay on fungicide-amended and non-amended water agar plates. Laboratory assays were conducted by measuring reductions in mycelial growth and spore germination of fungi on fungicide-amended media relative to the non-amended control. These three fungicides have different modes of action and metabolic targets; therefore, they require different procedures for measuring fungicide sensitivity. Inhibition of colony radial growth assay was used to test for sensitivity to metconazole and fluxapyroxad whereas sensitivity to pyraclostrobin was tested using inhibition of spore germination assay in this study. The experiments were conducted twice.

**Mycelial Growth Assay.** Sensitivity to metconazole and fluxapyroxad was tested using a mycelial growth assay on fungicide-amended and non-amended media. Mycelial plugs 5 mm in diameter were taken from the margin of a 1-week old culture on qPDA. Plugs were placed upside down in the center of fungicide-amended and non-amended PDA plates. Two replications

of each isolate and fungicide concentrations were prepared. Plugs were incubated for 4 days in the dark at 25°C to allow for mycelial growth. Two measurements of colony diameter were made at right angles to each other and the mean colony diameter was calculated and corrected by subtracting the diameter of the mycelial plug. Relative growth was calculated as the ratio between the corrected colony diameter on fungicide-amended medium and the corrected colony diameter on non-amended medium. The $EC_{50}$ value, the concentration that reduced the growth of mycelium to a value of 50% for each isolate was estimated based on linear regression of probit-transformed relative inhibition (1-RG) on $\log_{10}$-transformed fungicide concentration. The transformations were used to linearize the relationship between relative inhibition and the fungicide concentration so that linear regression could be used to fit a linear model to the relationship and enable prediction of the $EC_{50}$ value. The conditions for testing and duration of tests were standardized to ensure repeatability and accuracy of results.

**Spore Germination Assay.** Sensitivity to pyraclostrobin was tested using a spore germination assay. Mycelial plugs 5 mm in diameter were taken from 4-day-old revived cultures and were transferred using a sterile scalpel to plated V8 agar medium. For each isolate, this was replicated five times. Isolates of *C. cassiicola* were grown on V8 agar media for 10 days at 25 to 27°C under continuous fluorescent light to induce sporulation. This method was chosen after conducting several preliminary experiments with different media and light conditions (Onesirosan et al., 1975; Sharma and Bowen, 2016; Fernando et al., 2012). Conidial suspensions of each isolate were prepared by flooding the plates with 5 mL of a solution of sterile water and gently scraping the surface of the mycelia with a rod to dislodge the conidia. Conidial suspensions of 20,000 spores/mL from each individual isolate were calculated using a hemacytometer, and were then transferred onto fungicide-amended and non-amended water agar

plates. Two replicates of each isolate and treatment were prepared. After incubating at 25°C for 24 h, 100 spores per plate were examined microscopically and germination percentage was recorded. Relative germination (RG) was calculated as the percent germination on fungicide-amended medium divided by the percent germination of the same isolate on non-amended medium. A conidium was considered germinated if the length of the germ tube was equal to or greater than half the length of the conidium (Beckman and Payne, 1983). Relative inhibition (1-RG) was probit-transformed, and linearly regressed on log10-transformed fungicide concentration. Fungicide sensitivity for each isolate was expressed as the $EC_{50}$ value (the fungicide concentration that inhibits spore germination by 50% relative to the control), which was estimated from the linear regressions.

**Effect of strobilurin fungicide and SHAM on spore germination.** All 40 isolates of *C. cassiicola* were tested to determine whether salicylhydroxamic acid (SHAM) affected the response of spore germination to the fungicide pyraclostrobin. SHAM was added to fungicide-amended and non-amended water agar to prevent alternative oxidation pathways in the fungus from giving a false resistance or reduced sensitivity result (Ziogas et al., 1997). SHAM was dissolved in methanol and was incorporated with fungicide-amended and non-amended water agar plates at a concentration of 100 µg/mL of SHAM. The effect of SHAM at 100 µg/mL on spore germination was evaluated in combination with pyraclostrobin concentrations of 0-10 µg/mL as previously described.

**Data Analysis.** The $EC_{50}$ values were calculated with the PROC REG function in SAS (Version 9.3; SAS Institute Inc., Cary, NC). The $EC_{50}$ value for each isolate was estimated based on linear regression of probit-transformed relative inhibition (1-RG) on $log_{10}$-transformed fungicide concentration. The frequency distribution of $log_{10}$-transformed $EC_{50}$ values was tested

for normality using four tests: Shapiro-Wilk *W*, Kolmogorov-Smirnov *D*, Cramer-vol Mises $W^2$, and Anderson-Darling $A^2$ (PROC UNIVARIATE). Tukey's multiple comparisons test (HSD) was performed to compare the mean $\log_{10}$-transformed $EC_{50}$ values among fungicide sensitivity trials. The coefficient of variability (standard error/mean) of $\log_{10}$-transformed $EC_{50}$ values for individual isolates among all experimental repeats was calculated as a measure of fungicide sensitivity assay reproducibility. Paired *t* tests were performed to compare the effect of SHAM on individual isolates as well as to compare the mean $\log_{10}$-transformed $EC_{50}$ values of isolates grown on SHAM amended WA plates and those grown on plates without SHAM. Simple linear correlation coefficients were calculated (PROC CORR) to determine the relationship between the sensitivity to i) metconazole and fluxapyroxad, ii) metconazole and pyraclostrobin, and iii) fluxapyroxad and pyraclostrobin, and to evaluate the potential for cross-resistance between them.

**Results**

Coefficients of variation of $\log_{10}$-transformed $EC_{50}$ values of individual isolates among experimental repeats were less than 20% for all fungicides tested. This indicates that the $\log_{10}$-transformed $EC_{50}$ values for individual isolates were consistent among the experimental repeats. Thus, data from these individual experimental repeats were combined to determine the mean $EC_{50}$ value for each isolate and fungicide (Table 2.2). The frequency distribution of $EC_{50}$ values for metconazole (Figure 2.1) was log normal based on results of all four normality tests (Shapiro-Wilk *W*, Kolmogorov-Smirnov *D*, Cramer-vol Mises $W^2$, and Anderson-Darling $A^2$); $EC_{50}$ values ranged from 0.015 to 0.205 µg/mL with a mean of 0.072 µg/mL (Table 2.2). Similar to metconazole, the frequency distribution of $EC_{50}$ values for fluxapyroxad (Figure 2.2) was log normal based on results of all four normality tests; $EC_{50}$ values ranged from 0.001 to 0.126 µg/mL with a mean of 0.026 µg/mL (Table 2.2). Similar to results for metconazole, these tests revealed that $EC_{50}$ values for pyraclostrobin ranged from 0.013 to 0.200 µg/mL, with a mean of

0.064 µg/mL (Table 2.2). The four normality statistics did not reject the null hypothesis that the $EC_{50}$ values were normally distributed (Figure 2.3).

The mean $EC_{50}$ values of the 40 *C. cassiicola* isolates from cotton for metconazole and pyraclostrobin with SHAM were significantly higher than the mean $EC_{50}$ value for fluxapyroxad (Table 2.2). There was no statistically significant difference between the mean $EC_{50}$ values for metconazole and pyraclostrobin with SHAM (Table 2.2). Differences in sensitivity to all three fungicides in vitro were observed among the isolates. All the isolates were sensitive to the tested fungicides, but the sensitivity varied among the *C. cassiicola* isolates. Fluxapyroxad reduced radial growth by 50% at lower concentrations than metconazole and pyraclostrobin with SHAM.

The addition of SHAM at 100 µg/mL to media amended with pyraclostrobin had a significant effect on mean $EC_{50}$ values of *C. cassiicola* isolates (Table 2.3). Isolates grown on fungicide amended media without SHAM had higher $EC_{50}$ values compared to when grown on fungicide amended media with SHAM.

Despite the fact that the frequency distributions of $EC_{50}$ values for metconazole, fluxapyroxad and pyraclostrobin among *C. cassiicola* isolates were similar, the correlations among them were not significant (Table 2.4), with correlation coefficients (r) ranging from -0.078 to 0.006. No significant correlation was observed between $EC_{50}$ values for metconazole and fluxapyroxad, metconazole and pyraclostrobin or fluxapyroxad and pyraclostrobin (Table 2.4).

**Discussion**

Determining the baseline sensitivity is the first step in conducting monitoring programs to detect significant shifts in pathogen sensitivity to a fungicide. This is initiated to ensure efficacy of current fungicide spray programs, to recommend appropriate resistance management strategies and to monitor the effectiveness of these recommended practices. To our knowledge,

this study provides the first report of sensitivity of current populations of *C. cassiicola* from cotton to the DMI fungicide metconazole, the SDHI fungicide fluxapyroxad and to the QoI fungicide pyraclostrobin. Currently, DMIs are registered for use on many crops, to include cotton, providing a broad spectrum activity against numerous fungal pathogens. Metconazole is marketed as a product premixed with a QoI fungicide pyraclostrobin (Twinline). Pyraclostrobin is a registered QoI fungicide for use on cotton marketed as both individual (Headline) and as premixed products. Fluxapyroxad is an SDHI fungicide currently marketed as premixed product with QoI fungicide pyraclostrobin (Priaxor).

Triazole fungicides such as metconazole are ergosterol biosynthesis inhibiting fungicides. This class inhibits mycelial growth and the in vitro bioassay uses inhibition of colony radial growth to measure resistance/sensitivity to this fungicide (Secor and Rivera, 2012).

Conventional bioassays have been used to assess baseline sensitivity profiles to the SDHI fungicide boscalid using either mycelial growth assay or spore germination assay (Avenot and Michailides, 2007; Ishii and Nishimura, 2007; Avenot and Michailides, 2010). Considering both procedures can be used for SDHI fungicides, mycelial growth assay was chosen over spore germination assay for fluxapyroxad for ease of experimentation.

Strobilurin fungicides such as pyraclostrobin are QoIs that block electron transport through the mitochondrial system. This class of fungicides inhibits spore germination and therefore, the in vitro bioassay uses spore germination to measure resistance/sensitivity to this fungicide (Secor and Rivera, 2012).

Baseline sensitivity to metconazole has not been documented in *C. cassiicola* from cotton. However, it has been documented in many other Ascomycete fungi since its release in the market in 1992. In this study, *C. cassiicola* isolates exhibited a relatively narrow range of $EC_{50}$

values for metconazole (0.015-0.205 µg/mL) consistent with results of baseline range of $EC_{50}$ values for *Fusarium oxysporum* (0.0058 to 0.080 µg/mL with a mean of 0.038 µg/mL), *Fusarium graminareum* (0.006 to 0.080 µg/mL with a mean of 0.031 µg/mL), *Fusarium sp. nov*. (0.007 to 0.084 µg/mL with a mean of 0.0187 µg/mL) (Burlakoti et.al, 2010) and *Alternaria alternata* (0.04 to 0.48 µg/mL with a mean of 0.26 µg/mL) (Fonseka and Gudmestad, 2016) to metconazole. In contrast, it was very different from the wider range of $EC_{50}$ values for isolates of *Sclerotinia sclerotiorum* (0.05 to 1.64 µg/mL) (Ameen et al., 2012). This relatively narrow range of $EC_{50}$ values indicates that there is limited variation within the current population of *C. cassiicola* with respect to sensitivity to metconazole. This may be due to the limited exposure of the pathogen to the fungicide. Similar results were seen with baseline sensitivity studies previously mentioned for *Fusarium sp*. on sugarbeet and *Alternaria sp*. on potato, which were both tested prior to exposure to metconazole. In contrast, the relatively wider range of $EC_{50}$ values for isolates of *S. sclerotiorum*, also tested prior to exposure to metconazole, may be due to the possibility that the pathogen is naturally more tolerant to metconazole (Ameen et al., 2012). In addition, frequency distribution of $EC_{50}$ values for metconazole (Figure 2.1) was log-normal on all four normality tests, as is typical for DMI fungicides.

According to the frequency distribution of $EC_{50}$ values determined for metconazole (Figure 2.1), *C. cassiicola* isolates collected from cotton appear to be sensitive to this fungicide based on the criteria adapted from Edgington & Klew (1971). They considered the fungicide highly toxic if $EC_{50} < 1$ µg/mL (sensitive), moderately toxic if $1 < EC_{50} < 50$ µg/mL (moderately resistant) and non-toxic if $EC_{50} > 50$ µg/mL (resistant). For metconazole, the mycelial growth of only 24 of the 40 studied isolates was completely inhibited at 3.0 µg/mL. The criteria adapted for classifying the sensitivities were from a study that looked at fungitoxicity of benzimidazole

compounds to a wide spectrum of fungi. However, these same criteria were also used and adapted for testing sensitivity of *C. cassiicola* from soybean to DMI fungicides cypoconazole, epoxiconazole, flutriafol and tebuconazole (Avozani et al., 2014).

The $EC_{50}$ values for fluxapyroxad fell under the "sensitive category", ranging from 0.001 to 0.126 μg/mL with a mean $EC_{50}$ value of 0.026 μg/mL (Table 2.2). In comparison, baseline sensitivities of *Alternaria alternata* to fluxapyroxad (Avenot et al., 2014) revealed various levels of sensitivities that were separated into six different phenotypes. Highly sensitive *A. alternata* isolates had an $EC_{50}$ value of 0.001 μg/mL while sensitive isolates had $EC_{50}$ values ranging from 0.01 to 0.97 μg/mL, with a mean of 0.147 μg/mL. In contrast, *A. alternata* isolates with reduced sensitivity to fluxapyroxad in the same study had $EC_{50}$ values ranging from 1.01 to 4.98 μg/mL, with a mean of 2.383 μg/mL. Resistance to fluxapyroxad was also documented as low ($EC_{50}$ values of 5.53 and 5.8 μg/mL), moderate ($10.19< EC_{50}<91.07$) and high ($EC_{50}>100$). In this study, *C. cassiicola* isolates had a relatively narrow range of $EC_{50}$ values. This indicated limited variation with the current *C. cassiicola* population with respect to sensitivity to fluxapyroxad. Similar to metconazole, this may be due to the limited exposure of the pathogen to the fungicide, with its registration on cotton for target spot being as recent as 2015. Similarly, the highly sensitive baseline isolates of *A. alternata* from pistachio to fluxapyroxad were collected from pistachio orchards that had never been exposed to any SDHI fungicides. In contrast, the *A. alternata* isolates with resistance or reduced sensitivity to fluxapyroxad were collected from commercial pistachio orchards where boscalid, another SDHI fungicide, had previously been used. According to the Fungicide Resistance Action Committee (FRAC), the risk for resistance development to SDHI fungicides is estimated to be medium to high. Fluxapyroxad is a fungicide

from the SDHI group that has only been recently registered for use on cotton for target spot in 2015 (Hathorn, 2015).

In this study, when tested for sensitivity to fluxapyroxad, all isolates were observed to have complete inhibition of mycelial development at 3.0 μg/mL. Following the classification proposed by Edgington et al. (1971), all isolates were considered sensitive to this fungicide. This same classification was adapted and modified by Miyamoto et al. (2009) when testing sensitivities of *C. cassiicola* isolates from cucumber to the SDHI fungicide boscalid. Results from this same study on boscalid revealed existence of resistant isolates of *C. cassiicola* from cucumber with $EC_{50}$ values higher than 30 μg/mL. In a separate study on sensitivity of SDHI fungicides on *C. cassiicola* from tomato, resistant isolates and reduction in sensitivity were documented as well (MacKenzie et al., 2017). In this study, in 48 out of 49 assessments, when an isolate had reduced sensitivity to fluxapyroxad, the isolate was cross resistant with benzovidiflupyr (MacKenzie et al., 2017). Resistance to SDHI fungicides have been observed in other pathogens as well (Gudmestad et al., 2013). Such fungicide resistance could be the result of different exposure to the fungicides applied to the crop.

Though the *C.cassiicola* isolates from cotton are now sensitive to fluxapyroxad, caution should be taken to avoid overuse of the fungicide in order to minimize risk for fungicide resistance. Under field conditions, the level of control Priaxor (fluxapyroxad + pyraclostrobin) provides is superior to the level of control provided by other fungicides. Currently, this is the most efficacious registered fungicide for controlling target spot on cotton (Mehl et al., 2017). With the risk for resistance evolution to SDHI fungicides estimated as medium to high, growers should avoid overuse of these chemistries despite their efficacy in the field in order to avoid

resistance. The decision by BASF to market Priaxor as a combination of an SDHI and a QoI fungicide is a resistance management strategy designed to delay that risk.

In this study, the frequency distribution of $EC_{50}$ values was determined for pyraclostrobin for *C. cassiicola* isolates collected from cotton (Figure 2.3). According to Edgington et al. (1971), all 40 isolates appear to be sensitive to the fungicide (Table 2.1). Spore germination was completely inhibited on medium containing 10.0 μg/mL pyraclostrobin in 35 of the 40 isolates tested. In a different study where *C. cassiicola* isolates from soybean were tested for sensitivity to QoI fungicides picoxystrobin, pyraclostrobin, azoxystrobin and trifloxystrobin, classifications for sensitivities were modified and adapted from Leroux et al. (2010). In this same study, an isolate was considered sensitive if $EC_{50} < 0.16$ μg/mL; moderately sensitive if $EC_{50}$ is 0.16 to 1.0 μg/mL; highly resistant if $EC_{50} > 1.0$ μg/mL. When applying these modified criteria for this study, 38 out of 40 *C. cassiicola* isolates tested from cotton fell under the sensitive category. The remaining two isolates were considered moderately sensitive. The range and means of $EC_{50}$ values of *C. cassiicola* isolates for the QoI fungicide (Table 2.2) were also relatively narrow and similar with results of baseline sensitivity range in *Cercospora zeae-maydis* ($EC_{50}$ 0.0003 to 0.025 μg/mL with a mean of 0.0010 μg/mL) (Bradley and Pedersen, 2011). Again, this sensitivity of *C. cassiicola* isolates may be due to the limited exposure of the pathogen to the fungicide. The registration of pyraclostrobin (Headline) on cotton for target spot occurred in 2008 and the isolates tested were collected from 2013 to 2015. Similarly, the sensitive baseline isolates of *C. zeae-maydis* from corn to pyraclostrobin were collected from fields where QoI fungicides have never been applied.

Foliar applications of strobilurin fungicides are registered for cotton against target spot and other fungal diseases such as Alternaria leaf spot (*Alternaria brassicae, A. alternata*),

Ascochyta blight (*Nothophoma gossypiicola*.), Cercospora blight and leaf spot (*Cercospora gossypin*a) and Stemphylium leaf spot (*Stemphylium solani*). Due to proven efficacy, QoI fungicides have been used in management of target spot. However, widespread use of QoIs in cotton poses a threat because the potential for fungicide resistance in *C. cassiicola* populations appears to be high (Takeuchi et al., 2006; Miyamoto et al., 2009; Xavier et al., 2013). Under field conditions, one to two applications of pyraclostrobin (Headline) provided significantly better control of target spot than all other fungicides tested, except Priaxor (Walls et al., 2012; Hagan et al., 2017; Mehl et al., 2017). Although results of in-vitro fungicide sensitivity assays may not immediately translate into predictions of efficacy in the field, determining the baseline sensitivity is important and is the first step in inducting monitoring programs to detect significant shifts in pathogen sensitivity to a fungicide. These results are useful for establishment and subsequent monitoring of fungicide resistance management strategies (Russell, 2004).

