**Genetic diversity of the fungal pathogen *Corynespora cassiicola* and its fungicide resistance**

by

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## Abstract

*Corynespora cassiicola* C.T. Wei is a widespread phytopathogenic fungus that causes disease in tropical and subtropical regions in up to 400 plant species, including fruits, vegetables, ornamentals, forestry, and row crops. Disease symptoms can be observed on plant leaves, stems, roots, flowers, and fruits. Incidence of the disease, target spot, as well as severity has become more frequent across cotton and soybean producing countries, and significant yield losses can occur if it is not properly controlled. The fungus has a parasitic lifestyle coupled with a saprophytic and endophytic lifestyle which complicates disease management. The overall objective of this dissertation was to better understand the pathogen, its interaction with soybean plants, and risks associated with chemical control. The first chapter reviewed the literature on *C. cassiicola* focused on cotton and soybean, including pathogen biology and genetic diversity, and a detailed discussion on two essential IPM strategies emphasizing their importance to overcome the disease. The second chapter investigated the genetic diversity of *C. cassiicola* isolates from symptomatic leaves of cotton and soybean by studying the morphology, pathogenicity, and molecular phylogeny based on cassiicolin-encoding genes and four loci. The third chapter revealed the impact of *C. cassiicola* diversity on screening for resistance to target spot on soybean by comparing two screening methods: leaf wilting bioassay and plant inoculation. The fourth chapter determined the sensitivity profile of *C. cassiicola* isolates from cotton and soybean to five commercial fungicides, and if there is a fitness loss on *C. cassiicola* QoI-resistant isolates. Finally, the fifth chapter first reported *C. cassiicola* isolates from soybean in the United States with the G143A mutation in the cytochrome *b* gene that confers resistance to QoI fungicides. Altogether, the results of this dissertation provide useful insights for research on the management of target spot disease to reduce the risk of epidemics and yield losses.

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# Chapter 1. Review of Literature


## Abstract

*Corynespora cassiicola* (Berk. & Curt.) C.T. Wei is a phytopathogenic fungus that causes disease in a broad range of species of plants worldwide. Indeed, it is a known pathogen of cotton and soybean with relevant importance. Currently, there is an increasing demand for food and fiber to supply a growing world population, where crop production needs to be conducted with increased yields on existing farmable land while protecting the environment, especially in accordance with IPM strategies. In light of that, the present review provides an overview of research conducted on *C. cassiicola* in general but with a special focus on cotton and soybean provided whenever possible. The biology and diversity found among *C. cassiicola* isolates are presented. In addition, a more detailed discussion on two essential IPM strategies is provided emphasizing their importance to overcome the disease; the potential of the risk of fungicide resistance development with use of fungicides to control target spot, and the need for genetic control with resistant varieties. Therefore, this review should provide insights for management of target spot, reducing the risk of epidemics and yield losses.

# 1. Introduction

## 1.1 Overview

Target spot was reported as a soybean disease for the first time in the New World in 1945. It was reported as *Helminthosporium vignae* Olive and designated as a pathogen of cowpea (*Vigna sinensis* (L.) Endl.) and soybean (*Glycine max* (L.) Merr) in the United States (Olive et al., 1945). In 1950, the pathogen was reclassified to *Corynespora cassiicola* (Berk. & Curt.) C.T. Wei (Wei, 1950). In 1976, target spot on soybean was reported in Brazil (Almeida et al., 1976) and Argentina in late 1980s (Ploper and Ramallo, 1988). On cotton, *C. cassiicola* was reported in Alabama for the first time in 1959 (Jones, 1961). In 1995, Corynespora leaf spot was reported occurring on upland cotton (*Gossypium hirsutum* L.) in Brazil (Mehta et al., 2005). Recently, several publications reported the first occurrence and the re-emergence of the disease on cotton in China (Wei et al., 2014), in Brazil (Galbieri et al., 2014), and U.S. states, including Georgia (Fulmer et al., 2012), Alabama (Campbell et al., 2012; Conner et al., 2013), Louisiana (Price et al., 2015), and Tennessee (Butler et al., 2016). Target spot has been a concern for farmers and researchers due to its increasing occurrence especially on soybean (Godoy, 2015) and cotton (Sumabat et al., 2018a), where it causes great damage when not properly controlled. Disease incidence and severity have been increasing possibly due to monoculture farming, adoption of conservation tillage systems, the use of susceptible cultivars, lack of crop rotation and optimal weather patterns changes for the disease development (Avozani et al., 2014; Koenning et al., 2006). In 2004, atypical conditions of heavy rains due to hurricanes in South Carolina resulted in estimated yield loss from target spot on soybean of 20-40% in some locations (Koenning et al., 2006). High yield losses (18-32%) have been reported in Mississippi for susceptible soybean lines (Hartman, 2015). Annual losses worldwide of 10-30% are estimated on cotton caused by pests and diseases (Tarazi

et al., 2019). Yield losses up to 448 kg/ha of seed + lint yield have been estimated for cotton (Bowen et al., 2018), and up to 1009 kg/ha of yield losses for soybean (Faske, 2017).

Cotton (*Gossypium* spp.) is a perennial plant that is grown as annual crop in tropic and sub-tropical areas to produce natural fibers and seeds (Deguine et al., 2008). It is comprised of approximately 50 species worldwide, divided into diploids ($2n = 2x = 26$) from the Old World (Africa-Asia), such as *G. herbaceum* L. and *G. arboreum* L., and tetraploids ($2n = 4x = 52$) from the New World (Mesoamerica-South America), such as *G. barbadense* L., and *G. hirsutum* L. (OECD, 2010). Two species are the most cultivated species in the world: *G. hirsutum*, known as upland cotton or Acala cotton, and *G. barbadense* as Pima or Egyptian cotton (Deguine et al., 2008; OECD, 2010). Besides fiber production, cotton is commercially grown as an oilseed crop with seed oil content close to 15% (Hinze and Kohel, 2012). According to the estimates for 2020, 33 million hectares are planted worldwide, and 79% of all cotton in the world is produced in five countries. India was the world's leading producer of cotton in 2020, followed by China, United States, Brazil, and Pakistan (Table 1.1) (USDA, 2020). Detailed description of plant pathogens on cotton can be found on the second edition of the *Compendium of Cotton Diseases* but *C. cassiicola* has not been covered as a pathogen yet (Kirkpatrick and Rothrock, 2001).

Soybean (*G. max*) is an annual legume native to Eastern Asia that was adapted to grow in a wide range of soil conditions and climate zones (tropical, subtropical and temperate climates). It is one of the most profitable legumes in the world and has been used as soybean meal and oil (Hartman et al., 2011). The high protein (~40%) and oil content (~20%) of the soybean seeds are responsible for the extensive soybean cultivation (Dwevedi and Kayastha, 2011; Hartman et al., 2015; Phansak et al., 2016). As food demand increases globally with the increase of the world population, the challenge for the 21$^{st}$ century is to increase soybean yield and other plant species

(Stupar, 2010). Annually, the world has been facing the intensification of soybean production and new regions have become soybean producers. The top five world producers of soybean are Brazil, United States, Argentina, China, and India, and the area planted worldwide is 128 million hectares of soybean, according to the estimates of 2020. About 90% of all soybean is produced in these five countries (Table 1.1) (USDA, 2020). The expansion and the intensification of soybean cultivation, increases diseases affecting the crop, allowing the increase of pathogens (Hartman et al., 2011). The first *Soybean Disease Compendium* covered 50 diseases (Sinclair and Shurtleff, 1975) and recently, more than 200 pathogens are known to infect soybean plants (Hartman et al., 2015). As a result of the diseases' occurrence, soybean yield losses becomes more frequent. Genetic improvements and agronomic practices helped boost soybean yields (Hartman et al., 2011).

*Corynespora cassiicola* is becoming relevant and concerning as a plant pathogen precisely because of its increasing occurrence on several high economic value crops. Several strategies have been indicated to control target spot, such as effective fungicides, crop rotation, and resistant varieties. The identification of germplasm resistant to *C. cassiicola* isolates has not been successful (Teramoto et al., 2013) and the indiscriminate use of fungicides results in high production costs and is not an environmentally friendly approach. Besides that, *C. cassiicola* presents a high-risk to becoming resistant to fungicides (FRAC, 2019). The development of resistant/tolerant germplasm seems to be a feasible strategy for disease management (Fernando et al., 2009) but it is important to take into consideration the variability of *C. cassiicola* isolates in order to develop effective resistance to target spot. As the disease becomes more important on cotton and soybean, there is an increasing need of more research on the phytopathogenic fungus. Studies that deal with different aspects of the pathogen, interaction with host plants, molecular approaches focused on their

diversity, efficiency of chemical control and their risk of resistance, and alternative paths to integrated pest management (IPM) are required to overcome this disease.

### 1.2 Pathogen description

The genus *Corynespora* is in the kingdom Fungi, phylum Ascomycota, class Dothideomycetes, subclass Pleosporomycetidae, order Pleosporales and family Corynesporascaceae (Robert et al., 2005). *Corynespora cassiicola* is a widespread plant pathogenic fungus that produces target-shaped necrotic spots on plant leaves and on stems, roots, flowers, and fruits, and has been recorded in over 70 countries on more than 400 plant species including fruits, vegetables, grains, perennial crops, forestry and various ornamental plants (Farr and Rossman, 2020). *Corynespora cassiicola* is mostly reported as a necrotrophic fungus (Lopez et al., 2018) where the pathogen destroys the host cell to utilize host nutrients and overwinter on infected soybean debris and seeds (Almeida et al., 2001). It has also been described as an endophyte (Déon et al., 2012b; Dixon et al., 2009) and as a saprophyte (Cai et al., 2006). The pathogen has also been isolated from nematodes (Carris et al., 1986) and humans (Lv et al., 2011).

Colonies of *C. cassiicola* exhibit whitish gray mycelium in the first days, turning dark gray with age on PDA (Lopez et al., 2018). Variation of spore morphology occurs depending on the substrate from which spores are collected (MacKenzie et al., 2018). Conidiophores are erect, branched, brown-colored, single or in clusters with swollen basal cells, 1-20 pseudosepta, 4-11 x 44-135 µm. Conidia are solitary or in chains (2-6), smooth, brownish, central hilum at the basal end, 3-20 pseudosepta, straight or slightly curved, 7-22 x 39-520 µm (Hartman, 2015). The presence of a hilum is a distinctive feature of the conidia, which appear thicker and darker compared with the rest of the conidia (Ellis and Holliday, 1971). Chlamydospore formation was reported from *C. cassiicola* isolates, allowing the pathogen to survive in soil or plant debris under

unfavorable conditions or with the absence of the host for almost two years (Olive et al., 1945; Oliveira et al., 2012). Pathogens that survive on soybean debris constitute the source of primary inoculum (Almeida et al., 2001). Target spot is a polycyclic disease with a short reproduction cycle where the pathogen can complete many generations in a single growing season (Agrios, 2005; MacKenzie et al., 2018).

Free water was not essential for germination but promoted germination of *C. cassiicola* isolates from *Hevea brasiliensis,* and temperatures between 10-35°C (optimum of 30°C) induced higher sporulation of the fungus (Fernando et al., 2012). Soybean leaves with free moisture and relative humidity close to 80% or above favored foliar infection by *C. cassiicola* (Hartman, 2015). The pathogen isolated from tomato requires prolonged humidity and 16-44 h of leaf wetness for spore germination and disease development (MacKenzie et al., 2018). Disease severity was positively correlated with temperature from 20 to 30°C and relative humidity higher than 90% (Ortega-Acosta et al., 2020). Temperatures ranging from 25 to 30°C and relative humidity at 95% were optimum for spore germination of *C. cassiicola* isolates from tomato (Dutta et al., 2020).

A toxin produced by highly pathogenic *C. cassiicola* isolates was found affecting susceptible symptomatic tomato cultivars; however, expression was not seen in resistant tomato cultivars (Onesirosan et al., 1975). Later, the toxin was purified and characterized as a phytotoxin called cassiicolin (de Lamotte et al., 2007). Phytotoxins are chemical compounds that are toxic to plants; generally they have low molecular weight secondary metabolites capable of passing through a 0.22 µm filter, and cause plant cell death. Cassiicolin is a secreted glycosylated protein with 27 amino acids that was reported as crucial for pathogenicity (Déon et al., 2012a). The toxin cassiicolin was reported as an important effector of *C. cassiicola*, and is represented by six cassiicolin isoforms (Cas1-Cas6) from different isolates, various hosts and geographical origin

(Barthe et al., 2007; Déon et al., 2014; Wu et al., 2018). Recently an additional cassiicolin isoform (Cas7) was identified (Lopez et al., 2018). Isolates carrying *Cas1* gene were considered the most aggressive on *Hevea* pathogenicity tests (Déon et al., 2014); however it is possible that there are other disease effectors besides cassiicolin (Déon et al., 2012a).

### 1.3 Disease symptoms

On cotton, the pathogen can infect all above ground parts. Small circular spots are observed on the cotyledonary leaves, and seedling death can occur if the pathogen infects hypocotyls. Small circular to irregular spots (2-10 mm) reddish-brown in color can be observed on cotton leaves. Necrosis and a typical "target spot" symptom occur in older lesions. With the advance of pathogen infection, cotton leaves complete premature senescence and severe defoliation can occur. Optimum conditions for disease development and pathogen infection is found in the lower canopy because of higher humidity levels and proximity to the soil where primary inoculum can be found (Galbieri et al., 2014).

On soybean, the pathogen can infect leaves, stems, pods, seeds, hypocotyls, and roots. Small circular to irregular spots in shape, from specks to mature spots (> 10 mm) are reddish-brown in color. Frequently, leaf symptoms can be associated with a dull-green or yellowish-green halo with a typical "target spot" in the center. Leaf veins, petioles and stems can exhibit dark brown lesions with different shapes and sizes; lesions on pods are usually circular, depressed, and dark in the center with brown margins. Premature defoliation can occur in severe cases of infection under optimum conditions. Symptoms on roots can be observed as dark reddish-brown lesions that will turn dark violet-brown when the fungus sporulates (Godoy, 2015).

### 1.4 Diversity of *C. cassiicola* isolates

The genetic diversity of *C. cassiicola* isolates has been documented using different techniques. Different genes have been amplified to construct phylogenetic trees to understand the evolution and variability of *C. cassiicola* isolates, and finally to help build management strategies (Banguela-Castillo et al., 2020; Bentes et al., 2018; Déon et al., 2014; Dixon et al., 2009; Hieu et al., 2020; Nghia et al., 2008; Oktavia et al., 2017; Qi et al., 2011, 2009; Shimomoto et al., 2011; Shrestha et al., 2017; Silva et al., 2003; Sumabat et al., 2018a; Wu et al., 2019).

Dixon et al. (2009) conducted the most prominent study on *C. cassiicola*. Combined data of four loci (rDNA ITS, *caa5, ga4,* and *act1*) of 143 isolates of *C. cassiicola* from 68 different plant species was used for phylogenetic analysis. They observed six phylogenetic lineages (PL) correlated with pathogenicity, host plants, and growth rate but could not explain the geographical origin of isolates. In China, *C. cassiicola* isolates from *Hevea* were successfully differentiated from other hosts based on ISSR markers (Qi et al., 2009). A lack of diversity of *C. cassiicola* isolates from several hosts was observed in Japan based on pathogenicity profiles and multi-gene analyses (*β*-tubulin, EF-1α, calmodulin, and actin) (Shimomoto et al., 2011). No diversity was found by sequencing rDNA ITS or morphological characters of *C. cassiicola* isolates but ISSR markers were successful in differentiating isolates from *Hevea* and other host plants (Qi et al., 2011). Déon et al (2012) demonstrated that differences exist between aggressive and moderately aggressive isolates in their levels of a putative effector protein, cassiicolin. The phylogenetic diversity of *C. cassiicola* isolates from several hosts and geographical origins based on four combined loci (rDNA ITS, *caa5, ga4,* and *act1*) was strongly structured by the toxin class. The cassiicolin gene was expressed in the early phase of infection and six cassiicolin isoforms (Cas1, Cas2, Cas3, Cas4, Cas5 and Cas6) were identified. Curiously, isolates with no detectable *Cas* gene (Cas0) were able to cause disease in rubber tree, indicating the possibility of yet uncharacterized effector(s), plus the co-

existence in the same isolate of several effectors (Déon et al., 2014). A new cassiicolin isoform, Cas7 was identified and despite the discovery of other candidate effectors, cassiicolin still remains the only toxin characterized for *C. cassiicola* (Lopez et al., 2018). *Cas1* gene has only been found on *C. cassiicola* from *Hevea* (Déon et al., 2014).

SNP analysis of four *C. cassiicola* isolates from cotton and one isolate from soybean revealed eight unique multilocus genotypes, and a single clone of *C. cassiicola* was found to predominate in the southeastern United States. However, sampling was limited to only one location in Tennessee (Shrestha et al., 2017). Analysis of rDNA ITS sequences showed high genetic diversity among 23 *C. cassiicola* isolates from Indonesia, but clusters did not indicate correlation with host species and geographical origin. Five haplotypes were found based on three SNP's (Oktavia et al., 2017). No genetic diversity was found for *C. cassiicola* isolates from cotton in the southeastern United States, whereas higher diversity was found for isolates from soybean. Isolates of *C. cassiicola* clustered based on their host species but not on their geographical origin (Sumabat et al., 2018a). Using next-generation sequencing approach, 13 polymorphic microsatellite markers were developed on 265 *C. cassiicola* isolates revealing that isolates clustered based on their host species. Clearly, *C. cassiicola* populations from soybean in Brazil and U.S. were genetically similar but different from populations from cotton in the U.S. The genetic similarity is unclear on populations from cotton from Brazil (Sumabat et al., 2018b). Putative effectors were identified for 35 *C. cassiicola* isolates from five host plants (CCP used as a reference), and the effector-based classification was highly consistent with those six PL from Dixon's study (Lopez et al., 2018).

Genetic diversity based on iPBS markers and variability in pathogenicity was found among 69 *C. cassiicola* isolates from 16 hosts in China (Wu et al., 2019). The use of SRAP markers resulted in clusters of *C. cassiicola* isolates from *Hevea* highly supported by the geographical

region, while clusters based on ISSR markers of rDNA ITS highly supported the host origin of isolates (Hieu et al., 2020). Recently, an updated classification was proposed for *C. cassiicola* isolates based on combined phylogenetic analysis (*act*1, rDNA ITS, *ga4,* and *caa5*), resulting in eight major phylogenetic clades (PhL1-PhL8). In addition, the two-locus CAPS (cleaved amplified polymorphic sequence) based on *ga*4 and *caa*5 loci was described as a novel approach to easily differentiate isolates (Banguela-Castillo et al., 2020).

*Corynespora cassiicola* is an important pathogen for rubber trees and most studies have been conducted in this pathosystem, as discussed. The wide genetic diversity found on *C. cassiicola* isolates that infect rubber trees was confirmed (PhL1-PhL5, and PhL7). *Corynespora cassiicola* isolates from soybean exhibited higher genetic diversity (PhL1, PhL3, and PhL5), than isolates from cotton (PhL1) (Banguela-Castillo et al., 2020). Host specificity was reported among isolates of *C. cassiicola* and it has been demonstrated that there is variation in the aggressiveness of isolates (Banguela-Castillo et al., 2020; Déon et al., 2014; Dixon et al., 2009; Sumabat et al., 2018a). Despite the extensive studies on the genetic diversity of *C. cassiicola* isolates and their relationship with the geographical origin, and host origin of isolates, not enough is known about their relationship with morphological characters, and host specificity taking into consideration difference on the response of germplasm. Indeed, extensive studies are needed with a focus on *C. cassiicola* isolates from cotton and soybean to finally bring more insights into disease management.

### 1.5 The use of fungicides to control target spot

Disease and pest control is highly dependent on conventional pesticides, which makes it the most widely used strategy for crop protection (Tarazi et al., 2019). Chemical control by application of fungicides was considered the most frequently used tool in disease management in

agriculture, and have been used for over 200 years (Brent and Hollomon, 2007). Foliar fungicide application is known to be the most effective tool to control target spot (Ma et al., 2020); however, their extensive application and incorrect use have led to the progressive development of resistance to fungicides aggravated by ecological problems (Asadollahi et al., 2013; Tarazi et al., 2019). Currently, the Fungicide Resistance Action Committee (FRAC; www.frac.info/home) works to prolong the effectiveness of fungicides by providing fungicide resistance management guidelines which includes an updated list of risk of resistance development by fungal pathogens. *Corynespora cassiicola* was classified as a pathogen with high risk of development of resistance to fungicides (FRAC, 2019), and resistant *C. cassiicola* isolates from cucumber, soybean, and tomato to several fungicide groups have been reported worldwide (Avozani et al., 2014; Date et al., 2004; Ishii et al., 2007; Miyamoto et al., 2010, 2009; Rondon and Lawrence, 2019; Teramoto et al., 2017; Xavier et al., 2013).

In 2012, the loss of efficiency of fungicides (benzimidazole, triazole, and strobilurin) used to control target spot on soybean was observed in the field in Brazil (Godoy et al., 2012). At that time, few fungicide options were reported as available to control target spot on soybean (Avozani et al., 2014). Benzimidazoles and thiophanates are among Methyl Benzimidazole Carbamates (MBC, FRAC code 1) fungicides which have been classified with a high risk for developing resistance (FRAC, 2020). MBC fungicides are inhibitors of *β-tubulin* assembly in mitosis with direct effects on nuclear division, inhibiting mycelial growth (Date et al., 2004; Ishii et al., 2007), and mutations in the *β-tubulin* gene cause different resistance levels to benzimidazoles (Date et al., 2004; Duan et al., 2019; Li et al., 2020). Benzimidazole resistance has been reported for *C. cassiicola* on several crops (Avozani et al., 2014; Date et al., 2004; Teramoto et al., 2017; Xavier et al., 2013). A decrease in the effectiveness of thiophanate-methyl was observed on cucumber

with the emergence of *C. cassiicola* resistant isolates (Date et al., 2004). Highly non-sensitive soybean *C. cassiicola* isolates to carbendazim and thiophanate-methyl were reported in Brazil (Avozani et al., 2014; Teramoto et al., 2017; Xavier et al., 2013). Recently, a double mutation of *β-tubulin* conferring resistance to benzimidazoles was reported for *C. cassiicola* isolates. In addition, a strong cross resistance was observed among carbendazim, benomyl, and thiabendazole on *C. cassiicola* isolates as a result of different *β-tubulin* mutations (Duan et al., 2019).

Quinone outside Inhibitors (QoI, FRAC code 11) fungicides, also known as strobilurins are among the most used fungicides to control plant diseases (Bartlett et al., 2002). However, QoI fungicides pose a high risk of resistance development (Grasso et al., 2006), and worldwide more than 20 pathogens were recoded as QoI-resistant in 2006 (Ishii and Hollomon, 2015). Three target site mutations were reported in the cytochrome *b* gene (F129L, G137R, and G143A) that are known to confer different levels of resistance to QoI fungicides (Bartlett et al., 2002; Duan et al., 2019; Grasso et al., 2006; Ishii et al., 2001), and the mutation G143A is the most common in QoI-resistant pathogens (Ma and Michailides, 2005). QoI-resistant isolates of *C. cassiicola* is a serious problem in Japan, where the disease cannot be controlled with these fungicides (Ishii et al., 2007). In Brazil, *C. cassiicola* from soybean was described as highly non-sensitive to pyraclostrobin, azoxystrobin, trifloxystrobin, and picoxystrobin (Teramoto et al., 2017). Cross-resistance for *C. cassiicola* within different QoI fungicides is known to occur (Brent and Hollomon, 2007; Duan et al., 2019). QoI-resistant isolates of *C. cassiicola* isolates from soybean but not from cotton isolates were reported in the U.S. (Rondon and Lawrence, 2019). Later, QoI resistance among *C. cassiicola* isolates from tomato was reported as widespread in Florida U.S., leading to the reduction of QoI fungicides applications in tomato fields (MacKenzie et al., 2020).

Succinate-DeHydrogenase Inhibitors (SDHI, FRAC code 7) fungicides are respiratory inhibitor fungicides with a site-specific mode of action but with a different target site within the fungal mitochondria when compared with QoI fungicides (Hollomon, 2015). SDHI fungicides are considered to be at moderate to high risk of resistance development (FRAC, 2020), and resistance has been reported in about 15 fungal pathogens (Torriani et al., 2017). Resistance to boscalid was reported for *C. cassiicola* isolates from cucumber in Japan (Miyamoto et al., 2009), and the use of boscalid to control Corynespora leaf spot has been discouraged (Miyamoto et al., 2010). Highly non-sensitive *C. cassiicola* isolates from soybean were reported in Brazil to fluxapyroxad and boscalid fungicides (Teramoto et al., 2017). In China, the resistance of *C. cassiicola* isolates from cucumber to boscalid was found widespread, suggesting the SDHI fungicides should be banned in certain areas of the country to avoid the emergence of multiresistance (Zhu et al., 2019).

So far, resistance to DMI fungicides on *C. cassiicola* has not been reported (Zhu et al., 2020). Despite the low risk of resistance development to DMI fungicides, it is still important to follow basic strategies to delay fungicide resistance development (Ishii and Hollomon, 2015). A low risk of fungicide resistance development can be found on multisite fungicides (FRAC, 2020) which have a broad spectrum of disease control, known to have a protectant and non-systemic effect on plants (Hollomon, 2015). Dithiocarbamate (mancozeb, maneb and propineb), inorganic (copper and sulphur), and chloronitriles (chlorothanlonil) are good examples of multisite fungicides (Brent and Hollomon, 2007; FRAC, 2020). These fungicides are a good fit to use in combination with another mode of action fungicide, usually a systemic fungicide, to reduce the selection pressure of one fungicide and inhibit the growth of resistant populations (FAO, 2012). Protectant fungicides have been used to control *C. cassiicola* on tomato fields in Florida, avoiding $3.5 million in potential revenue lost (MacKenzie et al., 2018).

Despite the problems caused by fungicide resistance, plant diseases have a negative impact on yields in almost any crop, and under favorable conditions the pathogen has greater ability to cause damage if no control measure is applied (Ul Haq et al., 2020). Currently, the large scale of crop production is highly dependent on efficient fungicide applications. Thus, it is essential to follow technical recommendations for its use to prevent the development of fungicide resistance (Deising et al., 2008), and fungicide mixtures are recommended to prevent the development of fungicide resistance (Brent and Hollomon, 2007; FAO, 2012; Ghini and Kimati, 2000). A meta-analysis of fungicide efficacy on soybean target spot from 2012 to 2016 in Brazil demonstrated that the most effective fungicides were the mixtures fluxapyroxad + pyraclostrobin and epoxiconazole + fluxapyroxad + pyraclostrobin. These fungicides were able to reduce 76.2% and 75.7% of target spot severity compared to the non-treated control, respectively. The least effective fungicides were mancozeb (49.6%), azoxystrobin + benzovindiflupyr (46.7%), and carbendazim (32.4%) (Molina et al., 2019). From 2014/2015 to 2016/2017 in Argentina, one and two applications of epoxiconazole + fluxapyroxad + pyraclostrobin were the best treatments to control target spot on soybean, with an average control of 59% and 53.9% compared with the untreated control. The least effective treatments for control were pyraclostrobin + epoxiconazole (35%), and azoxystrobin + benzovindiflupyr (28.9%) (Reznikov et al., 2019). The application of fluxapyroxad + pyraclostrobin yielded the best target spot control on cotton in U.S. (Price et al., 2017). Azoxystrobin, pyraclostrobin, and the mixture pyraclostrobin + metconazole have been recommended to control target spot on cotton in Alabama, U.S. Triazoles and strobilurins fungicides can be applied preventatively at first bloom or on-demand when the disease first appears followed by a second application 14 to 21 days later (ACES, 2020).

