UNIVERSIDADE FEDERAL DE VIÇOSA

DEIVID SACON

MOLECULAR AND PHENOTYPIC CHARACTERIZATION OF THE SENSITIVITY OF *Corynespora cassiicola* TO SITE-SPECIFIC FUNGICIDES

VIÇOSA - MINAS GERAIS
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ABSTRACT

SACON, Deivid, M.Sc., Universidade Federal de Viçosa, July, 2020. **Molecular and phenotypic characterization of the sensitivity of *Corynespora cassiicola* to site-specific fungicides**. Advisor: Sérgio Hermínio Brommonschenkel.

Target spot caused by *Corynespora cassiicola* is an important leaf disease, capable of causing economic losses in several crops, including soybean and cotton. For the management of this disease, chemical control is major component, but site-specific fungicide resistance has become a major concern. Sequences of target gene of MBC (Methyl Benzimidazoles Carbamates), QoI (Quinone Outside Inhibitors), DMI (Demethylation Inhibitors) and SDHI (Succinate Dehydrogenase Inhibitors) fungicides in 79 *C. cassiicola* isolates obtained from soybean and cotton samples collected from Brazilian producing regions during 2017-2020 were characterized, and the impact of nucleotide changes leading to amino acid substitutions in the sensitivity to these fungicides was addressed by quantifying *in vitro* mycelial growth inhibition. Nucleotide polymorphism leading to E198A and F200Y substitutions in β-tubulin, G143A in CytB, B-H278Y and C- N75S / A in the SDH were found. G143A that usually leads to high level of resistance in fungi to QoIs was present in 87% of the isolates surveyed; F129L and G137R substitutions were not detected. The E198A substitution in β-tubulin provided greater resistance to carbendazim compared to F200Y. B-H278Y, C-N75S /A substitutions conferred cross resistance to the fungicides benzovindiflupir, fluxapyroxade and inpyrfluxam and provided similar resistance levels; however, a stronger effect on fluxapyroxad sensibility was verified. No variation in the DMI target site (*Cyp51*) nor resistance to the prothioconazole DMI was observed. Substitutions leading to multiple resistance to fungicides MBC, QoI, and SDHIs fungicides was identified in 20 isolates. Target site mutations leading to amino acid substitution in *C. cassiicola* specically associated with isolates from cotton or soybean were not detected. These results provide an overview of the current situation of fungicide resistance of *C. cassiicola* from cotton and soybean providing information for better chemical management of target spot in these crops.

Keywords: DMI. MBC. QoI. SDHI. Target spot.

RESUMO

SACON, Deivid, M.Sc., Universidade Federal de Viçosa, julho de 2020. **Caracterização molecular e fenotípica da sensibilidade de *Corynespora cassiicola* a fungicidas sítio-específicos**. Orientador: Sérgio Hermínio Brommonschenkel.

A mancha-alvo causada por *Corynespora cassiicola* é uma importante doença foliar, capaz de causar prejuízos econômicos em diversas culturas, incluindo soja e algodão. Para o manejo dessa doença, o controle químico é o componente principal, mas a resistência a fungicidas sítios específicos tornou-se uma grande preocupação. Sequências de genes alvos de fungicidas MBC (Metil Benzimidazóis Carbamatos), QoI (Inibidores Externos de Quinona), DMI (Inibidores da Demetilação) e SDHI (Inibidores da Succinato Desidrogenase) em 79 isolados de *C. cassiicola* obtidos a partir de amostras de soja e algodão coletadas de regiões produtoras brasileiras durante 2017-2020 foram caracterizados, e o impacto das alterações de nucleotídeos que levam a substituições de aminoácidos e a sensibilidade a esses fungicidas foi avaliado pela quantificação da inibição do crescimento micelial *in vitro*. Foi encontrado polimorfismo de nucleotídeo que leva a substituições E198A e F200Y na β-tubulina, G143A no CytB, B-H278Y e C-N75S / A na SDH. G143A que geralmente leva a alto nível de resistência em fungos a QoIs estava presente em 87% dos isolados pesquisados; Substituições F129L e G137R não foram detectadas. A substituição E198A na β-tubulina proporcionou maior resistência ao carbendazim em comparação com a F200Y. As substituições de B-H278Y, C-N75S / A conferiram resistência cruzada aos fungicidas benzovindiflupir, fluxapiroxade e inpyrfluxam e proporcionaram níveis de resistência semelhantes; no entanto, foi verificado um efeito mais forte na sensibilidade do fluxapiroxade. Não foi observada variação no local alvo do DMI (*Cyp51*) nem resistência ao DMI protioconazol. Substituições que levam a resistência múltipla aos fungicidas MBC, QoI e SDHIs foram identificadas em 20 isolados. Não foram detectadas mutações no sítio-alvo que levam à substituição de aminoácidos em *C. cassiicola* especificamente associada a isolados de algodão ou soja. Esses resultados fornecem uma visão geral da situação atual de resistência a fungicidas de *C. cassiicola* de algodão e soja, fornecendo informações para um melhor manejo químico da mancha-alvo nessas culturas.

Palavras-chave. DMI. MBC. QoI. SDHI. Mancha Alvo.

SUMMARY

1 INTRODUCTION

Corynespora cassiicola (Berk. & M. A. Curtis) C. T. Wei, is an anamorphic Dothideomycetes, type species of the genus that comprises more than 100 species. This species has a cosmopolitan behavior, occurring in subtropical and tropical countries (Dixon *et al*., 2009; Teramoto *et al*., 2017; Farr and Rossman, 2019). Besides infecting leaves, stems, and roots of more than 530 plant species from 380 genera, including monocots, dicots, ferns, and one cycad, this fungus has also been reported on nematode cysts and human skin and eyes (Boosalis and Hamilton, 1957; Carris and Glawe, 1986; Hyde *et al*., 2001; Yamada *et al*., 2013; Wang *et al*., 2019).

The pathogenicity profile and genetic variability of *C. cassiicola* is very wide. Some isolates are pathogenic to a wide range of hosts while others show some host specificity (Onesirosan *et al*., 1974; Cutrim and Silva, 2003; Dixon *et al*., 2009). In addition to, some isolates seem to be opportunists found associated with injuries (Volin and Pohronezny, 1989) or present on living nonsymptomatic tissue (Dixon *et al.* 2009). Recently, eight major phylogenetic clades (PhL1-PhL8) have been described based on a Bayesian inference analysis of sequences from four combined genomic loci from 245 isolates obtained 44 plant species, with prevalence of some lineages on some hosts such as tomato, papaya and soybean (Banguela-Castillo *et al*., 2020).

In soybean (*Glycine max* L. Merrill), cotton (*Gossypium hirsutum* L.) and other hosts the fungus causes the disease known as the target spot or Corynespora leaf spot. In soybean, the typical leaf symptoms of this disease are reddish-brown lesions, rounded to irregular, usually surrounded by yellow halos with a diameter of 10 to 15 mm. Reddish-brown lesions may also be found on petioles, pods and stems. Lesions usually develop concentric rings, first seen in the lower strata of the canopy, later spread throughout the plant (Galbieri *et al*., 2014; Godoy *et al*., 2016; Bowen and Hagan, 2018)

Some lesions are distinctly zonate, hence the common disease name. Yield losses are a result of lost leaf canopy which in turn reduces yield capability of the crop. Favorable conditions for target spot development commonly occur in Brazil from mid to late of the main crop season, at the beginning of the reproductive stage of the crop when the canopy closes (Teramoto *et al*., 2013). Under this conditions, potential yield losses of

soybean due to target spot ranges from 8% to 40.5%, depending on the cultivar (Edwards Molina *et al.,* 2019).

The symptoms of target spot or Corynespora leaf blight on cotton plants are similar to those observed on soybean plants. In the foliar phase of the disease, the symptoms first appear on the leaves in the lower canopy and spread upward through the canopy towards the shoot tips, characterized as small circular spots (Galbieri *et al.,* 2014; Goulart and Lamas, 2016).

The well-developed lesions are necrotic and show typical "target spot" (concentric rings) symptoms, with alternating light and dark brown bands and depression at the center of the lesion. Once multiple leaf spots appear, diseased leaves senesce, showing severe necrosis, and fall to the ground. Under favorable conditions it can severely reduce cotton plant canopy. Although the impact of this disease on cotton yield losses in Brazil is still not available, in other countries yield losses that ranges from 5 to 40% and lint reduction by 448 kg/ha have been reported (Hagan, 2014; Bowen and Hagan, 2018).

In Brazil, the widespread adoption of no-till cultivation practices, sowing of susceptible cultivars, the successive planting of susceptible crops and the use of susceptible cover crops to manage nematodes creates conditions for continuous survival and multiplication of *C. cassiicola* and selection for more adapted strains in different production environments (Edwards Molina *et al.*, 2019). Most of Brazil's cotton is planted as a second crop after soybean are harvested and still is not very understood the relationship of *C. cassiicola* populations from soybean and cotton, although different studies have shown that isolates from cotton and from soybean were pathogenic to both crops and no phenotypic and molecular differentiation between could be made (Galbieri *et al.,* 2014; Goulart and Lamas, 2016; Sumabat *et al*., 2018).

This production landscape also creates more favorable opportunity for evolution of isolates resistance to site-specific fungicides because fungicides with same mode of action (MOA) are being used to control different pathogens on soybean and cotton crops. In other countries, high frequency of resistance to MBC (Methyl Benzimidazoles Carbamates), QoI (Quinone Outside Inhibitors) and SDHI (Succinate Dehydrogenase Inhibitors) fungicides have been reported (Miyamoto *et al*., 2009; Duan *et al*., 2019; Rondon and Lawrence, 2019).

Resistance of isolates obtained from soybean, collected between 1997 and 2013 to MBC, QoI and DMI (Demethylation Inhibitors) were reported in Brazil by Teramoto *et*

al. (2017), based on *in vitro* sensitivity tests. More updated and detailed information is lacking, especially with relation to SDHI that were recently introduced into the market, although resistance to this group has been also observed in the soybean field trials and confirmed by the Fungicide Resistance Action Committee-FRAC (FRAC, 2020).

The resistance / insensitivity of fungi to fungicides can be conferred by different mechanisms, including mutations that lead to changes in the target enzyme conformation, reducing its affinity to the fungicide (Ma and Michailides, 2005; Rehfus *et al*., 2016; Teramoto *et al*., 2017; Duan *et al*., 2019). Studies describing the target-site mutations affecting fungicide sensitivity of Brazilian *C. cassiicola* isolates from soybean and cotton, their frequency, as well as the impact of these mutations in the background of different isolates are lacking, although brief reports from monitoring studies executed indicated the presence of mutations normally associated with resistance to these group of fungicides in other countries (FRAC, 2020).

Therefore, in this study, we have characterized the mutations of the target sites of MBC, QoI, SDHI and DMI fungicides in 79 *C. cassiicola* isolates from infected soybean and cotton collected during 2017-2020, and we have also determined the impact of these mutations on the *in vitro* sensitivity to these group of fungicides.

2 LITERATURE REVISION

2.1 *Corynespora cassiicola* and its importance on soybean and cotton crops

Soybean is a crop with multiple uses, ranging from human and animal food to production of lubricating oils, biodiesel and disinfectants. Its main use is for animal feed, due to its high protein content and low production cost, and is the most feed used to supply the animal's protein requirements. Due to the importance of this legume in human activities, soybean fields occupy approximately 126 million hectares in the world, of which 36.8 million are in Brazil, being the largest world producer of this legume. The average Brazilian crop yield is 3266 kg/ha, representing a total production of almost 120 million tons of grain per year (CONAB, 2020; USDA, 2020).

In turn, cotton is the most important fiber producing plant in the world. In addition to supplying fibers for the textile industry, it is also used for animal feed and edible oil production in a significant proportion. Cotton fields occupy an area of 32.7 million

hectares in the world, of which 1.7 million are in Brazil. The average Brazilian yield is 1723 kg/ha of plume, plus 2582 kg/ha of seed, representing a total production of 4.3 and 2.9 million tons of seed and plume, respectively.

Pests and disease occurrences are a factor that affects the crop yield ceiling for both cultures, a factor aggravated by the fact that they share pathogens (CONAB, 2020; USDA, 2020). In addition to, most of Brazil's cotton is planted as a second crop after soybean causing concerns to cotton and soybean producers, as both crops are attacked by *Corynespora cassiicola* (Schoch *et al*., 2009).

In soybean, cotton and other hosts the fungus causes the disease known as the target spot or Corynespora leaf spot. In soybean, the typical leaf symptoms of this disease are reddish-brown lesions, rounded to irregular, usually surrounded by yellow halos with a diameter of 10 to 15 mm. Reddish-brown lesions may also be found on petioles, pods and stems. Lesions usually develop concentric rings, first seen in the lower strata of the canopy, later spread throughout the plant (Galbieri *et al*., 2014; Godoy *et al*., 2016; Bowen and Hagan, 2018).

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The symptoms of target spot or Corynespora leaf blight on cotton plants are similar to those observed on soybean plants. In the foliar phase of the disease, the symptoms first appear on the leaves in the lower canopy and spread upward through the canopy towards the shoot tips, characterized as small circular spots (Galbieri *et al.,* 2014; Goulart and Lamas, 2016).

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not available, in other countries yield losses that ranges from 5 to 40% and lint reduction by 448 kg/ha have been reported (Hagan, 2014; Bowen and Hagan, 2018).

The first report of *C. cassiicola* was done in 1939 in China by Tai and Cheo (1937). In the American continent, more specifically in the United States, and first - reported in soybean in 1945 and in cotton in 1959 (Galbieri *et al*., 2014; Edwards Molina *et al*., 2019). In Brazil, the first identification of target spot symptoms was in 1974 in the state of Mato Grosso, followed by Paraná, in 1976 (Teramoto *et al*., 2013), Mato Grosso do Sul and Rio Grande do Sul in 1988 (Yorinori, 1989).

In cotton, the first report was recorded in 1995 in Mato Grosso, while in Mato Grosso do Sul its presence was confirmed in the 2011/12 crop season. Currently, the target spot occurs in all soybean and cotton-producing regions in the country (Edwards Molina *et al*., 2018; Godoy *et al*., 2018a). Its occurrence in soybean and cotton was also reported in the main producing regions abroad, especially in the Southeast and Center-South of the United States (Butler *et al*., 2016), Argentina (Edwards Molina *et al*., 2018) and China (Bowen and Hagan, 2018).

C. cassiicola has upright, branched conidiophores; in PDA colonies are white, gray or green; the mycelium is branched, with pale brown to hyaline septa and smooth walls. The conidia have size of 11.34 - 241, 94 µm x 2.58 - 11.17 µm and the number of septa can vary from 0 to 10 (Sajar, 2018). The conidia dispersion to neighboring plants occurs through rain splashes, while at long distance it occurs through the action of the wind. Prolonged relative humidity and water presence on the leaf surface for a period between 16 and 44 h have a fundamental role in spore germination. The pathogen is favored by higher temperatures, with the highest percentages of target spot severity in tomato plants in Florida was observed at temperatures between 28 and 32 °C (Mackenzie *et al*., 2018).

This species has a cosmopolitan behavior, occurring frequently in the subtropics and tropics infecting roots, stems and leaves of more than 407 plant species (Dixon *et al*., 2009; Teramoto *et al*., 2017; Farr and Rossman, 2019). This broad host range associated with the adoption of no-till practices, the successive sowing of susceptible host cultures the use of susceptible cultivars, the use of susceptible cover crops to manage nematodes and the loss of pathogen sensitivity to site-specific fungicides have contribute to increase the importance of the target spot in most of the Brazilian soybean and cotton-producing regions (Edwards Molina *et al*., 2018; Godoy *et al*., 2018a).

2.2 Target spot chemical control

Chemical control is a key component for the target spot management. In Brazil, there are 66 commercial products registered for the control of the target spot in soybean and six for cotton (AGROFIT, 2020). There is a variety of formulations, including multi-site alone, single-site alone, combination of multi-site and single-site, and two or three single-site fungicides combined. Different molecules from MBC (Methyl Benzimidazoles Carbamates), QoI (Quinone Outside Inhibitors), and SDHI (Succinate Dehydrogenase Inhibitors) fungicides, and DMI (Demethylation Inhibitors) site-specific fungicides are being used.

The control levels obtained with fungicides may vary depending on the products used and the cultivar of the host plant. A meta-analysis of target spot control in five soybean cultivars (BMX Power RR, M9144RR, TMG803, and NA5909 RG) and three fungicides applications, carried out between 2012 and 2016, revealed 32.4% control when the application was carried out with carbendazim (MBC); 46.7% with azoxystrobin (QoI) + benzovindiflupir (SDHI); 66.5% for prothioconazole (DMI) + trifloxystrobin (QoI); and 76.5% when the plots were sprayed with fluxapyroxade (SDHI) + pyraclostrobin (QoI) (Edwards Molina *et al*., 2019).

The large-scale use of these site-specific fungicides and the selection pressure favored by the succession of soybean/cotton crops and sharing of active ingredients between cultures for the control of the target spot, has accelerated the emergence of resistant populations (Tonin, 2014; Teramoto *et al*., 2017; Avozani *et al*., 2014 Duan *et al*., 2019).