The addition of SHAM at 100 µg/mL to media amended with pyraclostrobin had a significant effect on mean $EC_{50}$ values of *C. cassiicola* isolates (Table 2.3). Isolates grown on fungicide amended media without SHAM had higher $EC_{50}$ values compared to when grown on fungicide amended media with SHAM. This may indicate that *C. cassiicola* has the potential to utilize alternative respiration to overcome QoI fungicide inhibition in vitro. Similar results were observed in *C. cassiicola* isolates from tomato when tested for their sensitivity to azoxystrobin (MacKenzie et al., 2017). The addition of SHAM to fungicide-amended medium has been observed to increase the sensitivity of some plant pathogenic fungi to QoI fungicides (Wood and Hollomon, 2003).

Analysis of the mean $EC_{50}$ values revealed that $EC_{50}$ values for fluxapyroxad were significantly lower than $EC_{50}$ values for metconazole or pyraclostrobin. This suggests that the

current *C. cassiicola* population is more sensitive to fluxapyroxad compared to the other fungicides tested. This also suggests that fluxapyroxad would probably be effective at controlling *C. cassiicola* at lower rates than metconazole and pyraclostrobin. The data confirm the field observation by Hagan (2017) that Priaxor, a combination of fluxapyroxad + pyraclostrobin, often provides better disease control and superior yield response than other registered fungicides from different chemical classes.

The sensitivity of individual isolates to each of the three fungicides tested varied (Table 2.1). Isolates that had higher $EC_{50}$ values for one fungicide did not necessarily have high $EC_{50}$ values for the other two fungicides tested (Table 2.1). Thus, there is no evidence of cross-resistance as correlation analyses found no relationships of statistical significance (Table 2.4). Cross resistance is common between fungicides belonging to the same chemical class that share a similar mode of action but does not occur in all cases. One example is the case of SDHI fungicides where a lack of cross-resistance to fluopyram and an occurrence of cross-resistance to penthiopyrad in boscalid-resistant isolates were reported in *A. alternata* (Avenot et al., 2014). Previous reports of inconsistent relationship between sensitivities to fungicides with a similar mode of action make it clear that we cannot assume the existence of cross-resistance between fungicides of similar chemical class. In this study, although the range of sensitivities and mean sensitivities of *C. cassiicola* populations to these three different fungicides were similar, especially for metconazole and pyraclostrobin, sensitivities of an individual isolate varied among fungicides.

Since *C. cassiicola* is sensitive to the three fungicides evaluated in this study, with each fungicide having a different mode of action, a resistance management strategy can be utilized.

This can involve rotational use of these fungicides, providing timely applications of each chemical.

**Conclusions**

Until recently, cotton growers in the southeastern U.S. did not apply fungicides for the control of foliar diseases. However, since the recent outbreak of target spot in 2008, growers now turn to chemical treatments for aid. In Georgia, growers typically apply fungicides during the first and/or third week of bloom, or at the onset of disease. Repeated application of the same or chemically related fungicides on the same crop targeting the same pathogen can ultimately greatly increase the risk for resistance. Fungicides, used in rotation with, or mixed with chemicals with different modes of action and introduction of newer fungicides with high efficacy against *C. cassiicola* leaf spot is crucial to successful disease management. Considering the occurrence of fungicide resistance in *C. cassiicola* isolates collected from other hosts, *C. cassiicola* is a high-risk pathogen for fungicide resistance development. Therefore, development of resistance to various fungicides and fungicide classes should be monitored.

These sensitivities are not a true "baseline" as the opportunity for testing isolates without prior exposure to these three fungicides has passed. However, the current $EC_{50}$ ranges indicate a sensitive population. This will be a useful basis for comparison for future sensitivity studies. Future monitoring could document changes in the pathogen population sensitivity as growers continue to use these fungicides. This will subsequently help to predict impending resistance problems and to develop better disease management strategies.

## Literature Cited


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Table 2.1. List of *Corynespora cassiicola* isolates from cotton, year and place of isolation, and effective fungicide concentrations of metconazole, fluxapyroxad or pyraclostrobin that reduce mycelial growth or spore germination by 50% ($EC_{50}$ µg/mL)

| Isolate | Year | Location | | $EC_{50}$ µg/mL | | |
|---|---|---|---|---|---|---|
| | Isolated | County/Parish | State | Metconazole | Fluxapyroxad | Pyraclostrobin |
| CA | 2013 | Atkinson | GA | 0.030 | 0.024 | 0.029 |
| CB | 2013 | Bishop | GA | 0.150 | 0.053 | 0.067 |
| C Co | 2013 | Coffee | GA | 0.028 | 0.114 | 0.056 |
| CM-13 | 2013 | Mitchell | GA | 0.055 | 0.022 | 0.171 |
| Cpi-1 | 2013 | Pierce | GA | 0.044 | 0.032 | 0.039 |
| Cpi-2 | 2013 | Pierce | GA | 0.027 | 0.023 | 0.034 |
| Cpi-3 | 2013 | Pierce | GA | 0.018 | 0.022 | 0.074 |
| Cpi-4 | 2013 | Pierce | GA | 0.044 | 0.030 | 0.093 |
| Cpi-5 | 2013 | Pierce | GA | 0.114 | 0.013 | 0.128 |
| Cpi-6 | 2013 | Pierce | GA | 0.151 | 0.001 | 0.042 |
| Cpi-7 | 2013 | Pierce | GA | 0.023 | 0.024 | 0.053 |
| Cpi-8 | 2013 | Pierce | GA | 0.067 | 0.022 | 0.079 |
| Cpi-9 | 2013 | Pierce | GA | 0.113 | 0.03 | 0.013 |
| CTs-1 | 2013 | Madison | TN | 0.098 | 0.011 | 0.035 |
| CVA-5 | 2013 | Suffolk | VA | 0.088 | 0.011 | 0.026 |
| CW | 2013 | Ware | GA | 0.042 | 0.034 | 0.033 |
| FlM | 2013 | Jackson | FL | 0.045 | 0.015 | 0.068 |
| CLA-a | 2014 | Rapides | LA | 0.073 | 0.039 | 0.03 |
| CLA-b | 2014 | Rapides | LA | 0.034 | 0.007 | 0.052 |
| CLA-c | 2014 | Rapides | LA | 0.127 | 0.126 | 0.038 |
| CTNa-1 | 2014 | Dyer | TN | 0.097 | 0.075 | 0.054 |
| CTNb | 2014 | Dyer | TN | 0.054 | 0.032 | 0.099 |
| CTNc | 2014 | Dyer | TN | 0.017 | 0.007 | 0.015 |
| CTN2a-1 | 2014 | Madison | TN | 0.015 | 0.020 | 0.080 |
| BC-1 | 2015 | Tift | GA | 0.204 | 0.015 | 0.017 |
| CAL-1 | 2015 | Headland | AL | 0.205 | 0.018 | 0.179 |
| CAL-2 | 2015 | Baldwin | AL | 0.024 | 0.016 | 0.134 |
| CAL-2a | 2015 | Baldwin | AL | 0.050 | 0.013 | 0.075 |
| CAL-3 | 2015 | Elmore | AL | 0.037 | 0.038 | 0.068 |
| CAL-4 | 2015 | Macon | AL | 0.039 | 0.042 | 0.075 |
| CT-1 | 2015 | Tift | GA | 0.027 | 0.016 | 0.032 |
| CTGA_m1 | 2015 | Miller | GA | 0.172 | 0.009 | 0.079 |
| CTGA_S1_1 | 2015 | Seminole | GA | 0.032 | 0.019 | 0.015 |

Table 2.1 (continued) List of *Corynespora cassiicola* isolates from cotton, year and place of isolation, and effective fungicide concentrations of metconazole, fluxapyroxad or pyraclostrobin that reduce mycelial growth or spore germination by 50% ($EC_{50}$ µg/mL)

| Isolate | Year | Location | | $EC_{50}$ µg/mL | | |
|---|---|---|---|---|---|---|
| | Isolated | County/Parish | State | Metconazole | Fluxapyroxad | Pyraclostrobin |
| CTGA_S2_1 | 2015 | Seminole | GA | 0.056 | 0.016 | 0.072 |
| CTP-1 | 2015 | Mitchell | GA | 0.050 | 0.017 | 0.035 |
| EC-1 | 2015 | Emanuel | GA | 0.172 | 0.016 | 0.200 |
| GC-1 | 2015 | Tift | GA | 0.106 | 0.014 | 0.072 |
| SlM-1 | 2015 | Mitchell | GA | 0.020 | 0.010 | 0.014 |
| TCU-1 | 2015 | Thomas | GA | 0.125 | 0.008 | 0.029 |
| TCUa-1 | 2015 | Thomas | GA | 0.019 | 0.011 | 0.065 |

Table 2.2. Range and mean effective concentration of fungicides to inhibit mycelial growth and spore germination by 50% ($EC_{50}$) of forty *Corynespora cassiicola* isolates from cotton

| Fungicides | $EC_{50}$ µg/mL | | Method |
|---|---|---|---|
| | Range | Mean | |
| Metconazole | (0.015-0.205)[a] | 0.072A[b] | mycelial growth assay |
| Fluxapyroxad | (0.001-0.126) | 0.027B | mycelial growth assay |
| Pyraclostrobin | (0.013-0.200) | 0.064A | spore germination assay |

[a] Minimum and maximum mean $EC_{50}$ value of mycelium and spore inhibition for each fungicide.
[b] Means followed by the same uppercase letter are not significantly different according to Tukey's multiple comparisons test (HSD) ($P \leq 0.05$).

Table 2.3. Mean effective fungicide concentration of pyraclostrobin amended with 100 µg/mL of SHAM to inhibit spore germination by 50% ($EC_{50}$) of forty *Corynespora cassiicola* isolates.

| | No SHAM | SHAM 100 |
|---|---|---|
| | $EC_{50}$ µg/mL | |
| Pyraclostrobin | (0.014-0.199)[a] 0.071A[b] | (0.013-0.200) 0.064B |

[a] Minimum and maximum mean $EC_{50}$ value of spore inhibition for pyraclostrobin.

[b] Mean separation within row followed by the same uppercase letter are not significantly different according to *t*-test (LSD) ($P \leq 0.05$).

Table 2.4. Pearson correlation coefficients between $EC_{50}$ values for metconazole, fluxapyroxad and pyraclostrobin for 40 collected isolates of *Corynespora cassiicola* from cotton

| Fungicides | Metconazole | | Fluxapyroxad | | Pyraclostrobin | |
|---|---|---|---|---|---|---|
| | *r* | *P* value | *r* | *P* value | *r* | *P* value |
| Metconazole | - | - | 0.006 | 0.971 | 0.255 | 0.112 |
| Fluxapyroxad | 0.006 | 0.971 | - | - | -0.078 | 0.635 |
| Pyraclostrobin | 0.255 | 0.112 | -0.078 | 0.635 | - | - |

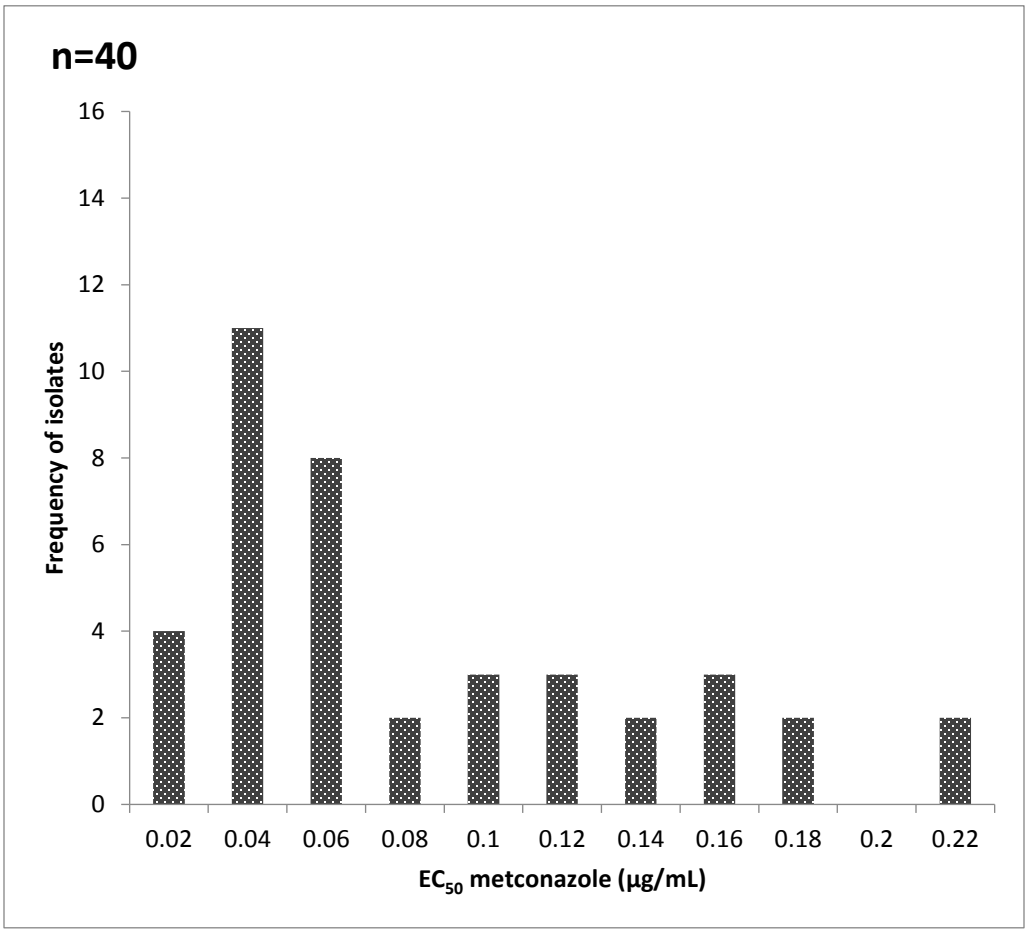


Figure 2.1. Frequency distribution of the effective fungicide concentration of metconazole to reduce mycelial growth by 50% ($EC_{50}$) of 40 *Corynespora cassiicola* isolates from cotton.

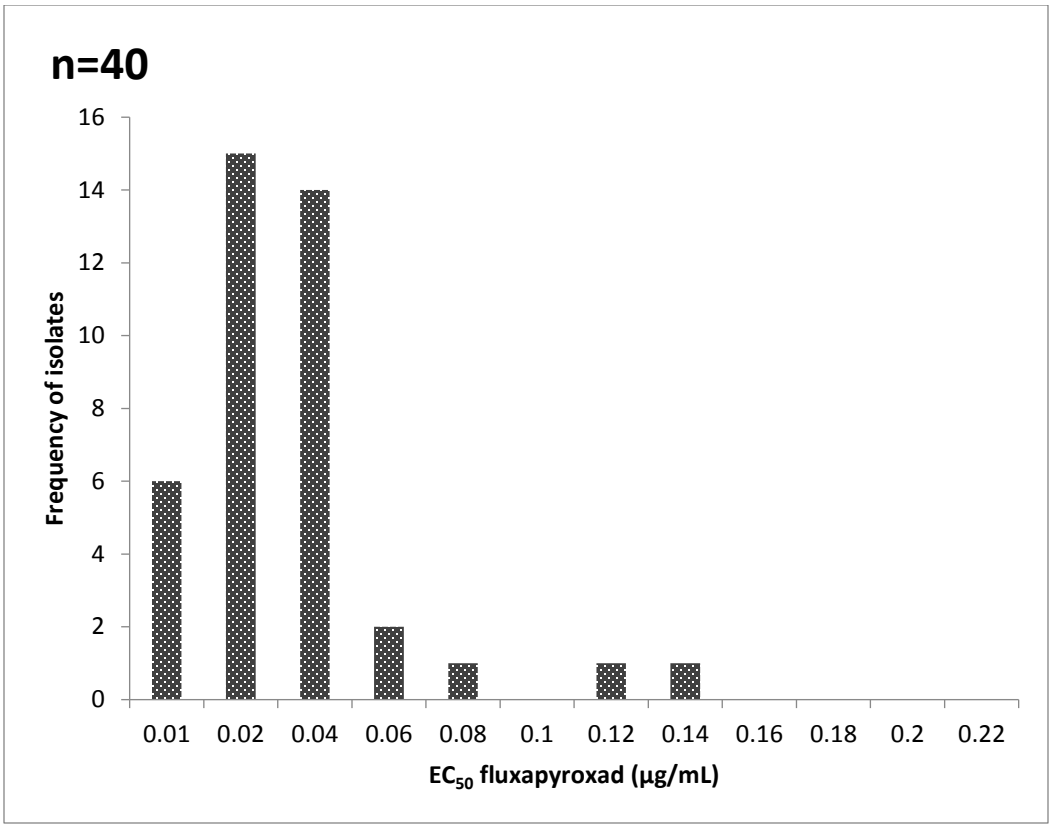


Figure 2.2. Frequency distribution of the effective fungicide concentration of fluxapyroxad to reduce mycelial growth by 50% ($EC_{50}$) of 40 *Corynespora cassiicola* isolates from cotton.

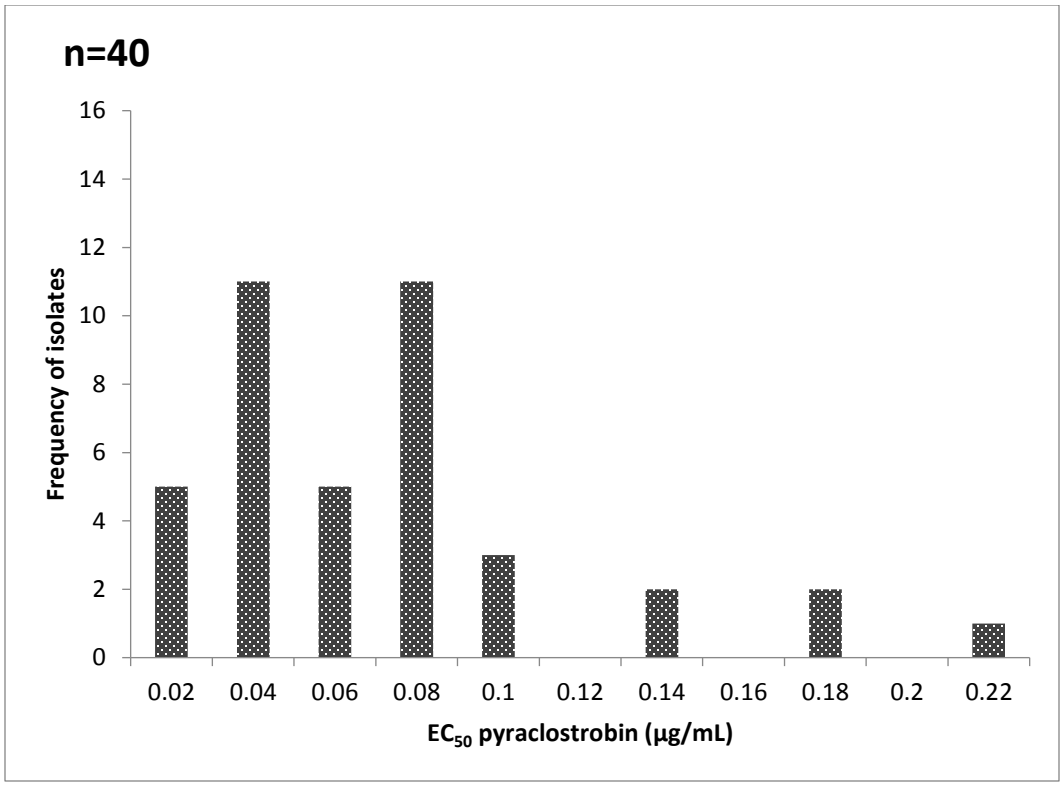


Figure 2.3. Frequency distribution of the effective fungicide concentration of pyraclostrobin to inhibit spore germination by 50% ($EC_{50}$) of 40 *Corynespora cassiicola* isolates from cotton.