Fungicide resistance has been described as a stable, heritable adjustment by the fungus to a specific fungicide, which results in reduced sensitivity to the fungicide by the fungus (Ma and Michailides, 2005). The resistance acquired by the specific pathogen to the fungicide is directly proportional to the applied doses, the frequency of application, the degree of coverage, the persistence in culture or in soil and the size of the treated area (Deising et al., 2008). Novel modes of action of fungicides are not easily discovered, consequently the use of a high risk resistance fungicide combined with a low risk resistance fungicide and the rotation with different fungicide groups are recommended to maintain the most effective available fungicides (Ishii and Hollomon, 2015). Site-specific fungicides such as strobilurins and carboximides are safer to non-target species, and are recommended to be included in the IPM programs; however, they generally possess a high risk of resistance development (Brent and Hollomon, 2007; Ishii et al., 2001). As described, the use of multisite fungicides seems to be a good fit to be used in combination with other mode of actions in the IPM. The reduction of *C. cassiicola* resistant isolates to different fungicides could be pursued by implementing the use of multisite fungicides to control target spot efficiently.

### 1.6 Genetic control for disease resistance

Information about genetic resistance of the host to *C. cassiicola* is quite limited and complete resistance to target spot has not been reported. Instead, foliar fungicides have been used as an alternative to control target spot on cultivars with low resistance levels or susceptibility to the disease to protect crop yield (Duan et al., 2019; Li et al., 2020; MacKenzie et al., 2018; Teramoto et al., 2013). The use of genetic resistance is a less expensive, safe and practical solution to control diseases being the foundation step of any IPM. Besides that, there is the benefit to the environment since the use of chemical control will be reduced or avoided (Khan et al., 2020).

Positive results from intensive screening in the breeding phase played an important role in *C. cassiicola* management on *Hevea* (Fernando et al., 2010). Therefore, the addition of genetic control through resistant varieties in the agricultural system is the key to overcome a disease, while it is economical and harmless to the environment (Palloix et al., 2009). Genetic control through resistant varieties should be implemented as a long-term strategy (Fernando et al., 2010). Improved varieties for disease resistance, in any crop, are essential for achieving higher yields, and usually are limited by abiotic and biotic factors (Hartman et al., 2011), and ideally screening for disease resistance should be done in every breeding program before commercial release (Silva et al., 1998). During the development of cultivars, breeders must select genotypes that contain promising resistance genes and combine them to guarantee the durability of resistance for as long as possible. Usually, resistance genes to specific diseases are obtained from exotic germplasm and transferred to susceptible elite genotypes (Palloix et al., 2009).

Plant breeding was extensively discussed as a technology option for feeding 10 billion people (Meyer et al., 2013), and breeding for disease resistance is an essential item to successfully reduce soybean yield losses (Hartman et al., 2011). Methods of germplasm screening for disease resistance is crucial for developing cultivars with good genetic resistance and to complement the classical breeding methods. The evaluation of germplasm for disease resistance is a vital step in breeding, which can be conducted under controlled or field conditions (Khan et al., 2020). Whenever soybean lines are being developed in a breeding program, favorable weather conditions for disease development may not occur under field conditions (Botha et al., 2009). Thus, there is a need for screening methods in controlled environments that will precisely reflect what is expected to happen in field environments (Kull et al., 2003). A screening method with a non-destructive approach would be an excellent alternative to find genetic resistance on a large scale but

compatibility of *in vitro* and *in vivo* assays were not found studying *C. cassiicola* isolates (Fernando et al., 2010). Perhaps the variability found in the pathogen and their host-specificity as previously described has not been considered appropriately.

No resistance was found when assessing soybean cultivars against *C. cassiicola* in Brazil (Teramoto et al., 2013). Although it is an 'old' disease on soybean, breeding programs have been focused on different diseases (Phytophthora root and stem rot, Sudden death syndrome, Soybean rust, Cercospora leaf blight, Frogeye leaf spot and Nematodes) (Hartman et al., 2011; Roth et al., 2020). Clearly, there has not been as much effort on cotton and soybean breeding to develop resistance to *C. cassiicola*, evidenced by the lack of an effective protocol to discern among susceptible and resistant germplasm. If there are no effective screening protocols under controlled conditions for the search of sources resistant to *C. cassiicola*, intensive screenings should be conducted in 'hot spots' of the disease. Diagrammatic scales to assess target spot severity in cotton (Fantin et al., 2018) and soybean (Soares et al., 2009) should be used to standardize results.

Taking into consideration the high risk of fungicide resistance development of *C. cassiicola*, genetic improvement for target spot resistance should be pursued as an important breeding objective to be added as an essential strategy in the IPM of cotton and soybean. More dedicated research efforts for genetic improvement of cotton and soybean varieties resistant to target spot are needed in the future. Therefore, the challenges of using genetic control in the IPM include finding sources of cotton and soybean resistant to *C. cassiicola*, the search of molecular markers associated with the disease resistance, and an appropriate screening method to validate all results.

## 2. Conclusions and future prospects

Over the years, we have seen an increasing demand for food and fiber to supply a growing world population, where crop production needs to be conducted with increasing yields on existing farmable land while protecting the environment (CropLife International, 2014). Thereby, the use of IPM should be pursued more than ever encompassing the maximum number of control measures. The importance of using IPM is not just for the disease discussed here but for every pest population as stated in the FAO definition of IPM (Vetek et al., 2017):

> "Integrated Pest Management (IPM) means the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and reduce or minimize risks to human and animal health and the environment. IPM emphasizes the growth of a healthy crop with the least possible disruption to agro-ecosystems and encourages natural pest control mechanisms" (p. 01).

Genetic resistance, crop rotation, fungicide, and nematicide seed treatments, mid-season fungicide sprays, and biocontrol were included in the IPM strategies recommended to manage diseases on soybean (Roth et al., 2020). Additionally, cultural practices are recommended such as the use of tillage, row spacing, plant population, and irrigation (Roth et al., 2020). The integration of crop rotation, destruction of plant residues, sanitary practices of seeds, germplasm genetic resistant to target spot, and fungicide applications would help with disease management and reduction of yield losses on cotton and soybean (Galbieri et al., 2014; Hartman et al., 2011). Tarazi et al. (2019) stated that the combination of biomolecules (microbial agents and biological control) with optimized microbiomes remains unexplored. The use of biotechnological solutions have been recommended to prevent losses caused by diseases and pests and to improve yields on cotton and other crops, by minimizing production costs, and providing environmentally friendly alternatives (Tarazi et al., 2019). The use of non-host cereal crops such as maize, millet, sorghum and wheat was suggested for crop rotation to help breaking the disease cycle of *C. cassiicola* (Galbieri et al.,

2014); however, the fact that *C. cassiicola* is a known saprophyte fungus (Cai et al., 2006) with the ability to survive in the soil or plant debris by the production of chlamydospores (Olive et al., 1945; Oliveira et al., 2012) cannot be forgotten. The importance of cultural practices on cotton was compared with the choice of disease resistant varieties, resulting in changes in the importance of different diseases (Deguine et al., 2008).

Since target spot has become a frequent disease in cotton and soybean fields, this review aimed to generate information to support the disease management program. Not all but some control options in the IPM were discussed here to improve and to better understand target spot management but there is a need of more applied research (Molina et al., 2019). Also, there is a need for research on developing disease prediction tools to anticipate possible pathogen threats and disease outbreaks (Dutta et al., 2020; Roth et al., 2020). If farmers and researchers could anticipate the optimum environmental conditions for target spot development, management practices could be implemented in time to reduce the risk of the disease.

In short, the most feasible way to overcome target spot should include the use of several IPM strategies to increase crops yield while protecting the natural environment. In these times where *C. cassiicola* has been causing disease outbreaks, it is essential to know how to overcome the disease by having a deep understanding of three aspects: the pathogen *per se*, its interaction with host plants, and the environment component; altogether, these aspects make up the known plant disease triangle as the foundation of plant disease management. These should help to better manage target spot on cotton and soybean, reducing the risk of epidemics and yield losses.

**Table 1.1** Top five production countries of cotton and soybean for the year 2020.

| Country/Region | Area (million ha) | Yield (kilograms per ha) | Production (million tons) | World production (rank) |
|---|---|---|---|---|
| **Cotton (*Gossypium* spp.)** | | | | |
| India | 13.40 | 487 | 6.53 | 1 |
| China | 3.25 | 1,826 | 5.93 | 2 |
| United States | 3.64 | 1,020 | 3.71 | 3 |
| Brazil | 1.55 | 1,686 | 2.61 | 4 |
| Pakistan | 2.20 | 614 | 1.35 | 5 |
| **World** | **32.94** | **775** | **25.52** | - |
| **Soybean (*Glycine max*)** | | | | |
| Brazil | 38.60 | 3,450 | 133.00 | 1 |
| United States | 33.60 | 3,490 | 117.38 | 2 |
| Argentina | 17.30 | 3,090 | 53.50 | 3 |
| China | 9.30 | 1,880 | 17.50 | 4 |
| India | 12.20 | 920 | 11.20 | 5 |
| **World** | **127.59** | **2,900** | **369.74** | - |

Source: USDA (2020).

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# Chapter 2. Genetic diversity of *Corynespora cassiicola* isolated from cotton and soybean and relationships with morphology and pathogenicity


## Abstract

*Corynespora cassiicola* is a fungal pathogen with increasing importance across cotton and soybean producing countries and is responsible for target spot disease in these crops. Here, we investigated the morphological characteristics, pathogenicity, and molecular phylogeny based on cassiicolin-encoding genes and four loci (rDNA ITS, *caa5, ga4,* and *act1*) of *Corynespora cassiicola* isolates. A total of 204 *C. cassiicola* isolates were obtained from symptomatic leaves of cotton and soybean sampled in the southern U.S. Mycelial growth rate (mm/day), concentration of spores (conidia/mL), conidia length and width, and number of pseudosepta were characterized for 12 *C. cassiicola* isolates. Pathogenicity assays were conducted with 12 *C. cassiicola* isolates from cotton and soybean on two soybean cultivars (RA-606 and NK S56-B7X). Phylogenetic analyses using nucleotide sequences of cassiicolin-encoding genes and four loci were performed with 32 *C. cassiicola* isolates from our collection, plus reference sequences obtained from GenBank. The phylogenetic tree based on cassiicolin-encoding genes resulted in six distinctive clades (Cas1 to Cas6) with some isolates carrying a combination of genes (Cas2+6). Phylogenetic analysis based on four loci placed most of the *C. cassiicola* isolates from cotton in a well-supported subclade, while *C. cassiicola* isolates from soybean were placed in different subclades. Host specialization was observed in the pathogenicity assays, where *C. cassiicola* isolates from soybean were the most aggressive. There was no indication that phylogenetic analyses correlates with morphology, pathogenicity, or geographical origin of isolates. Our molecular phylogenetic analysis suggests a higher genetic diversity for *C. cassiicola* isolates from soybean. Results generated from this study on *C. cassiicola* genetic diversity from cotton and soybean provides a useful insight to be used in the search of resistant germplasm to target spot.

## 1. Introduction

*Corynespora cassiicola* (Berk. & M. A. Curtis) C.T. Wei is a widespread plant pathogenic fungus that produces necrotic target spots on plant leaves, stems, roots, flowers, and fruits. As a cosmopolitan pathogen, *C. cassiicola* has been recorded all over the world in tropical and subtropical regions on more than 400 plant species including fruits, vegetables, grains, perennial crops, forest trees and various ornamental plants (Farr and Rossman, 2020). The fungus has also been identified with a saprophytic lifestyle from decaying matter and with an endophytic lifestyle from non-symptomatic leaves (Déon et al., 2012b; Dixon et al., 2009).

Disease management has been a concern in other countries due to the increasing occurrence of target spot on soybean (Godoy, 2015). In cotton (*Gossypium hirsutum* L.) and soybean (*Glycine max* L. Merr.), the disease commonly known as target spot has the potential to cause significant economic losses on host plants. Severe disease symptoms and significant yield losses can occur when the pathogen is not properly controlled (Hagan and Sikora, 2012; Koenning et al., 2006). Yield losses up to 448 kg/ha (400 lb/acre) of seed + lint yield have been estimated for cotton (Bowen et al., 2018), and up to 1009 kg/ha (900 lb/acre) of seed yield losses for soybean (Faske, 2017).

A toxin produced by highly pathogenic *C. cassiicola* isolates affecting susceptible cultivars of tomato was first reported in 1975; however, expression of the toxin was not seen in resistant tomato cultivars (Onesirosan et al., 1975). Later, it was found that the pathogenicity of *C. cassiicola* in rubber tree was mediated by cassiicolin, a small phytotoxic protein first purified from the pathogenic isolate CCP (Barthe et al., 2007; Breton et al., 2000; de Lamotte et al., 2007). Then, it was demonstrated that differences existed between aggressive and moderately aggressive isolates in their levels of a putative effector protein, cassiicolin (Déon et al., 2012a). Further studies found a variation in the cassiicolin gene for several isolates of *C. cassiicola* possibly related to

host range (Déon et al., 2014). The cassiicolin gene was found expressed in the early phase of infection (Déon et al., 2012a) and six cassiicolin isoforms (Cas1, Cas2, Cas3, Cas4, Cas5, and Cas6) were identified by PCR. The aggressive response of isolates were related to the type of isoform, where Cas1 was the most aggressive in rubber trees' pathogenicity assays (Déon et al., 2014). Some of the isolates from cotton had a combination of two *Cas* genes (Cas2+Cas6) and others had no detectable *Cas* gene (Déon et al., 2014).

Different techniques have been used to study the genetic characterization of *C. cassiicola,* such as RAPD, ISSR, AFLP, iPBS, and phylogeny. Several genes and partial genes have been amplified to construct phylogenetic trees to understand the evolution and variability of *C. cassiicola* isolates*,* and finally to build management strategies (Bentes et al., 2018; Déon et al., 2014; Dixon et al., 2009; Nghia et al., 2008; Oktavia et al., 2017; Qi et al., 2011, 2009; Shimomoto et al., 2011; Silva et al., 2003; Sumabat et al., 2018; Wu et al., 2019). Known genetic variability within *C. cassiicola* isolates is currently limited to rubber tree isolates. Five genetic groups were observed for 42 *C. cassiicola* isolates from several hosts and locations in Sri Lanka but no correlation between pathogenicity and RAPD groups was found (Silva et al., 2003). Additionally, at least two distinct groups of *C. cassiicola* were found to infect rubber trees in Malaysia using ISSR markers (Nghia et al., 2008). Dixon et al. (Dixon et al., 2009) conducted the most prominent study with the phylogenetic analysis using combined data of four loci (rDNA ITS, *caa5, ga4,* and *act1*) for 143 isolates of *C. cassiicola* from 68 different plant species. They observed six phylogenetic lineages (PL) correlated with pathogenicity, host plants, and growth rate but not with geographical origin. *Corynespora cassiicola* isolates from rubber tree were successfully differentiated from other hosts in China; authors concluded that clusters based on ISSR markers had a clear correlation to their original host (Qi et al., 2009). In Japan, a lack of diversity of *C.*

*cassiicola* isolates based on pathogenicity profiles and multi-gene analyses (*β*-tubulin, EF-1α, calmodulin, and actin genes) was observed (Shimomoto et al., 2011). A study based on ISSR analysis found that *C. cassiicola* isolates from rubber leaves clustered with a clear correlation with their original host but no correlation among morphological characters or phylogeny based on rDNA ITS only (Qi et al., 2011).

Déon et al. (2014) studied the diversity of the cassiicolin-encoding gene in *C. cassiicola* isolates from several hosts and geographical locations. The phylogenetic diversity based on four combined loci (Dixon et al., 2009) was strongly structured by the toxin class; however, the authors stated the possibility of yet uncharacterized effector(s), plus the co-existence in the same isolate of several effectors (Déon et al., 2014). The whole genome sequence (WGS) of four *C. cassiicola* isolates from cotton and one isolate from soybean were analyzed but the geographical origin of isolates was limited to only one location in Tennessee; a single clone of *C. cassiicola* was found to predominate in the southeastern United States (Shrestha et al., 2017). A high diversity among 23 *C. cassiicola* isolates from Indonesia was found but there was no correlation with host species or geographical origin using phylogeny based on rDNA ITS (Oktavia et al., 2017). Results from other study showed that 53 *C. cassiicola* isolates clustered based on host species without correlation with geographical origin. They also observed evidence of host specialization, and no genetic diversity for *C. cassiicola* isolates from cotton in the southeastern United States (Sumabat et al., 2018). Recently, a study using iPBS marker analysis confirmed the pathogenicity variability and genetic diversity in a study with 69 *C. cassiicola* isolates from 16 hosts in China; however, no pathogenic diversity was found for *C. cassiicola* isolates from rubber tree (Wu et al., 2019).

Despite several studies that tried to understand the variability of *C. cassiicola* isolates, there is no clear correlation between phylogenetic clades and intrinsic characteristics of the isolates,

such as geographic origin, pathogenicity or morphology. As target spot becomes more relevant as a plant disease due to its increasing occurrence on several high value crops, it is important to improve understanding about its pathogen, *Corynespora cassiicola* and its toxin, cassiicolin. The most feasible strategy for disease management seems to be the development of resistant/tolerant germplasm to the disease (Fernando et al., 2009). Development of effective resistance requires information about the variability of *C. cassiicola* isolates. Then, targeted isolates can be used in breeding programs for screening lineages. Ideally, screening for disease resistance should be included in every breeding program before commercial release of a cultivar (Silva et al., 1998).

The objective of this study was to understand the variability of *C. cassiicola* isolates from cotton and soybean based on morphology, pathogenicity, and phylogeny based on cassiicolin-encoding genes and four combined loci. Such data will provide information for disease management by identifying unique sources of *C. cassiicola* isolates that could be used in a breeding program.

## 2. Materials and methods

### 2.1 Isolates

Isolates of *C. cassiicola* were collected from cotton and soybean leaves showing symptoms typical of target leaf spot from fields in Alabama and Tennessee (Figure 2.1). Small amounts of mycelia and conidia from the surface of the lesions were directly transferred onto potato dextrose agar (PDA; Hardy Diagnostics, Santa Maria, CA) enriched with 50 mg/L of kanamycin. Symptomatic leaves were placed in plastic bags to induce fungal sporulation when it was not already present. When direct isolation was not successful, small pieces of leaf tissue from the edge of the necrotic lesions were surface-disinfected for 30 seconds in 70% ethanol, 1 min in 2% sodium hypochlorite, and then rinsed twice with sterile distilled water. Disinfected pieces of leaf tissues

were plated directly onto PDA enriched with 50 mg/L of kanamycin. Inoculated plates were incubated at 28 ± 2°C under a cycle of 12 h light/dark for colony growth, and pure colonies were selected to establish the *C. cassiicola* collection. Fungal identification was made based on morphological and reproductive characters (Ellis and Holliday, 1971).

### 2.2 Morphology

Of the 32 *C. cassiicola* isolates obtained in this study (Table 2.1), 12 isolates were selected for additional morphological characterization. Isolates were inoculated by placing one mycelial plug (7.0 mm) from a 10-day-old colony at the center of a Petri plate and incubated at 28 ± 2°C. Growth from different culture media: PDA and V8 agar (340 mL of V8 juice, 660 mL of water, 3 g of $CaCO_3$, 17 g of agar) (Dixon et al., 2009), and different light regimes: 12 h light/dark (12 h dark) and continuous dark (24 h dark) were recorded. Unbalanced data were used for different independent variables, and number of samples (*n)* were exhibited for each variable considered. Pictures were taken to record the characteristics of the mycelial growth after 10 days of incubation. The mycelial growth rate (mm/day) was determined by measuring colony diameter of each isolate along two perpendicular lines on PDA ($n$ = 571) and V8 agar ($n$ = 96) under 12 h dark ($n$ = 571) and 24 h dark ($n$ = 96) (Fernando et al., 2009). Data were collected when the mycelial growth of the first colony reached the borders of the Petri dish. To determine the concentration of spores (conidia/mL), each isolate of *C. cassiicola* was inoculated on PDA ($n$ = 143) and V8 agar ($n$ = 192) under 12 h dark ($n$ = 239) and 24 h dark ($n$ = 96) and allowed to grow for 10 days. For each isolate, six Petri dishes were flooded with 10 mL of sterile distilled water each and the colony surfaces were mechanically disturbed with a glass slide to release conidia. The conidia suspension was filtered through four layers of sterile gauze to remove most of the mycelia. The final volume of conidia suspension was centrifuged (2100 g for 10 min) to remove excess water, and the

suspensions were adjusted to 30 mL by adding sterile water, if necessary. The concentration of spores was determined under the microscope using a hemocytometer (Fernando et al., 2009). To determine the conidia morphology (width, length, and number of pseudosepta), each isolate of *C. cassiicola* was inoculated on PDA ($n$ = 600) and V8 agar ($n$ = 600) under 12 h dark only for 10 days. Twenty µL of the conidia suspension was placed on a clean glass slide, and the measurement of 100 conidia at 400x magnification were recorded by an inverted microscope: Nikon Eclipse T*i* connected with a CCD Nikon camera that transferred images to a computer with NIS-Element BR 3.10 program (Nikon Instruments Inc., Melville, NY).

Data analysis was done using RStudio, version 1.2.5033 (Free Software Foundation, Inc., Boston, MA). Graphical methods were used to check normality and homogeneity of variances of all independent variables. The variables of spore concentration and spore length were log transformed before Analysis of Variance (ANOVA) to obtain a normal distribution and to homogenize the variance. Independent variables (mycelial growth rate, spore concentration, length, width and number of pseudosepta) were subjected to a linear regression model; if statistically significant data were then subjected to ANOVA (R package 'car'), and mean separations were performed using Tukey's Honestly Significant Difference test (Tukey HSD) (R package 'agricolae'). The main effects "fungal isolate" (12 levels), "culture media" (2 levels), and "light regime" (2 levels) were tested as independent variables for mycelial growth and spore concentration data. For intrinsic characteristics of spores such as length, width, and number of pseudosepta, the main effects "fungal isolate" (12 levels) and "culture media" (2 levels) were tested as independent variables. Results were expressed as mean ± standard deviation (SD). Boxplots of the data were obtained by additionally using the R package 'ggplot2'.

### 2.3 Pathogenicity

Of the 32 *C. cassiicola* isolates obtained in this study (Table 2.1), 12 isolates were selected for the pathogenicity profile (same isolates from morphology studies). Each *C. cassiicola* isolate was inoculated on two soybean cultivars: RA-606 (Novartis Seeds, Minneapolis, Minnesota) ($n$ = 260) and NK S56-B7X (NK Seeds, Downers Grove, IL) ($n$ = 260). Seeds of the cultivar RA-606 were provided by the U.S. National Plant Germplasm System (NPGS) and were described as resistant to target spot. The cultivar NK S56-B7X is known as susceptible to target spot (Irby et al., 2018). Plant inoculations were conducted in the greenhouse at the Plant Science Research Center (PSRC) located at Auburn University, Auburn, AL. Trials were performed in 655 $cm^3$ polypropylene deepots D40L (Stuewe & Sons, Inc., Tangent, OR) filled with a mix of potting soil Pro-Mix BX (Premier Tech Horticulture, Quakertown, PA) and sterile Kalmia loamy sand soil (75:25 v/v), supplemented with 2.1 g/L of 18-6-12 Osmocote fertilizer (ICL Specialty Fertilizers, Tel Aviv-Yafo, Israel). Two soybean seeds were sown per deepot and thinned to one plant per deepot seven days after planting (DAP). Plants were watered daily as needed. Supplemental light of 1000-watt halide bulbs producing 110,000 lumens was supplied to maintain a day length of 14 hours per day. Greenhouse temperatures ranged from 24°C to 35°C.

The inocula of *C. cassiicola* were prepared by growing each isolate on PDA medium, incubated at 28 ± 2°C under 12 h dark for 10 days. Each *C. cassiicola* colony was flooded with 10 mL of sterile distilled water per Petri dish and the colony surfaces were mechanically disturbed with a glass slide to release conidia. The conidia suspension was filtered through four layers of sterile gauze to remove most of the mycelia. The concentration of conidia in the suspension was determined under a microscope using a hemocytometer. The conidia suspension was adjusted to $10^4$ conidia/mL. A professional spray bottle (Zep Inc., Atlanta, GA) was used to apply the conidia suspension of each *C. cassiicola* isolate to the adaxial and abaxial leaves of 25 days-old soybean

plants until runoff. Distilled water was used as a negative control. After inoculation, plants inoculated with the same isolate were covered with a transparent plastic bag for 72 h. After the incubation period, deepots were randomized and kept in the greenhouse for the duration of the trial. Symptoms were scored 20 days after inoculation using a disease rating system (Onesirosan, 1973) as follows: 0, no lesions on leaves or stems (no symptom); 1, weakly virulent or hypersensitive response (few to many nonexpanding pinpoint lesions); 2, moderately virulent (many expanding lesions, some coalescing, but not resulting in blight); and 3, highly virulent (lesions spreading to form large areas of dead tissue resulting in blighting). Trials were arranged in a randomized complete block design (RCBD) with five replications and repeated four times.

Data analysis was done using RStudio, version 1.2.5033 (Free Software Foundation, Inc., Boston, MA). Graphical methods were used to check normality and homogeneity of variances of the independent variable. Disease rating data were found to be non-normal, and non-parametric analyses were conducted using Kruskal-Wallis rank sum tests for multiple comparisons (R package 'agricolae') and Fisher's Least Significant Difference (LSD) *post-hoc* tests. Results were expressed as mean ± standard deviation (SD). Boxplots of the data were obtained by additionally using the R package 'ggplot2'.

### 2.4 DNA extraction, PCR amplification and sequencing

Mycelial plugs of each isolate were placed over a cellophane membrane onto a fresh PDA plate (Cassago et al., 2002). After 10 days of growth, mycelium was harvested and DNA was extracted from each isolate using a ZR Fungal/Bacterial MiniPrep$^{TM}$ kit (Zymo Research, California, USA). Extracted DNA concentration was measured using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) and stored at -20˚C before use.

Primers encompassing five available cassiicolin-encoding genes (Déon et al., 2014) and four additional loci (Dixon et al., 2009) were used for polymerase chain reaction (PCR) amplification (Table 2.2). PCR was performed on 20 ng/µL of *C. cassiicola* genomic DNA. A 50-µL reaction mix was prepared for each isolate containing 2 µL of DNA template, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 21 µL of deionized water, and 25 µL of JumpStart Taq ReadyMix (Sigma-Aldrich, St. Louis, MO). DNA amplification was conducted in a MultiGene DNA thermal cycler (Labnet International, Edison, NJ) with a program consisting of initial denaturation at 94˚C for 4 min; followed by 35 cycles at 95˚C for 30 s, 52˚C for 30 s, 72˚C for 45 s; and a final cycle at 72˚C for 5 min for all cassiicolin-encoding genes (Déon et al., 2014). For the primer pairs ITS1/ITS4, GA4F/GA4R, and CAA5F/CAA5R, the program consisted of initial denaturation at 94˚C for 3 min; followed by 30 cycles at 94˚C for 30 s, 58˚C for 30 s, 72˚C for 45 s; and a final cycle at 72˚C for 5 min. For the primer pairs ACT-512-F/ ACT-783-R, the program was identical to the previous except for an annealing temperature of 61˚C (Dixon et al., 2009). Amplified products were examined by electrophoresis in 1% (w/v) agarose gel stained with GelRed™ 10,000X in DMSO (Biotium Inc., Hayward, CA) in 1x TBE buffer and visualized under UV light to detect the presence of each target gene. A 1 kb Plus DNA ladder (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA) was used as the marker and a reaction without a DNA template was used as the negative control. Purified PCR products were Sanger sequenced by Eurofins MWG Operon LLC (Louisville, KY) in both directions. Forward and reverse nucleotide sequences were edited and assembled using BioEdit Alignment Editor (Tom Hall, Ibis Biosciences) to generate a consensus sequence.

