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Monografia apresentada à Universidade Federal de Lavras, como parte das exigências do Curso de Agronomia, para a obtenção do título de Bacharel.

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Dedico.

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ABSTRACT

Fungicide application is the main tool used to control foliar diseases of soybean. Depending on the mode of action, fungicides are considered to have a moderate to high risk for resistance development. Fungicide resistance is defined as the naturally occurring ability of an individual in a pathogen population to survive a fungicide treatment that would normally have effective control. This study aimed to screen populations of *Corynespora cassiicola* to monitor the emergence of fungicide resistance in Arkansas soybean fields. Symptomatic leaves with target spot were sampled from fields in six Arkansas counties. Leaf tissue was disinfected using 70% ethanol for 3 min and plated on acidified potato dextrose agar (APDA) summing 24 isolates of *C. cassiicola.* The collection of *C. cassiicola* isolates was used for an *in vitro* evaluation for fungicide resistance using poison plate method. For *In vitro* fungicide testing, clean isolates were cultured on potato dextrose agar (PDA) and maintained in an incubator at 26°C for seven days. A mycelium growth assay was used to calculate EC_{50} values (the effective concentration of fungicide that reduces mycelial growth by 50%) to the commercial-grade of azoxystrobin (22.9% a.i.; Syngenta) with doses ranging from 0 to 10 mg/L. Salicyhydroxamic acid (SHAM) was added in all doses to inhibit the alternative oxidase pathway. A 3.7mm plug was transferred to the center of the Petri plate (mycelium side down) with a specific fungicide concentration. Tests were conducted in an incubator with the temperature at 26°C in dark. The plates were completely randomized and designed with three replicates for each fungicide concentration. Each plate was an experimental unit, and plates were evaluated after five days. The colony diameters were averaged together, and the mean colony diameter was calculated. Analysis of the average colony diameter was analyzed with a log-logistic model in R (R version 4.1.0) using the Dose-Response Curve (DRC) program. All isolates had uniform reductions in mycelial growth, but no isolates were severely inhibited. The effective concentration for reducing 50% of the mycelium growth ranged from ≤ 0.001 to 10.0 mg/L ($\bar{X}$ = 1.88 mg/L, median = 0.85 mg/L) Based on the results, *C. cassiicola* isolates show lower sensitivity to QoI. All isolates were sequenced using specific primers for the *cytb* gene to detect the potential mutations associated with reduced sensitivity. Eighteen out of twenty-four isolates showed a mutation in the codon G143A changing the amino acid from glycine to alanine coffering resistance to QoI. Ongoing monitoring of fungicide resistance will aid the development of a resistance management program in Arkansas, which could help to address production challenges in the future.

Words keys: azoxystrobin; *Corynespora cassiicola;* fungicide resistance; soybean

RESUMO

A aplicação de fungicida é a principal ferramenta utilizada no controle de doenças foliares da soja. Dependendo do modo de ação, os fungicidas são considerados de risco moderado a alto para o desenvolvimento de resistência. A resistência a fungicidas é definida como a capacidade natural de um indivíduo em uma população de patógenos de sobreviver a um tratamento fungicida que normalmente teria um controle eficaz. Este estudo teve como objetivo rastrear populações de *Corynespora cassiicola* para monitorar a emergência de resistência em campos de soja do Arkansas. Folhas sintomáticas com mancha-alvo foram amostradas de campos em seis condados do Arkansas. O tecido foliar foi desinfetado com etanol 70% por 3 min e semeado em ágar batata dextrose acidificada (APDA) somando 24 isolados de *C. cassiicola*. A coleta de isolados de *C. cassiicola* foi utilizada para avaliação *in vitro* da resistência a fungicidas pelo método "poison". Para o teste de fungicida *in vitro*, isolados limpos foram cultivados em ágar batata dextrose (PDA) e mantidos em uma incubadora a 26°C por sete dias. Um ensaio de crescimento miceliar foi usado para calcular os valores de EC_{50} (a concentração efetiva de fungicida que reduz o crescimento de micélio em 50%) para o grau comercial de azoxistrobina (22,9% a.i.; Syngenta) com doses variando de 0 a 10 mg/L. O ácido salicihidroxâmico (SHAM) foi adicionado em todas as doses para inibir a via alternativa da oxidase. Um plugue de 3,7 mm foi transferido para o centro da placa de Petri (lado do micélio para baixo) com uma concentração específica de fungicida. Os testes foram conduzidos em uma incubadora com temperatura de 26°C no escuro. As placas foram inteiramente casualizadas e desenhadas com três repetições para cada concentração de fungicida. Cada placa era uma unidade experimental, e as placas foram avaliadas após cinco dias. Os diâmetros das colônias foram calculados em média juntos, e o diâmetro médio da colônia foi calculado. A análise do diâmetro médio da colônia foi analisada com um modelo log-logístico em R (R versão 4.1.0) usando o programa Dose-Response Curve (DRC). Todos os isolados tiveram reduções uniformes no crescimento micelial, mas nenhum isolado foi severamente inibido. A concentração efetiva para reduzir 50% do crescimento do micélio variou de ≤ 0,001 a 10,0 mg/L ($\bar{X}$ = 1,88 mg/L, mediana = 0,85 mg/L) Com base nos resultados, isolados de *C. cassiicola* apresentam menor sensibilidade à QoI. Todos os isolados foram sequenciados usando primers específicos para o gene cytb para detectar as potenciais mutações associadas à sensibilidade reduzida. Dezoito dos vinte e quatro isolados apresentaram uma mutação no códon G143A alterando o aminoácido de glicina para alanina, conferindo resistência a QoI. O monitoramento contínuo da resistência a fungicidas ajudará no desenvolvimento de um programa de gerenciamento de resistência no Arkansas, que pode ajudar a enfrentar os desafios de produção no futuro.

Palavras chaves: azoxystrobina, *Corynespora cassiicola*, Resistência à fungicidas, soja

SUMMARY

1. Literature Review

1.1 Overview

Soybean [Glycine max (L.) Merrill] is considered the most important oilseed and protein crop worldwide. Botanically, soybean is an annual legume plant in the Family Fabaceae (Hymowitz, 2008). It has been known that the use of domesticated soybeans could be as early as 2500-2300 B.C., though the first historical evidence places the emergence of soybean as a food crop in Northeastern China around 1700-1100 B.C. (Hartman et al. 2011). Today, most of the world's soybean is processed or crushed into soybean meal and oil. In World markets and trade, Argentina, Brazil and the United States are the main producers with around 362,947 million tons and a planted area of 127,842 million hectares (USDA, 2021). In 2020, the value of U.S. soybean exports to the world reached a record $25.7 billion, up nearly 40 percent ($7 billion) by value and up 23 percent (11.9 million tons) by volume from the prior year (USDA, 2021).

The worldwide importance of soybean pathogens and pests can directly affect the production and be responsible for yield losses. Unfavorable environments such as drought, light, temperature, humidity, and nutritional conditions can affect plant growth and development making the plants more susceptible to diseases or insect attacks. Diseases caused by biotic agents such as bacteria, fungi, and nematodes can be transmitted from an infected to a healthy plant and can cause diseases when conditions are favorable for infection and colonization (Hartman et al. 2011; Edwards Molina et al. 2022). For instance, biotic diseases in soybean production could reach an estimated loss of 59.9 million metric tons as reported in 2006 from the top eight soybean-producing countries in the world. This means that 28% of the crop was lost because of diseases (Hartman et al. 2016).