There is previously evidence of *C. cassiicola* resistance to fungicides from the group of demethylation inhibitors (DMIs) in Brazil and China; quinone outside inhibitors (QoI) in Brazil, China, Japan and the United States; succinate dehydrogenase (SDHI) inhibitors in Brazil, China and Japan; and β-tubulin (βTub) inhibitors in Brazil and China (Ishii *et al*., 2007; Miyamoto *et al*., 2009; Teramoto *et al*., 2017; Rondon and Lawrence, 2019; Duan *et al*., 2019; Zhu *et al*., 2019). These cases threaten the chemical control stability, justifying its classification as a high-risk pathogen for the development of fungicide resistance by the Fungicide Resistance Action Committee (FRAC, 2019).

Resistance/insensitivity to fungicides can be conferred by different mechanisms, including the alternative enzymes production, capable of replacing the target enzyme and

altering the target site, reducing the binding to the fungicide (Ma and Michailides, 2005; Rehfus *et al*., 2016; Teramoto *et al*., 2017; Duan *et al*., 2019).

2.3 Mutations and change in protein constitution

Mutation is any change in the genomic sequence, and is an important factor of genetic variability in the population. This change can occur through a point mutation of only one nucleotide, better known as single nucleotide polymorphisms (SNPs), or involve large fragments of the genome, causing chromosomal reorganizations (Rohner, 2016; Mizubuti, 2018).

Alteration of a single nucleotide, may or may not interfere with the corresponding amino acid in the protein constitution, when the mutation leads to an amino acid change it is non-synonymous, when the mutation does not interfere with the transcribed amino acid it is called a synonym (Choudhuri, 2014).

These population variations occur independently, most of the amino acid substitutions are harmful, bring penalties / interfere with fitness, or in some situations are neutral (Choudhuri, 2014).

Specifically, for plant pathogenic fungi; if these substitutions occur in site-specific fungicide target proteins, affecting the interaction with the active ingredient molecule in the field with the application of these fungicides for disease control, the selection pressure exerted by the fungicide on the pathogen population will select the variant (resistant) individuals. As the application of this fungicide is repeated, the frequency of resistant individuals in the population increases, due to the exclusion of the sensitive individuals and the selection of resistant variants (Choudhuri, 2014).

2.4 Resistance to the DMI fungicides

DMI (Demethylation Inhibitors) fungicides block demethylation of carbon-14 from lanosterol during the ergosterol biosynthesis by C14 desmethylase. Ergosterol, a 5,7-diene oxysterol, is the most abundant sterol in fungal cell membranes, where it regulates permeability and fluidity (Ma and Michailides, 2005; Zhu *et al*., 2020). These fungicides possess curative action, and are used to control fungi from Basidiomycotina and Ascomycotina subdivisions. DMI fungicides were used for the first time in agriculture in

the 1970s. In the following decade, there have been reports of problems regarding resistance to DMIs in various phytopathogens (Ma and Michailides, 2005).

Resistance to DMIs involves several mechanisms, which can act individually or in association. The most common mechanisms are: the modification of the C14 demethylase active site (Cyp51), reducing its affinity with the fungicide; the overexpression of the *Cyp51* gene and/or the reduction of the fungicide accumulation in the fungal cell due to the increased activity of the efflux pumps. In most site-specific fungicides, resistance is qualitative and is caused by the mutation of a single nucleotide in the gene that encodes the target of the fungicide, leading to changes in the target site. However, in the case of DMIs, resistance is quantitative, involving accumulation of several mutations (Trkulja *et al*., 2017).

Quantitative resistance to DMIs means that the development of resistance to this group does not result in the complete loss of sensitivity, but a gradual adaptation of the pathogen population, through the progressive selection of individuals with lower baseline sensitivity (Chong *et al*., 2019). Zhu *et al*. (2020) evaluated the sensitivity of 121 isolates collected from symptomatic cucumber leaves (*Cucumis sativus* L.) in the years of 2017 and 2018, in provinces of China where metconazole had never been used. The isolates showed EC_{50} (Effective Concentrative to cause growth inhibition by 50%) between 0.1 and 1.9 μg/mL, with a variation factor of 19.1, presenting a narrow sensitivity profile with no resistance. The sensitivity to metconazole did not correlate with the sensitivity to diphenoconazole, tebuconazole, myclobutanil and/or hexaconazole, showing different behaviors for each DMI fungicide.

In Brazil, the reduced sensitivity to fungicides in the DMI group was found in surveys carried out in the 2013 to 2019 growing season (FRAC, 2020). This reduction also was found in isolates collected in the states of Goiás, Mato Grosso, Mato Grosso do Sul, Maranhão, Minas Gerais, Tocantins, Paraná and Pará between 1997 and 2013, with the EC_{50} ranging from 0.16 μg/mL in sensitive isolates and 7 to 46 μg/mL in isolates classified as moderately resistant to prothioconazole and cyproconazole. In this survey, a highly resistant isolate for cyproconazole with EC_{50}> 100 μg/mL was identified. However, mutations in the *Cyp51* gene associated with resistance have not yet been identified, suggesting that other mechanisms may be responsible for the observed resistance (Teramoto *et al*., 2017).

2.5 Resistance to the SDHI fungicides

The SDHI (Succinate Dehydrogenase Inhibitors) fungicides inhibit the succinate dehydrogenase (SDH, or complex II) enzyme, which participates in the mitochondrial electron transport chain (Miyamoto *et al*., 2010). The active complex II consists of four SDH subunits: hydrophilic flavoprotein (SDHA), iron-sulfur protein (SDHB) and two lipophilic transmembrane C and D subunits (SDHC and SDHD). All SDHIs act in the ubiquinone binding site of the SDH enzyme (Avenot and Michailides, 2010).

Commercialization of this group started in 1966, with fungicides still considered to be of narrow-spectrum (so-called first generation, for example, carboxin) and exceptionally active against basidiomycetes. In 2003, the first carboxamide with broad spectrum activity (boscalid) (so-called second generation, other examples are: penthiopyrad, fluopyram) was made available and widely used in several cultures. Resistance to this active ingredient was first reported in 2007 for *Alternaria alternata* in pistachios (*Pistacia vera* L.) in the state of California, United States (Avenot *et al*., 2008; Mackenzie *et al*., 2018).

Molecular studies aimed at characterizing the mechanisms responsible for the acquisition of resistance to SDHI fungicides in *C. cassiicola* identified mutations that lead to amino acid substitutions in the SDHB (H278Y / R; I280V), SDHC (S73P; N75S) and SDHD (D95E; H105R; G109V) associated with resistance to boscalid, fluopyram, penthiopyrad and carboxin (Miyamoto *et al*., 2010; Zhu *et al*., 2019). In Brazil, the presence of H278Y mutations in the SDHB gene and N75S in the SDHC gene were observed in *C. cassiicola* isolates with reduced sensitivity to fluxapyroxade, collected from soybean in the states of Mato Grosso and Rio Grande do Sul states in the seasons 2016/17 and 2017/18 (FRAC, 2020).

A similar study conducted with isolates collected in the states of Goiás, Mato Grosso, Mato Grosso do Sul, Maranhão, Minas Gerais, Tocantins, Paraná and Pará between 1997 and 2013 and evaluated for sensitivity to DMIs was carried for SDHI (Teramoto *et al*., 2017), and resistance to fluxapyroxade was found in three isolates. These isolates collected in 1997, 1999 and 2001 presented EC_{50}> 90 µg/mL, while sensitive isolates presented EC_{50} between <0.16 and 0.63 µg/mL. None of the isolates showed resistance to fluopyram, emphasizing the need to study the behavior of isolates with these substitutions at the target site for new molecules belonging to this group that

are been registered for the management of this disease in soybean and cotton, to detect the occurrence of cross-resistance.

2.6 Resistance to QoI fungicides

The QoI (Quinone Outside Inhibitors) fungicides act on the complex III of respiration, at the binding site of the external quinone (Qe) in the cytochrome b (Cytb) in the electron transport chain, inhibiting cellular respiration. The most common mechanism of resistance to QoI is a change in the target site in the pathogen (Mackenzie *et al*., 2018).

Three mutations associated with loss of sensibility have been reported. A single nucleotide polymorphism (SNP) that leads to the substitution of the amino acid glycine for alanine at Cytb position 143 (G143A) confers complete resistance in several species of phytopathogens. The other mutations at position 129 (F129L) and 137 (G137R) confer different degrees of resistance (Mackenzie *et al*., 2018).

The mutation G143A of Cytb was reported for the first time in *C. cassiicola* isolates collected in soybean in the state of Alabama (USA), in 2019 (Rondon and Lawrence, 2019). Whereas in isolates from cucumbers grown in greenhouses in China, resistance was demonstrated in 619 analyzed isolates, five of which were collected between 2008 and 2012 and 613 between 2017 and 2018. The mutant isolates showed cross-resistance to azoxystrobin, fluoxastrobin, pyraclostrobin, phenaminstrobin, picoxystrobin and coumoxstrobin (Duan *et al*., 2019). The same mutation (G143A) was reported in *C. cassiicola* isolates collected from tomatoes, between 2015 and 2017 in Florida, providing complete resistance to the strobilurin fungicide, with EC_{50} greater than 100 μg/mL. Isolates with the F129L mutation showed intermediate resistance, with EC_{50} ranging from 7.7 to 36.7 μg/mL, but much higher than the EC_{50} observed in sensitive isolates, which was 0.2 to 1.8 μg/ml (Mackenzie *et al*., 2018; Mackenzie *et al*., 2020). The resistance factor for mutants of fungi with the variation in position 129 (F129L) is considered low compared to the variation in position 143 (G143A), while the substitution in position 137 (G137R) on resistance is not known, since its identification is rare, probably due to its impact on the overall fitness of the pathogen (Sierotzki, 2015).

In Brazil, *C. cassiicola* insensitivity to QoI, such as picoxystrobin, pyraclostrobin, azoxystrobin and trifloxystrobin was already reported in a study carried out with 34 isolates obtained from soybean in the states of Goiás, Mato Grosso, Mato Grosso do Sul, Maranhão, Minas Gerais, Tocantins, Paraná and Pará (Teramoto *et al*., 2017). The G143A

mutation was detected in soybean in monitoring studies carried out by FRAC in the 2015/16 and 2016/17 crop season, and studies carried out in 2018/19 showed a higher frequency of this mutation in isolates collected in seven Brazilian states that were monitored (MT, MS, GO, BA, MG, PR, TO) (FRAC, 2020).

2.7 Resistance to the MBC group

MBC (Methyl Benzimidazoles Carbamates) fungicides act by binding to the β-tubulin protein inhibiting the assembly of microtubules during nuclear division (Duan *et al*., 2019). Benzimidazole fungicides were introduced to agriculture for the first time in the 1970s. Since the beginning of its commercialization, there have been reports of 100 species of fungi with the development of resistance to fungicides in this group (Vela-Corcía *et al*., 2018).

Currently available MBC fungicides are benomyl, carbendazim, thiabendazole and thiophanate-methyl. They are registered in several countries of the world for a wide range of crops (cereals, fruit, vegetables, among others) with a broad spectrum of action, although they are not effective against oomycetes (Vela-Corcía *et al*., 2018).

Several point mutations associated with MBC resistance have been reported. These mutations occur at various positions in the gene encoding β-tubulin, resulting in changes in the amino acids at positions 6, 50, 167, 198, 200 and 240. However, mutations E198A / G / K / Q and F200Y are the most frequent (Duan *et al*., 2019).

Cross-resistance to different MBC compounds is generally observed, with the result that the other molecules that make up the class become inefficient, even without having been used on a large scale (Duan *et al*., 2019).

In China, individual mutations such as E198A have been reported, and three combinations of double mutations E198A + M163I; E198A + F167Y; and E198A + F200S associated with resistance in 619 isolates of *C. cassiicola* collected in cucumber in the years 2017 and 2018.

Double mutations were associated with higher levels of resistance to benzimidazoles. The highest EC_{50} values were observed in isolates with the E198A + F167Y substitutions (344.11 μg/mL for carbendazim, 175.31 μg/mL for benomyl and 74.33 μg/mL for thiabendazole), although the mutations E198A + F200S and E198A + M163I pairs show similar sensitivity, whereas the E198A substitution had a smaller impact on the sensitivity to carbendazim, benomyl and thiabendazole, with mean values

of EC_{50} 215.06, 92.08 and 40.44 µg/ml, respectively. Sensitive isolates collected in 2012 showed EC_{50} of 0.08 to 0.4 for the same benzimidazoles (Duan *et al*., 2019).

EC_{50} values > 1000 µg/mL to carbendazim and thiophanate-methyl have already been reported in isolates of *C. cassiicola* collected in soybean in Goiás, Mato Grosso, Tocantins, Mato Grosso do Sul, Paraná, Maranhão, Minas Gerais and Pará, between 2001 and 2013, while sensitive isolates presented EC_{50} of 0.16 µg/mL (Xavier *et al*., 2013; Teramoto *et al*., 2017).

The distribution of resistant isolates in cotton crops and their relationship with resistant isolates collected in soybean are not yet known, as well as the pattern of resistance of isolates obtained from these hosts, and underlying mutations associated with the resistance.

2.8 Molecular and pathogenic variability of *C. cassiicola*

The target spot has recently emerged as a serious disease in cotton, soybean and tomatoes in the United States, although its occurrence in these crops was already known without causing significant losses in productivity. The cause of this emergency in the United States is not yet known (Sumabat *et al*., 2018).

Population biology studies of emerging pathogens may help understand their progress in areas with a large population of host plants, especially when they are sown in sequence and share the same fungicidal molecules for control, as is the case of soybean and cotton in Brazil.

The phylogenetic analysis of 143 isolates of *C. cassiicola* from 68 species of plants collected in various regions of the world, showed six phylogenetic isolates widely distributed by the sampling regions, but correlated due to the host of origin (Dixon *et al*., 2009). This study included only one isolate from soybean in Brazil.

In another phylogenetic study with several isolates of *C. cassiicola*, including one cotton isolate and five isolates from soybean in Brazil (Déon *et al*., 2014), the importance of the cassiicoline toxin in the aggressiveness of the pathogen was observed. Of the six Brazilian isolates obtained from soybean (five) and cotton (one), one was not tested, two soybean isolates had the *cas2* gene, and two soybean and cotton isolates had the *cas2* and *cas6* genes.

The cluster analysis based on the sequencing of four genomic regions (ITS, actin and two hypervariable loci, *caa5* and *ga4*) of 53 isolates obtained from cotton, soybean

and tomato, in addition to cucumber, *Hydrangea* spp., *Mandevilla* spp., pepper and sesame with symptoms of target spot in the United States, revealed the formation of clusters related to the host of origin, with no geographical relationship (Sumabat *et al*., 2018).

Pathogenicity and virulence tests with forty isolates demonstrated the capacity for cross-infection, but greater aggressiveness was observed in the host of origin, with the exception of soybean isolates which were equally aggressive in soybean and cotton. In these tests, it was also observed that cotton was the most susceptible species among the four evaluated (soybean, cotton, tomato and cucumber) (Sumabat *et al*., 2018).

3 MATERIAL AND METHODS

3.1 Isolation and origin of isolates

Isolates of *Corynespora cassiicola*, were obtained by indirect isolation from soybean and cotton leaves with symptoms of target spot sampled from 2014 to 2020 from commercial fields throughout Brazil. Samples were taken at random from different Brazilian fields. Total samples were divided into three states: Bahia, Mato Grosso and Goiás. The leaf samples were herbarized and sent to the Laboratory of Genetics Genomic and interaction plant pathogen (LGGIPP) at Universidade Federal de Viçosa where the isolation was carried out from these samples. Isolates were maintained on potato dextrose agar (PDA) at 25°C without photoperiod and preserved with two different storage methods: (a) in cryovials with sterile water at 4°C, and (b) in 50% glycerol at −80°C.

3.2 DNA extraction

For DNA extraction, liquid cultures of isolates were initiated by placing small pieces of mycelium into 250 ml flasks containing 25 mL of YMS liquid medium (8 g of yeast extract L-1, 8 g of malt extract L-1, 8 g of sucrose L-1). The initial mycelium was carefully scraped with a sterile needle from the aerial mycelia of cultures growing on PDA plates. Liquid cultures were incubated at 25°C for four days. After incubation, mycelia were recovered with a sterile strainer, frozen in liquid nitrogen, and then macerated. The grinded mycelium was stored in 1.5 mL eppendorf tubes at -80 ºC. DNA

extraction and purification were performed following the NucleoSpin ™ DNA extraction kit protocol (Macherey-Nagel ™).

3.3 Amplification and sequencing of *ITS* and fungicide target genes

All isolates were characterized by sequencing ITS and six fungicide target genes: *β-tubulin, CytB, Cyp51, SdhB, SdhC* and *SdhD*. Oligonucleotides used in the amplification of the *β-tubulin, Cyp51, SdhB, SdhC* and *SdhD* were designed using *C. cassiicola* sequences deposited in the database of the National Biotechnology Information Center (NCBI) (Table 1). For *CytB* were used oligonucleotides described by Ishii et al. (2007); and for the *ITS* region those designed by Xie *et al*. (2008) (Table 1).