### 2.5 Phylogenetic analyses

The nucleotide sequence datasets for each target gene were aligned using CLUSTAL W (Thompson et al., 1994) in MEGA X (Kumar et al., 2018). Phylogenetic analysis of the cassiicolin-encoding genes was performed on 32 isolates from our collection, and sequences from 13 isolates were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/) for comparison (Table 2.3). The phylogenetic tree based on four loci was generated from the combined ribosomal DNA internal transcribed spacer (rDNA ITS), *ga4*, *caa5*, and *act1* of 32 isolates from our collection, and sequences of 11 isolates were retrieved from the GenBank database (Table 2.3). *Corynespora ligustri* (strain JYNZ) retrieved from GenBank database was selected as an outgroup for rooting purposes. Phylogenetic relationships were inferred using Maximum Likelihood (ML) method and General Time Reversible (GTR) model (Nei and Kumar, 2000) with uniform rates among sites in MEGA X (Kumar et al., 2018). For the heuristic search, initial trees were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and finally, the selection of the topology was made with superior log likelihood value. To determine the confidence for the internal nodes, the bootstrap method was used with 1,000 replicates.

## 3. Results

### 3.1 Isolates

Thirty-two isolates were sampled and identified as *C. cassiicola*, and the description of all isolates are given in Table 2.1. Of 32 *C. cassiicola* isolates from our collection, 12 isolates (37.5%) were obtained from cotton leaves from six locations (Fairhope, Macon County, Huntsville, and Brewton-AL, and Lincoln County-TN) and 20 isolates (62.5%) were obtained from soybean leaves from seven locations (Limestone County, Elmore County, Auburn, Eufaula, Brewton, Fairhope, and Macon County-AL). All cultures are maintained and stored at -80°C in the culture collection of the Department of Entomology and Plant Pathology at Auburn University (Auburn, Alabama, United States).

### 3.2 Morphology

Isolates of *C. cassiicola* exhibited a diverse set of colony shapes and colors depending on the culture media (PDA or V8 agar) or the light regime (12 h dark or 24 h dark) after 10 days at 28 ± 2°C (Figure 2.2). Colonies on PDA independently of the light regime were flat with moderate aerial mycelium (Figure 2.2A-B). On PDA under 12 h dark, the upper surface of the majority of colonies was light grey turning darker towards the center of the colony with a roundish shape; the reverse surface of the colonies was a creamy white, and dark grey to blackish in the center. Three isolates were slightly different under the same growing conditions. The upper surface of FHP01 and FHP22 isolates were whitish with margins pale brown to orange; and the reverse surface was light brown to orange turning dark brown towards the center. The upper surface of LIM14 isolate was whitish turning light grey towards the center, and olivaceous in the reverse surface (Figure 2.2A).

On PDA under 24 h dark, the upper surface of the majority of colonies was light grey turning darker towards the center of the colony with a roundish shape; the reverse surface of the colonies was creamy white, and dark grey to blackish in the center. Four isolates were slightly different under the same growing conditions. The upper surface of BRW03 isolate was pale grey to whitish, and pale white to medium grey in the reverse surface. The upper surface of FHP01 and FHP22 isolates were whitish with margins pale brown to orange; the reverse surface was light brown to orange turning dark brown towards the center. The shape of FHP01 colony was slightly irregular. While the upper surface of LIM14 isolate was light grey; the reverse surface olivaceous turning dark brown towards the center (Figure 2.2B).

Colonies on V8 agar independently of the light regime were thick and dense with abundant aerial mycelium. A wide variation of colony colors was observed for *C. cassiicola* isolates grown on V8 agar. For the majority of the isolates, the density of the culture medium made it difficult to differentiate between isolates based on the reverse surface (Figure 2.2C-D). On V8 agar under 12 h dark, the shape of the colony of BRW03 isolate was roundish with the upper surface white which was different from the other isolates. MAC01 and LIM14 isolates had a similar shape colony but with the upper surface light gray and the borders becoming medium gray towards the center. FHP01, FHP22, HSV01, HSV12, and LIM13 isolates exhibited the same shape colony but the upper surface was whitish to light gray. HSV01 and LIM13 isolates exhibited a sector formation with a different density of aerial mycelium. LIM02 was the only isolate with the upper surface greenish but with a slightly gray sector, but a roundish shape colony. ELM04, ELM06, and ELM07 isolates had an upper surface that was whitish in the borders becoming gray towards the center. However, an irregular colony shape was observed for ELM04 and ELM07 isolates, while a roundish shape was observed for ELM06 (Figure 2.2C).

On V8 agar under 24 h dark, isolates BRW03 and LIM14 had a roundish colony shape, with the upper surface whitish to light gray. MAC01 and FHP22 isolates exhibited the same colony shape, and the upper surface whitish with borders becoming medium gray towards the center. HSV01, HSV12, ELM04, and ELM07 isolates were very similar regarding the color of the upper surface, medium gray but slightly different regarding the colony shape; HSV01 with roundish shape, HSV12 with a slightly sector formation but still roundish, and isolates ELM04 and ELM07 with an irregular colony shape. Lastly, the isolate ELM06 exhibited a roundish colony shape, and upper surface light gray in the borders becoming medium gray towards the center (Figure 2.2D).

The average mycelial growth ranged from 6.59 ± 1.50 to 8.51 ± 0.97 mm/day for *C. cassiicola* isolates independently of culture media or light regime. HSV12 and LIM13 isolates had the highest mycelial growth rate, 8.51 ± 0.97, and 8.43 ± 1.00 mm/day, respectively; the lowest mycelial growth rate were observed for FHP01 and FHP22, 6.59 ± 1.50, and 7.11 ± 1.02 mm/day, respectively ($P < 0.0001$). Considering only the culture media, the mycelial growth of *C. cassiicola* grown on different culture media was significantly different ($P < 0.0001$). The mycelial growth rate on PDA was 7.53 ± 0.86 mm/day, while on V8 agar was 9.87 ± 1.07 mm/day. Under the light regime 12 h dark, mycelial growth of *C. cassiicola* was 7.67 ± 1.07 mm/day, while under 24 h dark was 9.07 ± 1.30 mm/day, also statistically significant ($P < 0.0001$). For mycelial growth no clear separation was observed among cotton and soybean *C. cassiicola* isolates. V8 media and 24 h dark supported the greatest mycelial growth of *C. cassiicola* isolates (Figure 2.3).

The spore concentration of *C. cassiicola* isolates independently of culture media or light regime ranged from $2.13 \times 10^4 \pm 1.87 \times 10^4$ to $4.78 \times 10^5 \pm 6.02 \times 10^5$ conidia/mL. LIM14 had the highest concentration of spores ($4.78 \times 10^5 \pm 6.02 \times 10^5$ conidia/mL), and LIM02 exhibited the least concentration of spores with $2.13 \times 10^4 \pm 1.87 \times 10^4$ conidia/mL ($P < 0.0001$). Considering

only the culture media, the spore concentration of *C. cassiicola* grown on V8 agar was 1.68 x $10^5$ ± 2.91 x $10^5$ conidia/mL, higher than on PDA which was 1.57 x $10^4$ ± 2.55 x $10^4$ conidia/mL ($P <$ 0.0001). Under the light regime 12 h dark, spore concentration of *C. cassiicola* was 1.19 x $10^5$ ± 2.42 x $10^5$ conidia/mL, while under 24 h dark was 6.31 x $10^4$ ± 2.06 x $10^5$ conidia/mL ($P \leq 0.05$). These results demonstrated that V8 media and 12 h dark are most likely to induce the highest amount of sporulation for *C. cassiicola* isolates (Figure 2.4).

For conidia morphology, length (µm), width (µm), and number of pseudosepta (unit) were analyzed (Figure 2.5). The average length of conidia ranged from 64.04 ± 33.13 µm for the FHP01 isolate to 36.75 ± 17.91 µm for the LIM02 isolate ($P < 0.0001$). When *C. cassiicola* was grown on PDA, conidia length was 44.19 ± 16.91 µm, while on V8 agar was 47.49 ± 22.79 µm ($P < 0.01$) (Figure 2.5A). Conidia length varies among *C. cassiicola* isolates, and isolates grown on V8 agar were significantly longer. The average width of conidia ranged from 8.03 ± 1.47 µm for the BRW03 isolate to 6.32 ± 0.90 µm for the ELM07 isolate ($P < 0.0001$). Width of *C. cassiicola* conidia was greater when grown on V8 agar (7.21 ± 1.34 µm), than on PDA (6.80 ± 1.27 µm) ($P < 0.0001$) (Figure 2.5B). Conidia width varies among *C. cassiicola* isolates, and isolates grown on V8 agar were significantly wider. The number of pseudosepta ranged from 3.37 ± 2.52 units for the FHP01 isolate to 1.49 ± 0.94 units for the FHP22 isolate ($P < 0.0001$). When *C. cassiicola* was grown on PDA, the number of pseudosepta was 1.88 ± 1.43 units, while on V8 agar was 2.42 ± 1.73 units ($P < 0.0001$). These results demonstrated that conidia morphology of *C. cassiicola* is highly affected, depending on the isolate or the culture media that they were grown before measurements. Conidia length and width, and number of pseudosepta were significantly greater when *C. cassiicola* isolates were grown in V8 media (Figure 2.5C).

### 3.3 Pathogenicity

Four pathogenicity tests were conducted on 12 isolates of *C. cassiicola* on two soybean cultivars: RA-606 and NK S56-B7X. Significant differences in symptoms were observed among the isolates (Kruskal-Wallis, df = 12, $\chi^2 = 255.83$, $P < 0.0001$) but not by cultivar (Kruskal-Wallis, df = 1, $\chi^2 = 3.56$, $P = 0.051$) (Figure 2.6). The results of the Kruskal-Wallis rank sum test based on pathogenicity demonstrated that isolate ELM06 was the most aggressive isolate, followed by ELM07, ELM04, LIM14, LIM02 ($P < 0.0001$) (Figure 2.6A). These five isolates were obtained from soybean plants, and the average disease rating ranged from 0.43 ± 0.59 to 1.40 ± 0.67. The average disease rating range for all isolates from cotton (BRW03, MAC01, FHP01, FHP22, HSV01, and HSV12) and one isolate from soybean (LIM13) was 0.05 ± 0.22 to 0.25 ± 0.49; statistically similar to the negative control (water) without any symptoms ($P < 0.0001$) (Figure 2.6A). Disease ratings did not statistically differ among the two soybean cultivars tested, RA-606 (0.41 ± 0.64) and NK S56-B7X (0.54 ± 0.74) ($P = 0.051$). Similar symptoms of target spot were visible on both soybean cultivars after inoculation with our *C. cassiicola* isolates (Figure 2.6B). Symptoms of *C. cassiicola* ELM06 isolate inoculated on RA-606 were observed in the lower leaves of the plants without advancing to the upper leaves (Figure 2.6C). Symptoms included small, circular and necrotic lesions with a yellow halo on the soybean leaves (Figure 2.6D), and small necrotic lesions on the stems (Figure 2.6E) were observed.

### 3.4 PCR amplification of cassiicolin-encoding genes

The primers covering the *Cas* sequences were able to amplify fragments around 750 bases of pairs on agarose gels. No amplification product was obtained from the controls, where water was used instead of DNA template. Of 32 *C. cassiicola* isolates from our collection analyzed in this study, we identified five isolates (15.6%) with the absence of cassiicolin-encoding genes

(Cas0), 12 isolates (37.5%) with Cas2, 5 isolates (15.6%) with Cas6, and 10 isolates (31.2%) with the combination of Cas2+6 (Table 2.1). Considering the total of 204 *C. cassiicola* isolates, Cas0 was represented by 3.9% of the cotton isolates, and 2.9% of the soybean isolates. Cas2 was represented by the majority of the *C. cassiicola* isolates which 36.8% were cotton isolates, and 10.8% were soybean isolates. Only 0.5% of the cotton isolates exhibited the combination Cas2+6, while soybean isolates exhibited 40.7% of the total. Cas6 was only represented by soybean isolates with 2.9% of the total. *Corynespora cassiicola* isolates from hydrangea were identified with Cas2 (1.5% to complete the total).

In this study, isolates of *C. cassiicola* sampled from cotton were identified with Cas0, Cas2, and the combination of Cas2+6. No *C. cassiicola* isolates sampled from cotton were identified with Cas6 alone. While isolates of *C. cassiicola* sampled from soybean were identified with Cas0, Cas2, Cas6, and the combination of Cas2+6 (Table 2.1). Soybean isolates were found to have higher diversity based on cassiicolin-encoding genes when compared with cotton isolates. For additional sequences extracted from the NCBI database, cassiicolin-encoding genes were indicated in Table 2.1 when available.

### 3.5 Phylogenetic analysis

DNA sequences of cassiicolin-encoding genes (Cas2 and Cas6) and four loci (rDNA ITS, *ga4*, *caa5*, and *act1*) were obtained for 32 isolates from our collection. Sequences were deposited in GenBank under accession numbers [MT820897 to MT820918] for Cas2, [MT820919 to MT820933] for Cas6, [MT820801 to MT820832] for *act1,* [MT820833 to MT820864] for *caa5,* [MT820865 to MT820896] for *ga4,* and [MT822647 to MT822678] for rDNA ITS (Table 2.3). The evolutionary analysis by ML based on cassiicolin-encoding genes involved 53 nucleotide sequences, and there were a total of 548 positions in the final dataset. *Corynespora cassiicola*

isolates from cotton and soybean obtained for this study grouped in two clades corresponding to Cas2 and Cas6, while reference isolates obtained from GenBank grouped in four clades according to their *Cas* gene (Cas1, Cas3, Cas4, and Cas5) (Figure 2.7). The Cas1 clade includes only *C. cassiicola* isolates from rubber tree, the reference pathogenic isolate CCP from Philippines, and the isolate CCAM3 from Cameroon (Déon et al., 2012a). The Cas2 clade includes isolates from our collection, in which nine isolates were sampled from cotton and 13 isolates were sampled from soybean. Besides isolates from our collection, Cas2 clade also comprises ATI17 isolate sampled from cotton in Brazil and four isolates sampled from soybean (ATI13, 493AA, 777AA, and RUD) (Déon et al., 2014). The Cas3 and Cas4 clades comprise only endophytic isolates of *C. cassiicola* sampled from rubber trees in Brazil (E70, E78, E79, and E139) (Déon et al., 2012b). The Cas5 clade also includes *C. cassiicola* isolates from rubber tree, CSB1 isolate from Malaysia and CSRI2 isolate from Sri Lanka (Déon et al., 2014). Finally, the Cas6 clade includes one cotton and 14 soybean isolates from our collection, in addition to ATI17, ATI13, and RUD reference isolates (Déon et al., 2014). Thirteen isolates were found to carry two cassiicolin-encoding genes (Cas2+6), being present on Cas2 and Cas6 clades simultaneously. Among those isolates, 10 were from our collection with nine isolates sampled from soybean and one isolate sampled from cotton. Five out of 32 isolates from our collection were excluded from the phylogenetic analysis because no cassiicolin-encoding gene was detected (Cas0).

The evolutionary analysis by ML based on four combined loci involved 44 nucleotide sequences, and there were a total of 1373 positions in the final dataset. The combined phylogenetic tree includes the JMP218 isolate from the phylogenetic lineage 1 (PL1) (Dixon et al., 2009), several isolates from the clade A (Déon et al., 2014), and several isolates from a third study (Sumabat et al., 2018) to determine placement of our isolates in the phylogenetic tree. The origin

and the cassiicolin-encoding gene were also indicated close to each isolate when known. Cotton and soybean isolates sampled for this study are contained in the PL1 and clade A based on the combined phylogenetic tree, which are included in five small clades that we consider as sister groups. These sister groups are defined by different ancestors in the base of the phylogenetic tree (Figure 2.8).

The major clade PL1 contains cotton and soybean isolates separated by different sister groups. The first sister group included two groups of isolates: (1) Isolates sampled from cotton in the southeastern U.S. (i.e. Alabama, Georgia, and Tennessee). These cotton isolates were not aggressive as the soybean isolates to the soybean cultivars tested in this study, which could suggest some host-specificity. They could be considered as genetically similar and were identified with Cas2 or Cas0. Interestingly two cotton isolates that weren't included in this subclade, were identified with the combination Cas2+6 (ATI17 and TEN01), indicating that cassiicolin-encoding genes could be part of the genetic evolution of these isolates; (2) Isolates sampled from soybean in Alabama that grouped with soybean isolates from Brazil (493AA, 777AA, and RUD) and one isolate from Georgia (SMR3), besides the one cotton isolate from Brazil (ATI17). The majority of the isolates here were identified with Cas2+6, except for one isolate with Cas6 (LIM14) and three isolates with Cas2 (493AA, 777AA, and ELM06). This includes the isolate ELM06 that had the highest disease rating average in this study (Figure 2.8).

Four different sister groups were formed with others soybean isolates and one cotton isolate. Two soybean pathogenic isolates from Alabama (ELM04 and ELM07) and one isolate from Brazil (ATI13) were grouped in the same sister group. Another group contained four isolates from Alabama (AUR03, FHP61, AUR02, and AUM01) with the majority identified with Cas6. Four soybean isolates from Alabama (FHP63, LIM02, LIM13, and ELM19) and one from

Tennessee (STs2) grouped together with the majority identified with Cas2. This group contained one *C. cassiicola* isolate (LIM13) that was sampled from soybean, however it was not aggressive as the other soybean isolates in the pathogenicity assays. Lastly, a single cotton isolate from Tennessee (TEN01) did not group with the other cotton isolates (Figure 2.8).

## 4. Discussion

Our analysis revealed *C. cassiicola* isolates within different sister groups in the phylogenetic tree but inside PL1 clade (Dixon et al., 2009) that corresponds to clade A (Déon et al., 2014). Both phylogenetic analyses conducted in this study based on cassiicolin-encoding genes and combined loci data indicated higher genetic diversity of *C. cassiicola* isolates collected in Alabama from soybean when compared with isolates collected from cotton. One distinct and well-supported clade was formed with those *C. cassiicola* isolates from cotton in the combined loci tree, except for the cotton isolate from Brazil (ATI17) and the cotton isolate from Tennessee (TEN01). The lack of diversity of populations of *C. cassiicola* from cotton sampled in the southeastern U.S. was previously reported (Shrestha et al., 2017; Sumabat et al., 2018). However, additional sampling of *C. cassiicola* isolates from cotton may reveal higher diversity, especially isolates with the combination of cassiicolin-encoding genes Cas2+6 that in our phylogenetic analysis fell in different subclades from the majority of cotton isolates suggesting a possible genetic diversity. *Corynespora cassiicola* isolates from soybean were found to be more genetically diverse in another study (Sumabat et al., 2018); however, unlike those authors, we couldn't correlate geographic origin clustering for our isolates. Such diversity should be considered in breeding programs when looking at target spot resistance to achieve effective and sustainable disease management strategies (Qi et al., 2011; Silva et al., 2003).

Of the five cassiicolin-encoding genes isoforms, two (Cas2 and Cas6) were amplified in our *C. cassiicola* isolates. The tree based on the *Cas* gene sequences with isolates from our collection and reference isolates from GenBank indicated the formation of six distinct clades; each clade corresponding to a different cassiicolin-encoding gene (Cas1 to Cas6). Cas1, Cas3, Cas4, and Cas5 clades placed only isolates from rubber tree, while *C. cassiicola* isolates from cotton and soybean were distributed in Cas2 and Cas6 clades. Ten *C. cassiicola* isolates from our collection were found to have a combination of different *Cas* genes (Cas2+6). These isolates were included in three different clades of the combined loci tree. The co-existence of different *Cas* genes in the same isolate was previously reported (Déon et al., 2014) where one isolate from cotton and two isolates from soybean were identified with that combination. In addition, five soybean isolates from our collection were identified with Cas6 gene alone that Déon et al. (2014) didn't identify in their studies after assessing 70 isolates from most of the *C. cassiicola* host plants. Most of the incongruences from the combined loci tree were found for those isolates identified with Cas0 that did not cluster together similar to the previous study (Déon et al., 2014) where Cas0 isolates were found in almost all clades. Disregarding those incongruences, few exceptions of the same *Cas* genes were found in different subclades of the combined tree demonstrating the contribution of these genes to the overall genetic structuration of *C. cassiicola* isolates in the phylogenetic tree. An important role in fungal biology and evolution could be linked to cassiicolin-encoding genes (Déon et al., 2014).

Pathogenicity assays showed that *C. cassiicola* isolates were more likely to cause disease symptoms on the same host where they were first isolated. Soybean isolates were more aggressive when inoculated on soybean plants, whereas *C. cassiicola* isolates from cotton were statistically similar to the negative control. These results corroborate with the idea of host specialization

reported by several authors (Dixon et al., 2009; Silva et al., 1998; Sumabat et al., 2018). However, further pathogenicity tests on different soybean and cotton cultivars are required to comprehensively evaluate the host specificity of *C. cassiicola* isolates obtained from cotton and soybean, especially after the inconsistency found for the soybean germplasm RA-606. According to the U.S. NPGS, the soybean germplasm RA-606 was described as resistant to target spot but we found compatible interactions after inoculation with *C. cassiicola* isolates from our collection. Three of the most aggressive isolates from the pathogenicity assays (ELM06, ELM07, and ELM04) were found to have different *Cas* genes (Cas2, Cas6, and Cas2+6 respectively) different from the pathosystem *C. cassiicola* on rubber tree where Cas1 was the most important *Cas* gene causing pathogenicity to the rubber tree (Déon et al., 2014; Lopez et al., 2018). Those authors suggested that it is possible the presence of effectors still not known or other compounds such as secondary metabolites may play an important role in pathogenicity. Cassiicolin, a necrotrophic toxin from the isolate CCP (Déon et al., 2014), is the only *C. cassiicola* effector characterized so far besides some possible candidate effectors (Lopez et al., 2018; Shuib et al., 2015).

The morphological characterization for the isolates analyzed in this study agrees with the descriptions of the fungus *C. cassiicola* (Ellis and Holliday, 1971). A high variation of morphological characteristics has been described among different isolates but also within a single isolate (Nghia et al., 2008; Qi et al., 2011; Silva et al., 1998). In our study, we did not find any distinguishable morphological characteristics among the 12 *C. cassiicola* isolates investigated, corroborating with results from a previous study (Qi et al., 2011). Identification based on morphology can be problematic, and other analyses, e.g., pathogenicity assays and phylogenetic analysis are needed. There was no correlation between the sister groups in the combined loci tree and pathogenicity assays or the morphological characters, besides the fact that 11 out of 12 *C.*

*cassiicola* isolates sampled from cotton clustered together in a unique subclade, and six isolates exhibited low disease ratings in the pathogenicity assays.

Our study gives insight into the complexity of the fungal pathogen *C. cassiicola*. Fungal pathogens continue to evolve in the environment, and new lineages of a certain species could arise to build a new disease model that we are not acquainted with. A better understanding of *C. cassiicola* populations can help with breeding programs by testing genetically distinct populations leading to the selection of resistant genotypes, which is directly linked to effective disease management.

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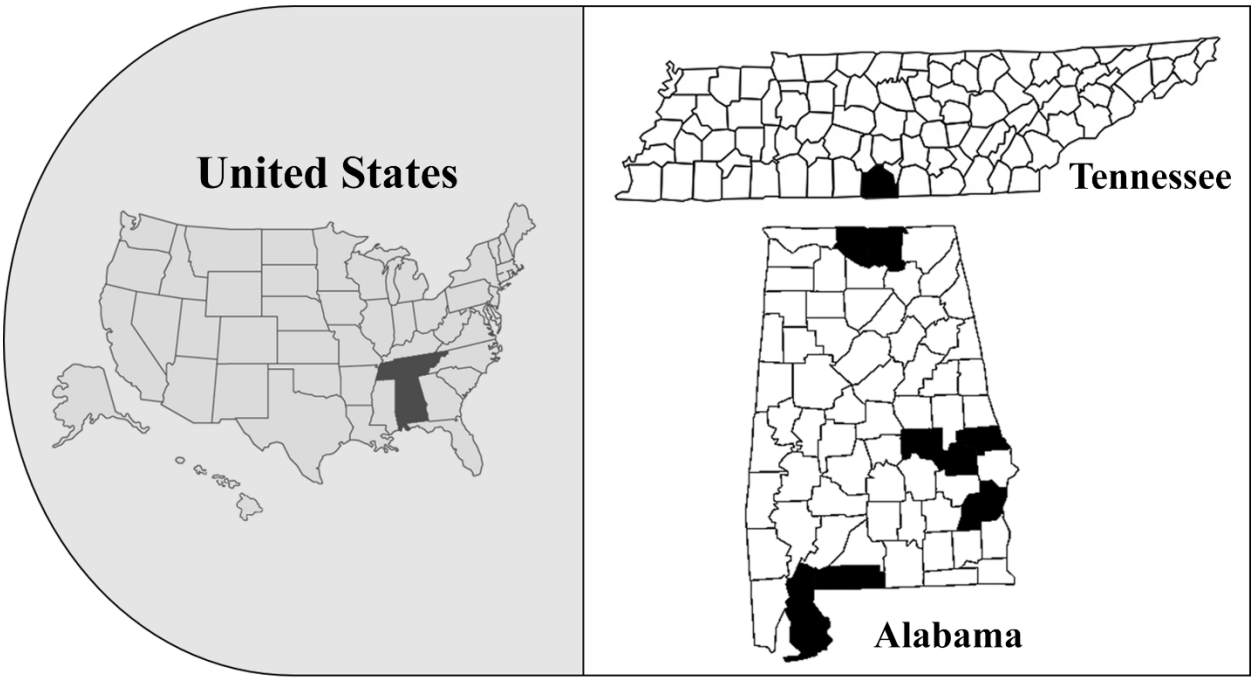


**Figure 2.1 Distribution map of the *Corynespora cassiicola* isolates sampled for this study**. Isolates were sampled from cotton and soybean plants in eight counties (colored in black) in Alabama and one county in Tennessee, U.S.