Some soybean diseases under conducive environmental conditions can cause major yield losses, however, these conditions vary from year to year. For example, severe losses caused by stem canker (caused by *Diaporthe aspalathi*, Castle. & Crous and *D. caulivora* [Athow & Caldwell] Santos, Vrandecic & Phillips) occurred frequently during the early 1980s due to the use of commercial cultivars lacking genetic resistance to the pathogen (Backman et al. 1985; Bradley et al. 2021). In 2012 and 2013, soybean rust was reported to caused approximately 40 to 60% yield losses in some soybean fields in Alabama (US) with estimated economic losses were upward of

$135,000 due to the lack timely fungicide applications (Delaney et al. 2018). Certain diseases are often observed in soybean plants but do not cause significant yield reduction. For instance, until 2018 the damage associated with target spot, caused by the ascomycete *Corynespora cassiicola,* was considered a minor issue. However, this scenario has changed in the last five years, nowadays, target spot is responsible for more than 500 bushels lost with an estimated economic loss of $5,000,000 (Bradley et al. 2021; Crop Protection Network, 2021).

In 1045, target spot was reported as a disease on soybean in North America. In this first moment, was reported as *Helminthosporium vignae* by Olive and designated as the pathogen of cowpea (Vigna sinesis (L.) Endl.) and soybean in the United States (Olive, 1945). In 1950, the pathogen was reclassified as *Corynespora cassiicola* (Berk. & Curt.) C.T. Wei (Wei, 1950). Target spot has been a concern for farmers, redoubling efforts on research focused on the pathogen due to its increasing occurrence, especially in soybean fields, and causing several high economic losses on crops (Godoy, 2015; Rondon, 2019). Several strategies have been proposed to improved control such as effective fungicides, crop rotation, and tolerant varieties. Currently, there are no resistance genes for target spot identified in soybean, hence no target spot resistant cultivars have been identified (Toulet et al., 2022). Therefore, identification of germplasm resistant to *C. cassiicola* isolates has been unsuccessful (Teramoto et al, 2013; Rondon, 2019). Without any target spot resistant cultivar available, the indiscriminate use of fungicide results in high production costs and increased management of the fungicide resistance. Besides that, *C. cassiicola* presents a high risk of becoming resistant to fungicides (FRAC, 2019). Since the disease becomes more significant in soybean, there is a rising need for more research on the biology and management of this phytopathogenic fungus.

1.2 Symptoms and Signs

Although target spot can affect stems, pods, seeds, hypocotyls, and roots, the most common symptoms in on the leaves. Leaf lesions are round to irregular in shape, reddish-brown in color, and from specks to mature spots (10 – 15 mm or more in diameter) (Godoy 2015). Lesions are frequently surrounded by dull-green or yellowish-green halos (Godoy 2015; Edwards Molina et al. 2022). Due to concentric halos formed on the leaf lesions, the disease was designated as "target spot" (Edwards Molina et al. 2022). One key factor about target spot epidemics is that symptoms are mostly observed in the lower part of the canopy (Faske 2016). However, conducive environmental conditions prevail - high relative humidity (>85%), and warm temperatures over a period of 5 to 7 days – symptoms can move up into the upper canopy and yield losses can prove to be significant (Faske 2016). Target spot is a characteristic example of the "light stealer" disease group because the coalescence of lesions can result in a decrease of the photosynthetic leaf area by the production of symptoms themselves in addition to leaf senescence that affects the diseases progression and occurs before soybean plants reaching physiological maturity (R8) (Boote et al. 1983). The damage to the photosynthetic leaf area is associated with a decrease in the plant's concentration of chlorophyll a and b (Fortunato et al. 2018)

Target spot can also infest areas on the pods, petioles, and stems in synergism with other foliar diseases that impact soybean production systems, such as frogeye leaf spot caused by *Cercospora sojina* and laboratory diagnosis analysis may be necessary to distinguish between the diseases. During long periods of rainfall and high humidity, spots may coalesce and cover the entire pod. In some cases, the pathogen penetrates the pod wall and produces small, blackish-brown lesions on the seeds (Godoy 2015).

While the symptoms associated with target spot at the field level are well-known, there can be some understated differences between symptom expression in the field and the symptoms present resulting from inoculation with fungal material in the greenhouse. In general, three different leaf lesion morphologies are observed as a result of *C. cassiicola* inoculations in greenhouse settings by using conidia for inoculations: a dark infection point surrounded by a chlorotic halo, a necrotic spot without a chlorotic halo, and brown-reddish specks restricted to the infection point (Edwards Molina et al. 2022)

1.3 Management

Fungicide applications for management of target spot are recommended either at the beginning pod (R3) and/or beginning seed (R5) stage with products that contained pre-mixes of fungicides with different modes of action. Field trials conducted in South America have shown the best control with a mixture of a QoI, DMIs, and SDHI (Edwards Molina et al. 2022). In Brazil, fungicides containing fluxapyroxad and prothioconazole have been found most efficient in controlling the disease and are recommended for susceptible cultivars (Godoy 2015; Edwards Molina et al. 2022; Faske 2016).

Management practices to reduce inoculum that consists of crop rotation using corn, grain sorghum, or rice. The pathogen overwinters in crop debris and the soil, producers should take into consideration this disease may be more problematic in fields with a history of the disease in cotton and soybean. Seed treatments and sowing seeds from disease-free plants could also help to manage the disease during the first weeks after planting (Madalosso 2017).

Variety selection is also important as some varieties are more susceptible to target spot than others (Faske 2016). Using most high-yielding soybean cultivars can help on compensating yield losses. Varieties that exhibited susceptibility and reduced yield to target spot should be avoided in fields with a history of target spot.

2. Introduction

Corynespora cassiicola (Berk. & M. A. Curtis) C. T. Wei the causal agent of target spot is an ascomycete fungus responsible for causing the diseases known as target spot on cotton and soybean plants. This fungus has been globally reported on more than 400 plant species including fruits, vegetables, grains, perennial crops, forestry, and various ornamental plants (Farr and Rossman, 2020) and the first report of target spot on soybean in the USA was in 1945 and a five year study on target spot at the Mississippi's Delta reported soybean yield losses ranging from 18 to 32% (Hartwing 1959). Target spot incidence and severity have been increasing possibly due to monoculture farming with the use of susceptible cultivars and optimal weather pattern favoring weather conditions for disease development (Edwards Molina et al. 2022; Avozani et al. 2014). Due to the increased incidence of target spots in soybean in the US, significant yield losses have been reported when the pathogen was not properly controlled (Bradley et al. 2021; Bowen et al. 2018). Without a cultivar with complete resistance to the target spot as a tool for the management of the disease, foliar fungicides may be used as an alternative to control target spot on cultivars with different levels of susceptibility (Edwards Molina et al. 2022; Duan et al. 2019).

Fungicides are the main tool used to control airborne pathogens such as *C. cassiicola* in soybean (Ma et al. 2020; Leadbeater et al. 2019). Farmers have had access to a range of effective chemicals that are active at low doses and provide a high level of disease control. The cost and difficulty of discovery and registration of new actives ingredients have led to a decreasing product pipeline. A gradually adverse regulatory environment has resulted in the withdrawal of many current active ingredients and the emergence of resistance to some of the most important classes of fungicides in many target pathogens is now compromising control (Lucas et al., 2015).

Depending on the mode of action, fungicides are considered to have a moderate to high risk for resistance development (Leadbeater et al. 2019). Fungicide resistance is defined as the naturally occurring inability of an individual in a pathogen population to survive a fungicide treatment that would normally have effective control (Leadbeater et al. 2019). Repeated application of fungicides and movement of resistant individuals over short and/or long distances are responsible to increase the fungicide resistance season by season (Leadbeater et al. 2019).