PCR reactions was performed in a final do 10 µL, using 6.3 µL of autoclaved milli-Q water, 1 µL of template DNA, 0.3 µL of each primer (at a concentration of 10µM), 0, 8 µL dNTPs (2.5µM), 1 µL Reaction Buffer 10 x; 0.2 µL of MgCl2 (25 mM); and 0.1 of the Taq DNA (Cellco).

The following temperature programmed of PCR reaction was used: an initial heating for 30 s at 95 °C was followed by 35 cycles at 95 °C for 30 s, extension with primer specific temperatures for 30 s (Table 1) and elongation at 72°C for 45 - 90 s, depending on the expected fragment size and a final elongation step at 72 °C for 5 min. PCR reactions were performed on the VeritiTM Thermal Cycler 96 wells thermocycler (Applied Biosystems).

Table 1. Oligonucleotide primers used in this study.

Oligonucleotides	Sequence 5'-3'	Temp. annealing (°C)	Gene	NCBI Acess	Amplicon (bp)
SDHB_F*	CTACCCAACAGCTCACTTGCC	62	*SdhB*	AB548738.1	990
SDHB_R	CACTCTTCTTCGCCATCCCACG				
SDHC_F	GACATCATCCACCACCCGCG	62	*SdhC*	AB548741.1	700
SDHC_R*	AACTCCGTCCCTTTTCCAGC				
SDHD_F	CGGTCTCTTCAGGCAGGC	62	*SdhD*	AB548743.1	890
SDHD_R*	TGCTCACTTTCCATCCCGC				
Cyp51_F1*	GCTCCCCTTCACTCCTGCTCGT	65	*Cyp51*	GCA003016335.1	1242
Cyp51_R2*	GTCGGGGAGGTTATTACTAGGTAG				
CytB_F*	GCGAATTCCTATTTAGTTGATTC	65	*CytB*[a]	-	526
CytB_R	GGTTACCTGATCCAGCTGTATC				
β-tub_F*	AGCCATCATGCGTGAGATTGTAC	60	*β-tubulin*	MH763695.1	1200
β-tub_R*	TCTACTCCTCAGCCTCGAGAG				
ITS1*	TCCGTAGGTGAACCTGCGG	62	*ITS*[b]	-	520
ITS4	TCCTCCGCTTATTGATATGC				

[a] Oligonucleotides obtained from the study by Ishii *et al*. (2007);
[b] Oligonucleotides obtained from the study by Xie *et al*. (2008);
* Oligonucleotides used in sequencing reactions

Sequencing reactions were performed using the BigDyeTM Terminator v 3.1 Cicle Sequencing kit (Applied Biosystems, USA) and specific oligonucleotides for each gene. Further assembly of sequences were done by the DNA Baser Sequence Assembler v. 3.5 (Heracle Biosoft). Alignment and translated amino acid sequences were done using the MEGA v. 5. software.

3.4 Protein homology modeling

Comparative modeling of the subunits of the *C. cassiicola* SDH protein was performed using the UCSF Chimera Software (Resource for Biocomputing, Visualization, and Informatics, University of California, San Francisco, USA). The SDH X-ray structure of SDH-carboxin complex from *Ascaris suum* (PDB 3VR8_C) was used as a structural template. The overall sequence identity with subunit C from *C. cassiicola* is 33.1%. Alignments of the amino acid sequence were performed using BLOSUM62 substitution matrix. The amino acid substitutions that generate resistance to SDHIs fungicides were implemented manually in the 3D model.

3.5 Determination of alternative respiration

Alternative oxidase (AOX) is a strobilurin-insensitive terminal oxidase that allows electrons from ubiquinol to bypass Complex III. Its synthesis is constitutive in some fungi but in many others is induced by inhibition of the main pathway. AOX provides a strobilurin-insensitive pathway for oxidation of NADH. Salicylhydroxamic acid (SHAM) is a characteristic inhibitor of AOX, and several studies have explored the potentiation of strobilurin activity by SHAM. (Ziogas *et al.,* 1997). In order to determine whether an alternative oxidation was present in *C. cassiicola* isolates chosen for the present study, spores and mycelium sensitivity to pyraclostrobin (Comet® - QoI), was evaluated in the presence and absence of salicylhydroxamic acid (SHAM, 100 µg/ml) (99% active, Sigma Aldrich) on five *C. cassiicola* isolates with previously characterized *cytB* gene in a preliminary experiment. Experiment was conducted once with four repetitions.

Serial dilutions of pyraclostrobin were made sterile distilled water. Afterwards, PDA was amended with pyraclostrobin at 0; 0.01; 0.1; 1; and 10 µg/ml for wild type

isolates, and 0; 0.156. 0.625; 2.5; 10; 40 and µg/ml for isolates with the G143A substitution.

For mycelial growth, petri dishes were filled with 20 mL of PDA media without and amended with different concentrations of the tested with and without SHAM. Mycelial plugs (4.5 mm in diameter) were cut from seven-day-old colony margin on PDA plates and placed on PDA plates amended with different fungicide concentrations. Plates were incubated in the dark at 25 °C for eight days (25 °C, in the dark), and colony diameters were measured using a Zaas precision digital caliper in two perpendicular directions and the mean value was used to estimate the EC_{50} values. The diameter of the circle (4.5 mm) was subtracted from the diameter of the colony, to determine growth after disk transfer.

For spore germination evaluation, the spore suspension was adjusted to $4x10^4$ spore/mL, and 120 µL of spore suspension of each isolate was added into micro plate of 96 wells, of each fungicide dilution described above (with and without SHAM). The plate was incubated for 12 hours in a shaker at 25 ° C, in dark and 90 rpm of agitation. For evaluation of the number of germinated spores, 80 µL of suspension in microplate was added to a slide and with the aid of an optical microscope 100 conidia were evaluated. Germination values were used to calculate the EC_{50}.

3.6 *In vitro* fungicide sensitivity testing

Because of scant sporulation in culture and following Duan *et al.* (2019), mycelial radial growth inhibition instead of spore germination assays were used for determining fungicide sensitivity. In order to calculate the effective fungicide concentration to inhibit of fungal radial growth for each isolate × fungicide combination, all fungicides were tested on isolates 32 isolates selected after molecular characterization.

Petri dishes were filled with 20 mL of PDA media without and amended with different concentrations of the tested. The following fungicide concentrations were used: pyraclostrobin (Comet® - QoI): 0; 0.001; 0.01; 0.1; 1; 10; 100 and 500 µg/mL; carbendazin (Carbomax 500 SC® - MBC): 0; 0.001; 0.01; 0.1; 1; 10; 100 and 500 µg/mL; prothioconazole (DMI): 0; 0.001; 0.01; 0.1; 0.5; 1; 10 µg/ml; fluxapyroxad, benzovindiflupyr and inpyrfluxam (SDHIs): 0; 0.5; 1; 5; 10; 50; 100 and 500 µg/mL.

Mycelial plugs (4.5 mm in diameter) were cut from seven-day-old colony margin on PDA plates and placed on PDA plates amended with different fungicide

concentrations. Plates were incubated in the dark at 25 °C for eight days (25 °C, in the dark), and colony diameters were measured using a digital caliper as previously described.

Experiment was conducted under a completely randomized design, with four replications for the SDHIs fungicides and three replications for the other groups. EC_{50} values (effective inhibitory concentration of 50% of the mycelial growth) and, also the MIC (minimum inhibitory concentration; for the purposes of the equation, 99.9% growth inhibition) were estimated. Resistant factors (RF) were calculated as the ratio of EC_{50} of isolates with mutation group/EC_{50} of wild type isolates group. RF determines how many times the mutation has increased resistance in the population due to the group of isolates without the mutation.

3.7 Statistical analysis

Dose–response curves, EC_{50} and the MIC values were generated for each isolate × fungicide combination (32 isolates). Function "drm" from "drc" package v. 2.5-12 (Ritz *et al*., 2015) was used in the R environment v. 3.6.3 (R Core Team, 2020) for nonlinear regression analysis. The models used were: LL.3 for pyraclostrobin, carbendazim and prothioconazole; w3 for benzovindiflupyr, fluxapyroxade and inpyrfluxam. The best-fit model for each fungicide was evaluated based on the log-likelihood, Akaike Information Criteria (AIC). T-student test unpaired was used to assess the difference in sensitivity between populations with and without G143A substitution in Cytochrome b. Normality and homogeneity of variance test for EC50 and MIC values was performed using the Shapiro-Wilk ($p \leq 0.05$) and Neill and Mathews ($p \leq 0.05$) test, respectively. The analysis of variance and the post-hoc Bonferroni were applied to compare the isolates groups with mutations in the *Sdhs* and *β-tubulin* genes. Cross-resistance to carboxamides was verified by using Spearman's correlation analysis of EC_{50} values. All statistical analyzes were realized using the R software (R Core Team, 2019).

4 RESULTS

4.1 Isolates and identify species confirmation by *ITS* sequencing

Monosporic culture isolation resulted in 57 isolates of *C. cassiicola* and 22 were made available by the company Tropical Melhoramento & Genética (TMG). In total, 79 isolates were used in this study, these 42 isolates were obtained from soybean and 37 from cotton (Table 2). All isolates were identified as *C. cassiicola* based on morphologic analyses of conidiophore and conidia and sequence analyses of internal transcribed spacer (ITS1/ITS4). All ITS sequences were used to run BLASTn searches. A BLAST search in GenBank revealed that all sequences showed 100% identity with *C. cassiicola*. These sequences were also aligned with *C. cassiicola* sequences recovered from soybean plants previously identified by Sumabat et al. (2018) (accession numbers MF320538.1 to MF320539.1) and showed >99.6% identity, confirming that our isolates belong to the species of *C. cassiicola*.

4.2 Identification and molecular characteristics of *C. cassiicola Cytochrome B, β-tubulin, Cyp51, Sdhb, Sdhc* and *Sdhd* gene

CytB, B-tubulin, Cyp51, Sdhb, Sdhc and *Sdhd* genes were amplified, comparison of the deduced amino acid sequences of the translated to these genes from the tested *C. cassiicola* isolates revealed prevalence of isolates with substitution in *CytB*, *β-tubulin*, *Sdhb* and *Sdhc* (Table 2). No variation was identified in the *Cyp51* among evaluated isolates (Table 2). *Cytb* sequences (526 bp) of 79 isolates analyzed showed that 69 isolates have non-synonymous mutation G353C in *Cytb* (GGT → GCT) leading to the G143A substitution. Of the 10 isolates without this alteration, 9 are from cotton, 8 to which were obtained from the same collection site. Only one wild type (without mutations that lead to resistance) isolate was obtained from soybean. Sequences with the G143A substitution, 41 were observed in soybean and 28 cotton isolates. In addition, *CytB* G353C mutation, six isolates from Mato Grosso also exhibit a C411T synonymous mutation (Supplementary Table 1).

Comparison of the deduced amino acid sequences of the translated *β-tubulin* gene sequences (1200 bp) obtained for 79 tested isolates revealed the prevalence of SNPs in 54 isolates in the gene compared to the reference wild type (Table 2). Two SNPs in special lead to amino acid substitutions associated with resistance to benzimidazoles in the

population analyzed: A593C (GAG → GCG) resulting in E198A substitution observed in 37 isolates from which 10 were obtained from cotton and 27 from soybean; T599A (TTC → TAC) resulting in F200Y substitution in 17 isolates from which 14 were obtained from cotton and three from soybean (Table 2).

Table 2. Description of the isolates obtained; host, obtaining year, origin location and amino acids substitution in the target genes of fungicides specific site.

Isolates	Location	Host	Year	Substitution in SDH subunits			Cyp51	CytB	β-tubulin
				SDHB	SDHC	SDHD			
CCUFV1	L.E.M.[1] – BA	cotton	-	WT	WT	WT	WT	G143A	F200Y
CCUFV2	L.E.M.[1] – BA	cotton	-	WT	WT	WT	WT	G143A	F200Y
CCUFV3	Barreiras – BA	cotton	-	WT	WT	WT	NT	G143A	F200Y
TMG506	Pedra Preta – MT	cotton	2014	WT	WT	WT	WT	G143A	F200Y
TMG542	MT	cotton	2014	WT	WT	WT	WT	G143A	F200Y
TMG543	MT	cotton	2014	WT	WT	WT	NT	G143A	F200Y
TMG544	MT	cotton	2014	WT	WT	WT	WT	G143A	F200Y
CCUFV4	Sinop-MT	soybean	-	WT	WT	WT	NT	G143A	E198A
CCUFV5	Rondonópolis – MT	soybean	-	WT	WT	WT	NT	G143A	E198A
CCUFV6	Rio Verde - GO	soybean	-	WT	WT	WT	NT	G143A	E198A
CCUFV7	Nova Mutum – MT	soybean	-	WT	WT	WT	WT	G143A	E198A
CCUFV8	Nova Mutum – MT	soybean	-	WT	WT	WT	WT	G143A	E198A
CCUFV9	Sinop - MT	soybean	-	WT	WT	WT	WT	G143A	E198A
CCUFV10	Nova Mutum – MT	soybean	-	WT	WT	WT	NT	G143A	E198A
TMG080	Sapezal - MT	soybean	2016	WT	WT	WT	NT	G143A	E198A
TMG082	P.G. [2]- MT	soybean	2016	WT	WT	WT	WT	G143A	E198A
TMG083	Nova Mutum - MT	soybean	2016	WT	WT	WT	NT	G143A	E198A
TMG109	Sorriso - MT	soybean	2016	WT	WT	WT	WT	G143A	E198A
TMG119	Correntina - BA	soybean	2016	WT	WT	WT	NT	G143A	F200Y
TMG121	Rondonópolis - MT	soybean	2018	WT	WT	WT	WT	G143A	E198A
TMG122	Matupibá - MT	soybean	2016	WT	WT	WT	WT	WT	WT
TMG557	Sapezal-MT	cotton	2018	WT	N75A	WT	WT	G143A	F200Y
TMG179	MT	soybean	2008	WT	WT	WT	NT	G143A	E198A
TMG547	Sapezal - MT	cotton	2018	WT	N75S	WT	NT	G143A	E198A
TMG549	Campo Verde - MT	soybean	2018	WT	WT	WT	NT	G143A	F200Y
TMG545	C.N.P.[3] - MT	cotton	2018	WT	N75S	WT	NT	G143A	E198A
TMG505	Rondonópolis - MT	cotton	2014	WT	WT	WT	NT	G143A	F200Y
TMG537	Rondonópolis - MT	cotton	2018	WT	WT	WT	WT	G143A	F200Y
TMG180	Palotina - MT	soybean	2018	WT	WT	WT	NT	G143A	E198A
TMG556	Sorriso - MT	cotton	2018	WT	WT	WT	WT	G143A	E198A
TMG507	Rondonópolis - MT	cotton	2017	WT	WT	WT	NT	G143A	F200Y
TMG548	Serra da Petrovina -M	cotton	2018	WT	WT	WT	NT	G143A	F200Y
CCUFV11	Rondonópolis - MT	cotton	2019	WT	WT	WT	NT	G143A	E198A
CCUFV12	Rondonópolis - MT	cotton	2019	WT	WT	WT	NT	G143A	WT
CCUFV13	Rondonópolis - MT	cotton	2019	WT	WT	WT	NT	WT	WT
CCUFV14	Rondonópolis - MT	cotton	2019	WT	WT	WT	NT	G143A	E198A
CCUFV15	Rondonópolis - MT	cotton	2019	WT	WT	WT	NT	G143A	F200Y
CCUFV16	Rondonópolis - MT	cotton	2019	WT	WT	WT	NT	G143A	WT
CCUFV17	Sorriso - MT	cotton	2019	WT	N75S	WT	NT	G143A	E198A
CCUFV18	C.N.P.[3]- MT	cotton	2019	WT	N75S	WT	NT	G143A	E198A

Continue...