# CHAPTER 3

# COMPARISON OF FUNGICIDE SENSITIVITY OF *CORYNESPORA CASSIICOLA* ISOLATES FROM CUCUMBER, HYDRANGEA, MANDEVILLA, PEPPER, SOYBEAN AND TOMATO TO ISOLATES PREVIOUSLY COLLECTED FROM COTTON

[1] Laurel, M. K. S., Kemerait, Jr., R. C., Brewer, M. T., Stevenson, K. L. & Newsom, L. J. To be submitted to *Cotton Science*.

**Abstract**

Target spot, caused by the fungus *Corynespora cassiicola*, is a serious disease of various ornamental, vegetable and field crops. Fungicides are used to control target spot. However, *C. cassiicola* has a history of developing resistance to effective fungicides. This poses a significant challenge to chemical control, presenting a need for resistance management strategies. To facilitate fungicide resistance monitoring, initial work tested baseline (current) isolates of *C. cassiicola* from cotton to metconazole (DMI), fluxapyroxad (SDHI) and pyraclostrobin (QoI). Each isolate was tested to determine the effective fungicide concentration ($EC_{50}$) at which mycelial growth or spore germination was inhibited by 50%. Further work compared fungicide sensitivity of 40 *C. cassiicola* cotton isolates to 20 *C. cassiicola* isolates collected from other hosts with presumed greater fungicide exposure (cucumber, pepper, soybean and tomato) and to those with presumed less exposure to fungicides (hydrangea and mandevilla). Mean $EC_{50}$ values of metconazole, fluxapyroxad and pyraclostrobin for *C. cassiicola* from cotton are 0.07, 0.03 and 0.06 µg/mL respectively. Mean $EC_{50}$ values of metconazole, fluxapyroxad and pyraclostrobin for *C. cassiicola* isolated from hosts with presumed greater fungicide exposure are 0.05, 0.02 and 0.04 µg/mL respectively and 0.03, 0.01 and 0.04 µg/mL for isolates from hosts with presumed less exposure to fungicides. Data showed an indication that with increased fungicide exposure, we are starting to see a shift in sensitivity.

**Introduction**

Target spot is an economically important disease of various ornamental, vegetable and field crops caused by the ascomycete *Corynespora cassiicola* (Berk. & M.A. Curtis). These crops include tomato (*Solanum lycopersicum*), rubber (*Hevea brasiliensis*), soybean (*Glycine max*), cucumber (*Cucumis sativus*) and cotton (*Gossypium hirsutum*) (Blasquez, 1972; Chee,

1988; Koenning et.al, 2006; Miyamoto et. al, 2009; Fulmer et.al, 2012). Target spot has been reported in all soybean growing regions of the U.S. Since 2014, this disease has been observed more frequently in Arkansas and the Mid-South (Faske, 2016). It has been of major concern for soybean growers due to the severe epidemic that ocvcured in 2016 in Mississippi (Allen, 2017). Based on pathogenicity tests conducted by Jones (1961), *C. cassiicola* isolates attacking cotton and soybean in the U.S. were identical. Similar results were obtained from pathogenicity tests conducted by Galbieri et al. (2014) indicating that *C. cassiicola* isolates attacking both cotton and soybean in Brazil belong to the same species of pathogen.

Previous studies have shown tremendous variation in the host range specificity among isolates of *C. cassiicola*. Some isolates were shown to have a wide host range beyond their host of origin. In a study conducted by Dixon et al. (2009), a pair of related isolates from cucumber could infect 2 to 5 other host species. In contrast, other isolates were specific to their host of origin. Host specificity among lineages and/or isolates has also been observed in *C. cassiicola* isolated from other hosts such as papaya (Dixon et al., 2009). In research conducted by Sumabat et al. (2015), significant differences in virulence were observed among 32 *C. cassiicola* isolates collected from different hosts and tested for pathogenicity on cotton, soybean, tomato and cucumber. Isolates originally from cotton were more virulent on cotton than were isolates from other hosts. Based on the study, *C. cassiicola* was shown to be more aggressive when used to inoculate the same host of origin. All isolates from cotton were most aggressive on cotton, but were less aggressive on other inoculated hosts. Similar results were observed for isolates from tomato and soybean.

Under environmental conditions of high relative humidity and extended periods of leaf wetness, the fungus causes target-like spots on leaves and fruit as well as premature defoliation

on numerous hosts. As the disease progresses, the spots become somewhat circular with light brown centers surrounded by dark brown margins (Blasquez, 1967). In the United States, target spot was first observed on cotton in Mississippi in 1961 (Jones, 1961) and subsequently not reported until 2005 when crop consultants in southwestern Georgia reported an unusual occurrence of leaf spot on cotton (Fulmer, 2012). In the past five years, target spot incidence has increased in cotton growing regions in Alabama (Campbell et al. 2012), North and South Carolina (Edmisten, 2012), Louisiana (Price et al. 2015b), and Tennessee (Butler et al. 2016). Initial inoculum for Corynespora leaf spot of cotton was originally believed to come from air-borne conidia from other hosts such as soybean or from crop debris in the soil. However, current studies by Sumabat et al. (2018) suggest this not true. Results of their study suggest that *C. cassiicola* responsible for emerging target spot epidemics in the southeastern United States are either due to the introduction of isolates that are host-specific or due to the evolution of more aggressive lineages on each host.

On soybeans, leaf lesions caused by *C. cassiicola* are reddish-brown, round to irregularly shaped and are frequently surrounded by a yellowish green halo. Larger spots on soybean leaves often develop distinct zonate patterns, hence the name target spot (Faske, 2016). In 2004, a large increase in incidence of target spot of soybean in North Carolina, South Carolina and Alabama was documented (Koenning et al., 2006). Although the disease is found in most soybean-growing countries, it has not been found to cause soybean yield loss in the southeastern United States (Koenning et al., 2006). Estimating yield impact by target spot on soybean has yet to be determined (Faske, 2016).

Target spot caused by *C. cassiicola* is also a concern for tomato growers. It is one of the most serious foliar and fruit diseases of tomato in Florida (Pernezny et al., 2002). Foliar

symptoms include small necrotic areas often surrounded by chlorotic halos. Fruit symptoms vary from small flecks to deeply pitted necrotic areas (Schlub et al., 2009). Yield losses due to direct fruit infections can vary greatly (Vallad et al., 2016). Losses of up to 11,800 kg/ha (30%) marketable yield have been documented in test plots where target spot has not been sufficiently controlled (Pernezny et al., 1996).

**Management**

Effective management of target spot involves a combination of cultural practices and chemical controls. Fungicides from the DMI, QoI and SDHI classes are of particular importance, as they are generally the most effective in controlling target spot on tomato (Schlub et al., 2009). Currently, demethylation inhibitor (DMI) fungicides, such as metconazole and flutriafol have been registered for management of target spot on cotton, either as stand-alone products (Topguard: flutriafol) or pre-mixed with other fungicides (Twinline: pyraclostrobin + metconazole) (Whitaker et al., 2017; Kelly, 2016; Hagan et al., 2013). These fungicides inhibit ergosterol synthesis which is important in the cell membrane structure of fungi (FRAC 2013). Succinate dehydrogenase inhibitor (SDHI) fungicides, such as boscalid and premixed (SDHI + QoI) fluxapyroxad + pyraclostrobin (Priaxor) are also registered commercially for the control of target spot on cucumber and cotton respectively (Whitaker et al., 2017; Kelly, 2016; Miyamoto et al., 2009). These fungicides interfere with fungal respiration by inhibiting the enzyme succinate dehydrogenase (SDH), in complex II of the mitochondrial electron transport chain (FRAC 2009). In addition, quinone outside inhibitor (QoI) fungicides, such as azoxystrobin (Quadris for cotton), premixed azoxystrobin + tetraconazole (Quadris Top for tomato) and pyraclostrobin (Headline for cotton; Cabrio for tomato), are recommended for control of target

spot as well (Pernezny et al., 2002). These fungicides interfere with the mitochondrial respiration, subsequently affecting spore germination and hyphal growth (Bartlett et al., 2002).

**Fungicide Resistance**

Fungicides of DMI, SDHI and QoI groups are registered to control target spot (Paret et al., 2015; Xavier et al., 2013). With intensive use of fungicides, fungal resistance is most likely to occur. "Fungicide resistance is the acquired and heritable reduction in sensitivity of a fungus to a fungicide that occurs as a result of the selection of insensitive members within a population" (FRAC 2016). The likelihood of resistance occurring depends on both biological and chemical factors, namely the pathogen and fungicide, respectively (Brent and Hollomon, 2007b). As growers turn to fungicides for control and management of target spot, sensitivity monitoring programs can be useful for detecting changes in the frequency of less sensitive or resistant isolates before control failure occurs. Establishment of baseline sensitivity is the first step toward a development of a fungicide sensitivity monitoring program and fungicide resistance management strategies (Russell, 2004). Baseline is defined as "a profile of the sensitivity of the target fungus to the fungicide constructed by using biological or molecular biological techniques to assess the response of previously unexposed fungal individuals or populations to the fungicide" (Russell, 2004). For QoI fungicides, sensitivity monitoring is mainly done by spore germination tests and additionally by PCR tests for the G143A mutation (Brent and Hollomon, 2007a). For other classes of fungicides, "various techniques are possible including mycelial growth inhibition, spore germination assays and germ tube elongation assays" (Russell, 2004). To obtain the fungicide sensitivity data, in vitro assays such as mycelial growth inhibition and inhibition of spore germination are conducted to determine the 50% effective fungicide concentration ($EC_{50}$) for each isolate. The $EC_{50}$ value corresponds to the dose that reduces the

growth of mycelium or spore germination to a value of 50% from the untreated control for each isolate (Russell, 2004). Monitoring allows for detection of an impending resistance situation. This is a vital area of resistance research; all knowledge of the distribution, evolution and impact of resistance in the field relies heavily on monitoring (Brent and Hollomon, 2007a).

According to the Fungicide Resistance Action Committee, DMI fungicides are considered to be medium-risk for resistance development (FRAC 2016). DMI resistance has been documented in pathogens causing over 30 fungal diseases including powdery mildew, apple scab and brown rot of stone fruits (McGrath, 2001; Köller et al., 1996). In one study, resistance of *C. cassiicola* isolates from soybean to carbendazim (MBC, FRAC Code 1), was observed from samples collected from Mato Grosso, Brazil (Xavier et al., 2013). In this same study, resistance of these isolates was observed to prothioconazole (DMI, FRAC Code 3); however, the $EC_{50}$ values for this DMI fungicide ranged from 0.47 μg/mL to 26.44 μg/mL (mean 5.02 μg/mL). This is noteworthy because an $EC_{50}$ of 1.0 to 50 μg/mL is considered as moderately resistant (Xavier et al., 2013). Some authors considered 1.0 μg/mL ($EC_{50}$) as a threshold for possible resistance to triazoles (Teramoto et al., 2011; Edgington and Klew, 1971).

SDHI fungicides pose a high-risk for resistance development (FRAC 2016). Resistance to SDHI fungicides has been documented in Japan where target spot is also problematic on cucumber despite availability of multiple fungicides. Isolates of *C. cassiicola* from cucumber collected in Japan were tested in vitro for their sensitivity to boscalid, an SDHI fungicide. Isolates without prior history of boscalid use had $EC_{50}$ values of 0.04 to 0.59 μg/mL. However, isolates with previous exposure to boscalid showed resistance, with $EC_{50}$ values ranging from 1.1 to 6.3 μg/mL for a moderately resistant group. A very highly resistant group had $EC_{50}$ values higher than 24.8 μg/mL (Miyamoto et al., 2009). In the United States, *C. cassiicola* isolates from

tomato collected in Florida from 2015 to 2017 were evaluated for fungicide sensitivity to seven respiration inhibitor fungicides including QoIs and SDHIs. The results revealed that about 75% of these isolates exhibited reduced sensitivity to one or more of the tested SDHI fungicides. In this study, 76 of 79 isolates that had reduced sensitivity to penthiopyrad were also cross resistant with boscalid. Moreover, in 48 of 49 assessments, when an isolate had reduced sensitivity to fluxapyroxad, the isolate was cross resistant with benzovindiflupyr (MacKenzie et al., 2017).

Currently, DMI and SDHI fungicide resistance monitoring in *C. cassiicola* is accomplished using in vitro mycelial growth fungicide sensitivity assays (Teramoto et al., 2017; Miyamoto et al., 2009). This methodology was chosen for DMI fungicides because triazoles (sterol demethylation inhibitor; DMI) target sterol 14α-demethylase (CYP51), an important enzyme in the ergosterol biosynthetic pathway (Köller, 1992; Price et al., 2015a). This process leads to the disruption of membrane structure and prevents active membrane transport due to a combination of two factors: the depletion of ergosterol in the cell and the accumulation of 14α-methylated sterols; resulting in fungistasis, as shown in inhibition of mycelial growth (Price et al. 2015a). SDHI fungicides, on the other hand, are inhibitors of succinate dehydrogenase in the mitochondrial respiration chain of numerous fungal pathogens and hence inhibit several stages in the life cycle of pathogens, including spore germination, mycelium and germ-tube growth (Burchett et al., 2017).

Similar to SDHI fungicides, QoI fungicides pose a high risk for resistance development according to the Fungicide Resistance Action Committee (FRAC 2016). QoI fungicides were introduced to the market around 1996 and QoI resistance of fungal pathogens was documented as early as 1998 (Bartlett et al., 2002). In 2012, a list of pathogens with field resistance to QoI fungicides was released (FRAC 2013). The list included a QoI resistant population of

*Cercospora sojina*, the causal agent of frog-eye leaf spot in soybean, which was documented in Tennessee in 2010 probably due to the long history for applying QoI fungicides for control of the disease (Zhang et al., 2012a; Zhang et al., 2012b). Moreover, isolates of *C. cassiicola* collected from tomato between 2015 to 2017 were tested for fungicide sensitivity. Ninety percent of these isolates were found resistant to the QoI fungicide azoxystrobin having $EC_{50}$ values greater than 10 μg/mL (MacKenzie et al., 2017).

Currently, QoI fungicide resistance monitoring in *C. cassiicola* from tomato and soybean is accomplished using in vitro conidial germination fungicide sensitivity assays with and without SHAM respectively (MacKenzie et al., 2017; Teramoto et al., 2017). Studies with azoxystrobin and pyraclostrobin have demonstrated that conidial germination is particularly sensitive to QoI fungicides (Bartlett et al., 2002). Additionally, in vitro studies have shown that some fungal plant pathogens can bypass the activity of respiration inhibiting fungicides through activation of an alternative respiratory pathway (Avila-Adame, 2002; Avila-Adame et al., 2003). Salicylhydroxamic acid (SHAM) can be added to QoI fungicide-amended medium to inhibit the alternative respiratory pathway that interferes with the activity of the fungicide (Wood and Hollomon, 2003). However, this alternative respiratory pathway does not play a practical and significant role in field resistance to QoI fungicides. The addition of SHAM to fungicide-amended medium has been observed to increase the sensitivity of some plant pathogenic fungi to QoI fungicides (Jin et al., 2009). In contrast, it has also been shown that SHAM has no effect in the sensitivity of other plant pathogenic fungi to QoI fungicides (Teramoto et al., 2017; Rebollar-Alviter et al., 2007).

A common approach to confirming resistance involves comparing sensitivities of isolates obtained from sites where performance declined with the sensitivities of isolates without prior

exposure to the fungicides in question. Where baseline sensitivity data do not exist, comparisons can be made between isolates obtained from at-risk sites with those collected from untreated areas (Ishi and Hollomon, 2015). The opportunity to test *C. cassiicola* isolates from cotton prior to fungicide exposure has already been missed. Therefore, we opted to compare current sensitivity of *C. cassiicola* isolates from cotton with that of *C. cassiicola* from other hosts with presumed greater fungicide exposure (soybean, cucumber, tomato, pepper) and to those with presumed less exposure to fungicides (hydrangea and mandevilla).

Hence, the objectives of this study were to compare sensitivity of *C. cassiicola* isolates from cotton to isolates collected from other hosts in their sensitivity to metconazole (DMI, FRAC Code 3), fluxapyroxad (SDHI, FRAC Code 7), and pyraclostrobin (QoI, FRAC Code 11). Focus was to compare the sensitivity of isolates from cotton to isolates from other hosts collected in Georgia which were expected to have different exposure to fungicides.

**Materials and Methods**

**Sampling, isolation, incubation and isolate maintenance.** *Corynespora cassiicola* was isolated from host species other than cotton symptomatic for target spot in the southeastern U.S. Five isolates were collected from hydrangea (*Hydrangea* spp.), two from mandevilla (*Mandevilla* spp.), two from tomato (*Solanum lycopersicum*), one from pepper (*Capsicum anum*), one from cucumber (*Cucumis sativus*) and nine from soybean (*Glycine max*) (Table 3.1). Isolates were obtained from leaves of these hosts with typical target spot lesions and small pieces of diseased tissue were placed on quarter strength potato dextrose agar (qPDA). For comparison, a total of 40 isolates of *C. cassiicola* was obtained from cotton symptomatic for target spot from Georgia and the southern U.S. Isolates were obtained from cotton leaves with typical target spot lesions and isolated on qPDA. Tissue was incubated at 25°C for 3 days in the dark to allow for

mycelial growth and sporulation. Plugs were taken from the edges of the mycelial growth from each isolate and transferred to qPDA as subcultures. After 7 days these active cultures were used in fungicide sensitivity assays. For long term storage, all isolates were maintained on filter paper discs as dried mycelium and stored at -20°C until further use. Stored isolates were recovered by placing a piece of filter paper with fungal mycelium on a fresh plate of qPDA and incubated at 25°C for 7 days in preparation for fungicide sensitivity assays.

**Fungicide-amended media preparation.** To test the sensitivity of *C. cassiicola* to selected fungicides, potato dextrose agar (PDA) cooled to 55°C was amended with 0, 0.0003, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0 or 3.0 µg of a.i./mL of technical grade metconazole or fluxapyroxad (BASF Corporation). Water agar (WA) was amended with 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0 or 10 µg of a.i./mL of technical grade pyraclostrobin (BASF Corporation) after being autoclaved and cooled to 55°C. Media were poured into sterile 9-mm-diameter sterile petri plates using approximately 30 mL per plate and allowed to solidify. In addition, salicylhydroxamic acid (SHAM) at 100 µg/mL dissolved in methanol was either added or not added to the WA. SHAM is used in QoI fungicide in vitro assays to prevent fungi from using an alternative respiration pathway (Ziogas et al., 1997).

**Fungicide sensitivity assays.** Sensitivity of each isolate to metconazole and fluxapyroxad was determined by using an in vitro mycelial growth assay on fungicide-amended and non-amended PDA plates. Sensitivity of each isolate to pyraclostrobin was determined through a spore germination assay on fungicide-amended and non-amended water agar. Laboratory assays were conducted by measuring reductions in mycelial growth and spore germination of fungi in the presence of fungicides through fungicide-amended media. These three fungicides have different modes of action and metabolic targets; therefore, they require

different procedures for measuring fungicide sensitivity. Inhibition of colony radial growth assay was used to test for sensitivity to metconazole and fluxapyroxad whereas sensitivity to pyraclostrobin was tested using inhibition of spore germination assay in this study. The experiments were conducted twice.