Quinone outside inhibitor (QoI, FRAC Code 11) is one of the fungicide groups commonly used to manage foliar diseases in soybean. Strobilurin fungicides are used widely to control many ascomycetes, basidiomycetes, and oomycetes. Strobilurin broad spectra of activity and their widespread usage have exerted significant selection pressure on many plant pathogen populations. The mode of action of QoI is by blocking the transfer of electrons at the Quinone "outside" site of the *cytochrome bc1 complex*. Molecular variation in the target site is one likely explanation that has been confirmed for QoI fungicides, which are themselves natural products of certain basidiomycete fungi. Alternatively, there may be redundancy in the protein target due to the presence of additional copies of the encoding gene (Lucas et al. 2015). The mechanisms of resistance are based mostly on mutations that occur in the *cytochrome b* gene, most frequently the G143A mutation, a Glycine (Gly) to Alanine (Ala) change in the position 143, but also the F129L mutation, a Phenylalanine (Phe) to Leucine (Leu) change at position 129, and G137R mutation, a Glycine (Gly) to Arginine (Arg) (Rondon & Lawrence, 2019; Sierotzki et al., 2007; Sierotzki & Stammler, 2019).

The use of fungicides continues rising to control diseases in soybean and monitoring the sensitivity of *C. cassiicola* to common foliar fungicides, especially QoIs, has not been determined in Arkansas. The effort will focus on using an *in vitro* method for monitoring *C. cassiicola* populations and establishing the effective concentrations that reduced 50% growth (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 control management strategies are effective (Bolton et al. 2013; Rondon and Lawrence 2019; MacKenzie et al. 2020a). Therefore, the objectives of this study were (i) to monitor the emergence of fungicides resistance against QoIs in Arkansas soybean fields and (ii) to determine if there is a fitness penalty in *C. cassiicola* isolates with the G143A mutation in the cytochrome b gene.

3. Materials and Methods

3.1 Collection and fungal isolation

A total 24 of *Corynespora cassiicola* were collected from six different Arkansas counties (Crawford, Desha, Le, Mississippi, St. Francis, and Washington). Isolations were isolated as singles spores from sporulating leaf lesions or by plating surface-sterilized symptomatic leaves and seeds on a general isolation media, consisting of acid potato dextrose agar (APDA; 12.0 g/L of agar, 20.0 g/L of dextrose, 4.0 g/L of potato) containing 75 mg/L of streptomycin sulfate and 1.5 ml of 10% acid lactic acid.

All *C.* cassiicola isolates were identified based on their morphology, containing obclavate to cylindrical pseudo septate conidia with a highly distinctive hilum on its end, or at the point of conidiophore attachment (MacKenzie et al., 2020). Confirmation of *C. cassiicola* was based on molecular characterization by sequencing the ribosomal DNA internal transcribed spacer (ITS). The genomic DNA of each isolate was used to perform PCRs with the primers ITS5/ITS4 (White et al. 1990). Total DNA was extracted using an Omega Fungal DNA Mini Kit. The final volume of the PCR was 25 µL containing: 2 µL of genomic DNA, 5x PCR Butter, 25 mM of $MgCl_2$, 25mM dNTPs, 10 µM of each primer, 100x BSA, and 5 U/µL *Taq* DNA. The PCR cycling conditions were initial denaturation at 95 °C for 3 min, thirty-five amplification cycles (95 °C, 60 s; 55 °C, 90 s; 72 °C, 90 s) with a final extension at 72 °C for 10 min. The amplified fragments were visualized by electrophoresis in 1% (w/v) agarose gel stained with EZ-Vision Bluelight DNA Dye 10,000x in 1x TAE butter and visualized under UV light to detect the presence of each target gene. A 1 kb Plus DNA ladder was used as the marker and a reaction without a DNA template was used as the negative control. Purified PCR products were submitted to Eurofins Genomics (Louisville, KY) for sequencing in both directions. Forward and reverse nucleotide sequences were edited and assembled using Geneious prime (version 2021.0) to generate a consensus sequence. The consensus sequence was used to run BLAST in NCBI.

3.2 *In vitro* mycelial growth sensitivity assay

A mycelium growth assay was used to calculate EC_{50} values (the effective concentration of fungicide that reduces mycelial growth by 50%) to the commercial grade of azoxystrobin (22.9% a.i.; Syngenta Crop Protection) each was used to prepare stock solution at a concentration of 2,500 mg/L. Serial dilutions in sterilized water were prepared for each concentration. Fungicide sensitivity was evaluated by determining mycelial growth on PDA amended with different concentrations of each dose. When PDA had been cooled to 57°C, azoxystrobin was added to PDA at 0, 0.001, 0.01, 0.1, 1, 10, and 50 mg/L, poured at approximately 20 ml per Petri dish (15 x 85 mm). Salicyhydroxamic acid (SHAM) at 60 mg/L was added in all doses to inhibit the alternative oxidase pathway (Bradley and Pedersen 2011).

Isolate plugs (3.7 mm) were taken from a clean colony and placed face down on fungicide-amended media and control plates (SHAM only and non-amended PDA). Plates were incubated for five days in the dark at 26 °C. The diameter of the mycelial growth was evaluated using a caliper to measure the growth in two perpendicular directions. Average colony diameters were transformed to relative growth using non-amended control (0 mg/L). This experiment consisted of three replicates and was conducted twice.

3.3 Sequence-based analyses of *cytb* gene

Genomic DNA from each isolate used to perform PCRs with primers cct-cytb-F (5'-TATTATGCGGGATGTAAATAATGG-3') and cct-cytb-R (5'-TAATGAGAAGAATCTATTTAATGTAGCA-3') (MacKenzie et al. 2020). PCR tubes were placed in a thermal cycler with an initial denaturation of 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s, completed by a final elongation at 72°C for 5 min. The amplified fragments were visualized by electrophoresis in 1% (w/v) agarose gel stained with EZ-Vision Bluelight DNA Dye 10,000x in 1x TAE butter and visualized under UV light to detect the presence of each target gene. A 1 kb Plus DNA ladder was used as the marker and a reaction without a DNA template was used as the negative control. Purified PCR products were submitted to Eurofins Genomics (Louisville, KY) for sequencing in both directions. Forward and reverse nucleotide sequences were edited and assembled using Geneious prime (version 2021.0) to

generate a consensus sequence. Sequences were aligned with the predicted cytb gene from *C. cassiicola* isolate ELM07, which is a resistant isolate from Alabama (NCBI Assembly accession number MN564895.1).

3.4 Data analysis

EC_{50} values for relative growth and active ingredient concentration were analyzed with a 4-parameter log-logistic model in R (R version 4.1.0) using the Dose-Response Curve (DRC) program. EC_{50} values calculated in R were used in JMP Pro 16 to build a histogram with the frequencies for the *C. cassiicola* population (n = 24) and box plots comparing the years.

To assess the fitness of isolates to azoxystrobin, their mycelial growth was measured on plate control (0 ppm) with three replicates and compared with QoI-resistant and QoI-sensitive (Rondon 2020).

4. Results

4.1 In vitro mycelial growth sensitivity assay

The sensitivity of 24 *C. cassiicola* isolates taken from soybean infected leaves in Arkansas were used to establish a baseline sensitivity to azoxystrobin. Mycelial growth inhibition of *C. cassiicola* isolates grown on fungicide-amended media was used to obtain EC_{50} values. None isolates were completely inhibited at 10 mg/L. The EC_{50} values of all the tested *C. cassiicola* isolates ranged from ≤ 0.001 to 10.0 mg/L (mean = 1.88 mg/L and median = 0.855 mg/L) (Figure 1). Five isolates showed higher EC_{50}. In addition to higher EC_{50} values, plates with higher ppm had larger percent growth than plates with lower ppm. Isolate 21-13 was the only one to show this abnormal growth between 0 and 1 mg/L (Figure 2). The range in EC_{50} between two QoI-resistant and QoI-sensitive isolates shows a difference in levels of sensitivity to the azoxystrobin (Figures 2 and 3).