**Table 2.1** Description of *Corynespora cassiicola* isolates used in this study.

| No. | Isolate identification | Geographical origin | Host plant | Sampling date | Toxin class |
|---|---|---|---|---|---|
| 1 | FHP01 | Fairhope, AL | *G. hirsutum* | 02/21/2017 | Cas0 |
| 2 | FHP22 | Fairhope, AL | *G. hirsutum* | 02/21/2017 | Cas2 |
| 3 | BRW03 | Brewton, AL | *G. hirsutum* | 08/01/2017 | Cas2 |
| 4 | LIM02 | Limestone County, AL | *G. max* | 08/11/2017 | Cas2 |
| 5 | LIM13 | Limestone County, AL | *G. max* | 08/11/2017 | Cas0 |
| 6 | LIM14 | Limestone County, AL | *G. max* | 08/11/2017 | Cas6 |
| 7 | ELM04 | Elmore County, AL | *G. max* | 09/07/2017 | Cas2+6 |
| 8 | ELM06 | Elmore County, AL | *G. max* | 09/07/2017 | Cas2 |
| 9 | ELM07 | Elmore County, AL | *G. max* | 09/07/2017 | Cas6 |
| 10 | MAC01 | Macon County, AL | *G. hirsutum* | 09/07/2017 | Cas2 |
| 11 | HSV01 | Huntsville, AL | *G. hirsutum* | 09/18/2017 | Cas2 |
| 12 | HSV12 | Huntsville, AL | *G. hirsutum* | 09/18/2017 | Cas0 |
| 13 | AUB25 | Auburn, AL | *G. max* | 10/25/2017 | Cas2+6 |
| 14 | AUM01 | Auburn, AL | *G. max* | 11/01/2017 | Cas6 |
| 15 | AUM05 | Auburn, AL | *G. max* | 11/01/2017 | Cas2+6 |
| 16 | AUR02 | Auburn, AL | *G. max* | 11/01/2017 | Cas6 |
| 17 | AUR03 | Auburn, AL | *G. max* | 11/01/2017 | Cas0 |
| 18 | AUR08 | Auburn, AL | *G. max* | 11/01/2017 | Cas2+6 |
| 19 | EUF15 | Eufaula, AL | *G. max* | 09/05/2018 | Cas2+6 |
| 20 | TEN01 | Lincoln County, TN | *G. hirsutum* | 09/07/2018 | Cas2+6 |
| 21 | BRW21 | Brewton, AL | *G. max* | 09/13/2018 | Cas2+6 |
| 22 | BRW35 | Brewton, AL | *G. hirsutum* | 09/13/2018 | Cas2 |
| 23 | FHP50 | Fairhope, AL | *G. max* | 10/19/2018 | Cas2+6 |
| 24 | FHP61 | Fairhope, AL | *G. max* | 10/19/2018 | Cas6 |
| 25 | FHP63 | Fairhope, AL | *G. max* | 10/19/2018 | Cas2 |
| 26 | MAC06 | Macon County, AL | *G. max* | 07/18/2019 | Cas2+6 |
| 27 | BRW47 | Brewton, AL | *G. hirsutum* | 07/24/2019 | Cas0 |
| 28 | BRW50 | Brewton, AL | *G. hirsutum* | 07/24/2019 | Cas2 |
| 29 | ELM19 | Elmore County, AL | *G. max* | 08/06/2019 | Cas2 |
| 30 | HSV21 | Huntsville, AL | *G. hirsutum* | 09/10/2019 | Cas2 |
| 31 | AUR10 | Auburn, AL | *G. hirsutum* | 09/12/2019 | Cas2 |
| 32 | AUR15 | Auburn, AL | *G. max* | 09/12/2019 | Cas2+6 |
| 33 | 493AA[x] | Brazil | *G. max* | - | Cas2 |
| 34 | 777AA[x] | Brazil | *G. max* | - | Cas2 |
| 35 | ATI13[x] | Brazil | *G. max* | - | Cas2+6 |
| 36 | ATI17[x] | Brazil | *G. hirsutum* | - | Cas2+6 |
| 37 | CAL-2[x] | Baldwin County, AL | *G. hirsutum* | - | - |
| 38 | CCAM3[x] | Cameroon | *Hevea brasiliensis* | - | Cas1 |
| 39 | CCP[x] | Philippines | *Hevea brasiliensis* | - | Cas1 |
| 40 | CSB1[x] | Malaysia | *Hevea brasiliensis* | - | Cas5 |

| | | | | | |
|---|---|---|---|---|---|
| 41 | CSRI2[x] | Sri Lanka | *Hevea brasiliensis* | - | Cas5 |
| 42 | CT1[x] | Tift County, GA | *G. hirsutum* | - | - |
| 43 | CTs1[x] | Madison County, TN | *G. hirsutum* | - | - |
| 44 | E139[x] | Brazil | *Hevea brasiliensis* | - | Cas4 |
| 45 | E70[x] | Brazil | *Hevea brasiliensis* | - | Cas3 |
| 46 | E78[x] | Brazil | *Hevea brasiliensis* | - | Cas3 |
| 47 | E79[x] | Brazil | *Hevea brasiliensis* | - | Cas4 |
| 48 | JMP218[x] | Brazil | *G. max* | - | - |
| 49 | RUD[x] | Brazil | *G. max* | - | Cas2+6 |
| 50 | SMR3[x] | Marion County, GA | *G. max* | - | - |
| 51 | STs2[x] | Madison County, TN | *G. max* | - | - |

[x] Reference sequences obtained from GenBank; accession numbers can be found in Table 2.3.

**Table 2.2** Description of the primers used for polymerase chain reaction (PCR) amplification.

| Target gene | Primers names | Sequences (5'…3') | Reference |
|---|---|---|---|
| Cas1 | CasF18 | CCCAAGATACATGTTTTGAATGT | Déon et al., |
| | CasR27 | CCACACAAAGCAAGATACAGAATGAGC | 2012a |
| Cas2 | CasF17 | GGATTTGCCTGAGATCCTA | Déon et al., 2014 |
| | CasR24 | CAAACAATGCTAACCAAACAAAC | |
| Cas3/Cas4 | CasF20 | GTCGGCTAACTTGGGAAAAACTCT | Déon et al., |
| | CasR28 | GCAGGAAGCAAAACACAGAACAAG | 2012b |
| Cas5 | CasF19 | CGGGGAGGTATCAGGTGTGAGATA | Déon et al., 2014 |
| | CasR26 | CAGAACAAGCCAAAAGAGAACTAC | |
| Cas6 | CasF16 | GCTTGATTTGCCTGTGAGATACT | Déon et al., 2014 |
| | CasR25 | AAAACGATGCTAAACAAAAGGA | |
| rDNA ITS | ITS1 | TCCGTAGGTGAACCTGCGG | White et al., |
| | ITS4 | TCCTCCGCTTATTGATATGC | 1990 |
| *gaa4* | GA4F | CCTGCTCCGACTTTGTTGAG | Dixon et al., |
| | GA4R | GTCTGGGAGCAGCAAAGACT | 2009 |
| *caa5* | CAA5F | GTCCACAAGTGGAACCTCGT | Dixon et al., |
| | CAA5R | CCTCGTCTGCCAGTTCTTCT | 2009 |
| *act1* | ACT-512-F | ATGTGCAAGGCCGGTTTCGC | Carbone and |
| | ACT-783-R | TACGAGTCCTTCTGGCCCAT | Kohn, 1999 |

**Table 2.3** GenBank accession numbers of sequences analyzed in this study.

| No. | Isolate identification | GenBank accession number | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ***act1*** | ***caa5*** | ***ga4*** | **rDNA ITS** | **Cas1** | **Cas2** | **Cas3** | **Cas4** | **Cas5** | **Cas6** |
| **1** | FHP01 | MT820825 | MT820851 | MT820883 | MT822665 | - | - | - | - | - | - |
| **2** | FHP22 | MT820826 | MT820852 | MT820884 | MT822666 | - | MT820910 | - | - | - | - |
| **3** | BRW03 | MT820823 | MT820841 | MT820873 | MT822655 | - | MT820902 | - | - | - | - |
| **4** | LIM02 | MT820829 | MT820859 | MT820891 | MT822673 | - | MT820915 | - | - | - | - |
| **5** | LIM13 | MT820830 | MT820860 | MT820892 | MT822674 | - | - | - | - | - | - |
| **6** | LIM14 | MT820831 | MT820861 | MT820893 | MT822675 | - | - | - | - | - | MT820931 |
| **7** | ELM04 | MT820821 | MT820846 | MT820878 | MT822660 | - | MT820906 | - | - | - | MT820926 |
| **8** | ELM06 | MT820824 | MT820847 | MT820879 | MT822661 | - | MT820907 | - | - | - | - |
| **9** | ELM07 | MT820822 | MT820848 | MT820880 | MT822662 | - | - | - | - | - | MT820927 |
| **10** | MAC01 | MT820832 | MT820862 | MT820894 | MT822676 | - | MT820916 | - | - | - | - |
| **11** | HSV01 | MT820827 | MT820856 | MT820888 | MT822670 | - | MT820913 | - | - | - | - |
| **12** | HSV12 | MT820828 | MT820857 | MT820889 | MT822671 | - | - | - | - | - | - |
| **13** | AUB25 | MT820801 | MT820833 | MT820865 | MT822647 | - | MT820897 | - | - | - | MT820919 |
| **14** | AUM01 | MT820802 | MT820834 | MT820866 | MT822648 | - | - | - | - | - | MT820920 |
| **15** | AUM05 | MT820803 | MT820835 | MT820867 | MT822649 | - | MT820898 | - | - | - | MT820921 |
| **16** | AUR02 | MT820804 | MT820836 | MT820868 | MT822650 | - | - | - | - | - | MT820922 |
| **17** | AUR03 | MT820805 | MT820837 | MT820869 | MT822651 | - | - | - | - | - | - |
| **18** | AUR08 | MT820806 | MT820838 | MT820870 | MT822652 | - | MT820899 | - | - | - | MT820923 |
| **19** | EUF15 | MT820814 | MT820850 | MT820882 | MT822664 | - | MT820909 | - | - | - | MT820928 |
| **20** | TEN01 | MT820820 | MT820864 | MT820896 | MT822678 | - | MT820918 | - | - | - | MT820933 |
| **21** | BRW21 | MT820809 | MT820842 | MT820874 | MT822656 | - | MT820903 | - | - | - | MT820925 |
| **22** | BRW35 | MT820810 | MT820843 | MT820875 | MT822657 | - | MT820904 | - | - | - | - |
| **23** | FHP50 | MT820815 | MT820853 | MT820885 | MT822667 | - | MT820911 | - | - | - | MT820929 |
| **24** | FHP61 | MT820816 | MT820854 | MT820886 | MT822668 | - | - | - | - | - | MT820930 |
| **25** | FHP63 | MT820817 | MT820855 | MT820887 | MT822669 | - | MT820912 | - | - | - | - |
| **26** | MAC06 | MT820819 | MT820863 | MT820895 | MT822677 | - | MT820917 | - | - | - | MT820932 |
| **27** | BRW47 | MT820811 | MT820844 | MT820876 | MT822658 | - | - | - | - | - | - |
| **28** | BRW50 | MT820812 | MT820845 | MT820877 | MT822659 | - | MT820905 | - | - | - | - |
| **29** | ELM19 | MT820813 | MT820849 | MT820881 | MT822663 | - | MT820908 | - | - | - | - |
| **30** | HSV21 | MT820818 | MT820858 | MT820890 | MT822672 | - | MT820914 | - | - | - | - |
| **31** | AUR10 | MT820807 | MT820839 | MT820871 | MT822653 | - | MT820900 | - | - | - | - |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **32** | AUR15 | MT820808 | MT820840 | MT820872 | MT822654 | - | MT820901 | - | - | - | MT820924 |
| **33** | 493AA | KF810701 | KF810771 | KF810841 | KF810907 | - | JF915156 | - | - | - | - |
| **34** | 777AA | KF810699 | KF810769 | KF810839 | KF810905 | - | JF915157 | - | - | - | - |
| **35** | ATI13 | KF810698 | KF810768 | KF810838 | KF810904 | - | JF915168 | - | - | - | JF915181 |
| **36** | ATI17 | KF810702 | KF810772 | KF810842 | KF810908 | - | JF915159 | - | - | - | JF915182 |
| **37** | CAL-2 | MF320357 | MF320401 | MF320454 | MF320507 | - | - | - | - | - | - |
| **38** | CCAM3 | - | - | - | - | JF915150 | - | - | - | - | - |
| **39** | CCP | - | - | - | - | JF915148 | - | - | - | - | - |
| **40** | CSB1 | - | - | - | - | - | - | - | - | JF915175 | - |
| **41** | CSRI2 | - | - | - | - | - | - | - | - | JF915180 | - |
| **42** | CT1 | MF320364 | MF320408 | MF320461 | MF320514 | - | - | - | - | - | - |
| **43** | CTs1 | MF320366 | MF320410 | MF320463 | MF320516 | - | - | - | - | - | - |
| **44** | E139 | - | - | - | - | - | - | - | JF915172 | - | - |
| **45** | E70 | - | - | - | - | - | - | JF915169 | - | - | - |
| **46** | E78 | - | - | - | - | - | - | JF915170 | - | - | - |
| **47** | E79 | - | - | - | - | - | - | - | JF915171 | - | - |
| **48** | JMP218 | FJ853020 | FJ852877 | FJ852734 | FJ852591 | - | - | - | - | - | - |
| **49** | RUD | KF810700 | KF810770 | KF810840 | KF810906 | - | JF915164 | - | - | - | JF915183 |
| **50** | SMR3 | MF320387 | MF320434 | MF320487 | MF320540 | - | - | - | - | - | - |
| **51** | STs2 | MF320394 | MF320442 | MF320495 | MF320548 | - | - | - | - | - | - |
| **52** | JYNZ | MF428254 | MF427972 | MF428113 | MF428395 | - | - | - | - | - | - |

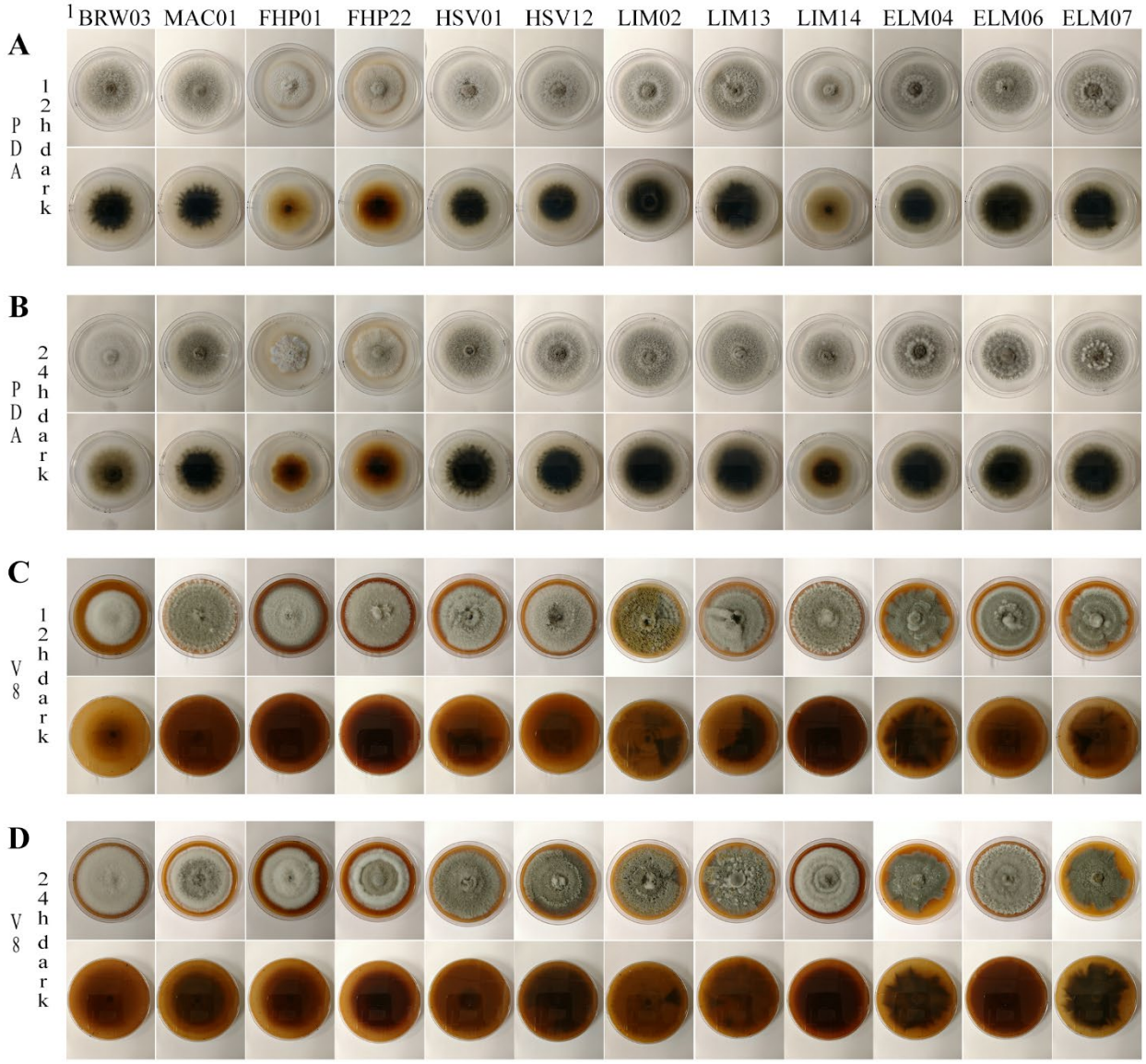


**Figure 2.2 Colony morphology of *Corynespora cassiicola* isolates.** Upper and reverse surface of colonies grown on PDA under 12 h of dark (A) and 24 h of dark (B) at 28 ± 2°C with 10 days of incubation. Upper and reverse surface of colonies grown on V8 agar under 12 h of dark (C) and 24 h of dark (D) at 28 ± 2°C with 10 days of incubation.
[1]*Corynespora cassiicola* isolates sampled from cotton (BRW03, MAC01, FHP01, FHP22, HSV01, and HSV12) and soybean (LIM02, LIM13, LIM14, ELM04, ELM06, and ELM07).

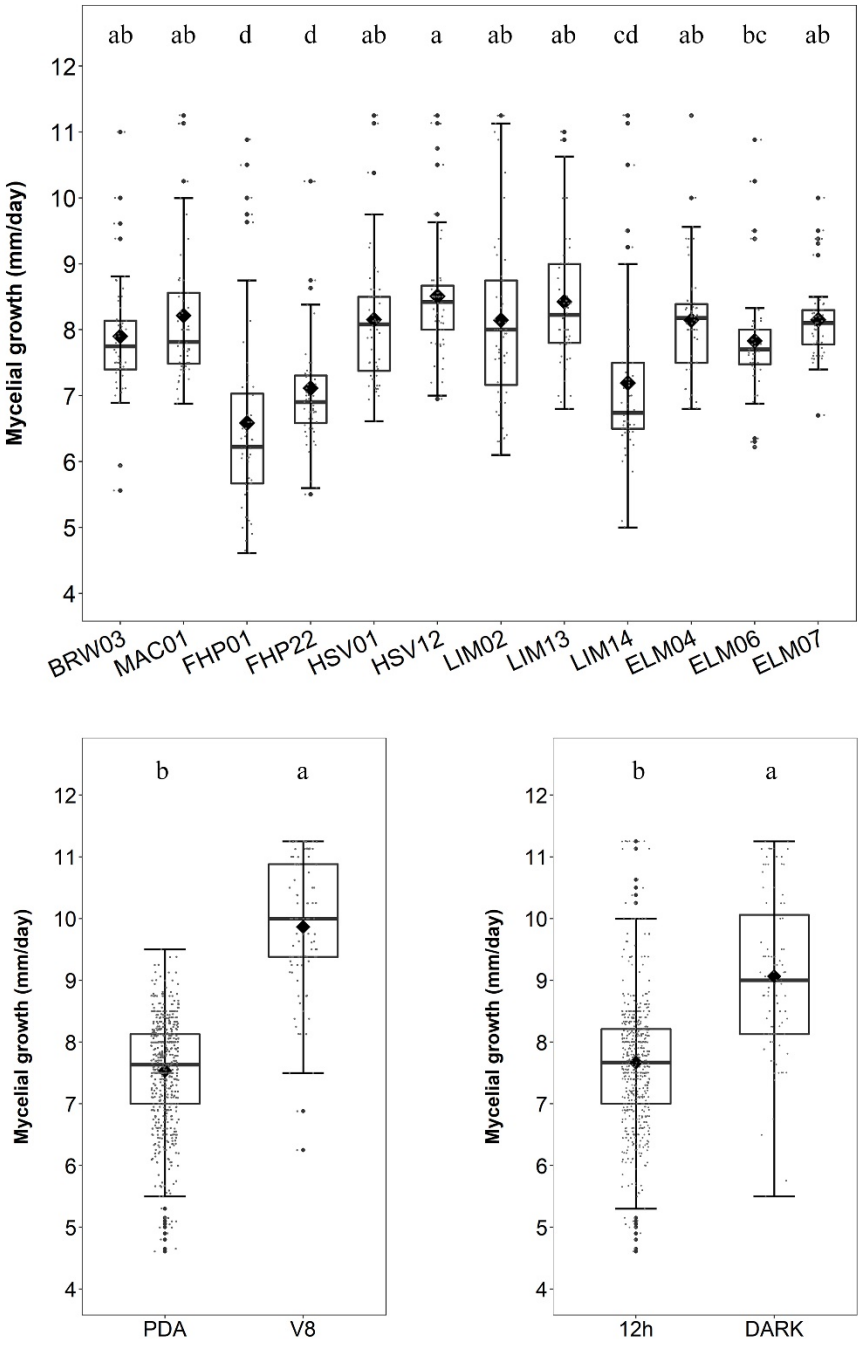


**Figure 2.3 Mycelial growth (mm/day) of *Corynespora cassiicola*.** Boxplots represents data of mycelial growth for each *C. cassiicola* isolate (upper panel), for mycelial growth of isolates grown in PDA and V8 agar (bottom left panel), and for mycelial growth of isolates grown in 12 h light/dark and only dark (bottom right panel). Black diamonds (♦) inside boxes show mean values, horizontal lines inside boxes mark the median value. Jitter (dots) inside each boxplot indicate the individual samples (*n*). Different letters above boxplots indicate statistically significant differences as defined by ANOVA and mean separations by Tukey's HSD test ($\alpha = 0.05$).

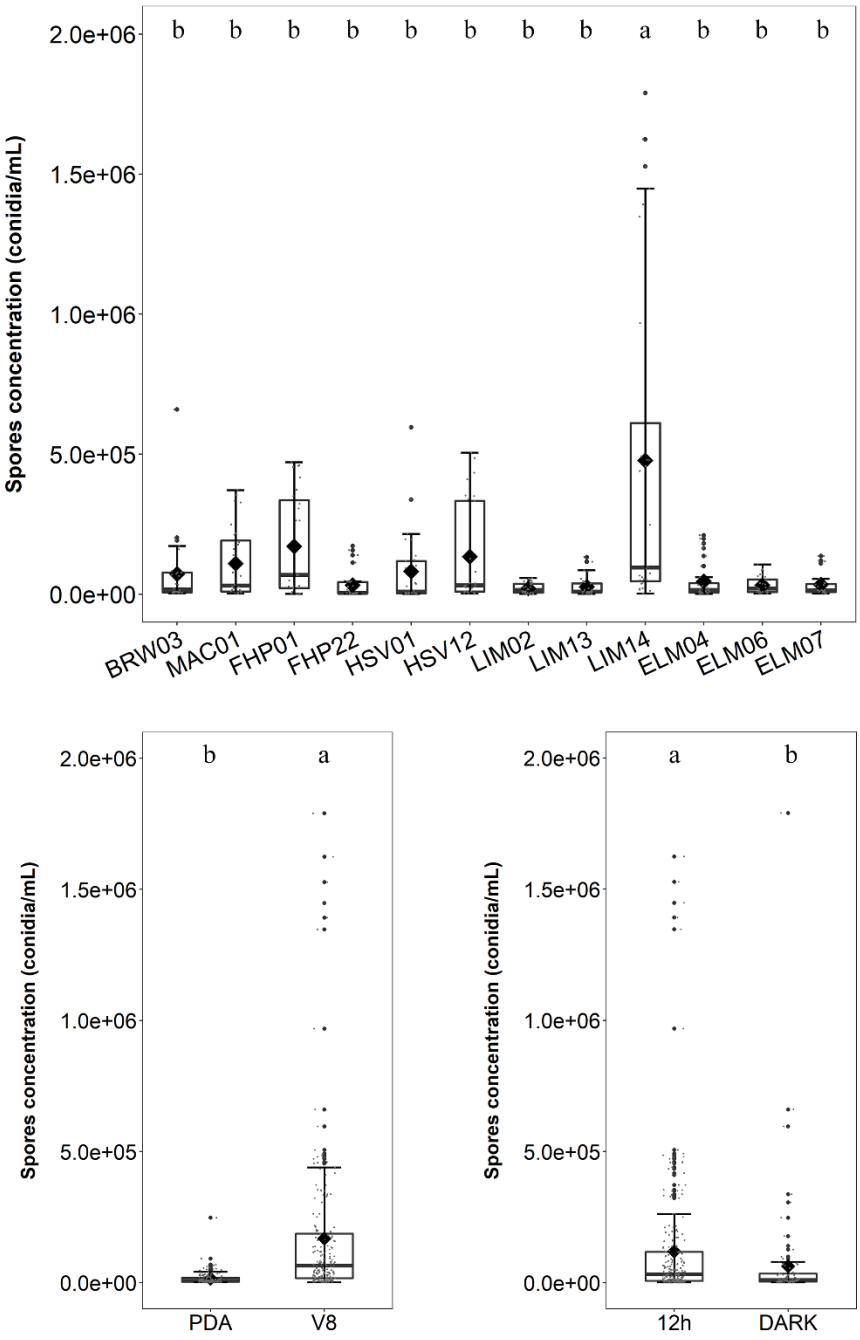


**Figure 2.4 Spore concentration (conidia/mL) of *Corynespora cassiicola*.** Boxplots represents data of spore concentration for each *C. cassiicola* isolate (upper panel), for spore concentration of isolates grown in PDA and V8 agar (bottom left panel), and for spore concentration of isolates grown in 12 h light/dark and only dark (bottom right panel). Black diamonds (♦) inside boxes show mean values, horizontal lines inside boxes mark the median value. Jitter (dots) inside each boxplot indicate the individual samples (*n*). Different letters above boxplots indicate statistically significant differences as defined by ANOVA and mean separations by Tukey's HSD test ($\alpha$ = 0.05).

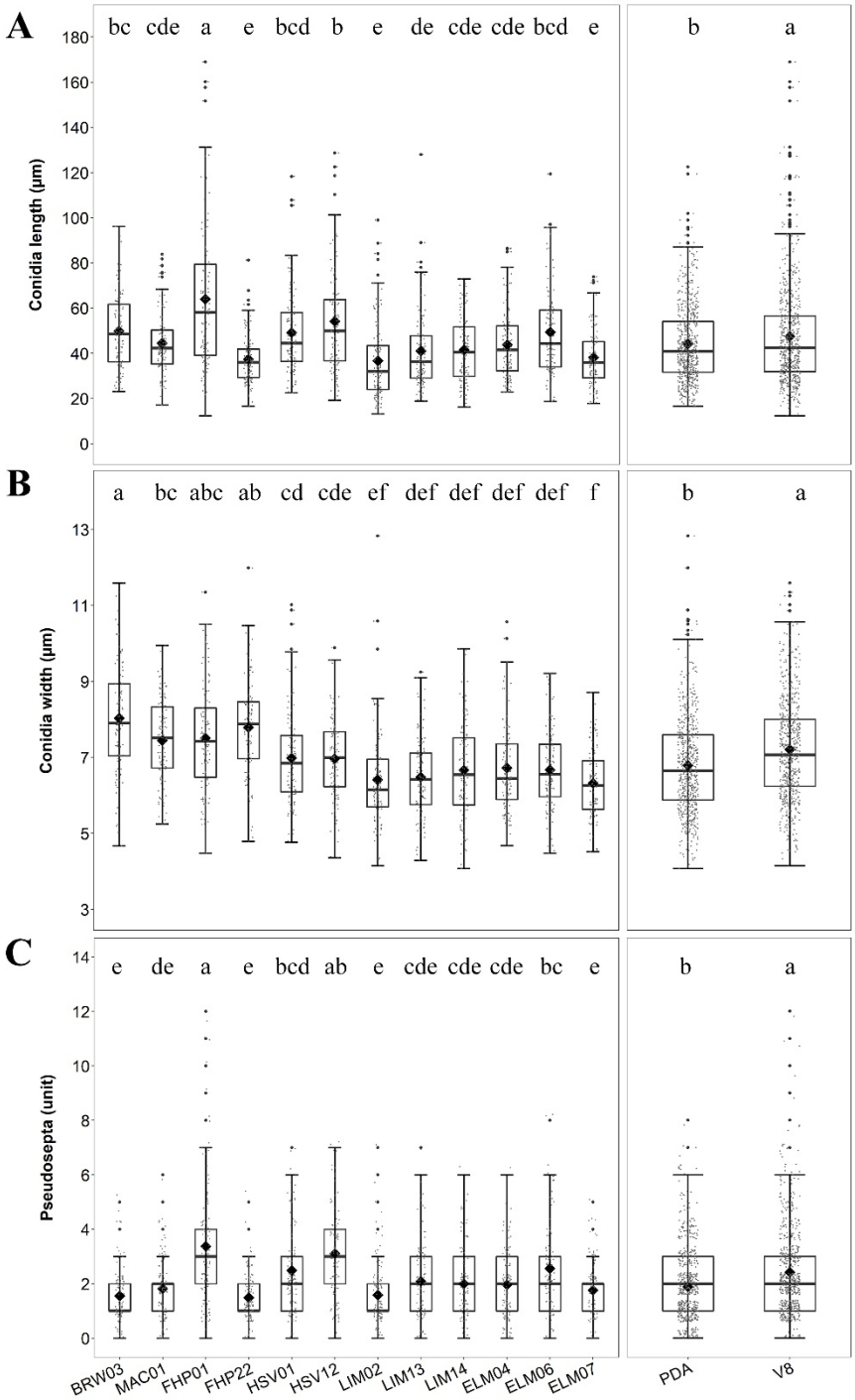


**Figure 2.5 Morphological measurements of *Corynespora cassiicola*.** Boxplots represent conidia length (µm) of each isolate, and conidia length (µm) for PDA and V8 agar (A). Conidia width (µm) of each isolate, and conidia width (µm) for PDA and V8 agar (B). Number of pseudosepta (unit) for each isolate, and number of pseudosepta (unit) for PDA and V8 agar (C). Black diamonds (♦) inside boxes show mean values, horizontal lines inside boxes mark the median value. Jitter (dots) inside each boxplot indicate the individual samples (*n*). Different letters above boxplots indicate statistically significant differences as defined by ANOVA and mean separations by Tukey's HSD test ($\alpha = 0.05$).