**Mycelial growth assay.** Sensitivity to metconazole and fluxapyroxad was tested using a mycelial growth assay on fungicide-amended and non-amended media. Five-millimeter-diameter mycelial plugs taken from the edge of a 7-day-old culture of each isolate were placed mycelium-surface down onto the center of the PDA plates amended with each concentration of the fungicides. The plates were then incubated at 25 to 27ºC for four days in the dark to allow for mycelial growth. Colony diameter was measured for each isolate, with the original diameter of the mycelial plug subtracted from each measurement. The average of the colony diameters measured in two perpendicular directions was used. Two replicates of each fungicide concentration were used per isolate. The experiment was performed twice. Relative growth was calculated as the ratio between the corrected colony diameter on fungicide-amended medium and the corrected colony diameter on non-amended medium. The effective concentration capable of inhibiting mycelial growth by 50% ($EC_{50}$) was estimated for each treatment by regression of the probit-transformed relative inhibition on $\log_{10}$-transformed fungicide concentration. The transformations were used to linearize the relationship between relative inhibition (1-RG) and the fungicide concentration so that linear regression could be used to fit a linear model to the relationship and enable prediction of the $EC_{50}$ value. The conditions for testing and duration of tests were standardized to ensure repeatability and accuracy of results.

**Spore Germination Assay.** Sensitivity to pyraclostrobin was tested using a spore germination assay. Mycelial plugs 5 mm in diameter were taken from 4-day-old revived cultures

and were transferred using a sterile scalpel to plated V8 agar medium. For each isolate, this was replicated five times. Isolates of *C. cassiicola* were grown on V8 agar media for 10 days at 25 to 27°C under continuous fluorescent light to induce sporulation. This method was chosen after conducting several preliminary experiments with different media and light conditions (Onesirosan et al., 1975; Sharma and Bowen, 2016; Fernando et al., 2012). From research conducted on sporulation of a *C. cassiicola* isolate from rubber, sporulation occurred when cultures were incubated under a few hours of UV light each day and that scraping off the mycelium from the culture did not enhance sporulation (Chee, 1988). Conidial suspensions of each isolate were prepared by flooding the plates with 5 mL of a solution of sterile water and gently scraping the surface of the mycelia with a rod to dislodge the conidia. Conidial suspensions of 20,000 spores/mL from each individual isolate were calculated using a hemacytometer, and 1mL of the spore suspension was then transferred onto fungicide-amended and non-amended water agar plates. Two replicates of each isolate and treatment were prepared. After incubating at 25°C for 24 h, 100 spores per plate were examined microscopically and germination percentage was recorded. Relative germination was calculated as the percent germination (RG) on fungicide-amended medium divided by the percent germination of the same isolate on non-amended medium. A conidium was considered germinated if the length of the germ tube was equal to or greater than half the length of the conidium (Beckman and Payne, 1983). Relative inhibition (1 minus RG) was probit-transformed and linearly regressed on $\log_{10}$-transformed fungicide concentration. Fungicide sensitivity for each isolate was expressed as the $EC_{50}$ value (the fungicide concentration that inhibits spore germination by 50% relative to the control) that was estimated from the linear regressions.

**Effect of strobilurin fungicide and SHAM on spore germination.** All 20 isolates were tested to determine whether salicylhydroxamic acid (SHAM) affected the response of spore germination to the fungicide pyraclostrobin. SHAM was added to fungicide-amended and non-amended water agar to prevent the alternative oxidation pathway in the fungus that could lead to a false resistance or reduced sensitivity result (Ziogas et al., 1997). SHAM was dissolved in methanol and was incorporated with fungicide-amended and non-amended water agar plates at a concentration of 100 μg/mL of SHAM. The effect of SHAM at 100 μg/mL on spore germination was evaluated in combination with pyraclostrobin concentrations of 0-10 μg/mL.

**Data Analysis.** The $EC_{50}$ values were calculated with the PROC REG function in SAS (Version 9.3; SAS Institute Inc., Cary, NC). The $EC_{50}$ value for each isolate was estimated based on linear regression of probit-transformed relative inhibition (1-RG) on $log_{10}$-transformed fungicide concentration. The frequency distribution of $log_{10}$-transformed $EC_{50}$ values was tested for normality using four tests: Shapiro-Wilk *W*, Kolmogorov-Smirnov *D*, Cramer-vol Mises $W^2$, and Anderson-Darling $A^2$ (PROC UNIVARIATE). Tukey's multiple comparisons test (HSD) was performed to compare the mean $log_{10}$-transformed $EC_{50}$ values among hosts among fungicide sensitivity trials. Paired *t* tests were performed to compare the effect of SHAM on individual isolates as well as to compare the mean $log_{10}$-transformed $EC_{50}$ values of isolates per host grown on SHAM amended WA plates and those grown on plates without SHAM.

**Results**

The metconazole $EC_{50}$ values for *C. cassiicola* isolates from hosts with presumed greater fungicide exposure (cucumber, pepper, soybean, tomato) ranged from 0.011 to 0.936 μg/mL with a mean of 0.047 μg/mL (Table 3.2 Fig. 3.1). The $EC_{50}$ values from hosts with predicted less exposure to fungicides (hydrangea and mandevilla) ranged from 0.019 to 0.076 μg/mL with a

mean of 0.026 µg/mL (Table 3.2 Fig. 3.1). The $EC_{50}$ values from cotton ranged from 0.015 to 0.205 µg/mL with a mean of 0.072 µg/mL (Table 3.2 Fig. 3.1). One soybean isolate (STs-1) had the highest $EC_{50}$ value of 0.936 µg/mL (Table 3.1). The mean $EC_{50}$ values for these three groups were not significantly different (*P*=0.252).

The fluxapyroxad $EC_{50}$ values for *C. cassiicola* isolates from hosts with presumed greater fungicide exposure (cucumber, pepper, soybean, tomato) ranged from 0.006 to 0.148 µg/mL with a mean of 0.020 µg/mL (Table 3.2 Fig. 3.2); from hosts with predicted less exposure to fungicides (hydrangea and mandevilla) $EC_{50}$ values ranged from 0.006 to 0.030 µg/mL with a mean of 0.012 µg/mL (Table 3.2 Fig. 3.2). The $EC_{50}$ values from cotton ranged from 0.001 to 0.126 µg/mL with a mean of 0.026 µg/mL (Table 3.2 Fig. 3.2). The mean $EC_{50}$ values for these three groups were not significantly different (*P*=0.432).

The $EC_{50}$ values of pyraclostrobin for inhibition of spore germination against *C. cassiicola* isolates from hosts with presumed greater fungicide exposure (cucumber, pepper, soybean, tomato) ranged from 0.019 to 0.182 µg/mL with a mean of 0.042 µg/mL (Table 3.2 Fig. 3.3); $EC_{50}$ values from hosts with predicted less exposure to fungicides (hydrangea and mandevilla) ranged from 0.019 to 0.121 µg/mL with a mean of 0.038 µg/mL (Table 3.2 Fig. 3.3). The $EC_{50}$ values from cotton ranged from 0.013 to 0.200 µg/mL, with a mean of 0.064 µg/mL (Table 3.2 Fig. 3.3). The mean $EC_{50}$ values for these three groups were not significantly different (*P*=0.555).

Isolates from soybean had larger $EC_{50}$ values for all fungicides tested compared to isolates from hydrangea although the differences were not significant. Isolates from soybean had larger $EC_{50}$ values for metconazole compared to isolates from cotton. When comparing $EC_{50}$ values for fluxapyroxad and pyraclostrobin, soybean and cotton isolates had similar $EC_{50}$ values.

The range of $EC_{50}$ values for metconazole and fluxapyroxad from soybean is greater than that of isolates from cotton whereas the range of $EC_{50}$ values from hydrangea is less than that of isolates from cotton (Table 3.4). For pyraclostrobin, the range of $EC_{50}$ values from soybean is less than that of isolates from cotton but greater than that of isolates from hydrangea whereas the range of $EC_{50}$ values from hydrangea is less than that of isolates from cotton.

The addition of SHAM at 100 µg/mL to media amended with pyraclostrobin had a significant effect on the mean $EC_{50}$ value of *C. cassiicola* isolates from tomato, for which addition of SHAM to the fungicide pyraclostrobin resulted in significantly lower mean $EC_{50}$ value (Table 3.3). The mean $EC_{50}$ values of *C. cassiicola* isolates from cucumber, pepper, soybean, hydrangea and mandevilla, with or without SHAM, were not significantly different (Table 3.3).

**Discussion**

*Corynespora cassiicola* was isolated from host species in addition to cotton from the southeastern U.S. symptomatic for target spot. Focus was to compare the sensitivity of isolates from cotton to isolates from other hosts collected in Georgia which were likely exposed to more or less fungicides than was cotton. Because it was a limited survey, we wanted to look at isolates from the same geographical location (Georgia) from various hosts, some with greater exposure to fungicides (soybean, cucumber, tomato and pepper) than others for which fungicides are not typically applied (hydrangea and mandevilla). However, we also looked at soybean isolates from Tennessee. Tennessee is important in this survey, both because of the long history for applying QoI fungicides for control of frog-eye leaf spot and because of the recent introduction of target spot in 2013 to the state (Butler et al., 2016).

From the frequency distribution of $EC_{50}$ values determined for metconazole (Figure 3.1), *C. cassiicola* isolates collected from various hosts appear to be sensitive to that fungicide. This was based on the criteria adapted from Edgington & Klew (1971), who considered a fungicide highly toxic to a fungal isolate with an $EC_{50} < 1$ µg/mL (sensitive), moderately toxic when $1 < EC_{50} < 50$ µg/mL (moderately resistant) and non-toxic when $EC_{50} > 50$ µg/mL (resistant). The criteria adapted for classifying the sensitivities were from a study that looked at fungitoxicity of benzimidazole compounds to a wide spectrum of fungi. However, these same criteria were also used and adapted for testing sensitivity of *C. cassiicola* from soybean to DMI fungicides cypoconazole, epoxiconazole, flutriafol and tebuconazole (Avozani et al., 2014). Based on these criteria, isolates from all hosts included in this study were sensitive to metconazole with the exception of a single soybean isolate. This particular soybean isolate was collected in Tennessee and had an $EC_{50}$ value of 0.936 µg/mL. This value is close to 1.0 µg/mL which separates "sensitive" from "moderately toxic" (Table 3.1). Some authors consider 1.0 µg/mL ($EC_{50}$) as a cut threshold for sensitivity to triazoles (Terramoto et al., 2011; Edgington and Klew, 1971). In Brazil, resistance to the methyl benzimidazole carbamate (MBC) carbendazim and decreased sensitivity to the demethylation inhibitor (DMI) prothioconazole ($EC_{50}$ ranged from 0.47 to 26.44 µg/mL with a mean of 5.02 µg/mL) on C. *cassiicola* isolated from soybean has been documented (Avozani et al., 2014; Xavier et al., 2013). Avozani (2014) studied the sensitivity of five *C. cassiicola* isolates from soybean to carbendazim and observed $EC_{50}$ values of 0.2 µg/mL, 0.26 µg/mL and >40 µg/mL for the remaining three isolates. Isolates from soybean, with presumed greater exposure to fungicides, had greater range and larger $EC_{50}$ values for metconazole, fluxapyroxad and pyraclostrobin compared to isolates from hydrangea which are expected to have least fungicide exposure (Table 3.4) although the differences were not

significant. In addition, the range of $EC_{50}$ values from soybean was greater than that of isolates from cotton for metconazole and fluxapyroxad (Table 3.4) however the differences were not significant. This is an indication that with increased exposure, shifts in fungicide sensitivity seem to have occurred.

Detection of decreased sensitivity of *C. cassiicola* to a fungicide is not unique to soybean isolates. In cucumber, Date et al. (2004) found *C. cassiicola* populations resistant to thiophanate-methyl and diethofencarb. In addition, *C. cassiicola* isolates collected from tomato in Florida over the period from 2015 to 2017 were evaluated for fungicide sensitivity to seven respiration inhibitor fungicides including QoIs and SDHIs. Nearly 86% of the isolates were sensitive to SDHI fluopyram (MacKenzie et al., 2017). Both tomato isolates evaluated in this current study were sensitive to all three fungicides tested, with $EC_{50}$ values ranging from 0.006 to 0.039 µg/mL (Table 3.1). Furthermore, according to the frequency distribution of $EC_{50}$ values determined for fluxapyroxad (Figure 3.2), *C. cassiicola* isolates collected from various hosts appear to be sensitive to fluxapyroxad. In the study conducted by MacKenzie et al. (2017), 55% of *C. cassiicola* isolates from tomato were still sensitive to the SDHI fluxapyroxad, while 45% were moderately resistant, having $EC_{50}$ values greater than 1.0 but less than 10 µg/mL.

In our study, $EC_{50}$ values determined for pyraclostrobin (Table 3.1) showed that all isolates were sensitive to the fungicide. However, in other studies resistance has been documented to QoI fungicides. Tomato *C. cassiicola* isolates from Florida collected from 2015 to 2017 were evaluated for fungicide sensitivity to seven respiration inhibitor fungicides to include QoI and SDHI classes. Results revealed that nearly 90% of the isolates were resistant to QoI fungicides azoxystrobin and fenamidone (MacKenzie et al., 2017). Having sampled only two tomato isolates, both from the state of Georgia and collected in 2013, it is not surprising to

find differences in fungicide sensitivity results. Both tomato isolates evaluated in the current study were sensitive to pyraclostrobin, having $EC_{50}$ values of 0.033 and 0.039 μg/mL. Furthermore, QoI resistance development has been documented in other fungal species such as *Stagonosporopsis spp.* (Stevenson et al., 2004), *Botrytis cinerea* (Banno et al., 2009), *Mycosphaerella fijiensis* (Sierotzki et al., 2000) and *Venturia inaequalis* (Lesniak et al., 2011). This underscores the importance of sensitivity monitoring for a fungal pathogen such as *C. cassiicola* that has the potential to rapidly develop resistance to single site mode of action fungicides such as pyraclostrobin.

When working with QoI fungicides in vitro, the site of action of QoI fungicides can be bypassed by the fungi through an alternative oxidase pathway which is inhibited by salicylhydroxamic acid (SHAM) (Ziogas et al., 1997). In this study, the addition of SHAM at 100 μg/mL to media amended with pyraclostrobin had a significant effect on mean $EC_{50}$ values of *C. cassiicola* isolates from tomato where isolates grown on fungicide amended media without SHAM had higher $EC_{50}$ values compared to when grown on fungicide amended media with SHAM (Table 3.3). The results were similar to studies on azoxystrobin sensitivity of *C. cassiicola* isolates from tomato in Florida (MacKenzie et al., 2017). However, there was no significant effect of SHAM in increasing the sensitivity of *C. cassiicola* isolates from cucumber, pepper, soybean, hydrangea and mandevilla to pyraclostrobin (Table 3.3). Still, *C. cassiicola* isolates from these 5 hosts, when grown on fungicide amended media without SHAM, had higher $EC_{50}$ values compared to when grown on fungicide amended media with SHAM. Similarly, on a study conducted by Teramoto et. al., (2017), they found that SHAM did not significantly affect the sensitivity of 4 *C. cassiicola* isolates from soybean to QoI fungicides.

In this study, fungicide sensitivity data for metconazole obtained from isolates with presumed reduced fungicide exposure (hydrangea and mandevilla) showed a much narrower range of sensitivity compared to *C. cassiicola* isolates collected from hosts with predicted greater exposure to fungicides (cucumber, pepper, soybean, tomato). In comparison, *C. cassiicola* isolates obtained from cotton had a broader range of sensitivities compared to isolates from hosts with presumed reduced fungicide exposure but had a narrower range when compared to isolates collected from hosts with presumed greater exposure to fungicides for metconazole and fluxapyroxad. These differences in range of sensitivities might have been due to the different history of fungicide exposure. Similar patterns in frequency distribution of fungicide sensitivity was observed for fluxapyroxad and pyraclostrobin but the differences in range was more apparent in the fungicide metconazole. This can be explained by the type of resistance one would expect to occur on these different classes of fungicides. The frequency of *C. cassiicola* SDHI and QoI-sensitive isolates follows the pattern of qualitative sensitivity distribution. Qualitative or single-step resistance occurs as the result of a single mutation in the target site which leads the sudden loss of product efficacy, as what is seen on respiration inhibitors (Brent and Hollomon 2007a; FRAC 2016). On the other hand, the frequency of *C. cassiicola* DMI-sensitive isolates follows the pattern of quantitative sensitivity distribution. Quantitative, or multi-step resistance, is a process where multiple mutations occurring in the target site result in a gradual shift from sensitivity to insensitivity in the pathogen population over several years as what has been observed in DMI fungicides (Brent and Hollomon 2007a; FRAC 2016).

Although this research included only a small number of isolates from other hosts, the results reinforce the occurrence of variations in fungicide sensitivity of *C. cassiicola* isolates to different classes of fungicides. A more detailed sampling representative of other hosts should be

conducted to better monitor and demonstrate shifts in sensitivity in future samplings. Continuous fungicide sensitivity monitoring is a very important resistance management technique because it allows for subsequent detection of population sensitivity shifts (Russell, 2004). Caution should be taken with frequency of fungicide applications. Single-site fungicides such as pyraclostrobin and fluxapyroxad pose greater risk for resistance and should be limited to only a few timely applications per year.

Lastly, compared to *C. cassiicola* isolates from cotton, these isolates from other hosts showed similar variability in fungicide sensitivity to metconazole, fluxapyroxad and pyraclostrobin. Isolates from soybean, with presumed greater fungicide exposure, had larger $EC_{50}$ values compared to isolates from hydrangea, which were presumed to have had the least fungicide exposure. The range of $EC_{50}$ values from soybean was greater than that of isolates from cotton and hydrangea. This is an indication that with increased fungicide exposure, we are starting to see a shift in sensitivity. Since resistance has been documented for *C. cassiicola* from other hosts, better management strategies for preventing resistance in the pathogen on cotton may be derived by looking into the studies conducted on these other crops affected by target spot.