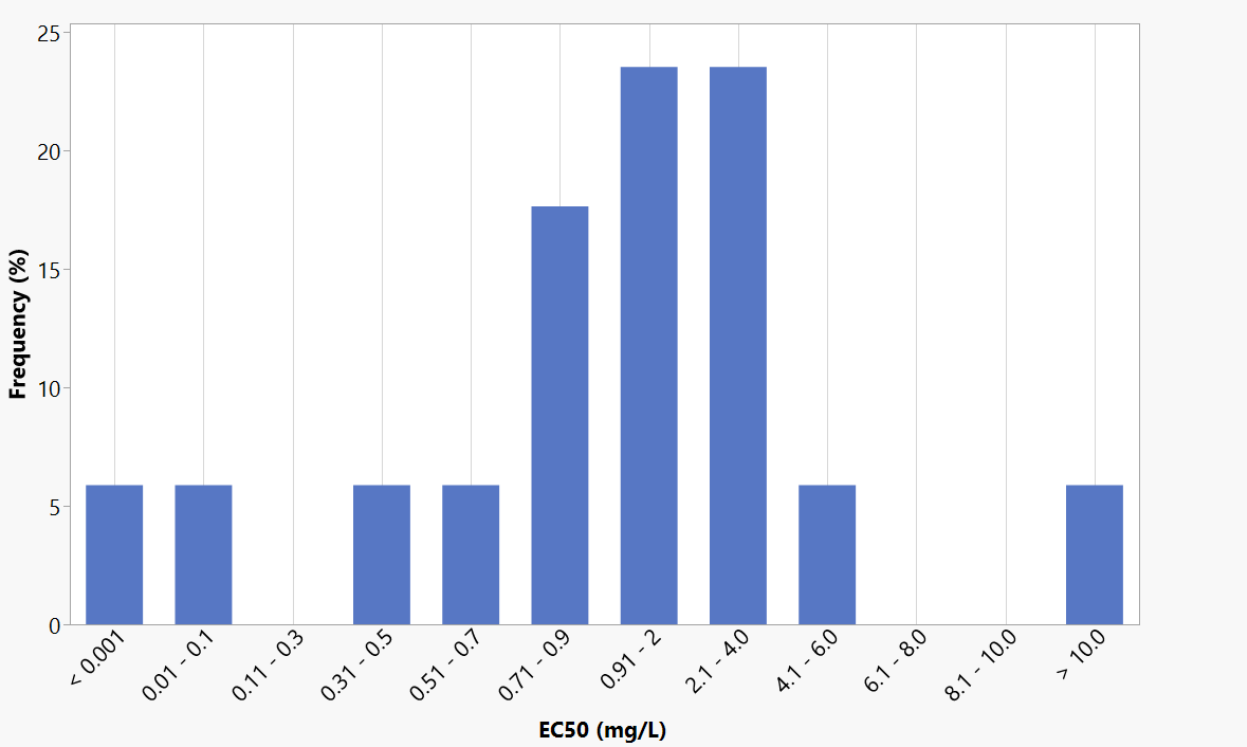

Figure 1. Frequency of EC_{50} values (Effective concentration of azoxystrobin that inhibits at least 50% of the fungal growth) for 24 isolates of *Corynespora cassiicola* in Arkansas soybeans.

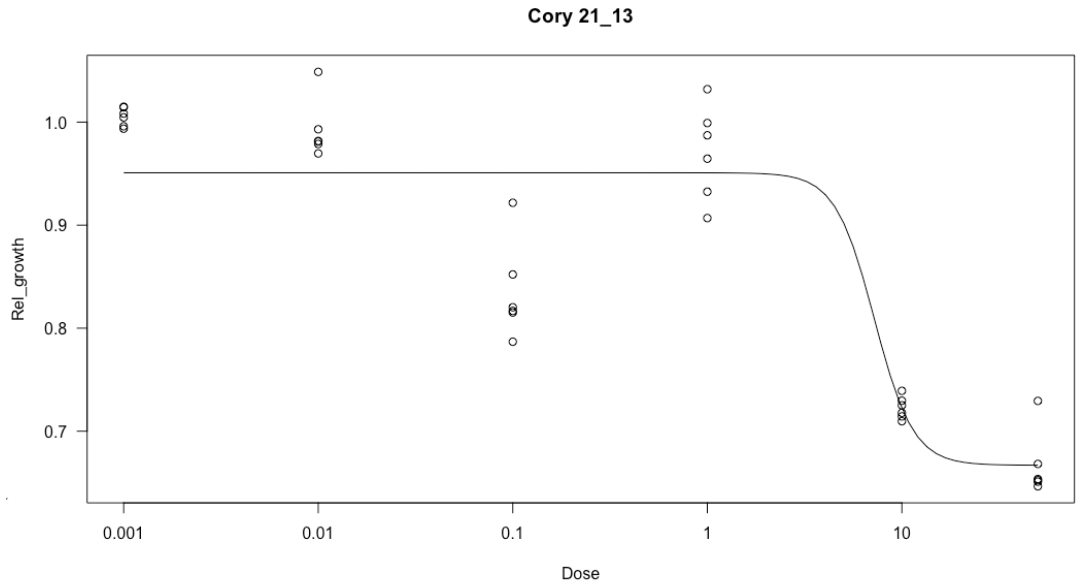

Figure 2. Dose-response curve for *C. cassiicola* QoI-resistant isolate 21-13 percent growth relative to the control challenged against Azoxystrobin concentrations. EC_{50} for isolate 21-13 was estimated at 7.21 mg/L.

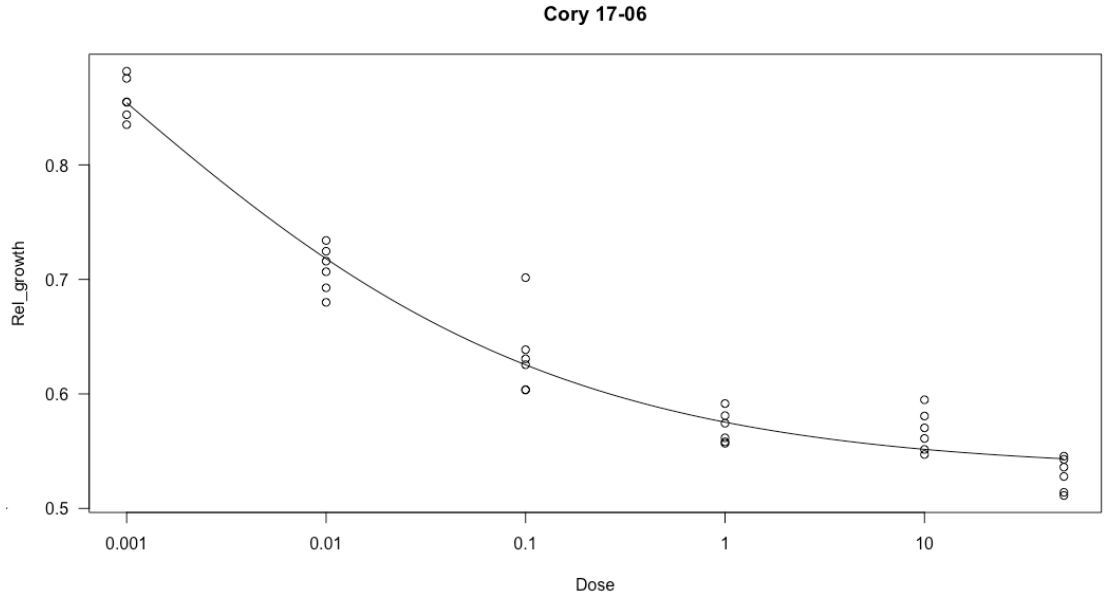

Figure 3. Dose-response curve for *C. cassiicola* QoI-sensitive isolate 17-06 percent growth relative to the control challenged against Azoxystrobin concentrations. EC_{50} for isolate 17-06 was estimated ≤0.001 (approx. 0.00066 mg/L).