Isolates	Location	Host	Year	Substitution in SDH subunits			Cyp51	CytB	β-tubulin
				SDHB	SDHC	SDHD			
CCUFV19	C.N.P.[3]- MT	cotton	2019	WT	N75S	WT	NT	G143A	E198A
CCUFV20	C.N.P.[3]- MT	cotton	2019	H278Y	WT	WT	NT	G143A	WT
CCUFV21	Campo Verde - MT	cotton	2019	WT	WT	WT	NT	G143A	F200Y
CCUFV22	Campo Verde - MT	cotton	2019	WT	N75S	WT	NT	G143A	E198A
CCUFV23	C.N.P.[3]- MT	cotton	2019	WT	N75S	WT	NT	G143A	E198A
CCUFV24	C.N.P.[3]- MT	cotton	2019	WT	WT	WT	NT	G143A	WT
CCUFV25	C.N.P.[3]- MT	soybean	2020	WT	WT	WT	WT	G143A	WT
CCUFV26	Sorriso - MT	soybean	2020	WT	WT	WT	WT	G143A	WT
CCUFV27	C.N.P.[3]- MT	soybean	2020	WT	N75S	WT	WT	G143A	E198A
CCUFV28	C.N.P.[3]- MT	soybean	2020	WT	N75S	WT	WT	G143A	F200Y
CCUFV29	C.N.P.[3]- MT	soybean	2020	WT	N75S	WT	WT	G143A	WT
CCUFV30	Sorriso - MT	soybean	2020	WT	WT	WT	WT	G143A	E198A
CCUFV31	C.N.P.[3]- MT	soybean	2020	WT	N75S	WT	WT	G143A	E198A
CCUFV32	Sorriso - MT	soybean	2020	WT	N75S	WT	WT	G143A	E198A
CCUFV33	Sorriso - MT	soybean	2020	WT	WT	WT	WT	G143A	WT
CCUFV34	L.R.V. [4]- MT	soybean	2020	WT	N75S	WT	WT	G143A	E198A
CCUFV35	C.N.P.[3]- MT	soybean	2020	WT	N75S	WT	WT	G143A	E198A
CCUFV36	Sorriso - MT	soybean	2020	WT	WT	WT	WT	G143A	WT
CCUFV37	Sorriso - MT	soybean	2020	WT	WT	WT	WT	G143A	WT
CCUFV38	L.R.V. [4]- MT	soybean	2020	WT	N75S	WT	WT	G143A	E198A
CCUFV39	C.N.P.[3]- MT	soybean	2020	WT	N75S	WT	WT	G143A	WT
CCUFV40	C.N.P.[3]- MT	soybean	2020	WT	N75S	WT	WT	G143A	WT
CCUFV41	C.N.P.[3]- MT	soybean	2020	H278Y	WT	WT	WT	G143A	E198A
CCUFV42	C.N.P.[3]- MT	soybean	2020	H278Y	WT	WT	WT	G143A	E198A
CCUFV43	Sorriso - MT	soybean	2020	WT	WT	WT	WT	G143A	WT
CCUFV44	Sorriso - MT	soybean	2020	WT	WT	WT	WT	G143A	E198A
CCUFV45	C.N.P.[3]- MT	soybean	2020	WT	N75S	WT	WT	G143A	E198A
CCUFV46	Sorriso - MT	soybean	2020	WT	N75S	WT	WT	G143A	E198A
CCUFV47	L.R.V. [4]- MT	soybean	2020	H278Y	WT	WT	WT	G143A	E198A
CCUFV48	Sorriso - MT	soybean	2020	WT	WT	WT	WT	G143A	WT
CCUFV49	Sorriso - MT	soybean	2020	WT	WT	WT	WT	G143A	WT
CCUFV50	Rondonópolis - MT	cotton	2020	WT	WT	WT	WT	WT	WT
CCUFV52	Rondonópolis - MT	cotton	2020	WT	WT	WT	WT	WT	WT
CCUFV53	Rondonópolis - MT	cotton	2020	WT	WT	WT	WT	WT	WT
CCUFV61	Rondonópolis - MT	cotton	2020	WT	WT	WT	WT	WT	WT
CCUFV62	Rondonópolis - MT	cotton	2020	WT	WT	WT	WT	WT	WT
CCUFV63	Rondonópolis - MT	cotton	2020	WT	WT	WT	WT	WT	WT
CCUFV67	Rondonópolis - MT	cotton	2020	WT	WT	WT	WT	WT	WT
CCUFV68	Rondonópolis - MT	cotton	2020	WT	WT	WT	WT	WT	WT

Legend: NT: Not tested; [1] L.E.M: Luiz Eduardo Magalhães; [2] P.G.: Porto dos Gaúchos; [3] C.N.P.: Campo Novo do Parecis; [4] L.R.V.: Lucas do Rio Verde.

PCR products was amplified from the *SdhB* (990 pb), *SdhC* (700 pb) and *SdhD* (890 pb) and analyzed for sequencing. The results showed three substitutions in 24 isolates, one in the SDHB subunit and two in the SDHC subunit. None amino acid change SDHD subunit were identified (Table 2). The presence of H278Y (CAC→TAC) substitutions were revealed in the SDHB subunit in four isolates (one was obtained from cotton and three from soybean) and N75S (AAC → AGC) in the SDHC subunit in 19 isolates (seven

were observed from cotton and 12 from soybean). Still in the SDHC subunit, N75A (AAC → GCC) substitution was identified in one isolate from cotton (Table 2). In *SdhB* and *Sdh*C 23 isolates exhibit mutations resulting in substitutions in agreement with some previous reports (Frac, 2020). Besides that, a new substitution, (N75A) in the SDHC subunit was also observed (Table 2). The highest proportion of isolates with substitutions in the subunits of the SDH enzyme was observed for the group obtained from soybean in the 2020 crop season, being present in 15 of the 24 isolates (Table 2).

New amino acid substitution N75A detected in the isolate of *C. cassiicola* resistant to SDHI, was analyzed for its location in the SDHC subunit of the SDH enzyme, to model its potential structural impact in the resistance to SDHI. The modeling was based on the X-ray structure of *Ascaris suum* (PDB 3VR8_C), as there is no X-ray structure of the *C. cassiicola* SDH available, the model had 33.1% identity. In the SDH homology model, the naturally conserved **haem b** group is represented in the figure, showing its proximity to the substitutions in position 75 (Figure 1).

Accumulation of substitutions (H278Y/N75S/N75A + G143A + F200Y/E198A) leading to simultaneous resistance to SDHI, QoI and MBC was confirmed in 20 isolates. Double substitutions in the same gene were not reported in the tested 79 *C. cassiicola* isolates (Table 2).

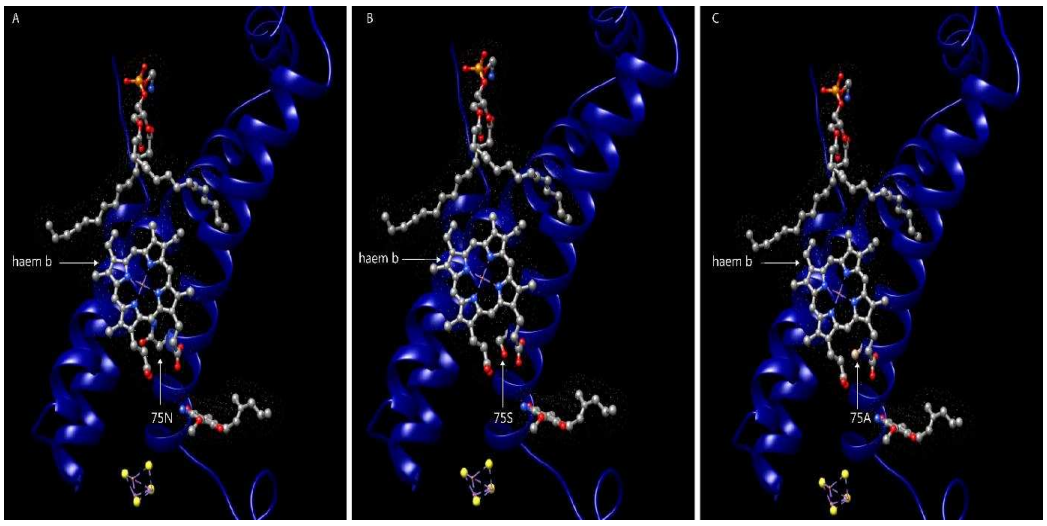

Figure 1. Homology model of *C. cassiicola* succinate dehydrogenase - subunit C, based on X-ray from *Ascaris suum* (PDB 3VR8_C) with substitutions at position 75 leading to SDHI resistance; **A**. wild type protein; **B**. N75S replacement previously described in the literature. **C**. N75A replacement.

4.3 Impact of *Cytb* mutation on the sensitivity to pyraclostrobin

There were no significant differences when comparing mycelial growth inhibition values with and without the addition of SHAM at a given fungicide concentration. SHAM addition also did not interfere with spore germination. This was observed for isolates with the G143A substitution (CCUFV32, CCUFV34 and TMG557), and for isolates without G143A substitution (CCUFV54 and CCUFV58) (Supplementary Figure 1).

The comparison of EC_{50} values obtained from mycelial growth and spore germination, demonstrated that G143A substitutions became the isolates less sensitive to pyraclostrobin in both evaluation methods. There was a greater effect in sensitivity of spores than in mycelia of isolates with and without the G143A substitution. (Supplementary Figure 2).

There was greater sensitivity to fungicide in spore germination, but sensitivity was determined by mycelial growth due to the difficulty in sporulation of some isolates. Therefore, it was assumed that for the isolates tested here AOX did not occur and SHAM was not used in subsequent experiments. Additionally, in a previous study, Teramoto *et al*. (2017) also did not observe any effect of SHAM in increasing the sensitivity of four *C. cassiicola* isolates to QoI fungicides.

So, to verify the sensitivity of the isolates with and without the identified substitutions, 32 isolates were tested for the sensitivity to pyraclostrobin through mycelial growth (Table 3). EC_{50} value of the isolates without the G143A substitution ranged from 1.4 to 13.4 μg/ml whereas the isolate that presented G143A had an EC_{50} between 9.5 and 95.0 μg/ml (Table 3). Mean EC_{50} of the isolates group with the substitution in position 143 was 34.5 μg/ml and for the group without mutation 6.8 μg/ml. Calculation of Resistance factor demonstrated a RF: 5.0 (Figure 2) for the group with the replacement G143A (Table 3; Supplementary Figure 3).

Considering the EC50 values, it was possible to validate the effect of G143A substitution on pyraclostrobin resistance by mycelial growth without the SHAM use, with a significant difference between groups of isolates (Figure 2). The dosages tested did not completely inhibit the growth of the fungus, as a result of which the MIC of all isolates was higher than the 500 μg/ml (Table 3).

Table 3. Sensitivity profile (EC50 and Minimum Inhibitory Concentration – MIC) of 32 *C. cassiicola* isolates to the QoI and MBC fungicides, and characterization of isolates in relation to the fungicide target protein CytB and B-tub.

Isolates	Pyraclostrobin		CytB	Carbendazim		β-tub
	EC_{50} (µg/mL)	MIC (µg/mL)		EC_{50} (µg/mL)	MIC (µg/mL)	
CCUFV26	64.4	>500	G143A	0.45	9.09	WT
CCUFV27	25.6	>500	G143A	>500	>500	E198A
CCUFV28	27.9	>500	G143A	>500	>500	F200Y
CCUFV29	20.2	>500	G143A	0.24	10.00	WT
CCUFV32	17.0	>500	G143A	>500	>500	E198A
CCUFV34	47.3	>500	G143A	>500	>500	E198A
CCUFV35	14.1	>500	G143A	>500	>500	E198A
CCUFV36	13.4	>500	G143A	0.04	10.00	WT
CCUFV38	14.5	>500	G143A	>500	>500	E198A
CCUFV39	21.9	>500	G143A	0.44	8.40	WT
CCUFV40	58.1	>500	G143A	0.09	0.20	WT
CCUFV41	56.1	>500	G143A	>500	>500	E198A
CCUFV42	28.4	>500	G143A	>500	>500	E198A
CCUFV45	21.5	>500	G143A	>500	>500	E198A
CCUFV46	30.8	>500	G143A	>500	>500	E198A
CCUFV47	40.0	>500	G143A	>500	>500	E198A
CCUFV48	61.8	>500	G143A	0,02	0,07	WT
CCUFV50	10.8	>500	WT	0,02	0,09	WT
CCUFV53	3.09	>500	WT	0,05	10.00	WT
CCUFV55	5.7	>500	WT	0,26	8,72	WT
CCUFV58	1.4	>500	WT	0,07	0,31	WT
CCUFV62	13.4	>500	WT	0,23	10,60	WT
TMG543	23.3	>500	G143A	>500	>500	F200Y
TMG557	20.1	>500	G143A	>500	>500	F200Y
TMG547	12.5	>500	G143A	>500	>500	E198A
TMG545	9.5	>500	G143A	>500	>500	E198A
CCUFV17	95.0	>500	G143A	>500	>500	E198A
CCUFV18	13.2	>500	G143A	>500	>500	E198A
CCUFV19	47.8	>500	G143A	>500	>500	E198A
CCUFV20	40.2	>500	G143A	0.03	1.43	WT
CCUFV21	35.7	>500	G143A	>500	>500	F200Y
CCUFV23	72.3	>500	G143A	>500	>500	E198A

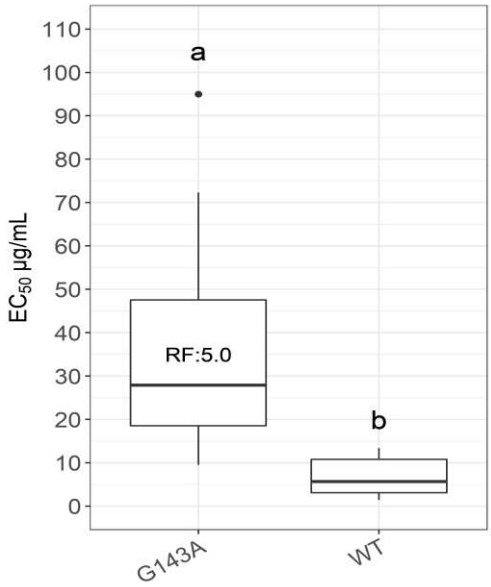

Figure 2. Comparison of the pyraclostrobin sensitivity profile (EC_{50} values) between the groups constituted of the WT (n = 5) and with the G143A substitution in CytB (n = 27) isolates by the t test ($p < 0.05$); the resistance factor (RF) is on the center of the box of the resistant group.

4.4 Impact of B-tubulin substitutions on *C. Cassiicola* to carbendazim sensitivity.

The analysis of sensitivity of 32 isolates of *C. cassiicola* to Carbendazim demonstrated that the EC_{50} value of the isolates without the E198A or F200Y substitutions ranged from 0.02 to 0.45 µg/ml, while isolates with E198A or F200Y displayed EC_{50}>500 µg/ml (Table 3; Figure 3). Considering the average EC_{50} of the isolates group without substitutions (0.16 µg/ml), the isolates with E198A or F200Y (EC_{50} >500) had RF greater than 3092. Wild type isolates showed high sensitivity to carbendazim, with low MIC (0.2 to 10 µg/ml), while the resistant groups exceed 500 µg/ml (Table 3).

To assess the difference in sensitivity between the wild type isolates and those carrying E198A or F200Y substitutions, the mycelial growth inhibition percentage at the dose of 100 µg/ml was used, since the EC50 values exceeded the highest dose used in the assay. The comparison of the groups using the Bonferroni test revealed less sensitivity in isolates with the E198A substitution (11.9%) compared to isolates with F200Y (43.3%). No mycelial growth was observed for wild type isolates (Figure 3; Supplementary Figure 4).

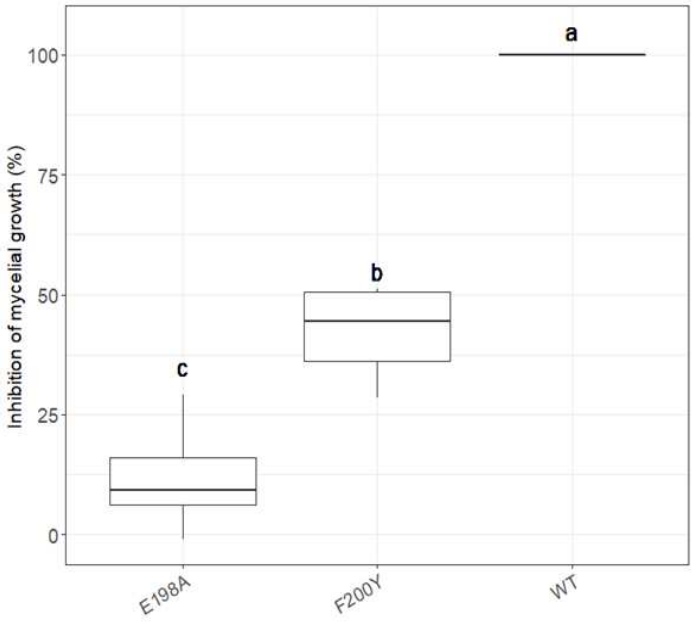

Figure 3. Comparison of the carbendazim sensitivity profile, through the values of reduction mycelial growth (%) at a dose of 100 µg/mL, between the groups constituted of the isolates: WT (n = 12) and isolates with F200Y (n = 4) and E198A (n = 16) substitution in β-tubulin, by the Bonferroni test ($p < 0.05$); groups with the same letter showed no difference.

4.5 Sensitivity profile of *C. Cassiicola* to prothioconazole

Since *Cyp51* sequence variation was not detected in the 48 isolates analyzed, we decided to determine the prothioconazole sensitivity profile. Differences could be associated with mechanisms not related to the target site mutations.

EC_{50} values of isolates ranged from 0.23 to 0.85 µg/ml. While the MIC values ranged from 0.76 to values greater than 10 µg/ml. Considering the isolate with the lowest EC_{50} (CCUFV23) and the one with the highest (CCUFV40), an RF of 3.6 can be observed. However, isolates with low EC_{50} did not always show low MIC. For example, while isolate CCUFV23 presented lower EC_{50} and lower MIC, isolate CCUFV42 presented EC_{50} of 0.35 µg/ml and MIC greater than 10 µg/ml. Eleven isolates had EC_{50} between 0.71 and 0.85 µg/ml, while only one were in the group between 0.10 and 0.30 µg/ml (Table 4; Figure 4; Supplementary Figure 5).