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Table 3.1. List of *Corynespora cassiicola* isolates, host of origin, year and place of isolation, and effective fungicide concentrations of metconazole, fluxapyroxad and pyraclostrobin that reduce 50% mycelial growth or spore germination ($EC_{50}$ μg/mL)

| Isolate | Host | Year Isolated | Location State | $EC_{50}$ (μg/mL) | | |
|---|---|---|---|---|---|---|
| | | | | Metconazole | Fluxapyroxad | Pyraclostrobin |
| CuC-1 | cucumber | 2013 | GA | 0.039 | 0.017 | 0.036 |
| GaAH-1 | hydrangea | 2013 | GA | 0.031 | 0.009 | 0.019 |
| GaAH-2 | hydrangea | 2013 | GA | 0.024 | 0.018 | 0.022 |
| GaNH-2 | hydrangea | 2013 | GA | 0.020 | 0.007 | 0.030 |
| HAL-1 | hydrangea | 2015 | AL | 0.020 | 0.012 | 0.120 |
| HGAm-1 | hydrangea | 2015 | GA | 0.021 | 0.006 | 0.044 |
| GaAHb-1 | mandevilla | 2013 | GA | 0.076 | 0.030 | 0.061 |
| GaAHb-2 | mandevilla | 2013 | GA | 0.019 | 0.009 | 0.029 |
| PE-2-1 | pepper | 2013 | GA | 0.026 | 0.016 | 0.038 |
| SStb | soybean | 2013 | GA | 0.013 | 0.012 | 0.120 |
| SMR-1 | soybean | 2013 | GA | 0.028 | 0.015 | 0.041 |
| STs-1 | soybean | 2013 | TN | 0.936 | 0.148 | 0.036 |
| STs-2 | soybean | 2013 | TN | 0.028 | 0.024 | 0.021 |
| STNa-1 | soybean | 2014 | TN | 0.151 | 0.020 | 0.019 |
| STNa-2 | soybean | 2014 | TN | 0.018 | 0.011 | 0.021 |
| STNb-1 | soybean | 2014 | TN | 0.028 | 0.014 | 0.181 |
| STNb-2 | soybean | 2014 | TN | 0.045 | 0.018 | 0.081 |
| STNc-1 | soybean | 2014 | TN | 0.185 | 0.018 | 0.035 |
| Tcf-1 | tomato | 2013 | GA | 0.085 | 0.058 | 0.033 |
| Tcl-1 | tomato | 2013 | GA | 0.011 | 0.006 | 0.039 |

Table 3.2. Mean effective concentration of fungicides to inhibit mycelial growth and spore germination by 50% ($EC_{50}$) of *Corynespora cassiicola* isolates from seven different hosts

| Fungicide | Hosts | Number of Isolates (n) | $EC_{50}$ (µg/mL) Range[a] | Mean[b] | Assay/ Method |
|---|---|---|---|---|---|
| Metconazole | cucumber, pepper, soybean, tomato | 13 | 0.011-0.936[a] | 0.123 A | mycelial growth |
| Metconazole | hydrangea, mandevilla | 7 | 0.019-0.076[a] | 0.030 A | mycelial growth |
| Metconazole | cotton | 40 | 0.015-0.205[a] | 0.072 A | mycelial growth |
| Fluxapyroxad | cucumber, pepper, soybean, tomato | 13 | 0.006-0.148[a] | 0.029 A | mycelial growth |
| Fluxapyroxad | hydrangea, mandevilla | 7 | 0.006-0.030[a] | 0.013 A | mycelial growth |
| Fluxapyroxad | cotton | 40 | 0.001-0.126[a] | 0.027 A | mycelial growth |
| Pyraclostrobin | cucumber, pepper, soybean, tomato | 13 | 0.019-0.182[a] | 0.054 A | spore germination |
| Pyraclostrobin | hydrangea, mandevilla | 7 | 0.019-0.121[a] | 0.047 A | spore germination |
| Pyraclostrobin | cotton | 40 | 0.013-0.200[a] | 0.064 A | spore germination |

[a] Minimum and maximum $EC_{50}$ value of mycelium and spore inhibition for each fungicide.
[b] Means within each fungicide followed by the same uppercase letter were not significantly different according to Tukey's honestly significant difference (HSD) ($P \leq 0.05$).

Table 3.3 Mean [a] effective fungicide concentration of pyraclostrobin amended with 100 µg/mL of SHAM to inhibit spore germination by 50% ($EC_{50}$) of twenty *Corynespora cassiicola* isolates from six different hosts

| Host | Number of Isolates | Pyraclostrobin $EC_{50}$ (µg/mL) | |
|---|---|---|---|
| | | No SHAM | SHAM 100 |
| cucumber | 1 | 0.039 A [b] | 0.036 A |
| pepper | 1 | 0.035 A | 0.035 A |
| soybean | 9 | 0.062 A | 0.062 A |
| tomato | 2 | 0.051 A | 0.036 B |
| hydrangea | 5 | 0.049 A | 0.047 A |
| mandevilla | 2 | 0.056 A | 0.045 A |

[a] Mean $EC_{50}$ values were calculated from four replicates for each isolate

[b] Mean separation within row followed by the same uppercase letter are not significantly different according to *t*-test (LSD) ($P \leq 0.05$).

Table 3.4. Mean [a] effective concentration of fungicides to inhibit mycelial growth and spore germination by 50% ($EC_{50}$) of *Corynespora cassiicola* isolates from soybean, cotton and hydrangea

| Host | Number of Isolates (n) | $EC_{50}$ µg/mL | | |
|---|---|---|---|---|
| | | Metconazole | Fluxapyroxad | Pyraclostrobin |
| soybean | 9 | 0.16 | 0.03 | 0.06 |
| hydrangea | 5 | 0.03 | 0.01 | 0.05 |
| cotton | 40 | 0.07 | 0.03 | 0.06 |

[a] Mean $EC_{50}$ values were calculated from four replicates for each isolate

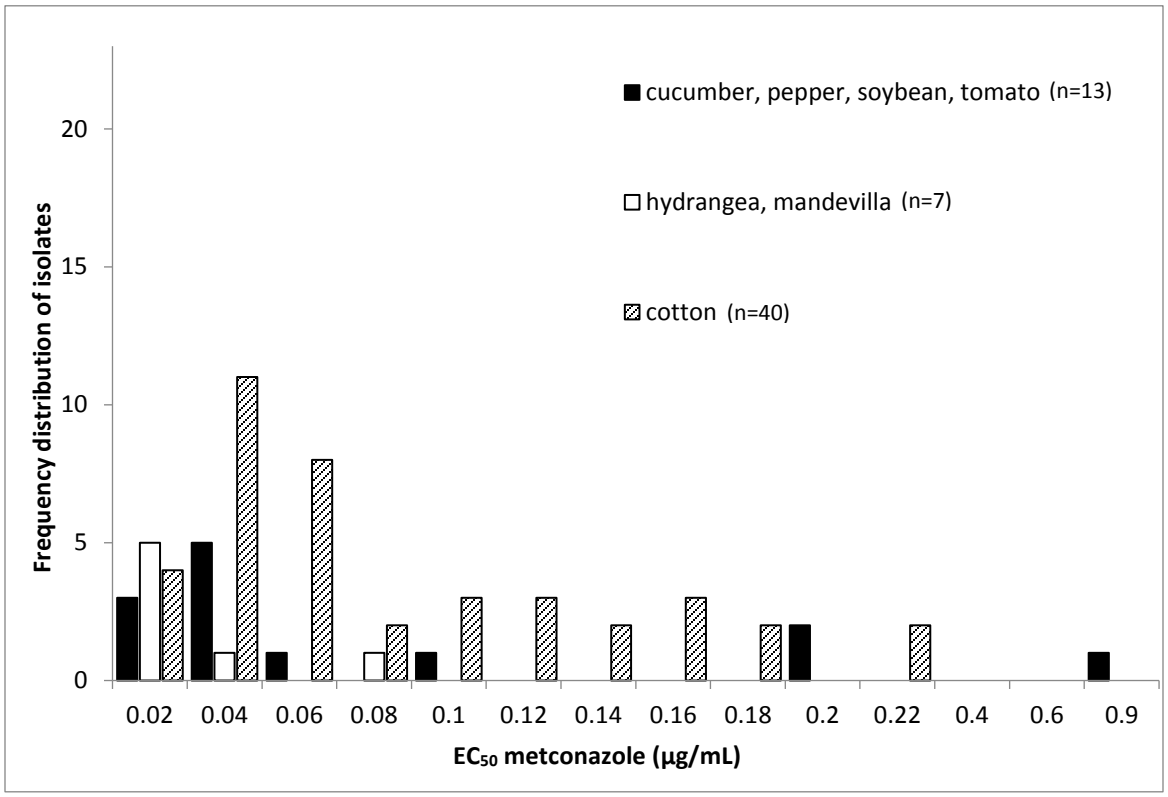


Figure 3.1. Frequency distribution of 50% effective fungicide concentration ($EC_{50}$) of metconazole for 40 *Corynespora cassiicola* isolates from cotton and 20 isolates from other hosts. 40 isolates collected from cotton from southeastern United States (striped bars) and 20 isolates of *Corynespora cassiicola* isolated from cucumber (1 isolate), pepper (1 isolate), soybean (9 isolates), tomato (2 isolates) with predicted greater fungicide exposure (black bars), and hydrangea (5 isolates), mandevilla (2 isolates) with predicted less exposure to fungicides (white bars), on PDA medium after a 4-day incubation at 25ºC in the dark.

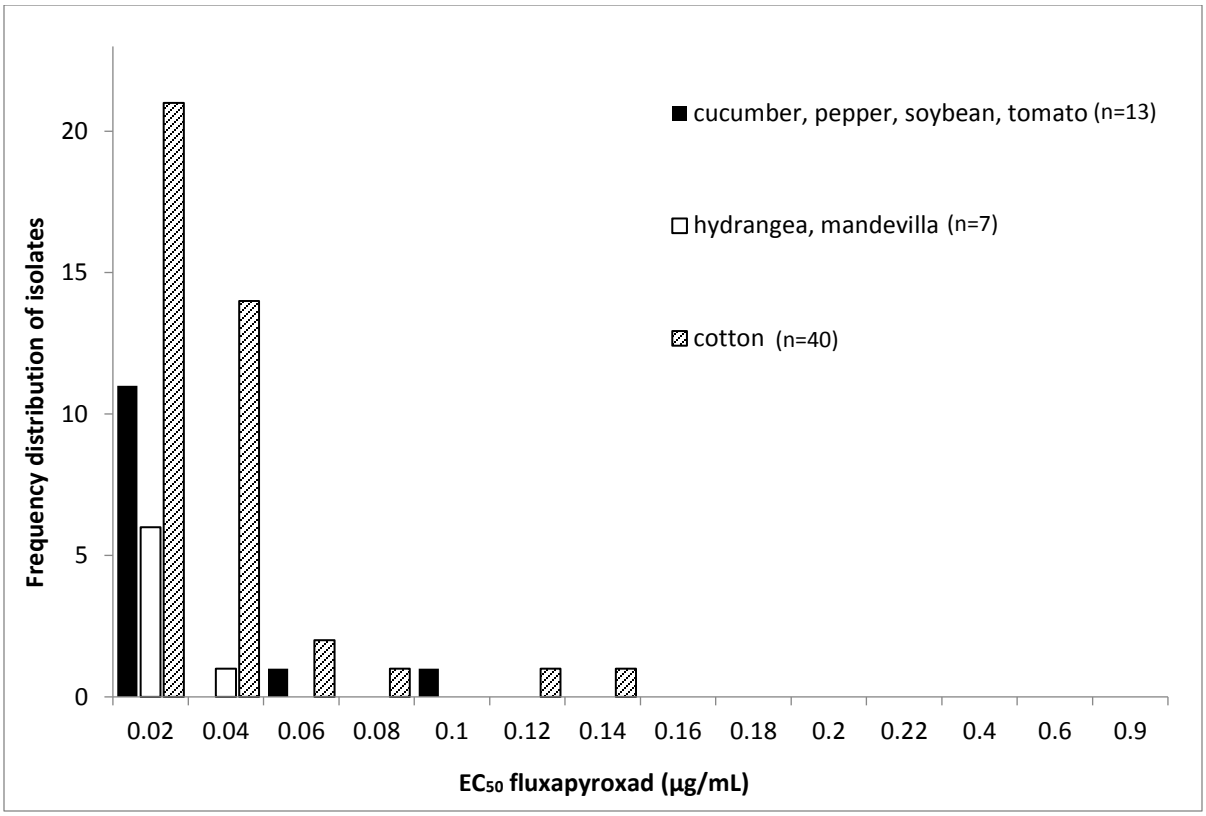


Figure 3.2. Frequency distribution of 50% effective fungicide concentration ($EC_{50}$) of fluxapyroxad for 40 *Corynespora cassiicola* isolates from cotton and 20 isolates from other hosts. 40 isolates collected from cotton from southeastern United States (striped bars) and 20 isolates of *Corynespora cassiicola* isolated from cucumber (1 isolate), pepper (1 isolate), soybean (9 isolates), tomato (2 isolates) with predicted greater fungicide exposure (black bars), and hydrangea (5 isolates), mandevilla (2 isolates) with predicted less exposure to fungicides (white bars), on PDA medium after a 4-day incubation at 25ºC in the dark.

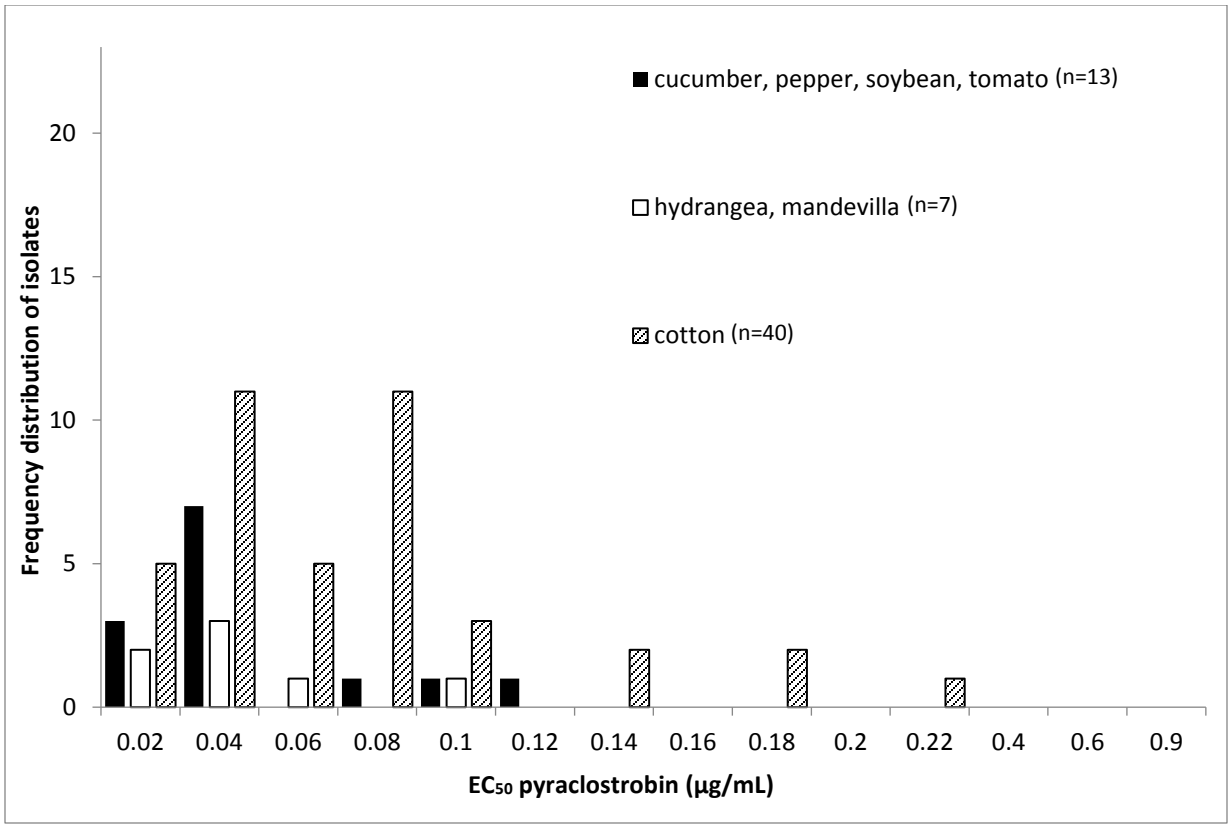


Figure 3.3. Frequency distribution of 50% effective fungicide concentration ($EC_{50}$) of pyraclostrobin for inhibition of spore germination against 40 *Corynespora cassiicola* isolates from cotton and 20 isolates from other hosts. 40 isolates collected from cotton from southeastern United States (striped bars) and 20 isolates of *Corynespora cassiicola* isolated from cucumber (1 isolate), pepper (1 isolate), soybean (9 isolates), tomato (2 isolates) with predicted greater fungicide exposure (black bars), and hydrangea (5 isolates), mandevilla (2 isolates) with predicted less exposure to fungicides (white bars), on PDA medium after a 4-day incubation at 25ºC in the dark.

# CHAPTER 4

# ASSESSMENT OF FUNGICIDES, VARIETIES AND PLANT GROWTH REGULATORS IN THE MANAGEMENT OF TARGET SPOT ON COTTON IN GEORGIA

## Introduction

Target spot of cotton, caused by the fungus *Corynespora cassiicola* (Berk & M.A. Curtis) C. T. Wei, is an economically important foliar disease that is of recent concern to cotton growers particularly in the southeastern United States. This emerging disease on cotton was recognized as a threat in southwestern Georgia in 2005 (Fulmer et al., 2012). Other states where target spot outbreaks have occurred include Alabama (Campbell et al., 2012; Conner et al., 2013), Louisiana (Price et al., 2015), Virginia, Tennessee (Butler et al., 2016), North Carolina (Edmisten, 2012), and South Carolina. In some situations, estimated yield losses in select cultivars exceeded 336 kg/ha seed cotton (Conner et al., 2013). The disease can spread rapidly and cause significant yield reductions in environmental conditions favorable for disease development. Environmental factors that favor leaf infection are prolonged conditions of high relative humidity and leaf wetness or free moisture provided by light rain or heavy dew as well as warm temperatures (Faske, 2016; Pernezny et al., 2002). Predicted losses, estimated at 5% in Alabama and Georgia, would amount to $70 million, and predicted losses estimated at 40% would be devastating to the cotton producers of these states (Conner et al., 2013; Hagan, 2014).

Target spot is an economically important disease on cotton elsewhere in the world as well. It has been observed on cotton in China in 2013 (Wei et al., 2014) and in Brazil in 2004 (Galbieri et al., 2014) where the disease was reported to limit production of cotton. In India,

target spot pathogenic to cotton bolls was observed as early as 1988 (Lakshmanan et al., 1990). In addition, *C. cassiicola* has a broad host range and is able to infect at least 530 different plant species including papaya (*Carica papaya* L.), tomato (*Solanum lycopersicum*), cucumber (*Cucumis sativus*), cowpea (*Vigna unguiculata*), soybean (*Glycine max),* and *Hydrangea* spp. (Bala, 1993; Koenning et al., 2006; Blazquez, 1972; Blasquez, 1967; Boosalis et al., 1957; Chase, 1993; Chase et al., 1986; Chase, 1984; Hansen et al., 1994; McGovern, 1994; McMillan et al., 1995; Miller, 1974; Raffel et al., 1999).

Following disease onset in cotton, premature defoliation often occurs after distinctive target-shaped lesions develop and spread throughout the canopy. As the disease progresses, the spots become somewhat circular with light brown centers surrounded by dark brown margins (Fulmer et al., 2012). Heavy defoliation, when it occurs late in the year, serves as harvest aid rather than negatively impacting yield. However, with early disease onset, yield losses of up to 200 lb/A (224 kg/ha) of lint has been noted (Hagan and Sikora, 2012).

Management of target spot requires an integration of both cultural practices and chemical methods. Production practices that may impact development of target spot include seeding rate, tillage and crop rotation (Hagan et al., 2015). Scouting for target spot is also of high importance. Cultural practices, as for many other diseases, have limited effectiveness on target spot management. An effective means of managing target spot is through the application of fungicides. Unfortunately, *C. cassiicola* has shown tremendous ability to adapt and become resistant, or less sensitive, to effective protectant and systemic fungicides as seen with target spot on soybean, tomato and cucumber (Teramoto et al., 2017; MacKenzie et al., 2017; Avozani et al., 2014; Teramoto et al., 2013; Miyamoto et al., 2009).