4.1.1 EC_{50} from different locations and years

Corynespora cassiicola isolates were collected from seven different counties in the Arkansas soybean field from 2016 to 2021. In 2016, isolates collected are considered QoI-sensitives with a lower EC_{50}. During 2017, the EC_{50} started to increase, and some isolates showed resistance. Isolates collected in Stuttgart, Marianna and Rohwer are considered QoI-resistant. However, isolates collected in Keiser (2017) and Kibler (2018) are sensitive.

In 2021, Mariana (EC_{50}median = 0.87 mg/L), Happy (EC_{50}median = 1.77 mg/L) and Rohwer (EC_{50}median = 1.07 mg/L) are the most counties with a higher number of the isolates QoI-resistant. Isolate 21-13 (figure 2) from Marianna showed a higher EC_{50} = 7.21 mg/L. The difference between EC_{50} and year can be seen in figure 4.

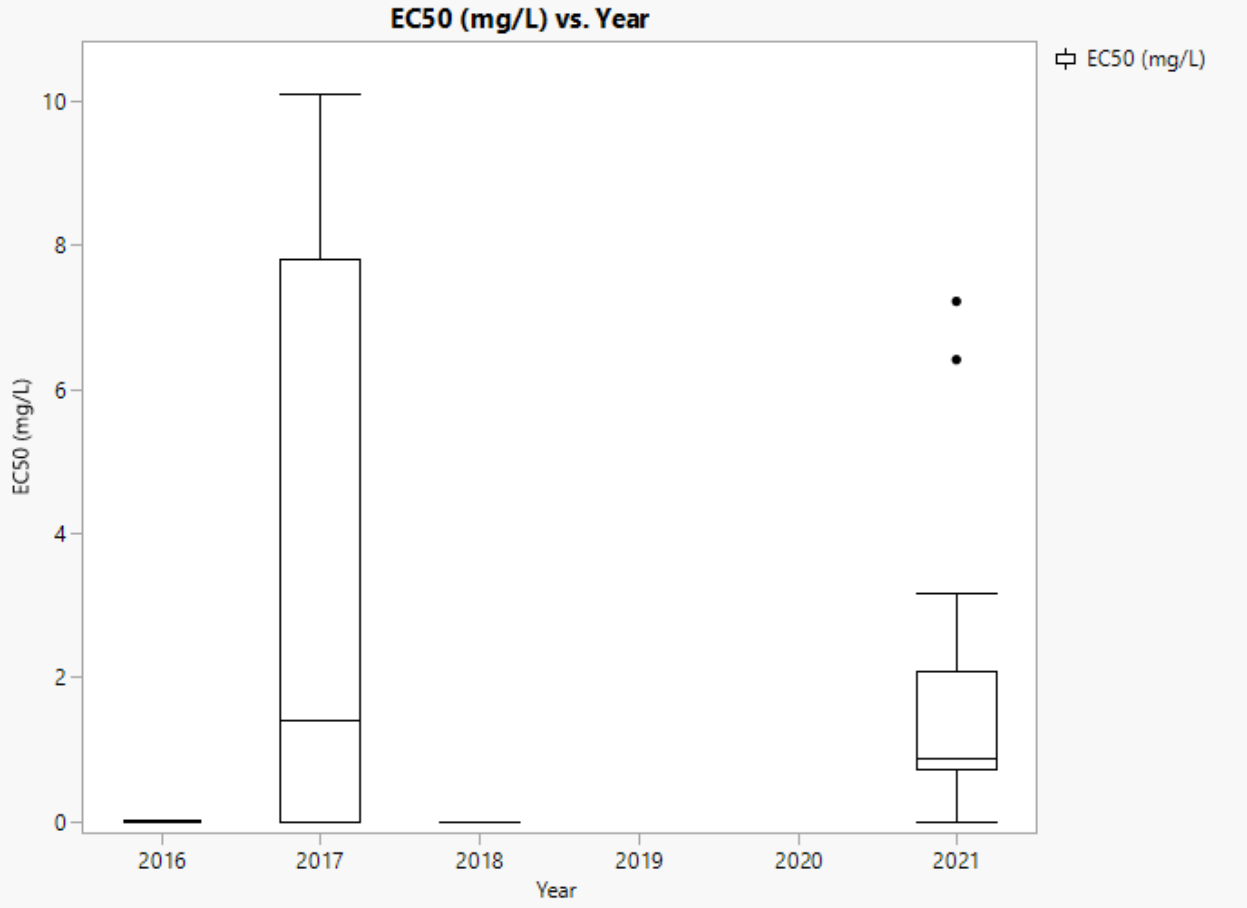

Figure 4: Distribution of EC_{50} values for *Corynespora cassiicola* isolates among years in Arkansas (2016, n = 2; 2017, n = 5; 2018, n = 1; 2021, n = 16).

4.2 Fitness assessment of *C. cassiicola* isolates

Mycelial growth of 24 isolates of *C. cassiicola* was measured on plate control (0 ppm) with three replications and compared between QoI-resistant and QoI-susceptible (Rondon 2020). Eighteen isolates classified as QoI-resistant and six were QoI-sensitive were used to identify potential fitness cost due to resistance. Significant differences in mycelial growth was observed among the isolates (df = 23, F = 37.7688 and P < 0.0001). Mean comparison of mycelial growth separated the isolates into statistical groups, and significant differences were observed in the mycelial growth of *C. cassiicola*. QoI-resistant isolates were placed in statistically different groups (Figure 5). We could not correlate the mycelial growth of *C. cassiicola* isolates with the presence of the G143A mutation (QoI-resistant) (Rondon, 2019).