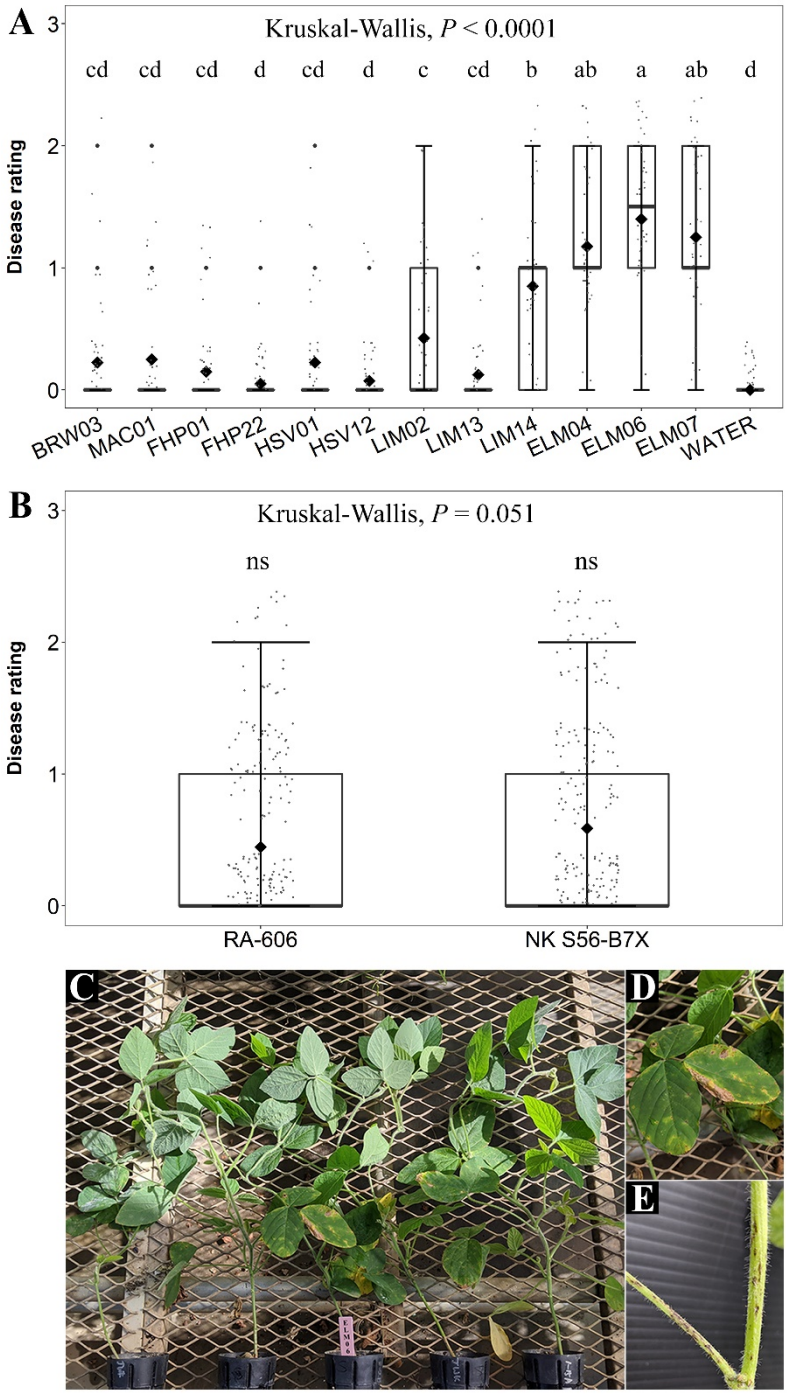


**Figure 2.6 Pathogenicity of *Corynespora cassiicola* isolates on soybean.** Disease rating after inoculation with 12 *C. cassiicola* isolates and the negative control (water) (A) on two soybean cultivars (RA-606 and NK S56-B7X) (B). The letters above boxplots represent significance groups as defined by Kruskal-Wallis test (LSD, α = 0.05). No significant differences are represented by ns. Black diamonds (♦) inside boxes show mean values, horizontal lines inside boxes mark the median value. Jitter (dots) inside each boxplot indicate the individual samples (*n*). Symptoms after inoculation with ELM06 isolate on RA-606 cultivar (C) showing details on the soybean leaves (D) and stems (E).

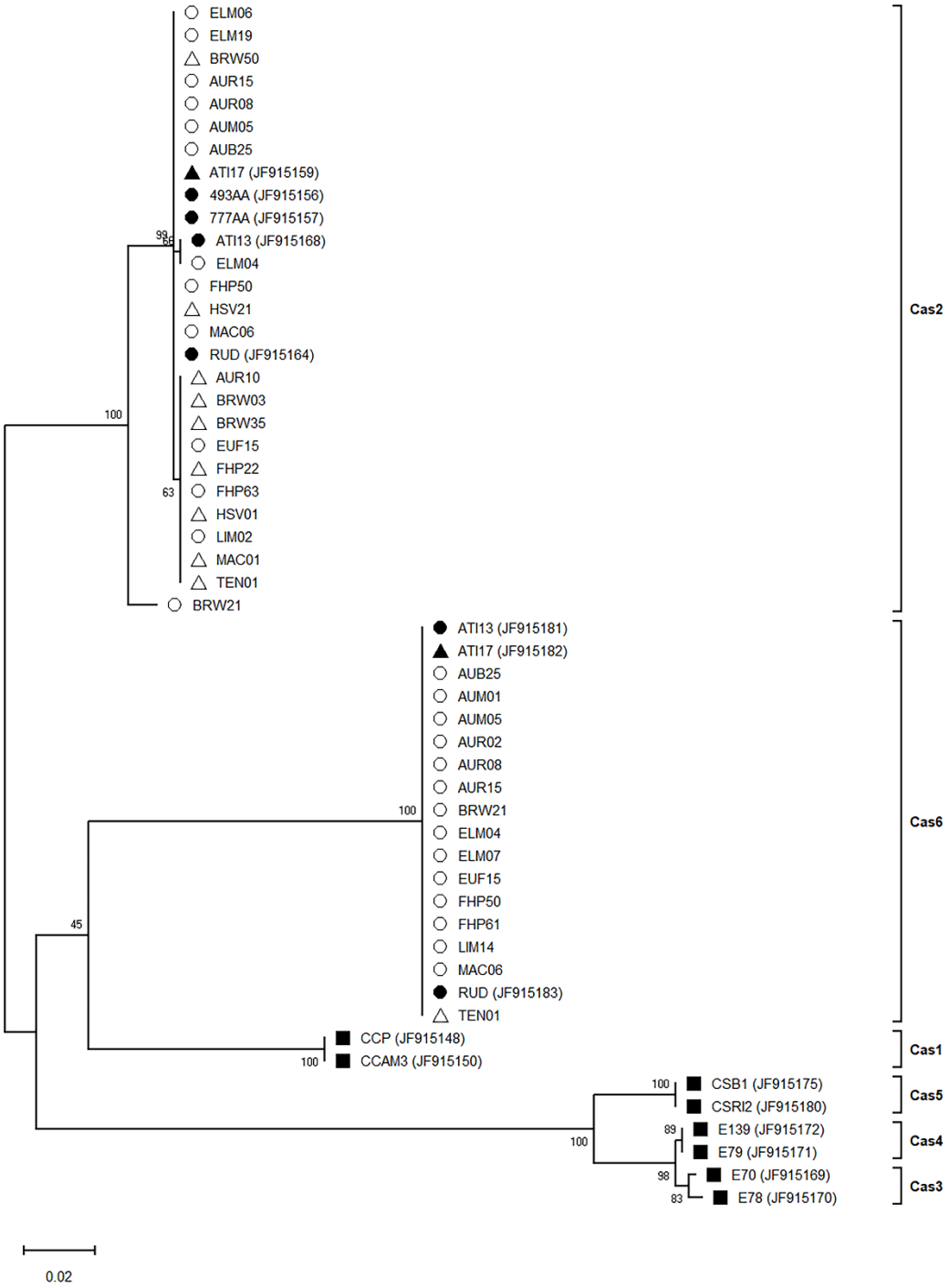


**Figure 2.7 Phylogenetic analysis of the cassiicolin-encoding genes from *Corynespora cassiicola* isolates.** The circle symbol represents isolates from soybean, triangle symbol represents isolates from cotton, and square symbol represents isolates from rubber tree; if colored in black, sequences were retrieved from GenBank database. The tree was inferred by using the Maximum Likelihood method and General Time Reversible model in MEGA X. The numbers at the branches indicate bootstrap majority consensus values on 1,000 replicates.

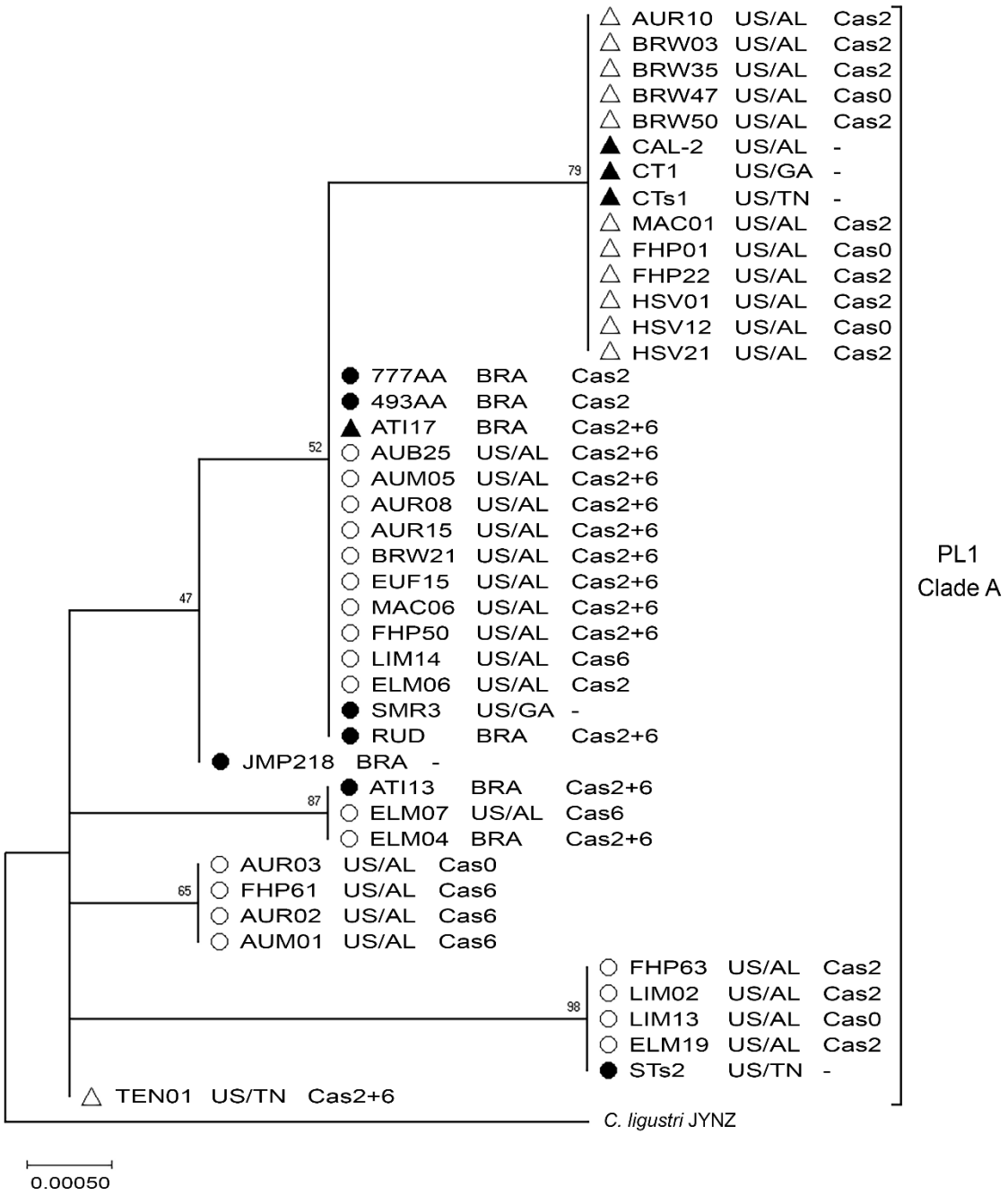


**Figure 2.8 Phylogenetic analysis of the combined data from four loci (*act1*, *caa5*, *ga4*, and rDNA ITS) sequences from *Corynespora cassiicola* isolates.** The circle symbol represents isolates from soybean, and triangle symbol represents isolates from cotton; if colored in black, sequences were retrieved from GenBank database. The tree was inferred by using the Maximum Likelihood method and General Time Reversible model in MEGA X. The numbers at the branches indicate bootstrap majority consensus values on 1,000 replicates. Tree was rooted using *C. ligustri* (strain JYNZ) as outgroup. Black bracket delineates the phylogenetic lineage (PL) (Dixon et al., 2009) and clade A (Déon et al., 2014).

# Chapter 3. The impact of *Corynespora cassiicola* variability on two screening methods for target spot resistance on soybean


## Abstract

The phytopathogenic fungus *Corynespora cassiicola* (Burk. & M.A. Curtis) C.T. Wei is known to cause target spot on soybean. Significant yield losses can occur if the disease is not properly controlled. The genetic diversity of the pathogen is well-documented and a toxin, cassiicolin, was reported and found to be related to disease resistance. However, screening methods are lacking for target spot resistance on soybean that accounts for pathogen diversity. Therefore, the main objective of this work was to understand the impact of the variability of *C. cassiicola* isolates sampled from cotton and soybean on screening for resistance to target spot on soybean by comparing the evaluation techniques of leaf wilting bioassay and plant inoculation. Leaf wilting bioassay using crude culture filtrates and plant inoculation with a spore solution were conducted with 12 *C. cassiicola* isolates from cotton and soybean on different soybean cultivars. Significant differences were found among isolates, cassiicolin-encoding genes, and soybean cultivar for the leaf wilting bioassay. Significant differences were found among isolates, cassiicolin-encoding genes, and host plant origin of the isolate for plant inoculation. However, results from the leaf wilting bioassay did not match the results from plant inoculation, indicating a low correlation among methods. The genetic diversity of *C. cassiicola* isolates clearly affects the results from leaf wilting and plant inoculation. Besides cassiicolin, a different profile of substances, metabolites, or toxins might be present on *C. cassiicola* isolates from cotton and soybean. Genetic resistance of soybean to target spot has been desired, though regardless of the screening methods it is essential to account for the diversity found on *C. cassiicola* isolates. These results indicate the need for more research on the role of cassiicolin in pathogenicity and aggressiveness of *C. cassiicola* from cotton and soybean, and the presence of other disease effectors in these isolates.

## 1. Introduction

Target spot is a fungal disease on soybean (*Glycine max*) caused by the phytopathogenic fungus *Corynespora cassiicola* (Burk. & M.A. Curtis) C.T. Wei. Disease symptoms include leaf lesions with roundish to an irregular shape, reddish-brown in color, frequently surrounded by yellowish-green halos; petioles and stems exhibit dark brown lesions with different shape and size; lesions on pods are usually circular, depressed, dark in the center with brown margins (Godoy, 2015). Severe disease symptoms and significant yield losses can occur when the pathogen is not properly controlled (Koenning et al., 2006), and yield losses up to 1009 kg/ha (900 lb/acre) have been estimated for soybean in the U.S. (Faske, 2017). In addition to soybean, up to 400 plant species have been reported as hosts to *C. cassiicola* worldwide (Farr and Rossman, 2020).

Onesirosan et al. (1975) reported that a highly pathogenic isolate of *C. cassiicola* produced a toxic substance in leaves of susceptible tomato cultivars but not in resistant cultivars. A toxin recovered from the *C. cassiicola* isolate CCP from rubber tree (*Hevea brasiliensis*), named as cassiicolin, was purified and biochemically characterized for the first time (Breton, 1997). The culture filtrate of the isolate CCP was highly toxic to the susceptible clone of rubber tree (PB 260) resulting in leaf wilting, and no symptoms were observed on the resistant clone (GT 1) (Breton et al., 2000). The behavior of a typical host-selective toxin (HST) was described for the purified cassiicolin, inducing cellular damages identical to the fungus inoculation on *Hevea* leaves and exhibiting the same host selectivity (Barthe et al., 2007). Cassiicolin was reported as an important effector of *C. cassiicola*, containing six cassiicolin isoforms (Cas1-Cas6) from different isolates sampled from various hosts and geographical origin (Barthe et al., 2007; Déon et al., 2014; Wu et al., 2018). Isolates carrying *Cas1* gene were considered the most aggressive on *Hevea* pathogenicity tests (Déon et al., 2014). The presence of other disease effectors besides cassiicolin

was suggested for another isolate (CCAM3) with medium aggressiveness besides CCP (Déon et al., 2012).

Toxins from *C. cassiicola* were found to be heavily involved in partial resistance of the *C. cassiicola*-rubber tree pathosystem (Barthe et al., 2007). Toxins produced by fungal pathogens are secondary metabolites well-known to be essential for pathogenicity, particularly when produced by necrotrophic fungi (Horbach et al., 2011). However, the majority of toxins produced by fungal pathogens are non-selective toxins, affecting a broad range of hosts. These toxins are not the primary determinant of pathogenicity (Walton, 1996); instead, they contribute to virulence and symptom development, while HST's affect virulence and pathogenicity (Breton et al., 2000). The use of toxins produced by *C. cassiicola* to screen for disease resistance has advantages over using fungal inoculations, such as control of the environment, faster results, and higher throughput (Breton et al., 2000).

The genetic diversity of *C. cassiicola* isolates has been documented (Banguela-Castillo et al., 2020; Déon et al., 2014; Dixon et al., 2009; Hieu et al., 2020; Sumabat et al., 2018), and that further complicates the search for resistant germplasm. Breeding for disease resistance is an essential item to successfully reduce soybean yield losses (Hartman et al., 2011), and methods of rapid screening for disease resistance are desired to complement the classical breeding methods. The choice of representative isolates based on their genetic diversity and the methodology used for screening are major problems faced by plant pathologists and plant breeders. The lack of studies on screening methodologies to select soybean germplasm resistant or tolerant to target spot make it difficult, forcing reliance upon field response to the disease. Positive results from intensive screening in the breeding phase play an important role in disease management, and the use of resistant or tolerant cultivars has been recommended as a long-term strategy to control the disease;

however, it is not always available (Fernando et al., 2010). Therefore, the main objective of this work was to understand the impact of *C. cassiicola* isolate variability on screening for resistance to target spot on soybean by comparing two methods: leaf wilting bioassay and plant inoculation.

## 2. Materials and methods

### 2.1 Fungal isolation and identification

Alabama isolates of *C. cassiicola* were recovered from cotton and soybean leaves showing typical target leaf spot symptoms. Direct isolation was used and small amounts of mycelia and conidia on the surface of the lesions were directly transferred onto potato dextrose agar (PDA; Hardy Diagnostics, Santa Maria, CA) enriched with 50 mg/L of kanamycin. Symptomatic leaves were placed in plastic bags to induce fungal sporulation when it was not already present. When direct isolation was not successful, small pieces of leaf tissue from the edge of the necrotic lesions were surface-disinfected for 30 seconds in 70% ethanol, 1 min in 2% sodium hypochlorite, and then rinsed twice with sterile distilled water. Disinfected pieces of leaf tissues were plated directly onto PDA enriched with 50 mg/L of kanamycin. Inoculated plates were incubated at 28 ± 2°C under 12 h of photoperiod for colony growth, and pure colonies were selected to establish the *C. cassiicola* collection. Fungal identification was made based on morphological and reproductive characters (Ellis and Holliday, 1971), and confirmed by sequencing the internal transcribed spacer using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Dixon et al., 2009). For long-term conservation, mycelium and spores were preserved at -80°C in 20% glycerol (Lopez et al., 2018).

### 2.2 Cassiicolin-encoding genes identification

Mycelial plugs of twelve *C. cassiicola* isolates (Table 3.1) were placed over a cellophane membrane onto a fresh PDA plate (Cassago et al., 2002). After 10 days of growth, mycelium was harvested and DNA was extracted from each isolate using a ZR Fungal/Bacterial MiniPrep™ kit (Zymo Research, California, USA). Extracted DNA concentration was measured using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) and stored at -20˚C before use. Primers encompassing five cassiicolin-encoding genes (Déon et al., 2014) were used for polymerase chain reaction (PCR) amplification. PCR was performed on 20 ng/µL of *C. cassiicola* genomic DNA. A 50-µL reaction mix was prepared for each isolate containing 2 µL of DNA template, 1 µL of forward primer (10 µM), 1 µL of reverse primer (10 µM), 21 µL of deionized water, and 25 µL of JumpStart Taq ReadyMix (Sigma-Aldrich, St. Louis, MO). DNA amplification was conducted in a MultiGene DNA thermal cycler (Labnet International, Edison, NJ) following the methodology described by Déon et al. (2014). To detect the presence of each cassiicolin-encoding gene, amplified products were examined by electrophoresis in 1% (w/v) agarose gel stained with GelRed™ 10,000X in DMSO (Biotium Inc., Hayward, CA) in 1x TBE buffer and visualized under UV light. A 1 kb Plus DNA ladder (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA) was used as the marker and a reaction without a DNA template was used as the negative control.

### 2.3 Crude culture filtrate preparation

Isolates were inoculated at the center of a PDA plate by placing one mycelial plug (7.0 mm) from a 10-day-old colony and incubated at 28 ± 2°C under 12 h of photoperiod. For crude culture filtrate (CCF) production, 100 mL of Potato Dextrose Broth (PDB) in 250 mL flasks was inoculated with one mycelial plug (7.0 mm diameter) from a 10-days-old culture of each *C. cassiicola* isolate. The inoculated flasks were incubated for 21 days, at room temperature (RT, 25

± 2°C), and 12 h of photoperiod on a bench top orbit environ shaker set at 160 rpm (Lab-Line Instruments Inc, Melrose Park, IL). The liquid culture with the mycelial growth was filtered through four layers of sterile gauze, following by a Whatman No. 1 filter paper, and finally filter-sterilized under vacuum through a set of Millipore membranes (0.40 and 0.22 µm). CCF of all *C. cassiicola* isolates were stored at 4˚C until used (Breton et al., 2000; Déon et al., 2012).

### 2.4 Leaf-wilting bioassay

Seeds of the cultivar Davis (Arkansas Agricultural Experiment Station, Fayetteville, AR) and RA-606 (Novartis Seeds, Inc., Minneapolis, Minnesota) were provided by the U.S. National Plant Germplasm System (NPGS). Resistance to target spot was registered for Davis (Caviness and Walters, 1966) and was described for RA-606 (USDA, 1983). The cultivars AG48X9 (Associated Seed Growers, Inc., Creve Coeur, CO) and NK S56-B7X (NK Seeds, Downers Grove, IL) are known as susceptible to target spot (Irby et al., 2018). Soybean plants were grown in the greenhouse at the Plant Science Research Center (PSRC) located at Auburn University, Auburn, AL. Trials were performed in 3100 $cm^3$ polypropylene round pots filled with a mix of potting soil Pro-Mix BX (Premier Tech Horticulture, Quakertown, PA) and sterile Kalmia loamy sand soil (75:25 v/v), supplemented with 2.1 g/L of 18-6-12 Osmocote fertilizer (ICL Specialty Fertilizers, Tel Aviv-Yafo, Israel). Two soybean seeds were sown per pot and thinned to one plant per pot seven days after planting (DAP). Plants were watered daily as needed. Supplemental light of 1000-watt halide bulbs was supplied to maintain a day length of 14 hours per day. Greenhouse temperatures ranged from 24°C to 35°C. For the leaf-wilting bioassay, trifoliate soybean leaves fully expanded from the greenhouse were immediately immersed in disposable culture tubes (13 x 100 mm) (VWR International, Radnor, PA) containing 5 mL of each CCF (Breton et al., 2000). Distilled water was used as a negative control. Culture tubes with trifoliate soybean leaves in the

CCF were incubated for 24 hours under RT, and 12 h of photoperiod. Each trial was conducted with two soybean germplasms, one tolerant and one susceptible to target spot. Trials were conducted five times for the cultivars Davis / AG48X9, and seven times for the cultivars RA-606 / NK S56-B7X. The experiment was arranged in a randomized complete block design where treatments were replicated four times within each block of soybean cultivars. The degree of leaf wilting at 24 h was rated in three wilting categories: (1) mild, (2) moderate, and (3) severe (Figure 3.1) (Fernando et al., 2010).

### 2.5 Plant inoculation

Seeds of RA-606 and NK S56-B7X soybean cultivars were sown in the greenhouse at PSRC. Polypropylene deepots D40L (Stuewe & Sons, Inc., Tangent, OR) were filled with a mix of potting soil Pro-Mix BX (Premier Tech Horticulture, Quakertown, PA) and sterile Kalmia loamy sand soil (75:25 v/v), supplemented with 2.1 g/L of 18-6-12 Osmocote fertilizer (ICL Specialty Fertilizers, Tel Aviv-Yafo, Israel). Soybean plants were thinned to one plant per deepot seven days after planting (DAP). Plants were watered daily as needed, and the light was supplemented with 1000-watt halide bulbs to maintain a day length of 14 h/day. Greenhouse temperatures ranged from 24°C to 35°C.

Isolates of *C. cassiicola* were grown in PDA medium and incubated at 28 ± 2°C under 12 h of photoperiod for 10 days. Each *C. cassiicola* colony was flooded with 10 mL of sterile distilled water per Petri dish and the colony surfaces were mechanically disturbed with a glass slide. The conidia suspension was filtered through four layers of sterile gauze, and the concentration of the suspension was determined under a microscope using a hemocytometer. The conidia suspension was adjusted to $10^4$ conidia/mL. The conidia suspension of each *C. cassiicola* isolate was sprayed on the adaxial and abaxial leaf surfaces of 25 days-old soybean plants until runoff. Distilled water

was used as a negative control. After inoculation, plants inoculated with the same isolate were covered with a transparent plastic bag for 72 h. After the incubation period, deepots were randomized and kept in the greenhouse for the duration of the trial. Symptoms were scored 20 days after inoculation using a disease rating system, ranging from 0 (no symptom) to 3 (highly virulent) (Onesirosan, 1973). Two trials were conducted in a randomized complete block design (RCBD) with five replications.

### 2.6 Data analysis

Data analysis was performed using RStudio, version 1.2.5033 (Free Software Foundation, Inc., Boston, MA). Graphical methods were used to check normality and homogeneity of variances of the independent variable. Leaf wilting and plant inoculation rating data were found to be non-normal, and non-parametric analyses were conducted on pooled data using Kruskal-Wallis rank-sum tests for multiple comparisons (R package 'agricolae') followed by Fisher's Least Significant Difference (LSD) post-hoc test with Holm adjustment ($\alpha = 0.05$). Results were expressed as mean ± standard deviation (SD). Boxplots of the data were obtained by additionally using the R package 'ggplot2'.

## 3. Results

Immersion of soybean trifoliates in *C. cassiicola* CCF resulted in wilting and interveinal chlorosis in 24 hours. The phytotoxicity of CCF showed significant differences among isolates and leaf wilting ratings ranged on average from 1.13 ± 0.40 to 2.05 ± 0.84, while ratings for plant inoculation ranged on average from zero (no disease) to 1.55 ± 0.51 (Table 3.1). Six isolates (FHP01, ELM06, BRW03, FHP22, ELM04, and LIM13) significantly increased leaf wilting compared with the negative control (water) ($\chi^2 = 158.88$, $P < 0.0001$), while five isolates (ELM06, ELM04, ELM07, LIM02, and LIM14) significantly increased ratings on plant inoculation

compared with negative control (water) ($\chi^2$ = 129.75, $P < 0.0001$). The most aggressive isolates for leaf wilting were not the same for plant inoculation.

The isoform of the cassiicolin-encoding gene that each isolate carries in their genome exhibited significant differences for leaf wilting ratings ($\chi^2$ = 8.84, $P < 0.05$). The average leaf wilting of isolates Cas0 (1.61 ± 0.79) was statistically higher than isolates Cas6 (1.37 ± 0.65). The leaf wilting average of isolates Cas2 and Cas2+6 were statistically similar (1.49 ± 0.76 and 1.52 ± 0.71, respectively) (Figure 3.2). However, the opposite was observed for plant inoculation ratings, where Cas6 and Cas2+6 exhibited statistically higher and similar ratings (1.27 ± 0.68, and 1.35 ± 0.49 respectively). Cas6 and Cas2+6 were statistically different from Cas2 (0.56 ± 0.71), and Cas0 exhibited the lowest ratings for plant inoculation (0.18 ± 0.39) ($\chi^2$ = 74.32, $P < 0.0001$) (Figure 3.3). The response of soybean to the presence or absence of cassiicolin differ among the tested methods: leaf wilting or plant inoculation.

No significant differences were observed for leaf wilting between the host plant origin of the isolate, whether *C. cassiicola* isolates were isolated from cotton or soybean symptomatic leaves ($\chi^2$ = 0.03, $P > 0.1$). *Corynespora cassiicola* isolates originally obtained from cotton exhibited a leaf wilting average on soybean of 1.52 ± 0.78, while isolates from soybean exhibited a leaf wilting average on soybean of 1.49 ± 0.73 (Figure 3.2). However, plant inoculation ratings of *C. cassiicola* isolates from soybean (1.03 ± 0.73) were statistically higher than isolates from cotton (0.27 ± 0.51) ($\chi^2$ = 67.38, $P < 0.0001$) (Figure 3.3). *Corynespora cassiicola* isolates exhibited a preference for the same host when inoculated on plants, but that was not observed for leaf wilting using CCF of *C. cassiicola*.

Significant differences were observed among leaf wilting of soybean cultivars ($\chi^2$ = 45.54, $P < 0.0001$). Davis exhibited a higher leaf wilting average (1.71 ± 0.77), while RA-606 exhibited

the lowest leaf wilting average (1.31 ± 0.60). Leaf wilting of AG48X9 and NK S56-B7X were statistically similar on average (1.44 ± 0.71 and 1.50 ± 0.78, respectively) (Figure 3.2). No significant differences were observed for ratings of plant inoculation among the cultivars RA-606 and NK S56-B7X (0.52 ± 0.70 and 0.68 ± 0.76, respectively) ($\chi^2 = 2.75$, $P > 0.1$) (Figure 3.3). The response of soybean cultivars to *C. cassiicola* differed according to the method assessed.

## 4. Discussion

A good agreement among leaf wilting assay and conidial inoculation of *C. cassiicola* on rubber tree clones was found by Breton et al. (2000); however, the same trend was not observed in our study. For the pathosystem *C. cassiicola*-soybean, we observed differences in isolate aggressiveness, soybean cultivar, and the cassiicolin-encoding gene for each methodology, leaf wilting, and plant inoculation but not with a clear correlation. That could be related to the fact that all studies with *C. cassiicola* from *Hevea* contained *Cas1* gene, while our isolates were found to express other toxin classes: Cas2, Cas6, Cas2+6, and Cas0 (with no detectable *Cas* gene). At this time, *Cas1* has only been found in *C. cassiicola* from *Hevea* (Déon et al., 2014). Cassiicolin was characterized as a small cysteine-rich glycoprotein produced by *C. cassiicola* isolate CCP and reported as a disease effector (Barthe et al., 2007; Breton et al., 2000; de Lamotte et al., 2007; Tran et al., 2016). Déon et al. (2014) demonstrated the most aggressive isolates on two cultivars of rubber tree had *Cas1* gene, suggesting that cassiicolin was the only *C. cassiicola* effector; although, some isolates without *Cas* gene (Cas0) were found to be moderately virulent to rubber tree. Tran et al. (2016) suggested that an isolate carrying *Cas1* gene (CCAM3) might share a common effector besides cassiicolin with isolates identified with Cas0. Lopez et al. (2018) identified around 45 putative secreted candidate effectors from CCP which may have some effect on disease

development. Ribeiro et al. (2019) demonstrated the essential role of cassiicolin for the virulence of CCP with a *Cas1* mutant, suggesting that cassiicolin is the only necrotrophic effector.

We demonstrated that isolates identified as Cas0 were among the most aggressive in the leaf wilting bioassay, suggesting that another disease effector is probably present. Despite inducing symptoms after plant inoculation, these isolates were not the most aggressive, indicating that the possible effector could not be responsible for the disease development on the host, or a necrotrophic effector such as the *Cas1* mutant. The absence of a cassiicolin-encoding gene (Cas0) did not affect the toxicity of the CCF in the leaf wilting bioassay. Isolates carrying *Cas2* or *Cas6* gene alone or in combination (*Cas*2+6) were the most aggressive for plant inoculation. These results indicate that a different profile of substances, metabolites, or toxins might be present in crude culture filtrates of *C. cassiicola* isolates from cotton and soybean. Host specificity has been reported among isolates of *C. cassiicola* and variation in aggressiveness among isolates has been demonstrated (Banguela-Castillo et al., 2020; Déon et al., 2014; Dixon et al., 2009; Sumabat et al., 2018). Our results support the variability found on *C. cassiicola* isolates since the response of isolates to both screening methods tested did not show a clear correlation. The variability of *C. cassiicola* isolates shown is a key to the successful search for resistance to target spot. The host specificity was clear only for the plant inoculation where isolates with the same host origin were the most aggressive. Perhaps, the apparatus to cause leaf symptoms are different for *C. cassiicola* isolates inoculated on the same host compared with isolates with a different host plant origin.

In addition, our results in the pathosystem *C. cassiicola*-soybean showed that no difference was found among soybean cultivars when inoculated with spore solution, which was different from the leaf wilting bioassay where one soybean cultivar (RA-606) exhibited the lowest leaf wilting rating. This cultivar was selected to be tested precisely because of its resistance to target spot

(USDA, 1983). In contrast, the cultivar Davis was considered resistant (Caviness and Walters, 1966) but exhibited the highest rating for the leaf wilting bioassay in this study. Certainly, these results emphasize that the resistance of soybean to target spot cannot rely on screening using only a few *C. cassiicola* isolates, and that resistance to target spot found in the past might not be fully accurate.

Studies on different pathogens have been conducted using culture filtrates against soybean germplasm to understand the role of toxins in the pathogen infection. Huang and Hartman (1998) demonstrated that it was possible to separate resistant and susceptible reactions in soybean using culture filtrate of *Fusarium solani* f. sp. *glycines*. Studying the same pathogen, Hartman et al. (2004) reported that soybean cuttings immersed in culture filtrates exhibited disease symptoms but not for culture filtrates of *F. solani* from other hosts, confirming the specificity of the pathotoxin. A lack of correlation of the effects induced on soybean by *Diaporthe phaseolorum* var. *caulivora* and the toxin was observed by Lalitha et al. (1989), suggesting that the toxin might not be a pathogenicity factor for the specificity of isolates. Abbas et al. (2019) studying toxins from *Macrophomina phaseolina* did not find phaseolinone in cell-free culture medium filtrates; however, a wide range of (-)-botryodiplodin levels were found from different isolates, with some of them highly phytotoxic to soybean. It was suggested that other toxins may be responsible for the pathogen infection for those isolates with low production of (-)-botryodiplodin. Fernando et al. (2010) stated that *in vitro* screening using CCF of *C. cassiicola* on *Hevea* should be used only to obtain preliminary data because they do not correlate with *in planta* observations. The sensitivity/tolerance of *Hevea* plants was shown to be cultivar-dependent for both techniques: inoculation with a spore solution and bioassays using purified cassiicolin (Déon et al., 2012). Several studies have been conducted with different pathogens on soybean that appear to be

functional for *in vitro* assessment; however, the pathosystem *C. cassiicola*-rubber tree seems to be more complex; likewise, it is possible that the pathosystem *C. cassiicola*-soybean is also complex.

In summary, the genetic diversity found in isolates of *C. cassiicola* from cotton and soybean exhibited different results in the response of soybean to the leaf wilting bioassay and plant inoculation evaluation methods. Our results suggest that there is a possibility that other effectors besides cassiicolin that play a role in the response of soybean to the *C. cassiicola* isolates. Research studying the pathosystem *C. cassiicola*-soybean is currently limited by the lack of an effective protocol to discern among susceptible and resistant soybean germplasm that encompass the variability found in the pathogen. The screening of soybean germplasm could be facilitated using non-destructive methods, such as the leaf wilting bioassay. Regardless of the methodology used to screen resistant soybean germplasm to target spot, it is important to account for the pathogen variability and their host-interaction. Questions still remain as to the role of cassiicolin in the pathogenicity and aggressiveness of *C. cassiicola* isolates from cotton and soybean, and further investigations are needed to demonstrate the presence of other disease effectors in those isolates.

**Figure 3.1 Leaf wilting scale.** Leaves of soybean illustrating the degree of wilting after 24 h of incubation at room temperature (25 ± 2°C). From left to right: (1) mild, (2) moderate, and (3) severe.

**Table 3.1** Results of the non-parametric analysis using Kruskal-Wallis test for the *in vitro* leaf wilting bioassay and plant inoculation at the greenhouse.

| Treatment | Host plant | Toxin class | Leaf wilting [x] | | Plant inoculation [y] | |
|---|---|---|---|---|---|---|
| | | | ***n*** | **Mean ± SD [z]** | ***n*** | **Mean ± SD [z]** |
| FHP01 | Cotton | Cas0 | 64 | 2.05 ± 0.84 a | 20 | 0.20 ± 0.41 d |
| ELM06 | Soybean | Cas2 | 88 | 1.90 ± 0.88 ab | 20 | 1.25 ± 0.79 ab |
| BRW03 | Cotton | Cas2 | 64 | 1.70 ± 0.87 abcd | 20 | 0.35 ± 0.67 d |
| FHP22 | Cotton | Cas2 | 64 | 1.66 ± 0.86 bcde | 20 | 0.10 ± 0.31 d |
| ELM04 | Soybean | Cas2+6 | 64 | 1.52 ± 0.71 bcdef | 20 | 1.35 ± 0.49 ab |
| LIM13 | Soybean | Cas0 | 88 | 1.47 ± 0.71 cdef | 20 | 0.20 ± 0.41 d |
| ELM07 | Soybean | Cas6 | 64 | 1.41 ± 0.64 cdefg | 20 | 1.55 ± 0.51 a |
| LIM14 | Soybean | Cas6 | 64 | 1.33 ± 0.67 defg | 20 | 1.00 ± 0.73 ab |
| HSV12 | Cotton | Cas0 | 64 | 1.25 ± 0.53 efg | 20 | 0.15 ± 0.37 d |
| HSV01 | Cotton | Cas2 | 64 | 1.22 ± 0.52 fg | 20 | 0.40 ± 0.60 cd |
| MAC01 | Cotton | Cas2 | 64 | 1.22 ± 0.58 fg | 20 | 0.40 ± 0.60 cd |
| LIM02 | Soybean | Cas2 | 64 | 1.17 ± 0.42 fg | 20 | 0.85 ± 0.59 bc |
| Negative control | - | - | 128 | 1.13 ± 0.40 g | 20 | 0.00 ± 0.00 d |
| **Chi-Square ($\chi^2$)** | | | | **158.88** | | **129.75** |
| ***P*-value** | | | | **<0.0001** | | **<0.0001** |

[x] Leaf wilting assays were conducted on Davis / AG48X9 and RA-606 / NK S56-B7X. Scale ratings for leaf wilting ranged from 1 to 3.

[y] Plant inoculations were conducted on RA-606 / NK S56-B7X. Scale ratings for plant inoculation ranged from 0 to 3.

[z] Mean ± SD followed by the same letter in the columns were not significantly different (Kruskal-Wallis test, $\alpha = 0.05$).

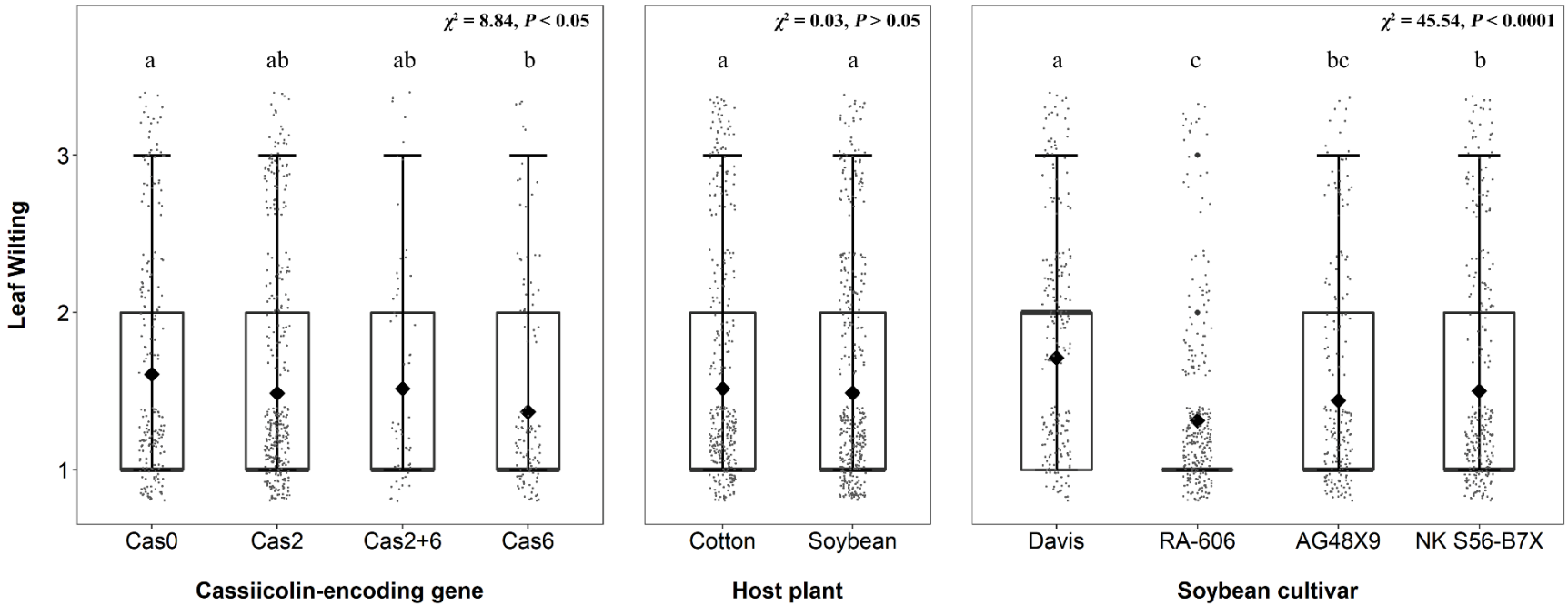


**Figure 3.2 Ratings of leaf wilting bioassay.** Boxplots represent data of leaf wilting bioassay ratings separated by cassiicolin-encoding gene of *Corynespora cassiicola* isolates (left panel), host plant of *C. cassiicola* isolates (middle panel), and soybean cultivar where leaf wilting was assessed (right panel). The letters above boxplots represent significance groups as defined by Kruskal-Wallis test (LSD, $\alpha = 0.05$). Black diamonds (♦) inside boxes show mean values, horizontal lines inside boxes mark the median value. Jitter (dots) inside each boxplot indicate the individual samples (*n*).

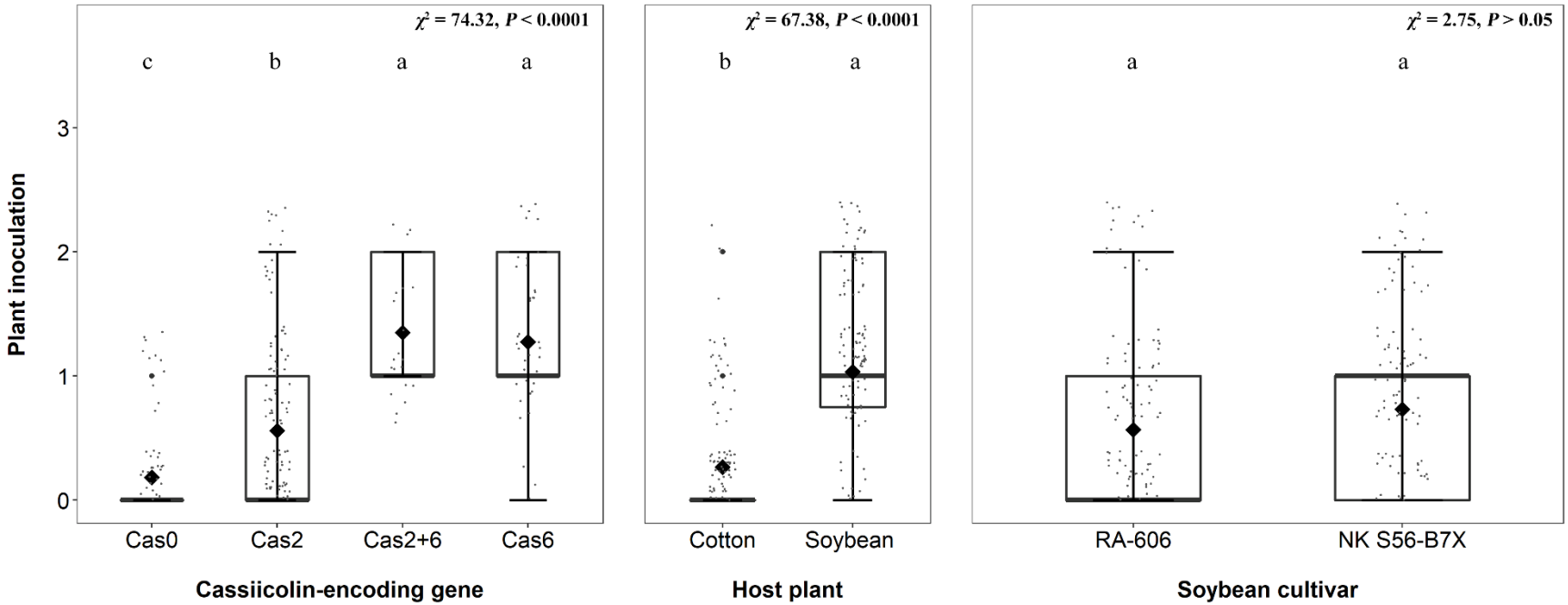


**Figure 3.3 Ratings of plant inoculation.** Boxplots represent data of plant inoculation ratings separated by cassiicolin-encoding gene of *Corynespora cassiicola* isolates (left panel), host plant of *C. cassiicola* isolates (middle panel), and soybean cultivar where *C. cassiicola* isolates were inoculated (right panel). The letters above boxplots represent significance groups as defined by Kruskal-Wallis test (LSD, $\alpha = 0.05$). Black diamonds (♦) inside boxes show mean values, horizontal lines inside boxes mark the median value. Jitter (dots) inside each boxplot indicate the individual samples (*n*).

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# Chapter 4. Fungicide sensitivity profiles of *Corynespora cassiicola* isolates from Alabama cotton and soybean fields to different fungicide groups


## Abstract

Target spot, caused by a fungal pathogen known as *Corynespora cassiicola*, is a disease on cotton and soybean plants with increasing importance. Fungicides are a crucial tool in disease management; however, there are reported cases of *C. cassiicola* resistance to different fungicide groups. The objectives of this study were (i) to determine the sensitivity profiles for *C. cassiicola* from cotton and soybean to thiophanate-methyl, prothioconazole, pyraclostrobin, mancozeb, and the combination fluxapyroxad + pyraclostrobin; (ii) to determine if there is a fitness loss on *C. cassiicola* isolates with the G143A mutation in the cytochrome *b* gene. Twelve *C. cassiicola* isolates were used to determine the $EC_{50}$ values using non-linear regression. The $EC_{50}$ values of *C. cassiicola* isolates ranged from 0.57 to 1.99 µg/mL ($\bar{X}$ = 0.93 µg/mL) for thiophanate-methyl, 0.31 to 2.73 µg/mL ($\bar{X}$ = 0.76 µg/mL) for prothioconazole, 4.61 to 76.48 µg/mL ($\bar{X}$ = 30.36 µg/mL) for pyraclostrobin, 4.20 to 18.63 µg/mL ($\bar{X}$ = 46.38 µg/mL) for mancozeb, and 0.14 to 1.28 µg/mL ($\bar{X}$ = 0.61 µg/mL) for the combination fluxapyroxad + pyraclostrobin. Thiophanate-methyl and prothioconazole were the only fungicides where cotton and soybean isolates did not exhibit statistically different $EC_{50}$ values. QoI-resistant isolates exhibited statistically higher $EC_{50}$ values than QoI-sensitive isolates for all fungicides, except for thiophanate-methyl. No fitness penalty was found, whether QoI-resistant or QoI-sensitive isolates. Our findings will be useful to monitor sensitivity of U.S. populations of *C. cassiicola* from cotton and soybean, and to facilitate fungicide resistance management through detection of shifts in fungicide sensitivity.

## 1. Introduction

*Corynespora cassiicola* (Berk. & M. A. Curtis) C.T. Wei is a widespread ascomycete fungus responsible for causing the disease known as target spot on cotton and soybean plants. This fungus has been reported worldwide on more than 400 plant species including fruits, vegetables, grains, perennial crops, forestry and various ornamental plants (Farr and Rossman, 2020). Disease management has been a concern in other countries due to increasing occurrence of target spot on soybean fields (Godoy et al., 2015), but recently concern about target spot on cotton has been rising (Sumabat et al., 2018). Target spot incidence and severity have been increasing possibly due to monoculture farming, no-tillage practices, the use of susceptible cultivars, and optimal weather pattern changes for disease development (Avozani et al., 2014; Koenning et al., 2006). Complete resistance to target spot has not been found, and foliar fungicides may be used as an alternative to control target spot on cultivars with low resistance levels or susceptibility to the disease (Duan et al., 2019; Li et al., 2020). Significant yield losses have been reported when the pathogen was not properly controlled (Bowen et al., 2018; Faske, 2017; Hagan and Sikora, 2012; Koenning et al., 2006).

Chemical control by application of fungicides is far the most frequently used tool in disease management in agriculture, and have been used for over 200 years (Brent and Hollomon, 2007). According to Ma et al. (2020), foliar fungicide application is known to be the most effective tool to control the disease caused by the fungus *C. cassiicola*. However, the progressive development of resistance to fungicides is aggravated by the incorrect use of the fungicide groups (Asadollahi et al., 2013). The Fungicide Resistance Action Committee (FRAC; https://www.frac.info/home) makes available a constantly updated list of risk of resistance development by fungal pathogens. Novel modes of action of fungicides are not easily discovered, consequently use of a high risk resistance fungicide combined with a low risk resistance fungicide and rotation with different

fungicide groups are recommended to maintain the most effective available fungicides (Ishii and Hollomon, 2015). Site-specific fungicides are safer to non-target species and recommended to be included in integrated pest management (IPM) programs, however, generally possess a high risk of resistance development (Ishii et al., 2001). Unfortunately, there are reported cases of *C. cassiicola* isolates from cucumber, soybean, and tomato resistant to fungicides with different mode of actions.

Methyl Benzimidazole Carbamates (MBC, FRAC code 1) fungicides are inhibitors of β-tubulin assembly in mitosis with direct effects on nuclear division, inhibiting mycelial growth. (Date et al., 2004; Ishii et al., 2007). Benzimidazoles and thiophanates are among MBC fungicides which have been classified with a high risk for developing resistance (FRAC, 2020), and benzimidazole resistance was reported for *C. cassiicola* on several crops (Avozani et al., 2014; Date et al., 2004; Teramoto et al., 2017; Xavier et al., 2013). Thiophanate-methyl was very effective for controlling target spot on cucumber but a decrease in the effectiveness was observed with the emergence of *C. cassiicola* resistant isolates (Date et al., 2004). DeMethylation Inhibitors (DMI, FRAC code 3) fungicides are inhibitors of sterol C-14 alpha-demethylation during ergosterol biosynthesis and were first used in the 1970's (Brent and Hollomon, 2007). Triazoles are among the most used and known chemical group of DMI fungicides and even with their intense use over 50 years, few cases of resistance to them have been reported (Brent and Hollomon, 2007; Fan et al., 2013; FRAC, 2020). They are classified as fungicides with a medium risk of resistance development (FRAC, 2020).

Quinone outside Inhibitors (QoI, FRAC code 11) fungicides, also known as strobilurins were first sold in 1996 and pyraclostrobin was released by BASF in 2002. Given the efficiency of strobilurins fungicides, in about four years their sales reached over 10% of the global fungicide

market (Bartlett et al., 2002). However, QoI fungicides pose a high risk of resistance development (Grasso et al., 2006) with the first field resistance reported in wheat in 1998 (Ishii et al., 2001). In 2006, more than 20 pathogens had been recorded as QoI-resistant worldwide (Ishii and Hollomon, 2015). Three target site mutations were reported in the cytochrome *b* gene (F129L, G137R, and G143A) that are known to confer different levels of resistance to QoI fungicides (Bartlett et al., 2002; Duan et al., 2019; Grasso et al., 2006; Ishii et al., 2001). QoI resistance of *C. cassiicola* is a serious problem in Japan, where resistant isolates cannot be controlled (Ishii et al., 2007). Succinate-DeHydrogenase Inhibitors (SDHI, FRAC code 7) fungicides are also respiratory inhibitor fungicides like QoI's with a site-specific mode of action but with a different target site within the fungal mitochondria (Hollomon, 2015). These fungicides have been classified as a high risk of resistance development (FRAC, 2020), and resistance has been reported in about 15 fungal pathogens (Torriani et al., 2017). Resistance to boscalid, a SDHI fungicide, was already reported in *C. cassiicola* on cucumber in Japan (Miyamoto et al., 2009), and use of boscalid to control Corynespora leaf spot has been discouraged (Miyamoto et al., 2010).

The use of fungicides continues to rise to control diseases on cotton and soybean, and *in vitro* sensitivity of *C. cassiicola* isolates associated with cotton and soybean in the United States have not been determined. It is vital to monitor *C. cassiicola* populations with $EC_{50}$ values for their degree of sensitivity to one or more fungicides to facilitate the detection of shifts in the sensitivity of *C. cassiicola*, and to determine if resistance strategies are effective (Brent and Hollomon, 2007; Emmitt et al., 2018; Russell, 2004). Therefore, the objectives of this study were (i) to determine the baseline sensitivity profiles of *C. cassiicola* isolates from cotton and soybean including QoI-resistant isolates to thiophanate-methyl, prothioconazole, pyraclostrobin, mancozeb, and the

combination of fluxapyroxad + pyraclostrobin; (ii) to determine if there is a fitness loss on *C. cassiicola* isolates with the G143A mutation in the cytochrome *b* gene.

## 2. Materials and methods

### 2.1 Fungal isolation and identification

Isolates of *C. cassiicola* were recovered from cotton and soybean leaves showing typical target leaf spot symptoms collected from Alabama. Direct isolation was used and small amounts of mycelia and conidia on the surface of the lesions were directly transferred onto potato dextrose agar (PDA; Hardy Diagnostics, Santa Maria, CA) enriched with 50 µg/mL of kanamycin. Symptomatic leaves were placed in plastic bags to induce fungal sporulation when it was not already present. When direct isolation was not successful, small pieces of leaf tissue from the edge of the necrotic lesions were surface-disinfected for 30 seconds in 70% ethanol, 1 min in 2% sodium hypochlorite, and then rinsed twice with sterile distilled water. Disinfected pieces of leaf tissues were plated directly onto PDA enriched with 50 µg/mL of kanamycin. Inoculated plates were incubated at 28 ± 2°C under a cycle of 12 h light/dark for colony growth, and pure colonies were selected to establish the *C. cassiicola* collection. Fungal identification was made based on morphological and reproductive characters (Ellis and Holliday, 1971), and confirmed by sequencing the internal transcribed spacer using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Dixon et al., 2009).

### 2.2 Fungicides

All fungicides tested in this study were commercial formulations, with one having more than one active ingredient (Table 4.1). Fungicides were individually dissolved in sterile distilled water to prepare stock solutions (1,000 and 10,000 µg/mL) immediately before use.

### 2.3 *In vitro* fungicide sensitivity

Twelve isolates of *C. cassiicola* from cotton ($n$ = 6) and soybean ($n$ = 6) were selected to test the fungicide sensitivity in the *in vitro* bioassay. Four of these 12 isolates were reported as QoI-resistant previously (Rondon and Lawrence, 2019). Isolates were inoculated by placing one mycelial plug (7.0 mm) from a 10-day-old colony at the center of a PDA plate and incubated at 28 ± 2°C under a cycle of 12 h light/dark. For the *in vitro* bioassay, experiments were performed using the methods previously described with minor modifications (Ishii et al., 2007). Cooled PDA media, enriched with 50 µg/mL of kanamycin, was amended with six fungicide concentrations (0.01, 0.1, 0.5, 1, 10 and 100 µg/mL of active ingredient) and poured into Petri plates. PDA plates amended with fungicides were inoculated with one mycelial plug (7.0 mm) taken from the edges of growing PDA cultures. PDA plates without the addition of fungicide were used as control. Inoculated plates were incubated at 28 ± 2°C under a cycle of 12 h light/dark to determine the effect of each fungicide on mycelial growth. No salicylhydroxamic acid (SHAM) to inhibit the alternative respiratory pathway was added to the media, since Teramoto et al. (2017) didn't find any effect of SHAM when studying the sensitivity of *C. cassiicola* isolates to QoI fungicides. Mycelial growth was determined by measuring colony diameter of each plate along two perpendicular lines when the first colony reached the borders of the plate. The diameter of the mycelial plugs for each plate was subtracted before calculating the average of the two measurements for each plate. The percent growth inhibition due to the fungicide treatments at

different concentrations was calculated as follows: [(dc – dt) / dc] x 100, where dc = average diameter of fungal colony in control, and dt = average diameter of fungal colony in fungicide treatment (Ishii et al., 2007). The percent growth inhibition was used to calculate the $EC_{50}$ values (fungicide concentration that inhibited 50% of the mycelial growth) for each isolate-fungicide and were expressed in µg/mL. The experiment was a completely randomized design with four replicates of each isolate-fungicide concentration combination. A Petri dish was used as an experimental unit and two independent experiments were conducted for each fungicide.

### 2.4 Fitness assessment of *C. cassiicola* QoI-resistant isolates

To assess the fitness of *C. cassiicola* isolates resistant to pyraclostrobin, their mycelial growth was measured on fungicide-free PDA plates with four replications and compared with *C. cassiicola* isolates sensitive to the same mode of action (Zhang and Bradley, 2017). The isolates were cultured as described above, and mycelial growth measurements were obtained for all isolates. The experiments were conducted in a completely randomized design and repeated six times.

### 2.5 Data analysis

The $EC_{50}$ values were estimated by the Gauss-Newton iterative method in the non-linear regression procedure using PROC NLIN in SAS 9.4 (SAS Institute Inc., Cary, NC). Diagnostics plots were generated to check for normality and equal variance assumptions. Data from two trials for each fungicide were combined for statistical analysis representing eight replications per isolate-fungicide concentration. $EC_{50}$ values for each fungicide were subjected to analysis of variance using PROC GLM, and means were separated with Tukey's HSD test ($\alpha = 0.05$). Two-sample Student's t-tests were performed using PROC TTEST ($\alpha = 0.05$) for detecting significant $EC_{50}$ by the origin of the isolates (cotton or soybean) for each fungicide. Mycelial growth data were

combined after visual diagnostics of normality/equal variance assumptions and subjected to analysis of variance using PROC GLM, and means were separated with Tukey's HSD test ($\alpha$ = 0.05).

## 3. Results

### 3.1 *In vitro* fungicide sensitivity

The sensitivity of 12 *C. cassiicola* isolates obtained from cotton and soybean infected leaves in Alabama were tested to establish a baseline sensitivity to thiophanate-methyl, prothioconazole, pyraclostrobin, mancozeb, and the combination of fluxapyroxad + pyraclostrobin (Table 4.1). Mycelial growth inhibition of *C. cassiicola* isolates grown on fungicide amended media was used to obtain $EC_{50}$ values (Table 4.2). The $EC_{50}$ values of all the tested *C. cassiicola* isolates ranged from 0.57 to 1.99 µg/mL ($\bar{X}$ = 0.93 µg/mL) for thiophanate-methyl; from 0.31 to 2.73 µg/mL ($\bar{X}$ = 0.76 µg/mL) for prothioconazole; from 4.61 to 76.48 µg/mL ($\bar{X}$ = 30.36 µg/mL) for pyraclostrobin; from 4.20 to 18.63 µg/mL ($\bar{X}$ = 46.38 µg/mL) for mancozeb; and finally, ranged from 0.14 to 1.28 µg/mL ($\bar{X}$ = 0.61 µg/mL) for the combination fluxapyroxad + pyraclostrobin (Table 4.2).

For the fungicide thiophanate-methyl, no difference was found for the mean $EC_{50}$ for *C. cassiicola* isolated from cotton ($\bar{X}$ = 0.82 [0.75-0.90; 95% C.L.] µg/mL) or soybean ($\bar{X}$ = 1.03 [0.79-1.28; 95% C.L.] µg/mL) ($P$ = 0.1006). Again, no difference was found for the mean $EC_{50}$ for *C. cassiicola* isolated from cotton ($\bar{X}$ = 0.62 [0.49-0.76; 95% C.L.] µg/mL) or soybean ($\bar{X}$ = 0.89 [0.59-1.20; 95% C.L.] µg/mL) ($P$ = 0.110) for the fungicide prothioconazole. The mean $EC_{50}$ for *C. cassiicola* isolated from cotton ($\bar{X}$ = 12.50 [7.83-17.17; 95% C.L.] µg/mL) was statistically lower than the mean $EC_{50}$ for *C. cassiicola* isolated from soybean ($\bar{X}$ = 48.22 [37.21-59.23; 95% C.L.] µg/mL) ($P < 0.0001$) for pyraclostrobin. The mean $EC_{50}$ for *C. cassiicola* isolated from

cotton ($\bar{X}$ = 6.30 [5.51-7.09; 95% C.L.] µg/mL) was statistically lower than the mean $EC_{50}$ for *C. cassiicola* isolated from soybean ($\bar{X}$ = 10.56 [8.72-12.39; 95% C.L.] µg/mL) ($P < 0.0001$) for mancozeb. *Corynespora cassiicola* isolated from cotton exhibited statistically lower $EC_{50}$ mean ($\bar{X}$ = 0.36 [0.30-0.42; 95% C.L.] µg/mL) than *C. cassiicola* isolated from soybean ($\bar{X}$ = 0.86 [0.75-0.96; 95% C.L.] µg/mL) ($P < 0.0001$) for the combination fluxapyroxad + pyraclostrobin (Figure 4.1). These results suggested that *C. cassiicola* isolated from cotton and soybean does not have the same behavior for fungicides with different modes of action.

No difference was found in the mean $EC_{50}$ for QoI-resistant isolates ($\bar{X}$ = 0.87 [0.64-1.10; 95% C.L.] µg/mL) and QoI-sensitive isolates ($\bar{X}$ = 0.96 [0.80-1.11; 95% C.L.] µg/mL) ($P = 0.543$) when amended with thiophanate-methyl. For all other fungicides, QoI-resistant isolates always exhibited statistically higher mean $EC_{50}$ when compared with QoI-sensitive isolates. When amended with prothioconazole, the mean $EC_{50}$ for QoI-resistant isolates was $\bar{X}$ = 1.15 (0.72-1.59; 95% C.L.) µg/mL; and for QoI-sensitive isolates was $\bar{X}$ = 0.56 (0.46-0.66; 95% C.L.) µg/mL ($P = 0.0106$). For pyraclostrobin amended media, the mean $EC_{50}$ for QoI-resistant isolates was $\bar{X}$ = 65.24 (52.60-77.88; 95% C.L.) µg/mL; and for QoI-sensitive isolates was $\bar{X}$ = 12.92 (9.22-16.62; 95% C.L.) µg/mL ($P < 0.0001$). For mancozeb amended media, the mean $EC_{50}$ for QoI-resistant isolates was $\bar{X}$ = 11.98 (9.61-14.35; 95% C.L.) µg/mL; and for QoI-sensitive isolates was $\bar{X}$ = 6.65 (5.81-7.49; 95% C.L.) µg/mL ($P = 0.0001$). When amended with the combination fluxapyroxad + pyraclostrobin, the mean $EC_{50}$ for QoI-resistant isolates was $\bar{X}$ = 1.02 (0.90-1.14; 95% C.L.) µg/mL; and for QoI-sensitive isolates was $\bar{X}$ = 0.40 (0.35-0.46; 95% C.L.) µg/mL ($P < 0.0001$) (Figure 4.2). These results suggested that a possible correlation can be found between isolates with higher $EC_{50}$ values for pyraclostrobin (QoI-resistant) and isolates with other mutations which exhibit higher $EC_{50}$ values for fungicides other than thiophanate-methyl.

### 3.2 Fitness assessment of *C. cassiicola* QoI-resistant isolates

Mycelial growth of 12 *C. cassiicola* isolates were compared. Among those isolates, four were QoI-resistant, and eight were QoI-sensitive (Rondon and Lawrence, 2019). Significant differences in mycelial growth was observed among the isolates ($df = 11$, $F = 41.63$, $P < 0.0001$). Mean comparison of mycelial growth divided the isolates into several statistical groups, and significant differences was observed for mycelial growth of *C. cassiicola* isolates. QoI-resistant isolates of *C. cassiicola* were placed in statistically different groups (Figure 4.3). We could not correlate mycelial growth of *C. cassiicola* isolates with the presence of G143A mutation (QoI-resistant).

## 4. Discussion

*Corynespora cassiicola* isolates obtained from symptomatic soybean leaves in the U.S. were confirmed to have the G143A mutation in the cytochrome *b* gene, which confers resistance to QoI fungicides (Rondon and Lawrence, 2019). In this study, baseline sensitivities of the same 12 *C. cassiicola* isolates obtained from cotton and soybean infected leaves in Alabama were established for thiophanate-methyl, prothioconazole, pyraclostrobin, mancozeb, and the mixture fluxapyroxad + pyraclostrobin. Results for each fungicide are compared with the literature, and some useful insights for fungicide resistance management are highlighted.

Thiophanate-methyl, one of the MBC fungicides, a group of β-tubulin inhibitors that have been extensively used to control several fungal pathogens exhibit a positive cross-resistance among MBC fungicides (carbendazim, benomyl, thiabendazole, fuberidazole, and thiophanate-methyl) (Duan et al., 2019; FRAC, 2020; Ma and Michailides, 2005). Our results demonstrated that 75% of *C. cassiicola* isolates from Alabama U.S. exhibited $EC_{50} < 1.0$ µg/mL, while only 3 isolates had an $EC_{50} > 1.0$ µg/mL, regardless of whether isolates were obtained from cotton or

soybean. Our results were equivalent to the $EC_{50}$ of wild-type strains of *C. cassiicola* in cucumber, where those isolates were extremely sensitive to benzimidazoles (Duan et al., 2019). Highly non-sensitive soybean *C. cassiicola* isolates to carbendazim were reported in Brazil with $EC_{50}$ > 165 µg/mL (Xavier et al., 2013). Avozani et al. (2014) observed sensitive *C. cassiicola* isolates from soybean with $EC_{50}$ < 1.0 µg/mL for carbendazim, while sensitivity loss was observed for those *C. cassiicola* isolates with $EC_{50}$ > 40 µg/mL for the same fungicide. $EC_{50}$ > 556 µg/mL for carbendazim and $EC_{50}$ > 294 µg/mL for thiophanate-methyl was found in Brazil for *C. cassiicola* isolates (Teramoto et al., 2017). Benzimidazole-resistant isolates were reported with $EC_{50}$ values > 192 µg/mL for carbendazim, $EC_{50}$ values > 78 µg/mL for benomyl, and $EC_{50}$ values > 18 µg/mL for thiabendazole (Duan et al., 2019). Even though *C. cassiicola* isolates from cotton and soybean in the U.S. did not exhibit reduced sensitivity to benzimidazoles, we do not recommend the use of MBC fungicides as a sole fungicide to control target spot; if needed, the MBC fungicide should be used in a mixture with other modes of action that are effective to control *C. cassiicola*. Resistance of *C. cassiicola* to MBC fungicides generated by mutations in the β-tubulin gene cause different resistant levels to benzimidazoles (Date et al., 2004; Duan et al., 2019; Li et al., 2020). Duan et al. (2019) warned that with the use of higher doses of benzimidazoles, a single mutation in the β-tubulin gene will evolve super-resistance (multiple mutations) that confer high levels of resistance. This led the authors to suggest that the use of benzimidazoles to control *C. cassiicola* in cucumber should be restricted in China.

Despite the intensive use of DMI fungicides to control target spot, there are few reports about the resistance of *C. cassiicola* to DMI fungicides (FRAC, 2020) indicating that they still are a good option for disease control. In this study, we observed $EC_{50}$ values for *C. cassiicola* isolates from Alabama U.S. to prothioconazole within the range of what was found in previous studies in

Brazil. The highest $EC_{50}$ value to prothioconazole was 2.73 µg/mL for a soybean *C. cassiicola* isolate, and no significant difference was found whether the isolate was obtained from cotton or soybean. Xavier et al. (2013) studied the sensitivity of 24 *C. cassiicola* isolates from soybean in Brazil to prothioconazole and observed a range of $EC_{50}$ values from 0.47 to 26.44 µg/mL ($\bar{X}$ = 5.02). Avozani et al. (2014) also reported the sensitivity of five *C. cassiicola* soybean isolates in Brazil to four DMI fungicides (cyproconazole, epoxiconazole, flutriafol, and tebuconazole), where the mean $EC_{50}$ values ranged from 0.77 to 20.32 µg/mL. Later, Teramoto et al. (2017) reported $EC_{50}$ values of 34 Brazilian soybean *C. cassiicola* isolates ranging from <0.16 to 46.44 µg/mL to prothioconazole, and ranging from <0.16 to 100 µg/mL to cyproconazole. According to the criteria used by Teramoto et al. (2017), isolates should be considered as moderately sensitive (MS, $EC_{50}$ = 0.16-1.0 µg/mL) and sensitive (S, $EC_{50}$ < 0.16 µg/mL) to DMI. Russell (2004) stated that cross-resistance can be present between DMI fungicides active against the same fungus. Usually China is the first place to report resistance of *C. cassiicola* isolates to different fungicides because of their intensive use of fungicides to control cucumber Corynespora leaf spot; however, so far no *C. cassiicola* isolate with resistance to DMI fungicides has been reported in China (Zhu et al., 2020). Based on our results of $EC_{50}$ values for DMI fungicides, we understand that they remain effective against *C. cassiicola* without reduced sensitivity, and could continue to be used to control target spot on cotton and soybean. Although, it is still important to follow basic strategies to delay fungicide resistance development (Ishii and Hollomon, 2015).

*Corynespora cassiicola* poses a moderate to high risk to develop resistance against single-target fungicides like DMI's and QoI's (FRAC, 2020). The emergence of *C. cassiicola* isolates resistant to pyraclostrobin (QoI fungicide) (Rondon and Lawrence, 2019) will become a limitation to the management of target spot in the field. Among all three mutations found in the cytochrome

*b* gene, the mutation G143A is the most common in QoI-resistant pathogens (Ma and Michailides, 2005), which is also associated with high levels of resistance to QoI fungicides (Duan et al., 2019). F129L and G137R mutations are responsible for low to moderate levels of resistance (Duan et al., 2019). Among 34 *C. cassiicola* isolates sampled from soybean in Brazil, Teramoto et al. (2017) reported $EC_{50}$ < 0.16 µg/mL for 10 isolates, considering them as sensitive to pyraclostrobin. Only one isolate was considered as highly non-sensitive with an $EC_{50}$ = 36.55 µg/mL. Additionally, 14 isolates exhibited $EC_{50}$ > 28 µg/mL to azoxystrobin, 21 isolates exhibited $EC_{50}$ > 28 µg/mL to trifloxystrobin, and 10 isolates with $EC_{50}$ > 28 µg/mL to picoxystrobin. All of them were considered as highly non-sensitive to QoI fungicides (Teramoto et al., 2017). According to Brent and Hollomon (2007), fungicides belonging to the same chemical group can show cross-resistance for *C. cassiicola* within different QoI fungicides, which Duan et al. (2019) also reported. All four *C. cassiicola* isolates with the G143A mutation reported in Rondon and Lawrence (2019) exhibited statistically higher $EC_{50}$ values for pyraclostrobin ($EC_{50}$ > 50 µg/mL) and the combination of fluxapyroxad + pyraclostrobin ($EC_{50}$ > 0.73 µg/mL) in this study. The high values of $EC_{50}$ for *C. cassiicola* isolated from soybean ($\bar{X}$ = 48.22 µg/mL) found in this study suggest loss of sensitivity to pyraclostrobin in Alabama, U.S. but not for *C. cassiicola* isolated from cotton ($\bar{X}$ = 12.50). Fungicides applications to control diseases is a common practice in soybean fields, but the same practice was not common on cotton fields until recently. This can be observed by the $EC_{50}$ values of *C. cassiicola* from cotton and soybean to pyraclostrobin. $EC_{50}$ values > 100 µg/mL were found for azoxystrobin on *C. cassiicola* isolates from tomato in Florida (MacKenzie et al., 2020). Consequently, QoI fungicide applications to control *C. cassiicola* was reduced, aiming to limit the spread of resistance development in tomato fields in Florida (MacKenzie et al., 2020).

The same *C. cassiicola* QoI-resistant isolates (LIM14, ELM04, ELM06, and ELM07) with the highest $EC_{50}$ values to pyraclostrobin, exhibited the highest values of $EC_{50}$ to the combination fluxapyroxad + pyraclostrobin. However, $EC_{50}$ values to the fungicide mixture were < 1.5 µg/mL in our study. Teramoto et al. (2017) reported the sensitivity of *C. cassiicola* soybean isolates to fluxapyroxad (SDHI fungicide) with $EC_{50}$ < 1 µg/mL for the majority of the isolates (85%); however, 3 isolates exhibited $EC_{50}$ > 91 µg/mL to fluxapyroxad and one isolate with $EC_{50}$ > 100 µg/mL to boscalid. These isolates were classified as highly non-sensitive to SDHI fungicides. Zhu et al. (2019) reported sensitive isolates with $EC_{50}$ = 0.92 to 2.12 µg/mL, and highly-resistant isolates with $EC_{50}$ > 50 µg/mL for boscalid. Fungicide resistance in *C. cassiicola* has developed in a short period of time when SDHI was used as a sole fungicide, causing severe problems in the disease management (Zhu et al., 2019). As single-site respiration inhibitors, SDHI fungicides are considered to be at moderate to high risk of resistance, analogous to the QoI fungicides (FRAC, 2020). Our results suggest that the combination of fungicides with different modes of action provide an adequate control of the pathogen but SDHI should not be used as a sole fungicide. Fungicide mixtures are recommended to prevent the development of fungicide resistance (Brent and Hollomon, 2007; FAO, 2012; Ghini and Kimati, 2000).

Multisite fungicides are classified with a low risk of development of fungicide resistance, and dithiocarbamates fungicides (mancozeb, maneb and propineb) are among them (Brent and Hollomon, 2007; FRAC, 2020). Multisite activity fungicides are widely used because of their broad spectrum of disease control, and are known to have a protectant and non-systemic effect on plants. Thus, they cannot control pathogens after their establishment on plants (Hollomon, 2015). Even with extensive use over the years, resistance development has not been an issue for protectant fungicides since their mode of action acts in different sites (multisite) on the target pathogen, and

hence it will interfere on many metabolic processes of the pathogen (Hollomon, 2015; Ishii and Hollomon, 2015). These fungicides are a good fit to be used in combination with another mode of action fungicide, usually a systemic fungicide to reduce the selection pressure on one fungicide, and inhibit the growth of resistant populations (FAO, 2012). According to MacKenzie et al. (2018), the control of *C. cassiicola* on tomatoes in Florida relies on constant applications of protectant fungicides which avoid $3.5 million in potential revenue lost in fields without protectant fungicides applications. Here, we demonstrated that the majority of *C. cassiicola* isolates exhibited $EC_{50}$ < 10 µg/mL, with two of them (17%) with $EC_{50}$ > 12 µg/mL for mancozeb. *Cercospora* species from soybean in Argentina exhibited $EC_{50}$ > 10 µg/mL to the fungicide mancozeb (Sautua et al., 2020). Torres-Calzada et al. (2015) studying sensitivity of *Colletotrichum truncatum* to mancozeb found only two isolates with intermediate resistance ($EC_{50}$ > 20 µg/mL), while 90% of the isolates were found to be sensitive to mancozeb ($EC_{50}$ < 10 µg/mL). The use of alternative fungicide classes such as multisite fungicides could be an additional method of reducing selection pressure on populations of *C. cassiicola*.

The fungicides studied here have different mechanisms of action, thus there is no reason of having cross-resistance relationship with fungicides that are not closely related to their mechanism of action (Brent and Hollomon, 2007); however, it seems that *C. cassiicola* QoI-resistant isolates always exhibited higher $EC_{50}$ values for other fungicides, other than thiophanate-methyl. Further investigations are necessary to confirm this trend. The fitness of fungicide-resistant isolates was described as important to develop helpful anti-resistance strategies because the competitive ability of these isolates defines their persistence in the fungal population when there is no fungicide selection pressure (Ishii, 2015). The identification of characteristics associated with resistant isolates is essential for fungicide resistance risk assessment (Ma et al., 2018). No correlation was

observed between the mycelial growth and sensitivity to pyraclostrobin, with no clear separation of QoI-resistant and -sensitive isolates. These results suggest that there is no fitness penalty of *C. cassiicola* isolates from cotton and soybean associated with resistance to pyraclostrobin based on mycelial growth. Investigations are still needed on different resistance mechanisms that *C. cassiicola* might express to other fungicides groups. Torriani et al. (2017) emphasized that extra research is required to know the possible fitness cost associated with fungicide resistance. Zhang and Bradley (2017) found no difference between QoI-resistant and –sensitive isolates of *Cercospora sojina* for sporulation and radial growth. Previous studies with *Phytophthora capsici* mutants carrying the G137R mutation exhibited an equal fitness compared with sensitive isolates. These mutants can remain competitive and under selection pressure by azoxystrobin might occupy a dominant position in the field population (Ma et al., 2018).

In general, fungicides can effectively control sensitive but not resistant pathogens populations, which will be predominant in the field population over time (Ma and Michailides, 2005). The management of fungicide resistance cannot prevent the evolution of resistance in fungal pathogen populations, but it can decrease the occurrence of new cases of resistance, and delay resistance development to preserve the efficacy of fungicides (FAO, 2012). An increase in use of fungicides to control diseases on cotton and soybean will increase the selection pressure toward a shift in reduced sensitivity for *C. cassiicola* and other foliar pathogens. Therefore, it is important to follow strictly anti-resistance strategies and promote alternative disease management practices (Brent and Hollomon, 2007). To avoid the rapid development of *C. cassiicola* populations non-sensitive to fungicides, single-site fungicides should be applied in combination with fungicides that have different modes of action, and the number of applications should be limited for each crop cycle (Ghini and Kimati, 2000). Additionally, we recommend that

applications with QoI fungicides should be avoided when not combined with another mode of action in areas where resistant populations have been reported.

The importance of baseline data for fungal pathogens was stated by Russell (2004) as essential to explain shifts in sensitivity, and further to provide evidence that resistant populations were responsible for the disease control failures. Our study characterized $EC_{50}$ values of *C. cassiicola* isolates for five fungicides on cotton and soybean in the United States, and these values can be used as a reference for further studies. Furthermore, it is imperative to develop disease-resistant varieties, use crop rotation, and even possible biological control options. These strategies will complement the management of target spot in the field in combination with chemical control, prolonging the life expectancy of fungicides.

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**Table 4.1** Description of commercial formulation of fungicides tested in this study.

| Fungicide classification | Active ingredient (%) | Commercial product | Manufacturer |
|---|---|---|---|
| MBC Thiophanate (Group 1) | Thiophanate-methyl 45% | Topsin 4.5 FL | UPL |
| DMI Triazole (Group 3) | Prothioconazole 41.0% | Proline 480 SC | Bayer CropScience |
| QoI Strobilurin (Group 11) | Pyraclostrobin 23.6% | Headline EC | BASF |
| Dithiocarbamate (Group M3) | Mancozeb 58.1% | Manzate Pro-Stick | UPL |
| SDHI Carboxamides (Group 7) + QoI Strobilurin (Group 11) | Fluxapyroxad 14.33% + Pyraclostrobin 28.58% | Priaxor 500 SC | BASF |

**Table 4.2** Sensitivity of *Corynespora cassiicola* isolates obtained from symptomatic cotton and soybean leaves to five fungicides.

| Isolate | Origin | $EC_{50}$ (µg/mL)[x] | | | | |
|---|---|---|---|---|---|---|
| | | Thiophanate-methyl | Prothioconazole | Pyraclostrobin | Mancozeb | Fluxapyroxad + Pyraclostrobin |
| BRW03 | Cotton | 0.81 bc | 0.72 bc | 4.61 d | 6.05 bc | 0.41 cdef |
| FHP01 | Cotton | 1.06 bc | 0.50 c | 9.73 d | 4.96 c | 0.57 cde |
| FHP22 | Cotton | 0.98 bc | 1.50 b | 18.50 cd | 4.20 c | 0.25 ef |
| HSV01 | Cotton | 0.57 c | 0.37 c | 12.32 cd | 5.86 bc | 0.14 f |
| HSV12 | Cotton | 0.63 c | 0.34 c | 6.60 d | 7.43 bc | 0.29 def |
| MAC01 | Cotton | 0.90 bc | 0.32 c | 23.24 bcd | 9.31 bc | 0.52 cde |
| ELM04[y] | Soybean | 0.58 c | 0.52 c | 75.50 a | 9.24 bc | 1.08 a |
| ELM06[y] | Soybean | 0.71 c | 0.87 bc | 59.02 ab | 11.85 b | 1.00 ab |
| ELM07[y] | Soybean | 0.64 c | 0.49 c | 49.96 abc | 8.21 bc | 1.28 a |
| LIM02 | Soybean | 1.99 a | 0.43 c | 14.51 cd | 5.44 bc | 0.59 cd |
| LIM13 | Soybean | 0.72 c | 0.31 c | 13.85 cd | 9.97 bc | 0.47 cde |
| LIM14[y] | Soybean | 1.55 ab | 2.73 a | 76.48 a | 18.63 a | 0.73 bc |
| **Mean** | | 0.93 | 0.76 | 30.36 | 46.38 | 0.61 |
| **CV (%)[z]** | | 53.40 | 63.47 | 76.93 | 46.38 | 31.66 |
| **F value** | | 6.12 | 17.23 | 10.75 | 8.17 | 26.30 |

[x] LS-mean of $EC_{50}$ values (estimated fungicide concentration that inhibited 50% of the mycelial growth) followed by the same letter in the columns were not significantly different in Tukey's HSD test ($P < 0.05$).

[y] Isolates with G143A mutation that confers resistance to QoI fungicides (Rondon and Lawrence, 2019).

[z] Coefficient of variation.

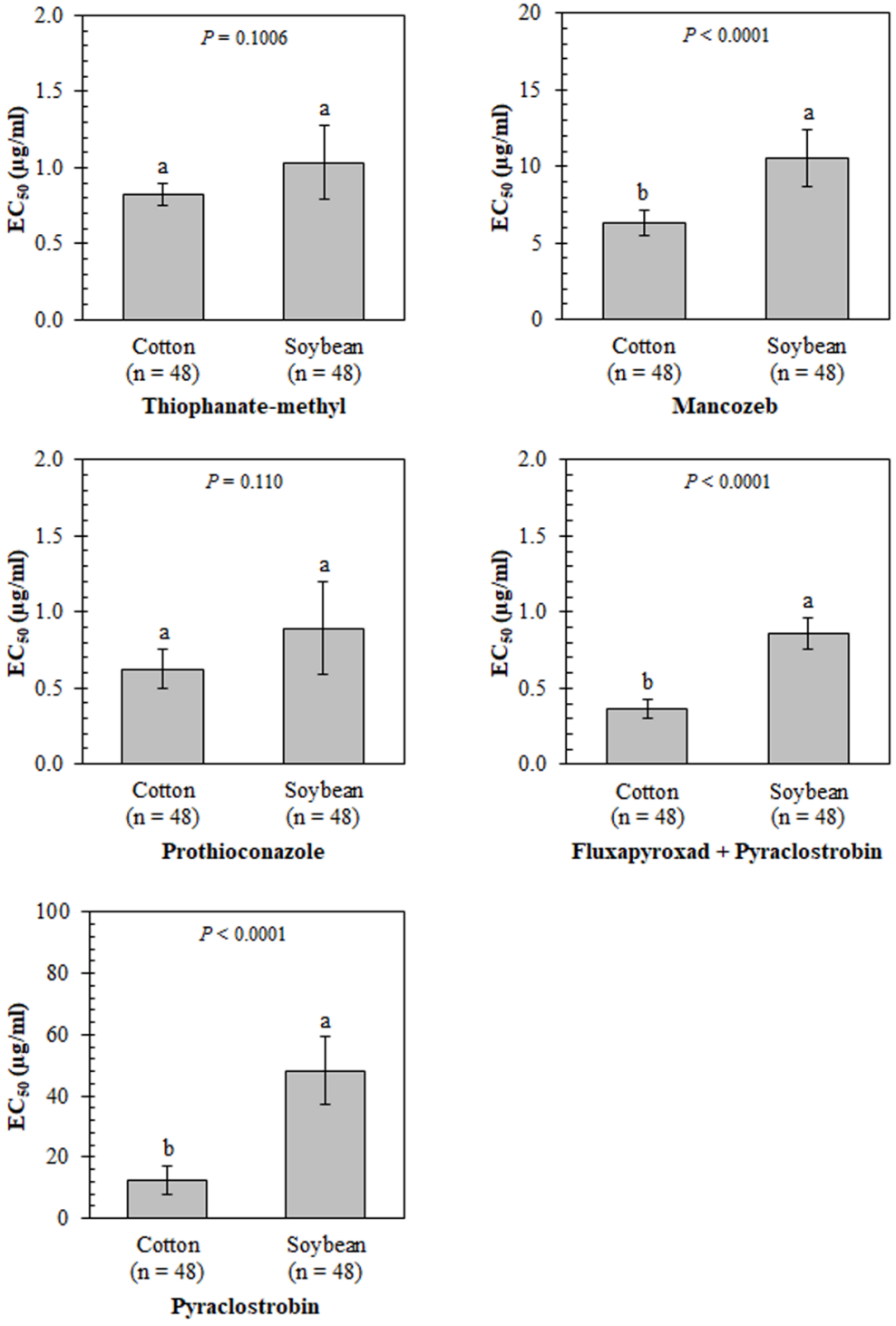


**Figure 4.1 $EC_{50}$ values of *Corynespora cassiicola* isolates for each fungicide, separated by origin of isolation (cotton or soybean).** Data represent means of replicate samples, vertical bars indicate 95% C.L., and *n* the sample size. Bars labeled with different letters with each origin of isolation are significantly different according to two-sample Student's t-test ($\alpha = 0.05$).

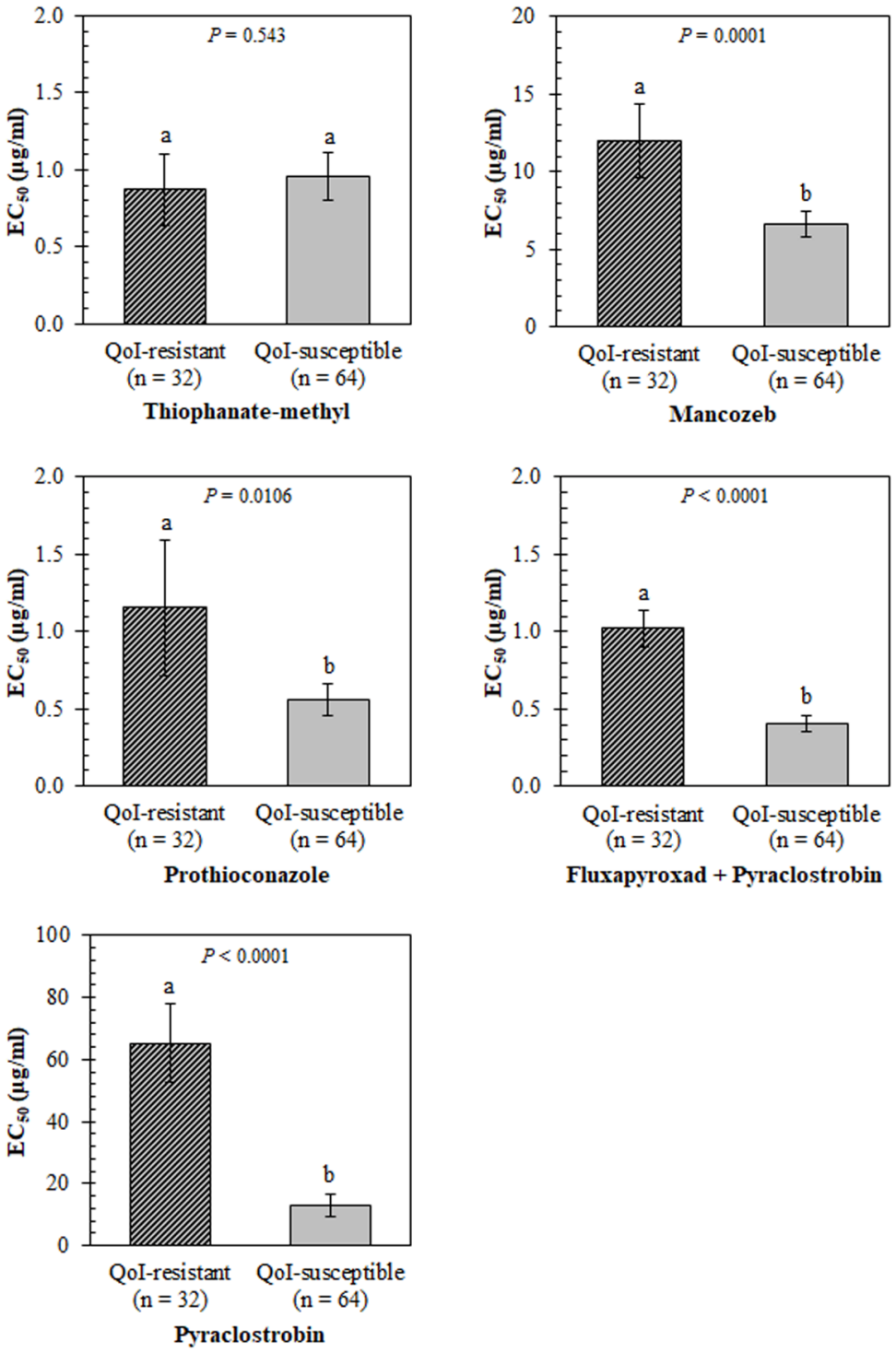


**Figure 4.2 $EC_{50}$ values of *Corynespora cassiicola* isolates for each fungicide, separated by the reaction to the QoI fungicide.** QoI-resistant represent isolates detected with G143A in the cytochrome *b* gene (bars with diagonal stripes), and QoI-susceptible represent isolates with C143A (wild type) (Rondon and Lawrence, 2019). Data represent means of replicate samples, vertical bars indicate 95% C.L., and *n* the sample size. Bars labeled with different letters with each origin of isolation are significantly different according to two-sample Student's t-test ($\alpha = 0.05$).

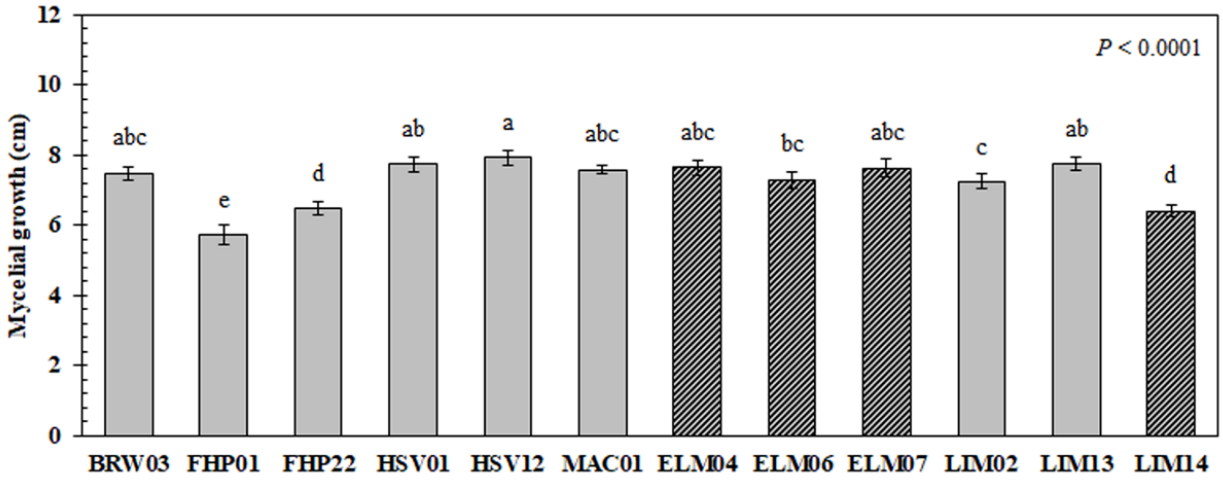


**Figure 4.3 Mycelial growth on fungicide-free PDA of *Corynespora cassiicola* isolates.** QoI-resistant isolates are highlighted with diagonal stripes inside the bars, while QoI-sensitive are represented by gray bars only. Data represent means of replicate samples ($n$ = 28), and vertical bars indicate 95% C.L. Bars labeled with different letters are significantly different according to Tukey's HSD test ($\alpha = 0.05$).

# Chapter 5. *Corynespora cassiicola* isolates from soybean in Alabama detected with G143A mutation in the cytochrome *b* gene


## Abstract

*Corynespora cassiicola* C.T. Wei is a widespread plant pathogenic fungus that causes target-shaped necrotic spots on plant leaves and on stems, roots, flowers, and fruits and has been recorded worldwide on up to 400 plant species. The disease is known as Corynespora leaf spot or target spot on cotton and soybean. A single DNA fragment (600 bp) was amplified using described primers, and based on cytochrome *b* gene nucleotide sequences, four out of 12 isolates of *C. cassiicola* were found to have a mutation that replaces the codon 143 from GGT to GCT, resulting in an amino acid change from glycine to alanine (G143A). All four isolates were sampled from soybean plants located in north (LIM14) and central (ELM04, ELM06, and ELM07) Alabama. No other point mutation on cytochrome *b* gene was found for six *C. cassiicola* isolates sampled on cotton (BRW03, MAC01, FHP01, FHP22, HSV01, and HSV12) or on other two isolates sampled on soybean (LIM02 and LIM13). Other known mutations were found in our isolates. According to the Fungicide Resistance Action Committee, *C. cassiicola* rapidly developed resistance to fungicides and is an example of a pathogen that must be classified as a high risk of developing resistance to a different fungicide class. To the best of our knowledge, the present study is the first to report a G143A mutant in *C. cassiicola* from field populations in the United States.

## 1. Introduction

*Corynespora cassiicola* (Berk. & M.A. Curtis) C.T. Wei is a widespread plant pathogenic fungus causing target necrotic spots on plant leaves, stems, and fruits, is worldwide on up to 400 plant species, and known as Corynespora leaf spot or Target spot on cotton and soybean. In the southeastern U.S. genetically distinct populations of *C. cassiicola* were found causing target spot epidemics on cotton and soybean (Sumabat et al., 2018).

Disease management has been a concern due to increasing occurrence of target spot (Godoy, 2015). Severe disease symptoms and significant yield losses occur with this pathogen, especially during rainy seasons. Yield losses of 18 to 32% have been documented on soybeans in the U.S. (Godoy, 2015). Fungicides have been a crucial tool in disease management; however, there are reported cases of *C. cassiicola* isolates from tomato, cucumber, and soybean resistant to systemic fungicides (Fungicide Resistance Action Committee – FRAC, www.frac.info/publications). *Corynespora cassiicola* is considered a high-risk pathogen for development of fungicide resistance (FRAC, 2019), and mutations associated with QoI-resistance have been detected in the cytochrome *b* (*cytb*) gene based on three amino acid substitutions: G143A, F129L, and G137R (Duan et al., 2019). G143A mutation has been characterized for *Cercospora sojina* (Mathew et al., 2019) in the U.S. but not for *C. cassiicola*.

Fungicides are used to control cotton and soybean diseases; QoI-resistant populations of *C. cassiicola* might be present in the U.S. For this reason, the objective was to assess the occurrence of point mutations in the *cytb* gene associated with QoI-resistance from Alabama isolates of *C. cassiicola*.

## 2. Material and methods

Symptomatic leaves were randomly collected from cotton and soybean plants (Figure 5.1A-B) across Alabama in 2017/2018. Isolates of *C. cassiicola* were obtained by direct isolation on PDA containing 0.005% kanamycin. PDA plates were incubated at room temperature (RT, 25 ± 2°C), then pure colonies were obtained to establish the *C. cassiicola* Alabama collection (Figure 5.1C-D). All isolates were identified as *C. cassiicola* based on conidiophore and conidia morphology (Figure 5.1E-F) and ITS sequencing (ITS1/ITS4).

Total DNA was extracted using a ZR Fungal/Bacterial MiniPrep™ kit. To identify nucleotide point mutation on the *cytb* gene, fragments were amplified from total DNA using PCR primers described by Duan et al. (2019). Purified PCR products were Sanger sequenced by Eurofins MWG Operon LLC (Louisville, KY), and nucleotide sequences were edited and aligned using BioEdit Alignment Editor (Tom Hall, Ibis Biosciences). Sequences were deposited in GenBank under accession numbers MN564884-MN564895. QoI-sensitive (C6-2) and QoI-resistant (ST-20S-1) sequences of *C. cassiicola* (Ishii et al. 2007) were included to illustrate the nucleotide point mutation.

## 3. Results

A single DNA fragment (600 bp) was amplified using described primers, and based on *cytb* nucleotide sequences, 4 out of 12 isolates of *C. cassiicola* were found to have a mutation that replaces the codon for amino acid 143 from GGT to GCT, resulting in an amino acid change from glycine to alanine (G143A) (Figure 5.2). These isolates originated from soybeans located in north (LIM14) and central (ELM04, ELM06, ELM07) Alabama (Figure 5.3). The G143A mutation was not found on isolates of *C. cassiicola* from cotton plants. No other point mutations on *cytb,* such as F129L and G137R, were found in our isolates.

## 4. Discussion

According to (FRAC, 2019), the short development of resistance to different fungicide class of *C. cassiicola* on soybean is one example of a pathogen that must be classified as a threatening. The amino acid change on *cytb* from glycine to alanine at position 143 (G143A) is known to confer high levels of resistance to QoI fungicides, while F129L and G137R mutations are responsible for low to moderate levels of resistance (Duan et al. 2019).

QoI fungicides have a single-site mode of action and are extensively applied to manage multiple diseases in field crops. Given the high-risk of *C. cassiicola* to develop fungicide resistance (Duan et al., 2019; Ishii et al., 2007), the management of fungicide resistance will be a major challenge. Knowing that field populations of *C. cassiicola* have mutations associated with QoI-resistance, it will be necessary to monitor the spread of resistant isolates to manage resistance development. To the best of our knowledge, this study is the first to report G143A mutants in *C. cassiicola* from field populations from soybean in the U.S.

## 5. Literature cited


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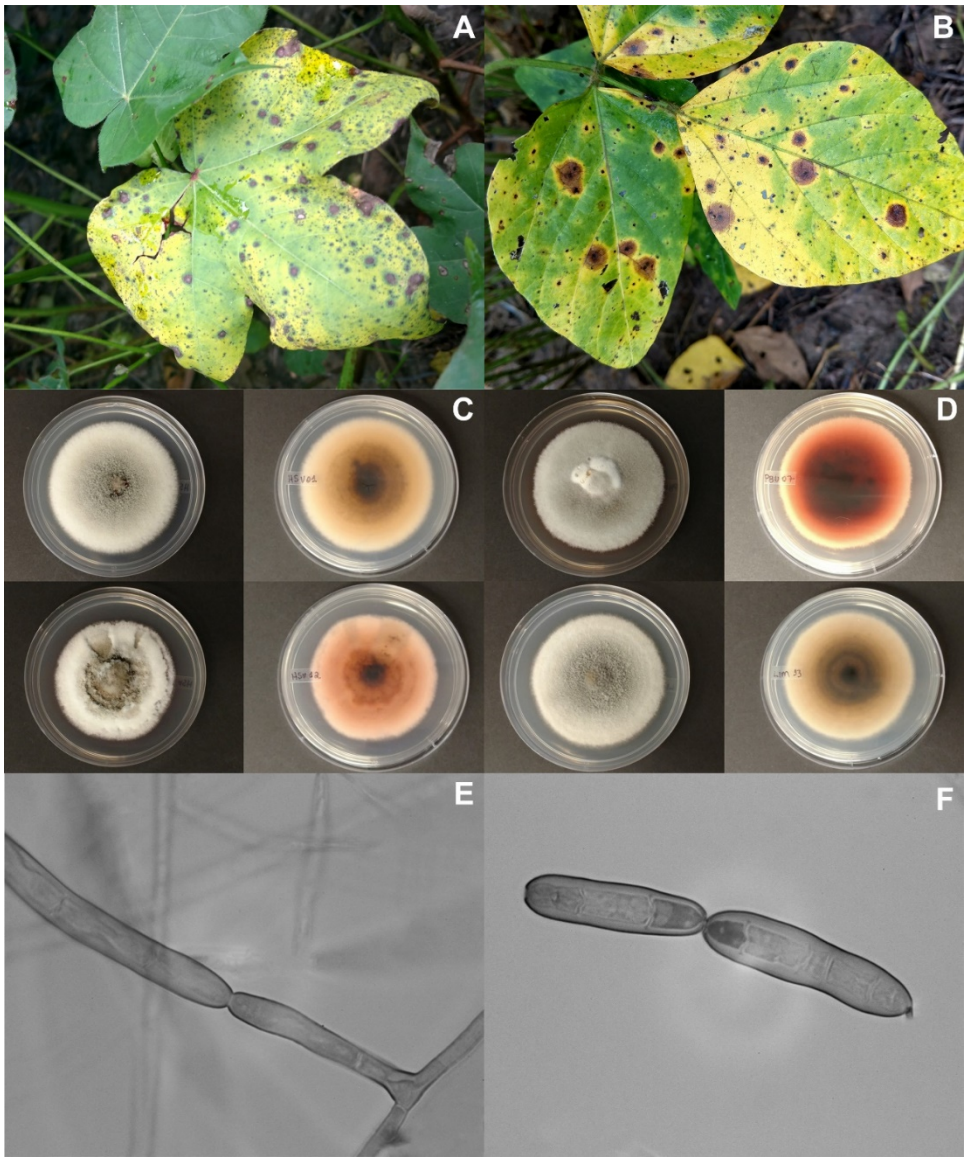


**Figure 5.1 Target spot and its causal agent, *Corynespora cassiicola*.** Disease symptoms on cotton (A) and soybean (B) leaves; Top and bottom of PDA plates with pure colonies of *C. cassiicola* obtained from cotton (C) and soybean (D) to establish a new collection from Alabama; Detail of the *C. cassiicola* conidiophore (E) and conidia (F) morphology.

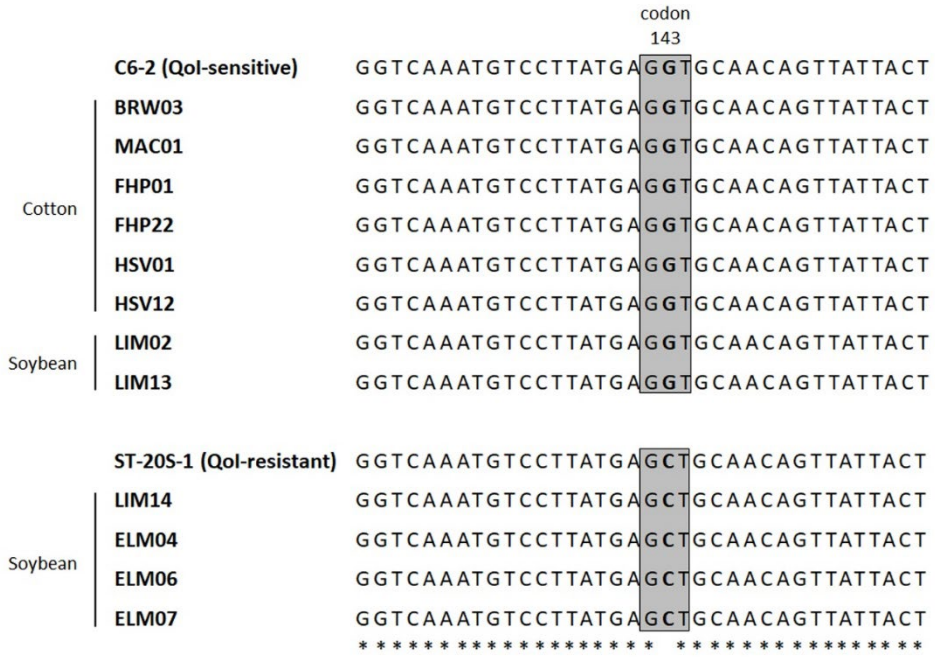


**Figure 5.2 Partial nucleotide sequences of the cytochrome *b* gene.** Twelve isolates of *Corynespora cassiicola* are represented, plus two isolates used as a reference, QoI-sensitive (C6-2) and QoI-resistant (ST-20S-1) from Ishii et al. (2007). Dark gray highlighted areas represent codon 143 and the amino acid substitution (G143A) that occurs as a result of the point mutation shown in bold (G**G**T → G**C**T).

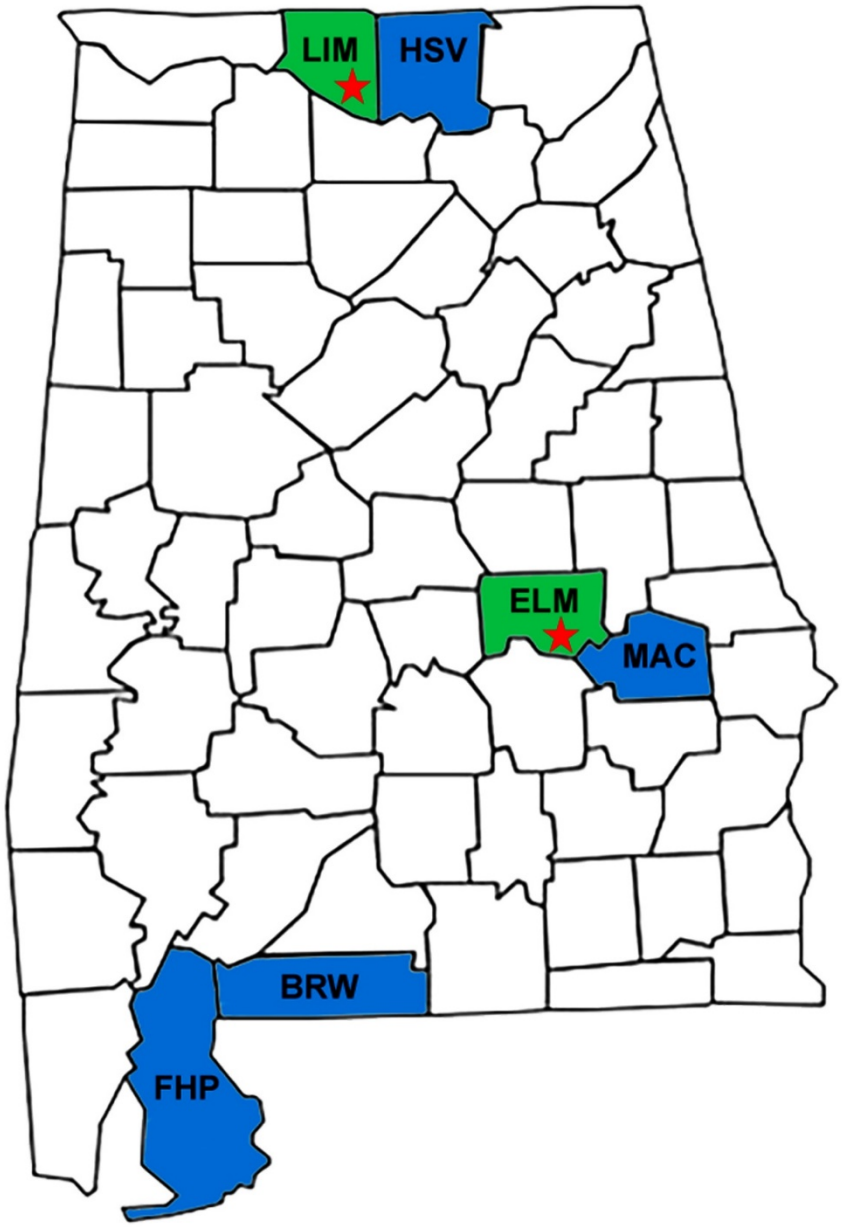


**Figure 5.3 Distribution map of the *Corynespora cassiicola* from Alabama U.S.** Isolates were sampled from cotton (blue colored) and soybean (green colored). Abbreviations on the map represent the initial names of the isolates sampled. Red stars represent sampled locations where isolates were found with the G143A mutation.