Currently, there are no known resistant cotton varieties to target spot. However, cotton varieties differ greatly in their susceptibility to the disease (Hagan, 2014). In a 2014 trial in Alabama, significant differences in target-spot induced defoliation were observed among cotton varieties and fungicide treatments (Hagan et al., 2015). Phytogen 499 WRF was observed to have significantly higher target spot incited defoliation levels compared to other cotton varieties and similar results were reported previously (Hagan et al., 2012; Hagan, 2013; Hagan, 2014).

Until recently, fungicides were not used to control cotton foliar diseases. However such use has become more common in recent years due to the damage and losses from target spot and introduction of new fungicides. Today, quinone outside inhibitor (QoI), demethylation inhibitor (DMI), and QoI-DMI premix fungicides are registered to manage foliar diseases of cotton. In 2007, the QoI fungicide pyraclostrobin (Headline; BASF Corporation, Research Triangle Park, NC) was the first foliar fungicide labeled in cotton. In 2008, azoxystrobin (Quadris; Syngenta Crop Protection, Greensboro, NC) was labeled for foliar disease prevention and boll rot management. These two fungicides, ranging from a single to double applications provided control of target spot by interfering with the mitochondrial respiration, subsequently affecting spore germination and hyphal growth (Bartlett et al., 2002). Demethylation inhibitor (DMI) fungicides, such as metconazole and flutriafol (Topguard) have been registered for management of target spot, either as stand-alone products or pre-mixed with other fungicides (Twinline: pyraclostrobin + metconazole) (Whitaker et al., 2017; Kelly, 2016; Hagan et al., 2013). These fungicides inhibit sterol synthesis which is important in the cell membrane structure of fungi (FRAC 2013). Recently, combination of a succinate dehydrogenase inhibitor (SDHI) fungicide and a QoI fungicide, fluxapyroxad + pyraclostrobin (Priaxor) was also registered commercially for the control of target spot (Whitaker et al., 2017; Kelly, 2016). SDHI fungicides interfere with

fungal respiration by inhibition of the enzyme succinate dehydrogenase (SDH) in complex II of the mitochondrial electron transport chain (FRAC 2016). Other SDHI fungicides registered for the control of target spot on other hosts include boscalid, fluopyram, and penthiopyrad (Vallad et al., 2016; Miyamoto et al., 2009).

Currently, fungicides labeled for application in cotton include azoxystrobin (Quadris), pyraclostrobin (Headline), pyraclostrobin + metconazole (Twinline), pyraclostrobin + fluxapyroxad (Priaxor), and azoxystrobin + benzovindiflupyr (solatenol) (Elatus), flutriafol (Topguard), and prothioconazole + trifloxystrobin (Stratego YLD). Tebuconazole is labeled for use on cotton, but specifically for control of southwestern rust (Hagan and Sikora, 2012).

Fungicide penetration into the dense canopy is a concern, therefore, placement and timing of fungicide applications is important. A fungicide program should be initiated before canopy closure to allow for appropriate coverage of the leaves (Whitaker et al., 2018). Based on field trials conducted in Georgia, fungicide applications that most consistently reduced premature defoliation were those made during the first and third week of bloom. Applications made in the third week of bloom seem most critical (Walls et. al, 2012). Additionally, fungicide efficacy could be improved by directing the spray into the lower and mid canopy to improve leaf coverage (Hagan et al., 2015).

Another management strategy to reduce the impact of target spot is through the application of plant growth regulators (Kelly, 2016). Cotton is an indeterminate crop that grows vegetatively and reproductively at the same time (Mao et al., 2014). Excessive vegetative growth often results negatively on the yield of the crop (Eaton, 1955). Plant growth regulators are used extensively in cotton production to inhibit the negative effects of excessive vegetative growth on the indeterminate crop (Mao et al., 2014). This allows for cotton to produce sufficient vegetative

growth to support fruiting bodies without allowing the plant to become rank. Various chemistries can be used but the most commonly utilized chemical worldwide is mepiquat chloride (Dodds et al., 2010). Mepiquat chloride is a gibberellin biosynthesis inhibitor that is known to control morphological growth by reducing leaf area, controlling plant height, and reducing internode length (Mao et al., 2014). The result is a more compact plant that allows adequate airflow and penetration of light into the canopy (Reddy et al., 1990).

Despite previous studies conducted on management of target spot, there is still much to learn about the disease and how to effectively control it. In order to meet the need for applied research to better understand how to manage the disease, field trials at multiple locations were conducted. This was designed to minimize premature defoliation and subsequent yield loss from target spot. Specifically, the objectives of this study were to i) investigate the management of target spot with registered fungicides, ii) determine the optimum number and timing of fungicide applications, and iii) evaluate the susceptibility of cotton varieties to the disease. This study was conducted from 2014 to 2017. The effects of using PGR (plant growth regulator) treatments were also assessed during the final growing season.

## Materials and Methods

**Locations and experimental design.** A total of six field trials were conducted in Georgia at the University of Georgia Stripling Irrigation Research Park in Camilla and Attapulgus Research and Education Center, from 2014 to 2017. These two fields were chosen because target spot was previously reported as problematic in these locations. In the 2014 trial in Attapulgus, two different cotton cultivars were planted, PHY 499 WRF, known to be high yielding but very susceptible to target spot, and DPL 1137 B2RF, which is considered less susceptible to the disease. In 2015 and 2016, these two different cotton cultivars, PHY 499 WRF and DPL 1137

B2RF were also used. However, in 2017, PHY 490 WRF and DPL 1646 B2RF were planted because PHY 499 WRF seeds were not available.

For the 2014 to 2016 trials, the experimental design used was a split plot with a factorial arrangement of cultivar and fungicide treatments. Cotton cultivars PHY 499 WRF and DPL 1137 B2RF were the whole plot treatment and fungicide programs were the split-plot treatment. Four replications of treatments were included. Fungicide treatments consisted of 1 or 2 applications of Headline SC (6 fl. oz. / A), Topguard (7 fl. oz. / A), Priaxor (4 fl. oz. / A), Quadris SC (6 fl. oz. / A) and a nontreated control.

In 2017, experiments were designed as a split-plot, with four treatment replications, where the cotton cultivar was the whole-plot treatment factor and fungicide/PGR treatment was the sub-plot treatment factor. Cotton cultivars planted were PHY 490 WRF and DPL 1646 B2RF was used. Fungicide treatments consisted of one and two applications of Priaxor (4 fl. oz. / A). A nontreated control was included. Also included were standard (2 applications; 8 fl. oz. / A, 16 fl. oz. / A) and aggressive (3 applications; 16 fl. oz. / A, 16 fl. oz. / A, 24 fl. oz. / A) applications of the plant growth regulator mepiquat chloride.

For all trials, individual fungicide treatments were applied to plots that consisted of 4 rows spaced 3 feet apart by 40 feet in length. Plots were maintained using established management practices in each experimental station. Recommended rates of herbicide, insecticide and fertilizer of the Georgia Cooperative Extension System were applied to all plots. Plots were irrigated via an overhead irrigation system as needed.

**Fungicide Applications.** *2014-2016 Trials***.** One to two applications of fungicides were made in each study at two week intervals at 1$^{st}$ and 3$^{rd}$ week of bloom using a Lee Spider Spray Trac sprayer (Lee Company, Idalou, TX) at a pressure of 40 psi to achieve a spray volume of 15

gal/A. Plots were 40 feet in length and four rows wide spaced 3 feet apart (center two rows were treated and rated). Fungicides in these trials included Headline SC (6 fl. oz. / A, pyraclastrobin), Topguard (7 fl. oz. / A, flutriafol ), Priaxor (4 fl. oz. / A, fluxapyroxad + pyraclostrobin), Quadris SC (6 fl. oz. / A, azoxystrobin) and a non-treated control and ranged from a single application to two applications.

*2017 Trials.* Fungicides were applied using a Lee Spider Spray Trac sprayer (Lee Company, Idalou, TX) at a pressure of 40 psi to achieve a spray volume of 15 gal/A. Plots were 40 feet in length and four rows wide spaced 3 feet apart (center two rows were treated and rated). Only one fungicide was included in these trials, Priaxor (4 fl. oz. / A, fluxapyroxad + pyraclostrobin) and a non-treated control. Fungicides were applied once or twice. The effect of plant growth regulators was evaluated in 2017. Standard (2 applications; 8 fl. oz. / A, 16 fl. oz. / A) and aggressive (3 applications; 16 fl. oz. / A, 16 fl. oz. / A, 24 fl. oz. / A) applications of mepiquat chloride were included with the treatments.

In the 2015 and 2017 trials, fungicide treatments were applied at disease onset for the single fungicide application treatments. For double fungicide application treatments, fungicides were applied at disease onset and again 2weeks later. In the 2014 and 2016 trials, fungicide treatments were applied based upon vegetative and reproductive growth stages, and included 1$^{st}$ week of bloom (B1) and 3$^{rd}$ week of bloom (B3).

**Disease Assessment.** In all trials, defoliation was visually assessed in each plot. Defoliation was assessed on a scale from 0 to 100% with 0 = no defoliation and 100 = completely defoliated. Disease severity was visually estimated as the percent leaf area affected with target spot (average for entire plot) on a 0 to 100% scale with 0 = no leaf spot/target spot and 100 = entire leaf area covered with target spots. Disease severity and defoliation were

assessed three times in the 2014 and 2017 trials and were assessed twice in the 2015 and 2016 trials during mid to late season amounting to two to three assessments per location. Area under the disease severity progress curve (AUDSPC) and area under the disease defoliation progress curve (AUDDPC) were calculated from repeated severity and defoliation assessments using the trapezoidal method (Shaner and Finney, 1977). AUDSPC and AUDDPC were calculated as for area under the disease progress curve (AUDPC)

$$\text{AUDPC} = \sum_{i=1}^{n-1} (\frac{y_i + y_i + 1}{2})(t_i + 1 - t_i)$$

where, *t* is the time in days after planting for each assessment, *y* is disease severity or defoliation and *n* is the number of assessments.

**Plot Yields.** Yield data was also recorded at the end of the season for each location. Plots were mechanically harvested and seedcotton per plot were weighed individually, then combined and averaged by treatment.

**Statistical Analysis.** Data were analyzed separately for each trial. The generalized linear mixed model procedure (PROC GLIMMIX) in SAS (version 9.4, SAS Institute, Cary, NC) was used to determine the effect of cultivar, fungicide treatment, number of fungicide applications and interaction among the factors on final disease severity, final disease defoliation, AUDSPC, AUDDPC and yield. For all trials, variety and fungicide treatment were considered as fixed effects, with replication and replication x variety as random effects. The differences in the least square means were tested by Tukey's multiple comparisons test. Main effects of cultivar or fungicide treatment were evaluated when there was no significant interaction between cultivars and fungicide treatment for each variable. The residuals were tested for normality using four tests: Shapiro-Wilk *W*, Kolmogorov-Smirnov *D*, Cramer-vol Mises $W^2$, and Anderson-Darling

$A^2$ in PROC UNIVARIATE. When data violated the assumptions of normality, transformations were used. The natural log transformation was used for final disease severity, final disease defoliation, AUDSPC and AUDDPC. Back-transformed means of all transformed variables are presented in the results. Contrast statements in SAS (version 9.4, SAS Institute, Cary, NC) were used to determine the effect of number of fungicide applications.

**Results**

**2014 Field Season.** The effects of cultivars, fungicide treatments and number of fungicide applications on target spot and cotton yield in this trial are presented in Table 4.1. In the 2014 trial in Attapulgus, Phytogen 499 WRF proved to be most susceptible to target spot, having significantly greater final disease severity ($P$=0.0280) and greater final defoliation ($P$=0.0001) compared to Deltapine 1137 B2RF. The AUDSPC and AUDDPC for target spot were significantly greater in Phytogen 499 WRF than in Deltapine 1137 B2RF. Fungicide treatment had a significant effect on AUDSPC and AUDDPC values (Table 4.1). Headline and Priaxor significantly reduced AUDSPC and AUDDPC values compared to the nontreated control. Total yield was not significantly different between varieties nor between numbers of fungicide applications. However, yields of Deltapine 1137 B2RF were numerically higher than Phytogen 499 WRF. Fungicide treatment had a significant effect on yield with Headline applied at 1$^{st}$ and 3$^{rd}$ week of bloom producing the greatest amount in yield. Number of fungicide applications significantly affected final defoliation ($P$=0.0508) where two fungicide applications have significantly less final defoliation compared to single application of fungicides tested.

**2015 Field Season.** The effects of varieties, fungicide treatments and number of fungicide applications on target spot and cotton yield in this trial are presented in Table 4.2. The AUDSPC for target spot was significantly greater in Phytogen 499 WRF than in Deltapine 1137

B2RF (*P*=0.0259) but final disease severity and defoliation were similar on the two cultivars. No significant differences in disease severity, defoliation or yield were found among fungicide treatments. Although fungicide effect was not significant, 1 or 2 applications of Priaxor or Quadris were the best treatments in the trial in terms of severity, defoliation and yield, on both single and double applications. The AUDSPC for target spot was significantly different between single and double fungicide applications (*P*=0.0372). Yield of Phytogen 499 WRF was numerically higher than that of Deltapine 1137 B2RF, yet the difference was not significant.

**2016 Field Season.** In Attapulgus, the effects of varieties, fungicide treatments and number of fungicide applications on target spot and cotton yield are presented in Table 4.3. Although not significantly different, it is interesting to note that the hypothesized less susceptible variety Deltapine 1137 B2RF had greater AUDSPC and AUDDPC values than Phytogen 499 WRF. Fungicide treatment had significant effects on final disease severity, final disease defoliation, AUDSPC and AUDDPC values. Two applications of Priaxor tended to result in significantly reduced AUDSPC and AUDDPC values compared to all the other treatments and to the nontreated control. There was no statistically significant difference in yield between varieties or among treatments. However, two applications of Quadris and Priaxor resulted in greatest numerical yield.

In Stripling, the effects of varieties, fungicide treatments and number of fungicide applications on target spot and cotton yield are presented in Table 4.4. The AUDSPC, AUDDPC and final defoliation for target spot were significantly greater in Phytogen 499 WRF than in Deltapine 1137 B2RF. There was a significant variety by fungicide interaction for final disease severity (*P*=0.0027) so varieties within each treatment were compared separately. In all but one fungicide treatments, final disease severity was numerically greater in Phytogen 499 WRF

compared to Deltapine 11327 B2RF. The opposite effect was observed in two applications of Topguard where final disease severity was numerically greater in Deltapine 1137 B2RF compared to Phytogen 499 WRF but the difference was not significant. No significant differences in defoliation were found among fungicide treatments for target spot but there was a significant effect of fungicide on AUDSPC ($P$=0.0211). Two applications of Priaxor or Quadris had significantly lower AUDSPC compared to the nontreated control. Plot yields were not significantly different between varieties and among fungicide treatments. Two applications of Priaxor yielded highest numerically.

**2017 Field Season.** In Attapulgus, the effects of varieties, fungicide treatment and plant growth regulators on severity, defoliation and yield for target spot were evaluated. No significant differences in AUDSPC or AUDDPC were found between varieties Phytogen 490 WRF and Deltapine 1646 B2RF but there were significant differences in final defoliation and yield. The best level of control both for severity and defoliation was achieved with two applications of Priaxor in combination with aggressive PGR (mepiquat chloride). All treatment combinations had significantly lower disease severity and defoliation compared to the nontreated control ($P$=<.0001). Fungicide applications had a significant effect on target spot severity, defoliation and yield and there were few fungicide x variety interactions (Table 4.5). There was a significant variety by fungicide interaction for final disease defoliation ($P$=<.0001) where in Phytogen 499 WRF, 2 applications of Priaxor significantly decreased final defoliation compared to 1 application of Priaxor or the nontreated control. However, in Deltapine 1137 B2RF, 1 application of Priaxor resulted in numerically lowest levels of final defoliation compared to 2 applications of Priaxor and was significantly different from the nontreated control. Overall, AUDSPC and AUDDPC significantly decreased with increasing fungicide applications compared with the

nontreated control, although the difference between a single application of Priaxor versus double applications was not significant (Table 4.5). Plant growth regulators (PGR) significantly reduced final disease defoliation (*P*=0.0258), AUDSPC (*P*=0.0273) and AUDDPC (*P*=0.0006) values compared to the nontreated control (Table 4.5) but did not have a significant effect on yield. There was a cotton variety x fungicide interaction on yield. Yield of the variety Phytogen 490 WRF was not significantly affected by fungicide treatment. However, yield was significantly affected by fungicide treatment on the cotton variety Deltapine 1646 B2RF; both single and double applications of Priaxor produced significantly greater yields compared to the plots that did not receive any fungicide treatment (*P*=0.0016). Plant growth regulators did not significantly affect yield (*P*=0.1408) although with increase rate and frequency of use of PGR, numerical increases in yield were noted.

In Stripling, the effects of varieties, fungicide treatments and application of plant growth regulators on target spot and cotton yield are presented in Table 4.6. No significant differences in severity, defoliation or yield were found between varieties Phytogen 490 WRF and Deltapine 1646 B2RF. However, all fungicide treatments significantly lowered disease severity and defoliation compared to the nontreated control (*P*=<.0001). Final disease severity, final defoliation, AUDSPC and AUDDPC significantly decreased with fungicide applications compared with the nontreated control. There was no significant difference between 1 and 2 applications, except for AUDDPC. Lowest defoliation was achieved with two applications of Priaxor but final disease defoliation was not significantly different between 1 and 2 Priaxor applications (Table 4.6). Both single and double applications of Priaxor significantly increased yield compared to plots that did not receive any fungicide treatment. Plant growth regulators did not have a significant effect on disease severity, defoliation or yield. However, it is interesting to

note that in this trial, plots that received PGRs have numerically higher final disease severity, final defoliation, AUDSPC, AUDDPC and lower yield values compared to the nontreated control.

**Discussion**

This study looked at integrated strategies for managing target spot of cotton. This research compared one and two application programs of selected registered fungicides for control of target spot and yield response on two cotton cultivars. The effects of plant growth regulators in controlling the disease and on yield were also assessed. Overall, AUDDPC season-long defoliation rankings mirrored those for final target spot (%) defoliation. Similarly, lower AUDDPC and final defoliation values were noted for the DPL 1137 B2RF variety as compared with the higher values for Phytogen 499 WRF (Fig. 4.1). Overall, disease severity and defoliation were greater on Phytogen 499 WRF than Deltapine 1137 B2RF. Since complete genetic resistance is not yet available for commercial varieties, tolerant varieties are an effective tool for managing target spot in cotton. As previously reported (Hagan et al., 2017; Hagan et al., 2016; Hagan et al., 2015), Phytogen 499 WRF is one of the most susceptible cotton varieties to target spot. Over the three year study and four field trials, typically (%) defoliation values were numerically higher for Phytogen 499 WRF than Deltapine 1137 B2RF and in two of the four trials, the difference was significant (Fig. 4.1). Although it has been hypothesized that Phytogen 499 WRF is more susceptible to target spot, this may result from the variety's growth habit. It has a full canopy architecture and rank growth, creating a microenvironment that favors development of target spot. However, in the 2017 trials, where Phytogen 490 WRF and Deltapine 1646 B2RF were tested, differences in variety were not as apparent in terms of disease. In addition, yield response to cotton variety was not significant across all years.