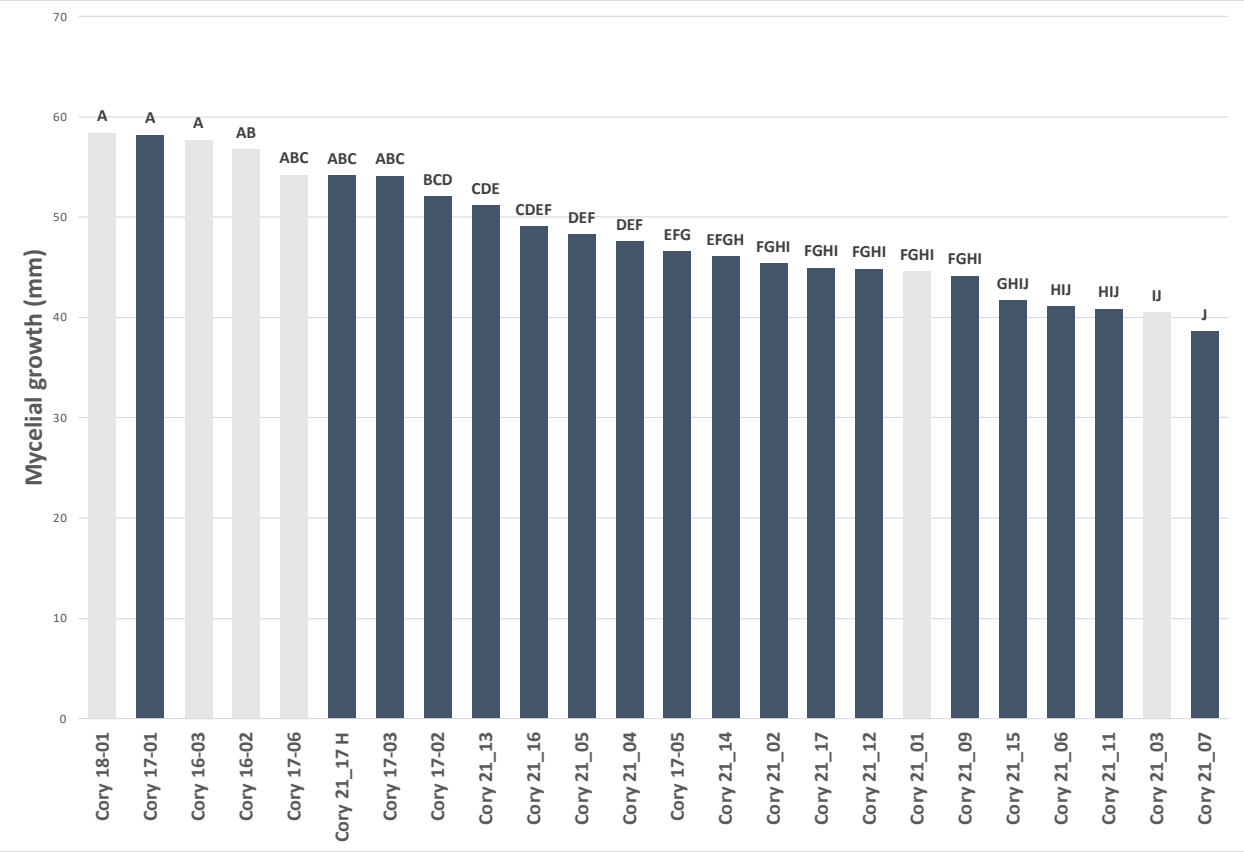

Figure 5. Mycelial growth on PDA added fungicide of *C. cassiicola* isolates. QoI-resistant isolates are highlighted, while QoI-sensitive is represented by light gray only. Data represent the means of replicating samples. Bars labeled with different letters are significantly different according to Tukey's HSD test ($\alpha = 0.05$).

4.3 G143 Mutation in the cytochrome b gene

The fungi DNA was amplified using primers cc-cyt-F and cc–cytb-R (MacKenzie et al. 2020) and based on *cytb* nucleotide sequences, six isolates are characterized as QoI-sensitive (Figure 6) and eighteen are QoI-resistant (Figure 7). A mutation in *C. cassiicola* was identified that replaces the codon for amino acid 143 from GGT to GCT, developing in an amino change from glycine to alanine (G143A). These isolates originated from soybean fields located in the Delta Region, northwest of Arkansas, including four counties (Happy, Marianna, Stuttgart, and Rohwer). Any other mutations on *cytb*, F129L, and G137R, were not found in our isolates.

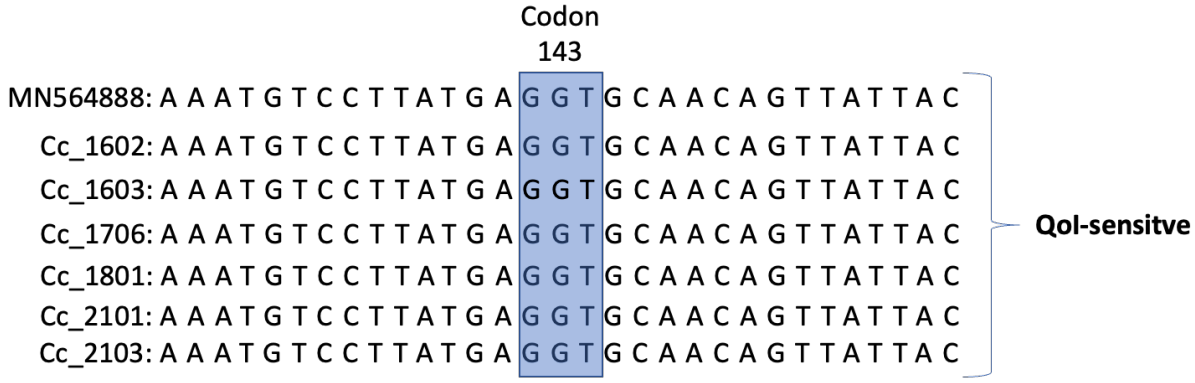

Figure 6. Partial nucleotide sequences of the *cytochrome b* gene of 6 isolates of Corynespora cassiicola plus one isolate used as a reference, QoI-sensitive (MN564888) from Rondon et al. (2019).

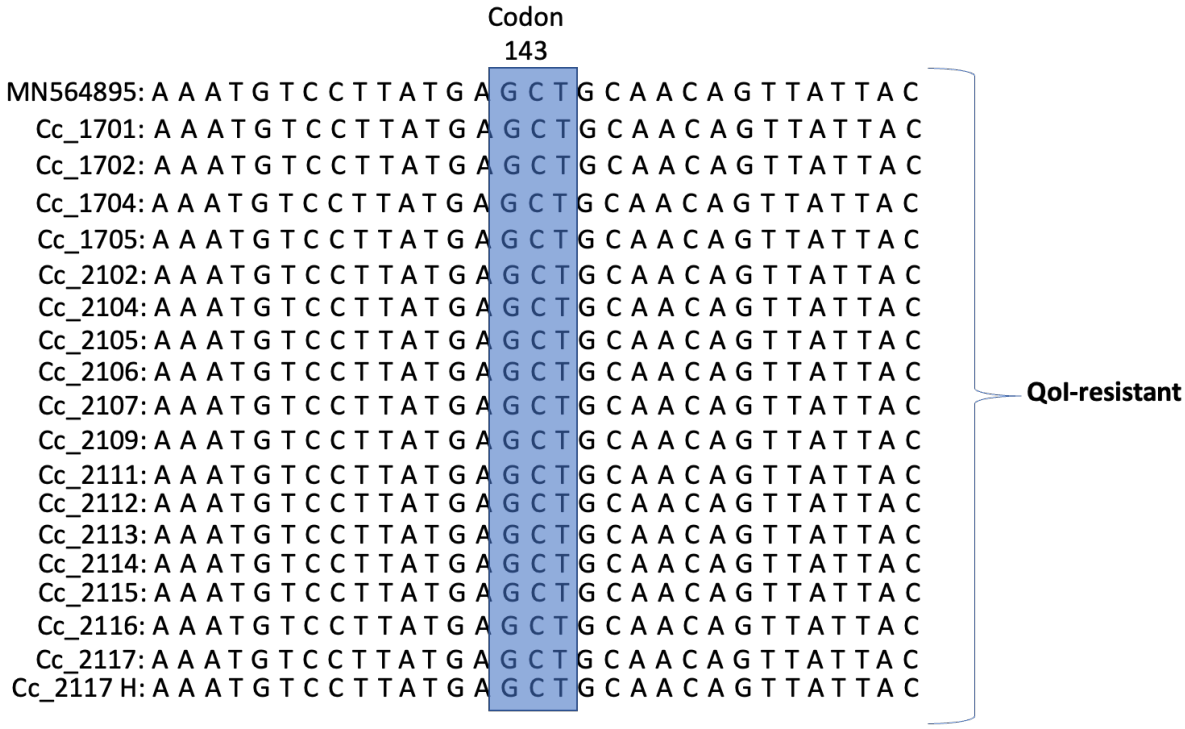

Figure 7. Partial nucleotide sequences of the *cytochrome b* gene of 18 isolates of Corynespora cassiicola plus one isolate used as a reference, QoI-resistant (MN564895) from Rondon et al. (2019).

5. Discussion

Target spot started to be considered as an important disease after 2018 in the U.S. Before this year, yield losses caused by *C. cassiicola* were minor issues and grouped with "other diseases" such as Alternaria seed rot, taproot decline, and black root rot due to the lower damages (Bradley et al. 2021). In 2018, the total estimated soybean yield losses caused by target spot in 29 states of the U.S. was 910 bushels in thousands (Bradley et al. 2021).