Table 4. Sensitivity profile (EC50 and Minimum Inhibitory Concentration – MIC) of 32 *C. cassiicola* isolates to the prothioconazole fungicide, and characterization of isolates in relation to the fungicide target gene DMI, *Cyp51*.

Isolates	Prothioconazole		*Cyp 51*
	EC_{50} (µg/mL)	MIC (µg/mL)	
CCUFV26	0.46	4.46	WT
CCUFV27	0.49	0.76	WT
CCUFV28	0.62	1.71	WT
CCUFV29	0.54	>10	WT
CCUFV32	0.83	2.19	WT
CCUFV34	0.65	1.97	WT
CCUFV35	0.73	2.62	WT
CCUFV36	0.59	2.03	WT
CCUFV38	0.35	>10	WT
CCUFV39	0.63	3.37	WT
CCUFV40	0.85	3.20	WT
CCUFV41	0.81	2.11	WT
CCUFV42	0.35	>10	WT
CCUFV45	0.32	3.22	WT
CCUFV46	0.82	2.25	WT
CCUFV47	0.66	2.32	WT
CCUFV48	0.51	0.77	WT
CCUFV50	0.82	3.97	WT
CCUFV53	0.76	2.89	WT
CCUFV55	0.75	5.00*	WT
CCUFV58	0.75	2.38	WT
CCUFV62	0.50	1.00	WT
TMG543	0.74	5.00*	NT
TMG557	0.37	10.00*	WT
TMG547	0.35	10.00*	NT
TMG545	0.57	3.44	NT
CCUFV17	0.77	>10	NT
CCUFV18	0.45	5.00*	NT
CCUFV19	0.66	>10	NT
CCUFV20	0.37	1.26	NT
CCUFV21	0.69	1.99	NT
CCUFV23	0.23	1.52	NT

*When the equation estimated MIC dose higher than the dose at which growth of the plaque isolate was observed, it was decided to determine the MIC as the minimum dose used that inhibited mycelial growth.

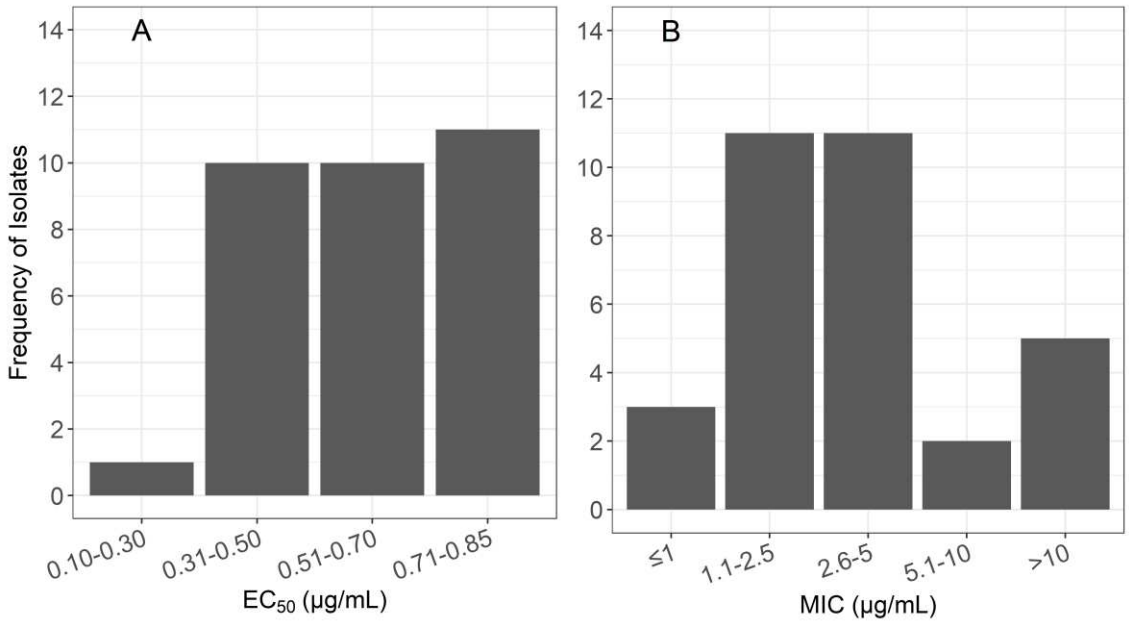

Figure 4. Sensitivity distribution of 32 *C. cassiicola* isolates to prothioconazole based on inhibition of mycelial growth (from Table 4); **A**) The EC_{50} values represent the effective concentration that inhibited 50% of mycelial colony growth; **B**) Minimum Inhibitory Concentration (MIC).

The amplitude between the isolates with the highest and lowest EC_{50} is small, without a drastic loss of sensitivity being observed. For MIC, most isolates had complete inhibition between 1.1 and 5 µg/ml (Figure 4).

4.6 Impact of SDH substitutions on the sensitivity of *C. cassiicola* to carboxamides

Sensitivity the *C. cassiicola* isolates groups with different SDH substitutions were compared in relation to mycelial growth at different concentrations of benzovindiflupyr, fluxapyroxad and inpyrfluxam fungicides.

For the benzovindiflupyr fungicide, the EC_{50} value of wild type isolates ranged from 0.7 to 2.1 µg/mL, whereas the isolates that presented the H278Y substitution in the subunit SDHB, an EC_{50} between 4.3 to 23.2 µg/mL was observed; isolates with N75S substitution in the SDHC subunit showed EC_{50} between 7.6 to 36.0 µg/mL and the isolate with N75A substitution in the SDHC subunit showed EC_{50} of 12.2 µg/mL (Table 5).

For the fluxapyroxad fungicide, the EC_{50} variation was broader, wild type isolates ranged from 0.06 to 7.1 µg/mL, whereas isolates that presented the H278Y mutation in

the SDHB subunit presented EC_{50} between 29.3 to 296.8 µg/mL; isolates with N75S substitution in the SDHC subunit showed between 7.2 and 170.9 µg/mL and the isolate with N75A substitution in the SDHC subunit showed EC_{50} of 9.8 µg/mL (Table 5; Supplementary Figure 6).

In contrast for the fungicide inpyrfluxam, variation within each group of isolates was narrower. EC_{50} of wild type isolates varied between 1.1 and 2.2 µg/mL; isolates with the H278Y substitution in the SDHB subunit between 7.8 and 21.9 µg/mL; and with the N75S substitution in the SDHC subunit between 3.4 and 46.0 µg/mL. The isolate with the N75A substitution in the SDHC subunit had an EC50 of 10.1 µg/mL. This mutation conferred stable resistance phenotype to the fungicides tested with a range from 9.8 to 12.2 µg/mL (Table 5; Supplementary Figure 7).

Furthermore, comparisons between the isolates groups (Bonferroni test, $p < 0.05$) reveled a reduction in the sensitivity due to substitutions in the SDHB and SDHC subunits for the benzovindiflupyr, fluxapyroxad and inpyrfluxam fungicides. The group with the N75A mutation in the SDHC subunit was not evaluated due to the fact that it contained only one isolate. However, the sensitivity of this isolate is similar to those with mutations already reported in the literature (Figure 5).

Isolates with substitutions in the SDHB and SDHC subunits presented variations in the resistance phenotype depending upon the fungicide analyzed. There are also variations within each isolate's groups regarding the impact of substitutions in the SDHB and SDHC subunits, but all of them are more resistant to fungicides compared to wild type isolates. For benzovindiflupyr, the substitutions H278Y, N75A and N75S conferred resistance factors of 11.6, 10.2 and 15.6, respectively; whereas, for fluxapyroxad: 45.7, 3.7 and 21.7; and for inpyrfluxam: 9.9, 6.6 and 9.6 (Figure 5).

Although the substitutions SDHB and SDHC subunits had magnitude of impact on resistance phenotype that was SDHI molecule-dependent, they are all capable of providing cross resistance to the different SDHI molecules evaluated (Figure 6).

Table 5. Sensitivity profile (EC_{50}) of 32 *C. cassiicola* isolates to the benzovindiflupyr, fluxapyroxad and inpyrfluxam fungicides, and characterization of isolates in relation to the fungicide target protein SDH subunits (SDHB, SDHC and SDHD).

Isolates	EC_{50} (µg/mL)			Substitution in SDH subunits		
	Benzovindiflupyr	Fluxapyroxad	Inpyrfluxam	SDHB	SDHC	SDHD
CCUFV26	2.1	4.3	1.6	WT	WT	WT
CCUFV27	33.3	113.2	9.1	WT	N75S	WT
CCUFV28	10.5	97.8	18.4	WT	N75S	WT
CCUFV29	34.4	143.6	8.2	WT	N75S	WT
CCUFV32	27.3	170.9	46.0	WT	N75S	WT
CCUFV34	36.0	21.0	17.1	WT	N75S	WT
CCUFV35	19.4	35.8	11.6	WT	N75S	WT
CCUFV36	1.85	7.1	1.6	WT	WT	WT
CCUFV38	11.2	50.0	15.1	WT	N75S	WT
CCUFV39	11.9	69.8	9.2	WT	N75S	WT
CCUFV40	25.9	155.3	21.7	WT	N75S	WT
CCUFV41	23.2	77.6	16.0	H278Y	WT	WT
CCUFV42	22.8	296.8	21.9	H278Y	WT	WT
CCUFV45	23.1	14.1	10.5	WT	N75S	WT
CCUFV46	9.5	40.2	8.4	WT	N75S	WT
CCUFV47	4.3	29.3	15.0	H278Y	WT	WT
CCUFV48	0.97	2.6	1.3	WT	WT	WT
CCUFV50	1.5	3.1	1.3	WT	WT	WT
CCUFV53	0.97	2.2	2.2	WT	WT	WT
CCUFV55	0.64	1.5	2.2	WT	WT	WT
CCUFV58	1.0	2.4	1.1	WT	WT	WT
CCUFV62	1.0	2.4	1.7	WT	WT	WT
TMG543	0.7	0.63	1.1	WT	WT	WT
TMG557	12.2	9.8	10.1	WT	N75A	WT
TMG547	23.0	7.9	13.1	WT	N75S	WT
TMG545	7.8	7.4	7.2	WT	N75S	WT
CCUFV17	7.6	13.5	22.7	WT	N75S	WT
CCUFV18	15.1	14.8	14.3	WT	N75S	WT
CCUFV19	12.6	7.2	3.4	WT	N75S	WT
CCUFV20	5.2	75.8	7.8	H278Y	WT	WT
CCUFV21	1.5	0.06	1.2	WT	WT	WT
CCUFV23	9.8	7.7	13.2	WT	N75S	WT

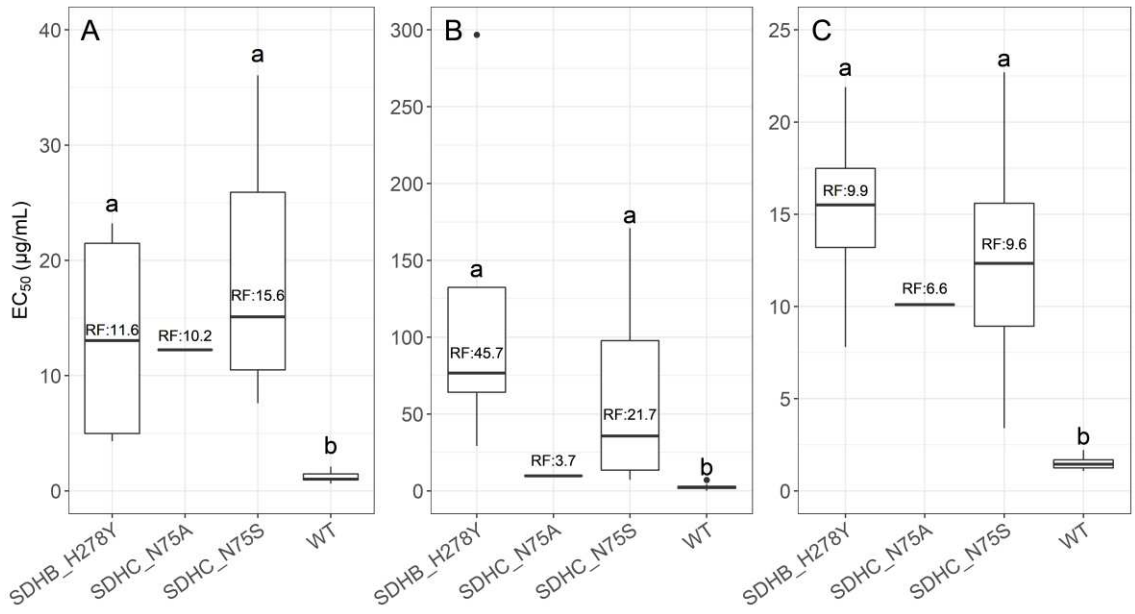

Figure 5. Evaluation of the resistance level attributed to the *C. cassiicola* group in terms of the present substitution by comparison between groups with wild type (WT) isolates (n=10), and with substitution in the SDHB - H278Y (n=4), SDHC - N75A (n=1) and SDHC-N75S (n=18) subunits, using EC_{50} values (from Table 5) by the Bonferroni test ($p < 0.05$); For the fungicides benzovindiflupyr (A); fluxapyroxad (B) and inpyrfluxam (C); Under the N75A mutation in the SDHC subunit, the test was not applied due to having only one isolate. Follows in the center of each box the resistance factor (RF) of the group with each mutation.

Great sensitivity variability was observed for MIC values. For wild type isolates growth were not observed in concentrations greater than 100 µg/mL for benzovindiflupyr, 14.7 to 180.1 µg/mL for fluxapyroxad and 20.8 to 98.4 µg/mL for inpyrfluxam (Table 6).

Regardless of the substitutions in the subunits of the SDH enzyme, resistant isolates did not grow at a dose of 500 µg/mL for the benzovindiflupyr and inpyrfluxam fungicides. Isolates with substitution N75S or H278Y grew at the dose of 100 µg/mL, but did not grow at the dose of 500 µg/mL for benzovindiflupyr. The N75S substitution presented MIC between 140.1 and 500 µg/mL for inpyrfluxam; H278Y substitution, between 254.4 and 500 µg/mL for inpyrfluxam. MIC of 500 µg/mL for both fungicides was observed for the isolate with the N75A substitution. For fluxapyroxad, all resistant isolates were able to resist and grow at 500 µg/mL (Table 6).

Five isolates were selected, being one wild type, two with the N75S substitution and two with H278Y to be evaluate the inhibition of mycelial growth in plates with higher doses of fluxapyroxad (0; 12.5; 50; 200; 4000 µg/mL). Even with the increase in the concentration of fungicide in the medium, it was not possible to observe complete inhibition of the isolates with the mutation in the SDHB and SDHC subunits, while the sensitive isolate achieved complete mycelial growth inhibition at a concentration of 50 µg/mL (Figure 7).

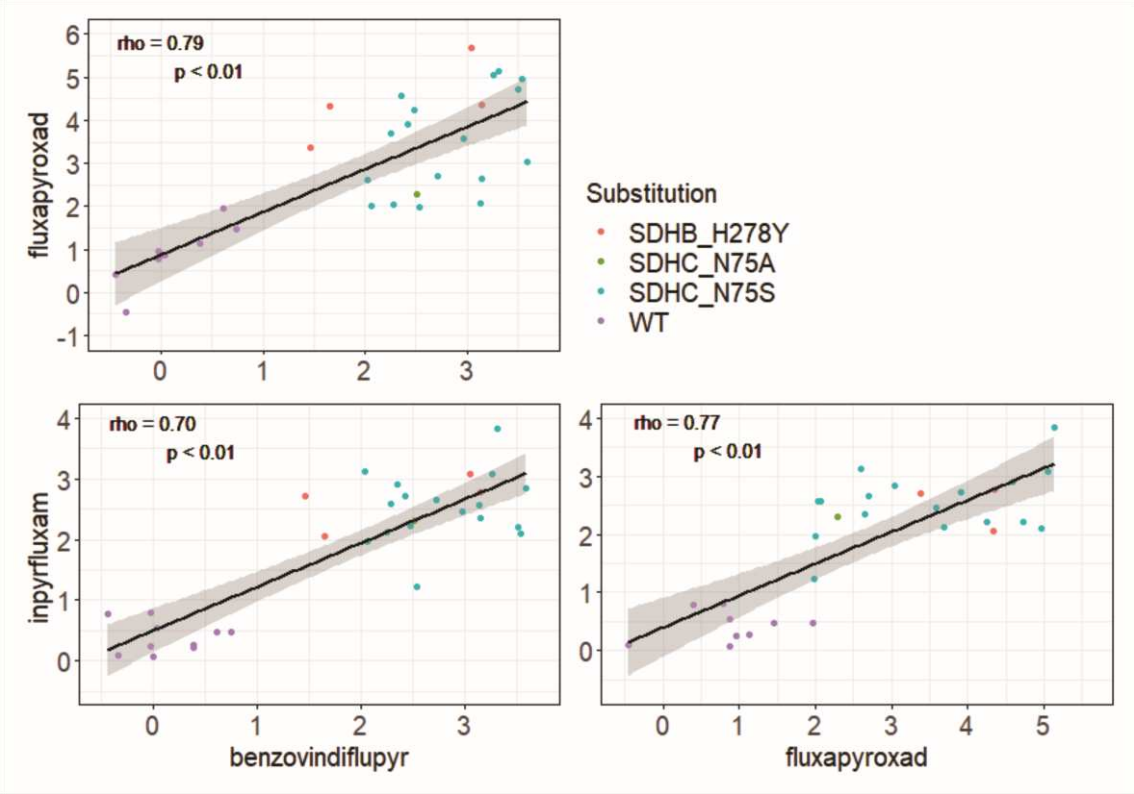

Figure 6. Evaluation of the cross resistance conferred by substitutions in the SDHB and SDHC subunits of *C. cassiicola* isolates to benzovindiflupyr, fluxapyroxade and inpyrfluxam fungicides (Table 5) by the Spearman's correlation; sensitivity measured as EC_{50} (µg/mL) values were expressed with $\log_{10}$ scale; $p < 0.01$ means the correlation were statistically significant.