Deltapine 1137 B2RF yields were greater than those of Phytogen 499 WRF in three out of four trials although the differences were not significant. It was only in the 2015 Attapulgus trial, where disease pressure was lowest, that Phytogen 499 WRF resulted in greater numerical increase of yield compared to Deltapine 1137 B2RF, but again, the difference was not significant. In addition, the field trial conducted in 2015 in Attapulgus had an extended period without rain during the growing season (Table 4.7). These warmer and drier days were unfavorable for the development of target spot epidemics (Hagan, 2017). AUDSPC and AUDDPC and final disease severity were noticeably lower compared to other years, and differences could not be detected among fungicide treatments.

Fungicides delayed disease progress and decreased overall defoliation. Fungicide selection significantly impacted target spot defoliation in four out of six field trials conducted in this study. When compared with the nontreated control, two applications of Priaxor gave better season-long target spot control as indicated by lowest AUDDPC values. However, significant yield responses to fungicide applications were not common. It was only in the 2014 trial in Attapulgus and in the 2017 trials in Attapulgus and Stripling that there were significant treatment effects on yield. In 2014, two applications of Headline produced the greatest yield, which was significantly different from the nontreated control (Table 4.1). However, yield did not differ among fungicide treatments. In 2017, both single and double applications of Priaxor significantly increased yield compared to plots that did not receive any fungicide treatment. Two applications of Priaxor resulted in greater numerical increase in yield compared to one Priaxor application although the difference was not significant. For all other field trials, there was no significant difference in yield across treatments although two applications of Priaxor consistently resulted in highest numerical yield. This is similar to results of fungicide efficacy trials conducted on target

spot on tomatoes in Florida (Paret et al., 2015) and on soybeans in Brazil (Teramoto et al., 2017) where fungicide programs reduced disease but did not have a significant effect on yield. It has been noted that the impact of *C. cassiicola* on yield has been difficult to gauge (Hagan and Sikora, 2012).

Results in this study validate what has been observed in previous years where applications of registered fungicides Headline, Quadris, Twinline, Priaxor and Elatus have been effective at suppressing target spot development but did not consistently result in a significant yield increase. However, in some instances, beneficial yield results were reported as an outcome of fungicide applications when disease pressure is high or in places where cotton in intensively managed (Hagan, 2017). Results of a multi-year regional project on management of target spot performed in 8 locations showed significant yield response following two applications of Priaxor in two states, Alabama and Tennessee (Mehl et al., 2017). This provides evidence that economic benefits may exist when making fungicide applications to cotton fields where heavy disease pressure is observed.

In this study, Priaxor was the most efficacious registered fungicide for target spot in cotton. The number of applications did not greatly influence Priaxor efficacy. In trials where use of fungicides had a significant effect on the disease, despite better disease control compared to the nontreated control, other registered fungicides do not seem to provide the same level of disease control as Priaxor.

The effects of plant growth regulators (PGRs) were also tested in two trials in this study. In the 2017 Attapulgus trial, PGRs significantly reduced final disease defoliation, AUDSPC and AUDDPC values compared to the nontreated control (Table 4.5). However, PGRs did not have a significant effect on yield although numerical increase in yield were noted in plots that received

PGR treatments compared to the nontreated control. In the 2017 Stripling trial, PGRs did not have a significant effect on all the variables tested. Previous research on PGRs and cotton response in terms of yield have been inconsistent (Dodds, 2017). In some cases, a yield increase from PGR application can be observed whereas in others, a decrease occurs; however, in most cases, no effect on yield has been observed (Dodds, 2017).

**Conclusions**

Managing target spot of cotton requires timely scouting for disease, variety and fungicide selection. In terms of varietal selection, PHY 499 WRF appears to be more susceptible compared to DPL 1137 B2RF. Varieties PHY 490 WRF and DPL 1646 B2RF do not differ in risk for disease. Two applications of Priaxor gave the best season-long target spot control as indicated by lowest AUDDPC values. However, growers should be aware that even though a fungicide may limit defoliation, it may not benefit yield. Whether cotton would benefit from a fungicide application to protect from target spot needs to be assessed on a field by field basis. In terms of use of plant growth regulators, both 2017 trials showed no significant effect on yield. In one trial, PGR significantly reduced severity and defoliation compared to the nontreated control. However, on the other trial, PGR effect on target spot was not significant. Hence, there is still much need for applied research on effects of PGR on target spot.

Lastly, over usage of fungicides in cotton, just like in any other crop, may increase selection pressure for resistance development. Due to a lack of consistent significant increase in yield, fungicides should not be applied to cotton without first assessing risk to disease.

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Table 4.1. Effect of variety, fungicide treatment and number of fungicide applications on target spot of cotton in Attapulgus, Georgia in 2014.

| Treatment | | FDS [a e] | FDD [a e] | AUDSPC [b e] | AUDDPC [b e] | Yield [e] |
|---|---|---|---|---|---|---|
| **Cotton Variety** | | (%) | (%) | | | (lbs/A) |
| PHY 499 WRF | | 22.60 A [d] | 19.82 A | 638.22 A | 361.03 A | 931.57 A |
| DPL 1137 B2RF | | 18.90 B | 2.37 B | 533.67 B | 56.34 B | 1041.48 A |
| *P*(α=0.05) | | 0.0280 | 0.0001 | 0.0083 | 0.0180 | 0.4498 |
| **Fungicide Treatment** | | | | | | |
| Headline | 1st WB [c] | 18.91 bc [d] | 3.86 bc | 515.01 f | 74.14 c | 948.96 b |
| Topguard | 1st WB | 23.00 ab | 9.82 ab | 632.77 ab | 206.73 ab | 1026.13 ab |
| Priaxor | 1st WB | 19.14 bc | 6.80 bc | 548.45 def | 129.11 bc | 1132.11 ab |
| Quadris | 1st WB | 21.15 abc | 8.28 abc | 603.57 bcd | 157.05 bc | 982.37 ab |
| Headline | 1st WB + 3rd WB | 19.68 bc | 4.63 bc | 557.67 cdef | 140.06 bc | 1292.05 a |
| Topguard | 1st WB + 3rd WB | 22.07 ab | 6.25 bc | 618.89 abc | 130.19 bc | 797.47 b |
| Priaxor | 1st WB + 3rd WB | 17.32 c | 3.18 c | 523.87 ef | 80.48 c | 923.38 b |
| Quadris | 1st WB + 3rd WB | 20.70 abc | 6.92 bc | 585.25 bcde | 150.54 bc | 954.01 ab |
| Nontreated | | 25.14 a | 24.48 a | 687.77 a | 363.81 a | 868.93 b |
| *P*-value | | <.0001 | <.0001 | 0.0003 | 0.0194 | 0.0039 |
| **Application** | | 0.3640 | 0.0508 | 0.8728 | 0.6529 | 0.5971 |

[a] FDS= final disease severity (%), FDD= final disease defoliation (%).

[b] Area under the disease severity progress curve (AUDSPC) and area under the disease defoliation progress curve (AUDDPC) were calculated with three assessment dates.

[c] WB= week of bloom.

[d] Means in the same column followed by the same uppercase letter or lowercase letter are not significantly different based on Tukey's honestly significant difference test (α=0.05).

[e] Data are combined across varieties or across fungicide treatments when there are no variety-treatment interactions.

Table 4.2. Effect of variety, fungicide treatment and number of fungicide applications on target spot of cotton in Attapulgus, Georgia in 2015.

| Treatment | | FDS [a e] | FDD [a e] | AUDSPC [b e] | AUDDPC [b e] | Yield [e] |
|---|---|---|---|---|---|---|
| **Cotton Variety** | | (%) | (%) | | | (lbs/A) |
| PHY 499 WRF | | 1.74 A [d] | 0.89 A | 22.02 A | 9.20 A | 1608.17 A |
| DPL 1137 B2RF | | 0.42 A | 0.13 A | 2.23 B | 0.42 A | 1519.18 A |
| *P*-value | | 0.0500 | 0.0992 | 0.0259 | 0.0780 | 0.1299 |
| **Fungicide Treatment** | | | | | | |
| Headline | 1st WB [c] | 1.07 a [d] | 0.87 ab | 7.36 a | 3.06 a | 1549.56 a |
| Topguard | 1st WB | 2.12 a | 0.32 ab | 38.30 a | 3.43 a | 1588.12 a |
| Priaxor | 1st WB | 0.49 a | 0.19 ab | 3.40 a | 1.58 a | 1388.47 a |
| Quadris | 1st WB | 1.10 a | 0.27 ab | 14.73 a | 0.53 a | 1693.62 a |
| Headline | 1st WB + 3rd WB | 0.99 a | 0.65 ab | 3.18 a | 5.84 a | 1503.05 a |
| Topguard | 1st WB + 3rd WB | 0.81 a | 0.10 b | 5.06 a | 1.44 a | 1509.85 a |
| Priaxor | 1st WB + 3rd WB | 0.71 a | 0.25 ab | 3.68 a | 0.79 a | 1625.56 a |
| Quadris | 1st WB + 3rd WB | 0.47 a | 0.35 ab | 3.13 a | 4.71 a | 1643.71 a |
| Nontreated | | 1.46 a | 1.71 a | 22.75 a | 12.71 a | 1571.11 a |
| *P*-value | | 0.3810 | 0.0454 | 0.1160 | 0.0633 | 0.3889 |
| **Application** | | 0.2266 | 0.7102 | 0.0372 | 0.5273 | 0.8002 |

[a] FDS= final disease severity (%), FDD= final disease defoliation (%).

[b] Area under the disease severity progress curve (AUDSPC) and area under the disease defoliation progress curve (AUDDPC) were calculated with two assessment dates.

[c] WB= week of bloom.

[d] Means in the same column followed by the same uppercase letter or lowercase letter are not significantly different based on Tukey's honestly significant difference test ($\alpha$=0.05).

[e] Data are combined across varieties or across fungicide treatments when there are no variety-treatment interactions.

Table 4.3. Effect of variety, fungicide treatment and number of fungicide applications on target spot of cotton in Attapulgus, Georgia in 2016.

| Treatment | | FDS [a e] | FDD [a e] | AUDSPC [b e] | AUDDPC [b e] | Yield [e] |
|---|---|---|---|---|---|---|
| **Cotton Variety** | | (%) | (%) | | | (lbs/A) |
| PHY 499 WRF | | 1.89 A [d] | 12.26 A | 32.78 A | 260.01 A | 2309.81 A |
| DPL 1137 B2RF | | 1.83 A | 14.60 A | 39.24 A | 295.63 A | 2529.91 A |
| *P*-value | | 0.9435 | 0.3040 | 0.8187 | 0.3757 | 0.2240 |
| **Fungicide Treatment** | | | | | | |
| Headline | 1st WB [c] | 2.93 ab [d] | 12.71 ab | 82.62 ab | 302.58 bc | 2375.38 a |
| Topguard | 1st WB | 2.12 ab | 15.02 ab | 39.90 abcd | 292.56 bc | 2222.74 a |
| Priaxor | 1st WB | 0.71 b | 12.76 ab | 28.69 bcd | 249.16 bc | 2488.82 a |
| Quadris | 1st WB | 3.10 ab | 10.73 ab | 62.18 abc | 216.33 c | 2316.62 a |
| Headline | 1st WB + 3rd WB | 1.22 ab | 15.08 a | 21.39 cd | 286.48 bc | 2452.70 a |
| Topguard | 1st WB + 3rd WB | 3.07 ab | 21.19 a | 87.38 ab | 408.20 ab | 2127.23 a |
| Priaxor | 1st WB + 3rd WB | 0.36 b | 4.15 b | 4.45 e | 109.57 d | 2644.51 a |
| Quadris | 1st WB + 3rd WB | 1.00 ab | 11.00 ab | 17.38 d | 230.95 c | 2655.10 a |
| Nontreated | | 4.98 a | 32.65 a | 97.95 a | 683.01 a | 2495.64 a |
| *P*-value | | 0.0013 | 0.0007 | 0.0002 | <.0001 | 0.1325 |
| **Application** | | 0.0802 | 0.4759 | 0.0049 | 0.3721 | 0.2243 |

[a] FDS= final disease severity (%), FDD= final disease defoliation (%).

[b] Area under the disease severity progress curve (AUDSPC) and area under the disease defoliation progress curve (AUDDPC) were calculated with two assessment dates.

[c] WB= week of bloom.

[d] Means in the same column followed by the same uppercase letter or lowercase letter are not significantly different based on Tukey's honestly significant difference test ($\alpha$=0.05).

[e] Data are combined across varieties or across fungicide treatments when there are no variety-treatment interactions.

Table 4.4. Effect of variety, fungicide treatment and number of fungicide applications on target spot of cotton in Stripling, Georgia in 2016.

| Treatment | | FDS [a] | | FDD [a e] | AUDSPC [b e] | AUDDPC [b e] | Yield [e] |
|---|---|---|---|---|---|---|---|
| **Cotton Variety** | | (%) | | (%) | | | (lbs/A) |
| PHY 499 WRF | | - | | 36.40 A | 67.25 A | 525.05 A | 1502.37 A |
| DPL 1137 B2RF | | - | | 7.04 B | 29.58 B | 111.90 B | 1548.72 A |
| *P*-value | | 0.0185 | | 0.0104 | 0.0050 | 0.0030 | 0.2709 |
| **Fungicide Treatment** | | PHY 499 WRF | DPL 1137 B2RF | | | | |
| Headline | 1st WB [c] | 3.12 abc [d] | 1.71 bc | 17.29 a | 40.52 bc | 181.65 a | 1551.14 a |
| Topguard | 1st WB | 3.00 abc | 1.63 bc | 15.35 a | 42.43 bc | 256.62 a | 1472.87 a |
| Priaxor | 1st WB | 3.31 abc | 1.83 bc | 16.93 a | 45.21 abc | 214.03 a | 1538.89 a |
| Quadris | 1st WB | 5.31 a | 1.91 abc | 20.48 a | 59.98 a | 386.44 a | 1495.33 a |
| Headline | 1st WB + 3rd WB | 4.70 ab | 2.13 abc | 14.86 a | 52.18 ab | 194.57 a | 1483.76 a |
| Topguard | 1st WB + 3rd WB | 2.14 abc | 3.12 abc | 15.38 a | 47.02 abc | 218.31 a | 1523.92 a |
| Priaxor | 1st WB + 3rd WB | 3.90 ab | 1.00 c | 13.49 a | 33.32 c | 219.28 a | 1587.22 a |
| Quadris | 1st WB + 3rd WB | 2.42 abc | 1.91 abc | 9.91 a | 35.02 c | 187.92 a | 1542.30 a |
| Nontreated | | 3.84 ab | 1.66 bc | 29.01 a | 53.04 ab | 432.31 a | 1534.47 a |
| *P*-value | | 0.2773 | | 0.1187 | 0.0211 | 0.0819 | 0.8183 |
| **Application** | | 0.7951 | | 0.0836 | 0.1644 | 0.2289 | 0.5717 |

[a] FDS= final disease severity (%), FDD= final disease defoliation (%).

[b] Area under the disease severity progress curve (AUDSPC) and area under the disease defoliation progress curve (AUDDPC) were calculated with two assessment dates.

[c] WB= week of bloom.

[d] Means in the same column followed by the same uppercase letter or lowercase letter are not significantly different based on Tukey's honestly significant difference test ($\alpha$=0.05).

[e] Data are combined across varieties or across fungicide treatments when there are no variety-treatment interactions.

Table 4.5. Effect of variety, fungicide treatment and plant growth regulator on target spot of cotton in Attapulgus, Georgia in 2017.

| Treatment | FDS [a h] | | FDD [a] | AUDSPC [b h] | AUDDPC [b h] | Yield | |
|---|---|---|---|---|---|---|---|
| **Cotton Variety** | (%) | | (%) | | | (lbs/A) | |
| PHY 490 WRF | 23.89 A [g] | | 30.27 B | 426.96 A | 654.30 A | 1670.71 B | |
| DPL 1646 B2RF | 26.25 A | | 35.97 A | 433.84 A | 741.42 A | 2332.27 A | |
| *P*-value | 0.4898 | | 0.0013 | 0.8955 | 0.2360 | 0.0027 | |
| **Fungicide Treatment** | | PHY 490 WRF | DPL 1646 B2RF | | | PHY 490 WRF | DPL 1646 B2RF |
| Nontreated | 41.04 A [g] | 47.94 A | 49.17 A | 714.00 A | 1119.59 A | 1634.41 C | 2073.64 B |
| Priaxor (1) [c] | 16.04 B | 25.71 C | 27.60 BC | 300.26 B | 513.82 B | 1649.84 C | 2377.65 A |
| Priaxor (2) [d] | 18.13 B | 19.01 D | 31.84 B | 276.95 B | 460.18 B | 1727.88 C | 2545.54 A |
| *P*-value | <.0001 | | <.0001 | <.0001 | <.0001 | 0.0016 | |
| **PGR** | | | | | | | |
| Nontreated | 23.96 A [g] | | 36.39 A | 466.00 A | 789.97 A | 1919.82 A | |
| PGR standard [e] | 28.13 A | | 32.09 B | 444.88 A | 651.92 B | 2019.19 A | |
| PGR aggressive [f] | 23.13 A | | 30.86 B | 380.32 B | 651.70 B | 2065.47 A | |
| *P*-value | 0.1071 | | 0.0258 | 0.0273 | 0.0006 | 0.1408 | |

[a] FDS= final disease severity (%), FDD= final disease defoliation (%).

[b] Area under the disease severity progress curve (AUDSPC) and area under the disease defoliation progress curve (AUDDPC) were calculated with three assessment dates.

[c] Priaxor (1) = (pyraclostrobin + fluxapyroxad) one application, 4floz/A, BASF Corporation.

[d] Priaxor (2) = (pyraclostrobin + fluxapyroxad) two applications, 4floz/A, BASF Corporation.

[e] PGR standard = mepiquat chloride (0.35 lbs. a.i./gal) 2 applications ( 8 floz/A @ pinhead and 16 floz/A 3 weeks later).

[f] PGR aggressive = mepiquat chloride (0.35 lbs. a.i./gal) 3 applications ( 16 floz/A @ pinhead, 16 floz/A 3 weeks later and 24 floz/A 3 weeks later).

[g] Means in the same column followed by the same uppercase letter are not significantly different based on Tukey's honestly significant difference test ($\alpha$=0.05).

[h] Data are combined across varieties or across fungicide treatments when there are no variety-treatment interactions.

Table 4.6. Effect of variety, fungicide treatment and plant growth regulator on target spot of cotton in Stripling, Georgia in 2017.

| Treatment | FDS [a h] | FDD [a h] | AUDSPC [b h] | AUDDPC [b h] | Yield [h] |
|---|---|---|---|---|---|
| **Cotton Variety** | (%) | (%) | | | (lbs/A) |
| PHY 490 WRF | 24.04 A [g] | 42.78 A | 373.33 A | 661.59 A | 1898.09 A |
| DPL 1646 B2RF | 28.00 A | 42.89 A | 433.33 A | 928.41 A | 1813.19 A |
| *P*-value | 0.4543 | 0.9750 | 0.4611 | 0.0993 | 0.3320 |
| **Fungicide Treatment** | | | | | |
| Nontreated | 37.50 A [g] | 60.00 A | 577.50 A | 1273.78 A | 1626.54 B |
| Priaxor (1) [c] | 19.28 B | 35.00 B | 302.50 B | 651.44 B | 1892.14 A |
| Priaxor (2) [d] | 21.28 B | 33.50 B | 330.00 B | 459.78 C | 2048.23 A |
| *P*-value | <.0001 | <.0001 | <.0001 | <.0001 | 0.0013 |
| **PGR** | | | | | |
| Nontreated | 24.89 A [g] | 39.61 A | 386.67 A | 713.39 A | 1905.75 A |
| PGR standard [e] | 24.78 A | 43.89 A | 385.00 A | 839.56 A | 1859.17 A |
| PGR aggressive [f] | 28.39 A | 45.00 A | 438.33 A | 832.06 A | 1801.99 A |
| *P*-value | 0.4262 | 0.1843 | 0.4405 | 0.2519 | 0.6206 |

[a] FDS= final disease severity (%), FDD= final disease defoliation (%).

[b] Area under the disease severity progress curve (AUDSPC) and area under the disease defoliation progress curve (AUDDPC) were calculated with three assessment dates.

[c] Priaxor (1) = (pyraclostrobin + fluxapyroxad) one application, 4floz/A, BASF Corporation.

[d] Priaxor (2) = (pyraclostrobin + fluxapyroxad) two applications, 4floz/A, BASF Corporation.

[e] PGR standard = mepiquat chloride (0.35 lbs. a.i./gal) 2 applications ( 8 floz/A @ pinhead and 16 floz/A 3 weeks later).

[f] PGR aggressive = mepiquat chloride (0.35 lbs. a.i./gal) 3 applications ( 16 floz/A @ pinhead, 16 floz/A 3 weeks later and 24 floz/A 3 weeks later).

[g] Means in the same column followed by the same uppercase letter or lowercase letter are not significantly different based on Tukey's honestly significant difference test ($\alpha$=0.05).

[h] Data are combined across varieties or across fungicide treatments when there are no variety-treatment interactions.

Table 4.7. Weather conditions at field sites for cotton growing seasons from 2014 to 2017.

| Year | Location | Month | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | **May** | **Jun** | **Jul** | **Aug** | **Sep** | **Oct** | **Nov** |
| | Mean Monthly Temperature (°F) | | | | | | | |
| 2014 | Attapulgus, GA [a] | 72.5 | 79.5 | 79.9 | 81.8 | 77.6 | 67.8 | 52.8 |
| 2015 | Attapulgus, GA | 74.9 | 80.3 | 82.7 | 81.6 | 76.3 | 69.1 | 65.5 |
| 2016 | Attapulgus, GA | 73.0 | 79.9 | 82.4 | 81.6 | 78.5 | 70.1 | 61.3 |
| 2016 | Camilla, GA [b] | 73.7 | 80.6 | 83.4 | 82.7 | 79.3 | 70.8 | 61.4 |
| 2017 | Attapulgus, GA | 73.1 | 77.7 | 90.5 | 80.9 | 76.4 | 70.0 | 60.4 |
| 2017 | Camilla, GA | 73.6 | 78.4 | 71.6 | 81.7 | 76.8 | 70.0 | 60.1 |
| | Monthly rain fall (in) | | | | | | | |
| 2014 | Attapulgus, GA | 3.19 | 2.27 | 4.51 | 0.61 | 7.65 | 3.07 | 5.91 |
| 2015 | Attapulgus, GA | 1.42 | 4.01 | 3.54 | 2.51 | 8.40 | 0.55 | 6.03 |
| 2016 | Attapulgus, GA | 2.96 | 5.81 | 5.83 | 5.00 | 5.54 | 2.23 | 0.42 |
| 2016 | Camilla, GA | 2.42 | 7.60 | 3.11 | 8.15 | 4.53 | 0.24 | 0.76 |
| 2017 | Attapulgus, GA | 3.72 | 10.56 | 4.2 | 4.3 | 4.26 | 2.5 | 0.43 |
| 2017 | Camilla, GA | 5.37 | 7.24 | 3.7 | 7.1 | 3.35 | 3.47 | 0.54 |

[a] Historical data in Attapulgus Research and Education Center retrieved from the Georgia Automated Environmental Monitoring Network (http://www.georgiaweather.net/).

[b] Historical data in Stripling Irrigation and Research Park retrieved from the Georgia Automated Environmental Monitoring Network (http://www.georgiaweather.net/).

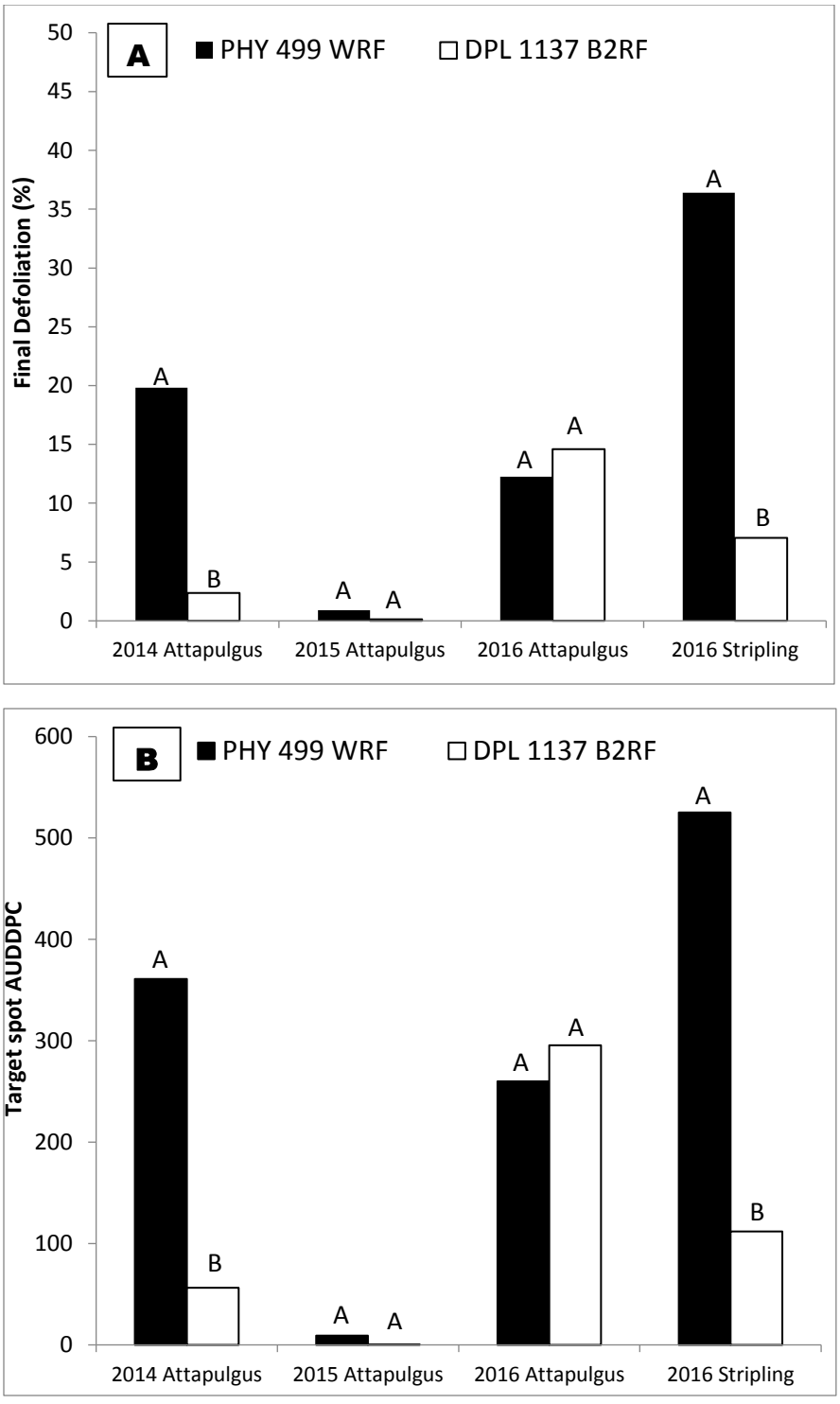


Figure 4.1. Target spot A) final (%) defoliation and B) AUDDPC season-long defoliation values as impacted by variety. Means in each figure followed by the same letter are not significantly different according to Tukey's honestly significant difference test.

# CHAPTER 5

## SUMMARY AND CONCLUSIONS

Target spot of cotton caused by *Corynespora cassiicola* (Berk. & M.A. Curtis) is an important emerging foliar disease of cotton in the southeastern United States. In Georgia in 2015, an estimated combined damage and cost of control of 3.8 million dollars were due to target spot (Kemerait Jr., 2017). With lack of availability of resistant cultivars as well as growers' decision to plant cotton in consecutive growing season, fungicides may be necessary to control foliar diseases such as target spot and to increase yields. However, repetitive use of fungicides may ultimately lead to failure of efficacy due to development of fungicide resistance. Therefore, sensitivity monitoring of *C. cassiicola* isolates in vitro is crucial for monitoring for shifts in sensitivities to fungicides used for control of target spot. In addition, it is known that some *C. cassiicola* isolates are virulent on several hosts while others are highly host-specialized. It has been documented that *C. cassiicola* isolates from other hosts have developed resistance or some level of decreased sensitivity to fungicides from the DMI, QoI and SDHI classes. For these reasons, it is equally important to look at fungicide sensitivities of *C. cassiicola* isolates from other hosts of origin. Moreover, evaluation of management of target spot through cultivar selection, application of fungicides, and number of fungicide applications as well as use of plant growth regulators is also of great importance.

In chapter 2, the results of this study indicate that *C. cassiicola* isolates from cotton are sensitive to fungicides metconazole (DMI, FRAC Code 3), fluxapyroad (SDHI, FRAC Code 7) and pyraclostrobin (QoI, FRAC Code 11). However, these sensitivities vary among isolates. The

differences observed in the $EC_{50}$ values of *C. cassiicola* isolates tested in vitro offered an early indication that resistance can occur in the future or that variable sensitivity is already present. The $EC_{50}$ values observed for these sensitive isolates fell between 0.015 to 0.205 μg/mL for metconazole, similar to the values reported by Burlakoti et al. (2010) on Fusarium species; 0.001 to 0.126 μg/mL for fluxapyroxad, similar to baseline sensitivities of *Alternaria alternata* to this SDHI fungicide (Avenot et al., 2014); and 0.013 to 0.200 μg/mL for pyraclostrobin, similar with results of baseline sensitivity range in *Cercospora zeae-maydis* (Bradley and Pedersen, 2011).

Furthermore, the mean $EC_{50}$ values of the 40 *Corynespora cassiicola* isolates from cotton for metconazole and pyraclostrobin were significantly higher than the $EC_{50}$ value for fluxapyroxad. There was no statistical difference between the mean $EC_{50}$ values for metconazole and pyraclostrobin. Differences in sensitivity to all three fungicides in vitro were observed among the isolates. All the isolates were sensitive to the tested fungicides, but the sensitivity varied among the *C. cassiicola* isolates. Fluxapyroxad reduced radial growth by 50% at lower concentrations compared to metconazole and pyraclostrobin. The addition of SHAM at 100 μg/mL to media amended with pyraclostrobin had significant effect on mean effective concentration of the fungicide to inhibit spore germination by 50% ($EC_{50}$) of *Corynespora cassiicola* isolates. This may indicate that *C. cassiicola* has the potential to utilize alternative respiration to overcome QoI fungicide inhibition in vitro.

Despite similarity in the frequency distribution of $EC_{50}$ values for metconazole, fluxapyroxad and pyraclostrobin among *C. cassiicola* isolates, the correlations among them were non-significant, with *r* ranging from -0.078 to 0.006. No significant correlation was observed for $EC_{50}$ levels for metconazole and fluxapyroxad, metconazole and pyraclostrobin or fluxapyroxad and pyraclostrobin. There is no evidence of cross-resistance among fungicides.

Although these data do not represent a true baseline because the window of opportunity of testing isolates without prior exposure to these three fungicides has passed, the $EC_{50}$ ranges are representative of a sensitive population. This will be a useful basis for comparison for future sensitivity studies. Future sensitivity monitoring could measure changes in the pathogen population sensitivity as growers continue to use these fungicides. This will allow greater insight into the management of target spot. Furthermore, continuous monitoring of variation in fungicide sensitivity of these three chemical classes for *Corynespora cassiicola* populations from cotton is essential to help determine risk for resistance development. This will subsequently help manage impending resistance problems by developing better disease management strategies. As for the products tested, no case of field resistance in *C. cassiicola* has currently been reported on cotton in the southeastern United States.

In the third chapter, frequency distribution of $EC_{50}$ values of metconazole, fluxapyroxad and pyraclostrobin for colony growth/spore germiantion inhibition against *Corynespora cassiicola* isolates from hosts with presumed greater fungicide exposure (cucumber, pepper, soybean, tomato) ranged from 0.011 to 0.936 μg/mL (metconazole), 0.006 to 0.148 μg/mL (fluxapyroxad), and 0.019 to 0.182 μg/mL (pyraclostrobin); from hosts with presumed less exposure to fungicides (hydrangea and mandevilla) $EC_{50}$ values ranged from 0.019 to 0.076 μg/mL (metconazole), 0.006 to 0.030 μg/mL (fluxapyroxad), and 0.019 to 0.121 μg/mL (pyraclostrobin). Compared to *C. cassiicola* isolates from cotton, these isolates from other hosts showed similar variability in fungicide sensitivity to metconazole, fluxapyroxad and pyraclostrobin. In addition, other studies have documented *C. cassiicola* isolates from other hosts showing resistance to the three classes of fungicides tested. Isolates from soybean, with presumed greater fungicide exposure, had larger $EC_{50}$ values compared to isolates from

hydrangea which are presumed to have least fungicide exposure. The range of $EC_{50}$ values from soybean is greater than that of isolates from cotton and hydrangea for metconazole and fluxapyroxad. This is an indication that with increased fungicide exposure, a shift in sensitivity may occur. Since resistance is already documented for *C. cassiicola* from other hosts, better management strategies for preventing resistance on target spot on cotton may be derived by looking into the studies conducted on these other crops affected by target spot.

Although this research included a small number of isolates from other hosts, the results reinforce the occurrence of variations in fungicide sensitivity of *C. cassiicola* isolates to different classes of fungicides. A more detailed sampling representative of other hosts should be conducted to better compare with, monitor and demonstrate shifts in sensitivity in future samplings. These results also indicate that monitoring the effectiveness of these fungicides in the field is of increasing importance especially in the years to come, as a trend towards possible isolate insensitivity to metconazole was noticed in this small sample size having a soybean isolate with an $EC_{50}$ value of 0.936 µg/mL.

Continuous fungicide sensitivity monitoring is a very important management technique because it allows for subsequent detection of population sensitivity shifts (Russell, 2004). Caution should be taken with frequency of fungicide applications. Single-site fungicides such as pyraclostrobin and fluxapyroxad pose greater risk for resistance and should be limited to only a few applications per year.

In chapter 4, research conducted on the variety selection, application of foliar fungicides, and number of fungicide applications as well as effect of plant growth regulators on cotton has determined benefits in disease reduction. In 2014 to 2016, Phytogen 499 WRF has been documented to have more disease severity and defoliation compared to the hypothesized less

susceptible Deltapine 1137 B2RF. In terms of yield, when disease pressure was high such as in five out of six trials in this study, Deltapine 1137 B2RF out-yielded Phytogen 499 WRF. This result is similar to the results presented by Mehl et al. (2017) where Deltapine1137 B2RF had higher yields compared to Phytogen 499 WRF at 3 locations with high disease pressure. On the other hand, Phytogen 499 WRF had higher yields at 2 locations where there is lower disease or later disease onset (Mehl et al., 2017). Similarly, in the 2015 Attapulgus trial, where disease pressure was lowest, Phytogen 499 WRF resulted in greater numerical increase of yield compared to Deltapine 1137 B2RF, however, the difference was not significant.

When considering fungicide applications, all fungicides reduced severity and defoliation; however, two applications of Priaxor (pyraclostrobin + fluxapyroxad) resulted in significantly least amount of disease severity and defoliation and greatest numerical increase in terms of yield although not significantly different among treatments. Currently, fluxapyroxad + pyraclostrobin (Priaxor) is the most efficacious registered fungicide for controlling target spot on cotton (Hagan, 2017; Mehl et al., 2017).

In the 2017 trials, two different varieties were evaluated, namely Phytogen 490 WRF and Deltapine 1646 B2RF. Unlike the previous trials using Phytogen 499 WRF and Deltapine 1137 B2RF, there were no varietal response in severity and defoliation in the 2017 trials. In terms of treatments, all treatment combinations have significantly lower disease severity and defoliation compared to the nontreated control ($P$=<.0001). Least amount of target spot severity and defoliation was achieved with two applications of Priaxor. In Stripling, both single and double applications of Priaxor significantly increased yield compared to plots that did not receive any fungicide treatment ($P$=0.0013). However, in Attapulgus, there was a cotton variety x fungicide interaction where application of fungicide did not have a significant effect on yield on the variety

Phytogen 490 WRF. On the other hand, yield was significantly affected by fungicide treatment on the cotton variety Deltapine 1646 B2RF where both single and double applications of Priaxor significantly increased yield compared to plots that did not receive any fungicide treatment ($P$=0.0016).

This just shows the complexity in assessing effects of fungicide program on cotton yield in fields with target spot. Moreover, target spot will be most severe in fields with rank growth. The risk of target spot can be reduced by careful management of growth of the crop (Whitaker et al., 2017). However, in terms of use of plant growth regulators, both 2017 trials showed no significant effect on yield. In one trial, PGR significantly reduced severity and defoliation compared to the nontreated control. Plant growth regulators did not significantly affect yield ($P$=0.1408) although with increase rate and frequency of use of PGR, numerical increases in yield were noted. However, on the other trial, PGR effect on target spot was not significant on all variables tested. Hence, there is still much need for applied research on effects of PGR on target spot.

Overall, two applications of Priaxor gave the best season-long target spot control as indicated by lowest AUDDPC values. However, growers should be aware that even though a fungicide may limit defoliation, it will not consistently benefit yield potential. Whether cotton would benefit from a fungicide application to protect from target spot needs to be assessed on a field by field basis.

Fungicides were effective for target spot disease control in terms of severity and defoliation. However, yield improvements were usually not significantly different between fungicides and the untreated control although double applications of Priaxor (pyraclostrobin + fluxapyroxad) tended to be numerically greater. Still, there were some instances where beneficial

yield results were reported as an outcome of fungicide applications when disease pressure is high, as seen in 3 out of 6 trials in this study. A multi-year regional evaluation of control of target spot (Mehl et al., 2017) showed fungicides significantly impacted yield at 3 out of 8 locations. Priaxor treatments had the greatest yields with 144 to 394 lb/A increase over control. Moreover, 2 fungicide applications increased yield more than 1 application in the Alabama and Tennessee locations. Similar to the results of this study, number of applications did not matter at the other 6 loccations (Mehl et al., 2017).

In conclusion, when disease pressure was high and rapid defoliation occurred, Deltapine 1137 B2RF yielded better than Phytogen 499 WRF. There was a varietal response in terms of controlling the disease where Phytogen 499 WRF resulted in greater disease severity and defoliation ratings compared to Deltapine 1137 B2RF. Fungicide applications significantly reduced defoliation and numerically increased yield over the untreated control. A double application of Priaxor seems to be the best fungicide treatment to control target spot of cotton and expect greater yield response. Lastly plant growth regulator mepiquat showed no significant effect on yield. There is still much to learn about the effects of controlling the growth of the plant in relation to target spot.

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