In Arkansas, target spot of soybean was a minor disease. Since 2014, this disease has been observed more frequently and during the 2016 cropping season, it was one of the most widespread and severe foliar diseases of soybean in the state (Faske 2016). Yield and economic losses caused by *C. cassiicola* on soybean between 2018 and 2021 were approximately 511,000 bushels valued at U$5,000,000 (Crop Protection Network, 2021). In Arkansas, there were several reports of significant defoliation (50-80%) that contributed to at least 15-20 bushels/acre loss in yield (Faske 2016).

Several factors can be responsible for the upcoming target spot such as climate change and difficulty to manage the disease. Ideal conditions for disease development such as excess moisture, high relative humidity (>85%), and warm temperatures over a period of 5 to 7 days cause this fungus to prevail and infect (Faske 2020). According to FRAC (2019), the increased development of resistance of *C. cassiicola* to different fungicide classes on soybean is rated with a high risk of resistance due to development of resistance in short time span. Strobilurin resistance is an example of this, the mutation at position G143A changing amino acid on *cytb* gene from glycine to alanine is known to confer high resistance to QoI fungicides (Duan et al. 2019; Rondon and Lawrence 2019). Besides that, QoI fungicides are one of the most applied fungicides to manage diseases in different crops. With higher exposure to the product and misuse of fungicide, the resistance can appear and evolve rapidly, making the control of diseases a big challenge.

Isolates of *Corynespora cassiicola* obtained from symptomatic soybean leaves in different Arkansas counties were confirmed to have the G143A mutation in the cytochrome b gene, which confers resistance to QoI fungicides. Results of the *in vitro* and PCR assay showed that 18 out of the 24 Arkansas *C. cassiicola* isolates were resistant to QoI fungicides, which means 75% of the isolates are resistant. Most of the resistant isolates showed EC_{50} range from 0.6 to 7 mg/L ($\bar{X}$ = 2.27 mg/L; median = 1.15 mg/L). Among 14 isolates of *C. cassiicola* isolates sampled from

soybean in Brasil, Teramoto et al. (2017) reported EC_{50} > 28 mg/L for azoxystrobin, and all of them were considered highly tolerant to QoI fungicides (Teramoto et al. 2017). In addition, a study on *C. cassiicola* isolated from tomato exhibited EC_{50} > 100 mg/L for azoxystrobin in Florida (MacKenzie et al. 2020b). The EC50 values found in this study for *C. cassiicola* suggests loss sensitivity for azoxystrobin in Arkansas on soybean.

Comparing the different regions of Arkansas where the isolates were collected, the most QoI-resistant population in 2021 are in Happy, Marianna, Stuttgart and Rohwer, the counties that are in Delta Region, main soybean region production. QoI-sensitive isolates were not found in Rohwer, Happy and Marianna, only in Kibler (2018), Keiser (2017), and Stuttgart (2017). Combining information on the increasing damage caused by *C. cassiicola* in Arkansas and the emergence of fungicide resistance, these two factors may be intertwined, but further investigations are necessary to confirm this trend. Generally, fungicides can successfully control sensitive but not resistant pathogens populations, which over time will be predominant population in the field (Ma and Michailides 2005). The management of fungicide resistance cannot stop the evolution of resistance in fungal pathogens populations, but it can decline the occurrence of new cases and delay resistance development to preserve the efficacy of fungicides (Staetz 2012).

The fitness of fungicide resistant isolates was defined as important aspect to developing helpful anti-resistance approaches. Isolates with a fitness cost will alter their competitive capacity and it defines their persistence in the fungal population when there is no fungicide pressure (Ishii and Hollomon 2015). The identification of characteristics associated with resistant isolates is essential for fungicide resistance risk assessment (Ma et al. 2018). In this study, was no observed correlation between mycelial growth and sensitivity to azoxystrobin, with no clear separation of QoI-sensitive and resistant isolates. These results suggest there is no association of *C. cassiicola* isolates with resistance to azoxystrobin based on mycelial growth. Deising et al. n.d. found no difference between QoI-resistant and sensitive isolates of *Cercospora sojina* for sporulation and radial growth and accentuated those extra experiments are essential to know the possible fitness penalty associated with fungicide resistance. A study conducted with *Phytophthora capsici* with a mutation in the codon G137R, exhibited an equal fitness compared with sensitive isolates, which indicates that there could be no fitness cost associated. Therefore, a mutation can be associated with the ability to compete and survive under selection pressure by azoxystrobin occupying a dominant position in the field population, but this is not always the case (Ma et al. 2018).

Knowing that field populations of *C. cassiicola* have mutations with QoI-resistance, it is necessary to monitor the frequency and rate of fungicide applications to avoid resistance. It is also important to search for other tools for integrating management of diseases. To avoid the increase and rapid development of tolerant *C. cassiicola* populations, multi-site fungicides should be consider, applying those in combination with a single site that has different modes of action could help to delay resistance. Besides that, fungicide application should be limited and without unnecessary exposure. Disease prediction can help to understand how the infections occur according to the factors such as weather, host, and pathogen and make effective management of the disease.

6. Final consideration

The development of fungicide resistance for *C. cassiicola* in Arkansas soybean fields can be a major issue to manage, since the disease events are becoming more frequent and yield losses can be more than 50% in one season under conducive weather. The issue of having *C. cassiicola* population that is tolerant could exacerbate the problem. Monitoring is essential to explain shifts of sensitivity over the years and provide evidence that resistant populations were responsible for the disease control failures. The objective of our study was to understand how the fungicide resistance for target spot correlates with the constant increase of the disease in Arkansas soybean fields and how we can improve our knowledge for effective control without unnecessary exposure to the fungicides. Furthermore, it is necessary to develop new methods and research that can help farmers to manage diseases such as cultivar resistance, new molecules, biological control, and disease prediction that considers climate change. These strategies will help the management of target spot in the field in combination with chemical control, prolonging the life expectancy of fungicides. We hope to continue researching to help the Arkansas farmers better manage soybean diseases and produce with sustainability.

7. Conclusion

Considering the concern about target spot and the emergence of this disease in Arkansas soybean fields, it is a good time to review what we know about it and what steps we might consider for the upcoming cropping season. There is a need for applied research to better understand how to manage this disease. However, management can be improved based on what is known about other foliar diseases. Variety selection is important as some varieties were more susceptible to target spot than others, therefore, soybean varieties that were observed to be susceptible to target spot should be avoided in fields with a history of target spot.

Cultural practices that consist of rotation with corn, grain sorghum, or rice will reduce the amount of inoculum for the subsequent crop. The pathogen overwinters in crop debris and the soil, producers should take into consideration that this disease may be more problematic in fields with a history of the disease in cotton and soybean.

Planting and production practices promoting a quick canopy closer may be at higher risk for target spot development, which needs further investigation. Though most universities in North America have focused on frogeye leaf spot and Cercospora leaf blight, there have been some fungicide trials on target spot in South America.

In these trials, SDHI and DMIs fungicides have been more efficient in controlling *Corynespora cassiicola.* Such trials are needed, but laboratory experiments are being conducted to determine the effectiveness of these and other commonly used fungicides to defeat fungal growth from isolates collected in Arkansas.

Furthermore, because target spot begins in the lower canopy, fungicide timing is essential for plant protection. Suppressing diseases development before it has a chance to move up the canopy and utilizing enough water volume to carry the fungicide into the canopy are important factors in managing this disease.

This study is the first to report G143A mutation in *C. cassiicola* from field populations of soybean in Arkansas in the U.S.

8. References

Avozani, A., Reis, E. M., and Tonin, R. B. 2014. Sensitivity loss by corynespora cassiicola, Isolated from soybean, To the fungicide carbendazim. Summa Phytopathologica. 40:273–276.

Bolton, M. D., Rivera, V., and Secor, G. 2013. Identification of the G143A mutation associated with QoI resistance in Cercospora beticola field isolates from Michigan, United States. Pest Management Science. 69:35–39.