Table 6. Sensitivity profile (Minimum Inhibitory Concentration - MIC) of 32 *C. cassiicola* isolates to the benzovindiflupyr, fluxapyroxad and inpyrfluxam fungicides, and characterization of isolates in relation to the fungicide target proteins SDH subunits (SDHB, SDHC and SDHD).

Isolates	MIC (µg/mL)			Substitution in SDH subunits		
	Benzovindiflupyr*	Fluxapyroxad	Inpyrfluxam	SDHB	SDHC	SDHD
CCUFV26	100	143.3	21.2	WT	WT	WT
CCUFV27	500	>500	278.0	WT	N75S	WT
CCUFV28	500	>500	485.3	WT	N75S	WT
CCUFV29	500	>500	500*	WT	N75S	WT
CCUFV32	500	>500	500*	WT	N75S	WT
CCUFV34	500	>500	463.0	WT	N75S	WT
CCUFV35	500	>500	500*	WT	N75S	WT
CCUFV36	100	59.2	25.9	WT	WT	WT
CCUFV38	500	>500	500*	WT	N75S	WT
CCUFV39	500	>500	500*	WT	N75S	WT
CCUFV40	500	>500	218.4	WT	N75S	WT
CCUFV41	500	>500	500*	H278Y	WT	WT
CCUFV42	500	>500	500*	H278Y	WT	WT
CCUFV45	500	>500	500*	WT	N75S	WT
CCUFV46	500	>500	500*	WT	N75S	WT
CCUFV47	500	>500	500*	H278Y	WT	WT
CCUFV48	50	35.5	20.8	WT	WT	WT
CCUFV50	100	36.0	92.1	WT	WT	WT
CCUFV53	100	17.6	20.9	WT	WT	WT
CCUFV55	100	34.3	26.5	WT	WT	WT
CCUFV58	100	180.1	98.4	WT	WT	WT
CCUFV62	100	104.2	72.1	WT	WT	WT
TMG543	50	14.5	23.9	WT	WT	WT
TMG557	500	>500	500*	WT	N75A	WT
TMG547	500	>500	500*	WT	N75S	WT
TMG545	500	>500	140.1	WT	N75S	WT
CCUFV17	500	>500	339.1	WT	N75S	WT
CCUFV18	500	>500	500*	WT	N75S	WT
CCUFV19	500	>500	249.8	WT	N75S	WT
CCUFV20	500	>500	254.4	H278Y	WT	WT
CCUFV21	500	14.7	31.2	WT	WT	WT
CCUFV23	500	>500	178.6	WT	N75S	WT

* The model estimated MIC values above the doses that inhibited 100% of the growth in the plate. Thus, it was preferred to use the dose that inhibited growth as a cut-off point for values estimated beyond.

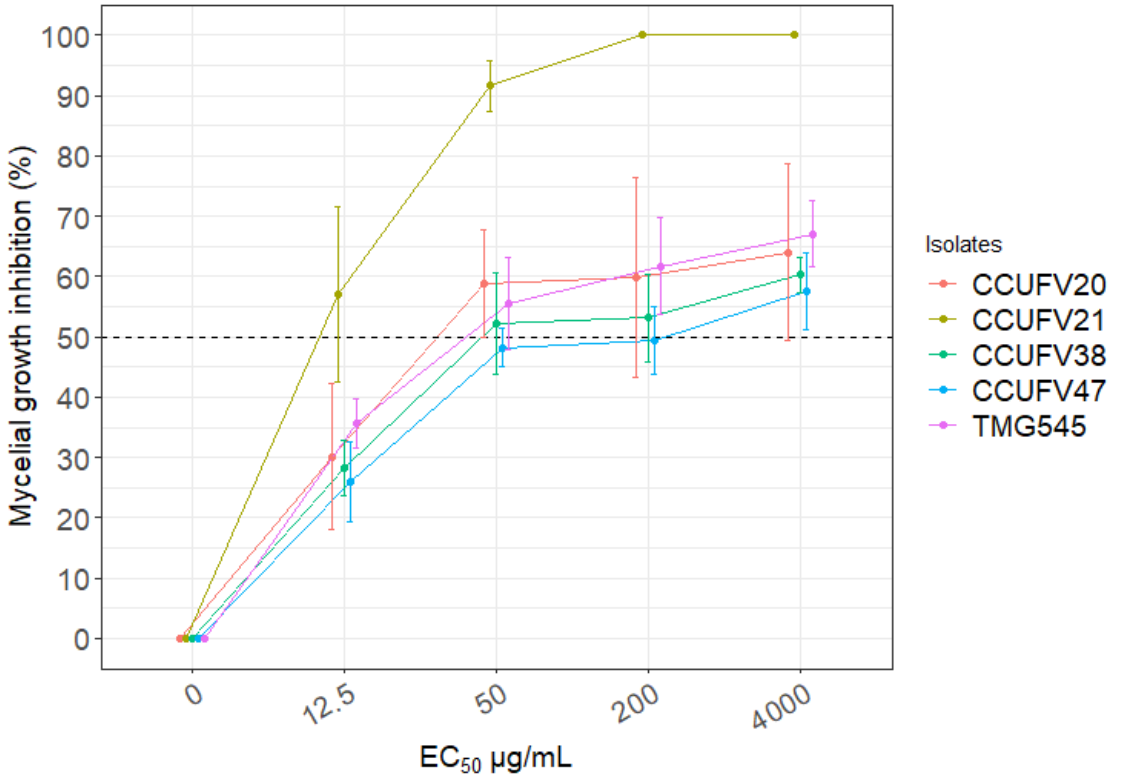

Figure 7. Determination of the total inhibition dose by evaluating the mycelial growth inhibition (%) of *C. cassiicola* in different doses (µg/mL) of the fungicide fluxapyroxad; the isolates used were CCUFV21 (wild-type); CCUFV20 and CCUFV47 (with H278Y substitution), and CCUFV38 and TMG545 (with N75S substitution); the bars represent the confidence interval above or below the average.

5 DISCUSSION

This work provided a characterization molecular update and phenotypic characterization of fungicide sensibility of *C. cassiicola* isolates collected both in soybean and cotton fields. Wide spread occurrence of amino acid substitution led to resistance to MBC, QoI and SDHI fungicides.

Resistance of Brazilian isolates of *C. cassiicola* from soybean to MBC, QoI and DMI were reported by Teramoto *et al.* (2017). However, sensibility to new SDHIs have not been described at this moment. FRAC have been monitoring and isolates with reduced sensibility to SDHI were detected in fungi population collected during the 2017/2018 soybean crop season. Genetic characterization of these populations detected the presence of mutations leading to substitution in the subunits B (H278Y) and C (N75S) in SDH,

which are known to be associated with *C. cassiicola* resistance to SDHI (FRAC, 2018). However, an updated detailed and simultaneous profiling of all site-specific fungicide registered to control target spot was lacking. Likewise, *C. cassiicola* isolates obtained from cotton field have not been fully contemplated in studies, since the target spot disease is still an emerging disease in this crop. Cotton succession with soybean, long cycle in the field and the employment of the same fungicides used in soybean to control leaf diseases of cotton, could impact the management of diseases in soybean, including target spot.

Nucleotide changes leading to E198A and F200Y substitutions in *β*-tubulin, which resulted in a strong resistance to carbendazim, were detected in 68% of the isolates evaluated (54 isolates) with EC_{50} greater than 500 μg/ml. Curiously, 86% of the isolates with the F200Y substitution were obtained from cotton, only 14% from soybean plants. At a dose of 100 μg/ml, isolates with E198A substitution had greater mycelial growth compared to those with the F200Y substitution, demonstrating a important contribution of E198A to reduced sensitivity to benzimidazoles, also observed by Duan *et al*., (2019) in *C. cassiicola* populations from greenhouse-cultivated cucumber.

Double substitution in the β-tubulin was reported for *C. cassiicola* isolates from cucumber in China (Duan *et al.*, 2019), which was not detected in Brazilian isolates. Among Chinese isolates individual E198A substitution and double substitutions in (E198A + M163I; E198A + F167Y; and E198A + F200S) have been reported, associated with resistance in 100% of *C. cassiicola* isolates collected in cucumber in the years 2017 and 2018. E198A substitution alone in Chinese isolates had a smaller impact on carbendazim sensitivity than reported double mutations, but even so it was able to provide an high EC_{50} (215.6 μg/ml), since EC_{50} sensitive isolates collected in 2012 ranged from 0.08 to 0.4 μg/ml (Duan *et al.*, 2019). A smaller frequency of MBC resistance was observed compared to QoI resistance. It could be result to less spread used of this fungicide group in soybean and cotton, because of the availability of other more effective active ingredient and reduced used for target control after resistance issues were raised.

Cytb substitution G143A that usually leads to high level of resistance in fungi to QoIs was present in 87% of the isolates surveyed. Substitutions F129L and G137R were not detected. G143A substitution has the ability to confer strong cross-resistance patterns for six QoIs with distinct structural constitution: azoxystrobin, fluoxastrobin,

pyraclostrobin, phenaminstrobin, picoxystrobin and coutoxystrobin (Adkison *et al*., 2012; Duan *et al*., 2019).

QoI sensitivity can also be diminished by the induction of alternative respiratory pathway in response to the action of inhibitors such as QoI fungicides (Ishii *et al*., 2007; Jin Li *et al.,* 2009). This alternative pathway, which is active in the presence of an alternative oxidase, was identified as the cause for relatively low QoI sensitivities of fungal mycelia (Olaya and Koller, 1999; Mackenzie *et al*., 2018).

Germinating spores of several plant-pathogenic fungi were found to be highly sensitive to QoIs, the standard procedure to test QoI sensitivity is based on assessment of the relative conidia germination in the presence of QoI fungicide and SHAM, a competitive inhibitor of the alternative oxidase. This procedure would allow to detect reduced sensitivity due to alteration in the target site, without the interference of the alternative respiration.

We found that the altered respiratory pathway does not contribute to sensitivity when in the QoI pyraclotrobin presence, both in mycelial growth and in spore germination inhibition. Additionally, we also report a greater fungitoxicity of pyraclostrobin on spores than to mycelium in isolates with the G143A substitution. EC50 in spore germination was also lower for the WT isolates, but not statistically significant.

Based on previous genotypic characterization, the isolates assayed were previously classified as WT, which showed similar sequence with isolate type MH763695.1, or mutants, that one that showed any type of mutation in genomic regions analyzed. In addition, due to variable sporulating capacity of different isolates *C. cassiicola* studied and not effect of SHAM in increasing the sensitivity to QoI fungicides, also described by Teramoto *et al.,* (2017), we decided to access the sensitivity of *C. cassiicola* to pyraclostrobin evaluating the mycelial growth inhibition without SHAM in growth media. This procedure allows us to directly address the impact of G143A on QoI sensitivity, and demonstrated that mutants exhibit a resistance factor of 5.0 to pyraclostrobin when compared with wild type isolates. This resistance factor to pyraclostrobin is smaller than RF previously reported to azoxystrobin and pyraclostrobin (Duan *et al.,*2019; Mackenzie *et al*., 2018).

Pyraclostrobin exhibit high fungitoxicity already reported by Duan *et al*., (2019), and compose two of the six formulations registered to control *C. cassiicola* in cotton and in 13 of the 66 labeled to soybean. It was already demonstrated that G143A substitution

in *C. cassiicola* confers strong cross-resistance patterns for six QoIs with distinct structural constitution, azoxystrobin, fluoxastrobin, pyraclostrobin, phenaminstrobin, picoxystrobin and coutoxystrobin (Adkison *et al*., 2012; Duan *et al.,* 2019). Therefore, our findings of reduced sensibility to pyraclostrobin can be translated to others QoIs registered for target spot control on soybean and cotton.

Three substitutions amino acid: H278Y in the SDHB subunit and N75S and N75A in the SDHC subunit, isolates obtained from cotton and soybean were found, demonstrating to be present independent of host analyzed. H278Y (SDHB subunit) and N75S (SDHC subunit) substitutions had been already reported in Brazilian isolates of *C. cassiicola*, collected from soybean in Mato Grosso and Rio Grande do Sul states in the 2016/17 and 2017/18 crop seasons, with reduced sensitivity to fluxapyroxad (FRAC, 2018). These and additional mutations that lead to amino acid substitutions in the SDHB (H278Y/R; I280V), SDHC (S73P; N75S), and SDHD (D95E; H105R; G109V) SDH subunits associated to SDHI boscalid resistance had previously been reported in *C. cassiicola* (Miyamoto et al., 2010; Zhu et al., 2019). Only mutation leading to N75A substitution in the SDHC subunit was discovered in this work. Similar to H278Y and N75S substitutions, N75A substitution conferred cross-resistance phenotype to the tested SDHIs fungicides.

H278Y and N75S had stronger effect on fluxapyroxad, compared to benzovindiflupyr and inpyrfluxam. These three carboxamides share the carboxamide structure (R_1-CO-NH-R_2) that is required for full fungicidal activity and belong to class of SDHI (pyrazole carboxamide) (FRAC, 2020). This differential impact may be related to the structure of these fungicides' molecules and the way that they interact and bind to the ubiquinone binding-site (Q-site) through hydrogen bonds. In this regard, it interesting to note that both benzovindiflupyr and inpyrfluxam were more fungitoxic to *C. cassiicola* than fluxapyroxad, and site-directed mutagenesis of SDHB residues of from *B. cinerea* indicates that the level of SDH inhibition depends on the affinity of each SDHI for its target (Lalève *et al*., 2014). Besides that, H278Y was present in Japanese *C. cassiicola* isolates highly resistant to boscalid, a SDHI fungicide, found by Miyamoto *et al*. (2010) in cucumber.

Some SDH substitutions are not directly involved in binding to SDHIs, but are located close to the residues that form the binding cavern, as is the case of the N86S for *Zymoseptoria tritici* and N75S in *Pyrenophora teres* substitutions in the SDHC subunit

(Rehfus *et al*., 2016; Rehfus, 2018). These substitutions can cause a structural rearrangement inside of binding cavern, interfering in SDH-SDHI interactions. Many substitutions found in *P. teres* isolates resistant to resistant are located in the SDHC and SDHD subunits, near to the haem b group of the SDH enzyme (C-G79R, C-H134R, C-S135R, D-G138V and D-H134R). Therefore, substitutions found in position 75 for *C. cassiicola* could be equivalent to substitution previously described to *Z. Tritici* and *P. teres*, since they are also near to the haem b group. The role of haem b group in the SDH enzyme activity is still not clear (Rehfus, 2018; Maklashina *et al*., 2010).

Inpyrfluxam is a new carboxamide that is under registration to control soybean rust and other soybean fungal disease. In field trials, inpyrfluxam combined with tebuconazole, achivied 80% control of Asian soybean rust in the 2017/18 and 2018/19 crop seasons, and in mixture with prothioconazole delivered 79% control in the 2018/19 crop season (Godoy *et al*., 2018; Godoy *et al*., 2019). Occurrence of substitutions that affects the sensitivity to this SDHI brings concerns related to its efficiency durability after registration and highligths

We did not detect any mutation leading to amino acid substitutions known to be associated with DMI resistance. RF of 3.6 was observed among the isolates, considering the lowest and highest EC_{50}. A higher frequency of isolates had EC_{50} between 0.51-0.90 µg/ml, but no resistant individuals were observed in the evaluated sample. A broader sensitivity profile was observed by Xavier *et al.* (2013) and Teramoto *et al*. (2017), with EC_{50} values <0.16 to 46.4 for prothioconazole in isolates collected from soybean in several Brazilian states between 1996 and 2013, with RFs values higher than the ones observed in this work. If the resistance mechanism affects the fitness of the pathogen, the frequency of individuals, even though resistant in the population, tends not to increase, and obtaining these isolates in the sampling becomes more difficult.

These results indicated that DMIs remains still an effective tool to manage target spot. However, its extended use in many different commercial formulations may accelerate the selection of *C. cassiicola* with higher level of DMI resistance associated with resistance to other site-specific fungicides, considering that nineteen sampled isolates have been identified as resistant to QoI, MBCs and SDHIs fungicides.

Our findings illustrated the challenge of fungicide resistance in large scale soybean and cotton production in Brazil. Fungicide resistance is a natural population phenomenon and the most effective way to prevent or manage fungicide resistance is a proactive

approach using diverse disease control tactics aiming to reduce selection pressure. Doing what it takes to delay fungicide resistance development is a good alternative comparing to pay the price of resistance once it occurs.

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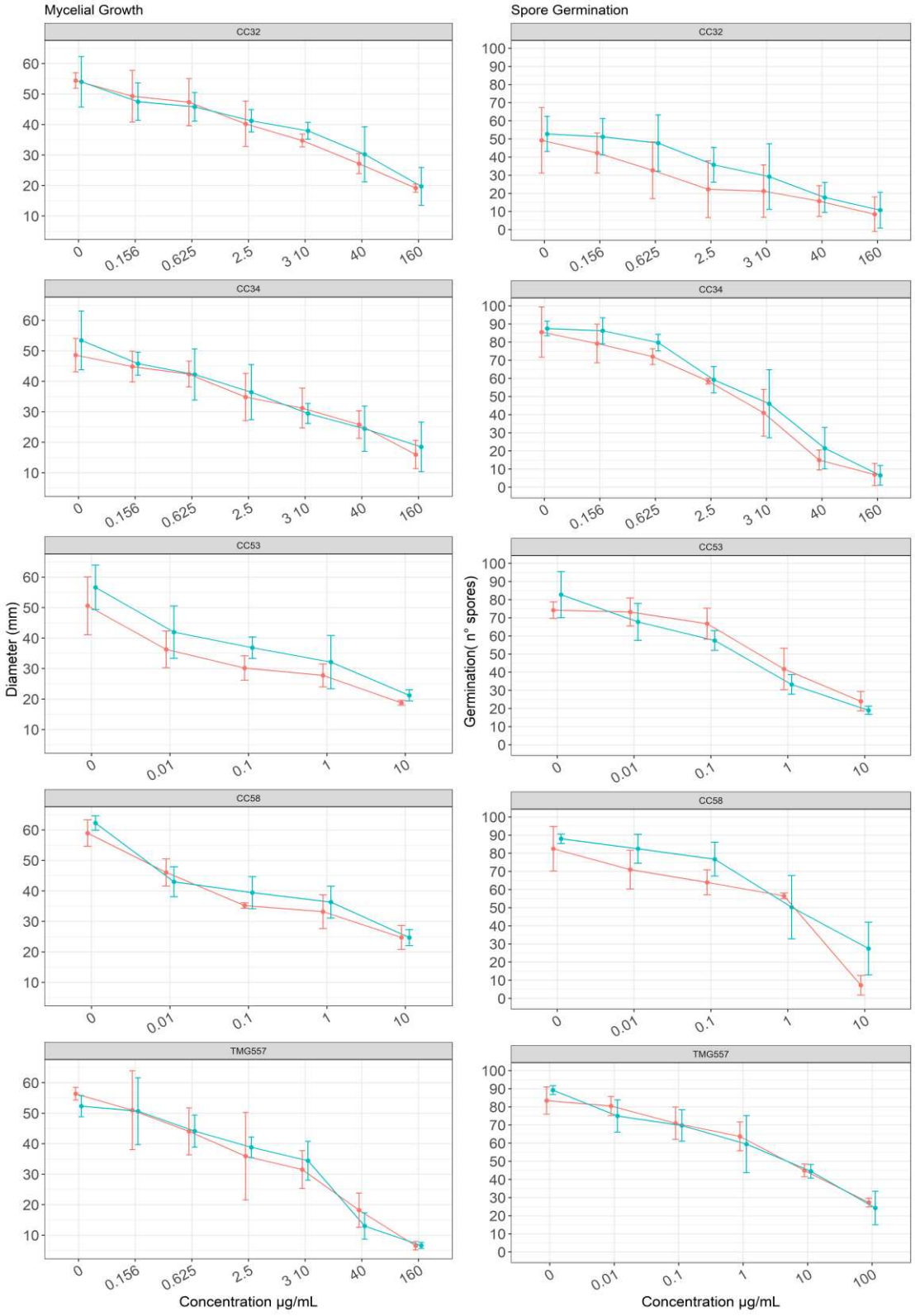
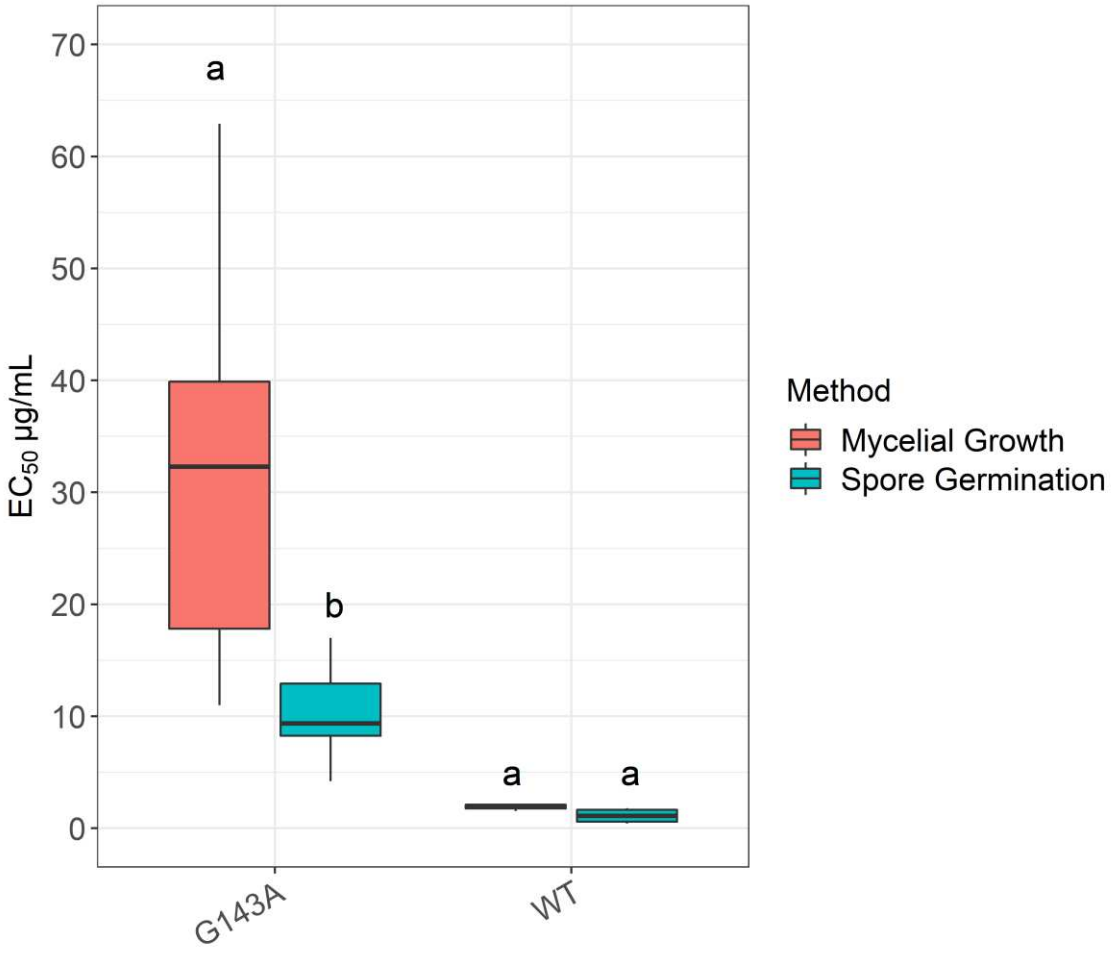
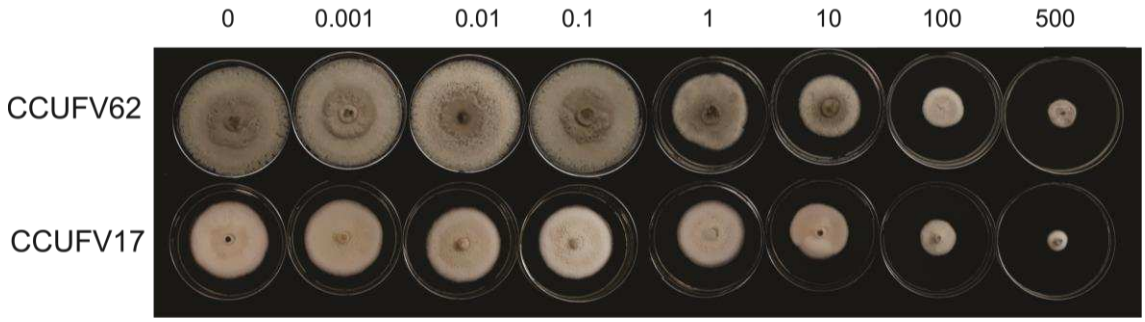
7 SUPPLEMENTARY INFORMATION



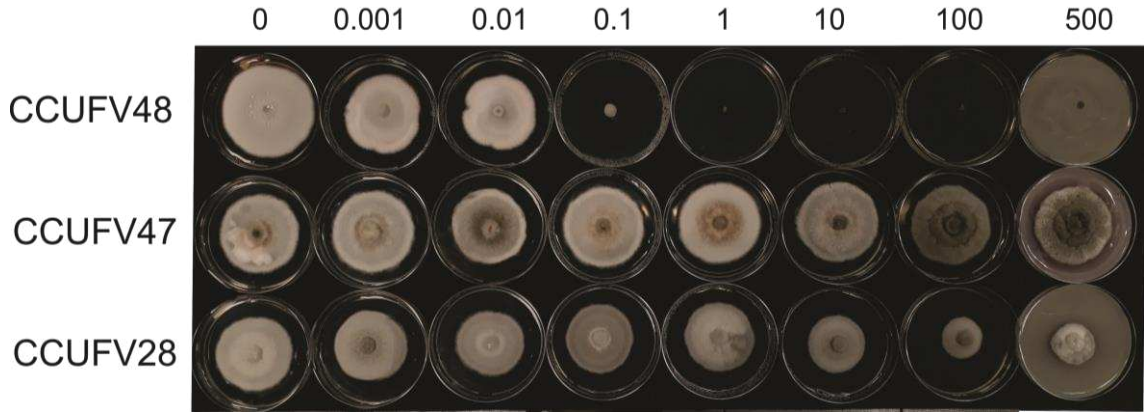
Supplementary Figure 1. Dose-response curves from effect of SHAM on mycelium growth and spore germination of assessing sensitivity in sensitive (CCUFV54 and CCUFV58) and resistant (CCUFV32, CCUFV34 and TMG557) isolates of the *C. cassiicola* to the fungicide pyraclostrobin; the bars represent the confidence interval above or below the average; fungicide concentration with SHAM (red line); fungicide concentration without SHAM (blue line).



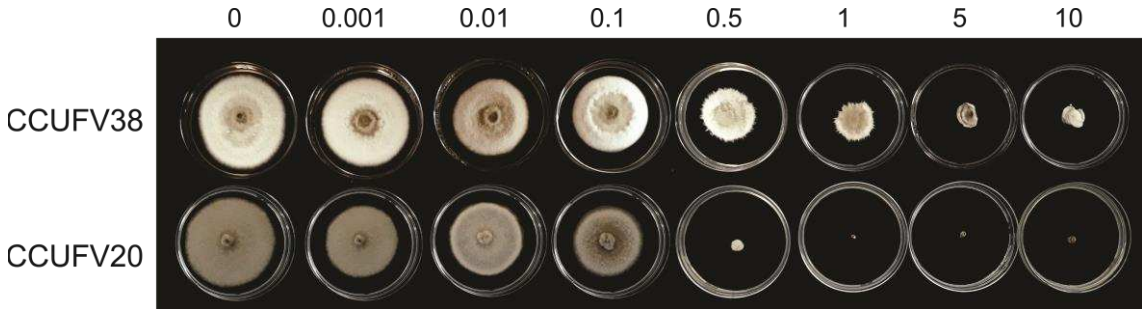
Supplementary Figure 2. Sensitivity comparation of mycelium and spores (EC_{50}) of wild type (CCUFV54 and CCUFV58) and G143A resistant (CCUFV32, CCUFV34 and TMG557) *C. cassiicola* isolates to piraclostrobin by the Bonferroni test ($p < 0.05$).



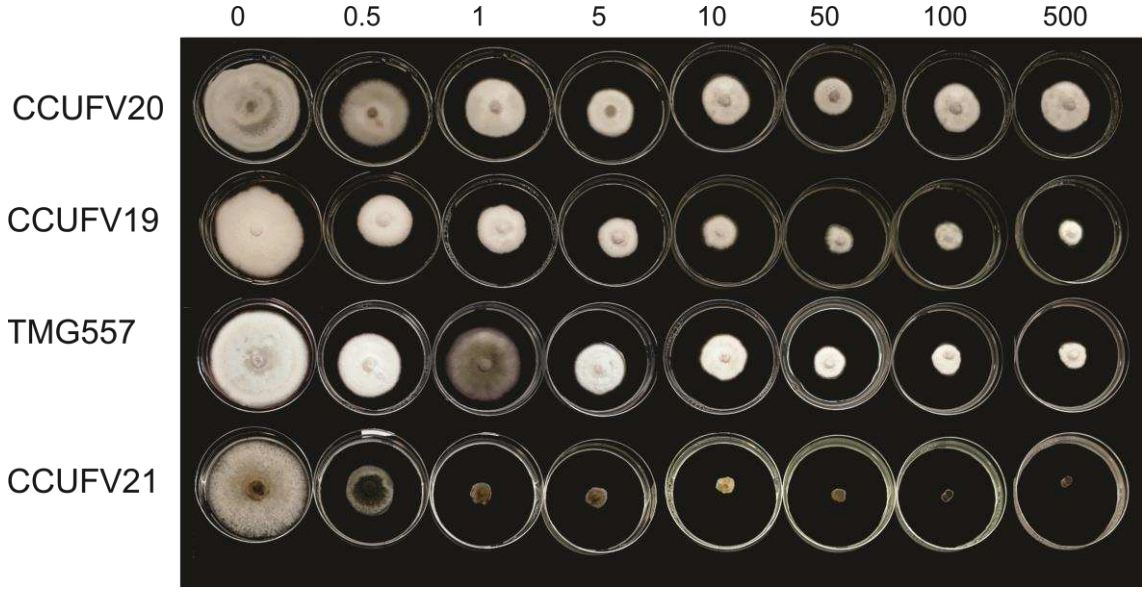
Supplementary Figure 3. Colony growth inhibition on potato dextrose agar (PDA), amended with eight different concentrations of fungicide pyraclostrobin (expressed in µg/mL), after eight days of incubation at 25 °C for isolates CCUFV62 (WT), CCUFV17 (G143A).



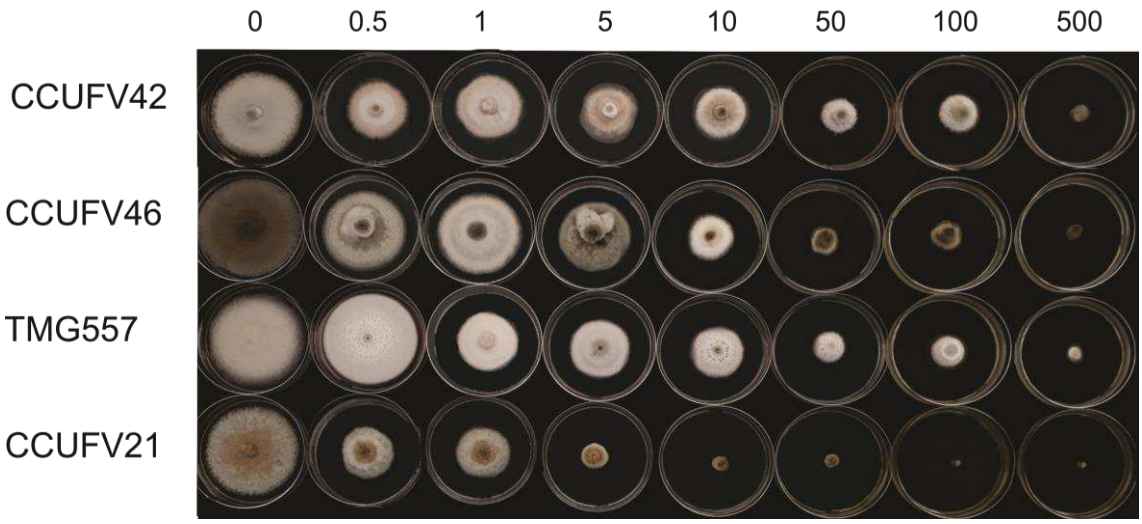
Supplementary Figure 4. Colony growth inhibition on potato dextrose agar (PDA), amended with eight different concentrations of fungicide carbendazim (expressed in µg/mL), after eight days of incubation at 25 °C for isolates CCUFV48 (WT), CCUFV47 (E198A) and CCUFV28 (F200Y).



Supplementary Figure 5. Colony growth inhibition on potato dextrose agar (PDA), amended with eight different concentrations of fungicide prothioconazole (expressed in µg/mL), after eight days of incubation at 25 °C for isolates CCUFV38 and CCUFV20 (WT).



Supplementary Figure 6. Colony growth inhibition on potato dextrose agar (PDA), amended with eight different concentrations of fungicide fluxapyroxad (expressed in µg/mL), after eight days of incubation at 25 °C for isolates CCUFV20 (H278Y), CCUFV19 (N75S), TMG557 (N75A) and CCUFV21 (WT).



Supplementary Figure 7. Colony growth inhibition on potato dextrose agar (PDA), amended with eight different concentrations of fungicide inpyrfluxam (expressed in µg/mL), after eight days of incubation at 25 °C for isolates CCUFV42 (H278Y), CCUFV46 (N75S), TMG557 (N75A) and CCUFV21 (WT).

Supplementary Table 1. Substitution in gene (exon and intron) and protein of fungicide targets (*SdhB, SdhC, SdhD, β-tub*, *CytB* and *ITS*) of *C. cassiicola* isolates.

Isolate	Exon														Intron															*Cyp51*	*ITS*		Protein				
	Sdhb									*Sdhc*			*Sdhd*		*Btub*					*CytB*			*Sdhb*			*Sdhc*			*Sdhd*					SDHB	SDHC	CytB	Btub	
	252	255	323	569	596	950	985	194	318	319	383	605	87	519	593	599	822	882	927	353	411	477	679	687	158	219	262	292		39	489	278	75	143	198	200
CCUFV1_c	A	T	T	C	A	C	G	T	A	A	T	C	C	C	A	A	C	C	C	C	C	T	A	C	G	C	C	G	WT	T	G	H	N	A	E	Y
CCUFV2_c	A	T	T	C	A	C	G	T	A	A	T	C	T	C	A	A	C	C	C	C	C	T	A	T	G	C	C	G	WT	T	G	H	N	A	E	Y
CCUFV3_c	G	C	T	T	G	C	G	T	A	A	T	C	T	C	A	A	C	C	C	C	C	C	C	T	G	C	C	G	NT[1]	T	G	H	N	A	E	Y
TMG506_c	A	T	T	C	A	C	G	T	A	A	T	C	T	C	A	A	C	C	C	C	C	T	A	T	G	C	C	G	WT	T	G	H	N	A	E	Y
TMG542_c	A	T	T	C	A	C	G	T	A	A	T	C	T	C	A	A	C	C	C	C	C	T	A	T	G	C	C	G	WT	T	G	H	N	A	E	Y
TMG543_c	A	T	T	C	A	C	G	T	A	A	T	C	T	C	A	A	C	C	C	C	C	T	A	C	G	C	C	G	NT	T	G	H	N	A	E	Y
TMG544_c	A	T	T	C	A	C	G	T	A	A	T	C	T	C	A	A	C	C	C	C	C	T	A	C	G	C	C	G	WT	T	G	H	N	A	E	Y
CCUFV4_s	G	C	T	T	G	C	G	T	A	A	C	T	T	T	C	T	C	T	C	C	C	C	C	C	G	T	A	C	NT	T	G	H	N	A	A	F
CCUFV5_s	G	C	T	T	G	C	G	T	A	A	C	T	T	T	C	T	C	T	C	C	C	C	C	C	G	T	A	C	NT	T	G	H	N	A	A	F
CCUFV6_s	G	C	T	T	G	C	G	T	A	A	C	T	T	T	C	T	C	C	C	C	C	C	C	C	G	T	A	C	NT	T	G	H	N	A	A	F
CCUFV7_s	G	C	T	T	G	C	G	T	A	A	C	T	T	T	C	T	C	T	C	C	C	C	C	C	G	T	A	C	WT	T	G	H	N	A	A	F
CCUFV8_s	G	C	T	T	G	C	G	T	A	A	C	T	T	T	C	T	C	T	C	C	C	C	C	C	G	T	A	C	WT	T	G	H	N	A	A	F
CCUFV9_s	G	C	T	T	G	C	G	T	A	A	C	T	T	T	C	T	C	T	C	C	C	C	C	C	G	T	A	C	WT	T	G	H	N	A	A	F
CCUFV10_s	G	C	T	T	G	C	G	T	A	A	C	T	T	T	C	T	C	T	C	C	C	C	C	C	G	T	A	C	NT	T	G	H	N	A	A	F
TMG080_s	G	C	T	T	G	C	G	T	A	A	C	T	T	T	C	T	T	T	C	C	C	C	C	C	G	T	A	C	NT	C	G	H	N	A	A	F
TMG082_s	G	C	T	T	G	C	G	C	A	A	T	C	T	T	C	T	C	T	C	C	C	C	C	C	A	T	A	C	WT	T	G	H	N	A	A	F
TMG083_s	G	C	T	T	G	C	C	T	A	A	C	T	C	T	C	T	C	T	C	C	C	C	C	C	G	T	A	C	NT	T	G	H	N	A	A	F
TMG109_s	G	C	T	T	G	C	G	T	A	A	T	C	T	C	C	T	C	C	C	C	C	C	C	C	A	T	A	C	WT	T	G	H	N	A	A	F
TMG119_s	G	C	T	T	G	C	G	T	A	A	C	T	T	T	A	A	C	T	C	C	C	C	C	C	G	T	A	C	NT	T	G	H	N	A	E	Y
TMG121_s	G	C	G	T	G	C	G	T	A	A	C	T	T	T	C	T	C	T	C	C	C	C	C	C	G	T	A	C	WT	T	G	H	N	A	A	F

Isolate	Exon														Intron																					Cyp51	ITS		Protein				
	Sdhb									*Sdhc*				*Sdhd*	*Btub*						*Cyt_B*				*Sdhb*		*Sdhc*			*Sdhd*													
	252	255	323	569	596	950	985	194	318	319	383	605	87	519	593	599	822	882	927	353	411	477	679	687	158	219	262	292		39	489	SDHB 278	SDHC 75	Cyt B 143	Btub 198	Btub 200						
TMG122_s	G	C	T	T	G	C	G	T	A	A	T	C	T	T	A	T	C	C	C	G	C	C	C	C	A	T	A	C	WT	T	G	H	N	G	E	F						
TMG557_c	G	C	T	T	G	C	G	T	G	C	C	T	T	T	A	A	C	T	C	C	C	C	C	C	T	G	T	A	C	WT	T	G	H	A	A	E	Y						
TMG179_s	G	C	T	T	G	C	G	T	A	A	C	T	T	T	C	T	T	T	C	C	C	C	C	C	T	G	T	A	C	NT	T	C	H	N	A	A	F						
TMG547_c	A	T	T	C	A	C	G	T	A	G	T	C	T	C	C	T	C	C	C	C	C	T	A	T	G	C	C	G	NT	T	G	H	S	A	A	F						
TMG549_s	A	T	T	C	A	C	G	T	A	A	T	C	T	C	A	A	C	C	C	C	C	T	A	T	G	C	C	G	NT	T	G	H	N	A	E	Y						
TMG545_c	A	T	T	C	A	C	G	T	A	G	T	C	C	C	C	T	C	C	C	C	C	T	A	T	G	C	C	G	NT	T	G	H	S	A	A	F						
TMG505_c	A	T	T	C	A	C	G	T	A	A	T	C	T	C	A	A	C	C	C	C	C	T	A	C	G	C	C	G	NT	T	G	H	N	A	E	Y						
TMG537_c	A	T	T	C	A	C	G	T	A	A	T	C	T	C	A	A	C	C	C	C	C	T	A	T	G	C	C	G	WT	T	G	H	N	A	E	Y						
TMG180_s	G	C	G	T	G	C	G	T	A	A	C	T	T	T	C	T	T	T	C	C	C	C	C	T	G	T	A	C	NT	T	G	H	N	A	A	F						
TMG556_c	A	T	T	C	A	C	G	T	A	A	T	C	C	C	C	T	C	C	C	C	C	T	A	T	G	C	C	G	WT	T	G	H	N	A	A	F						
TMG507_c	A	T	T	C	A	C	G	T	A	A	T	C	T	C	A	A	C	C	C	C	C	T	A	T	G	C	C	G	NT	T	G	H	N	A	E	Y						
TMG548_c	A	T	T	C	A	C	G	T	A	A	T	C	T	C	A	A	C	C	C	C	C	T	A	T	G	C	C	G	NT	T	G	H	N	A	E	Y						
CCUFV11_c	A	T	T	C	A	C	G	T	A	A	T	C	C	C	C	T	C	C	C	C	C	T	A	C	G	C	C	G	NT	T	G	H	N	A	A	F						
CCUFV12_c	A	T	T	C	A	C	G	T	A	A	T	C	C	C	A	T	C	C	C	C	C	T	A	T	G	C	C	G	NT	T	G	H	N	A	E	F						
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CCUFV14_c	A	T	G	C	A	C	G	T	A	A	T	C	T	C	C	T	C	C	C	C	C	T	A	C	G	C	C	G	NT	T	G	H	N	A	A	F						
CCUFV15_c	A	T	T	C	A	C	G	T	A	A	T	C	C	C	A	A	C	C	C	C	C	T	A	T	G	C	C	G	NT	T	G	H	N	A	E	Y						
CCUFV16_c	G	C	T	T	G	C	G	T	A	A	T	C	T	T	A	T	C	T	T	C	C	C	C	T	G	T	A	C	NT	T	G	H	N	A	E	F						
CCUFV17_c	A	T	T	C	A	C	G	T	A	G	T	C	T	C	C	T	C	C	C	G	C	T	A	C	G	C	C	G	NT	T	G	H	S	G	A	F						
CCUFV18_c	A	T	G	C	A	C	G	T	A	G	T	C	T	C	C	T	C	C	C	C	C	T	A	T	G	T	A	C	NT	T	G	H	S	A	A	F						

Isolate												Exon													Intron					ITS		Protein				
				Sdhb						*Sdhc*			*Sdhd*			*Btub*			*Cyt_B*			*Sdhb*			*Sdhc*		*Sdhd*		*Cyp51*			SDHB	SDHC	Cyt B	Btub	
	252	255	323	569	596	950	985	194	318	319	383	605	87	519	593	599	822	882	927	353	411	477	679	687	158	219	262	292		39	489	278	75	143	198	200
CCUFV19_c	G	C	T	T	G	C	G	T	A	G	T	C	T	C	C	T	C	C	C	C	C	C	C	T	G	C	C	G	NT	T	G	H	S	A	A	F
CCUFV20_c	A	T	T	C	A	T	G	T	A	A	T	C	C	C	A	T	C	C	C	C	C	T	A	C	G	C	C	G	NT	T	G	Y	N	A	E	F
CCUFV21_c	A	T	T	C	A	C	G	T	A	A	T	C	C	C	A	A	C	C	C	C	C	T	A	T	G	C	C	G	NT	T	G	H	N	A	E	Y
CCUFV22_c	G	C	T	T	G	C	G	T	A	G	C	T	T	T	C	T	C	T	C	C	C	C	C	T	G	T	A	C	NT	T	G	H	S	A	A	F
CCUFV23_c	A	T	T	C	A	C	G	T	A	G	T	C	T	C	C	T	C	C	C	C	C	T	A	T	G	C	C	G	NT	T	G	H	S	A	A	F
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CCUFV32_s	G	C	T	T	G	C	G	T	A	G	C	T	T	T	C	T	T	T	C	C	C	C	C	C	G	T	A	C	WT	T	G	H	S	A	A	F
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CCUFV34_s	G	C	T	T	G	C	G	T	A	G	C	T	T	T	C	T	T	T	C	C	C	C	C	T	G	T	A	C	WT	T	G	H	S	A	A	F
CCUFV35_s	G	C	T	T	G	C	G	T	A	G	T	C	T	T	C	T	T	T	C	C	C	C	C	T	G	T	A	C	WT	T	G	H	S	A	A	F
CCUFV36_s	A	T	T	C	A	C	G	C	A	A	T	C	T	T	A	T	C	C	C	C	T	T	A	C	A	T	A	C	WT	C	G	H	N	A	E	F
CCUFV37_s	A	T	T	C	A	C	G	C	A	A	T	C	T	T	A	T	C	C	C	C	C	T	A	T	A	T	A	C	WT	T	G	H	N	A	E	F
CCUFV38_s	G	C	T	T	G	C	G	T	A	G	C	T	T	T	C	T	T	T	C	C	C	C	C	T	G	T	A	C	WT	T	G	H	S	A	A	F
CCUFV39_s	A	T	T	C	A	C	G	C	A	G	T	C	T	T	A	T	C	C	C	C	T	T	A	C	A	T	A	C	WT	T	G	H	S	A	E	F

Isolate	Exon																					Intron							Cyp51	ITS		Protein				
	Sdhb							*Sdhc*					*Sdhd*	*Btub*						*Cyt_B*		*Sdhb*			*Sdhc*	*Sdhd*						SDHB	SDHC	Cyt B	Btub	
	252	255	323	569	596	950	985	194	318	319	383	605	87	519	593	599	822	882	927	353	411	477	679	687	158	219	262	292		39	489	278	75	143	198	200
CCUFV40_s	A	T	T	C	A	C	G	C	A	G	T	C	T	T	A	T	C	C	C	C	T	T	A	C	A	T	A	C	WT	T	G	H	S	A	E	F
CCUFV41_s	G	C	T	T	G	T	G	T	A	A	C	T	C	T	C	T	T	T	C	C	C	C	C	T	G	T	A	C	WT	T	G	Y	N	A	A	F
CCUFV42_s	G	C	T	T	G	T	G	T	A	A	C	T	T	T	C	T	T	T	C	C	C	C	C	C	G	T	A	C	WT	T	G	Y	N	A	A	F
CCUFV43_s	A	T	T	C	A	C	G	C	A	A	T	C	T	T	A	T	C	C	C	C	C	T	A	C	A	T	A	C	WT	T	G	H	N	A	E	F
CCUFV44_s	G	C	T	T	G	C	G	T	A	A	C	T	C	T	C	T	T	T	C	C	C	C	C	C	G	T	A	C	WT	T	G	H	N	A	A	F
CCUFV45_s	G	C	T	T	G	C	G	T	A	G	C	T	T	T	C	T	T	T	C	C	C	C	C	C	G	T	A	C	WT	T	G	H	S	A	A	F
CCUFV46_s	G	C	G	T	G	C	G	T	A	G	C	T	T	T	C	T	T	T	C	C	C	C	C	T	G	T	A	C	WT	T	G	H	S	A	A	F
CCUFV47_s	G	C	T	T	G	T	G	T	A	A	C	T	T	T	C	T	T	T	C	C	C	C	C	T	G	T	A	C	WT	T	G	Y	N	A	A	F
CCUFV48_s	A	T	T	C	A	C	G	C	A	A	T	C	C	T	A	T	C	C	C	C	T	T	A	C	A	T	A	C	WT	T	G	H	N	A	E	F
CCUFV49_s	A	T	T	C	A	C	G	C	A	A	T	C	C	T	A	T	C	C	C	C	T	T	A	C	A	T	A	C	WT	T	G	H	N	A	E	F
CCUFV50_c	G	C	T	T	G	C	G	T	A	A	T	C	C	T	A	T	C	T	T	G	C	C	C	C	G	T	A	C	WT	T	G	H	N	G	E	F
CCUFV52_c	G	C	T	T	G	C	G	T	A	A	T	C	T	T	A	T	C	T	T	G	C	C	C	C	G	T	A	C	WT	T	G	H	N	G	E	F
CCUFV53_c	G	C	T	T	G	C	G	T	A	A	T	C	T	T	A	T	C	T	T	G	C	C	C	C	G	T	A	C	WT	T	G	H	N	G	E	F
CCUFV61_c	G	C	T	T	G	C	G	T	A	A	T	C	C	T	A	T	C	T	T	G	C	C	C	C	G	T	A	C	WT	T	G	H	N	G	E	F
CCUFV62_s	G	C	T	T	G	C	G	T	A	A	T	C	T	T	A	T	C	T	T	G	C	C	C	C	G	T	A	C	WT	T	G	H	N	G	E	F
CCUFV63_c	G	C	T	T	G	C	G	T	A	A	T	C	T	T	A	T	C	T	T	G	C	C	C	C	G	T	A	C	WT	T	G	H	N	G	E	F
CCUFV67_c	G	C	T	T	G	C	G	T	A	A	T	C	T	T	A	T	C	T	T	G	C	C	C	C	G	T	A	C	WT	T	G	H	N	G	E	F
CCUFV68_c	G	C	T	T	G	C	G	T	A	A	T	C	C	T	A	T	C	T	T	G	C	C	C	C	G	T	A	C	WT	T	G	H	N	G	E	F

[1]Acronym "NT" represents that the gene has not been sequenced.