Boote, K. J., Jones, J. W., Mishoe, J. W., and Berger, R. D. 1983. Coumpling Pest to Crop Growth Simulators to Predict Yield Reductions.

Bowen, K. L., Hagan, A. K., Pegues, M., Jones, J., and Miller, H. B. 2018. Epidemics and yield losses due to corynespora cassiicola on cotton. Plant Disease. 102:2494–2499.

Bradley, C. A., Allen, T. W., Sisson, A. J., Bergstrom, G. C., Bissonnette, K. M., Bond, J., et al. 2021a. Soybean Yield Loss Estimates Due to Diseases in the United States and Ontario, Canada, from 2015 to 2019. Plant Health Progress. 22:483–495.

Bradley, C. A., Allen, T. W., Sisson, A. J., Bergstrom, G. C., Bissonnette, K. M., Bond, J., et al. 2021b. Soybean Yield Loss Estimates Due to Diseases in the United States and Ontario, Canada, from 2015 to 2019. Plant Health Progress. 22:483–495.

Bradley, C. A., and Pedersen, D. K. 2011. Baseline sensitivity of Cercospora zeae-maydis to quinone outside inhibitor fungicides. Plant Disease. 95:189–194.

Deising, H. B., Fraaije, B., Mehl, A., Oerke, E.-C., Sierotzki, H., and Stammler, G. *Modern Fungicides and Antifungal Compounds VIII Proceedings of the 18th International Reinhardsbrunn Symposium, April 24-28, 2016, Friedrichroda, Germany*.

Delaney, M., ArchMiller, A. A., Delaney, D. P., Wilson, A. E., and Sikora, E. J. 2018. Effectiveness of fungicide on soybean rust in the southeastern United States: A meta-analysis. Sustainability (Switzerland). 10.

Duan, Y., Xin, W., Lu, F., Li, T., Li, M., Wu, J., et al. 2019. Benzimidazole- and QoI-resistance in Corynespora cassiicola populations from greenhouse-cultivated cucumber: An emerging problem in China. Pesticide Biochemistry and Physiology. 153:95–105.

Edwards Molina, J. P., Navarro, B. L., Allen, T. W., and Godoy, C. v. 2022. Soybean target spot caused by Corynespora cassiicola: a resurgent disease in the Americas. Tropical Plant Pathology. Available at: https://link.springer.com/10.1007/s40858-022-00495-z.

Faske, T. 2016. Arkansas Soybeans: Target Spot - What Do We Know?

Faske, T. 202AD. Target spot begins movement to midwest.

Faske, T., Kirkpatrick, T., Zhou, J., and Tzanetakis, I. *Soybean Diseases - Arkansas Soybean Handbook - Chapter 11 - MP197*.

Fortunato, A. A., Debona, D., Aucique-Pérez, C. E., Fialho Corrêa, E., and Rodrigues, F. A. 2018. Chlorophyll a fluorescence imaging of soya bean leaflets infected by Corynespora cassiicola. Journal of Phytopathology. 166:782–789.

Godoy, C. V. 2015. *Target Spot, Compendium of Soybean Diseases and Pests*. Fifth edition. eds. G.L. Hartaman, J. C. Rupe, E.J. Sikora, L.L Domier, J.A. Davis, and K.L. Steffey. St. Paul: American Phytopathological Society.

Hartman, G. L., Rupe, J. C., Sikora, E. J., Domier, L. L., Davis, J. A., and Steffey, K. L. 2016. *Compendium of Soybean Diseases and Pests*. 5th ed.

Hartman, G. L., West, E. D., and Herman, T. K. 2011. Crops that feed the World 2. Soybean-worldwide production, use, and constraints caused by pathogens and pests. Food Security. 3:5–17.

Hartwing, E. 1959. Effect of target spot on yield of soybeans. Plant Disease.

Ishii, H., and Hollomon, D. W. 2015. *Fungicide resistance in plant pathogens - Principles and a guide to practical management*.

Leadbeater, A., T. McGrath, M., A. Wyenandt, C., and L. Stevenson, K. 2019. An Overview of Fungicide Resistance and Resistance Management: History and Future Trends. In *Fungicide Resistance in North America* , eds. Katherine L. Stevenson, Margaret T. McGrath, and Christian A. Wyenandt. St. Paul: The American Phytopathological Society, p. 1–26.

Lucas, J. A., Hawkins, N. J., and Fraaije, B. A. 2015a. The Evolution of Fungicide Resistance. Advances in Applied Microbiology. 90:29–92.

Lucas, J. A., Hawkins, N. J., and Fraaije, B. A. 2015b. The Evolution of Fungicide Resistance. Advances in Applied Microbiology. 90:29–92.

Ma, D., Jiang, J., He, L., Cui, K., Mu, W., and Liu, F. 2018. Detection and characterization of qoi-resistant phytophthora capsici causing pepper phytophthora blight in china. Plant Disease. 102:1725–1732.

Ma, D., Jiang, J., Zhu, J., Zhang, L., Li, B., Mu, W., et al. 2020. Evaluation of sensitivity and resistance risk of corynespora cassiicola to isopyrazam and mefentrifluconazole. Plant Disease. 104:2779–2785.

Ma, Z., and Michailides, T. J. 2005. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. Crop Protection. 24:853–863.

MacKenzie, K. J., Xavier, K. v., Wen, A., Timilsina, S., Adkison, H. M., Dufault, N. S., et al. 2020a. Widespread QoI fungicide resistance revealed among corynespora cassiicola tomato isolates in Florida. Plant Disease. 104:893–903.

MacKenzie, K. J., Xavier, K. v., Wen, A., Timilsina, S., Adkison, H. M., Dufault, N. S., et al. 2020b. Widespread QoI fungicide resistance revealed among corynespora cassiicola tomato isolates in Florida. Plant Disease. 104:893–903.

Madalosso, M. G. 2017. Mancha-alvo (Corynespora cassiicola).

Oilseeds: World Markets and Trade. Available at: https://public.govdelivery.com/accounts/USDAFAS/subscriber/new.

PlantDisease69n08_641.

Rondon, M. N. 2020. *Genetic diversity of the fungal pathogen Corynespora cassiicola and its fungicide resistance*.

Rondon, M. N., and Lawrence, K. S. 2019. Corynespora cassiicola isolates from soybean in Alabama detected with G143a mutation in the Cytochrome b gene. Plant Health Progress. 20:247–249.

Sierotzki, H., Frey, R., Wullschleger, J., Palermo, S., Karlin, S., Godwin, J., et al. 2007. Cytochrome b gene sequence and structure of Pyrenophora teres and P. tritici-repentis and implications for QoI resistance. Pest Management Science. 63:225–233.

Sierotzki, H., and Stammler, G. 2019. *Resistance of Plant Pathogens to QoI Fungicides (FRAC Code 11)*.

Soja em números (safra 2020/21). 2021.

Staetz, C. A. 2012. *International Code of Conduct on the Distribution and Use of Pesticides Guidelines on Prevention and Management of Pesticide Resistance*.

Teramoto, A., Machado, T. A., dos Santos, L. M., Volf, M. R., Meyer, M. C., and da Cunha, M. G. *Reação de cultivares de soja à Corynespora cassiicola*. Available at: www.sbfito.com.br.

Teramoto, A., Meyer, M. C., Suassuna, N. D., and da Cunha, M. G. 2017. Sensibilidade de corynespora cassiicola isolado de soja a fungicidas in vitro e controle químico de mancha-alvo da soja no campo. Summa Phytopathologica. 43:281–289.

Toulet, M. L., Neira, D. A., Escobar, M., Pardo, E. M., Arias, M. E., Ploper, L. D., et al. 2022. Morphological and pathogenic characterization of Corynespora cassiicola isolates reveals specific genotypic interactions in soybean. Plant Pathology.

White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications.