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VARIABILIDADE GENÉTICA E SENSIBILIDADE DE *CERCOSPORA KIKUCHII*, *COLLETOTRICHUM TRUNCATUM* E *CORYNESPORA CASSIICOLA* A FUNGICIDAS

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Tese apresentada ao Programa de Pós-Graduação em Agronomia, da Universidade Estadual de Londrina, área de concentração em Fitossanidade/Fitopatologia.

Orientadora: Drª Maria Isabel Balbi-Peña
Coorientadora: Drª Cláudia Vieira Godoy

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DEDICA

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“Peçam, e lhes será dado; busquem, e encontrarão; batam, e a porta lhes será aberta. Pois todo o que pede, recebe; o que busca, encontra; e àquele que bate, a porta será aberta” (LUCAS 11:9-10).”

MELLO, FLÁVIA ELIS DE. **Variabilidade genética e sensibilidade de *Cercospora kikuchii*, *Colletotrichum truncatum* e *Corynespora cassiicola* a fungicidas.** 2019. 232 f. Tese de Doutorado em Agronomia – Universidade Estadual de Londrina, Londrina, 2019.

RESUMO

Os fungicidas metil benzimidazol carbamato (MBC), inibidores da quinona externa (IQe), inibidores da desmetilação (IDM) e inibidores da succinato desidrogenase (ISDH) são utilizados para o controle de doenças causadas por *Cercospora kikuchii*, *Colletotrichum truncatum* e *Corynespora cassiicola* na cultura da soja no Brasil. No entanto, devido a aplicações excessivas, indivíduos variantes presentes nas populações destes fungos podem ser selecionados, resultando em menor sensibilidade a fungicidas anteriomente eficazes. Os principais objetivos deste trabalho foram: i) determinar os níveis de sensibilidade ou resistência aos fungicidas das diferentes espécies de fungo; ii) avaliar *in vitro* a resistência cruzada das populações de *C. kikuchii* e *C. cassiicola* aos fungicidas do grupo MBC (carbendazim e tiofanato-metílico) e IQe (azoxistrobina, picoxistrobina e piraclostrobina); iii) determinar a concentração efetiva para inibir 50% (CE50) da população de *C. cassiicola* aos fungicidas carbendazim (MBC), piraclostrobina (IQe) e protioconazol (IDM); iv) caracterizar os possíveis polimorfismos nos genes-alvo *cyp51,* β-tubulina, citocromo *b* (*cyt b*), e *S*dh (subunidades b, c, d) e sua relação com a resistência; v) analisar as variações heterozigóticas das mutações e vi) estudar a diversidade genética entre isolados sensíveis e resistentes a partir das variações obtidas nos diferentes genes analisados. Os fungos foram isolados de folhas de soja coletados em diferentes safras e regiões de cultivo. A resistência cruzada foi testada pelo método de crescimento micelial em meio de cultura utilizando a dose discriminatória de 10 µg/mL dos fungicidas carbendazim, tiofanato-metílico, azoxistrobina, picoxistrobina e piraclostrobina para o fungo *C. cassiicola*. A CE50 foi analisada por meio do método de microtitulação colorimétrica com absorbância de 540 nm. Os polimorfismos nos genes-alvos dos fungicidas foram analisados com base no sequenciamento de nova geração, com uma profundidade de 30.000 reads, no equipamento Illumina MiSeq. A CE50 de *C. cassiicola* variou entre 0 e 100 µg/mL para os três fungicidas testados. Houve resistência cruzada positiva entre os ingredientes ativos que pertencem aos grupos químicos MBC e IQe para *C. kikuchii* e *C. cassiicola.* Na caracterização molecular de *C. kikuchii* para o gene β-tubulina foram encontradas apenas mutações sinônimas para todos os isolados analisados. Para *C. truncatum* foi encontrada a mutação A244E. Já, para *C. cassiicola* foram identificadas as mutações S168Y, I189V, E198A, F200Y e S275N. No caso do gene *cyt b*, a análise dos polimorfismos para os três fungos analisados, identificou a substituição de glicina para alanina na posição 143 (G143A). As mutações F129L e G137R não foram encontradas para nenhuma das três espécies avaliadas. A análise molecular do gene *cyp51* identificou as mutações Q161H, N178D, P180S, P200L, K217R, E274D, S279N, E281K, T290S, I299V, S363N, K442Q e L503V para isolados de *C. kikuchii*, 44 mutações não sinônimas para *C. truncatum* e as mutações S279N e I299V para *C. cassiicola*. Para os genes Sdh (subunidades a, b, c, d) foram encontrados 23 pontos de mutação para o gene SdhA e 25 mutações para o gene SdhB em *C. truncatum*. Para *C. kikuchii* foram observadas mutações sinônimas para SdhA e 14 substituições para o gene SdhB: H17L, G27A, T167M,

P192L, E200D, N290K, I320N, I434V, A436V, D447N, H448R, E454Q, N513S e Q569K. Para *C. cassiicola* foram identificadas mutações nas posições 318, 454, 461, 556, (A-E318D, I454V, A461S, Y556F), 34, 39, 260 (B-A34V, V39I, K260R), 37, 61, 85, 163, (C-Q37H, S61T, G85S, I163V) e 126 (D-V126I), respectivamente. Apesar, do elevado polimorfismo observados nos genes que conferem resistência a fungicidas, para os três fungos analisados não houve correlação entre os isolados fenotipicamente classificados com resistentes e os isolados mutantes. Portanto, novos estudos devem ser realizados para confirmar se as novas mutações observadas nesse estudo estão correlacionadas com a resistência a fungicidas. Os isolados considerados menos sensíveis aos fungicidas, foram os isolados que apresentam as mutações G143A, E198A e F200Y, que já estavam descritas na literatura. Mutações múltiplas aos fungicidas MBC e QoI foram relatadas em *C. cassiicola*, sendo a mutações G143A + E198A a mais frequente. Variantes homozigóticas e heterozigóticas foram encontradas em todas as espécies e em todos os genes analisados. A alta diversidade genética encontradas neste estudo são responsáveis pela alta variabilidade genética dos isolados de *C. kikuchii* e *C. cassiicola*. Além disso, mais estudos devem ser realizados para confirmar se os novos polimorfismos encontrados nessa pesquisa, conferem resistência a fungicidas.

Palavras-chave: Antracnose, crestamento foliar de cercóspora, mancha-alvo da soja, mancha púrpura da semente, resistência a fungicidas.

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ABSTRACT

Methyl benzimidazole carbamate (MBC), quinone oxidase inhibitors (QoI), demethylation inhibitors (DMI) and succinate dehydrogenase inhibitors (SDHI) are fungicides groups used to control *Cercospora kikuchii*, *Colletotrichum truncatum* and *Corynespora cassiicola* in soybean fields in Brazil. However, due to excessive applications, variant individuals present in the natural populations of these fungi were selected, resulting in a lower sensitivity to the fungicides previously effective. Therefore, the main goals were: i) to determine the sensitivity or resistance levels to the fungicides of the different fungus species; ii) to evaluated cross-resistance *in vitro* of *C. kikuchii* and *C. cassiicola* to the fungicides MBC (carbendazim and thiophanate methyl) and QoI (azoxystrobin, picoxystrobin and pyraclostrobin); iii) to define the effective concentration to inhibition 50% (EC50) of spore germination of *C. cassiicola* to the fungicides carbendazim (MBC), pyraclostrobin (QoI) and prothioconazole (DMI); iv) to characterize the possible polymorphisms in the target genes *cyp51*, β-tubulin, cytochrome *b* (*cyt b*), and Sdh (subunits b, c, d) and their correlation with resistance; v) to analyze heterozygous variations of mutations and vi) to study genetic diversity in sensitive and resistant isolates from the variations obtained in the different genes analyzed. Fungi were isolated from soybean leaflets collected in fields in different growing seasons. The EC50 sensitivity of isolates *C. cassiicola* was analyzed by colorimetric microtiter method, using absorbance with wavelengths at 540 nm. Cross-resistance was tested for MBC and QoI fungicides by radial growth assay in amended medium using discriminatory dose (10 μg/mL) of carbendazim, thiophanate-methyl, azoxystrobin, picoxystrobin and pyraclostrobin fungicides. The isolates were sequenced using next generation sequencing approach with 30,000 reads using Illumina MiSeq equipment. The EC50 of *C. cassiicola* ranged from 0 to 100 μg / mL for the three fungicides tested. There was a high positive cross-resistance between the active ingredients of the MBC and QoI chemical groups for *C. kikuchii* and *C. cassiicola*. Molecular characterization of *C. kikuchii* for the β-tubulin gene was found only for the identification of all isolates. For *C. truncatum*, the A244E mutation was found. For *C. cassiicola* were found the S168Y, I189V, E198A, F200Y and S275N mutations. Sequencing of the *cyt b* gene for the three species analyzed identified the glycine mutations for alanine at position 143 (G143A). The F129L and G137R mutations were not found for any species. Molecular analysis of the *cyp51* gene revealed mutations Q161H, N178D, P180S, P200L, E274D, S279N, E281K, T290S, I299V, S363N, K442Q and L503V for *C. kikuchii*, 44 nonsynonymous mutations for *C. truncatum* and S279N and I299V mutations for *C. cassiicola*. For the Sdh genes (subunits a, b, c, d), 23 point mutation were found for SdhA gene and 25 mutations for the SdhB gene *in C. truncatum*. The mutations observed for SdhB in *C. kikuchii* were: H17L, G27A, T167M, P192L, E200D, N290K, I320N, I434V, A436V, D447N, H448R, E454Q, N513S and Q569K. For *C. cassiicola* were found substitutions at codon 318, 454, 461, 556, (A-E318D, I454V, A461S, Y556F), 34, 39, 260 (B-A34V, V39I, K260R), 37, 61, 85, 163, (C-Q37H, S61T, G85S, I163V) and 126 (D-V126I), respectively. Despite, the high polymorphism observed in the genes that confer fungicide resistance, for three fungi analyzed, no correlation had been observed between isolates phenotypically classified as resistant and the isolates which showed mutations. Therefore, further studies should be performed to confirm if the new mutations found in this study are correlate with fungicide resistance. Isolates with less sensitive to fungicides, were the isolates that showed the mutations G143A, E198A and F200Y, which were already been described in the literature. Multiple mutations to MBC and QoI fungicides have been reported in *C. cassiicola*, being the G143A + E198A the most frequent. Homozygous and heterozygous variants were found for the three species. High genetic diversity found in this study, are responsible for the high genetic variability of the isolates of *C. kikuchii* and *C. cassiicola*. In addition, further studies should be conducted to confirm whether the new polymorphisms found in this research confer resistance to fungicides.

Keywords: Anthracnose, cercospora leaf blight, fungicide resistance, purple seed stain, target spot.

SUMÁRIO

1 INTRODUÇÃO

A soja [*Glycine max* (L.) Merrill] é uma das principais culturas que garante a sustentabilidade econômica da atividade agrícola no Brasil, aumentando a cada ano sua área de cultivo. Em razão disso, constata-se um aumento na intensidade de doenças na cultura devido a monocultura, utilização de cultivares suscetíveis e uniformidade genética da espécie (TECNOLOGIAS, 2013). Os agroquímicos, dentre eles os fungicidas, têm efetiva participação para evitar perdas ocasionadas pelas doenças. Contudo, em virtude da intensificação da aplicação destes produtos o seu uso tem se tornado uma preocupação constante para a produção agrícola nacional, tanto devido aos possíveis impactos ambientais como, a crescente ineficiência dos mesmos para muitos patógenos.

Os fungos *Cercospora kikuchii* [(Tak. Matsumoto & Tomoy.) M.W. Gardner (crestamento foliar de cercóspora e mancha púrpura da semente)], *Colletotrichum truncatum* [(Schwein.) Andrus & W.D. Moore (antracnose)] e *Corynespora cassiicola* [Berk. & M. A. Curtis C. T. Wei. (mancha-alvo da soja)] são patógenos que estão amplamente disseminados nas principais regiões de cultivo de soja no Brasil. Sob condições edafoclimáticas favoráveis, as doenças de final de ciclo (DFCs), causadas pelos fungos *C. kikuchii* e *C. truncatum*, podem ocasionar perdas de rendimento que variam entre 36 % a 100 % (ALMEIDA; 2005), enquanto, o uso de cultivares suscetíveis ao fungo *C. cassiicola,* perdas de produtividade podem chegar a até 50% (GODOY et al., 2017).

As estratégias de manejo para essas doenças envolvem principalmente o controle químico (KLINGELFUSS; YORINORI, 2001). Na safra 2016/17, aproximadamente 56% dos fungicidas utilizados no Brasil, foram aplicados em soja, gerando gastos de U$ 3,2 bilhões (SINDIVEG, 2017). O número médio de aplicações no Estado Paraná na safra de 2016/2017 foi de 4 aplicações, visando principalmente o controle da ferrugem-asiática (*Phakopsora pachyrhizi*) (BARROS et al., 2017). No entanto, todos os outros fungos que infectam a soja também são expostos nessas aplicações. Os principais modos de ação dos fungicidas utilizados na cultura da soja são fungicidas metil benzimidazol carbamatos (MBC), inibidores de quinona externa (IQe), os inibidores de desmetilação (IDM) e os inibidores de succinato desidrogenase (ISDH) (GODOY; MEYER, 2014).

Os fungicidas IDM representam um importante modo de ação no controle de doenças. No entanto, o uso intensivo em várias culturas levou a seleção de isolados menos sensíveis a este grupo químico (SCHMITZ et al., 2014; KLOSOWSKI et al., 2016a; KLOSOWSKI et al., 2016b). Os mecanismos de resistência para IDMs são variáveis, mas os três mecanismos principais envolvem uma ou mais mutações de ponto, superexpressão do gene *cyp51* durante a formação de esteróis, redução intracelular do fungicida e da sua acumulação mediada por um aumento da energia de regulação de transportadores de efluxo (BRENT, 2011; ZIOGAS; MALANDRAKIS, 2015). Mutações pontuais foram descrita em várias espécies de fungos incluindo as substituições V136A/C, Y137F, A379G, I381V, S524T em *Zymoseptoria tritici* (HEICK; JUSTESEN; JØRGENSEN, 2017) e as mutações mais comuns Y134F, Y136F e Y137F relatadas para *Blumeria graminis* (WYAND; BROWN, 2005) e *Mycosphaerella* sp. (CAŇAS-GUTIERREZ et al., 2009; CHONG et al., 2010; LEROUX; WALKER, 2011; COOLS; FRAAJIE, 2013)

Resistência aos fungicidas MBC foi primeiramente relatada em 1969 e, desde o início da sua comercialização, pelo menos 130 espécies de fungos desenvolveram resistência a esse grupo (FRAC, 2014). Os fungicidas comerciais desta classe atuam inibindo a montagem dos microtúbulos do fuso durante a divisão nuclear ligando-se à proteína β-tubulina (SCHROEDER; PROVVIDENTI, 1969; GEORGOPOULOS; DOVAS, 1973; DAVIDSE, 1986; DAVIDSE; ISHII, 1995). Populações resistentes apresentaram diversas mutações na proteína β-tubulina. As mutações ocorrem em vários códons como 6, 50, 167, 198, 200 e 240. No entanto, as mutações E198A / G / K / Q e F200Y são as mais frequentes (KOENRAADT; JONES 1992; MA; MICHAILIDES, 2005). Resistência dos fungos *C. cassiicola*, (AVOZANI, 2011; TERAMOTO et al., 2012; XAVIER et al., 2013) isolado de folhas de soja, e de *C. kikuchii* aos fungicidas MBCs (PRICE III et al., 2015), tem sido relatada nos últimos anos no Brasil e no mundo, em diferentes regiões produtoras.

Os fungicidas IQe têm atividade para a maioria das classes de fitopatógenos e, por sua vez, atuam interferindo a respiração mitocondrial do complexo III (complexo do citocromo bc1) (BRENT, 1995; SIEROTZKI, 2015). Três substituições de aminoácidos no citocromo b (*cyt b*) relacionadas à resistência a IQe têm sido relatados em diversos fungos: G143A (substituição do aminoácido glicina por alanina), F129L (substituição do aminoácido fenilalanina por leucina) e G137R (substituição do aminoácido glicina por arginina). Isolados que apresentam a mutação F129L ou G137R expressam resistência moderada (parcial) enquanto isolados que apresentam a mutação G143A expressam altos níveis de resistência (completa), sendo na maioria das vezes associada com falha de controle no campo (FERNÁNDEZ-ORTUÑO et al., 2008). A mutação G143A em *C. sojina* (ZENG et al., 2015; STANDISH, et al., 2015) e a mutação F129L em *P. pachyrhizi* (KLOSOWSKI, 2016b) foram recentemente observadas em regiões produtoras de soja.

Os fungicidas inibidores da succinato desidrogenase (complexo II, succinato-ubiquinona redutase), ISDH também conhecido como carboxamidas, começaram a ser aplicados na cultura da soja no Brasil em 2013 (GODOY, MEYER, 2014). A enzima SDH, alvo dos fungicidas, atua bloqueando o sítio de ligação de ubiquinona da mitocôndria. Esta enzima é composta por quatro subunidades (A, B, C e D) e o local de ligação dos inibidores ocorrem nas subunidades B, C e D. Apesar da recente introdução, no Brasil, dessa nova geração de fungicidas, isolados de *P. pachyrhizi* apresentaram mutações no gene S*dh*C (C- I86F) em apenas 2 anos de exposição ao fungicida (SIMÕES et al., 2018). Adicionalmente, diversos casos têm sido relatados em todo o mundo, incluindo *C. cassiicola* em pepinos (MIYAMOTO et al., 2009; ISHII et al., 2011), *Sclerotinia. sclerotiorum* em canola (GLAETTLI; STAMMLER; SCHLEHUBER, 2009) e *Alternaria solani* em batata (GUDMESTAD et al., 2013).

Com a intensificação do uso de fungicidas no Brasil torna-se necessário monitorar e elucidar os mecanismos de resistência das principais classes de fungicidas aplicados na cultura da soja, a fim de fornecer informações importantes para redefinir as estratégias de manejo de doenças de forma a evitar aplicações desnecessárias e a consequente seleção de variantes resistentes e perda da tecnologia em um curto prazo. Desta forma, os objetivos do estudo foram: i) determinar os níveis de sensibilidade ou resistência aos fungicidas das diferentes espécies de fungo; ii) avaliar *in vitro* a resistência cruzada das populações de *C. kikuchii* e *C. cassiicola* aos fungicidas do grupo MBC (carbendazim e tiofanato-metílico) e IQe (azoxistrobina, picoxistrobina e piraclostrobina); iii) determinar a concentração efetiva para inibir 50% (CE_{50}) da população de *C. cassiicola* aos fungicidas carbendazim (MBC), piraclostrobina (IQe) e protioconazol (IDM); iv) caracterizar os possíveis polimorfismos nos genes-alvo β-tubulina, citocromo *b* (*cyt b*), *cyp51* e S*dh* (subunidades b, c, d) e sua relação com a resistência em populações de *C. kikuchii*, *C. truncatum* e *C*. *cassiicola* v) analisar as variações heterozigóticas das mutações e vi) estudar a diversidade genética entre isolados sensíveis e resistentes a partir das variações obtidas nos diferentes genes analisados.

A tese será apresentada na forma de artigo científico:

Artigo A: “Fungicide resistance and genetic diversity of *Cercospora kikuchii* isolates for *cyp51, cyt b*, β-tubulin and SdhB genes in soybean”

Artigo B: “Molecular detection of fungicide resistance of *Colletotrichum truncatum* for *cyp51*, *cyt b*, β-tubulin and *S*dhB genes in soybean in Brazil”

Artigo C: “Mechanisms of fungicide resistance and genetic diversity of *Corynespora cassiicola* isolates from soybean based on the polymorphisms of β-tubulin, *cyt b*, *cyp51*, SdhB, SdhC and SdhD target genes”

2 REVISÃO DE LITERATURA

2.1 Histórico da soja

Originária do continente asiático, a soja [*Glycine max* (L.) Merril] tem como centro de origem a região nordeste da China (MA, 1984). A soja começou a ser cultivada há aproximadamente 2.500 anos, mas, no continente americano, o cultivo desta oleaginosa ocorreu somente em 1879 (MA, 1984; FORCELINI et al., 2004). Atualmente, mais de 80% da produção mundial concentra-se nos países deste continente, sendo os principais países produtores os Estados Unidos (117 milhões de toneladas), o Brasil (115 milhões de toneladas) e a Argentina (40 milhões de toneladas) (USDA, 2018). Na safra 2017/2018, a produção mundial foi de aproximadamente 334 milhões de toneladas (USDA, 2018).

No Brasil, a cultura foi introduzida no Estado da Bahia no ano de 1882, contudo, foi no Estado do Rio Grande do Sul que a cultura passou a ser cultivada em larga escala (BLACK, 2000). Atualmente, a soja é amplamente cultivada no país, principalmente, devido ao fácil manejo, condições favoráveis ao cultivo e à utilização de novas tecnologias no campo que impulsionaram o desenvolvimento da cultura. Tais fatores foram fundamentais para o Brasil se consolidar na safra de 2017/2018 como sendo o principal exportador mundial de soja, com exportação de aproximadamente 73 milhões de toneladas de grãos (USDA, 2018).

Segundo dados do sexto levantamento de safra de grãos, a produção nacional de soja na safra de 2017/2018 foi de 113.024,6 milhões de toneladas, atingindo uma produtividade média nacional de 3.225 kg ha^{-1} (CONAB, 2018). O Estado do Mato Grosso destaca-se por apresentar a maior produção nacional (61.986,5 milhões de toneladas), seguido pelo Estado do Paraná (40.851,4 milhões de toneladas) e pelo Estado do Rio Grande do Sul (35.516,6 milhões de toneladas) (CONAB, 2018).

Contudo, dentre os principais fatores que afetam o custo de produção da soja, estão os problemas fitossanitários. Aproximadamente 40 doenças que incidem na cultura da soja já foram identificadas no Brasil (TECNOLOGIAS, 2013). As doenças fúngicas apresentam graves implicações para o setor. Estima-se que perdas registradas devido à ferrugem-asiática, principal doença da cultura, variam, entre 13% a 70%, dependendo do estádio em que afeta as plantas e do nível de severidade da doença (ABAG, 2015).

2.2 Crestamento foliar de cercóspora e mancha púrpura da semente

2.2.1 Origem e histórico da doença

O fungo *C. kikuchii* (Matsumoto & Tomoyasu) M.W. Gardner, foi primeiramente relatado na Coréia no ano de 1921 (SUZUKI, 1921) e posteriormente, nos Estados Unidos no ano de 1924 (GARDNER, 1925). Inicialmente a doença foi descrita como mancha púrpura da semente (GARDNER, 1925). Contudo, Walters (1978) relatou o aparecimento de sintomas nas folhas superiores de plantas de soja, e denominou de crestamento foliar de cercóspora.

Com isto, a infecção pelo fungo *C. kikuchii* resultou em dois diferentes complexos: crestamento foliar de cercóspora e mancha púrpura da semente, ambas as doenças são causadas pelo fungo *C. kikuchii*, que causam redução da produtividade e a qualidade de produção, respectivamente. (MURAKISHI, 1951; WILCOX; ABNEY, 1973, ROY; ABNEY, 1976). A ocorrência da mancha púrpura da semente é maior em climas tropicais devido a temperaturas mais quentes e ambiente úmido (SINCLAIR; BACKMAN, 1989). Por isso, no Brasil, o fungo *C. kikuchii* esteve durante muito tempo associado ao agente causal da doença mancha púrpura da semente (MIYASAKA, 1958).

Contrariamente ao entendimento atual, estudos recentes de análise filogenética de *Cercospora* spp. mostraram a ocorrência de múltiplos agentes causais associados ao complexo de doenças crestamento foliar de cercóspora e mancha púrpura da semente, sendo estes relatados, como prováveis representantes de novas espécies, ainda a serem descritas (SOARES et al., 2015).

2.2.2 Etiologia

As espécies de *Cercospora* estão entre os mais destrutivos fitopatógenos no mundo (TO-ANUN; HIDAYAT; MEEBOON, 2011). Mais de 3000 espécies deste gênero infectam um grande número de hospedeiros, incluindo leguminosas, gramíneas e plantas ornamentais (POLLACK, 1987).

A grande maioria de *Cercospora* spp., incluindo *C. kikuchii*, apresentam a fase sexual desconhecida, embora, a fase teleomórfica, *Mycosphaerella,* tenha sido identificada para algumas espécies deste gênero, como a *Mycosphaerella arachidis*, agente causal da doença mancha da folha no amendoim (CHUPP, 1954; CORLETT, 1991).

Cercospora kikuchii pertence ao reino Fungi, filo *Ascomycota*, subfilo *Pezizomycotina*, Classe *Dothideomycetes*, subclasse *Dothideomycetidae*, ordem *Mycosphaerellales* e família *Mycosphaerellaceae* (FUNGORUM, 2018a). Este fungo caracteriza-se por produzir conidióforos de coloração marrom em pequenos feixes (CHUPP, 1954). Os conídios são hialinos aciculiformes e apresentam a base truncada e vértices afilados (CHUPP, 1954).

A produção de esporos de *C. kikuchii* é uma tarefa laboriosa. A esporulação abundante do fungo muitas vezes ocorre somente no tecido do hospedeiro (MATSUMOTO, 1928; MURAKISHI, 1951). Diversos meios de cultura foram testados para induzir a esporulação tais como: decocção de folha de cenoura (KILPATRICK, 1956), batata dextrose e ágar (ROY; ABNEY, 1976); ágar de soja (VATHAKOS; WALTERS, 1979) V-8 (EL-GHOLL et al., 1982); contudo, os resultados destes trabalhos não foram conclusivos.

2.2.3 Ciclo da doença e epidemiologia

A sobrevivência do patógeno ocorre em restos culturais e sementes infectadas (MANANDHAR; HARTMAN, 2008). Sob condições ambientais naturais, este fungo é capaz de sobreviver em restos culturais por até seis meses e em condições ambientais favoráveis o período de sobrevida pode chegar a até quatro anos (KILPATRICK, 1956; BAIRD et al., 1997). A invasão do patógeno nos tecidos vegetais ocorre pela atividade necrotrófica do fungo (MATSUMOTO; TOMOYASU, 1925).

A disseminação do patógeno no campo inicia-se pelo cultivo de sementes contaminadas que, em seguida, infectam os cotilédones (SAKAI, 2000). Na soja, a aceleração do desenvolvimento da doença, ocorre em condições ambientais, como chuvas torrenciais acompanhadas de ventos, em razão da maior liberação dos conídios que infectam a parte aérea e gradualmente propagam-se por toda planta (FUJITA, 1990; SAKAI, 2000).

A infecção de *C. kikuchii* ocorre pela aderência do apressório no tecido vegetal e através da penetração direta do tubo germinativo no tegumento da semente por meio de poros superficiais (CHEN; LYDA; HALLIWELL, 1979; ORTH; SCHUH, 1992), e/ ou através dos estômatos das folhas (UPCHURCH et al., 1991). A alta umidade relativa durante esse processo e o molhamento foliar permitem que o tubo germinativo continue a germinar (SCHUH, 1992). O tempo de infecção para o fungo *C. kikuchii* é de 24 horas em condições de temperaturas de até 25 ºC e um período de molhamento foliar entre 24-36 horas (SCHUH, 1991). Temperaturas acima de 30º C e molhamento foliar menor do que 18 horas, são desfavoráveis para a infecção (SCHUH, 1991). Após a infecção, inicia-se a etapa de colonização, que ocorre nos espaços intercelulares do tecido parenquimático (MATSUMOTO; TOMOYASU, 1925; FUJITA, 1990; ORTH; SCHUH, 1992).

Durante os processos de desenvolvimento e reprodução, o patógeno produz uma substância fotossensível denominada de cercosporina (MACRI; VIANELLO, 1979; DAUB, 1982; DAUB; BRIGGS, 1983). Esta substância é ativada pela luz, produzindo o superóxido de oxigênio. Em consequência, ocorre aumento da atividade das espécies reativas em oxigênio, que são capazes de peroxidar os lipídeos da célula, levando a rigidez celular, a fuga de electrólitos da membrana e consequentemente a morte celular (DAUB, 1982; DAUB; BRIGGS, 1983).

2.2.4 Sintomatologia

O complexo de doenças, crestamento foliar de cercóspora e mancha púrpura da semente ocorre no hospedeiro de forma simultânea ou separadamente (ORTH; SCHUH, 1994), causando sintomas no hipocótilo, caule, vagem, folha, pecíolo e sementes (LEHMAN; 1950).

No hipocótilo e no caule, os sintomas característicos são manchas vermelhas superficiais, geralmente, limitadas ao córtex da planta. Em situações de baixa fertilidade do solo, o fungo é capaz de necrosar a medula da planta (ALMEIDA et al., 2005).

Nas vagens, os sintomas são caracterizados por pontuações de coloração vermelha que evoluem para manchas de coloração castanho-avermelhadas. Com a infecção da vagem, o fungo atinge a semente e causa a doença MPS (ALMEIDA et al., 2005).

Na semente, esta doença é caracterizada por pontos ou manchas irregulares de coloração que varia de rosa ao roxo escuro, e que pode cobrir toda a área do tegumento da semente (SCHUH, 1990). A toxina cercosporina, produzida pelo fungo, é responsável pela coloração característica desta doença (KUYAMA; TAMURA, 1957).

Nas folhas, o crestamento foliar de cercóspora é identificado pelo bronzeamento ou pontuações de coloração castanho-avermelhadas, que podem coalescer e se estender por toda a superfície. Como consequência, ocorre a desfolha precoce que resulta em perdas de rendimento (SCHUH, 1990; ALMEIDA et al., 2005).

2.2.5 Estratégias de controle

As doenças crestamento de cercóspora e mancha purpura da semente são consideradas doenças de final de ciclo (EMBRAPA, 1999). Entre as principais recomendações de controle estão a utilização de sementes sadias, o tratamento de sementes, a incorporação de restos culturais, rotação e sucessão de culturas com espécies não suscetíveis e a aplicação de fungicidas (SINCLAIR; BACKMAN, 1989; SINCLAIR, 1993; EMBRAPA, 1999).

A principal ferramenta utilizada para o controle da doença é a aplicação de fungicidas. Até o momento, 174 produtos formulados foram registrados para soja para o controle desta doença (AGROFIT, 2018). Devido à pressão de seleção imposta pelas constantes aplicações de fungicidas, diversas espécies de *Cercospora* vêm, desenvolvendo algum tipo de resistência. Resistência a fungicidas de diferentes grupos químicos já foi relatada em *C. beticola* (BIRLA et al., 2012; BOLTON; RIVERA; SECOR, 2013), *C. sojina* (ZHANG et al., 2012; STANDISH, et al., 2015; ZENG et al., 2015) e *C. kikuchii* (IMAZAKI et al., 2006; PRICE III, et al., 2015).

2.3 Antracnose

2.3.1 Origem e histórico da doença

O primeiro relato do *Colletotrichum* spp., como agente causal da antracnose na soja, ocorreu na Coréia em 1917, por Takimoto sendo primeiramente identificado por Hemmi como *Colletotrichum glycines* Hori (fase anamórfica) e *Glomerella glycines* (fase teleomórfica) (LEHMAN; WOLF, 1926).

Muitas espécies do gênero *Colletotrichum* foram identificadas na cultura da soja (LEHMAN; WOLF, 1926; TIFFANY; GILMAN, 1954; ROY, 1982; SINCLAIR, 1982). Recentemente a espécie *C. cliviae* que infecta a cultura da soja foi relatada no Brasil (DIAS et al., 2018). Contudo, a espécie mais comumente associada com antracnose em soja é denominada de *C. truncatum* (Schw.) Andrus & Moore (ANDRUS; MOORE; 1935; HYDE et al., 2009).

No Brasil a doença foi primeiramente relatada no ano de 1961 no Estado do Rio Grande do Sul e foi considerada uma das principais doenças da soja, especialmente na região do Cerrado (ARAÚJO, CAFÉ-FILHO, CUPERTINO, 1988; ALMEIDA et al., 2005). Na safra de 2010, a doença ressurgiu na região Centro-Norte do Brasil e recentemente é responsável por perdas de rendimento de aproximadamente 90 kg / ha de grãos a cada 1% de aumento na incidência da doença (DIAS et al., 2016).

Em 1994, perdas recordes de produtividade ocasionada por esta doença foram registradas nos principais países produtores de soja, atingindo cerca de 77.500 toneladas no Brasil, seguidos por Estados Unidos, com 71.400 toneladas e Argentina com 36.700 toneladas (WRATHER et al., 1997). Danos significativos devido a antracnose, foram relatadas em lavouras comerciais no Estado do Tocantins na safra 2011/2012, indicando ineficiência de fungicidas para o manejo da antracnose (DIAS et al., 2016).

2.3.2 Etiologia

O fungo *C. truncatum* pertence ao reino Fungi, filo *Ascomycota*, subfilo Pezizomycotina, classe *Sordariomycetes*, subclasse *Hypocreomycetidae*, ordem *Glomerellales* e família *Glomerellaceae.* A fase sexuada é classificada no gênero *Glomerella* e possui sinônimos como *Vermicularia truncata* Schwein. (1832), *C. dematium* f. *truncatum* (Schwein.) (1957) e *Glomerella truncata* (Schwein.) C. L. Armstr. & Banniza (2006) (FUNGORUM, 2018b).

A espécie *C. truncatum* pertence ao grupo de *Colletotrichum* com conídios curvos (DAMM et al., 2009). Este fungo caracteriza-se por formar acérvulos pretos, com setas pigmentadas e septadas (MANANDHAR; HARTMAN, 1999). Os conídios podem medir de 19,5 a 24 µm x 2 a 2,5 µm são fusiformes e apresentam extremidades afiladas (DIAS, 2014).

2.3.3 Ciclo da doença e epidemiologia

A sobrevivência do patógeno ocorre em restos culturais e sementes infectadas (ATHOW, 1973; SINCLAIR; BACKMAN, 1989), plantas daninhas e em mais de 300 espécies de plantas hospedeiras (HARTMAN; MANANDHAR; SINCLAIR, 1986; MCLEAN; ROY, 1988; SINCLAIR; BACKMAN, 1989; FARR; ROSSMAN, 2018). A invasão do fungo nos tecidos do hospedeiro ocorre pela atividade hemibiotrófica, sendo esta dividida em fase biotrófica e necrotrófica. A fase biotrófica tem duração de 48 horas e caracteriza-se por ser assintomática e pelo desenvolvimento de hifas primárias multiseptadas (LATUNDE-DADA; LUCAS, 2007). Em seguida, inicia-se a fase necrotrófica, caracterizando pela produção de hifas secundárias que invadem os tecidos adjacentes do hospedeiro (LATUNDE-DADA; LUCAS, 2007).

A disseminação do patógeno ocorre pela deposição dos esporos no tecido do hospedeiro, por meio da ação de respingos de gotas de chuva que incidem nos acérvulos, fragmentando a massa de esporos (MEYER; KLEPKER, 2007). Os conídios depositados no tecido vegetal fixam-se por meio de apressórios e o tubo germinativo penetra no tecido injuriado de forma direta (AGRIOS, 2005). Assim, o transporte do fungo até a parte aérea da planta, inicia-se por uma semente contaminada (NEERGARD, 1979), que infecta o tecido do hospedeiro, dando início ao processo de infecção sistêmica.

O ciclo primário da infecção ocorre pelos ascósporos e conídios produzidos em plantas hospedeiras ou em restos de cultura (AGRIOS, 2005). Enquanto, a infecção secundária ocorre pela produção de conídios produzidos na infeção primária, sob condições climáticas favoráveis (AGRIOS, 2005). As condições climáticas ideais são molhamento foliar prolongado, precipitações regulares e frequentes, alta umidade relativa do ar e temperatura acima de 25 ºC (ALMEIDA et al., 2005). Nessas condições, os níveis de perdas podem chegar a 100% (ALMEIDA et al., 2005).

Após, inicia-se o processo de colonização do tecido do hospedeiro através do crescimento micelial do patógeno nos espaços intercelulares (AGRIOS, 2005). Portanto, no começo da colonização, as células do hospedeiro ainda permanecem viáveis (BHADAURIA et al., 2013). Com aumento da reprodução do patógeno ocorre alterações na expressão de genes elicitores, os quais induzem a morte celular (BHADAURIA et al., 2013). Com isso, novos acérvulos são formados, produzindo grande volume de conídios que são disseminados pelo efeito de respingos de chuva, iniciando um novo ciclo de infecção (AGRIOS, 2005).

2.3.4 Sintomatologia

Os sintomas na cultura da soja podem ser observados em praticamente todos os estágios de desenvolvimento, causando deterioração de sementes, morte de plântulas e infecção sistêmica em toda parte aérea (GOULART, 1997). Devido à grande gama de hospedeiros os sintomas são variados, mas geralmente, formam-se lesões necróticas deprimidas nas hastes, vagens, pecíolos e folhas que progridem para necrose (SINCLAIR; BACKMAN, 1989).

O principal sintoma da antracnose é a queda e o apodrecimento de vagens (MEYER; KLEPKER, 2007). Os sintomas iniciais nas vagens são estrias ou manchas claras de formato arredondado que evoluem para manchas de coloração castanho-escura a negra (MEYER; KLEPKER, 2007). As vagens infectadas no início de sua formação podem comprometer a produção de grãos. A qualidade da semente é afetada em infecções tardias, causando danos em pré e pós-emergência (SINCLAIR; BACKMAN, 1989; HARTMAN; SINCLAIR; RUPE, 1999; HENNING et al., 2005).

Os sintomas em sementes contaminadas são manchas deprimidas de coloração castanho-escura (MCGEE, 1986). Os sinais são observados pela presença de acérvulos típicos e inúmeras setas de coloração escura (GOULART, 1997). Em regiões de clima tropical, têm-se observado aumento considerável da perda de viabilidade, durante o armazenamento das sementes, principalmente em períodos de alta pluviosidade na colheita (GOULART, 1997; HENNING et al., 2005).

De forma geral, em clima temperado, a incidência deste patógeno em sementes de soja é baixa (MCGEE, 1986).

Nas hastes, pecíolos e ramos florais, os sintomas são manchas negras ligeiramente deprimidas e brilhantes e sintomas de cancro nos pecíolos (MEYER; KLEPKER, 2007). Nas folhas são observadas lesões necróticas de coloração castanho-escuro ou pretas sobre as nervuras e enrolamento foliar (HAMAWAKI et al., 2002; MEYER; KLEPKER, 2007).

2.3.5 Estratégias de controle

As principais medidas de controle para a antracnose são rotação de culturas, utilização de sementes sadias, manejo equilibrado da adubação potássica do solo, utilização de espaçamento adequado evitando o adensamento, resistência genética e o tratamento químico com fungicidas (MEYER; KLEPKER, 2007).

A principal estratégia de controle empregada para esta doença, tem sido o controle químico. Inicialmente, os fungicidas registrados para o controle de antracnose em soja, eram aplicados no tratamento de sementes (PICININI; FERNANDES, 2000). Contudo, diversos estudos destacaram a eficiência de fungicidas sistêmicos em aplicações foliares, sendo os principais grupos químicos inibidores de quinona externa e metil benzimidazol carbamato (MEYER; RODACKI, 2005; GAWADE, et al., 2009; DA SILVA PESQUEIRA, BACCHI, GAVASSONI, 2016). Até o momento, 51 fungicidas comerciais foram registrados para o controle da antracnose em soja (AGROFIT, 2018). No Brasil, devido ao uso excessivo de fungicidas aplicados, populações resistentes de *Colletotrichum* spp. frequentemente são relatadas em diversas culturas (TANAKA; PASSOS; BETTI; 1997; PERES et al.; 2004; SARTORATO; 2006; SARTORI, 2007; DA SILVA LOPES et al., 2018).

2.4 Mancha-alvo da soja

2.4.1 Origem e histórico da doença

Corynespora cassiicola (Berk. & M. A. Curtis), Wei (1950), agente causal da mancha-alvo, é originário do continente asiático. Tai e Cheo, 1937 e Teng, 1939 relataram que a doença foi primeiramente observada na China, em folhas de soja coletadas entre os anos de 1936 e 1939. Inicialmente o patógeno foi identificado como *Cercospora vignicola* Kawamura. Contudo, alguns anos após, em 1945, Liu identificou no Japão, o mesmo fungo no feijão e denominou de *Helminthosporium vignae* (LIU, 1948).

A primeira constatação da doença no continente americano ocorreu nos Estados Unidos no ano de 1945, sendo descrita pelos autores Olive; Bain e Lefebvre (1945), que relataram a presença de *Helminthosporium vignae,* em feijão-caupi e soja. No Brasil, o primeiro relato da ocorrência da mancha-alvo foi no Estado de Mato Grosso no ano de 1974 e posteriormente, no Estado do Paraná, no ano de 1976 (ALMEIDA et al., 1976). Em 1988 a doença foi também constatada nos Estados de Mato Grosso do Sul e Rio Grande do Sul (YORINORI, 1989). Atualmente, a doença ocorre em todas as regiões de cultivo de soja no país (GODOY et al., 2016).

2.4.2 Etiologia

O fungo *C. cassiicola* pertence ao reino Fungi, filo *Ascomycota*, subfilo *Pezizomycotina*, classe *Dothideomycetes*, subclasse *Pleosporomycetidae*, ordem *Pleosporales* e família *Corynesporascaceae* (FUNGORUM, 2018c). *Corynespora cassiicola* é um fungo cosmopolita e inespecífico. Desde os primeiros relatos da doença, foram observadas mais de 390 espécies de plantas hospedeiras do patógeno cultivadas em mais de 80 países de clima tropical e subtropical (ELLIS, 1971; SILVA et al., 1995; FARR; ROSSMAN, 2018).

Corynespora cassiicola apresenta conidióforos eretos, ramificados, de coloração que varia de pálida a marrom (ELLIS, 1971). Segundo Gasparotto e Pereira (2012) os conídios e conidióforos podem variar morfologicamente, de acordo, com a umidade do ambiente em que são produzidos. De forma geral, *C. cassiicola* caracteriza-se por apresentar septos, que variam até número máximo de 20, e que podem medir de 110-850 µm ou 44-350 µm de comprimento por 4-11 µm de largura (ELLIS, 1971; HARTMAN; SINCLAR; RUPE, 1999).

2.4.3 Ciclo da doença e epidemiologia

Corynespora cassiicola caracteriza-se por ser um patógeno hemibiotrófico (SINCLAIR; BACKMAN, 1989). O ciclo do patógeno se inicia a partir da sobrevivência do fungo em sementes (SINCLAIR; BACKMAN, 1989) e em mais de 390 hospedeiros alternativos (FARR; ROSSMAN, 2018). Na ausência do hospedeiro a sobrevivência do patógeno pode chegar a até dois anos (KUROZAWA; PAVAN; REZENDE, 2005).

A disseminação dos conídios a curtas distâncias ocorre por meio de respingos de chuva para plantas vizinhas (SINCLAIR; BACKMAN, 1989; SILVA; CAMPOS; SILVA, 2008), enquanto que à longa distância pela ação do vento (ALMEIDA et al., 2005; SILVA; CAMPOS; SILVA, 2008). Os conídios depositados na superfície da folha fixam-se por meio de apressórios e germinam sob condições de prolongado molhamento foliar e temperaturas que variam de 22 ºC a 30 ºC (SINCLAIR, 1982).

Durante a colonização o patógeno produz toxinas, denominada cassiicolina, que matam os tecidos adjacentes ao ponto de infeção e assim, extraem nutrientes do hospedeiro para sua sobrevivência e reprodução, formando um novo ciclo de infecção (FORCELINI, 2010). Os primeiros sintomas da doença são visíveis de 5 a 7 dias após a penetração do patógeno no hospedeiro (AGRIOS, 2005).

2.4.4 Sintomatologia

Após a colonização do tecido do hospedeiro, os sintomas da mancha-alvo são observados em toda parte aérea da planta, nas raízes, hastes e vagens. Nas folhas, as lesões iniciais caracterizam-se por uma pontuação parda no centro e anéis concêntricos de coloração mais escura ao redor da lesão (ALMEIDA et al., 2005). Com o progresso da doença, as lesões iniciais evoluem para grandes manchas circulares, de coloração castanho-clara a castanho escura, atingindo até 2 cm de diâmetro (ALMEIDA et al., 2005). A alta severidade da doença em cultivares suscetíveis e a rápida expansão da lesão, ocasionam a queda prematura de folhas, lesões necróticas em pecíolos e nas hastes, abertura, exposição e geminação dos grãos nas vagens (SINCLAIR; BACKMAN, 1989).

Nas raízes, os sintomas são manchas de coloração vermelho arroxeada no tecido cortical, evoluindo para uma coloração negra (AVOZANI, 2011). Os sintomas reflexo da infecção radicular são observados pelo amarelecimento das folhas e maturação prematura dos grãos (ALMEIDA et al., 2005). Em solos úmidos, o fungo produz conidióforos e conídios ao redor das raízes, facilitando a identificação do patógeno pelos sinais após a morte da planta (ALMEIDA et al., 2005).

2.4.5 Estratégias de controle

As medidas de controle para *C. cassiicola* têm sido baseadas no manejo integrado, com o objetivo de manter a população do patógeno abaixo do limiar de dano econômico. As estratégias recomendadas para esta doença são: o uso de cultivares resistentes, o tratamento de sementes, a rotação/sucessão de culturas com milho e outras espécies de gramíneas e a aplicação de fungicidas (ALMEIDA et al., 2005).

Os ingredientes ativos registrados para controle da mancha-alvo em soja são os fungicidas do grupo dos MBC, dos IQe, dos IDM, e os ISDH. Atualmente, 48 fungicidas comerciais estão registrados para o controle da mancha-alvo em soja (AGROFIT, 2018). Contudo, devido a frequente aplicação destes fungicidas, populações resistentes a *C. cassiicola* têm sido reladas em diversas culturas (DATE et al., 2004; ISHII et al., 2007; HUANG et al., 2013; XAVIER et al., 2013; AVOZANI; REIS; TONIN, 2014; TERAMOTO et al., 2017).

2.5 Fungitoxicidade e sensibilidade

Fungicidas são substâncias químicas de origem natural ou sintética que aplicadas às plantas protegem-nas da penetração e/ou do desenvolvimento de fungos patogênicos. Nem todos os fungicidas têm ação direta nos fungos. Alguns atuam inibindo o crescimento micelial ou a esporulação do patógeno. Portanto, não são todas as substâncias químicas que são tóxicas aos fungos. Se um fungo for sensível a um fungicida, este apresenta fungitoxicidade, caso contrário, este será considerado insensível. A fungitoxicidade é definida pela capacidade desta substância química ser tóxica ao fungo em baixas concentrações. A fungitoxicidade é uma característica relacionada à molécula química, enquanto, a sensibilidade está atrelada ao patógeno (REIS; REIS; FORCELINI, 2007).

O valor de toxicidade de uma substância química é determinado pela sua concentração efetiva capaz de inibir 50% do crescimento micelial (CE_{50}) ou da germinação de esporos. A CE_{50} é específica e constante tanto para uma substância química quanto para o patógeno. Um baixo valor de CE_{50} indica uma alta ação fungicida (REIS; REIS; FORCELINI, 2007).

Edgington, Khew e Barron (1971) definiram os seguintes critérios para enquadrar as substâncias fungicidas em relação à fungitoxicidade**:** substância que apresenta CE_{50} <1 μg mL = alta fungitoxicidade e sensibilidade; CE_{50} entre 1 μg mL e 10 μg mL^{-1} = moderadamente fungitóxica, CE_{50} entre 10 μg mL^{-1} e 50 μg mL^{-1} = baixa fungitoxicidade e insensibilidade; e CE_{50}>50 μg mL^{-1} = não fungitóxico e insensibilidade ao fungicida.

Alterações para valores maiores de CE_{50} de um fungicida, no mesmo local e ao longo do tempo, poderá indicar seleção do patógeno alvo direcionado para a insensibilidade àquela molécula química (REIS; REIS; FORCELINI, 2007).

2.6 Resistência de fungos a fungicidas

A resistência a fungicidas pode ser definida como resultado da adaptação de uma espécie fúngica a um fungicida devido a uma mudança genética estável e hereditária, levando a reprodução e disseminação de populações mutantes com reduzida sensibilidade ao fungicida (DELP; DEKKER, 1985). A redução na sensibilidade do patógeno à substância química poderá ser parcial ou total (REIS; REIS; FORCELINI, 2007).

Os fatores genéticos envolvidos na resistência de fungos a fungicidas dependem dos seguintes fatores: número de loci envolvidos, o número de variantes alélicas em cada locus, a existência e relevância da relação entre os alelos dominante ou recessivos dos fungos resistentes e do tipo selvagem (BORCK; BRAYMER, 1974) e as interações aditivas ou sinérgicas entre os genes de resistência (ANGELINI; POLLASTRO; FARETA, 2015).

Genes responsáveis pela resistência a fungicidas estão localizados nos cromossomos nucleares ou em determinantes genéticos extracromossômicos (ANGELINI; POLLASTRO; FARETA, 2015). Desta forma, a posição nuclear ou citoplasmática dos genes, determinam os padrões de herança genética. Para os genes nucleares a herança genética é biparental clássica, ou seja, o zigoto recebe um alelo de um gene de cada progenitor. Enquanto, para os genes extracromossômicos a herança genética, é definida como não-mendeliana, a qual caracteriza-se por ser uma herança uniparental, sendo geralmente, de herança genética materna (GRIFFITHS, 1996; ANGELINI; POLLASTRO; FARETA, 2015). A maioria dos genes de resistência a fungicidas estão localizados nos cromossomos nucleares (ANGELINI; POLLASTRO; FARETA, 2015).

A resistência a fungicidas poderá resultar em mutações no sítio-alvo bioquímico de um único gene (GEORGOPOULOS, 1988), ou adição (KALAMARAKIS et al., 1991; LASSERON-de FARANDRE; DABOUSSI; LEROUX, 1991) ou interações sinergísticas (MOLNA; HORNOK; PESTI, 1985) entre muitos genes mutantes, fazendo com que a ligação do fungicida seja menos efetiva, ou até que não ocorra (HOLLOMON, 2012).

A resistência monogênica e oligogênica são causadas por um ou poucos genes, respectivamente. A resistência monogênica proporciona alterações qualitativas no fenótipo, caracterizando-se pela fácil distinção entre isolados sensíveis e resistentes. A resistência monogênica foi relatada para os fungicidas MBC, IQe e ISDH. Por outro lado, a resistência poligênica é causada por uma mutação em vários genes. Esta mutação tem pouco efeito no fenótipo e causa uma redução insignificante na sensibilidade de um fungicida. No entanto, as diversas mutações contribuem, para um efeito aditivo, produzindo um aumento do nível de resistência (ANGELINI; POLLASTRO; FARESTA, 2015). No campo, o resultado é uma diminuição quantitativa da sensibilidade a um fungicida com uma mudança lenta, contínua e gradual da população de fungos (ANGELINI; POLLASTRO; FARESTA, 2015). A resistência poligênica foi relatada para os fungicidas que atuam como IDM.

2.7 Mecanismos de resistência dos fungicidas da classe IDM

Os fungicidas IDM representam um importante grupo de fungicidas sistêmicos para o controle dos fungos das classes *Ascomycetos*, *Basidiomycetos* e os *Deuteromycetos* (LOEFFLER; BUTTERS; HOLLOMON, 1984; PONTZEN; POPPE; BERG, 1990; KÖLLER, 1992). Os primeiros fungicidas desta classe começaram a serem usados na agricultura na década de 1960 e, a partir de então, mais de 40 moléculas dos fungicidas IDM foram desenvolvidas para o controle de fitopatógenos. Os derivados dos compostos comerciais imidazóis e triazóis, denominado de azóis, são os mais importantes fungicidas utilizados na agricultura (ZIOGAS; MALANDRAKIS, 2015). Os fungicidas IDM agem na membrana celular inibindo a desmetilação do C14 durante a formação do esterol, principal componente da membrana celular dos fungos (ZIOGAS; MALANDRAKIS, 2015).

O mecanismo de resistência para IDMs é poligênico podendo expressar-se individualmente ou associados. Estes mecanismos de resistência incluem: mutações de ponto, modificações no sítio-alvo na desmetilação do C14 (gene *cyp51*) resultando na redução da afinidade do fungicida e a super expressão do gene alvo durante a formação do ergosterol, redução do acúmulo intracelular de fungicida mediado pelo aumento da regulação de transportadores de efluxo e múltiplos parálogos do gene alvo (BRENT, 2011; ZIOGAS; MALANDRAKIS, 2015).

Devido a genética da resistência aos IDMs ser poligênica, o desenvolvimento de resistência a este grupo químico, não leva a uma completa perda de controle da doença e alta frequência de populações resistentes são observados somente após uma adaptação gradual (GISI et al., 2000).

A mutação mais frequente observada para o gene *cyp51* é a substituição da tirosina (Y) na posição 134, 136 e 137 do códon por uma fenilalanina (F) (BECHER; WIRSEL, 2012). Estas mutações foram relatadas em *Uncinula necator* (DELYE, LAIGRET; CORIO-COSTET, 1997) e *Blumeria graminis* (WYAND; BROWN, 2005) a protioconazol e *Mycosphaerella* sp. resistente ao propiconazol (CAŇAS-GUTIERREZ et al., 2009; CHONG et al., 2010; LEROUX; WALKER, 2011; COOLS; FRAAJIE, 2013).

No Brasil, o uso intensivo de fungicidas na cultura da soja trouxe como consequências a seleção de populações de *P. pachyrhizi* menos sensíveis aos IDM a partir de 2007 (GODOY, 2012). A menor sensibilidade aos fungicidas deste grupo químico foram relatadas após 2007 em populações de *P. pachyrhizi* por Schmitz et al., (2014) e Klosowski et al., (2016a) e também em populações de *C. cassiicola* por Xavier et al., (2013) e Teramoto et al. (2017).

2.8 Mecanismos de resistência dos fungicidas da classe do MBC

Os fungicidas do grupo MBC estão divididos em três classes: benzimidazois, N-phenilcarbamatos e benzamidas. Os fungicidas comerciais desta classe atuam inibindo a montagem dos microtúbulos do fuso durante a divisão nuclear ligando-se à proteína β-tubulina (SCHROEDER; PROVVIDENTI, 1969; GEORGOPOULOS; DOVAS, 1973; DAVIDSE, 1986; DAVIDSE; ISHII, 1995). Os microtúbulos são formados por dímeros proteicos de α e β-tubulina. A função do microtúbulo na divisão do núcleo depende diretamente da montagem reversível da tubulina em polímeros de microtúbulo. Muitos fungicidas atuam interrompendo a montagem dos microtúbulos ou impedindo a desmontagem dos dímeros de proteínas na divisão nuclear dos eucariotos (YOUNG, 2015).

Os fungicidas benzimidazois foram introduzidos pela primeira vez em 1960 e representam o marco inicial dos problemas de resistência (DELP, 1981). Desde o início da sua comercialização, pelo menos 130 espécies de fungos desenvolveram resistência a esse grupo (FRAC, 2014). Este composto controla uma grande gama de patógenos das classes dos *Ascomycetos* e *Basidiomycetos*, contudo são inativos aos *Oomycetos* (YOUNG, 2015). Este composto tem sido utilizado na cultura da soja principalmente para o controle de doenças de final de ciclo, contudo sua eficiência no campo tem sido baixa (GODOY et al., 2013). Resistência do fungo *C. cassiicola*, isolado de folhas de soja, a MBCs tem sido relatada nos últimos anos no Brasil em diferentes regiões produtoras (AVOZANI, 2011; TERAMOTO et al., 2012; XAVIER et al., 2013). Atualmente os fungicidas comerciais utilizados na cultura da soja são: carbendazim, tiabendazol e tiofanato-metílico (AGROFIT, 2018).

Segundo o Comitê de Ação Antirresistência, os fungicidas benzimidazóis são classificados com alto risco de desenvolver resistência (FRAC 2013). Populações resistentes apresentaram diversas mutações na proteína β-tubulina que conferem resistência aos benzimidazois. Estas mutações ocorrem em vários códons como 6, 50, 167, 198, 200 e 240. No entanto, as mutações E198A / G / K / Q e F200Y são as mais frequentes (KOENRAADT; JONES 1992; MA; MICHAILIDES, 2005). Em mutantes resistentes, a resistência cruzada geralmente ocorre entre os diferentes compostos de benzimidazois e, com isso, a resistência a um composto acarreta a perda da eficácia para todas as outras classes de benzimidazois (DELP, 1987).

2.9 Mecanismos de resistência dos fungicidas da classe do IQe

Os fungicidas inibidores da respiração mitocondrial do complexo III (complexo do citocromo bc1) são um dos mais importantes grupos de fungicidas por controlarem uma grande gama de fitopatógenos (BRENT, 1995). Estes fungicidas são classificados em IQe, IQoS e QiI, diferenciando-se pelas ligações distintas no citocromo b (*cyt b*). O maior grupo é o IQe que tem atividade para a maioria das classes de *Deuteromycemetos*, *Ascomycetos*, *Basidiomycetos* e *Oomycetos* (SIEROTZKI, 2015).

Os primeiros componentes químicos extraídos do fungo *Strobilurus tenacellus* e a descoberta da estrobirulina como um fungicida ocorreu em 1977 (ANKE, 1995). Contudo, a comercialização ocorreu somente em 1996 (BRASSEUR; SARIBAŞ; DALDAL, 1996). Logo após a introdução destes fungicidas, foram detectados isolados resistentes de *Podospora fusca*, agente causal do míldio pulverulento das cucurbitáceas, em diversos países (BRASSEUR; SARIBAŞ; DALDAL, 1996; FERNÁNDEZ-ORTUÑO et al., 2008). O grupo IQe inclui 20 compostos subdivididos em 9 classes químicas. As estrobilurinas recomendas para o manejo de fitopatógenos na cultura da soja são: azoxistrobina, cresoxim-metílico, picoxistrobina, piraclostrobina, metominostrobina e trifloxistrobina (AGROFIT, 2018).

O modo de ação deste grupo ocorre pela inibição da transferência de elétrons no sítio Qo (sítio de oxidação quino externa) do complexo do citocromo bc1 da cadeia respiratória mitocondrial. Com isso, ocorre um bloqueio no fluxo de elétrons reduzindo a produção de ATP nas células do fungo (BECKER et al., 1981). Nem todas as mutações observadas no gene *cyt b* têm um forte efeito na resistência, algumas apresentam um efeito somente na atividade enzimática (SIEROTZKI, 2015). Os fatores de resistência são altamente dependentes da espécie e do efeito do fungicida no campo (SIEROTZKI, 2015). As mutações para resistência ao IQe G143A, F129L e G137R são as mais frequentemente observadas.

A primeira mutação trata-se de uma substituição pontual do aminoácido glicina na posição 143 por um aminoácido alanina no gene *cyt b* (G143A), sendo esta considerada uma mutação altamente resistente e mais frequentemente relatada entre os fitopatógenos (SIEROTZKI, 2015). Em *Cercospora sojina* agente causal da mancha olho-de-rã em soja, mutações G143A foram relatadas no Estado do Tennessee nos EUA (ZENG et al., 2015; STANDISH, et al., 2015).

A segunda mutação é a substituição descrita em *cyt b* é a substituição do aminoácido fenilalanina por leucina na posição 129 (F129L). Para esta mutação os fatores de resistência são considerados mais baixos, ou seja, supõe-se que tem um efeito mais forte na adaptabilidade da população do patógeno em sobreviver ao ambiente em isolados resistentes comparando-se com a mutação G143A, logo com menor chance de ocorrência (ESSER et al., 2004; ORR, 2009; SIEROTZKI et al., 2007). Contudo, a mutação F129L pode afetar a performance do fungicida IQe quando a população se encontra em grande número (PASCHE; WHARAM; GUDMESTAD, 2002). Esta mutação foi detectada para os fungos *Alternaria solani* (PASCHE; PICHE; GUDMESTAD, 2005), *Pyrenophora teres* (SEMAR et al., 2007) e *Phakopsora pachyrhizi* (KLOSOWSKI, 2016b).

A última mutação é uma substituição do aminoácido glicina por uma arginina na posição 137 (G137R). Não se sabe como esta afeta a ligação fungo-fungicida e seria a mais rara, provavelmente é a mutação que apresenta o maior custo adaptativo para o patógeno (SIEROTZKI, 2015).

2.10 Mecanismos de resistência dos fungicidas da classe do ISDH

Os fungicidas inibidores da succinato desidrogenase (complexo II, succinato-ubiquinona redutase), ISDH também conhecido como carboxamidas, foram utilizados pela primeira vez na década de 1960 para o controle de doenças causadas por *Basidiomycetos* (AVENOT; MICHAILIDES, 2010). Devido às doenças e ao espectro limitado das carboxamidas de “primeira geração”, à resistência em escala comercial mantiveram-se limitadas à poucas culturas/patossistemas (FRAC, 2014). O fungicida boscalide, lançado no ano de 2003, foi o primeiro composto ISDH com amplo espectro de ação, para vários fungos da classe *Ascomycetos*. (STAMMLER et al., 2007; STAMMLER et al., 2008). Recentemente a combinação de boscalide com os compostos benzovindifupir, bixafen, fluxapiroxade, isofetamida, isopirazam, penflufen, penthiopirad e sedaxano expandiu a segunda geração de moléculas ISDH, que tem sido registrada e lançada para diferentes alvos biológicos na agricultura (STAMMLER et al., 2015).

No Brasil, dois ingredientes ativos ISDH foram registrados em misturas com IQe para a cultura da soja, em 2013 e em 2014 (fluxapiroxade e benzovindiflupir, respectivamente), (GODOY, MEYER, 2014). A enzima alvo dos inibidores SDH é a succinate desidrogenase (SDH, chamado complex II) que age bloqueando o sítio de ligação da ubiquinona na mitocôndria. Os SDH são constituídos por quatro subunidades (A, B, C e D) e o local de ligação dos inibidores SHD ocorrem nas subunidades B, C e D (STAMMLER et al., 2015).

As mutações já relatadas em *B. cinerea* foram P225L / F / T ou B-H272Y / R / L / V e em *Pyrenophora teres* nas diferentes locais das subunidades B (B-H277Y), C (C-N75S, C-G79R, C-H134R, C-S135R) e D (D-D124N / E, D-H143R, D-D145G) (STAMMLER et al., 2015). Devido à especificidade do seu modo de ação, mutações no ponto alvo reduzem a sensibilidade em todas as três subunidades, causando a seleção de populações de fungos resistentes no campo (FRAC, 2014). Apesar da recente introdução dessa nova geração de fungicidas no Brasil, após dois anos, populações de *P. pachyrhizi* apresentaram mutações no gene SdhC (C-I86F) (SIMÕES et al., 2018). Além disso, outros 14 patógenos no mundo, incluindo fungos como *C. cassiicola*, em pepino (MIYAMOTO et al., 2009; ISHII, et al., 2011), *Sclerotinia sclerotiorum* em canola (GLAETTLI; STAMMLER; SCHLEHUBER, 2009) e *Alternaria solani*, em batata (GUDMESTAD et al., 2013) apresentaram resistência a essa molécula.

2.11 Resistência cruzada

A resistência cruzada ocorre quando a mudança em um fator genético resulta em resistência a diferentes fungicidas (DEKKER, 1976). Desta forma, a resistência cruzada pode ser classificada em resistência cruzada positiva e resistência cruzada negativa. A resistência cruzada positiva é comumente observada entre compostos que apresentam o mesmo modo de ação (BRENT; HOLLOMON 2007 a, b), enquanto a resistência cruzada negativa ocorre quando uma mutação causa resistência a um fungicida e aumenta a sensibilidade a outros químicos com o mesmo modo de ação (BRENT; HOLLOMON, 2007 a, b). A resistência cruzada positiva foi relatada para os fungicidas IQe (orisastrobin, azoxistrobin e metominostrobin) em *Magnaporthe oryzae* (MIYAGAWA; FUJI, 2013), MBC (carbendazim e thiabendazol) em *B. cinerea* (LEROUX et al., 1999) e ISDH (fluopiram, carboxin) em *B. cinerea* (LALÈVE et al.; 2014). Resistência cruzada negativa foi observada para MBC (dietahofencarb, difenilamina e diclorano) também em *B. cinearea* (LEROUX et al., 1999).

3. ARTIGO A: “Fungicide resistance and genetic diversity of *Cercospora kikuchii* isolates for *cyp51, cyt b*, β-tubulin and SdhB genes in soybean”

ABSTRACT

Cercospora leaf blight and purple stain of seed are a complex of disease caused by *Cercospora kikuchii*. The disease affect leaves and seeds in soybean (*Glycine max* (L.) Merr.) and often is managed by fungicide application of quinone outside inhibitors (QoI) and methyl benzimidazole carbamate (MBC) and recently succinate dehydrogenase inhibitor (SDHI). However, excessive fungicide applications, have contribute to development of resistant populations. The aims of this study were to characterized sensitive and resistant *C. kikuchii* isolates and analyzed cross-resistence using mycelial growth inhibition in amended media using discriminatory dose (10 µg mL^{-1}) of azoxystrobin, picoxystrobin, pyraclostrobin, carbendazim and thiophanate-methyl. We also performed *C. kikuchii* fungicides gene target *cyp51*, β-tubulin, *cyt b*, SdhA and SdhB using Illumina sequencing. The sequencing data was analysed the genetic diversity of *C. kikuchii* isolates. Cross-resistance was observed inside the groups QoI and MBC fungicides. A total of 69% and 71% of the isolates tested for QoI and MBC fungicides were phenotype as resistant. For *cyp51* gene it was detected 13 mutations: Q161H, N178D, P180S, P200L, K217R, E274D, S279N, E281K, T290S, I299V, S363N, K442Q and L503V. Despite the high polymorphism found for the *cyp51* gene, further studies should be perfomed to determine if these new mutations confer DMI fungicide resistance. For *cyt b* gene was observed only G143A mutations. In addiction, 75% of *C. kikuchii* isolates from soybean in Brazil carried G143A mutation. The mutations F129L and G137R were not found. Molecular characterization of MBC and SdhA fungicide revealed only synonyms mutations for all isolates analysed. In total 14 substitutions for SdhB were found: H17L, G27A, T167M, P192L, E200D, N290K, I320N, I434V, A436V, D447N, H448R, E454Q, N513S and Q569K. High genetic diversity of *C. kikuchii* between sensitive and resistant isolates confirmed that genetic mutations found in this study are responsible for the high genetic variability of the isolates. Therefore, anti-resistance strategies have to be implemented for efficiency recovery of QoI to control *Cercospora kikuchii* in soybean in Brazil.

Keywords: Cercospora leaf blight, purple seed stain; QoI and MBC.

3.1 INTRODUCTION

Soybeans (*Glycine max* (L.) Merr.) are the main grain produced in Brazil with sowing area of 35,046 mil hectares in the last crop season, representing about 60% of the area with grains during the summer crop season (CONAB, 2018). However, due to the favorable climatic conditions such as, high temperatures and relative humidity, this conditions enable the occurrence of diseases like *Cercospora kikuchii* complex. The *C. Kikuchii* complex includes purple stain of seed (GARDNER, 1925) and Cercospora leaf blight (WALTERS, 1978) and represent a complex of species. Recently, some undescribed *Cercospora* spp were associated to infected soybean in Brazil (SOARES et al., 2015). This pathogen is widely disseminated in main Brazilian soybean producing regions. Under high severity and favorable climate conditions, losses can reach 30 % up to 50% (WARD-GAUTHIER et al., 2015).

The disease complex, cercospora leaf blight and purple seed stain can occur in the host at the same time or separatelly (ORTH; SCHUH, 1994), causing symptoms in the hypocotyl, stem, pods, leaves, petiole and seeds (LEHMAN, 1950). Typical symptoms in leaves have a tanning or reddish-brown colors, which can extend throughout the surface leading to early defoliation (ALMEIDA et al., 2005). Symptoms in seed, are characterized by irregular pink to dark purple spots (SCHUH, 1990). Cercosporin toxin is responsible for pink-dark color characteristic of this disease (KUYAMA; TAMURA, 1957). High severity of cercospora leaf blight and purple seed stain causing reduction of germination and seed quality (WILCOX, ABNEY, 1973; YEH; SINCLAIR, 1981). Cercospora leaf blight and purple seed stain are management using cultural and chemical control. The use of healthy seeds, cultural remains incorporation, rotation or succession with non-susceptible crop species and sowing cultivars with resistant are the most economic and effective tools (SINCLAIR, BACKMAN, 1989; SINCLAIR, 1993; EMBRAPA, 1999). However, few commercially soybean cultivars are resistant to cercospora leaf blight and purple seed stain in Brazil (FINOTO et al., 2011). Therefore, fungicides application, as seed treatments and foliar, have being used as main management to control *C. kikuchii* in soybean.

In total 174 formulation products are labeled for soybean to control cercospora leaf blight and purple seed stain in Brazil (AGROFIT, 2018). Many of the commonly used fungicides are demethlylation inhibitors (DMI), quinone outside inhibitor (QoI), methyl benzimidazole carbamate (MBC) and recentely carboxamides succinate dehydrogenase inhibitor (SDHI) chemical groups.

Reduction in efficiency control has also been observed for DMI fungicides. This chemical group is used in pre-mixtures for the control of *C. kikuchii*. DMI fungicides acts on the cell membrane by inhibiting C14 demethylation during formation of the sterol, the main component of the fungal cell membrane (ZIOGAS; MALANDRAKIS, 2015). The mechanisms of resistance of DMI are variable, however, the main mutation occurs at the target site of the cytochrome P450 14-α sterol demethylase (*cyp51*) gene (SCHMITZ et al., 2014; ZIOGAS; MALANDRAKIS, 2015). The most frequent mutations in the *cyp51* gene observed for *Phakopsora pachyrhizi* are tyrosine (Y) substitution at position 134, 136 and 137 of the codon for a phenylalanine (F), substitution a lysine (K) for arginine (R) at the position (I) by threonine (T) at position 475 (BECHER; WIRSEL, 2012; SCHMITZ et al., 2014; KLOSOWSKI et al., 2015). In addition to these point mutations, overexpression of the *cyp51* gene is a mutation present for *P. pachyrhizi* (SCHMITZ et al., 2014). In Brazil, the excessive use of fungicides on soybean crops led to *P. pachyrhizi* populations less sensitive to DMI fungicides since 2007 (GODOY, 2012; SCHMITZ et al., 2014). Field isolates of *C. beticola* populations resistant to DMI were observed in sugar beet in Greece (KARAOGLANIDIS; IONNIDIS; THANOSSOULOPOULOS, 2000) and Serbia (BUDAKOV et al., 2014; FRAC, 2017).

MBC chemical group, benzimidazoles and thiophanates, as high risk of fungicide resistance (FRAC, 2017). In Brazil, thiabendazole and thiophanate-methyl, have been used in soybean seeds treatments to control *C. kikuchii* since 1990s (HENNING; KRZYZANOWSKI; FRANÇA NETO, 1991). This fungicide group acts by inhibiting the microtubules assembly during nuclear division by binding to β-tubulin protein (SCHROEDER; PROVVIDENTI, 1969; GEORGOPOULOS; DOVAS, 1973; DAVIDSE 1986; DAVIDSE; ISHII, 1995). The molecular mechanism was first observed in *Botrytis cinerea* and this mutation involved a point mutation in at codon 198 or 200 in β-tubulin gene, causing resistance to carbendazim and thiophanate-methyl. Addicionally, MBC resistante fungi isolates have exhbited cross resistance between different benzimidazoles chemical class (DELP, 1987). Resistant populations to MBC were observed in different isolates of *Cercospora* species and in different field crops. *C. apii* in celery (BERGER, 1973; FRAC, 2017); *C. arachidicola* in peanut (CLARK; BACKMAN; RODRIGUEZ-KABANA, 1974; LITTRELL, 1974; FRAC 2017) and *C. beticola* in sugar beet (GEORGOPOULOS; DOVAS, 1973; BUDAKOV et al., 2014; FRAC, 2017). Resistant population to MBC in *C. kikuchii* were observed in Japan (IMAZAKI et al., 2006) and in USA (PRICE, 2015).

The specific action of QoI fungicide is in mitochondrial respiration by the inhibition of electron transfer at Qo site of the cytochrome bc1 (*cyt b*) complex III (BECKER et al., 1981). The mechanism of QoI resistance is target site-based and involves mutations in the mitochondrial *cyt b* gene. Molecular mechanisms of resistance have been detected in several phytopathogenic fungi. The most commum mutations are amino acid substitution from glycine to alanine at position 143 (G143A), from phenylalanine to leucine at position 129 (F129L), and from glycine to arginine at position 137 (G137R) (GISI et al., 2002). Independente of the three QoI mutations occur, cross resistance is present in all QoI fungicide group for the vast majority isolates (SIEROTZKI, 2015). The mutation G143A are frequent and already have been reported in population in *Cercospora* spp. worldwide. The G143A mutation was reported in *C. sojina* (FRAC, 2011; ZENG et al., 2015) and *C. beticola* (BOLTON; RIVERA; SECOR, 2013) in United States and in Italy in *C. beticola* (BIRLA et al., 2012). Field resistant populations of *C. kikuchii* fungi to QoI fungides was recently observed in soybean production regions in United States (PRICE III, et al., 2015; ALBU et al., 2016) and recently, in Bolivia (SAUTUA et al., 2019).

SDHI fungicides began to be marketed in Brazil in the years of 2013 and 2014 and represent the newest class of fungicides registered to control *P. pachyrhizi* (GODOY; MEYER, 2014). However, all the remain fungi that infect the crop as late season disease are exposed to this such application. Addicionally, resistant populations of *P. pachyrhizi* (SIMÕES et al., 2018) were observed in Brazil. SDH fungicides act on complex II by blocking the ubiquinone binding site in the electron transport chain in the mitochondria. The target enzyme of SDH inhibitors is succinate dehydrogenase, consisting of four subunits (A, B, C and D). However, the binding site of SDHI inhibitors occur in the B, C and D subunits. The molecular mechanisms of resistance of sdhs genes occur due to the different amino acid substitutions in different subunits. SDH mutation regions are highly conserved regions among species (SIMÕES et al., 2018). According to Simões et al., 2018 the

(C-I86F) mutation in *P. pachyrhizi* is the most probable mutation for resistance to SDHI fungicides. In addition, it did not observe any relevant mutation for *P. pachyrhizi* for the SdhB and SdhD genes.

Therefore, due to the frequent historic of fungicide resistance to control *C. kikuchii*, with a combination of susceptible cultivars and repeated application with the same mode of action, monitoring the resistance of fungicides becomes essential for the management of the disease. Therefore, the study we defined sensitive *in vitro* of *C. kikuchii* isolates to the fungicides carbendazim and thiophanate methyl (MBC) and azoxystrobin, picoxystrobin, pyraclostrobin (QoI), and using the same experiment, we analysed cross-resistance into MBC and QoI active ingredients. We also characterize the possible polymorphisms to determine the point mutations involved QoI and MBC fungicide groups and their correlation with resistance. These data allow us to test the hypothesis that resistant isolates have impact on the genetic diversity.

3.2 MATERIALS AND METHODS

3.2.1 Isolation and origin of isolates

In total, 56 isolates of *C. kikuchii* from soybean were collected in the following Brazilian states: Federal District (n = 2), Goiás (n = 7), Mato Grosso (n = 12), Maranhão (n = 3), Minas Gerais (n=6), Pará (n= 1) ,Paraná (n = 17), Tocantins (n = 1), Roraima (n=1), and unknown (n=6).

The isolates collected in the crop seasons of 2015 and 2016 were isolated from soybean seeds with typical symptoms of purple spot. Isolation was performed directly (ALFENAS et al., 2007). The fungi tissues were placed in Petri dishes containing potato-dextrose-agar (PDA) culture medium (4 g L^{-1} potato extract, 20 g L^{-1} dextrose, 15 g L^{-1} agar, pH 5.6 ± 0.2). The identification of the species occurred by comparing the morphological characteristics of colonies of *C. kikuchii*.

The isolates belonging to the Embrapa Soybean Mycological Collection were isolated from stem (602, 607, 609, 611), pods (610) and leaves (611, 615, 616, 675, 1158, 1160,1185, 1192, 1429 and 1625).

3.2.2 Cross-resistance *in vitro*

In order to classify the sensitive and resistant fungi, the methodology of inhibition of mycelial growth in amended medium, with a discriminatory dose was used.

The isolates were cultivated in PDA culture medium at 24 ºC under photoperiod of 12 h / 12 h for 7 days. Petri dishes with 15 mm diameter were equally divided into four subunits containing two isolates with two replicates. Each isolate was composed of 4 replicates. Mycelial discs with 4 mm diameter from the colonies border were transferred to Petri dishes containing PDA medium and fungicides.

The fungicides were diluted in autoclaved distilled water and mixed with the PDA culture medium. The final concentration of active ingredients (a.i.) was 10 µg / mL^{-1}. The following commercial formulations were used: MBC: [carbendazim (50% a.i.; Bendazol®, Adama Ltd.), thiophanate-methyl (50% a.i.), Cercobin 500 SC®, Iharabras S. A Industries Chemicals)] and QoI [azoxystrobin (25% a.i. Priori®, Syngenta Crop Protection Ltd.), picoxystrobin (25% a.i. Oranis®, DuPont S.A.). The experiment was repeated twice.

Five days after incubation at 24 °C ± 2 °C and 12 h: 12 h photoperiod was determined the percentage of control of each fungicide by mycelial growth (mean diameter of two diametrically opposite measures). Cross-resistance was analyzed comparing the percentage of control between fungicides with the same mode of actions.

3.2.3 DNA extration

For DNA extration three mycelium discs of 7 mm were added into potato-dextrose (BD) liquid medium (4 g L^{-1} potato extract, 20 g L^{-1} dextrose) during 7 to 10 days. Thus, the mycelial mass was filtered through vacuum filtration. The dried mycelium were frozen in liquid nitrogen, macerated and stored in a 1.5 mL microcentrifuge tube at -80 ºC. The DNA was extracted following the modified CTAB extraction.

The macerated tissue was homogenized with 1000 µL of extraction buffer (1M Tris HLC (pH 8.0), 0.5M EDTA (pH 8.0), 5M NaCl, 0.1% β-mercaptoethanol, 200 µL CTAB to 5%) and incubated in a warm bath at 65 ° C for 60 minutes, with shaking and inversion of the microcentrifuge tubes every 15 minutes. The samples were centrifuged at 6000 rpm for 10 minutes. 700 µL of the supernatant was collected and transferred to a new 1.5 mL microcentrifuge tube. Then 700 µL of phenol: chloroform solution: iso-amyl alcohol (24: 24: 1) was added. After further centrifugation at 10,000 rpm for 5 minutes, 700 µL of the supernatant was transferred to a new microcentrifuge tube. For DNA precipitation, 500 µl of isopropanol was added and then the samples were homogenized in vortex and incubated at -20 ° C for 30 minutes. The DNA pellet was washed with 70% ethanol and resuspended in 60 µl of mili-q water + 6 µl of sodium acetate (3M) and 120 µl of 100% ethanol and was stored at 4 °C. Thus, the samples were centrifuged at a speed of 14,000 rpm for 5 minutes. The supernatant was discarded and pellet was washed again with 70% ethanol and dried. Samples were resuspended in 80 µL of mili-q + RNAse water at the final concentration of 80 µg / mL. DNA quality was assessed by electrophoresis on 1% agarose gel.

3.2.4 Primers design, PCR and fragments purification

The primers pairs β-tubulin (Genbank / EMBL access AY856374.1) and *cyt b* (Genbank / EMBL access EF176921.1), were designed using sequences of *C. beticola*. The primers pair for SdhA gene used here were described previously by Miyamoto; Ishii e Tomita (2010). The SdhB (Genbank / EMBL access AB548738.1) was designed using sequences of *Mycosphaerella graminicola*.

The amplicons length range from 716 to 1764 and were estimated by identification of the main mutations reported previously at literature. To achieve this, the primers forward and reverse were design to cover the maximum size of each the gene analysed.

The primers described were designed using the Primer 3 plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The specificity of the primers was in silico tested by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Primer pairs forward (F) and reverse (R) were described as follows: MBC [(primer MBC_F) 5’-CTGCATTCTGGCAGACCAT-3’; (primer MBC_R) 5’-TGAACTGGTCACCGACACG-3’]; QoI [(primer QoI_F) 5’-ATGGTTCTTACAAAGCACCTAGAA-3’; (primer QoI_R) 5’-

CACCCAATTGCATCAATAATAAGA-3’]; DMI [(primer DMI_F), 5’-ACGTCTTCACCTTCATTCTGC-3’ (primer DMI_R) 5’-GCCTCTCCCACTTCACAACA-3’]; S*dh* subunit B [(primer *Sdh*B_F) 5’-GAGCACACCAAGGAGCCTAT-3’; (primer *Sdh*B_R) 5’-GCACAACTGCTCCCTCTTG-3’].

PCR reactions for the target genes were prepared to a final volume of 50 µL containing 100 ng DNA from each sample, 1 µM of each primer, 10 µM dNTPs (Thermo Fischer Scientific), 5 X Phusion HF Buffer (Thermo Fischer Scientific) and 0.5 U Phusion DNA Polymerase (Thermo Fischer Scientific). The optimum annealing temperature was determined for each primer (Supplementary table S3.1). Annealing temperature ranged from 57ºC to 63ºC, followed by extension time which ranged from 30 to 60s and 26 to 35 cycles for amplification. All PCR reactions were performed on the MWG Biotech Inc Primus 96 thermal cycler. The PCR products were separated by electrophoresis using a 1% agarose gel and visualized under UV 360 nm light.

The PCR amplicons products of each genes were pooled by isolate for all samples for each gene were mixed and then purified using Nucleospin gel and Clean-up PCR (Macherey-Nagel) following the manufacturer's recommendations. The amplicon pools were quantified with the Qubit® (Thermo Fischer Scientific) for a final concentration of 3 ng / µL. After, the amplicon sequences were sent to Institute of Clinical Molecular Biology (IKMB) for processing.

3.2.5 NGS library preparation and genome association analysis

The Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) was used to prepare the amplicon library following the manufacture’s instructions. The final 56 libraries were distributed into four lanes on a flow cell for sequencing on an Illumina HiSeq 2500 system, utilizing a 150 bp paired-end reads length with an expected coverage of 30X.

The initial base calling, barcodes/adaptors trimming, quality filtering of the reads generated with the Illumina analysis pipeline (Fastq format) were performed using Trimmomatic 0.36 software (BOLGER; LOHSE; USABEL, 2014) and checked by FastQC software (ANDREWS et al., 2010). High-quality reads generated from all *C. kikuchii* genes sequenced from isolates were concatenated and *de novo* assembled with Trinity software (HAAS et al., 2013). The *C. kikuchii* genes of interest were identified by a blas2seq analyse (TATUSOVA; MADDEN, 1999), applying *cyt* b and β-tubulin, sequences from close related fungi species. The two *C. kikuchii* genes sequences were used, together with available sequence information from NCBI and MycoCosm - JGI database (https://genome.jgi.doe.gov/programs/fungi/index.jsf), to build the complete reference sequence for these genes (Supplementary table S3.1). *C. kikuchii cyt b* and β-tubulin gene structure were predicted using Softberry software (SOFTBERRY Inc., NY). The summary in silico method workflow is available in figure 3.1.

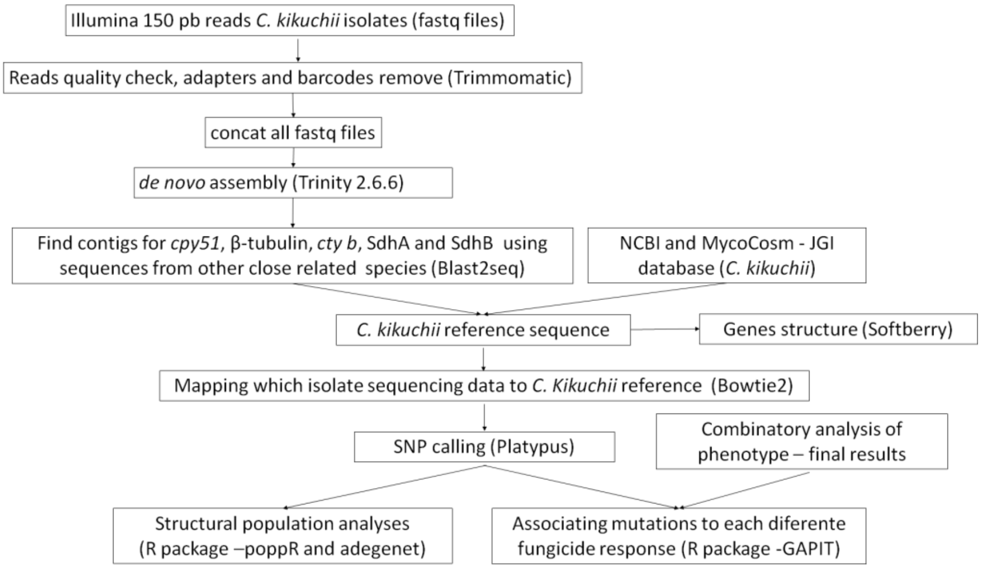

Figure 3.1. *In silico* analysis summary workflow for genetic characterization of *cyp51*, β-tubulin, *cty b*, SdhA and SdhB genes from *C. kikuchii* isolates.

High-quality reads were then aligned, by isolate, to the obtained *C. kikuchii* reference sequence for the five genes using the software bowtie2 (LANGMEAD; SALZBERG, 2012). The mapped reads were processed using Picard tools version 2.11 (Java, http://broadinstitute.github.io/picard/) to remove duplicate values, and a binary file of the extension bam representing the assembled genome of each sequenced isolate was generated. For SNP/InDel calling, we used Platypus version 0.8.1 (RIMMER et al., 2014) with minimum for two reads coverage. InDels were removed and >20% of missing data were removed from variant calling file (vcf) sing Vcftools version 0.1.12b (DANECEK et al. 2011) and remaining missing data were imputed with software Beagle 4 (AYRES et al., 2011).

3.2.6 QoI Sanger sequencing for validation

To validate NGS methodology 27 *C. kikuchii* isolates were randomly selected and sequenced using the primers described for *cyt b* gene. PCR reactions were prepared to a final volume of 50 µL containing 100 ng DNA from each sample, 1 µM of each primer, 10 µM dNTPs (Thermo Fischer Scientific), 5 X Phusion HF Buffer (Thermo Fischer Scientific) and 0.5 U Phusion DNA Polymerase (Thermo Fischer Scientific). PCR cycles were 35 cycles of initial denaturation at 98 °C for 10s, annealing at 57 °C for 60s, elongation at 72 °C for 30s and final extension for 8 minutes at 72 °C.

PCR products were adjusted to 25 ng / µL in the Nanodrop TM OneC spectrophotometer and were purified using Nucleospin gel and Clean-up PCR (Macherey-Nagel) following the manufacturer's recommendations. All PCR reactions were performed on the MWG Biotech Inc Primus 96 thermal cycler.

Sanger PCR reaction consisted of 0.6 µL of alkaline phosphatase (FAP) (Thermo Fisher Scientific) (1 U/ mL) enzyme, Exonuclease I (Thermo Fisher Scientific) (20 U / µL) 0.15 µL, 8 µL PCR products and 1.25 µL of dH2O (ChemSolute). The final volume was performed at 10 µL. The digestion reaction consisted of 10 min of purification at 37 ° C and 15 min inactivation of the enzymes at 75 ° C. The reaction consisted of 0.7 µL of Big Dye Terminator v3.1 (Applied Biosystems), 1.5 µL of Big Dye Terminator v3.1, 1.5 µL 5X sequencing buffer (Applied Biosystems), 1 µL (3,2 µM) of each primer pairs and 4.8 µL of dH_2O (ChemSolute). Sequencing amplifications program were carried out under following conditions: 1 min of initial denaturation at 96 °C; 25 cycles of denaturation of 10 s at 96 °C, annealing of 5 s at 50 °C and extension of 4 min at 60 °C; followed by a final extension at 10 °C. The sequencing was performed using 3730xl DNA Analyzer (Applied Biosystems, Foster, CA).

The nucleotide sequences were aligned with the reference sequence (NCBI/ access EF176921.1) of *C. beticola* mitochondrial *cyt b* gene and translated into amino acid sequences using Bioedit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

3.2.7 Genetic analysis

The genome association and prediction integrated tool (GAPIT) package in R (LIPKA, et al., 2012) was used to analyse phenotype and genotype association. GAPIT analyses were performed using a mixed linear model (MLM) statistical method.

Population genetic analyses were performed using ‘poppr’ (KAMVAR et al. 2014) and “adegenet” R packages (JOMBART, 2008). Minimum spanning network was applied to visualize population structure by haplotypes according to their origin location using the bitwise’s genetic distance. Minimum spanning network relationship was analysed for all five genes and also separated per gene. An UPGMA (SCHLIEP, 2010) distance tree was build to identify similarity into *C. kikuchii* groups, genetically related using poppr package (KAMVAR et al. 2014).

3.3 RESULTS

3.3.1 Phenotype and *in vitro* bioassay of cross-resistance in MBC and QoI

Isolates with mycelial growth inhibition (M.G.I.) less than 50% were considered resistant. These analysis revealed that azoxystrobin, picoxystrobin, pyraclostrobin (QoI) from 2013 were classified as resistant and had low ability (<50%) to control for the most of the isolates from 2013 onwards (Figure 3.2). A total of 69% and 71% of the isolates tested for QoI and MBC fungicides were phenotype as resistant.

Cross-resistance between carbendazim and thiophanate-methyl (MBC) and among azoxystrobin, picoxystrobin and pyraclostrobin (QoI) fungicides was confirmed by high positive linear correlation between M.G.I. of each compaired fungicides (R^2 0.8 to 0.96*)* (Figure 3.3).

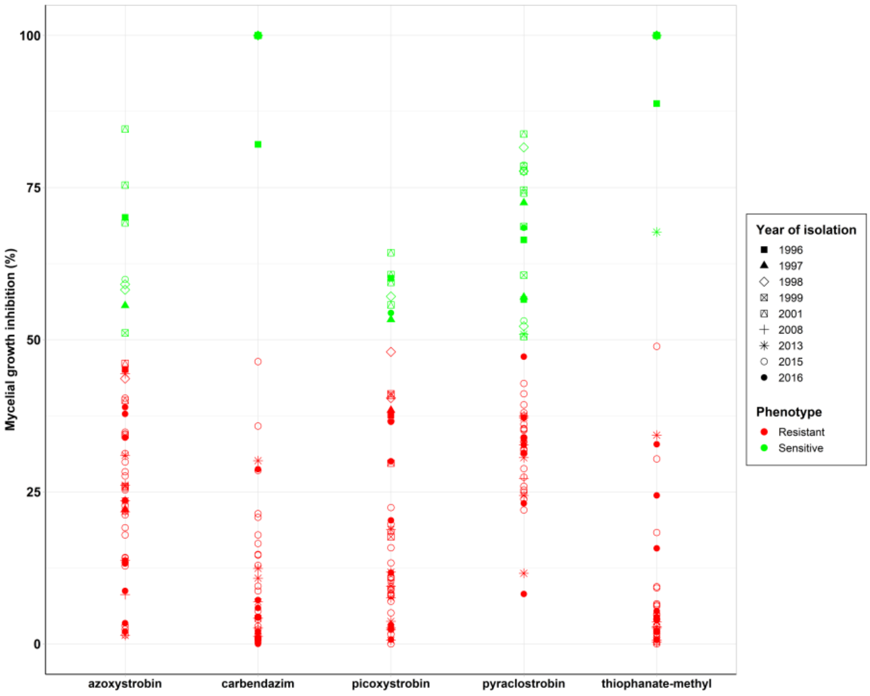

Figure 3.2. *Cercospora kikuchii* mycelial growth inhibition using discriminatory dose at 10 µg/mL to carbendazim and thiophanate-methyl, azoxystrobin, picoxystrobin, pyraclostrobin fungicides

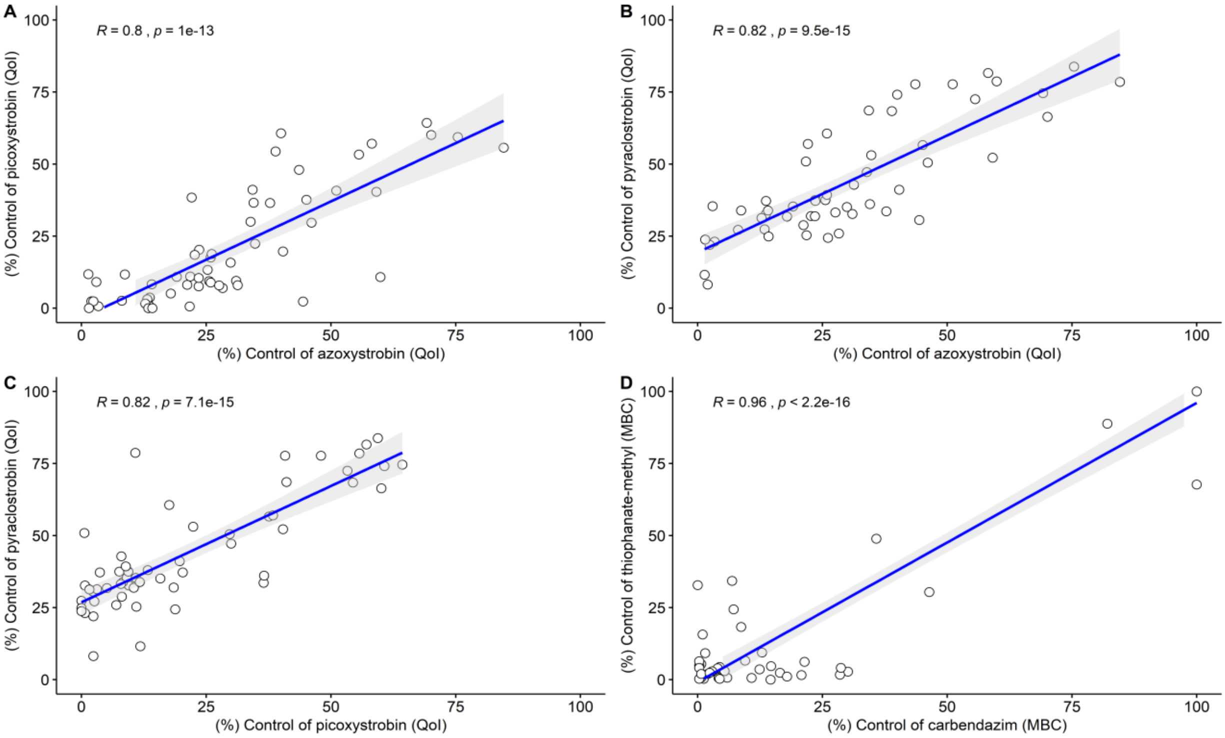

Figure 3.3 Cross-resistance among carbendazim and thiophanate-methyl (MBC) and azoxystrobin, picoxystrobin and pyraclostrobin (QoI) fungicides of *Cercospora kikuchii* isolates from soybean fields in Brazil**.**

3.3.2 Molecular characterization of *cyp51,* β-tubulin, *cyt b* and S*dh* (subunits a, b, c, d) fungicide target genes

Molecular characterization of *C. kikuchii* resulted in 218 synonymous and nonsynonymous mutations of which, were found 65 mutations for *cyp51* gene, 55 mutations for β-tubulin gene, 7 for *cyt b* gene*,* 4 for SdhA, 87 for SdhB (Supplementary table S.3.2). The genes structure of *C. cassiicola* in *cyp51*, β-tubulin and *cyt b* fungicide resistance target genes resulted in a 2187 bp, 3856 bp and 1161 bp fragment length, respectively (Figure 3.4).

A total 13 nonsynonymous mutations were found in *cyp51* gene: Q161H, N178D, P180S, P200L, K217R, E274D, S279N, E281K, T290S, I299V, S363N, K442Q and L503V. Among these, heterozygous variant in *C. kikuchii* isolates were observed at codon N178D, P180S, P200L, E274D, S279N, E281K, T290S, I299V, S363N, K442Q (Table 3.1, Supplementary figure S.3.6). In addition, 52 synonymous mutations were identified (Supplementary table S.3.2).

In the β-tubulin target was not found nonsynonymous mutations (Table 2, Supplementary figure S.3.6). In total, 55 synonymous mutations were found (Supplementary table S.3.2).

Homozygous variation was found at position 143 and was detected for 42 isolates. In this mutation, were replaced in the nucleotide sequence of GGT (sensitive) to GCT (resistant) at amino acid position 143 (Table 3.1). No isolates contained the F129L or G137R mutation (Table 3.1, Supplementary figure S.3.6). In addition to these mutations, six synonymous mutations were identified (Table 3.1, Supplementary figure S.3.6). In our study, we also analyzed the mutations in the SdhA gene. It was found a total of 4 synonymous mutations (Table 3.1, Supplementary figure S.3.6).

A total of 14 substitutions for SdhB were found: H17L, G27A, T167M, P192L, E200D, N290K, I320N, I434V, A436V, D447N, H448R, E454Q, N513S and Q569K. In addition to these mutations, 73 synonymous mutations were identified (Supplementary table S.3.2).

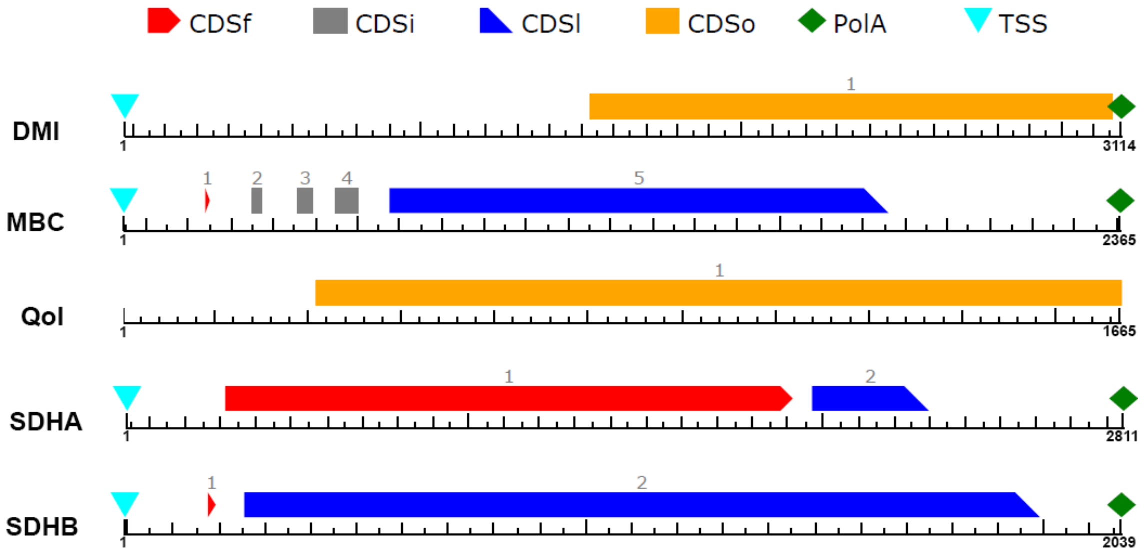

Figure 3.4 Prediction of *cyp51* (DMI), β-tubulin (MBC), *cyt b* (QoI), SdhA and SdhB genes using genomic DNA of *Cercospora kikuchii* isolates. CDSf - First (Starting with Start codon); CDSi - internal (internal exon), CDSl - last coding segment, ending with stop codon); CDSo: coding sequence, solo - predicted gene with a single exon; PolA: polyadenylation signal sequence (AATAAA) and TSS - Position of transcription start (TATA-box position and score).

3.3.3 QoI sequencing validation using Sanger sequencing method

To verify the homozygous or heterozygous variation in the *cyt b* gene 27 isolates of *C. kikuchii* were randomly selected for validation using Sanger sequencing method. Among those, six isolates were considered wild (Supplementary figure S.3.7). The homozygous variations observed in the NGS were also observed at nucleotide amino acid chromatogram results (Supplementary figure S3.6, Supplementary figure S3.8). Chromatogram graphs (wave DNA) confirmed that homozygous variant at codon position 143 of *C. kikuchii* DNA sequencing (Supplementary figure S3.8).

Table 3.1. Codon position origin, reference and altered alleles in fungicide gene targets (DMI, QoI, and SdhB) of *Cercospora kikuchii* isolates.

Ref. Pos.		483	532	538	600	650	822	836	843	868	895	1088	1324	1507	428	50	81	500	575	600	869	960	1300	1307	1339	1343	1360	1538	1705
Ref. Allele[1]		G	A	C	T	A	G	G	A	A	A	G	A	C	G	A	C	C	C	G	A	T	A	C	G	A	G	A	C
Alt. Allele		T	G	T	C	G	C	A	G	T	G	A	C	G	C	T	G	T	T	T	G	C	G	T	A	G	C	G	A
		DMI													QoI	SdhB													
Code /origin[2]	Year	Q161H	N178D	P180S	P200L	K217R	E274D	S279N	E281K	T290S	I299V	S363N	K442Q	L503V	G143A	H17L	G27A	T167M	P192L	E200D	N290K	I320N	I434V	A436V	D447N	H448R	E454Q	N513S	Q569K
1158 (PR)	2013	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	*
1160 (PR)	2013	*	G	T	C	*	C	A	G	*	G	*	C	G	C	T	*	*	*	*	*	*	*	*	*	*	*	G	*
1166 (PR)	2013	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
1185 (PR)	2013	*	G	T	C	*	C	A	G	A|T	G	*	C	G	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
1192 (TO)	2013	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	*
1429 (RR)	2013	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	C|A
1625 (MT)	2016	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
1642 (DF)	2016	*	G	T	C	*	C	A	G	A|T	G	*	C	G	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
1643 (DF)	2016	*	G	T	C	*	C	A	G	A|T	G	*	C	G	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
1656 (PA)	2016	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	C|A
1742-1 (MT)	2016	T	G	T	C	*	*	*	A|G	*	A|G	A	C	G	C	*	*	*	*	*	*	*	*	*	*	*	*	*	C|A
432_1 (MT)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
432_2 (MT)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	*
432_3 (MT)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	*
432_4 (MT)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
435_2 (MT)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	T	*	*	*	*	*	*	*	*	*	*	A
435_5 (MT)	2015	*	G	T	C	*	C	A	G	A|T	G	*	C	G	C	T	*	*	*	*	*	*	*	*	*	*	*	*	C|A
435_6 (MT)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
469_1 (GO)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
469_2 (GO)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	C|A
469_2A (GO)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A

Ref. Pos.		483	532	538	600	650	822	836	843	868	895	1088	1324	1507	428	50	81	500	575	600	869	960	1300	1307	1339	1343	1360	1538	1705
Ref. Allele*		G	A	C	T	A	G	G	A	A	A	G	A	C	G	A	C	C	C	G	A	T	A	C	G	A	G	A	C
Alt. Allele		T	G	T	C	G	C	A	G	T	G	A	C	G	C	T	G	T	T	T	G	C	G	T	A	G	C	G	A
		DMI													QoI	SdhB													
Code /origin[2]	Year	Q161H	N178D	P180S	P200L	K217R	E274D	S279N	E281K	T290S	I299V	S363N	K442Q	L503V	G143A	H17L	G27A	T167M	P192L	E200D	N290K	I320N	I434V	A436V	D447N	H448R	E454Q	N513S	Q569K
474_1 (GO)	2015	*	G	T	C	G	*	A	G	A|T	G	A	C	G	C	A|T	*	*	*	*	*	*	*	*	*	*	*	*	C|A
474_2 (GO)	2015	*	G	*	C	G	*	A	G	A|T	G	A	C	G	C	T	*	*	*	*	*	*	*	*	*	*	*	*	*
499_1 (GO)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	*
515188_1 (Unk)	Unk	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	C|A
515188_2 (Unk)	Unk	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
573_4 (MG)	2015	*	G	*	C	G	*	A	G	A|T	G	A	C	G	C	*	C|G	*	C|T	G|T	A|G	T|C	A|G	C|T	G|A	A|G	G|C	A|G	C|A
576_1 (MG)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
576_2 (MG)	2015	*	G	*	C	G	*	A	G	A|T	G	A	C	G	C	A|T	*	*	*	*	*	*	*	*	*	*	*	*	C|A
576_3 (MG)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	C|A
576_4 (MG)	2015	*	G	*	C	G	*	A	G	A|T	G	A	C	G	C	*	C|G	*	T	T	G	C	G	T	A	C		*	C|A
576_5 (MG)	2015	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
600 (PR)	1996	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C|A
601 (Ukn)	1996	*	*	*	*	*	*	*	*	*	*	A	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*
602 (PR)	1997	*	*	*	*	*	*	*	*	*	*	A	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*
603 (MA)	1997	*	G	T	C	*	C	A	G	A|T	G	*	C	G	*	T	*	*	*	*	*	*	*	*	*	*	*	G	*
604 (MT)	1998	*	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A
605 (MT)	1998	*	*	T	C	*	C	A	G	A|T	G	*	C	G	*	T	*	*	*	*	*	*	*	*	*	*	*	G	*
606 (PR)	1998	*	*	*	*	*	*	*	*	*	*	A	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*
607 (MA)	1999	*	G	T	C	*	C	A	G	A|T	G	*	C	G	*	T	*	*	*	*	*	*	*	*	*	*	*	G	*
608 (PR)	1999	*	G	T	C	*	C	A	G	A|T	G	*	C	G	*	T	*	*	*	*	*	*	*	*	*	*	*	G	*
609 (MA)	1999	*	G	C|T	T|C	*	C	A	G	*	G	G|A	C	G	*	T	*	*	*	*	*	*	*	*	*	*	*	G	*
611 (GO)	2001	*	*	*	*	*	*	*	*	*	*	A	*	*	C	A|T	*	*	*	*	*	*	*	*	*	*	*	*	C|A
612 (Unk)	2001	*	G	C	C*	*	C	A	G	A|T	G	*	C	G	*	T	*	*	*	*	*	*	*	*	*	*	*	G	C|A
613 (PR)	2001	*	*	*	*	*	*	*	*	*	*	A	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*
615 (MT)	2001	*	*	*	*	*	*	*	*	*	*	A	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*
616 (PR)	2001	*	G	C	C	*	C	*	G	A|T	G	*	C	G	*	T	*	*	*	*	*	*	*	*	*	*	*	G	*
675 (PR)	2008	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A

Ref. Pos.		483	532	538	600	650	822	836	843	868	895	1088	1324	1507	428	50	81	500	575	600	869	960	1300	1307	1339	1343	1360	1538	1705
Ref. Allele*		G	A	C	T	A	G	G	A	A	A	G	A	C	G	A	C	C	C	G	A	T	A	C	G	A	G	A	C
Alt. Allele		T	G	T	C	G	C	A	G	T	G	A	C	G	C	T	G	T	T	T	G	C	G	T	A	G	C	G	A
		DMI													QoI	SdhB													
Code /origin[2]	Year	Q161H	N178D	P180S	P200L	K217R	E274D	S279N	E281K	T290S	I299V	S363N	K442Q	L503V	G143A	H17L	G27A	T167M	P192L	E200D	N290K	I320N	I434V	A436V	D447N	H448R	E454Q	N513S	Q569K
E4_1 (PR)	2016	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	*
E4_4 (PR)	2016	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	*
E4_5 (PR)	2016	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	C|A
Embrapa_1 (PR)	2016	*	*	*	C	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	*
Embrapa_2 (PR)	2016	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	*	*	*	*	*	*	*
Faxinal_5 (PR)	2016	*	*	*	*	*	*	*	*	*	*	A	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	*	A
G16407_3 (Unk)	2016	*	*	*	*	*	*	*	*	*	*	A	*	*	C	T	*	*	*	*	*	*	G|A	*	*	*	*	*	*
G16407_4 (Unk)	2016	*	G|A	C|T	T|C	*	G|C	G|A	A|G	*	A|G	G|A	A|C	C|G	C	T	*	*	*	*	*	*	A	*	*	*	*	*	*

[1]The symbol (*) represents the same allele of the reference sequence; **[2]**(PR) Paraná; (TO) Tocantins; (RR) Roraima; (MT) Mato Grosso; (DF) Distrito Federal; (PA) Pará; (GO) Goiás; (Unk) Unknown; (MG) Minas Gerais; (MA) Maranhão;

3.3.4 Phenotype association of *cyt b* fungicide target gene and mutations

To identify *cyt b* phenotype association with genotype, we performed genome association using M.G.I. phenotype data.

We observed one association above the Bonferroni threshold adjusted for α = 0.05 (Figure 3.5). The synonymous substitution in the gene , *cyt b* G143A (p-value 0.00022) showed high associations between phenotype and genotype (Figure 3.5).

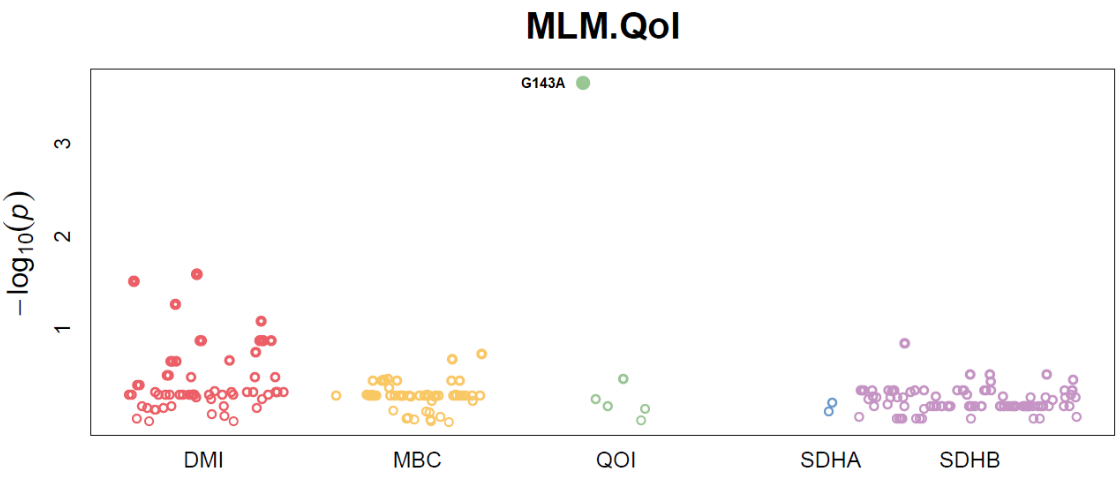

Figure 3.5. Manhattan plot of the genome association (GA) of *C. kikuchii* isolates for *cyt b* gene fungicide resistance target gene. GA mapping was performed using a mixed linear model (MLM). Significant associations were identified for the mutation G143A.

3.3.5 Genetic relatedness of *C. kikuchii* from soybean based on the pholymorfims on the fungicide target genes *cyp51*, β-tubulin, *cyt b*, SdhA and SdhB

Data from the bitwise gene length matrices per gene were analyzed using a UPGMA distance tree. The number of clusters (K) ranged from 4 for gene SdhA and 6 for genes *cyp51*, β-tubulin, *cyt b*, SdhB. The number of clusters, which is the best K value, was determined using Bayesian Information Criterion (BIC).

Cluster analysis for the *cyp51* gene separed the isolates 609 and G16407_4 into cluster 2 (orange) and these isolates showed heterozygose variation at mutations P180S and P200L (Supplementary figure S.3.6). The isolate 1742_1 was aggruped into cluster 6 (yellow) and was the only *C. kikuchii* isolate which showed mutation at position Q161H and heterozygose variation at mutations E281K and I299V. Cluster 4 (pink) were aggruped the isolates 1642, 612, 1185, 1643, 435_5, 616, 605, 607, 608, 1160, 603 which showed the mutations N178D, P180S, P200L, E274D, S279N, E281K and I299V, K442Q and L503V (Supplementary figure S.3.6, Figure 3.6).

For QoI fungicides sensitive isolate were separated into cluster 3 (dark blue) and cluster 5 (light green). The others cluster shared the mutation G143A which resulting QoI fungicide resistance (Figure 3.7).

The genetic diversity for β-tubulin (MBC) and SdhA genes were divided into four and six clusters, respectively. However, all the isolates showed for these genes, revealed only synonimus mutations for all isolates analysed. In this case, the clusters is indicating that there have been some changes at β-tubulin and SdhA gene loci according to geographic origins (Figure 3.7 and Figure 3.8).

The genetic diversity for Sdh*B* into sensitive and resistant isolates were distributed between six clusters. Cluster 1 (dark green) separates the isolates 573_4 and 576_4. These isolates showed the same mutations at positions G27A, P192L, E200D, N290K, I320N, I434V, A436V, D447N, H448R and Q569K. The isolate 576_4 revealed homozygouse variantion, for all mutations except at Q569K, while in the opposite the isolate 573_4 showed only heterozygose variation (Supplementary figure S.3.6). The isolates 612, 609, 1160, 603, 613, 605, 607 and 608 were seapared into cluster (light green) and these isolates differ from the others because, showed mutations at N513S mutation (Figure 3.9).

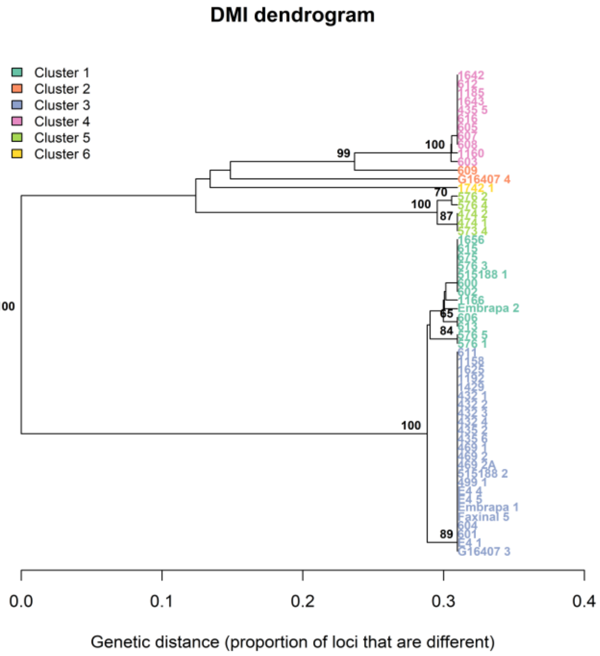

Figure 3.5 UPGMA clustering dendrogram of *C. kikuchii* isolates for *cyp51* fungicide resistance target gene. Distance is based on genetic similarity.

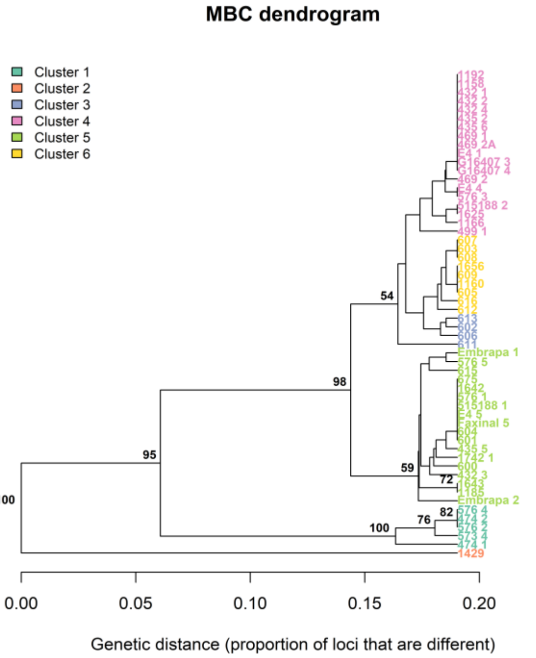

Figure 3.6. UPGMA clustering dendrogram of *C. kikuchii* isolates for β-tubulin fungicide resistance target gene. Distance is based on genetic similarity.

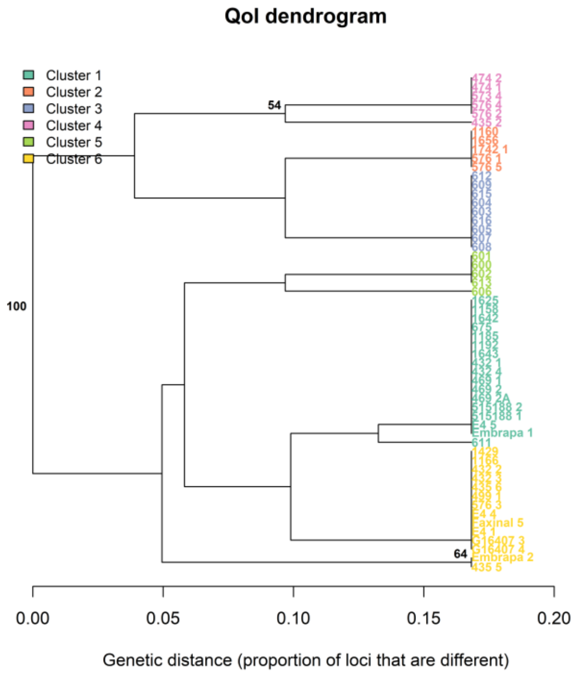

Figure 3.7. UPGMA clustering dendrogram of *C. kikuchii* isolates for *cyt b* fungicide resistance target gene. Distance is based on genetic similarity.

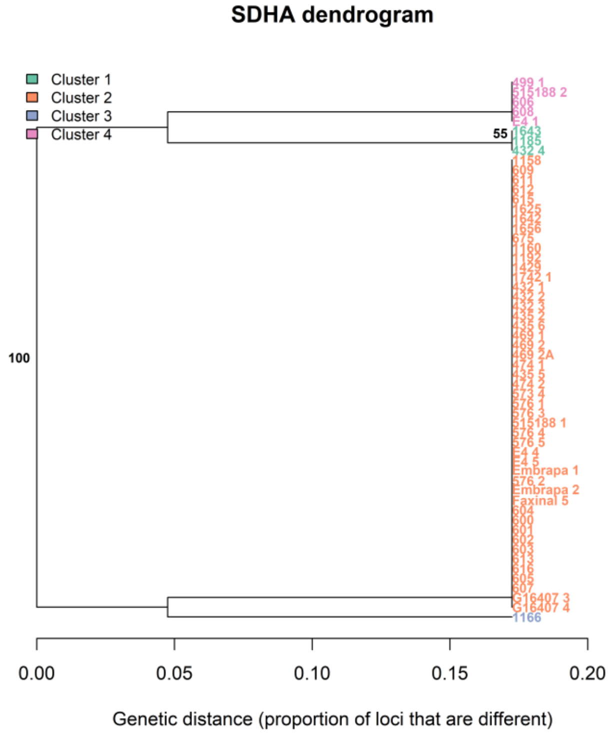

Figure 3.8. UPGMA clustering dendrogram of *C. kikuchii* isolates for SdhA fungicide resistance target gene. Distance is based on genetic similarity.

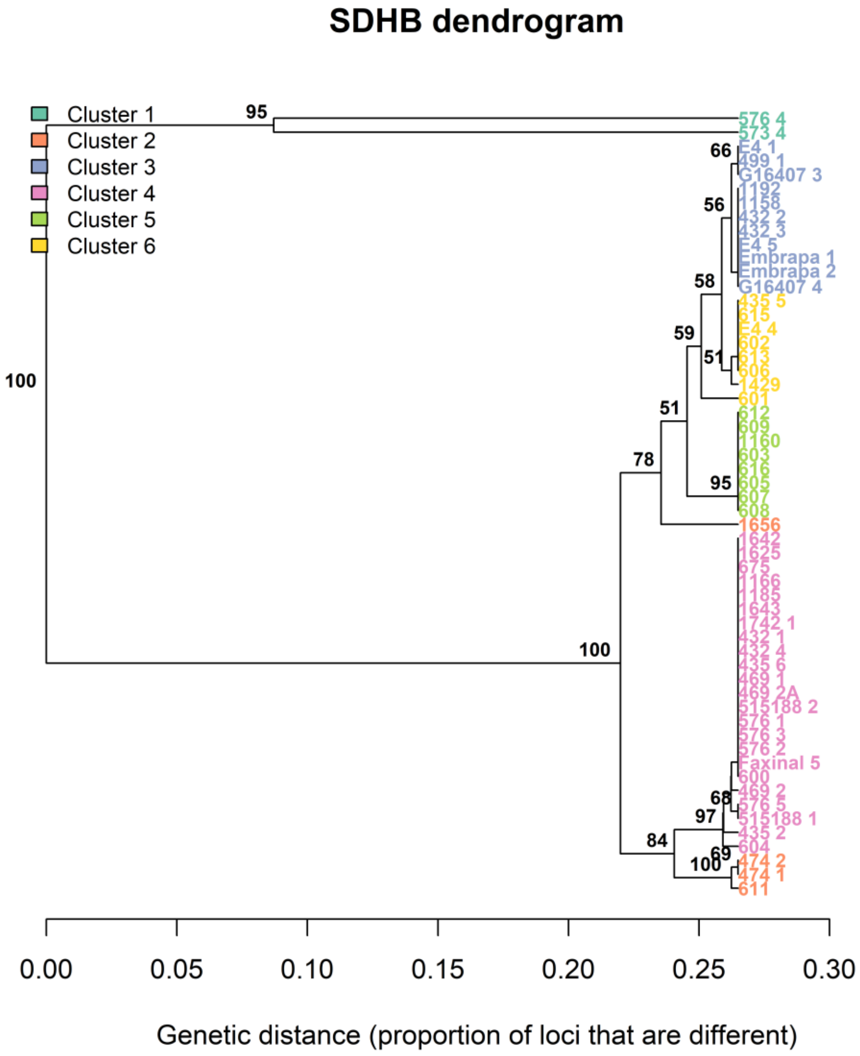

Figure 3.9. UPGMA clustering dendrogram of *C. kikuchii* isolates for *S*dhB fungicide resistance target gene. Distance is based on genetic similarity**.**

5.3.6 Relationship among *Cercospora kikuchii* genotypes

The minimum spanning network of *C. kikuchii* distinguished the most frequent genectic group and were placed close to one another (Figure 3.10). The global network showed from DF, GO, MA, MG, MT, PA, PR, RR and TO were distributed all across the network and were clustered around the most frequent genotype. The isolates from MA and PR 607 + 608, DF and PR 1185 + 1643, PR and TO, 1158 + 1192 and GO and MT, 432_1, 432_4, 469_1, 469_2A share the same haplotypes. Bitwise genetic distance in the minimum spanning network varied from 0.002 to 0.181 (Figure 3.10).

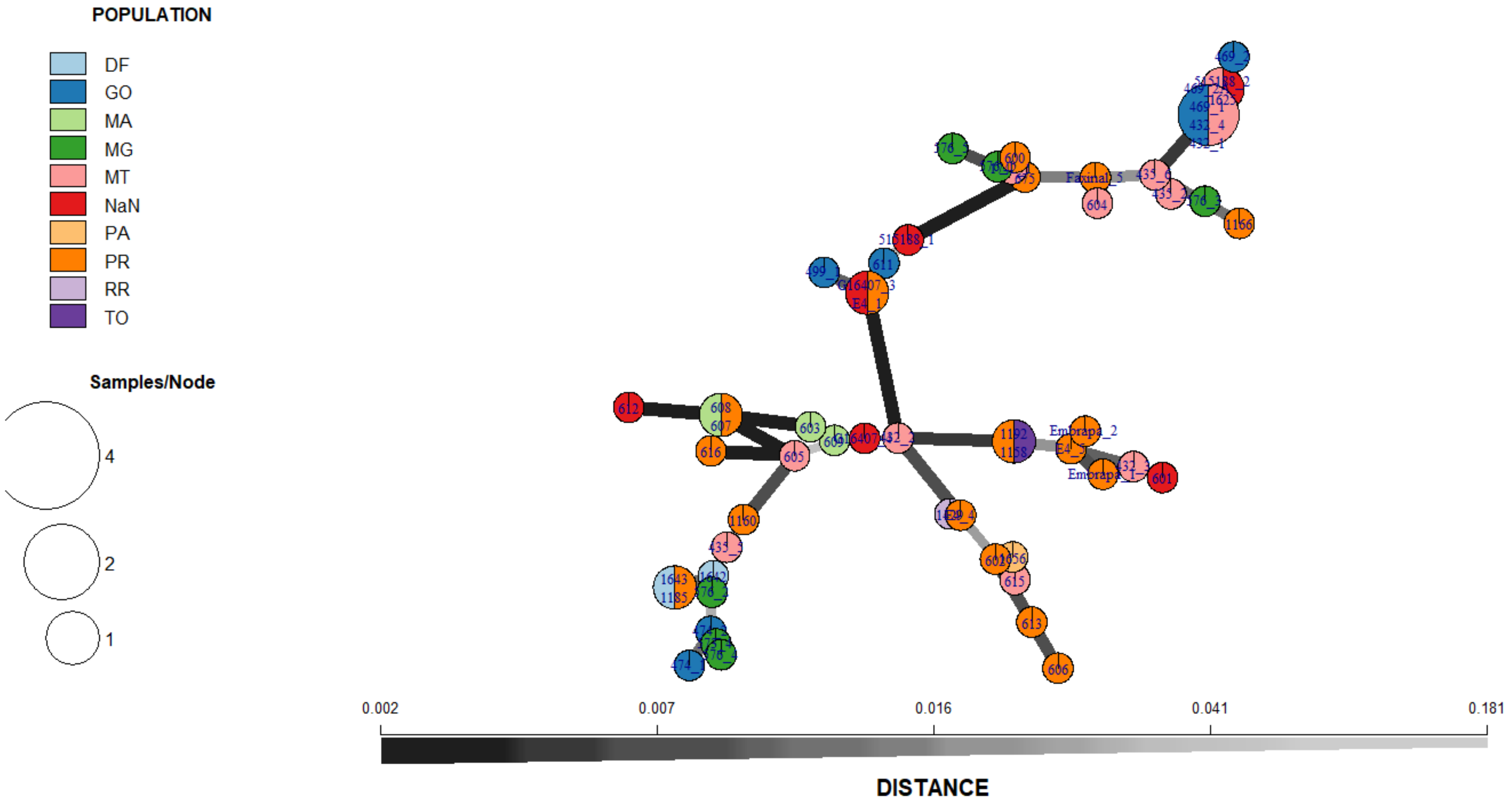

Figure 3.10. Median spanning network of *C. kikuchii* from soybean for fungicide resistance target genes *cyp51*, β-tubulin, *cyt b*, SdhA and SdhB. Each circle represents a haplotype. Node sizes are proportional to the number of isolates origin. Coloured slices represent different geographic origin.

5.3.7 Relationship among *C. kikuchii* genotypes separete per gene to fungicide resistance target genes

The genetic diversity for *cyp51* sensitive and resistant isolates was distributed into 15 haplotypes groups. The isolates 1742_1 is locate in one haplotype and showed mutations showed the Q161H, N178D, P180S, P200L, E281K, I299V, S363N, K442Q and L503V. The isolates aggruped in the haplotype (n=9) showed mutations at positions N178D, P180S, P200L, E274D, S279N, E281K, T290S, I299V, K442Q and L503V. The most frequent haplotypes (n=24 and n=9) agrruped isolates with different mutations (Figure 3.11).

The genetic diversity for β-tubulin (MBC) and SdhA genes were divided into four and six haplotypes, respectively. However, all the isolates showed for these genes, revealed only synonyms mutations for all isolates analysed. In this case, the clusters in indicating that there have been some changes at β-tubulin and SdhA gene loci according to geographic origins. The most frequent haplotypes for β-tubulin (n = 12 and n=8) and for SdhA are (n=47 and n=3) and both did not show any nonsynymous mutation (Figure 3.12, Figure 3.13).

In total 10 haplotypes were found for *cyt b* gene. Three haplotypes represents sensitive samples, collected before the intensive use of fungicide (n=9 and n=4, N=1 (606). All the others haplotype showed mutation at G143A (Figure 3.14).

The minimum spanning network for SdhB separates in 16 haplotypes. The isolates 612, 609, 1160, 603, 613, 605, 607 and 608 were separed in one haplotype and all these isolates revealed N513S mutation. The most frequent haplotype (n = 18), which showed different mutations (Figure 3.15).

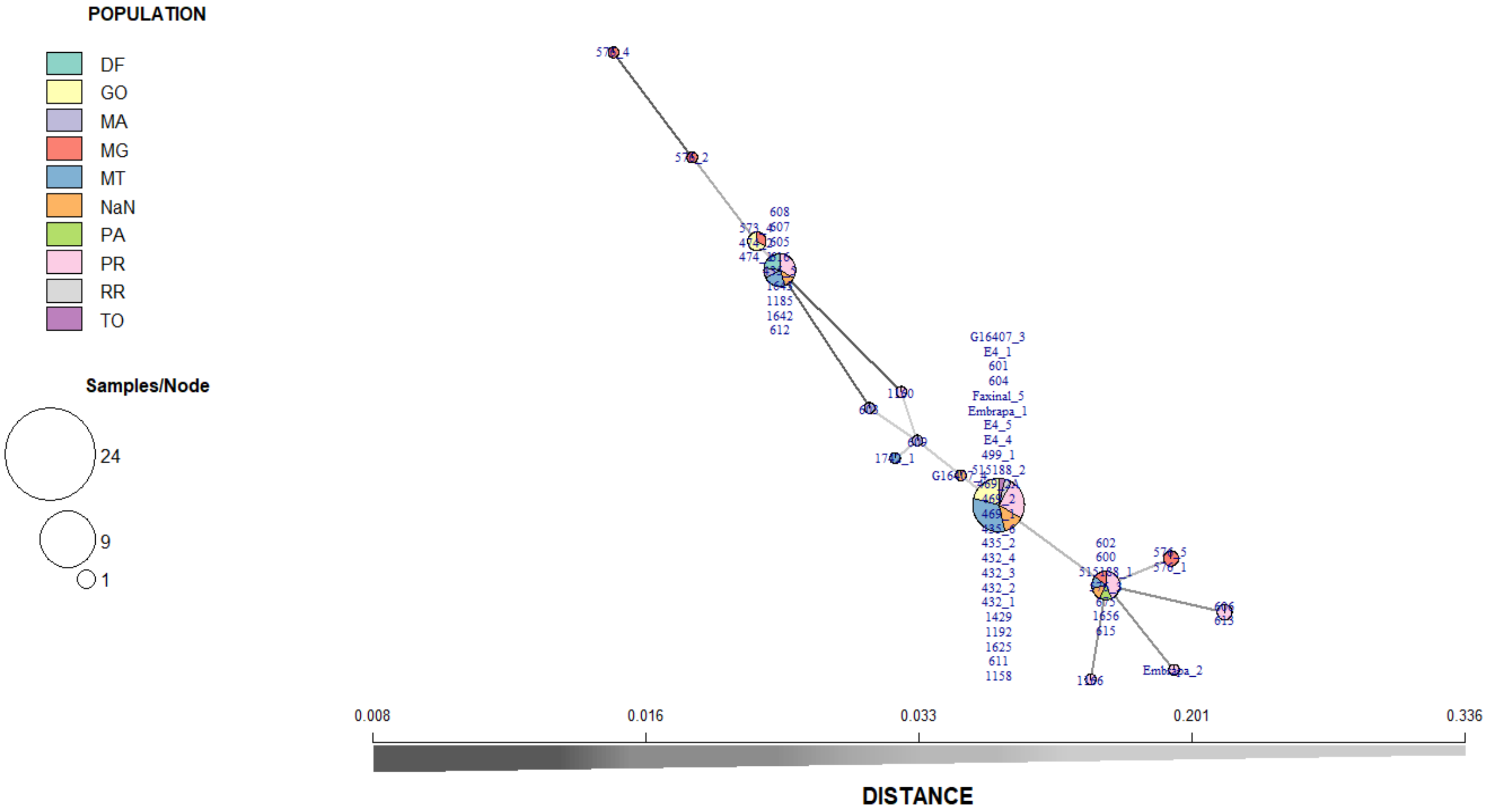

Figure 3.11 Median joining network of *C. kikuchii* from soybean for fungicide resistance target genes *cyp51.* Each circle represents a haplotype. Cicle size is proportional to the number of isolates origin into the haplotypes. Coloured slices represent different geographic origin.

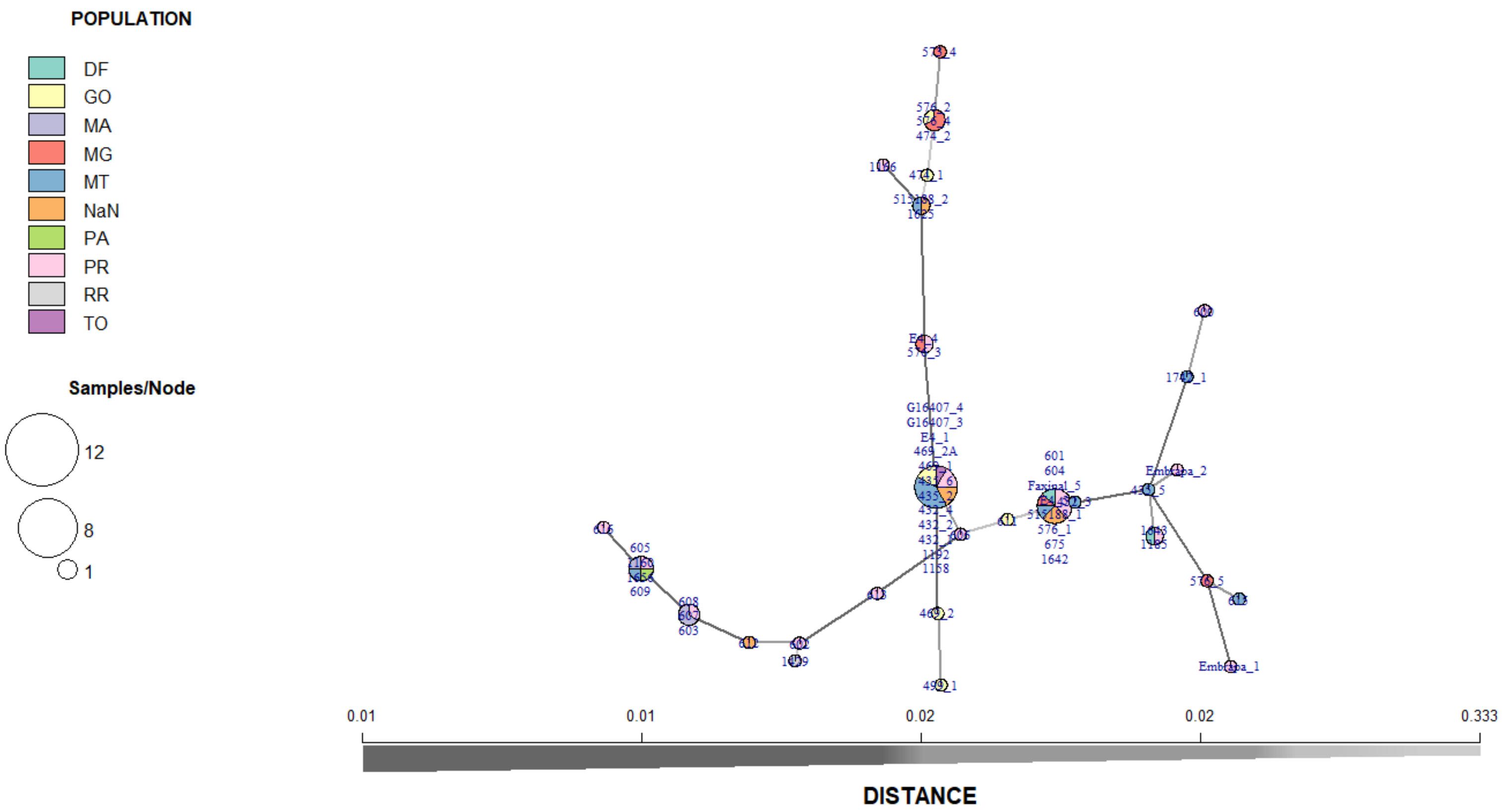

Figure 3.12. Median joining network of *C. kikuchii* from soybean for fungicide resistance target genes β-tubulin. Each circle represents a haplotype. Cicle size is proportional to the number of isolates origin into the haplotypes. Coloured slices represent different geographic origin.

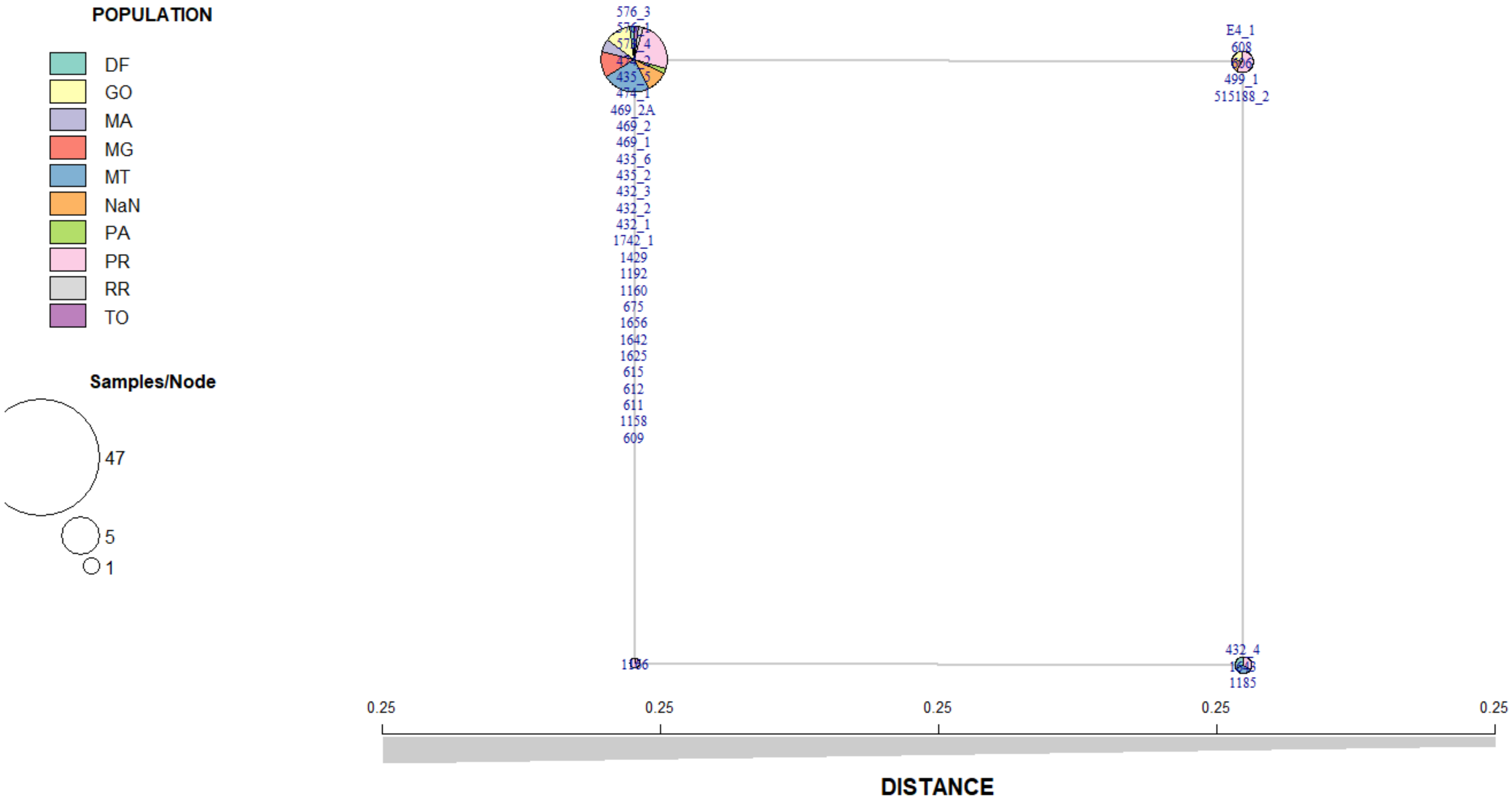

Figure 3.13. Median joining network of *C. kikuchii* from soybean for fungicide resistance target genes SdhA. Each circle represents a haplotype. Cicle size is proportional to the number of isolates origin into the haplotypes. Coloured slices represent different geographic origin.

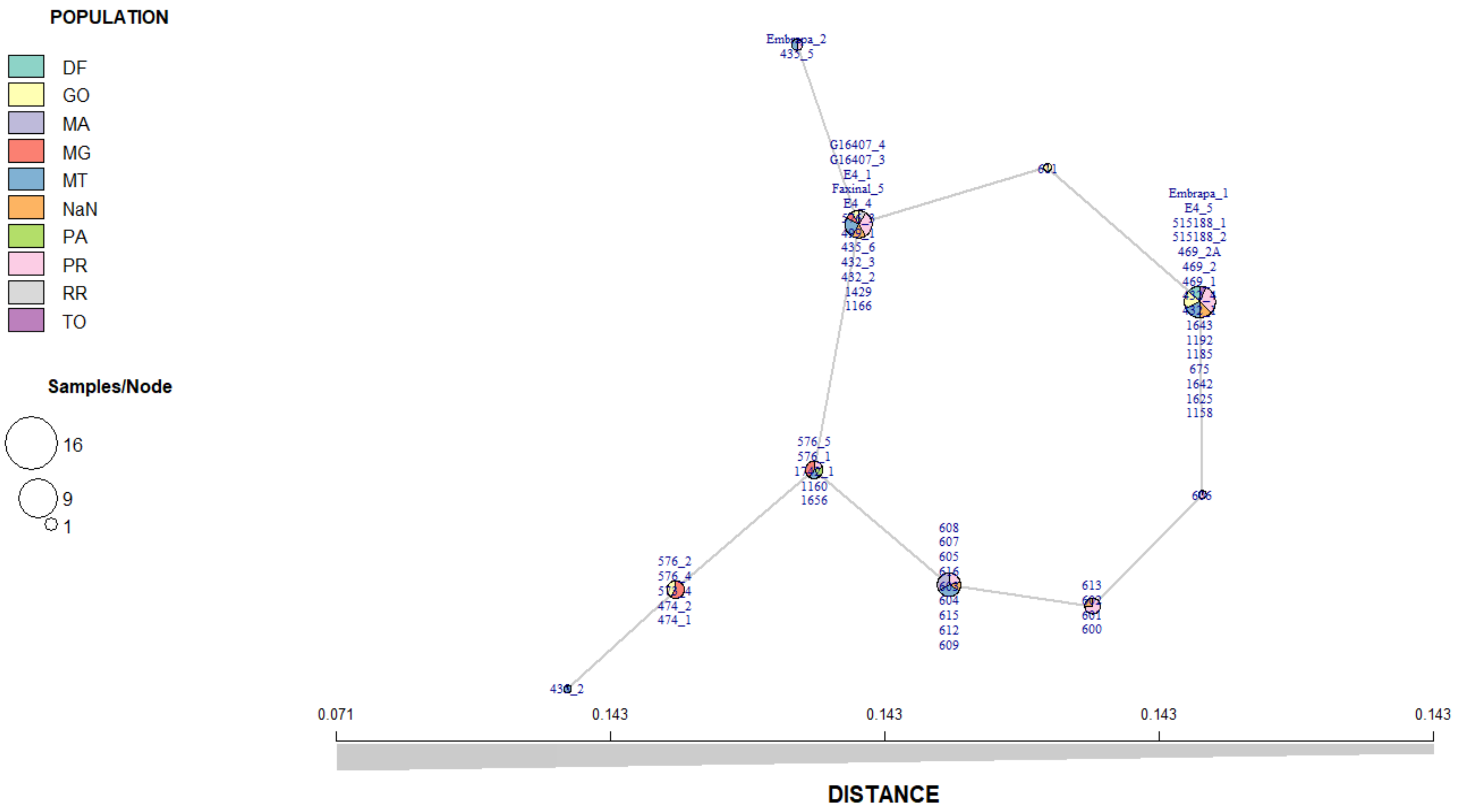

Figure 3.14. Median joining network of *C. kikuchii* from soybean for fungicide resistance target genes *cyt b*. Each circle represents a haplotype. Cicle size is proportional to the number of isolates origin into the haplotypes. Coloured slices represent different geographic origin.

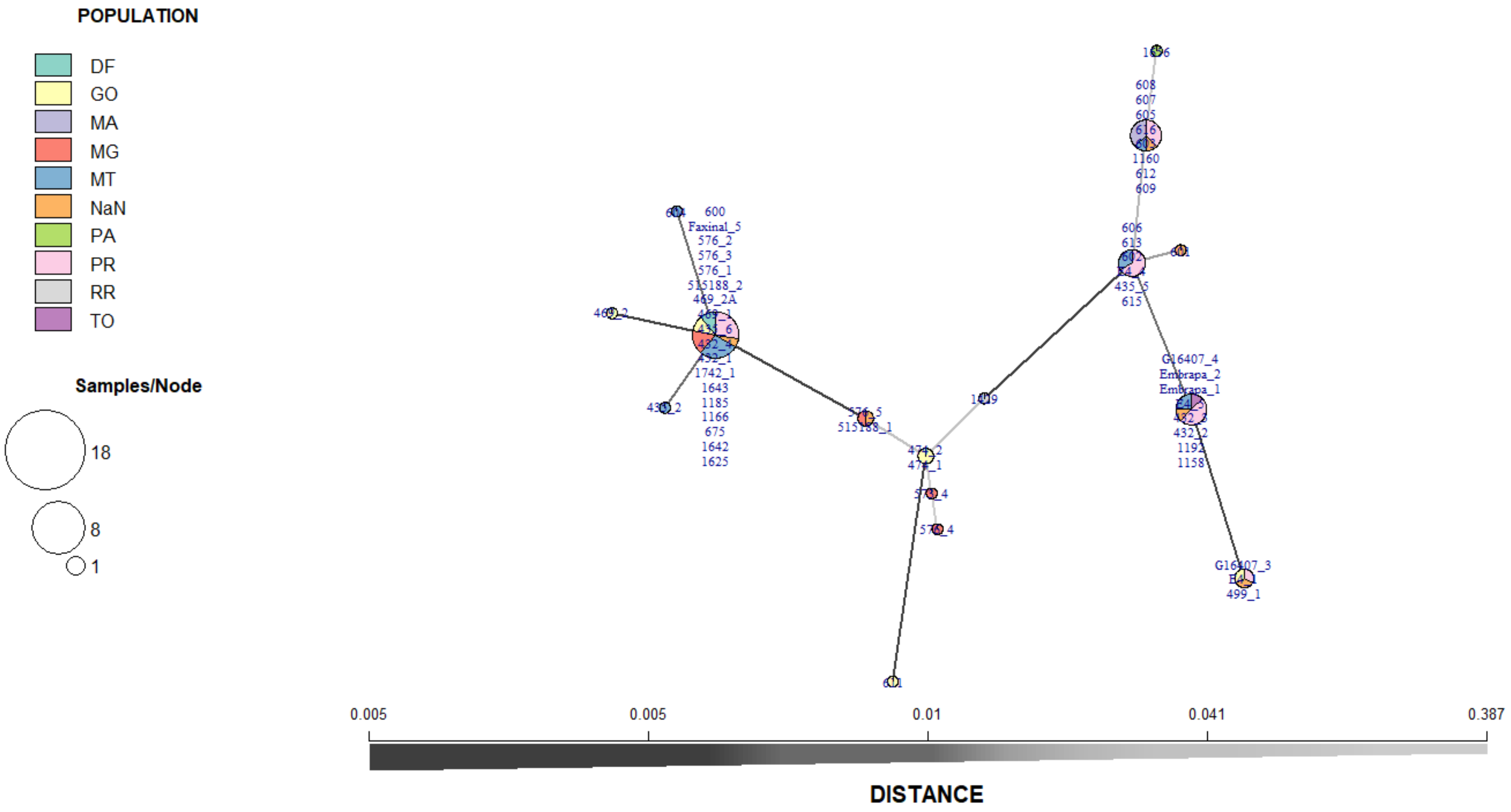

Figure 3.15. Median joining network of *C. kikuchii* from soybean for fungicide resistance target genes SdhB. Each circle represents a haplotype. Cicle size is proportional to the number of isolates origin into the haplotypes. Coloured slices represent different geographic origin.

3.4 DISCUSSION

In this research, we analyzed sensitive and resistant *C. kikuchii* isolates and their cross-resistance in soybean to QoI and MBC fungicides. This is the first next generation sequencing study in soybean to identify fungicide resistance in *C. kikuchii* to fungicide target genes for *cyp51,* β-tubulin, *cyt b* and SdhB. Using genome associated prediction integrated tool we identfiied that G143A mutation is the most significant mutation in *cyt b* gene in *C. kikuchii*. Clustering analysis showed that resistant and sensitive populations to fungicide target genes *cyp51,* β-tubulin, *cyt b* and SdhB were genetically distinct. Using next generation sequencing we could confirm heterozygose variant is occoring in *C. kikuchii* in *cyp51* and SdhB genes. In addition we also characterized sequencing variability of *C. kikuchii* to fungicide target genes. We identfied that G143A mutation is the most significant mutation in *cytb* gene in *Cercospora kikuchii*.

Sensitive and resistant phenotypes of *C. kikuchii* isolates could be distinguished using M.G.I. using a discriminatory dose at 10 μL/mL^{-1}. Previous research from *in vitro* bioassay suggest that alternative oxidation (AOX), can occurs in *Cercospora* spp. and consequently, avoinding the effects of QoI fungicides *in vitro* (ZIOGAS; BALDWIN; YOUNG, 1997). AOX is inhibited by salicylhydroxamic acid (SHAM) or propyl gallate (PG). Studies evaluating radial growth assay in QoI fungicides of *C.kikuchii* from soybean have shown that in a concentration of 20 µg/mL SHAM reduce colony growth by approximately 80% (PRICE et al., 2015). Alternatively, no significant diffferences were observed when comparing individual assay with and wihout the addition of PG inhibitor (PRICE et al., 2015). Therefore, any AOX inhibitor was used in this assay.

Results from cross-resistance obtained by *in vitro* in mycelial growth test, using discriminatory doses, showed positive correlation of *C. kikuchii* isolates exposed to QoI (azoxystrobin x picoxystrobin, picoxystrobin x pyraclostrobin, azoxystrobin x pyraclostrobin) and MBC (carbendazim x thiophanate-methyl) fungicide groups. Similar results were found in *C. kikuchii* (PRICE et al., 2015) and recently in *C. cassicola* in cucumber in China (DUAN et al., 2019).

Although the non-disruptive nature of *cyp51* gene mutations, 13 point mutations were identified for *cyp51* target gene. Therefore, further studies should be performed to verify if these mutations found can be correlated in a synergistic way to provide the lowest sensitivity to DMI fungicides.

In this study, we only found synonymous mutations for all *Cercospora* spp isolates analyzed for β-tubulin gene. Despite that, the mycelial growth assay for several isolates were not controlled at the 10 μg / mL^{-1} and were classified as resistant. Due to differences and low correlation between phenotyping and sequencing, it is concluded that the discriminatory dose at 10 μg / mL^{-1} was not efficient to identify sensitive and mutant isolates to MBC fungicides.

A total of 75% of *C. kikuchii* showed the mutation G143A. All the isolates present only homozygose variant for this mutation, which was confirmed by Sanger sequencing chromatogram wave length. The mutations F129L and G137R were not found. The results of this study indicate that QoI resistant *C. kikuchii* isolates occur thourghout soybean field in Brazil in soybean. The G143A mutation has been reported in soybean including, *C. sojina* in the United States (ZENG et al., 2012; STANDISH et al., 2015; ZENG et al., 2015) and *C. kikuchii* (PRICE et al., 2015, SAUTUA, 2019).

Dispite do not confer fungicide resitance SdhA gene had analized. For the gene we just found synonimmus mutantios. Analysing the mechanisms of resistance in SdhB gene we found 14 substitutions: H17L, G27A, T167M, P192L, E200D, N290K, I320N, I434V, A436V, D447N, H448R, E454Q, N513S and Q569K. The most frequent mutation was in Q569K, which were found in 32 isolates, followed by the N513S substitution, found in 9 isolates. For all mutations in SdhA and SdhB genes, homozygous and heterozygous variations were observed. This genomic characterization has also been reported in *Rizoctonia solani* (MU et al., 2013) and in *R. cerealis* (SUN et al., 2017).

The mutations found in this study, resulted in variability among populations to DMI, MBC, QoI and SDHI genes confirming that genetic mutations of fungicides target genes are responsible for the genetic variability among the populations. In this study, we showed that most of genetic variation was caused by frequent sprays of fungicides, with the same mode of action, in the field and resulted in a local directional selection pressure.

The results of this study indicate that QoI resistant *C. kikuchii* isolates occur thourghout in Brazil in soybean fields. In addition, cross-resistance were also confirmed into QoI active ingredients. This study also revelead possible point mutations in *cyp51* gene at Q161H, N178D, P180S, P200L, K217R, E274D, S279N, E281K, T290S, I299V, S363N, K442Q and L503V. In SdhB gene we also found that could be associated with possible potential mutations in sdhb gene H17L, G27A, T167M, P192L, E200D, N290K, I320N, I434V, A436V, D447N, H448R, E454Q, N513S and Q569K, which further studies will be needed to verify if these mutations are correlated with DMI fungicide resistance. In addition, heterozygous variant could be found in *cyp51* and SdhB genes. Only synonymous mutations were found in MBC gene. Therefore, anti-resistance strategies have to be implemented for efficiency recovery of QoI to control *C.kikuchii* in soybean in Brazil.

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3.5 REFERENCES

AGROFIT 2018. Sistema de agrotóxicos fitossanitários. Available at: http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. Accessed on March, 20, 2018.

ALBU, S.; PRICE, T.; DOYLE, V.; PADGETT, B.; SCHNEIDER, R. The G143A mutation is responsible for strobilurin fungicide resistance in *Cercospora* cf. *flagellaris*, a leaf blight and purple seed stain pathogen of Louisiana soybean. **Plant Health Progress**, v. 17, n. 3, p. 197-197, 2016.

ALFENAS, A. C.; FERREIRA, F. A.; MAFIA, R. G.; GONÇALVES, R. C. **Isolamento de fungos fitopatogênicos**. Métodos em fitopatologia. Viçosa: Ed. da UFV, p. 54, 2007.

ALMEIDA, A. M. R.; FERREIRA, L. P.; YORINORI, J. T.; SILVA, J. F. V.; HENNING, A. A.; GODOY, C. V.; COSTAMILAN, L. M.; MEYER, M. C. Doenças da soja. In:

KIMATI, H.; AMORIM, L.; REZENDE, J. A. M.; BERGAMIN FILHO, A.; CAMARGO, L. E. A. **Manual de Fitopatologia: doenças de plantas Cultivadas**. 4. ed. vol. 2. São Paulo: Editora Agronômica Ceres. 570-588 p, 2005.

ANDREWS, S. **FastQC: a quality control tool for high throughput sequence data** (2010). Disponível em: http://www.bioinformatics.babraham.ac.uk/projects/fastqc. Accessed in: March. 2018.

AYRES, D. L.; DARLING, A., ZWICKL, D. J.; BEERLI, P.; HOLDER, M. T.; LEWIS, P. O.; RAMBAUT, A. BEAGLE: an application programming interface and high-performance computing library for statistical phylogenetics. **Systematic biology**, v. 61, n. 1, p. 170-173, 2011.

BECHER, R.; WIRSEL, S. G. R. Fungal cytochrome P450 sterol 14α-demethylase (CYP51) and azole resistance in plant and human pathogens. **Applied Microbiology Biotechnology**, v.95, p. 825–840, 2012.

BECKER, W. F.; VON JAGOW, G.; ANKE, T.; STEGLICH, W. Oudemansin, strobilurin A, strobilurin B and myxothiazol: new inhibitors of the bc1 segment of the respiratory chain with an E-ßmethoxyacrylate system as common structural element. **FEBS Lett**, v.132, p. 329–333, 1981.

BERGER, R. D. Infection rates of *Cercospora apii* in mixed populations. **Phytopathology**, v. 63, p. 535-537, 1973.

BIRLA, K.; RIVERA-VARAS, V.; SECOR, G. A.; KHAN, M. F.; BOLTON, M. D. Characterization of cytochrome b from European field isolates of *Cercospora beticola* with quinone outside inhibitor resistance. **European journal of plant pathology**, v.134, n.3, p. 475-488, 2012.

BOLGER, A.M.; LOHSE, M.; USADEL, B. Trimmomatic: a flexible trimmer for Illumina sequence data**. Bioinformatics**, v. 30, n. 15, p. 2114-2120, 2014.

BOLTON, M. D.; RIVERA, V.; SECOR, G. Identification of the G143A mutation associated with QoI resistance in *Cercospora beticola* field isolates from Michigan, United States. **Pest management science**, v. 69, n.1, p. 35-39, 2013.

BORKERT, C. M.; YORINORI, J. T.; CORRÊA-FERREIRA, B. S.; ALMEIDA, A. M. R.; FERREIRA, L. P.; SFREDO, G. J. Seja o Doutor da sua Soja. **Informações Agronômicas**, Piracicaba, n. 66, p. 16, 1994.

BUDAKOV, D.; NAGL, N.; STOJŠIN, V.; BAGI, F.; DANOJEVIĆ, D.; NEHER, O. T.; TAŠKI-AJDUKOVIĆ, K. Sensitivity of *Cercospora beticola* isolates from Serbia to carbendazim and flutriafol. **Crop Protection**, v. 66, p. 120-126, 2014.

CLARK, E. M.; BACKMAN, P. A.; RODRIGUEZ-KABANA, R. *Cercospora arachidicola* and *Cercosporidium personatum* tolerance to benomyl and related fungicides in Alabama peanut fields. **Phytopathology**, v. 64, p.1476–1477, 1974.

CONAB- COMPANIA NACIONAL DE ABASTECIMENTO. Acompanhamento da safra brasileira de grãos, v. 5 - Safra 2017/18, n.6 - Sexto levantamento, março 2018.

DANECEK, P., AUTON, A., ABECASIS, G., ALBERS, C. A., BANKS, E., DEPRISTO, M. A.; MCVEAN, G. The Variant Call Format and VCFtools. **Bioinformatics**, v. 27, n. 15, p. 2156-2158, 2011.

DAVIDSE, L. C. Benzimidazole fungicides: mechanism of action and biological impact. **Annual Review of Phytopathology**, v.24, p.43–65, 1986.

DAVIDSE, L.; ISHII, H. Biochemical and molecular aspects of benzimidazoles, N -phenylcarbamates and N -phenylformamidoximes and the mechanisms of resistance to these compounds. **Modern selective fungicides**, p. 305–322, 1995.

DELP, C. J. Benzimidazole and related fungicides. Modern selective fungicides: properties, applications, mechanisms of action. Edi, H. Lyr in cooperation with H. Buchenhauer, 1987.

DUAN, Y.; XIN, W.; LU, F.; LI, T.; LI, M.; WU, J.; WANG, J.; ZHOU, M. BENZIMIDAZOLE-and QoI-resistance in *Corynespora cassiicola* populations from greenhouse-cultivated cucumber: An emerging problem in China. **Pesticide biochemistry and physiology**, v. 153, p. 95-105, 2019.

EMBRAPA. Recomendações técnicas para a cultura da soja no Paraná 1999/2000. Londrina. Embrapa Soja. (Embrapa Soja. Documentos, 131). 1999.

FINOTO, E.L. CARREGA, W.C.; SEDIYAMA, T.; ALBUQUERQUE, J.A.A.; CECON, P.R.; REIS, M.S. Efeito da aplicação de fungicida sobre caracteres agronômicos e severidade das doenças de final de ciclo na cultura da soja. **Revista Agro@mbiente,** Centro de Ciências Agrárias - Universidade Federal de Roraima, Boa Vista, Rr, v. 1, n. 5, p.44-49, abr. 2011. Trimestral.

FRAC**.** LIST OF PLANT PATHOGENIC ORGANISMS RESISTANT TO DISEASE CONTROL AGENTS 2017. Accessed in: 20 March, 2018.

FRAC-Fungicide Resistance Action Committee 2011. List of plant pathogenic organisms resistant to disease control agents (including FRAC Code numbering). Online document available at http://www. frac. info. Crop Life International, Brussels, Belgium.

GARDNER, M. W. Indiana plant diseases. **Proceeding Indiana Academy Science**, v. 35, p. 237-257, 1925.

GEORGOPOULOS, S. G.; DOVAS, C. Occurrence of *Cercospora beticola* strains resistant to benzimidazole fungicides in northern Greece**. Plant Disease**, v. 57, p. 321–324, 1973.

GISI, U.; SIEROTZKI, H.; COOK, A.; MCCAFFERY, A. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. **Pest management science**, v. 58, n. 9, p. 859-867, 2002.

GODOY, C. V. Risk and management of fungicide resistance in the Asian soybean rust fungus *Phakopsora pachyrhizi*. **Fungicide resistance in crop protection: Risk and management,** p. 87-95, 2012.

GODOY, C. V.; MEYER, M. C. Informativo fundação meridional. 2014. Available at: http://fundacaomeridional.com.br/artigos/2014/11/10/resistencia-a-fungicidas-na-cultura-da-soja. Accessed in September 01, 2015.

HAAS, B. J., PAPANICOLAOU, A., YASSOUR, M., GRABHERR, M., BLOOD, P. D., BOWDEN, J.; MACMANES, M. D. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. **Nature protocols**, v. 8, n. 8, p. 1494, 2013.

HENNING, A. A.; KRZYZANOWSKI, F. C.; FRANÇA NETO, J. B. Eficácia do tratamento de sementes de soja com fungicidas no controle dos principais fungos fito-patogênicos transmitidos pela semente. **Informativo ABRATES**, v. 1, n. 4, p. 98, 1991.

IMAZAKI, I.; IIZUMI, H.; ISHIKAWA, K.; SASAHARA, M.; YASUDA, N.; KOIZUMI, S. Effects of thiophanate-methyl and azoxystrobin on the composition of *Cercospora kikuchii* populations with thiophanate-methyl-resistant strains. **Journal of General Plant Pathology**, v. 72, n. 5, p. 292-300, 2006.

ISHII, H.; FOUNTAINE, J.; CHUNG, W. H.; KANSAKO, M.; NISHIMURA, K.; TAKAHASHI, K.; OSHIMA, M. Characterisation of QoI-resistant field isolates of *Botrytis cinerea* from citrus and strawberry. **Pest Management Science**, v. 65, n. 8, p. 916-922, 2009.

JOMBART, T. adegenet: a R package for the multivariate analysis of genetic markers. **Bioinformatics**, v. 24, n. 11, p. 1403-1405, 2008.

KAMVAR, Z. N.; TABIMA, J. F.; GRÜNWALD, N. J. Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. **PeerJ**, v. 2, p. e281, 2014.

KARAOGLANIDIS, G. S.; IONNIDIS, P. M.; THANOSSOULOPOULOS, C. C. Reduced sensitivity of *Cercospora beticola* isolates to sterol demethylation inhibiting fungicides. **Plant Pathology**, v. 49, p. 567–572, 2000.

KLOSOWSKI, A.C.; MAY DE MIO, L. L.; MIESSNER, S.; RODRIGUES, R.; STAMMLER G. Detection of the F129L mutation in the cytochrome b gene in *Phakopsora pachyrhizi.* **Pest Management Science**, v.72, n.6, p.1211–1215, 2015.

KUYAMA, S.; TAMURA, T. Cercosporin. A pigment of *Cercosporina kikuchii* Matsumoto et Tomoyasu. I. Cultivation of fungus, isolation and purification of pigment. **Journal of the American Chemical Society**, v. 79, n. 21, p. 5725-5726, 1957.

LANGMEAD, B.; SALZBERG, S. L. Fast gapped-read alignment with Bowtie 2. **Nature methods**, v. 9, n. 4, p. 357, 2012.

LEHMAN, S. G. Purple stain of soybean seeds. **North Carolina Agriculture Experimental Station Bulletin**, v. 369, p. 1-11. 1950.

LIPKA, A. E., TIAN, F., WANG, Q., PEIFFER, J., LI, M., BRADBURY, P. J.; ZHANG, Z. GAPIT: genome association and prediction integrated tool. **Bioinformatics**, v. 28, n. 18, p. 2397-2399, 2012.

LITTRELL, R. H. Tolerance in *Cercospora arachidicola* to benomyl and related fungicides. **Phytopathology**, v. 64, p.1377–1378, 1974.

MIYAMOTO, T.; ISHII, H.; TOMITA, Y. Occurrence of boscalid resistance in cucumber powdery mildew in Japan and molecular characterization of the iron–sulfur protein of succinate dehydrogenase of the causal fungus. **Journal of General Plant Pathology**, v. 76, n. 4, p. 261-267, 2010.

MU, W.; LI, B.; CHEN, C.; LIU, X.; HAO, J. Molecular mechanisms of thifluzamide resistance in *Rhizoctonia solani* (Abstr.). ***Phytopathology*** 104(Suppl. 1):S1.4, 2013.

ORTH, C. E.; SCHUH, W. Resistance of 17 soybean cultivars to foliar, latent, and seed infection by *Cercospora kikuchii.* **Plant disease**, v. 78, n. 7, p. 661-664, 1994.

PRICE III, P. P.; PURVIS, M. A.; CAI, G.; PADGETT, G. B.; ROBERTSON, C. L.; SCHNEIDER, R. W.; ALBU, S. Fungicide resistance in *Cercospora kikuchii*, a soybean pathogen. **Plant disease**, v. 99, n. 11, p. 1596-1603, 2015.

RIMMER, A., PHAN, H., MATHIESON, I., IQBAL, Z., TWIGG, S. R., WILKIE, A. O. VEAN, G. M.; LUNTER, G. Integrating mapping-, assembly-and haplotype-based approaches for calling variants in clinical sequencing applications. **Nature genetics**, v. 46, n. 8, p. 912, 2014.

SAUTUA, F. J.; SEARIGHT, J.; DOYLE, V. P.; PRICE III, P. P.; SCANDIANI, M. M.; CARMONA, M. A. The G143A Mutation Confers Azoxystrobin Resistance to Soybean Cercospora Leaf Blight in Bolivia. **Plant Health Progress**, v. 20, n. 1, p. 2-3, 2019.

SCHLIEP, K. P. phangorn: phylogenetic analysis in R. **Bioinformatics**, v. 27, n. 4, p. 592-593, 2010.

SCHMITZ, H. K.; MEDEIROS, C. A.; CRAIG, I. R.; STAMMLER, G. Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. **Pest management science**, v. 70, n. 3, p. 378-388, 2014.

SCHROEDER, W. T.; PROVVIDENTI, R. Resistance to benomyl in powdery mildew of cucurbits. **Plant Disease Report**, v. 53, p. 271-275, 1969.

SCHUH, W. Cercospora blight, leaf spot, and purple seed stain. **Compendium of soybean diseases**, p. 17-18, 1990.

SIEROTZKI, H. Respiration Inhibitors: Complex III. In: Ishii H, Hollomon D, editors. Fungicide Resistance in Plant Pathogens: **Principles and a Guide to Practical Management**. Japan: Springer p. 119-143, 2015.

SIMÕES, K.; HAWLIK, A.; REHFUS, A.; GAVA, F.; STAMMLER, G. First detection of a SDH variant with reduced SDHI sensitivity in *Phakopsora pachyrhizi.* **Journal of Plant Diseases and Protection**, v. 125, n. 1, p. 21-26, 2018.

SINCLAIR, J. B. Control of seedborne pathogens and diseases of soybean seeds and seedlings. **Pest Management Science**, v. 37, n.1, p. 15-19, 1993.

SINCLAIR, J. B.; BACKMAN, P. A. Compendium of soybean Disease. 3 ed. Edition St Paul, Minnesota. **American Phytopathological Society**, p. 106. 1989.

SOARES, A. P. G.; GUILLIN, E. A.; BORGES, L. L.; DA SILVA, A. C.; DE ALMEIDA, Á. M.; GRIJALBA, P. E.; GOTLIEB, A. M.; BLUHM, B. H; DE OLIVEIRA, L. O. More *Cercospora* species infect soybeans across the Americas than meets the eye. **PLoS One**, v. 10, n. 8, p. e0133495, 2015.

STANDISH, J. R.; TOMASO-PETERSON, M.; ALLEN, T. W.; SABANADZOVIC, S.; ABOUGHANEM-SABANADZOVIC, N. Occurrence of QoI fungicide resistance in Cercospora sojina from Mississippi soybean. **Plant disease**, v. 99, n. 10, p. 1347-1352, 2015.

SUN, H. Y.; LU, C. Q.; LI, W.; DENG, Y. Y.; CHEN, H. Homozygous and heterozygous point mutations in succinate dehydrogenase subunits b, c and d of *Rhizoctonia cerealis* conferring resistance to thifluzamide. **Pest management science**, v. 73, n. 5, p. 896-903, 2017.

TATUSOVA, T. A.; MADDEN, T. L. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. **FEMS microbiology letters**, v. 174, n. 2, p. 247-250, 1999.

WALTERS, H. J. Cercospora leaf blight of soybeans. **Phytopathology News**, v. 12, p. 165-166, 1978.

WARD-GAUTHIER, N.A.; SCHNEIDER, R.W.; CHANDA. A.; SILVA, E.C.; PRICE III, P.P.; CAI, G. Cercospora leaf blight and purple seed stain. In: HARTMAN, G.L.;

RUPE, J.C.; SIKORA, E.J.; DOMIER, L.L.; DAVIS, J.A.; STEFFEY, K.L. (Ed.). Compendium of soybean diseases and pests. 5. ed. Saint Paul: APS Press, 2015. p. 37-40.

WILCOX, J. R.; ABNEY T. S. Purple seed stain of soybean. **Phytopathology**, v. 63, p. 796-797, 1973.

YEH, C. C.; SINCLAIR, J. B. Effect of *Cercospora kikuchii* on soybean seed germination and its interaction with *Phomopsis* sp. **Phytopathology**, v. 105, p. 265-270, 1981.

ZENG, F.; ARNAO, E.; ZHANG, G.; OLAYA, G.; WULLSCHLEGER, J.; SIEROTZKI, H.; MING, R.; BLUHM; B. H.; BOND; J.P.; FAKHOURY, A. M.; BRADLEY, C. A. Characterization of quinone outside inhibitor fungicide resistance in *Cercospora sojina* and development of diagnostic tools for its identification. **Plant disease**, v. 99, n. 4, p. 544-550, 2015.

ZIOGAS, B. N., BALDWIN, B. C.; YOUNG, J. E. Alternative respiration: a biochemical mechanism of resistance to azoxystrobin (ICIA 5504) in Septoria tritici. **Pesticide Science**, v. 50, n. 1, p. 28-34, 1997.

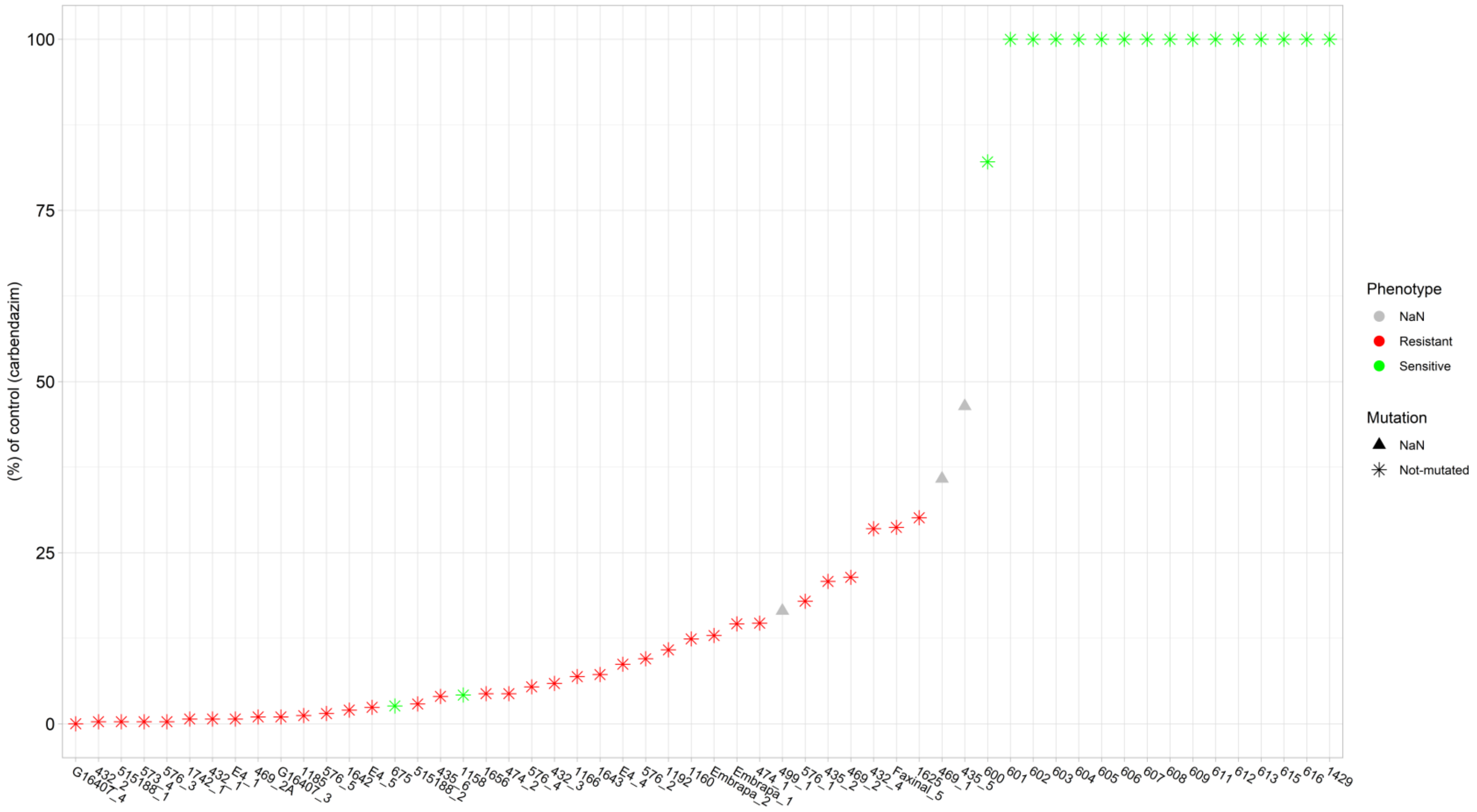
ZIOGAS, B. N.; MALANDRAKIS, A. A. Sterol Biosynthesis Inhibitors: C14 Demethylation (DMIs). In: Ishii H, Hollomon D, editors. **Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management**. Japan: Springer, p. 201-216, 2015.

3.6 SUPPLEMENTARY INFORMATION

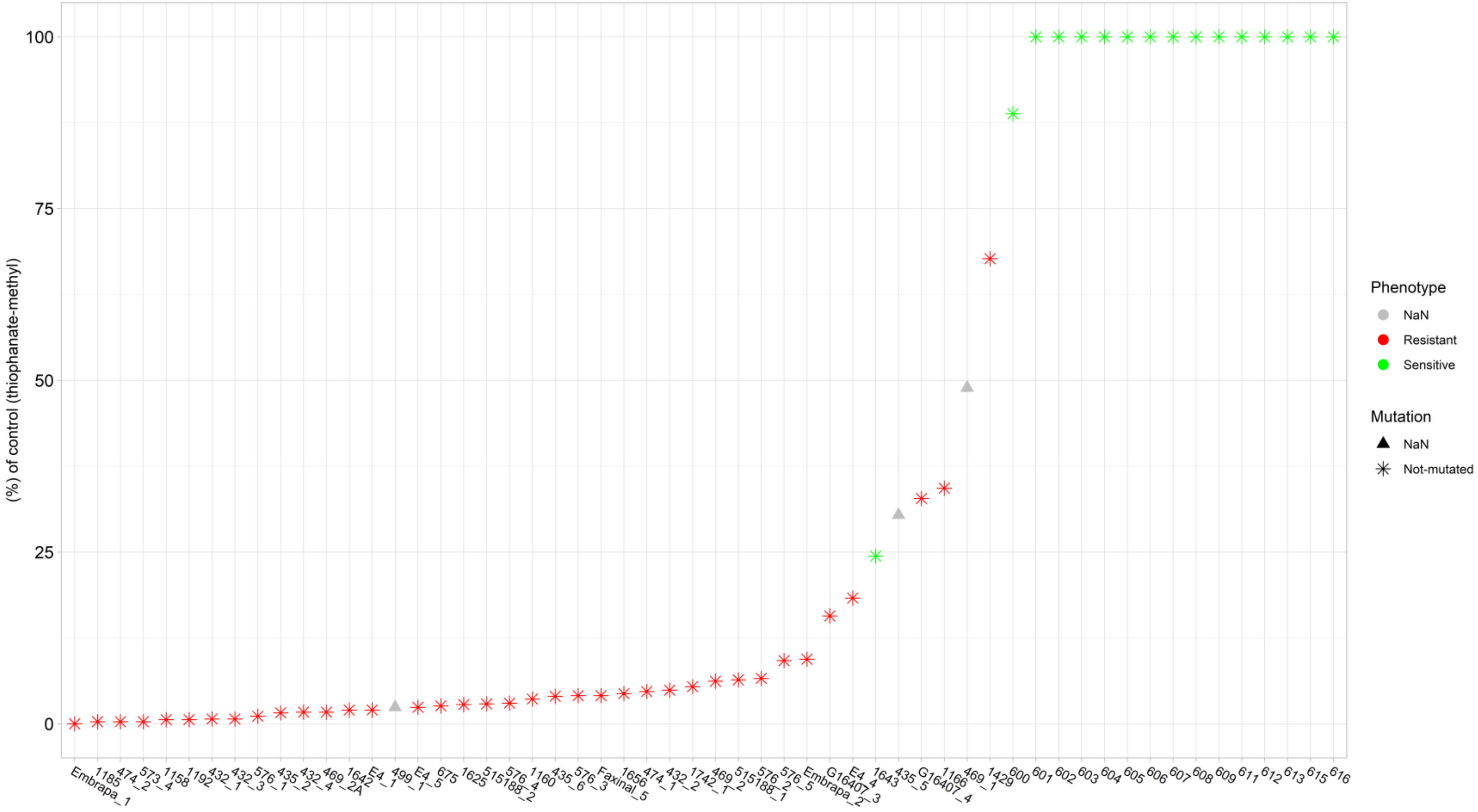
Supplementary table S.3.1. Primer pairs sequence and PCR conditions used to identification of point mutation in *C. kikuchii* isolates from soybean in *cyp51,* β-tubulin, *cyt b*, SdhA and SdhB.

Gene	Primer_F	Anneling position	Primer_R	Anneling position	Annealing temperature (ºC)	amplicon size (bp)
cyp51 (DMI)	ACGTCTTCACCTTCATTCTGC	1710	GCCTCTCCCACTTCACAACA	3052	63	1342
β-tubulin (MBC)	CTGCATTCTGGCAGACCAT	399	TGAACTGGTCACCGACACG	1627	63	1228
cyt b (QoI)	ATGGTTCTTACAAAGCACCTAGAA	701	CACCCAATTGCATCAATAATAAGA	1417	57	716
Sdh*A* (SdhA)[1]	CTCGTGGTGAGGGTGGTTACCT	1489	CGCTTGAAAGGTGGAACAGC	2494	63	1005
Sdh*B* (Sdh*B*)	GAGCACACCAAGGAGCCTAT	954	GCACAACTGCTCCCTCTTG	1145	63	1764

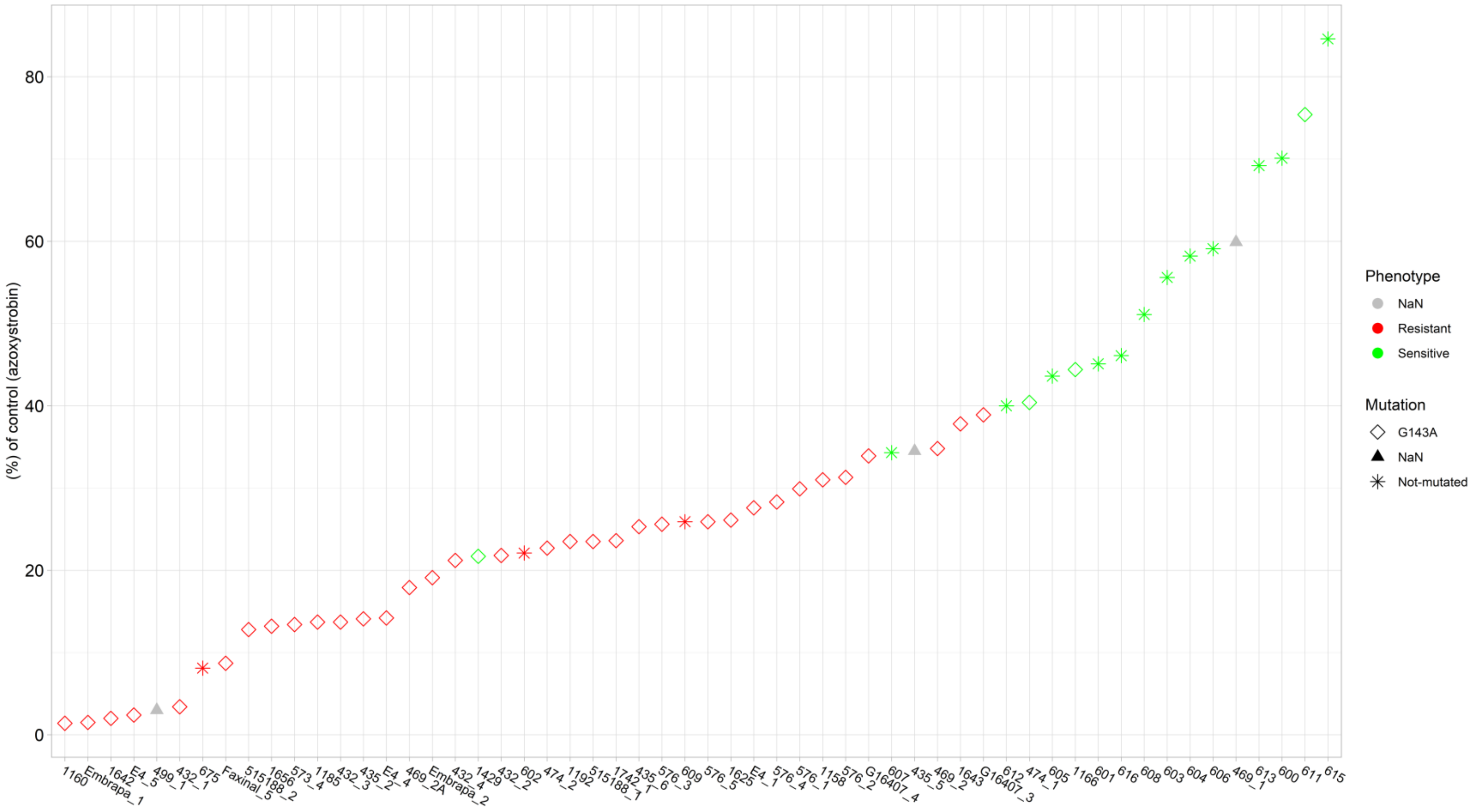
[1] Miyamoto et al.,2010.



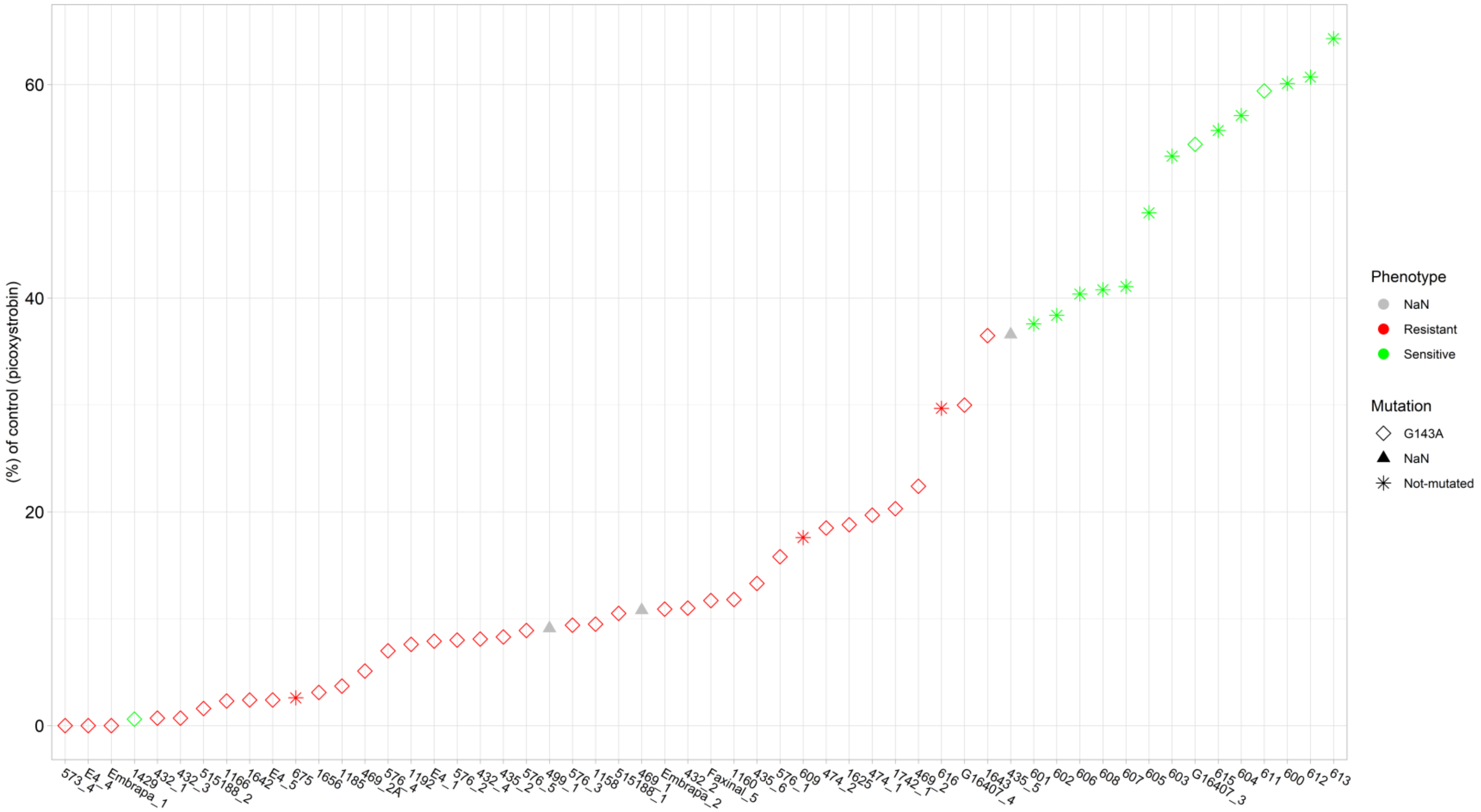
Suplementary figure S.3.1. Percentage of carbendazim control in *C. kikuchii* isolates from soybean. Colors scale represents isolate classification into different phenotype. Different geometric shapes represent the nonsynonymous mutation to the fungicide carbendazim (MBC).



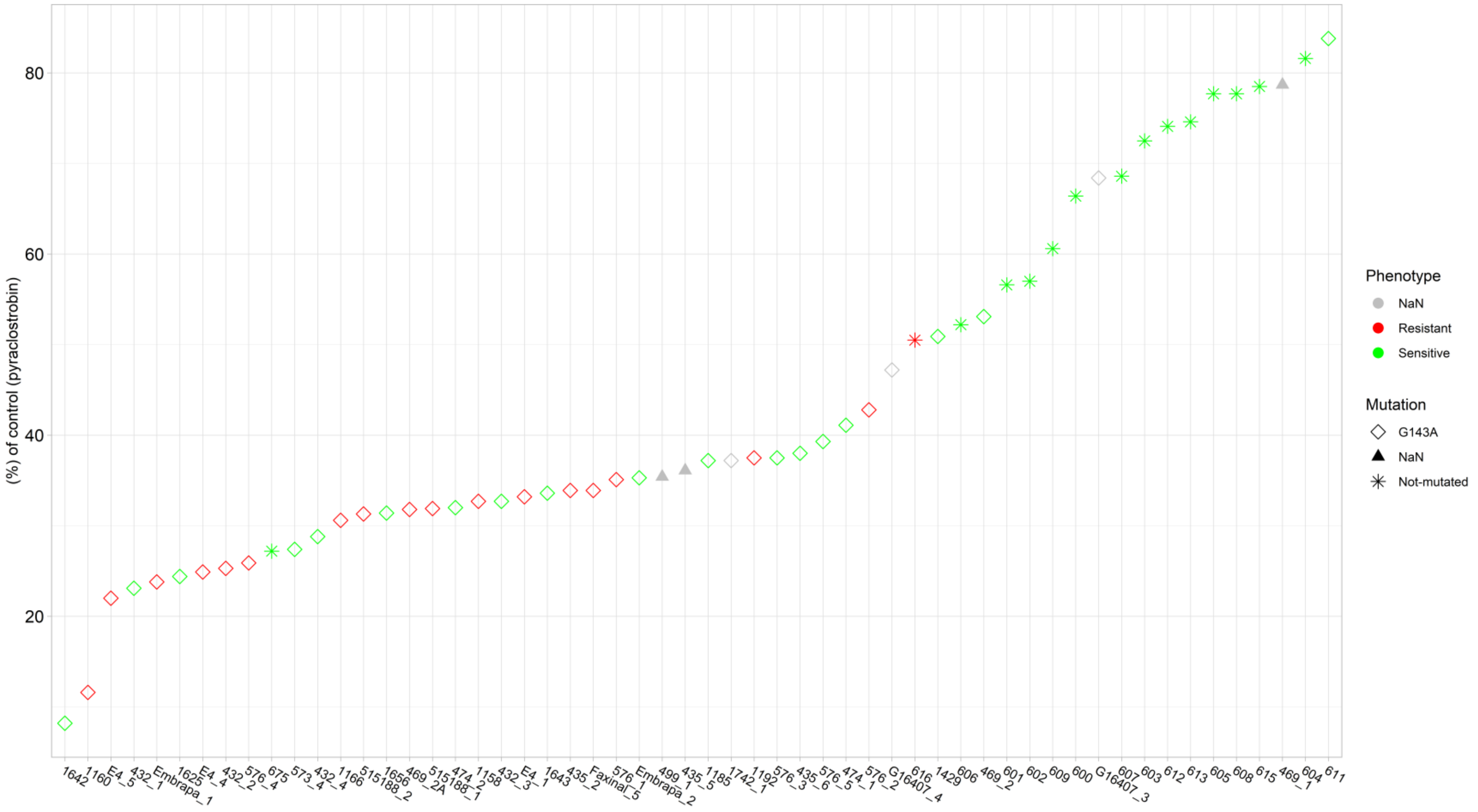
Suplementary figure S.3.2. Percentage of thiophanate-methyl control in *C. kikuchii* isolates from soybean. Colors scale represents isolate classification into different phenotype. Different geometric shapes represent the nonsynonymous mutation to the fungicide thiophanate-methyl (MBC).



Suplementary figure S.3.3. Percentage of azoxystrobin control in *C. kikuchii* isolates from soybean. Colors scale represents isolate classification into different phenotype. Different geometric shapes represent the nonsynonymous mutation to the fungicide azoxystrobin (QoI).



Suplementary figure S.3.4. Percentage of picoxystrobin control in *C. kikuchii* isolates from soybean. Colors scale represents isolate classification into different phenotype. Different geometric shapes represent the nonsynonymous mutation to the fungicide picoxystrobin (QoI).



Suplementary figure S.3.5. Percentage of picoxystrobin control in *C. kikuchii* isolates from soybean. Colors scale represents isolate classification into different phenotype. Different geometric shapes represent the nonsynonymous mutation to the fungicide pyraclostrobin (QoI).

Supplementary table S.3.3. Codon position, amino acid change and nonsynonymous and synonymous mutations for *cyp51* (DMI), β-tubulin (MBC), *cyt b* (QoI) and SdhA and SdhB fungicide target genes of *C. kikuchii* from soybean fields.

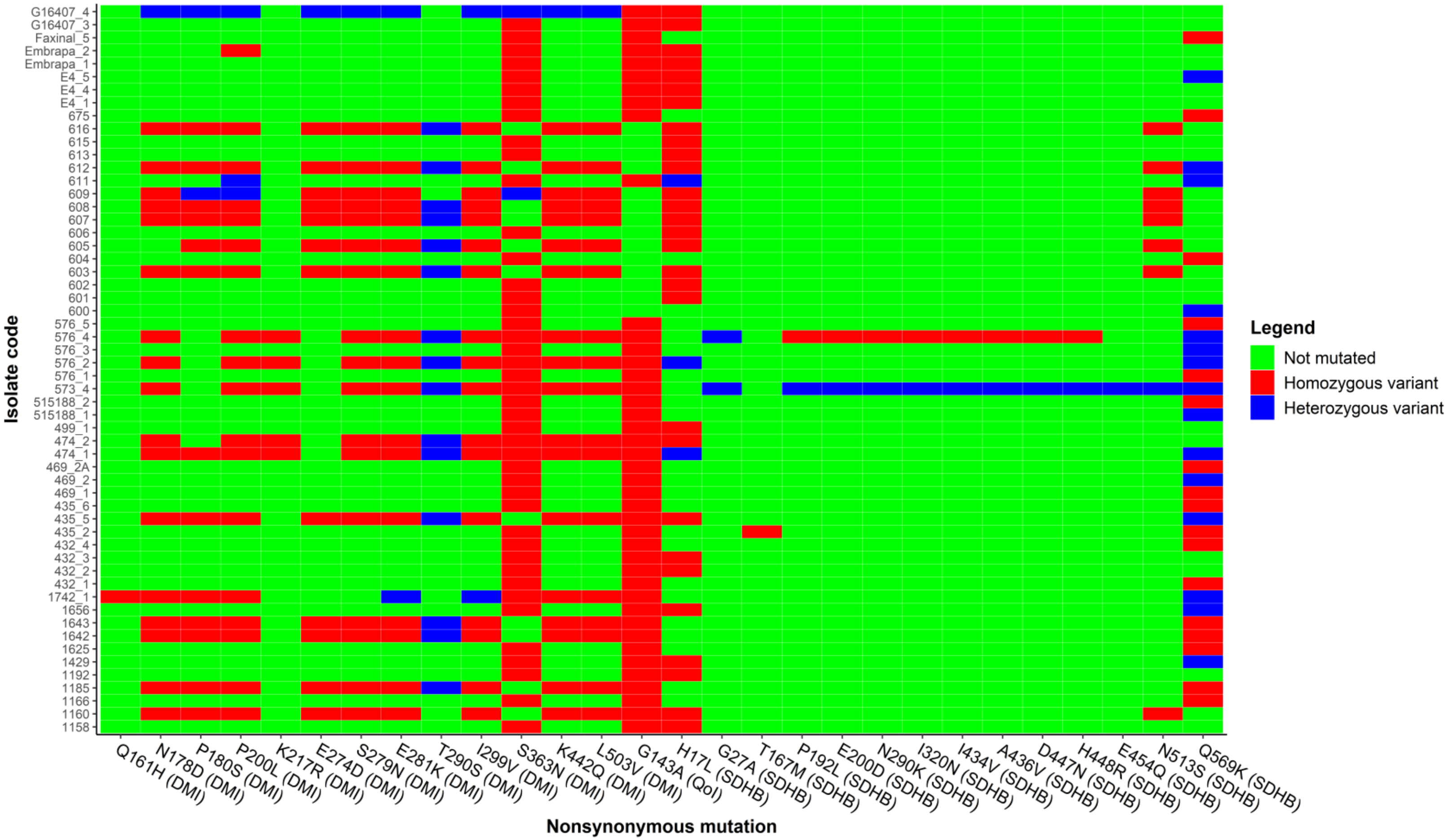
DMI				DMI				DMI			
Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations	
		non-syn	syn			non-syn	syn			non-syn	syn
321	GAC/GAT	-	D	657	GAC/GAT	-	D	984	CTA/CTG	-	L
342	CTG/CTC	-	L	663	ACA/ACC	-	T	990	CTT/CTG	-	L
360	GAA/GAG	-	E	672	GAA/GAG	-	E	1014	GAC/GAT	-	D
384	ACT/ACC,ACG	-	T	696	GGT/GGC	-	G	1074	GAG/GAA	-	E
387	CCG/CCA	-	P	702	ACG/ACC	-	T	1088	AGC/AAC	S363N	-
405	GTC/GTT	-	V	729	GCA/GCG	-	A	1092	CTC/CTG	-	L
426	TCC/TCA	-	S	756	GAT/GAC	-	D	1134	TTA/TTG	-	L
468	ACC/ACA,ACT	-	T	807	AGA/AGG	-	R	1149	CCG/CCC	-	P
483	CAG/CAT	Q161H	-	822	GAG/GAC	E274D	-	1164	CTT/CTC	-	L
528	TCC/TCT	-	S	831	TCG/TCA	-	S	1167	CGC/CGT	-	R
532	AAC/GAC	N178D	-	836	AGT/AAT	S279N	-	1221	ACG/ACC, ACT	-	T
538	CCA/TCA	P180S	-	843	GAA/AAA	E281K	-	1275	CCC/CCA	-	P
558	TCG/TCC	-	S	849	GAC/GAT	-	D	1324	AAG/CAG	K442Q	
600	CCT/CTC	P200L	-	864	CTA/CTG	-	L	1338	CTG/CTC	-	L
615	GCT/GCC	-	A	868	ACA/TCA	T290S	-	1344	CCC/CCG	-	P
627	CTG/CTC	-	L	895	ATT/GTT	I299V	-	1353	GTC/GTT	-	V
642	GTC/GTT	-	V	906	CGA/CGG	-	R	1365	GTT/GTC, GTG	-	V
650	AAA/AGA	K217R	-	969	TCT/TCC	-	S	1377	AAA/AAG	-	K

DMI				MBC				MBC			
Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations	
		non-syn	syn			non-syn	syn			non-syn	syn
1389	GGG/GGC	-	G	202	TTG/CTG	-	L	525	GTC/GTG	-	V
1396	CTG/TTG	-	L	222	GAC/GAT	-	D	537	GTT/GTC	-	V
1401	GTG/GTC	-	V	234	GCC/GCT	-	A	546	CCC/CCA	-	P
1407	AAG/AAA	-	K	240	CCT/CCA	-	P	561	CTC/CTG	-	L
1446	CGT/CGC	-	R	246	GGC/GGT	-	G	588	TCC/TCT	-	S
1470	TTT/TTC	-	F	252	CTC/CTT	-	L	630	ATC/ATT	-	I
1503	ACC/ACT	-	T	261	CCC/CCA	-	P	645	CTC/CTG	-	L
1507	CTT/GTT	L503V	-	279	GGT/GGC	-	G	669	GGT/GGC	-	G
1512	CGA/CGG	-	R	324	GAG/GAA	-	E	684	CTC/CTT	-	L
1521	AAA/AAG	-	K	339	GTC/GTG	-	V	690	TCC/TCT	-	S
1569	AGC/AGT	-	S	348	GTC/GTT	-	V	705	GGC/GGT	-	G
				375	GAG/GAA	-	E	711	ACC/ACA, ACT	-	T
				384	GAC/GAT	-	D	717	TGC/TGT	-	C
				393	CAG/CAA	-	Q	723	CGT/CGC	-	R
				417	CTC/CTT	-	L	729	CCC/CCA,CG	-	P
				429	ACC/ACT	-	T	750	CTC/CTG	-	L
				447	ACG/ACC,ACT	-	T	759	CTC/CTT	-	L
				468	CGT/CGC	-	R	777	CCC/CCA,CCG	-	P
				480	CCC/CCA	-	P	783	CCC/CCA	-	P
				486	CGT/CGC	-	R	798	TTC/TTT	-	F
				498	ACC/ACA	-	T	867	CTT/CTC	-	L

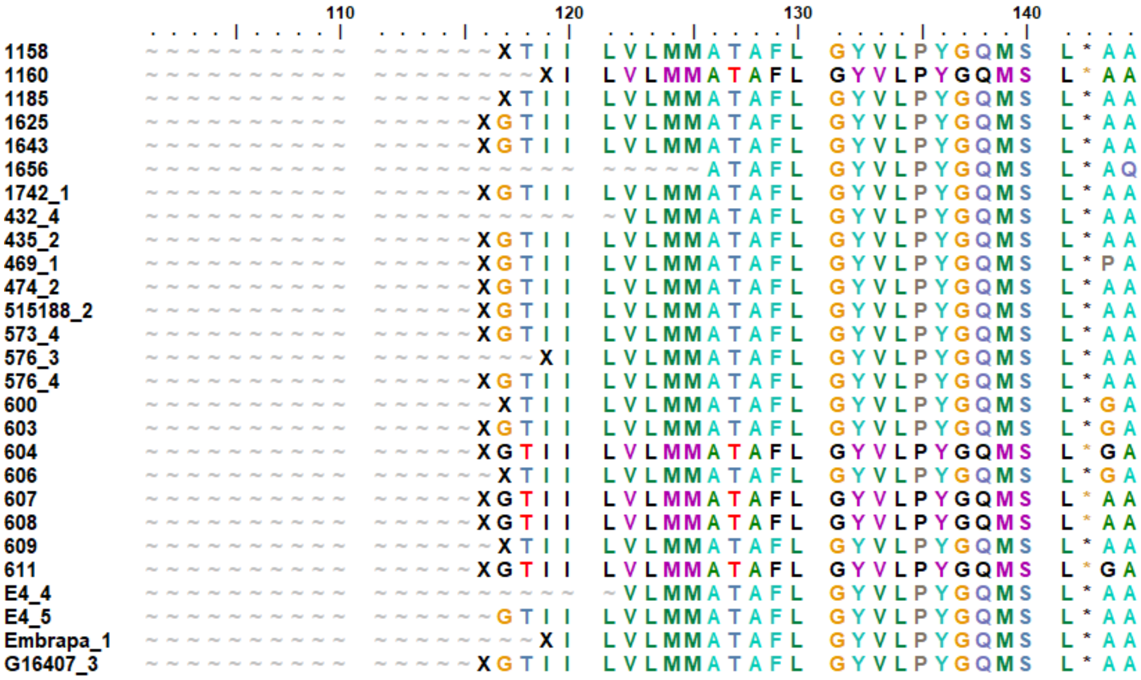
MBC				QoI				SdhA			
Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations	
		non-syn	syn			non-syn	syn			non-syn	syn
888	CCG/CCC	-	P	289	GTA/ATA	V97I	-	1128	CCA/CCC,CCT	-	P
894	AAC/AAT	-	N	428	GGT/GCT/	G143A	-	1206	CAT/CAC	-	H
903	GCC/GCT	-	A	531	AAT/AAC	-	N	1236	TAT/TAC	-	Y
921	AAC/AAT	-	N	627	AAT/AAC	-	N	1356	TCA/TCC,TCT	-	S
927	CGT/CGC	-	R	754	TTA/CTA	-	L				
954	CGC/CGT	-	R	897	GCG/GCT	-	A				
957	GGC/GGA	-	G	931	CTA/TTA	-	L				
975	GGA/GGG	-	G								
999	GTG/GTC	-	V								
1056	GCA/GCC	-	A								
1059	CTC/CTG	-	L								
1119	TCG/TCC	-	S								
1131	CTC/CTG,CTT	-	L								

SdhB				**SdhB**				**SdhB**			
Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations	
		non-syn	syn			non-syn	syn			non-syn	syn
50	CAC/CTC	H17L	-	500	ACG/ATG	T167M	-	966	AGT/AGC	-	S
81	GGC/GCC	G27A	-	552	GCC/GCT	-	A	987	CTG/CTT	-	L
84	AAG/AAA	-	K	575	CCA/CTA	P192L		1002	CCC/CCT	-	P
114	CAT/CAC	-	H	594	AAT/AAC	-	N	1032	TTG/TTA	-	L
204	GTC/GTT	-	V	600	GAG/GAT	E200D	-	1038	GTG/GTC	-	V
210	CCC/CCG	-	P	639	GAA/GAG	-	E	1041	AAT/AAC	-	N
228	ATA/ATC	-	I	645	GTT/GTG	-	V	1113	CAT/CAC	-	H
240	CTA/CTG	-	L	681	GGC/GGA,GGT	-	G	1119	AAC/AAT	-	N
252	TTC/TTT	-	F	691	TTG/CTG	-	L	1137	CCT/CCC	-	P
258	GGG/GGC	-	G	696	CAG/CAA	-	Q	1143	TCA/TCT	-	S
279	ATT/ATC	-	I	705	GGC/GGT	-	G	1149	ATT/ATC	-	I
282	GGT/GGG	-	G	714	GGT/GGC	-	G	1176	TCT/TCC	-	S
300	AGC/AGT	-	S	768	GGT/GGA	-	G	1194	AAT/AAC	-	N
315	GGC/GGG	-	G	813	TTC/TTT	-	F	1212	ATC/ATT	-	I
324	ACA/ACC	-	T	822	GAA/GAG	-	E	1221	GTT/GTG	-	V
330	TTT/TTC	-	F	846	TTG/TTA	-	L	1233	TTC/TTT	-	F
339	GAA/GAG	-	E	864	CCG/CCC	-	P	1239	GAC/GAT	-	D
343	CTG/TTG	-	L	869	AAC/AAG	N290K	-	1300	ATT/GTT	I434V	-
390	CAA/CAG	-	Q	873	TTT/TTC	-	F	1307	GCT/GTT	A436V	-
426	GGC/GGT	-	G	879	CCA/CCG	-	P	1317	ATA/ATC	-	I
463	CGA/AGA	-	R	885	ACT/ACC	-	T	1329	GAA/GAG	-	E
483	AAG/AAA	-	K	912	CCT/CCG	-	P	1339	GAT/AAT	D447N	-
498	GTC/GTT	-	V	960	ATT/AAC	I320N	-	1343	CAT/CGT	H448R	-

SdhB			
Codon position	Amino acid change	Mutations	
		non-syn	syn
1350	CAA/CAG	-	Q
1360	GAA/CAA	E454Q	
1377	AAA/AAG	-	K
1386	CTG/CTA	-	L
1422	CGC/CGT	-	R
1434	CCG/CCT	-	P
1443	ACA/ACG	-	T
1464	AAC/AAT	-	N
1482	CCC/CCT	-	P
1491	GCC/GCT	-	A
1509	TCC/TCT	-	S
1538	AAT/AGT	N513S	-
1629	TAC/TAT	-	Y
1635	GCA/GCG	-	A
1641	GTG/GTA	-	V
1674	CGG/CGA	-	R
1686	AAG/AAA	-	K
1705	CAA/AAA	Q569K	-



Suplementary figure S.3.6. Heatmap of nonsynonymous mutations *cyp51*, β-tubulin, *cyt b*, SdhA and SdhB genes, for fungicide target site resistance in *C. kikuchii* isolates. Colors on the map represent genetic variations for each isolate.



```
                            110         120          130          140
            ....|....|  ....|....|  ....|....|  ....|....|  ....
1158        ~~~~~~~~~~  ~~~~~~XTII  LVLMMATAFL  GYVLPYGQMS  L*AA
1160        ~~~~~~~~~~  ~~~~~~~~XI  LVLMMATAFL  GYVLPYGQMS  L*AA
1185        ~~~~~~~~~~  ~~~~~~XTII  LVLMMATAFL  GYVLPYGQMS  L*AA
1625        ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*AA
1643        ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*AA
1656        ~~~~~~~~~~  ~~~~~~~~~~  ~~~~~ATAFL  GYVLPYGQMS  L*AQ
1742_1      ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*AA
432_4       ~~~~~~~~~~  ~~~~~~~~~~  ~VLMMATAFL  GYVLPYGQMS  L*AA
435_2       ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*AA
469_1       ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*PA
474_2       ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*AA
515188_2    ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*AA
573_4       ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*AA
576_3       ~~~~~~~~~~  ~~~~~~~~XI  LVLMMATAFL  GYVLPYGQMS  L*AA
576_4       ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*AA
600         ~~~~~~~~~~  ~~~~~~XTII  LVLMMATAFL  GYVLPYGQMS  L*GA
603         ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*GA
604         ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*GA
606         ~~~~~~~~~~  ~~~~~~XTII  LVLMMATAFL  GYVLPYGQMS  L*GA
607         ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*AA
608         ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*AA
609         ~~~~~~~~~~  ~~~~~~XTII  LVLMMATAFL  GYVLPYGQMS  L*AA
611         ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*GA
E4_4        ~~~~~~~~~~  ~~~~~~~~~~  ~VLMMATAFL  GYVLPYGQMS  L*AA
E4_5        ~~~~~~~~~~  ~~~~~~GTII  LVLMMATAFL  GYVLPYGQMS  L*AA
Embrapa_1   ~~~~~~~~~~  ~~~~~~~~XI  LVLMMATAFL  GYVLPYGQMS  L*AA
G16407_3    ~~~~~~~~~~  ~~~~~XGTII  LVLMMATAFL  GYVLPYGQMS  L*AA
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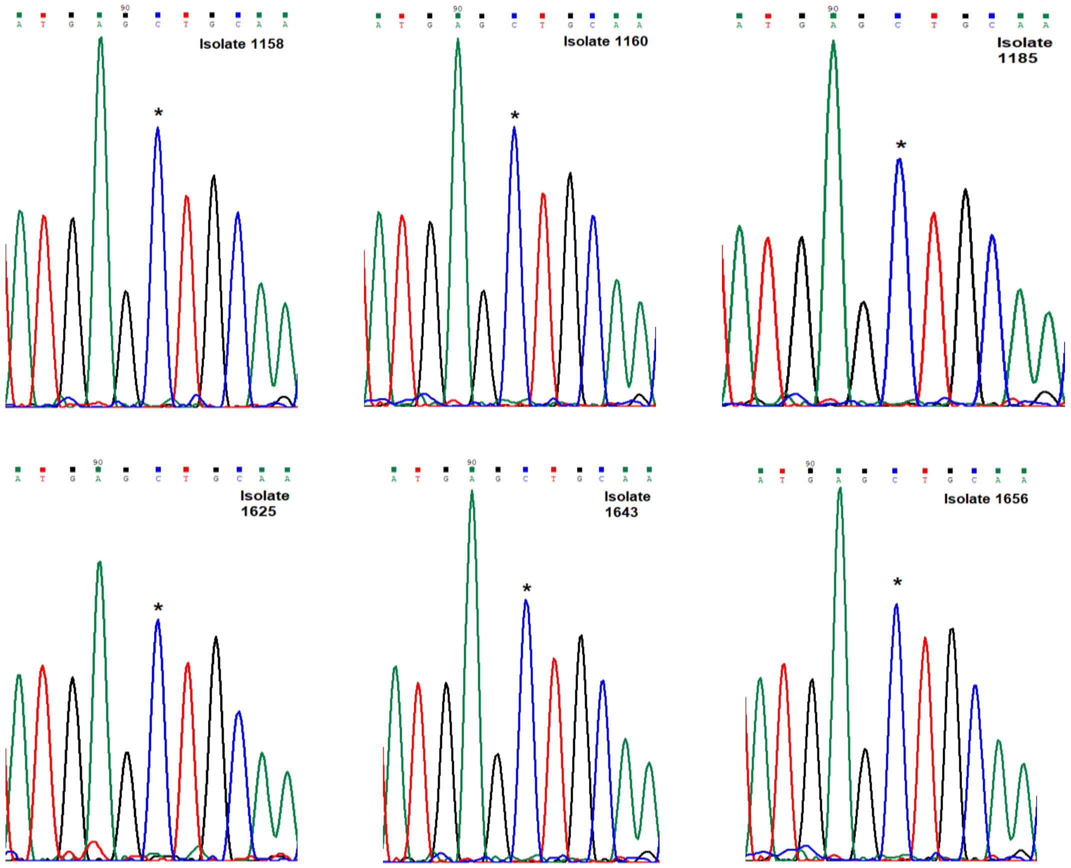
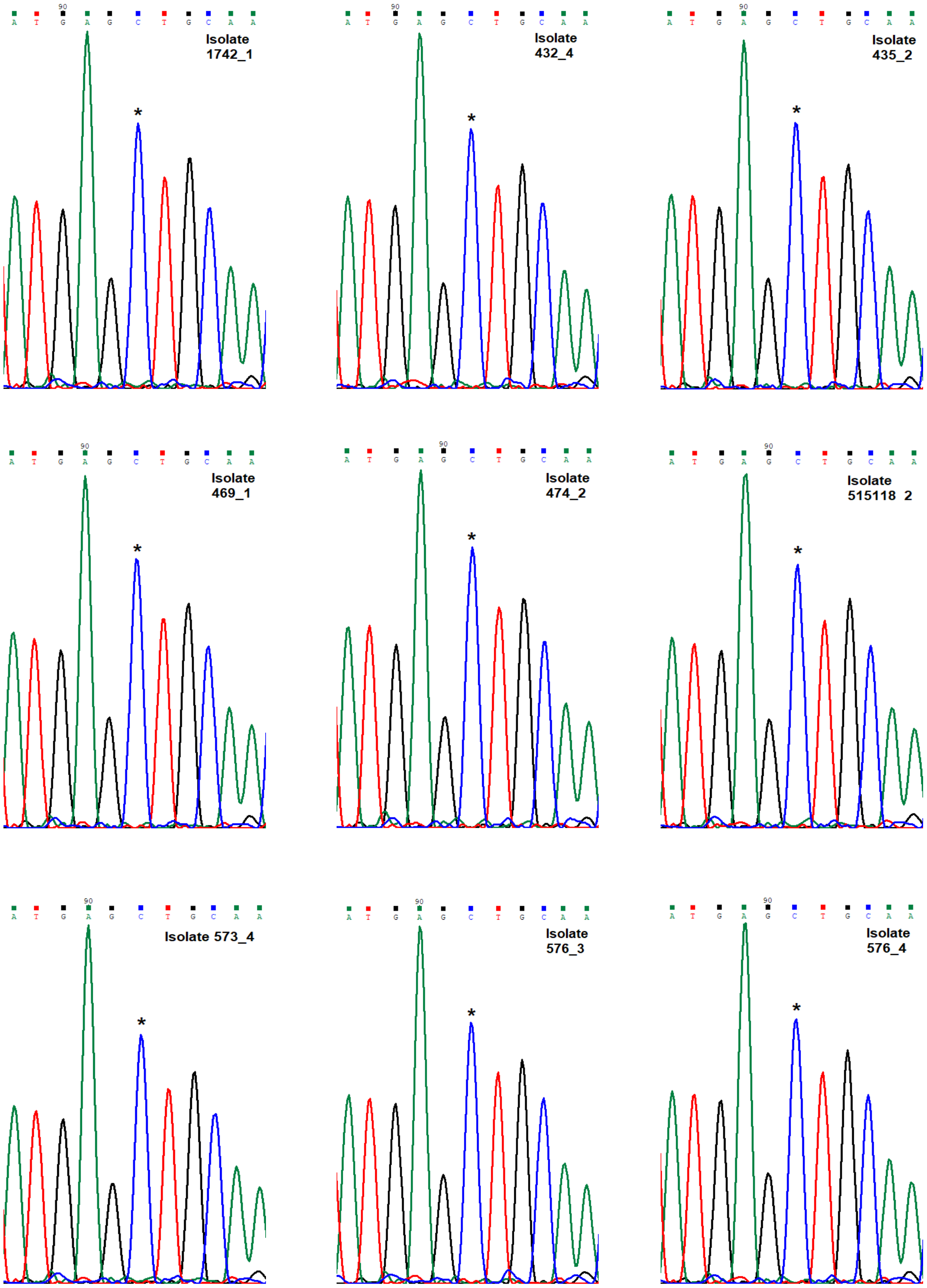
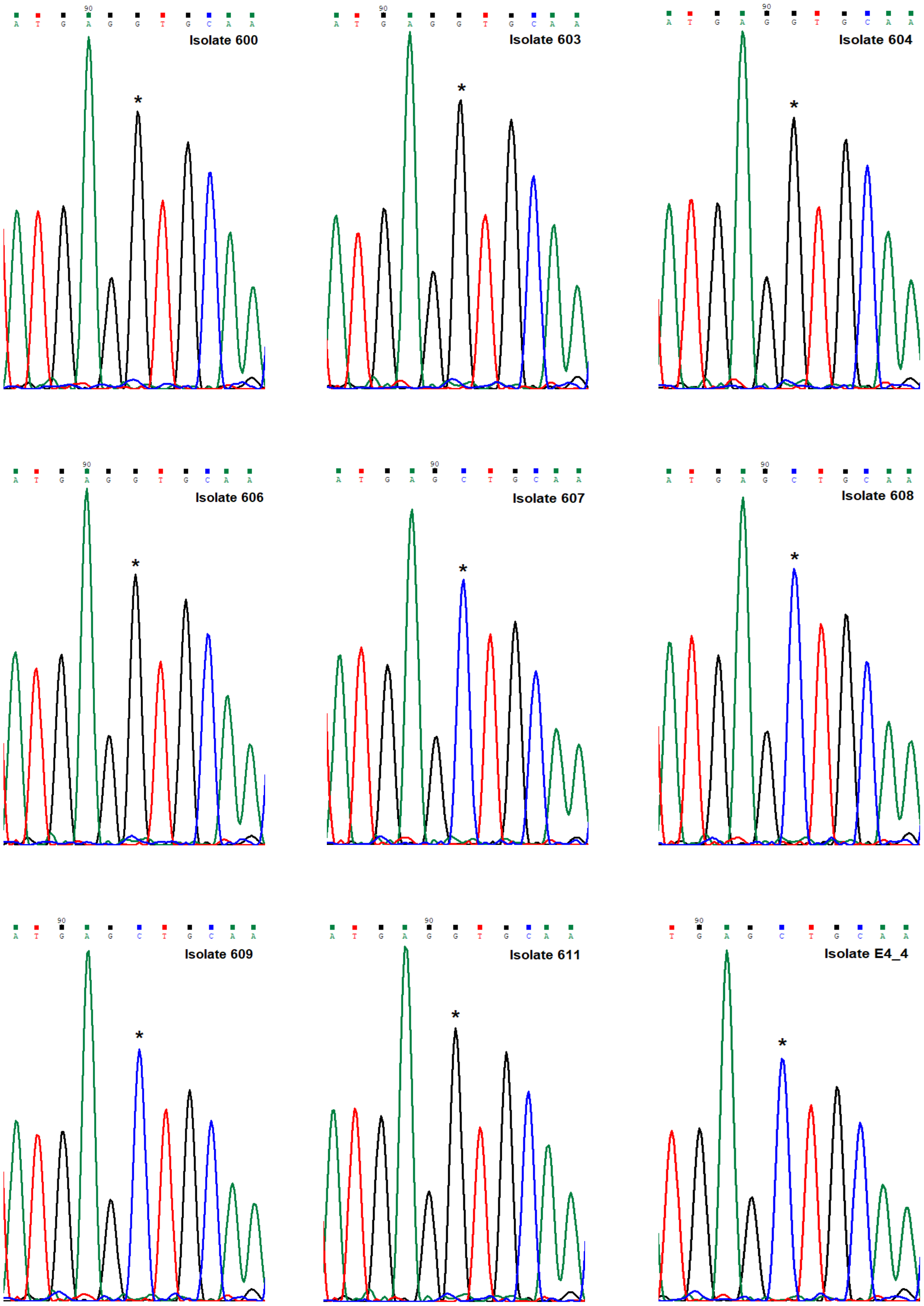
Suplementary figure S.3.7. Sanger sequencing in *cyt b* gene of *C. kikuchii* from soybean.



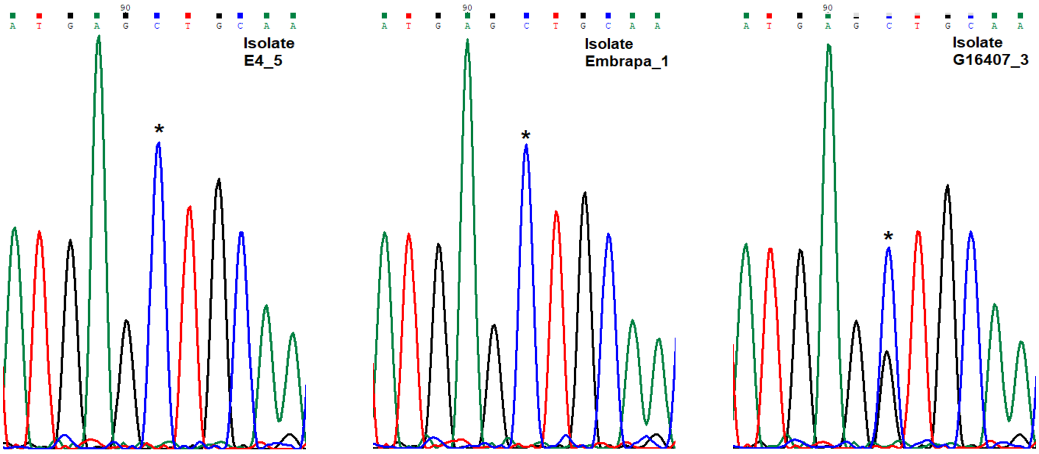
Suplementary figure S.3.8. Nucleotide chromatogram sequencing mutation from GGT in sensitive isolates to GCT in resistant *C. kikuchii* isolates in *cyt b* gene.



Suplementary figure S.3.8. Nucleotide chromatogram sequencing mutation from GGT in sensitive isolates to GCT in resistant *C. kikuchii* isolates in *cyt b* gene.



Suplementary figure S.3.8. Nucleotide chromatogram sequencing mutation from GGT in sensitive isolates to GCT in resistant *C kikuchii* isolates in *cyt b* gene.



Suplementary figure S.3.8. Nucleotide chromatogram sequencing mutation from GGT in sensitive isolates to GCT in resistant *C. kikuchii* isolates in *cyt b* gene.

4 ARTIGO B: "Molecular detection of fungicide resistance of *Colletotrichum truncatum* for *cyp51*, *cyt b*, β-tubulin and *S*dhB genes in soybean in Brazil"

ABSTRACT

Anthracnose, caused by the fungi *Colletotrichum truncatum* is an important disease, which affect soybean production. Fungicide application of demethlylation inhibitors (DMI), quinone outside inhibitors (QoI), methyl benzimidazole carbamate (MBC) and succinate dehydrogenase inhibitor (SDHI) has been widely used to managing this disease. However, due to the frequent application *C. truncatum* isolates can became resistante. The aims of this research were analyze polymorphisms in the target genes *cyp51*, *cyt b*, β-tubulin and SdhB. Using data from a priory study, effective concentration to inhibit 50% of fungal spore germination (EC50) to DMI, QoI and MBC were used to discriminate sensitive and resistant isolates. Molecular mechanisms of resistance were defined using next generation sequencing. For *cyp51*, it was detected 44 nonsynonymous mutation of isolates collected in 2013 year. Molecular characterization for MBC revealed A244E mutations. Molecular analysis detected heterozygous variation in 14 (50%) and 6 (21.4%) homozygous variation isolates, change from glycine to alanine at position 143 (G143A) for QoI target gene while other mutations (F129L and G137R) were not found. Were found 23 point mutation in *S*dhA and 25 in *S*dhB. Homozygous and heterozygous variants were found for *cyp51*, *cyt b*, β-tubulin and *S*dhB fungicide resistance genes. Therefore, these results discoveries may serve as the basis guides for new research management of fungicide resistance for *C. truncatum* in soybean in the future.

Keywords: Anthracnose, DMI, QoI, MBC, SDHI.

4.1 INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is one of the main Brazilian crops, promoting the economic sustainability of the agricutural, improving its cultivated area, year after year. Nevertheless, the large size of planted area with soybeans, and genetic uniformity, increase the occurrence of diseases, some of which have increased frequency and intensity (TECNOLOGIAS, 2013). The genus *Colletotrichum* comprises a large range of important plant pathogenic species that cause pre and postharvest diseases in cereals, grasses, fruits, legumes and crops in worldwide (SUTTON, 1992; WALLER, 1992). Anthracnose disease is caused by *Colletotrichum truncatum* (Schwein.) Andrus & D. Moore (ANDRUS; MOORE; 1935) is an important disease which causes considerable losses. Record losses of productivity caused by this disease, in the 1994 harvest, were recorded in the main soybean producing countries, reaching about 77,500 tons in Brazil (WRATHER et al., 1997). Under high relative humidity and temperatures between 18 ºC and 25 ºC, levels of losses can reach 100% (ALMEIDA; 2005).

The survival of the pathogen occurs in cultural remains, infected seeds (ATHOW, 1973; SINCLAIR; BACKMAN, 1989), weeds and in more than 300 species of host plant (SINCLAIR; BACKMAN, 1989; HARTMAN, MANANDHAR, SINCLAIR 1986; MCLEAN; ROY, 1988; FARR, ROSSMAN, 2017). The typical symptoms in soybeans, are necrotic sunken lesions on stems, pods, petioles and leaves that progress to necrosis and premature fall, causing seed deterioration, seedling death and systemic infection in aerial part (SINCLAIR; BACKMAN, 1989; GOULART, 1997). Anthracnose can be managed using crop rotation, healthy seeds, balanced management of soil potassium fertilization, use of adequate spacing, genetic resistance and chemical control (MEYER; KLEPKER, 2007). Although genetic resistance is more efficient, economical, there is no indication of comercial resistant cultivar, currently in Brazil (GALLI; PANIZZI; VIEIRA, 2006). In this way, the main effetive control strategy for this disease has been the fungicide application.

The fungicides registered for the control of anthracnose in soybean, were used in seeds treatments (PICININI; FERNANDES, 2000). However, several studies have highlighted the efficiency of systemic fungicides in foliar applications, the main chemical groups being quinone outside inhibitor (QoI) and methyl benzimidazole carbamate (MBC) (MEYER; RODACKI, 2005; GAWADE, et al., 2009, PESQUEIRA, BACCHI; GAVASSONI, 2016). In total 51 mixed formulations are labeled for soybean to control antracnose in Brazil (AGROFIT, 2018). Many of the commonly used fungicides are QoI, MBC, demethlylation inhibitors (DMI) and succinate dehydrogenase inhibitor (SDHI) groups.

The QoI fungicide acts inhibition mitocondrial respiration by binding to QoI center of cytochrome b (*cyt b*), a component of electron transport chain in complex III (BECKER et al., 1981). The mechanisms of resistance involves mutations an amino acid substitution from glycine to alanine at position 143 (G143A), from phenylalanine to leucine at position 129 (F129L), and from glycine to arginine at position 137 (G137R) (GISI et al., 2002). The G143A mutation in resistant field isolates have been described for anthracnose (*C. graminicola*) in bentgrass (*Agrostis stolonifera* L.), annual bluegrass (*Poa annua* L.) in Japan and United States (AVILA-ADAME; OLAYA; KÖLLER, 2003). The same point mutation has been reported for *C. cereale* in bentgrass and annual bluegrass in United States (YOUNG et al., 2010), in *C. siamense* resistant to azoxystrobin in peach and blueberry in United States (HU et al., 2015) and recently was observed in *C. gloeosporioides* in strawberry in China (WU et al., 2019). Addicionally, was observed in isolates expressing intermediate resistance had a F129L substitution (YOUNG et al., 2010). Reduced sensitivity of *C. trucantum* to QoI funcigides were initially observed *in vitro* (XAVIER-VALENCIO, 2017).

MBC, including carbendazim and thiophanate-methyl, have been the most commonly used fungicides for anthracnose control. However, benzimidazole-resistant isolates of *Colletotrichum* spp. have been commonly reported in Brazil in several commercial crops (TANAKA; PASSOS; BETTI; 1997; PERES et al.; 2004; SARTORATO; 2006; SARTORI, 2007; DA SILVA LOPES et al., 2018). Frac (Fungicide resistance action committee) classified MBC chemical group, benzimidazoles and thiophanates, as high risk of fungicide resistance (FRAC, 2018). This fungicide group acts by inhibiting the microtubules assembly during nuclear division by binding to β-tubulin protein (SCHROEDER; PROVVIDENTI, 1969; GEORGOPOULOS; DOVAS, 1973; DAVIDSE 1986; DAVIDSE; ISHII, 1995). The molecular mechanism was first observed in *Botrytis cinerea* and this mutation involved a point mutation at codon 198 or 200 in β-tubulin gene, causing resistance to benomyl (LUCK; GILLINGS, 1995). Resistance of *Colletotrichum* spp. has been reported in the last years as *C. truncatum* from pepper, papaya and physic nut were resisant to thiabendazole (TORRES-CALZADA et. al., 2015), *C. siamense* resistant to thiophanate-methyl in peach and blueberry in United States (HU et al., 2015) and recently, *C. siamense* and *C. fruticola* in strawberry and yam were resistant to carbenzim in China (HAN et al., 2019).

DMI fungicides are considered to be alternative to benzimidazole fungicides, and some of them have been label alone or as components of mixtures for the control of anthracnose. DMI inhibitors that have been used as agricultural fungicides include imidazoles and triazoles. DMI fungicides acts on the cell membrane by inhibiting C14 demethylation during formation of the sterol, the main component of the fungal cell membrane (ZIOGAS; MALANDRAKIS, 2015). The mechanisms of resistance of DMI are variable, however, the main mutation occurs at the target site of the cytochrome P450 14-α sterol demethylase (*cyp51*) gene (SCHMITZ et al., 2014; ZIOGAS; MALANDRAKIS, 2015). In *C. truncatum* in pepper the amino acid replaced at position 376 from methionine to leucine (M376L), at position 373 from histidine to asparagine (H373N) prevented the formation of the Fe – N coordinate bond between the heme iron active site and DMI chemical group, tebuconazole or myclobutanil, and apparently contributed to tebuconazole and myclobutanil insensitivity (ZHANG et al., 2017). In a recently genome analysis of *Colletotrichum* spp. revealed two potencial DMI targets, *cyp51A* and *cyp51B*. Both genes were identified in *C. truncatum* and other species. In *cyp51A* gene were found four amino acid variations L280Y, H238R, S302A and I366L and three variations H373N, M376L and S511T in *cyp51B* gene (CHEN et al., 2018).

SDHI fungicides began to be marketed in Brazil in the years of 2013 and 2014 (GODOY; MEYER, 2014). Since than, have been used a mixture of benzovindiflupyr, bixafen, fluxapyroxad with QoI, MBC and DMI chemical groups to control Asian soybean rust (*Phakospora pachyrhizi*). Despite its recent introduction, resistant populations of *P. pachyrhizi* (SIMÕES et al., 2018) already have been reported in Brazil. SDHI fungicides acts on complex II by blocking the ubiquinone binding site in the electron transport chain in the mitochondria. The target enzyme of SDH inhibitors is succinate dehydrogenase, consisting of four subunits (A, B, C and D) (HORSEFIELD; IWATA; BYRNE, 2004). However, the binding site of SDHI inhibitors occur in the B, C and D subunits (KEON; WHITE; HARGREAVES, 1991; BROOMFIELD; HARGREAVES, 1992; MATSSON et al., 1998; SKINNER et al., 1998; MATSSON; HEDERSTEDT, 2001; ITO et al., 2004; LI et al., 2006). The molecular mechanisms of resistance in fungi isolates can occur due to the different amino acid substitutions in different subunits as reported in *P. pachyrhizi* in (SIMÕES et al., 2018) *Podosphaera xanthii* (MIYAMOTO; ISHII; TOMITA, 2010) and *Corynespora cassiicola* (MIYAMOTO et al., 2010) However, in *C. gloesporioides*, *C. acutatum*, *C. cereale* and *C. orbiculare*, despite showed sensitivity to boscalid, fluxapyroxad and fluopyram in YBA agar medium and high polymorphisms into the target subunits SdhB, SdhC, SdhD no resistance mutations were found for *Colletotrichum* spp (ISHII et al., 2016).

Therefore, due to the frequent historic of fungicide resistance to control *C. truncatum*, with a combination of susceptible cultivars and repeated application with the same mode of action, monitoring the resistance of fungicides becomes essential for the management of the disease. In this study, we characterized polymorphisms in the target genes *cyp51,* β-tubulin, *cyt b* and *S*dhA, *S*dhB. These data allow us identify possible *C. truncatum* isolates resitante to DMI, QoI, MBC and SDHI fungicides in soybean.

4.2 MATERIALS AND METHODS

4. 2.1 Isolation and monosporic isolates

Soybean leaflets with symptoms of antracnose were collected in different soybean brazilian regions during 2010, 2011, 2012 and 2013 seasons. In total, 28 isolates of *C. truncatum* from soybean were isolated in the following Brazilian states: Paraná [(661-Francisco Beltrão)], [(895-Laranjeiras do Sul)], [(929-Cascavel)], [(938-Bela Vista do Paraíso)], [(991-Jataizinho)], [(313, 1176, 1177.3-Londrina)], [(1168-Borrazópolis)], [(1179-Bela Vista do Paraíso)], [(1813, 2013-Campo Mourão)], [(2213-Boa Esperança)], [(68.13-Cruzmaltina)], [(86.13, 8713-Tamarana)], [(88.13-Marilândia do Sul)], [(89.13-Faxinal)], [(95.13-Cruzmaltina)], [(97.13-Pitanga)], [(10013-Ponta Grossa)], Mato Grosso [(62.12-Sorriso)], [(59.13-Campo Novo Parecis)], Maranhão [(287-Balsas)], Roraima [(289-Boa Vista)], [(290-Boa Vista)] and [(280, 1713-Unknown)].

The fungi isolates were obtained by indirect isolation following the methodology described by ALFENAS et al., 2007. The fungi tissues were placed in Petri dishes containing potato-dextrose-agar (PDA) culture medium (4 g L^{-1} potato extract, 20 g L^{-1} dextrose, 15 g L^{-1} agar, pH 5.6 ± 0.2). The identification of the species occurred by comparing the morphological characteristics of spores of *C. truncatum* (XAVIER-VALENCIO, 2017).

The oldest isolates (287, 289, 290) belongs to Embrapa Soybean (Brazilian Agricultural Research Corporation) Mycological collection. These were isolated before the intensive use of fungicides, therefore, they were already being cultivated in Petri dishes.

In order to ensure genetic uniformity, monosporic isolates of *C. truncatum* were used. To develop monosporic isolates, Petri dishes with purified isolates were remained under continuous light during 7 days. The aerial mycelium formed in the colonies, was transferred to microcentrifuge tubes containing autoclaved distilled water. Aliquots of 50 µL to 100 µL of the diluted conidia suspension were pipetted and plated in agar-water culture medium (20 g L^{-1} agar) and incubated at 25 ± 2°C and photoperiod 12h:12h during 6 hours. Then, using a stereomicroscope microscope, the conidia germinated was transferred individually to Petri dishes with the PDA culture medium.

4.2.2 Baseline sensitivity to MBC, QoI and DMI

The EC50 values in this study were priori analysed by Xavier-Valencio (2017). Twenty-eight isolates were exposed to different concentrations of MBC, QoI and DMI fungicides to establish the effective concentration (EC50) to inhibit 50% of fungal sporulation. The oldest isolates, 287, 289 and 290, from 1992, 2004 and 2005, respectively, collected prior to the intensive use of fungicides were used for possible levels of comparison between susceptible and resistant isolates.

Spores of *C. truncatum* were exposed to the commercial formulations: MBC [(carbendazim (50% a.i. Bendazol®, Nortox)], QoI [(25% a.i. Pyraclostrobin Comet®; Basf)] and DMI [prothioconazole (25% de a.i Proline®; Bayer CropScience)].

A stock solution of 300 µg / mL was prepared to obtain the following concentrations of the fungicides: 0; 0.0032; 0.016; 0.08; 0.4; 2.0; 50.0 µg / mL. The experimental design was completely randomized.

An aliquot of 50 µL of the conidia suspension at 10^5 conidia / mL^{-1} and other of 50 µL of the different doses of the fungicides were mixed in each 96-well polystyrene microplate. In each microplate a “standard sample control” was added, which was amended with YBS medium (5 g yeast extract L^{-1}, 5 g bacto peptone L^{-1}, 10 g sodium acetate L^{-1}) + 50 µL of the different concentrations of the commercial formulations that was used to calculate the difference between final absorbance value of the “standard sample control” and the absorbance of the different doses of the fungicides + spores. For each isolate and fungicide concentration, four replicate wells were used. The experiment was performed once.

The microplates were sealed with adhesive film and incubated at 180 rpm in shaker for 40 minutes. After that, the microplates were conditioned in a growth chamber at 25 ºC and 12 h/12 h photoperiod for 7 days. The absorbance of each sample was measured on a microplate reader (ASYS, Eugendorf, Austria) with a wavelength of 540 nm. The linear regression model values were quantified following the methodology described by Xavier-Valencio (2017).

Based on the sensitivity differences of the isolates to the fungicides tested, the isolates were classified into three phenotypes: Highly Resistant (HR), $EC_{50} \geq 50$ µg mL^{-1}; Moderately Resistant (MR), 50 µg mL^{-1} $< EC_{50} <$ 1.0 µg mL^{1}; and Sensitive (S), $EC_{50} \leq 1.0$ µg mL^{-1} (EDGINGTON; KHEW; BARRON, 1971).

4.2.3 DNA extration

After the EC50 establishment, sensitive and resistant to fungicide monosporic isolates, were grown in potato and dextrose liquid medium for mycelial filtration. In the sequence, sample DNA were extracted for all samples.

Three mycelium discs of 7 mm were added into potato-dextrose (PD) liquid medium and grown (4 g L^{-1} potato extract, 20 g L^{-1} dextrose) during 7 to 12 days. Thus, the mycelial mass was filtered through vacuum filtration. The dried mycelium were frozen in liquid nitrogen, macerated and stored in a 1.5 mL microcentrifuge tube at -80 ºC. The DNA was extracted following the modified CTAB-DNA extraction.

The macerated tissue was homogenized with 1000 µL of extraction buffer (1M Tris HLC (pH 8.0), 0.5M EDTA (pH 8.0), 5M NaCl, 0.1% β-mercaptoethanol, 200 µL CTAB to 5%) and incubated in a warm bath at 65 ° C for 60 minutes, with shaking and inversion of the microcentrifuge tubes every 15 minutes. The samples were centrifuged at 6000 rpm for 10 minutes. 700 µL of the supernatant was collected and transferred to a new 1.5 mL microcentrifuge tube. Then 700 µL of phenol: chloroform solution: iso-amyl alcohol (24: 24: 1) was added. After further centrifugation at 10,000 rpm for 5 minutes, 700 µL of the supernatant was transferred to a new microcentrifuge tube. For DNA precipitation, 500 µl of isopropanol was added and then the samples were homogenized in vortex and incubated at -20 ° C for 30 minutes. The DNA pellet was washed with 70% ethanol and resuspended in 60 µl of mili-q water + 6 µl of sodium acetate (3M) and 120 µl of 100% ethanol and was stored at 4 °C. Thus, the samples were centrifuged at a speed of 14,000 rpm for 5 minutes. The supernatant was discarded and pellet was washed again with 70% ethanol and dried. Samples were resuspended in 80 µL of mili-q + RNAse water at the final concentration of 80 µg / mL. DNA quality was assessed by electrophoresis on 1% agarose gel.

4.2.4 Primers design, PCR and fragments purification

The primer pairs *cyp51* (Genbank / EMBL access XM_007287180.1), β-tubulin (Genbank / EMBL access M34491.1), *cyt b* (Genbank / EMBL access KM885303.1) and *S*dh*B* (Genbank / EMBL access XM_007596512.1) were designed using sequences of *C. gloeosporioides*, *C. graminicola*, *C. siamense*, and *C. fioriniae*, respectly, deposited at database of the National Center of Biotechnology Information (NCBI). For the *S*dh*A* gene were used KES 503 (5 'CTCGTGGTGAGGGTGGTTACCT 3') and KES 504 (5 'CGCTTGAAAGGTG GAACAGC 3') primer pairs described by Miyamoto; Ishii; Tomita,. 2010.

The amplicons length range from 808 to 1629 and were estimated by identification of the main mutations reported previously at literature. To achieve this, the primers forward and reverse were design to cover the maximum size of each the gene analysed.

The primers described were designed using the Primer 3 plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The specificity of the primers was compared by the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Primer pairs forward (F) and reverse (R) were described as follows: MBC [(primer MBC_F) 5'- GATCAAGATGGAGTATTCCGC-3'; (primer MBC_R) 5'-ATCTCATCCATGCCCTCTCC-3']; QoI [(primer QoI_F) 5'-

TGAAATTTCGGTTCCTTACTTG-3’; (primer QoI_R) 5’-GGCACTAAACATAGCAAGAACTCC-3’]; DMI [(primer DMI_F), 5’-TACACCGTCTTGACCACTCCT-3’ (primer DMI_R) 5’-CACGCTTCTCCCAGTAAATG-3’]; Sdh subunit B [(primer *S*dhB_F) 5’-CCTGCCGTCACTATTCCTC-3’; (primer *S*dhB_R) 5’-AAGCCATCTGCTTCTTGATCTC-3’].

PCR reactions for the target genes were prepared to a final volume of 50 µL containing 100 ng DNA from each sample, 1 µM of each primer, 10 µM dNTPs (Thermo Fischer Scientific), 5 X Phusion HF Buffer (Thermo Fischer Scientific) and 0.5 U Phusion DNA Polymerase (Thermo Fischer Scientific). The optimum annealing temperature was determined for each primer (Supplementary table S.1). Annealing temperature ranged from 57ºC to 63ºC, followed by extension time which ranged from 30 to 60s and 26 to 35 cycles for amplification. All PCR reactions were performed on the MWG Biotech Inc Primus 96 thermal cycler. The PCR products were separated by electrophoresis using a 1% agarose gel and visualized under UV 360 nm light.

The PCR amplicons for all samples for each gene were mixed and then purified using Nucleospin gel and Clean-up PCR (Macherey-Nagel) following the manufacturer's recommendations. The amplicon pools were quantified with the Qubit® (Thermo Fischer Scientific) for a final concentration of 3 ng / µL. After, the amplicon sequences were sent to Institute of Clinical Molecular Biology (IKMB) for processing.

4.2.5 NGS library preparation

The Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) was used to prepare the amplicon library following the manufacture’s instructions. The final 28 libraries were distributed into four lanes on a flow cell for sequencing on an Illumina HiSeq 2500 system, utilizing a 150 bp paired-end reads length with an expected coverage of 30X.

The initial base calling, barcodes/adaptors trimming, quality filtering of the reads generated with the Illumina analysis pipeline (Fastq format) were performed using Trimmomatic 0.36 software (BOLGER; LOHSE; USABEL, 2014) and checked by FastQC software (ANDREWS et al., 2010). High-quality reads generated from all *C. truncatum* genes sequenced from isolates were concatenated and *de novo* assembled with Trinity software (HAAS et al., 2013). The *C. truncatum* genes of interest were identified by a blas2seq analyse (TATUSOVA; MADDEN, 1999)*,* applying *cpy51*, *cyt b*, β-tubulin, *S*dhA and *S*dhB sequences from close related fungi species. The five *C. truncatum* genes sequences were used, together with available sequence information from NCBI and MycoCosm - JGI database (https://genome.jgi.doe.gov/programs/fungi/index.jsf), to build the complete reference sequence for these genes (Supplementary table S5.1). *C. truncatum cpy51*, *cyt b*, β-tubulin, *S*dhA and *S*dhB gene structure were predicted using Softberry software (SOFTBERRY Inc., NY) (Figure 4.1).

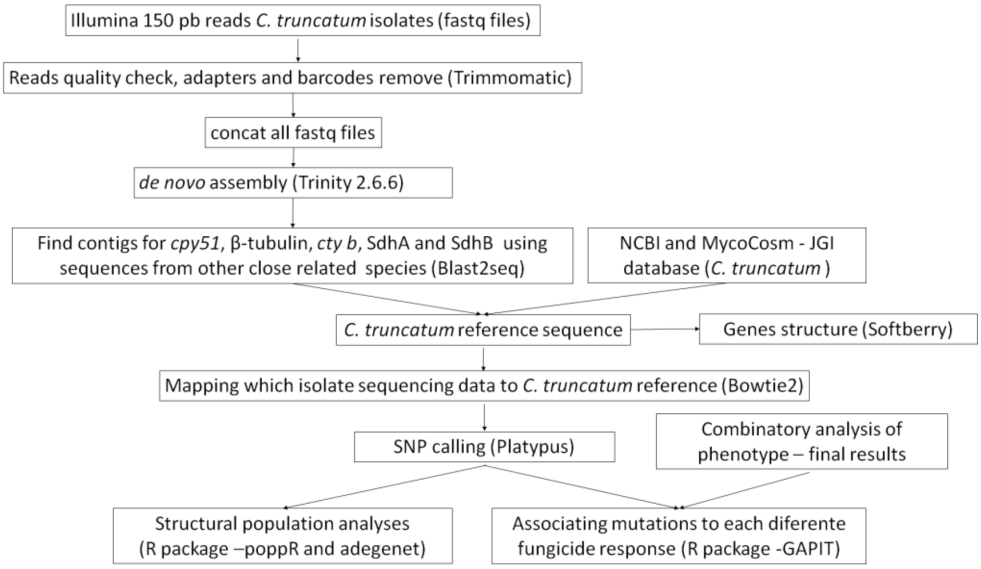


Figure 4.1. *In silico* analysis summary workflow for genetic characterization of *cpy51*, *β-tubulin*, *ctyb*, *S*dhA and *S*dhB genes from *C. truncatum* isolates.

High-quality reads were then aligned, by isolate, to the obtained *C. truncatum* reference sequence for the seven genes using the software bowtie2 (LANGMEAD; SALZBERG, 2012). The mapped reads were processed using Picard tools version 2.11 (Java, http://broadinstitute.github.io/picard/) to remove duplicate values, and a binary file of the extension bam representing the assembled genome of each sequenced isolate was generated. For SNP/InDel calling, we used Platypus version 0.8.1 (RIMMER et al., 2014) with minimum for two reads coverage. InDels were removed and >20% of missing data were removed from variant calling file (vcf) sing Vcftools version 0.1.12b (DANECEK et al. 2011) and remaining missing data were imputed with software Beagle 4 (AYRES et al., 2011).

4.2.6 QoI Sanger sequencing for validation

To validate NGS methodology 11 *C. truncatum* isolates were randomly selected and sequenced using the primers described for *cytb* gene. PCR reactions were prepared to a final volume of 50 µL containing 100 ng DNA from each sample, 1 µM of each primer, 10 µM dNTPs (Thermo Fischer Scientific), 5 X Phusion HF Buffer (Thermo Fischer Scientific) and 0.5 U Phusion DNA Polymerase (Thermo Fischer Scientific). PCR cycles were 35 cycles of initial denaturation at 98 °C for 10s, annealing at 57 °C for 60s, elongation at 72 °C for 30s and final extension for 8 minutes at 72 °C.

PCR products were adjusted to 25 ng / µL in the Nanodrop TM OneC spectrophotometer and were purified using Nucleospin gel and Clean-up PCR (Macherey-Nagel) following the manufacturer's recommendations. All PCR reactions were performed on the MWG Biotech Inc Primus 96 thermal cycler.

Sanger PCR reaction consisted of 0.6 µL of alkaline phosphatase (FAP) (Thermo Fisher Scientific) (1 U/ mL) enzyme, Exonuclease I (Thermo Fisher Scientific) (20 U / µL) 0.15 µL, 8 µL PCR products and 1.25 µL of dH2O (ChemSolute). The final volume was performed at 10 µL. The digestion reaction consisted of 10 min of purification at 37 ° C and 15 min inactivation of the enzymes at 75 ° C. The reaction consisted of 0.7 µL of Big Dye Terminator v3.1 (Applied Biosystems), 1.5 µL of Big Dye Terminator v3.1, 1.5 µL 5X sequencing buffer (Applied Biosystems), 1 µL (3,2 µM) of each primer pairs and 4.8 µL of dH_2O (ChemSolute). Sequencing amplifications program were carried out under following conditions: 1 min of initial denaturation at 96 °C; 25 cycles of denaturation of 10 s at 96 °C, annealing of 5 s at 50 °C and extension of 4 min at 60 °C; followed by a final extension at 10 °C. The sequencing was performed using 3730xl DNA Analyzer (Applied Biosystems, Foster, CA).

The nucleotide sequences were aligned with the reference sequence (NCBI/ access KM885303.1) of *C. siamense* mitochondrial *cytb* gene and translated into amino acid sequences using Bioedit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

4.2.7 Genetic analysis

Population genetic analyses were performed using ‘poppr’ (KAMVAR et al. 2014) and “adegenet” R packages (JOMBART, 2008). Minimum spanning network was applied to visualize population structure by haplotypes according to their origin location using the bitwise’s genetic distance. An UPGMA (SCHLIEP, 2010) distance tree was build to identify similarity into *C. truncatum* groups, genetically related using poppr package (KAMVAR et al. 2014).

4.3 RESULTS

4.3.1 EC50 of spore germination to DMI, MBC and QoI fungicides

The EC50 values in *C. truncatum* collected from soybean were determined previously (XAVIER-VALENCIO, 2017). The EC50 values of the isolates ranged from 0.051 to 4.67 µg / mL a.i. for prothioconazole (DMI), 1.89 to 2.93 µg / mL a.i. for carbendazim (MBC) and 0.36 to 5.32 µg / mL a.i. for pyraclostrobin (QoI) (Figure 4.2). The majority of isolates treated with carbendazim (89%) had being classified as moderately resistant or higly resistant. For pyraclostrobin 44% were considered sensitive, while 55% were considered moderately resistant. For prothioconazole spore germinnation of all the isolates were inhibited to 50 µg / mL a.i. (Figure 4.2, suplementary table S4.6.2.).

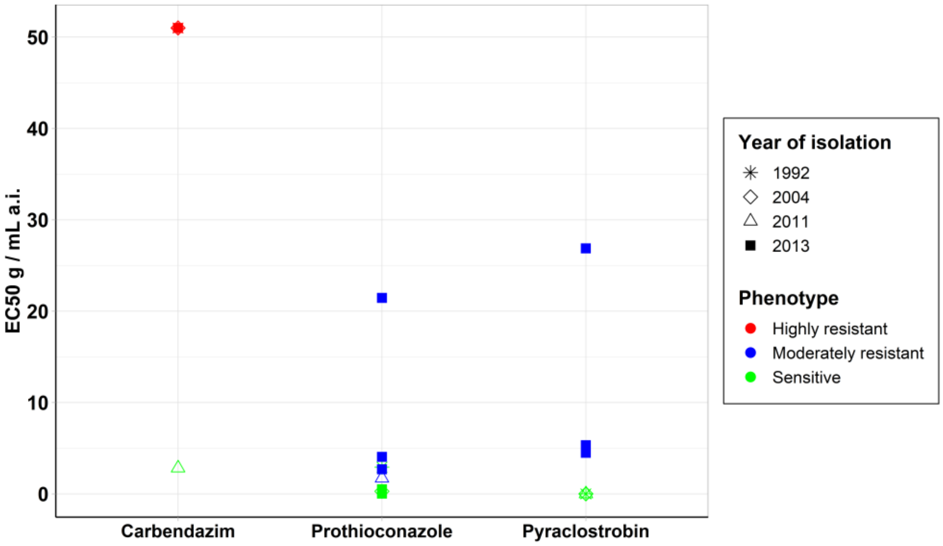


Figure 4.2. EC50 of *C. trucatum* isolates from soybean. Colors scale represents isolate classification into different phenotype. Different geometric shapes represents the fungicides carbendazim (MBC), prothioconazole (DMI) and pyraclostobin (QoI).

4.3.2 Molecular characterization of *cyp51,* β-tubulin, *cyt b*, SdhA and SdhB fungicide target genes

Molecular characterization of fungicide target genes from *C. truncatum* isolates resulted in 210 synonymous and nonsynonymous mutations of which, were found 56 mutations for *cyp51* gene, 4 mutations for β-tubulin gene, 29 for *cyt b gene,* 69 for SdhA and 52 for SdhB (Supplementary table S4.6.2). The genes structure of *C. truncatum* for *cyp51*, β-tubulin, *cyt b*, SdhA and SdhB fungicide resistance target genes resulted in a 1852 bp, 2074 bp, 1179 bp, 1737 bp and 1867 bp fragment length, respectively (Figure 4.3).

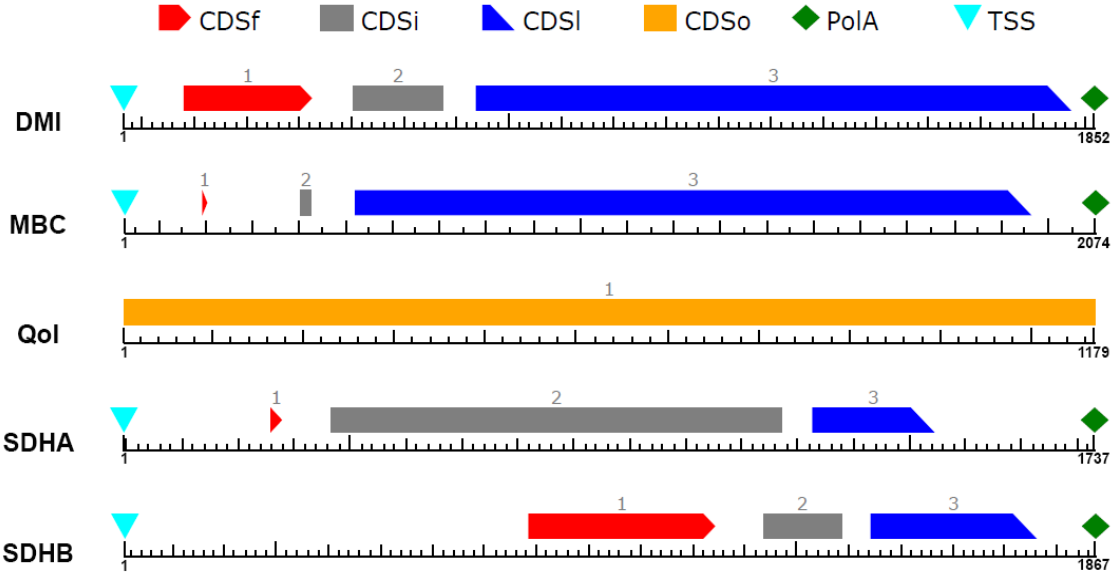


Figure 4.3. Prediction of *cyp51* (DMI), β-tubulin (MBC), *cyt b* (QoI) Sdh*A* and Sdh*B* genes using genomic DNA of *C. truncatum* isolates. CDSf - First (Starting with Start codon); CDSi - internal (internal exon), CDSl - last coding segment, ending with stop codon); CDSo: coding sequence, solo - predicted gene with a single exon; PolA: polyadenylation signal sequence (AATAAA) and TSS - Position of transcription start (TATA-box position and score).

Nucleotide sequencing for *cyp51* target site revelead 44 heterozygous variant at codon STOP192S, P194L, P196L, T200M, L204S, R229P, R229L, R230S, F236S, C259F, I264T, A266D, P268R, R277P, R277L, S279F, T302S, A307V, L312S, P321R, P321L, R349P,A353R, P354L, S356L, S358C, S358F, F377S, A379V, L409P, L413F, V425E, I446T, P466R, R474T, T483I, T486M, R495H, P496L, T497I, P502L, G506A, L512S, A531V. The T497I mutation was detected for isolate 1177.3 and 929. Heterozygose variant was found for all mutations (Figure 4.4).

In the β-tubulin target was found only one heterozygose mutations at codon A244E. Mutant isolates had nucleotide sequencing substitutions from (GCG/Alanine to GAC/ Glutamic acid at codon 244). The A244E mutation was detected 13 isolates: 10013, 1168, 1176, 1813, 2013, 2213, 280, 313,62.12,661,68.13, 86.13, 87.13 (Figure 4.5).

Heterozygous variation was found at position 143 and was detected for all isolates. G143A mutation were identified in 20 isolates, which means 71.43% of *C. truncatum* isolates. In this mutation, were replaced in the nucleotide sequence of GGT (sensitive) to GCT (resistant) at amino acid position 143. No isolates contained the F129L or G137R mutation (Figure 4.5).

In our molecular analysis, study we also analyzed the mutations in the *SdhA* gene. We found the follow heterozygous variations: F68Y, A76P, K178N, H185L, L188I, A189E, E207D, A213S, L302P, L303P, P304L, A307V, E312A, E312V, S321I, S321T, S329N, C332S, L333P, R335K, L338P, L354W (Figure 4.5).

For gene SdhB the nucleotide sequencing revelead heterozygous variant at codon G178R, P181A, P187S, P194A, H197Y, C199F, I223V, V233A, H240Y, V249M, L252V, STOP257R, H264Y, P266S, R282STOP, Q284STOP, T287S, D291H, P295S, D299H, STOP301Q, P307S, R313G, Q314STOP, Y316H (Figure 4.5).

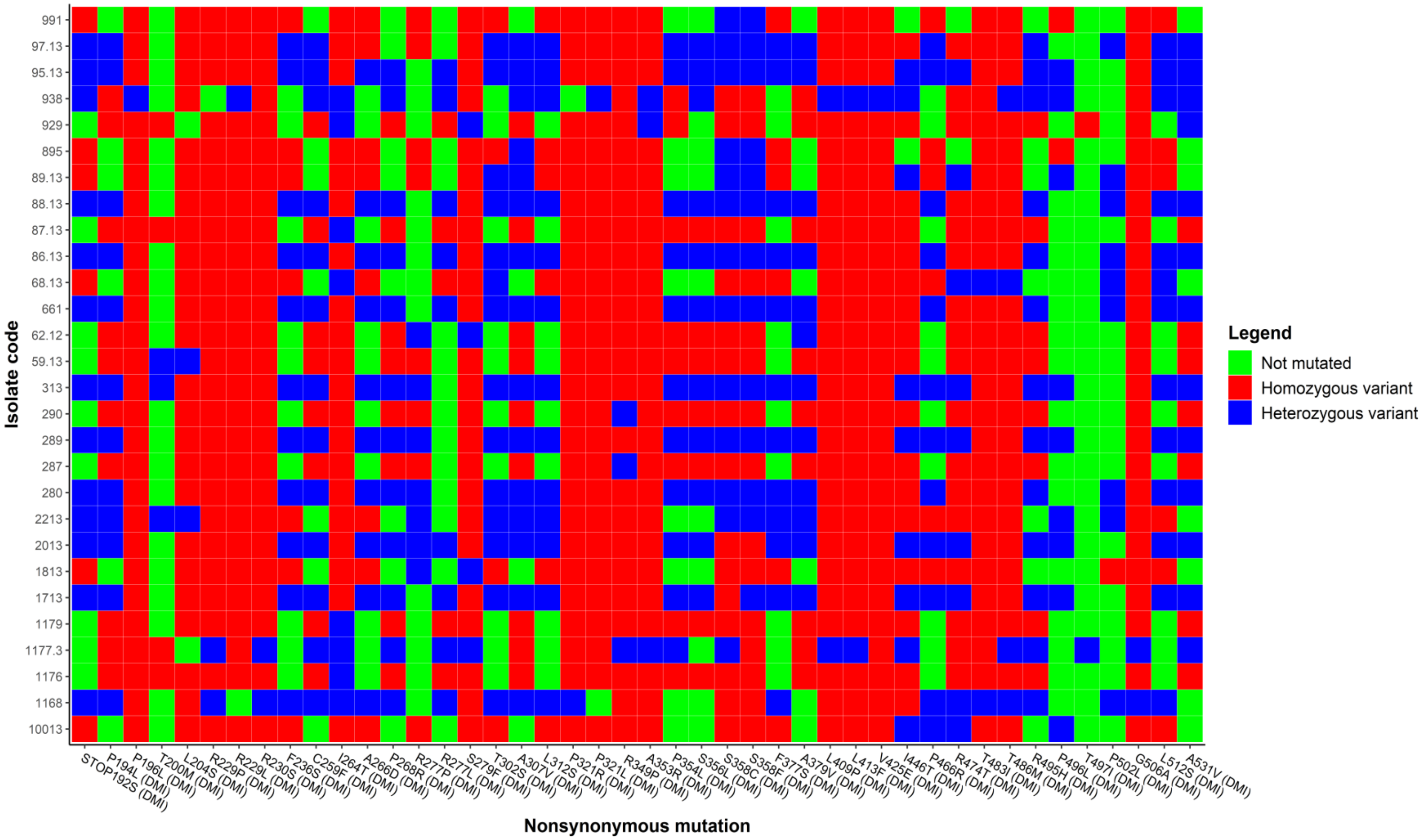


Figure 4.4. Heatmap of 44 nonsynonymous mutations *cyp51* (DMI) gene, for fungicide target site resistance in *C. truncatum* isolates. Colors on the map represent genetic variations for each isolate.

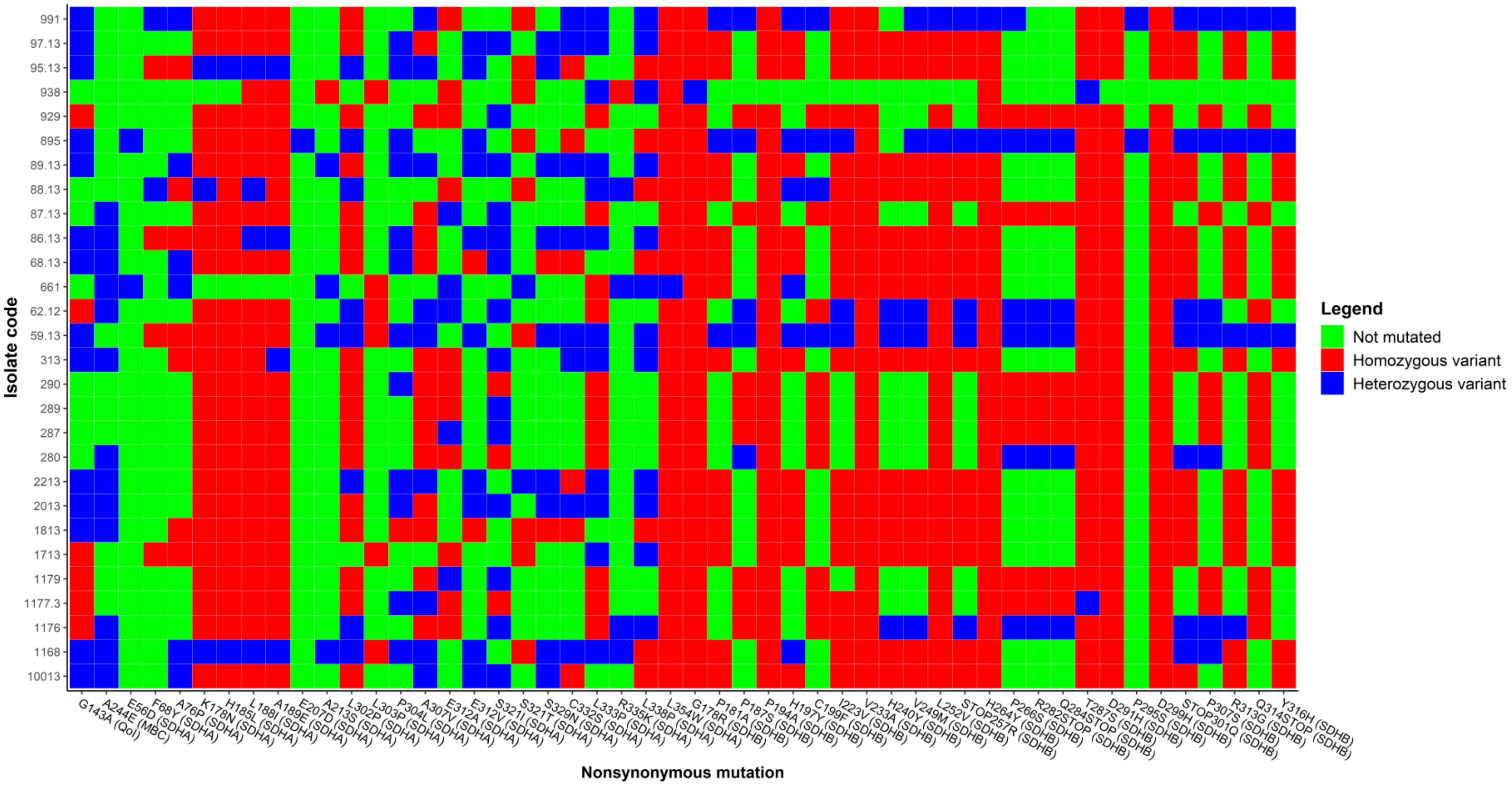


Figure 4.5. Heatmap of nonsynonymous mutations *cyt b*, β-tubulin, SdhA and SdhB genes for fungicide target site resistance in *C. truncatum* isolates. Colors on the map represent genetic variations for each isolate.

4.3.3 QoI sequencing validation using Sanger sequencing method

Sanger sequencing was performed for 11 *C. truncatum* isolates randomly selected for *cyt b* gene. Among those, the isolates 1813, 290, 87.13, 89.13, 895 and 938 were considered wild (Supplementary figure S4.6.1). The heterozygous variations observed in the NGS for the isolates 89.13, 1813 and 10013 were also observed at nucleotide amino acid chromatogram files (Supplementary figure S4.6.2). Analyzing chromatogram peaks in the region of position 143 it was possible to verify the occurrence of simultaneous peaks indicating the presence of the C alleles and G at codon 428 in 50% of the isolates analyzed (Supplementary figure S4.6.2). Divergent results were verified between the metologies analyzed. Cromatogr waves of Sanger sequencing showed the presence of noise and heterozygosis at codon G143A, which was clearly identified by the NGS**.**

4.3.4 Relationship among *C. truncatum* genotypes

The minimum spanning network of *C. truncatum* distinguished the most frequent genectic group and were placed close to one another (Figure 6). No isolate shared the same haplotype. The global network showed from Maranhão (MA), Paraná (PR) and Roraima (RR) were distributed all across the network and were clustered around the most frequent genotype. Bitwise genetic distance in the minimum spanning network varied from 0.008 to 0.343 (Figure 4.6).

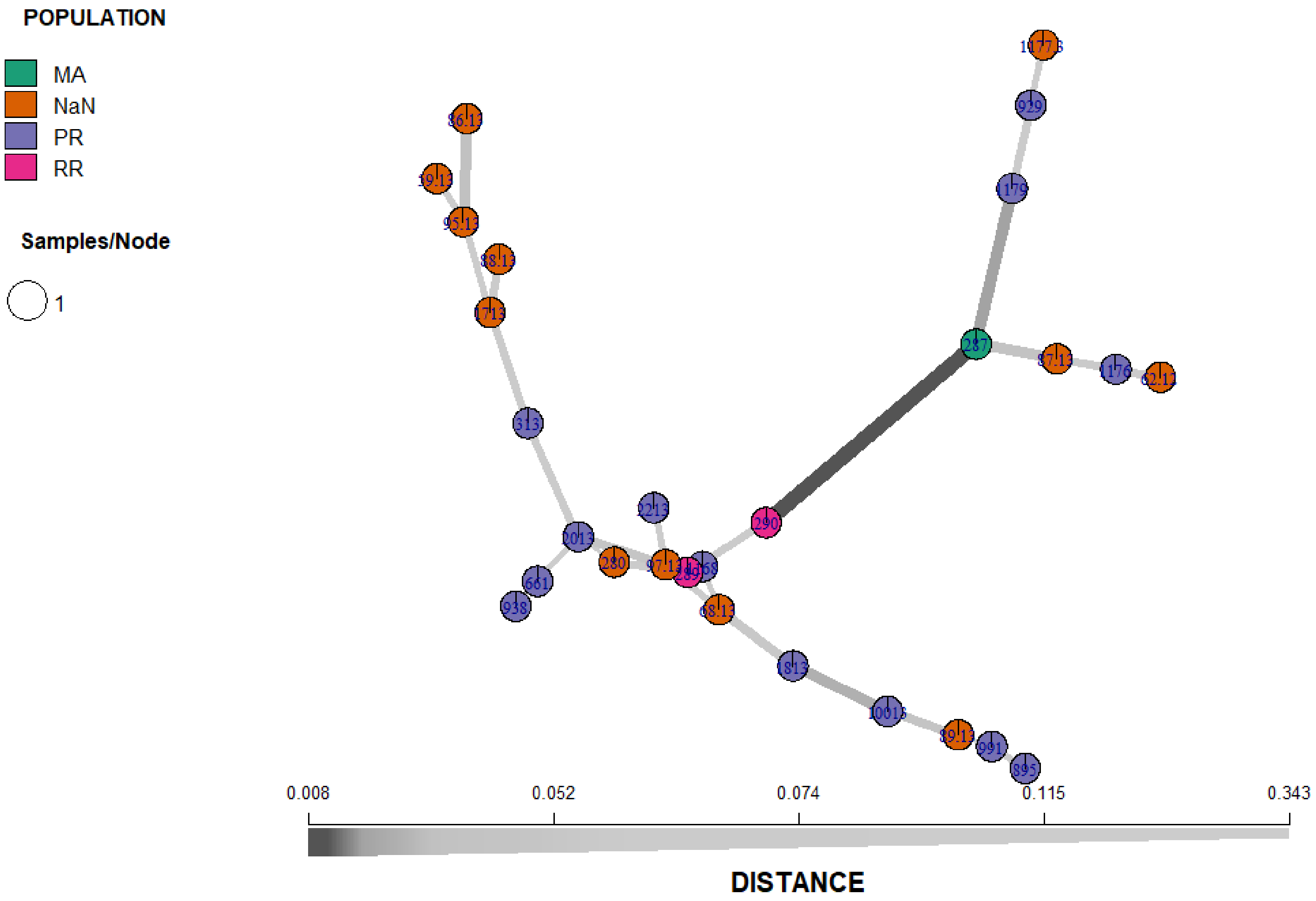


Figure 4.6. Median spanning network of *C. truncatum* from soybean for fungicide resistance target genes *cyp51*, β-tubulin, *cyt b*, SdhA and SdhB. Each circle represents a haplotype. Node sizes are proportional to the number of isolates origin. Coloured slices represent different geographic origin.

4.4 DISCUSSION

In this research, we analyzed possible point mutation in *C. truncatum* isolates from soybean to DMI, QoI and MBC and SDHI fungicides. This is the first next generation sequencing study in soybean to identify possible fungicide resistance in *C. truncatum* to fungicide resistance target genes for *cyp51,* β-tubulin, *cyt b* and SdhB. Using next generation sequencing we could confirm heteroplasmid is occuring in *C. truncatum* in *cyp51*, β-tubulin, *cyt b, S*dhA and *S*dhB genes.

Sequencing analysis revealed 44 possible point mutation in *cyp51* gene. However, all sample of *C. truncatum* were sensitive or moderately resistant to prothioconazole fungicide, therefore, the mutations did not correlate with prothyoconazol microtiter bioassay. The same results had been observed in *C. truncatum* collected from peach, soybean, citrus and begonia in China (CHEN et al., 2018). These phenomenon suggests that other unknown mechanisms different of point mutations may confer resistance to DMI in *Colletotrichum* spp (CHEN et al., 2018). In a study, of *C. truncatum* species *cyp51* sequencing revelead two *cyp51* genes, *cyp51*A e *cyp51*B (CHEN et al., 2018). An overexpression of *cyp*51A are more common in response to azole resistance, in fungi that carry both mutations *cyp51*A and *cyp51B* genes (BRUNNER et al., 2016).

Phenotype response of carbendazim bioassy was not correlate with genotype sequencing. Despite the most isolates classified as highly resistant, in this study, the main mutaitons E198A and F200Y were not found. One mutation was reported at codon 244, which change alanine to glutamic acid (A244E). This mutation could result MBC fungicide resistant and was observed in 46% of the isolates analysed.

The highest EC50 for pyraclostrobin value was 5.32 µg / mL a.i. and the isolates were classified as moderately resistant. Although, the point mutation at codon 143 in *cytb* gene that alter the glycine to alanine (G143A), were identified in more than 71% of *C. truncatum* isolates. And also, there was no correlation between phenotype methodology and genotype sequencing method for identify point mutation in *cytb* gene.

Analysing the mechanisms of resistance in sdhi genes we found 23 substitutions in SdhA and 25 *S*dhB genes. For all mutations in SDH genes, homozygous and heterozygous variations were observed. This genomic characterization has also been reported in *Rizoctonia solani* (MU et al., 2013) and in *R. cerealis* (SUN et al., 2017). High polymorphism into SdhB gene were also observed in *C. gloeosporioides*, *C. acutatum*, *C. cereale* and *C. orbiculare* however, no resistance mutations were found for *Colletotrichum* spp for SDHI fungicides (ISHII et al., 2016).

The minimum spanning network showed that *C. truncatum* mutate isolates to QoI, MBC and DMI is widely spread in soybean fields. Higher variability in *C. truncatum* support the premises that *cyp51*A can adapt more rapidly under selection pressure and *cyp51*B has more conserve function in *Colletotrichum* spp (CHEN, 2018).

This research shows possible new fungicide resistance mutation in β-tubulin gene at codon A244E. We also found in QoI fungicides G143A mutation in *cyt b* gene. These results suggest that isolates of *C. truncatum* to DMI, MBC and QoI at Brazilian soybean field and this increasing the risk for the selection pressure for resistant isolates. Despite, SdhA gene does not confer fungicide resistance substitutions was related 23 new point mutation. In addition, we also found 25 amino acid substitution in SdhB, could be associated with possible potential mutations, which confer SDHI fungicide resistance. In addition, heterozygous variants were confirmed for all genes analyzed. Therefore, these discoveries in *C. truncatum* in soybean may serve as the basis guides for new research management of fungicide resistance screening in the future.

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4.5 REFERENCES

ALFENAS, A. C.; FERREIRA, F. A.; MAFIA, R. G.; GONÇALVES, R. C. **Isolamento de fungos fitopatogênicos**. Métodos em fitopatologia. Viçosa: Ed. da UFV, p. 54, 2007.

AGROFIT 2018. Sistema de agrotóxicos fitossanitários. Available at: http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. Accessed in March, 25, 2018.

ALMEIDA, A. M. R.; FERREIRA, L. P.; YORINORI, J. T.; SILVA, J. F. V.; HENNING, A. A.; GODOY, C. V.; COSTAMILAN, L. M.; MEYER, M. C. Doenças da soja. In: KIMATI, H.; AMORIM, L.; REZENDE, J. A. M.; BERGAMIN FILHO, A.; CAMARGO, L. E. A. **Manual de Fitopatologia: doenças de plantas Cultivadas**. 4. ed. vol. 2. São Paulo: Editora Agronômica Ceres. 570-588 p, 2005.

ANDREWS, S. **FastQC: a quality control tool for high throughput sequence data** (2010). Disponível em: http://www.bioinformatics.babraham.ac.uk/projects/fastqc. Accessed in: March. 2018.

ANDRUS, C. F.; MOORE, W. D. *Colletotrichum truncatum* (Schw.), n. comb., on garden and lima beans. **Phytopathology**, 25, p.121-125, 1935.

ATHOW, K. L. Fungal diseases. In: Caldwell, B.E. (Ed.) Soybeans: Improvement, production and uses. **American Soc. Agronomy**, Madison, USA, p. 459-489, 1973.

AVILA-ADAME, C.; OLAYA, G.; KÖLLER, W. Characterization of *Colletotrichum graminicola* isolates resistant to strobilurin-related QoI fungicides. **Plant Disease**, v. 87, p.1426-1432, 2003.

AYRES, D. L.; DARLING, A., ZWICKL, D. J.; BEERLI, P.; HOLDER, M. T.; LEWIS, P. O.; RAMBAUT, A. BEAGLE: an application programming interface and high-performance computing library for statistical phylogenetics. **Systematic biology**, v. 61, n. 1, p. 170-173, 2011.

BECKER, W. F.; BECKER, W. F., VON JAGOW, G., ANKE, T., STEGLICH, W. Oudemansin, strobilurin A, strobilurin B and myxothiazol: new inhibitors of the bc 1 segment of the respiratory chain with an E-β-methoxyacrylate system as common structural element. **FEBS letters**, v. 132, n. 2, p. 329-333, 1981.

BOLGER, A.M.; LOHSE, M.; USADEL, B. Trimmomatic: a flexible trimmer for Illumina sequence data**. Bioinformatics**, v. 30, n. 15, p. 2114-2120, 2014.

BROOMFIELD, P. E.; HARGREAVES, J. A. A single amino-acid change in the iron-sulphur protein subunit of succinate dehydrogenase confers resistance to carboxin in *Ustilago maydis*. **Current genetics**, v. 22; n. 2, p. 117-121, 1992.

BRUNNER, P. C.; STEFANSSON, T. S.; FOUNTAINE, J.; RICHINA, V.; MCDONALD, B. A. A global analysis of CYP51 diversity and azole sensitivity in *Rhynchosporium commune*. **Phytopathology**, v. 106, n. 4, p. 355-361, 2016.

CHEN, S., WANG, Y., SCHNABEL, G., PENG, C. A., LAGISHETTY, S., SMITH, K., LUO, C.; YUAN, H. Inherent Resistance to 14α-Demethylation Inhibitor Fungicides in *Colletotrichum truncatum* Is Likely Linked to CYP51A and/or CYP51B Gene Variants. **Phytopathology**, v. 108, n. 11, p. 1263-1275, 2018.

DA SILVA LOPES, L. N.; SILVA, A. S.; DE OLIVEIRA PEREIRA, C. C.; DE MENEZES, I. P. P.; MALAFAIA, G.; DA PAZ-LIMA, M. L. Sensibilidade de Isolados de *Colletotrichum gloeosporioides* a Fungicidas. **Multi-Science Journal**, v.1, n.1, p. 106-114, 2018.

DANECEK, P., AUTON, A., ABECASIS, G., ALBERS, C. A., BANKS, E., DEPRISTO, M. A.; MCVEAN, G. The Variant Call Format and VCFtools. **Bioinformatics**, v. 27, n. 15, p. 2156-2158, 2011.

DAVIDSE, L. C. Benzimidazole fungicides: mechanism of action and biological impact. **Annual Review of Phytopathology**, v.24, p.43–65, 1986.

DAVIDSE, L.; ISHII, H. Biochemical and molecular aspects of benzimidazoles, N -phenylcarbamates and N -phenylformamidoximes and the mechanisms of resistance to these compounds. **Modern selective fungicides**, p. 305–322, 1995.

EDGINGTON, L. V.; KHEW, K. L.; BARRON, G. L. Fungitoxic spectrum of benzimidazole compounds. **Phytopathology**. v. 61, p. 42-44, 1971.

FARR, D. F.; ROSSMAN, A. Y. 2017. Fungal Databases, U.S. National Fungus Collections, ARS, USDA. Accessed on June 13, 2017, from https://nt.ars-grin.gov/fungaldatabases/

FRAC- Fungicide Resistance Action Committee 2018. **List of plant pathogenic organisms resistant to disease control agents.**

GALLI, J. A.; PANIZZI, R. C.; VIEIRA, R. D. Avaliação da resistência de variedades de soja a *Colletotrichum dematium* var. *truncata*. In: Congresso Brasileiro de Soja, 4, 2006, Londrina. Resumos. Londrina: Embrapa Soja, p.101, 2006.

GAWADE, D. B.; SURYAWANSHI, A. P.; PAWAR, A. K.; APET, K. T.; DEVGIRE, S. S. Field evaluation of fungicides, botanicals and bioagents against anthracnose of soybean. **Agricultural Science Digest**, v.29, n.3, p.174-177, 2009.

GEORGOPOULOS, S. G.; DOVAS, C. Occurrence of *Cercospora beticola* strains resistant to benzimidazole fungicides in northern Greece**. Plant Disease**, v. 57, p. 321–324, 1973.

GISI, U.; SIEROTZKI, H.; COOK, A.; MCCAFFERY, A. Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. **Pest management science**, v. 58, n. 9, p. 859-867, 2002.

GODOY, C. V.; MEYER, M. C. Informativo fundação meridional. 2014. Available at: http://fundacaomeridional.com.br/artigos/2014/11/10/resistencia-a-fungicidas-na-cultura-da-soja. Accessed on September 01, 2015.

GOULART, A. C. P. Fungos em sementes de soja: detecção e importância. EMBRAPA, Empresa Brasileira de Pesquisa Agropecuária, Centro de Pesquisa Agropecuária do Oeste, Ministério da Agricultura e do Abastecimento, vol 11, 1997.

HAAS, B. J., PAPANICOLAOU, A., YASSOUR, M., GRABHERR, M., BLOOD, P. D., BOWDEN, J.; MACMANES, M. D. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. **Nature protocols**, v. 8, n. 8, p. 1494, 2013.

HAN, Y. C.; ZENG, X. G.; XIANG, F. Y.; ZHANG, Q. H.; GUO, C.; CHEN, F. Y.; GU, Y. C. Carbendazim sensitivity in populations of *Colletotrichum gloeosporioides* complex infecting strawberry and yams in Hubei Province of China. **Journal of integrative agriculture**, v. 17, n. 6, p. 1391-1400, 2018.

HARTMAN, G. L., MANANDHAR, J. B.; SINCLAIR, J. B. Incidence of *Colletotrichum* spp. on soybeans and weeds in Illinois and pathogenicity of *Colletotrichum truncatum*. **Plant Disease**, v.70, n.8, p.780-782, 1986.

HORSEFIELD R.; IWATA S.; BYRNE B. Complex II from a structural perspective. **Current Protein & Peptide Science**, v.5, p.107–118, 2004.

HU, M. J.; GRABKE, A.; DOWLING, M. E.; HOLSTEIN, H. J.; SCHNABEL, G. Resistance in *Colletotrichum siamense* from peach and blueberry to thiophanate-methyl and azoxystrobin. **Plant disease**, v. 99, n. 6, p. 806-814, 2015.

ISHII, H.; ZHEN, F.; HU, M.; LI, X.; SCHNABEL, G. Efficacy of SDHI fungicides, including benzovindiflupyr, against *Colletotrichum* species. **Pest management science**, v.72, n.10, p.1844-1853, 2016.

ITO, Y.; MURAGUCHI, H.; SESHIME, Y.; OITA, S.; YANAGI, S. O. Flutolanil and carboxin resistance in *Coprinus cinereus* conferred by a mutation in the cytochrome b560 subunit of succinate dehydrogenase complex (Complex II). **Molecular Genetics and Genomics**, v.272, n.3, p. 328–335, 2004.

JOMBART, T. adegenet: a R package for the multivariate analysis of genetic markers. **Bioinformatics**, v. 24, n. 11, p. 1403-1405, 2008.

KAMVAR, Z. N.; TABIMA, J. F.; GRÜNWALD, N. J. Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. **PeerJ**, v. 2, p. e281, 2014.

KEON, J. P.; WHITE, G. A.; HARGREAVES, J. A. Isolation, characterization and sequence of a gene conferring resistance to the systemic fungicide carboxin from the maize smut pathogen, *Ustilago nuda*. **Current Genetics**, v.19, n.6, 475–481, 1991.

LANGMEAD, B.; SALZBERG, S. L. Fast gapped-read alignment with Bowtie 2. **Nature methods**, v. 9, n. 4, p. 357, 2012.

LI, J.; ZHOU, M.; LI, H.; CHEN, C.; WANG, J.; ZHANG, Y. A study on the molecular mechanism of resistance to amicarthiazol in *Xanthomonas campestris* pv. *citri.* **Pest Management Science**, v. 62, p. 440–445, 2006.

LUCK, J. E.; GILLINGS, M. R. Rapid identification of benomyl resistant strains of *Botrytis cinerea* using the polymerase chain reaction. **Mycological Research**, v. 99, n. 12, p. 1483-1488, 1995.

MATSSON, M.; ACKRELL, B. A.; COCHRAN, B.; HEDERSTEDT, L. Carboxin resistance in *Paracoccus denitrificans* conferred by a mutation in the membrane-anchor domain of succinate: quinone reductase (complex II). **Archives of Microbiology**, v. 170, n. 1, p. 27-37, 1998.

MATSSON, M.; HEDERSTEDT, L. The carboxin-binding site on Paracoccus denitrificans succinate: quinone reductase identified by mutations. **Journal of bioenergetics and biomembranes**, v.33, n. 2, p. 99-105, 2001.

MCLEAN, K. S.; ROY, K. W. Incidence of *Colletotrichum dematium* on prickly sida, spotted spurge and smooth pigweed and pathogenicity to soybean. **Plant disease**, v.72, n.5, p. 390-393, 1988.

MEYER, M. C.; KLEPKER, D. Manejo da Antracnose em soja. **Fitopatologia Brasileira**, v. 32, agosto 2007.

MEYER, M. C.; RODACKI, M. E. Efeito de duas aplicações de fungicidas no controle da ferrugem da soja, DFC e antracnose no Maranhão e Tocantins. In: Reunião de Pesquisa de Soja da Região Central do Brasil, 27, 2005, Cornélio Procópio. Resumos. Londrina: Embrapa Soja, p. 201-202, 2005.

MIYAMOTO, T.; ISHII, H.; STAMMLER, G.; KOCH, A.; OGAWARA, T.; TOMITA, Y.; KOBORI, S. Distribution and molecular characterization of *Corynespora cassiicola* isolates resistant to boscalid. **Plant Pathology**, v.59, n.5, p. 873-881, 2010.

MIYAMOTO, T.; ISHII, H.; TOMITA, Y. Occurrence of boscalid resistance in cucumber powdery mildew in Japan and molecular characterization of the iron–sulfur protein of succinate dehydrogenase of the causal fungus. **Journal of General Plant Pathology**, v. 76, n. 4, p. 261-267, 2010.

MIYAMOTO, T.; ISHII, H.; TOMITA, Y. Occurrence of boscalid resistance in cucumber powdery mildew in Japan and molecular characterization of the iron–sulfur protein of succinate dehydrogenase of the causal fungus. **Journal of General Plant Pathology**, v. 76, n. 4, p. 261-267, 2010.

MU, W.; LI, B.; CHEN, C.; LIU, X.; HAO, J. Molecular mechanisms of thifluzamide resistance in *Rhizoctonia solani* (Abstr.). ***Phytopathology*** 104(Suppl. 1):S1.4, 2013.

PERES, N. A. R.; SOUZA, N. L.; PEEVER, T. L.; TIMMER, L. W. Benomyl sensitivity of isolates of *Colletotrichum acutatum* and *C. gloeosporioides* from citrus**. Plant Disease**, v.88, n.2, p.125-130, 2004.

PESQUEIRA, A. S.; BACCHI, L. M. A.; GAVASSONI, W. L. Associação de fungicidas no controle da antracnose da soja no Mato Grosso do Sul. **Revista Ciência Agronômica**, v. 47, n. 1, p. 203-212, 2016.

PICININI, E. C.; FERNANDES, J. M. Doenças de soja: diagnose, epidemiologia e controle. Embrapa Trigo-Documentos, p.91, 2000.

RIMMER, A., PHAN, H., MATHIESON, I., IQBAL, Z., TWIGG, S. R., WILKIE, A. O. VEAN, G. M.; LUNTER, G. Integrating mapping-, assembly-and haplotype-based approaches for calling variants in clinical sequencing applications. **Nature genetics**, v. 46, n. 8, p. 912, 2014.

SARTORATO, A. S. " *In vitro*" sensitivity of isolated *Colletotrichum lindemuthianum* to fungicides. **Pesquisa Agropecuária Tropical,** v.*36,* n.3, p. 211, 2006.

SARTORI, J. E. Avaliação da sensibilidade in vitro de isolados de Colletotrichum lindemuthianum a fungicidas, 2007. Thesis (Mater degree in Agronomy)- Universidade Estadual Paulista Julio de Mesquita Filho- UNESP, Botucatu, SP, Brazil.

SCHLIEP, K. P. phangorn: phylogenetic analysis in R. **Bioinformatics**, v. 27, n. 4, p. 592-593, 2010.

SCHMITZ, H. K.; MEDEIROS, C. A.; CRAIG, I. R.; STAMMLER, G. Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. **Pest Management Science**, v. 70, n. 3, p. 378-388, 2014.

SCHROEDER, W. T.; PROVVIDENTI, R. Resistance to benomyl in powdery mildew of cucurbits. **Plant Disease Report**, v. 53, p. 271-275, 1969.

SIMÕES, K.; HAWLIK, A.; REHFUS, A.; GAVA, F.; STAMMLER, G. First detection of a SDH variant with reduced SDHI sensitivity in *Phakopsora pachyrhizi.* **Journal of Plant Diseases and Protection**, v. 125, n. 1, p. 21-26, 2018.

SINCLAIR, J. B.; BACKMAN, P. A. Compendium of soybean Disease. 3 ed. Edition St Paul, Minnesota: **American Phytopathological Society**, p.106, 1989.

SKINNER, W.; BAILEY, A.; RENWICK, A.; KEON, J.; GURR, S.; HARGREAVES, J. A single amino-acid substitution in the iron-sulphur protein subunit of succinate dehydrogenase determines resistance to carboxin in *Mycosphaerella graminicola*. **Current Genetics**, v. 34*,* n. 5, p. 393-398, 1998.

SUN, H. Y.; LU, C. Q.; LI, W.; DENG, Y. Y.; CHEN, H. Homozygous and heterozygous point mutations in succinate dehydrogenase subunits b, c and d of *Rhizoctonia cerealis* conferring resistance to thifluzamide. **Pest management science**, v. 73, n. 5, p. 896-903, 2017.

SUTTON, B. C. The genus *Glomerella* and its anamorph *Colletotrichum*. **The genus *Glomerella* and its anamorph *Colletotrichum***, p. 1-26, 1992.

TANAKA, M. A. S.; PASSOS, F. A.; BETTI, J. A. Resistência de Colletotrichum fragariae e C. acutatum ao benomyl na cultura do morango no Estado de São Paulo. **Scientia Agricola**, v. 54, n.3, p.139-146, 1997.

TATUSOVA, T. A.; MADDEN, T. L. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. **FEMS microbiology letters**, v. 174, n. 2, p. 247-250, 1999.

TECNOLOGIAS DE PRODUÇÃO DE SOJA– Região central do Brasil 2013. Londrina: Embrapa Soja, p. 265, 2013.

TORRES-CALZADA, C.; TAPIA-TUSSELL, R.; HIGUERA-CIAPARA, I.; MARTIN-MEX, R.; NEXTICAPAN-GARCEZ, A.; PEREZ-BRITO, D. Sensitivity of *Colletotrichum truncatum* to four fungicides and characterization of thiabendazole-resistant isolates. **Plant disease**, v. 99, n. 11, p. 1590-1595, 2015.

WALLER, J. M. *Colletotrichum* diseases of perennial and other cash crops. ***Colletotrichum*** **diseases of perennial and other cash crops**, p. 167-185, 1992.

WRATHER, J. A.; ANDERSON, T. R.; ARSYAD, D. M.; GAI, J.; PLOPER, L. D.; PORTA-PUGLIA, A.; YORINORI, J. T. Soybean disease loss estimates for the top 10 soybean producing countries in 1994. **Plant disease**, v.81, n.1, p.107-110, 1997.

WU, J. Y.; HU, X. R.; ZHANG, C. Q. Molecular detection of QoI-resistance in *Colletotrichum gloeosporioides* causing strawberry anthracnose based on loop-mediated isothermal amplification assay. **Plant Disease**, n. ja, 2019.

XAVIER-VALENCIO, S. A. Microtitulação colorimétrica para avaliar a sensibilidade de *Corynespora cassiicola* e *Colletotrichum* spp. a fungicidas e caracterização de isolados de *Colletotrichum* spp. 2017. Thesis (PhD in Agronomy)- State university of Londrina- UEL, Londrina, PR, Brazil.

YOUNG, J. R., TOMASO-PETERSON, M., TREDWAY, L. P.; DE LA CERDA, K. Occurrence and molecular identification of azoxystrobin-resistant *Colletotrichum cereale* isolates from golf course putting greens in the southern United States. **Plant Disease**, v. 94, p.751-757, 2010.

ZHANG, C.; DIAO, Y.; WANG, W.; HAO, J.; IMRAN, M.; DUAN, H.; LIU, X. Assessing the risk for resistance and elucidating the genetics of *Colletotrichum truncatum* that is only sensitive to some DMI fungicides. **Frontiers in microbiology**, v. 8, 1779, 2017.

ZIOGAS, B. N.; MALANDRAKIS, A. A. Sterol Biosynthesis Inhibitors: C14 Demethylation (DMIs). In: Ishii H, Hollomon D, editors. **Fungicide Resistance in**

Plant Pathogens: Principles and a Guide to Practical Management. Japan: Springer, p. 201-216, 2015.

4.6 SUPPLEMENTARY INFORMATION

Table S4.6.1. Primer pairs sequence and PCR conditions used to identification of point mutation in *Colletotrichum truncatum* isolates from soybean in *cyp51,* β-tubulin, *cyt b*, SdhA and SdhB to the fungicide resistance target genes.

Gene	Primer_F	Anneling position	Primer_R	Anneling position	Annealing temperature (ºC)	amplicon size (bp)	Reference
cyp51 (DMI)	TACACCGTCTTGACCACTCCT	535	CACGCTTCTCCCAGTAAATG	1781	58	1246	
β-tubulin (MBC)	GATCAAGATGGAGTATTCCGC	181	ATCTCATCCATGCCCTCTCC	1810	58	1629	
cyt b (QoI)	TGAAATTTCGGTTCCTTACTTG	88	GGCACTAAACATAGCAAGAACTCC	896	57	808	
*Sdh*A (*Sdh*A)	CTCGTGGTGAGGGTGGTTACCT	1489	CGCTTGAAAGGTGGAACAGC	2494	63	1005	Miyamoto et al., 2010
Sdh*B* (*Sdh*B)	CCTGCCGTCACTATTCCTC	836	AAGCCATCTGCTTCTTGATCTC	1749	63	913	

Table S4.6.2. Source of *Colletotrichum truncatum* isolates, EC50 µg mL^{-1} and phenotype to carbendazim, prothioconazole and pyraclostrobin.

Code[a]	**Year**	**Location**	**EC50 µg mL $^{-1}$ and phenotype**[b]					
			Carbendazim		**Prothioconazole**		**Pyraclostrobin**	
280	Unknown	Unknown	NT[c]		NT[c]		NT[c]	
287	22/05/1992	Balsas, Maranhão	> 50	HR	2.9326	MR	<0.0032	S
289	28/08/2004	Boa Vista, Roraima	> 50	HR	0.2926	S	<0.0032	S
290	01/02/2005	Boa Vista, Roraima	NT		NT		NT	
661	Unknown	Francisco Beltrão, Paraná,	NT		NT		NT	
895	02/2010	Laranjeira do Sul, Paraná,	NT		NT		NT	
929	Unknown	Cascavel, Paraná	NT		NT		NT	
938	05/04/2011	Bela Vista do Paraíso, PR	2.8299	MR	1.7331	MR	< 0.0032	S
991	15/02/2012	Jataizinho, Paraná	NT		NT		NT	
0313	05/02/2013	Londrina, Paraná	> 50	HR	0.0860	S	5.3280	MR
1168	18/02/2013	Borrazópolis, Paraná	NT		NT		NT	
1176	15/02/2013	Londrina, Paraná	NT		NT		NT	
1177	15/02/2013	Londrina, Paraná	NT		NT		NT	
1179	20/02/2013	Bela Vista do Paraíso, PR	> 50	HR	2.6917	MR	4.7764	MR
1813	17/01/2013	Campo Mourão, Paraná	NT		NT		NT	
2013	17/01/2013	Campo Mourão, Paraná	NT		NT		NT	
2213	17/01/2013	Boa Esperança, PR	> 50	HR	0.5430	S	4.4964	MR
6213	29/01/2013	Sorriso, Mato Grosso	NT		NT		NT	
6813	06/03/2013	Cruzmaltina, PR	> 50	HR	0.0334	S	4.8407	MR
8613	13/03/2013	Tamarana, Paraná	NT		NT		NT	
8713	13/03/2013	Tamarana, Paraná	NT		NT		NT	
8813	13/03/2013	Marilândia do Sul, Paraná	NT		NT		NT	
8913	13/03/2013	Faxinal, PR	> 50	HR	4.0583	MR	4.9294	MR
9513	20/03/2013	Cruzmaltina, Paraná	NT		NT		NT	
9713	20/03/2013	Pitanga, Paraná	NT		NT		NT	
10013	12/03/2013	Ponta Grossa, PR	>50	HR	2.1450	MR	2.6873	MR
1713	Unkown	Unkown	NT		NT		NT	
59/13	2013	Campo N. Parecis, MT	NT		NT		NT	

[a]EC$_{50}$ data source by Xavier-Valencio 2017; [b]Classification of EC$_{50}$ effective concentration to inhibit 50% of conidia germination for the classes of fungicides, carbendazim (MBC) pyraclostrobin (QoI) and prothioconazole (DMI) classification of the phenotypes in the categories, (S- Sensitive) EC$_{50}$ ≤1 µg / mL^{-1}; (MR- Moderately resistant) EC$_{50}$ 1 µg mL^{-1}<EC$_{50}$ < 50 µg / mL^{-1}; (HR-Highly resistant) EC$_{50}$ ≥ 50 µg / mL^{-1}; [c]NT- (Not tested).

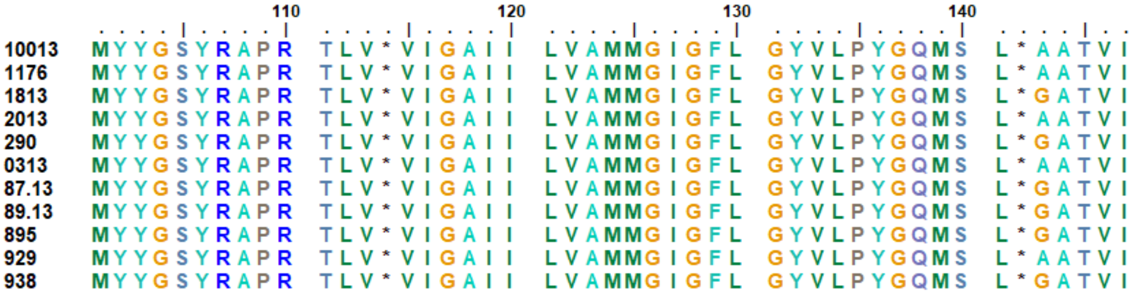
Supplementary table S4.6.3. Codon position, amino acid change and nonsynonymous and synonymous mutations for *cyp51* (DMI), β-tubulin (MBC), *cyt b* (QoI) and SdhA and SdhB fungicide target genes *Colletotrichum truncatum* from soybean fields.

DMI				**DMI**				**DMI**			
Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations	
		non-syn	syn			non-syn	syn			non-syn	syn
450	GAC/GAT	-	D	836	TCC/TTC	S279F	-	1230	TCC/TCA	-	S
575	TGA/TCA	STOP192S	-	900	TCA/TCG	-	S	1239	TTA/TTC	L413F	-
581	CCG/CTG	P194L	-	905	ACC/AGC	T302S	-	1268	TCC/TTC	S423F	-
587	CCT/CTT	P196L	-	920	GCC/GTC	A307V	-	1274	GTA/GAA	V425E	-
593	GTT/GCT	V198A	-	935	TTG/TCG	L312S	-	1337	ATC/ACC	I446T	-
599	ACG/ATG	T200M	-	953	TTA/TGA	L318STOP	-	1386	AGG/AGA	-	R
605	CTA/CCA	L202P	-	962	CCA/CGA,CTA	P321R,L	-	1397	CCA/CGA	P466R	-
611	TTG/TCG	L204S	-	1005	CCC/CCA	-	P	1421	AGG/ACG	R474T	-
686	CGA/CCA,CTA	R229P/L	-	1046	CGT/CCT	R349P	-	1443	AGG/AGA	-	R
690	AGA/AGC	R230S	-	1059	GCT/CGG	A353R	-	1448	ACT/ATT	T483I	-
699	AGA/AGG	-	R	1061	CCT/CTT	P354L	-	1457	ACG/ATG	T486MET	-
704	CCA/CCT	-	P	1067	TCA/TTA	S356L	-	1484	CGC/CAC	R495H	-
707	TTT/TCT	F236S	-	1073	TCC/TGT,TTT	S358C,F	-	1487	CCT/CTT	P496L	-
776	TGT/TTT	C259F	-	1082	CTC/CCC	L361P	-	1490	ACC/ATC	T497I	-
791	ATA/ACA	I264T	-	1094	AGA/AGC	R365S	-	1505	CCG/CTG	P502L	-
797	GCT/GAT	A266D	-	1130	TTC/TCC	F377S	-	1517	GGT/GCT	G506A	-
803	CCC/CGC	P268R	-	1136	GCG/GTG	A379V	-	1535	TTG/TCG	L512S	-
804	CCC/CCT	-	P	1214	GCC/GCT	-	A	1592	GCA/GTA	A531V	-
830	CGC/CCC,CTC	R277P,L	-	1226	CTA/CCA	L409P					

MBC				**QoI**				**QoI**			
Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations	
		non-syn	syn			non-syn	syn			non-syn	syn
726	ATC/TCC	-	S	114	GTC/GTA, GTT	-	V	753	GTT/GTA	-	V
731	GCG/GAG	A244E	-	153	GCA/GCC,GCT	-	A	774	TAC/TAT	Y	-
756	GCC/GCG	-	A	159	CAT/CAC	-	H	798	ACA/ACT	T	-
768	ATC/ATT	-	I	174	ATT/ATA	-	I	804	GCT/GCC,GCG	A	-
				283	TTA/CTA	-	L	834	CCT/CCA	P	-
				288	CAT/CAC	-	H	840	TAC/TAT	Y	-
				294	GGG/GGA	-	G	858	ATA/ATC	I	-
				318	TAT/TAC	-	Y				
				327	CCA/CCT	-	P				
				408	TAT/TAC	-	Y				
				428	GGT/GCT	G143A	-				
				432	GCA/GCT	-	A				
				483	ATT/ATA,ATC	-	I				
				573	GCT/GCC	-	A				
				582	GTT/GTA	-	V				
				591	CAC/CAT	-	H				
				597	ATA/ATC,ATT	-	I				
				609	GAC/GAT	-	D				
				618	GGG/GGA	-	G				
				642	TCA/TCT	-	S				
				696	ATA/ATT	-	I				
				726	AGC/AGT	-	S				

SDHA				SDHA				SDHA			
Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations	
		non-syn	syn			non-syn	syn			non-syn	syn
66	CTC/CTT	-	L	315	GTC/GTT	-	V	621	GAG/GAT	E207D	-
72	TCT/TCC	-	S	316	CTG/TTG	-	L	637	GCC/TCC	A213S	-
81	GAG/GAA	-	E	327	GTC/GTT	-	V	699	CAG/CAA	-	Q
84	CGC/CGA, CGT	-	R	342	GGT/GGC	-	G	705	TCC/TCT	-	S
93	GAG/GAA	-	E	363	ACC/ACT	-	T	711	GAT/GAC	-	D
135	GTC/GTT	-	V	405	GTT/GTC	-	V	720	GTT/GTC	-	V
138	GTC/GTT	-	V	423	GCT/GCC,GCG	-	A	783	ATC/ATT	-	I
147	TCG/TCC	-	S	426	TGC/TGT	-	C	798	CTT/CTC	-	L
168	GAG/GAC,GAT	E56D		432	GAG/GAA	-	E	810	CTT/CTC	-	L
171	GGC/GGA	-	G	447	TCT/TCC	-	S	816	CTC/CTT	-	L
195	GAC/GAT	-	D	459	GCT/GCG,GCC	-	A	905	CTT/CCT	L302P	-
203	TTC/TAC	F68Y		468	CTC/CTG,CTT	-	L	908	CTG/CCG	L303P	-
207	CTG/CTC,CTT	-	L	492	CTC/CTG	-	L	911	CCG/CTG	P304L	-
213	CTT/CTC	-	L	504	GGT/GGC	-	G	920	GCA/GTA	A307V	-
226	GCT/CCT	A76P	-	534	AAG/AAC	K178N		935	GAG/GCG,GTG	E312A,V	-
249	CTG/CTC	-	L	554	CAC/CTC	H185L		962	AGC/ACC,ATC	S321T,I	-
261	TCC/TCT	-	S	562	CTT/ATT	L188I		986	AGC/AAC	S329N	-
285	GGC/GGT	-	G	566	GCG/GAG	A189E		989	ACA/ACT	-	T
291	GAC/GAT	-	D	573	GAT/GAC	-	D	995	TGT/TCT	C332S	-
294	GTC/GTT	-	V	579	GGT/GGC	-	G	998	CTT/CCT	L333P	-
300	ACT/ACC	-	T	582	GCT/GCC	-	A	1004	AGA/AAA	R335K	-
309	ATT/ATC	-	I	600	CTT/CTC	-	L	1013	CTC/CCC	L338P	-
312	CCC/CCT	-	P	609	GTT/GTC	-	V	1061	TTG/TGG	L354W	-

SDHB				SDHB				SDHB			
Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations	
		non-syn	syn			non-syn	syn			non-syn	syn
81	CGC/CGT	-	R		510	CTA/CTT	L	871	GAT/CAT	D291H	-
114	CCT/CCC	-	P	532	GGA/CGA	G178R	-	883	CCG/TCG	P295S	-
120	ACA/ACC	-	T	541	CCA/GCA	P181A	-	895	GAT/CAT	D299H	-
129	CCC/CCG,CCT	-	P	559	CCG/TCG	P187S	-	901	TAA/CAA	STOP301Q	-
150	ATC/ATT	-	I	574	CTA/TTA	-	L	919	CCC/TCC	P307S	-
153	TAC/TAT	-	Y	580	CCA/GCA	P194A	-	937	CGG/GGG	R313G	-
165	CCC/CCG	-	P	589	CAC/TAC	H197Y	-	940	CAA/TAA	Q314STOP	-
180	GAG/GAA	-	E	596	TGC/TTC	C199F	-	946	TAT/CAT	Y316H	-
195	CAG/CAA	-	Q	598	CCC/CCT	-	P				
213	CTG/CTC	-	L	667	ATA/GTA	I223V	-				
222	ACC/ACT	-	T	698	GTT/GCT	V233A	-				
235	CTG/TTG	-	L	718	CAT/TAT	H240Y	-				
243	GCA/GCT	-	A	724	TTG/CTG	-	L				
265	CTG/TTG	-	L	745	GTG/ATG	V249MET	-				
279	CTT/CTC	-	L	754	CTA/GTA,TTA	L252V	-				
294	AGT/AGC	-	S	768	CTC/CTA	-	L				
297	TGC/TGT	-	C	769	TGA/CGA	STOP257R	-				
315	GGG/GGC	-	G	790	CAT/TAT	H264Y	-				
324	GCC/GCG	-	A	796	CCT/TCT	P266S	-				
333	ATT/ATC	-	I	844	CGA/TGA	R282STOP	-				
339	GGC/GGA	-	G	850	CAA/TAA	Q284STOP	-				
493	CCC/CCT	P	-	859	ACT/TCT	T287S	-				



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                         110                 120                 130                 140
        . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . . . . |  . . . . | . .
10013   M Y Y G S Y R A P R  T L V * V I G A I I  L V A M M G I G F L  G Y V L P Y G Q M S  L * A A T V I
1176    M Y Y G S Y R A P R  T L V * V I G A I I  L V A M M G I G F L  G Y V L P Y G Q M S  L * A A T V I
1813    M Y Y G S Y R A P R  T L V * V I G A I I  L V A M M G I G F L  G Y V L P Y G Q M S  L * G A T V I
2013    M Y Y G S Y R A P R  T L V * V I G A I I  L V A M M G I G F L  G Y V L P Y G Q M S  L * A A T V I
290     M Y Y G S Y R A P R  T L V * V I G A I I  L V A M M G I G F L  G Y V L P Y G Q M S  L * G A T V I
0313    M Y Y G S Y R A P R  T L V * V I G A I I  L V A M M G I G F L  G Y V L P Y G Q M S  L * A A T V I
87.13   M Y Y G S Y R A P R  T L V * V I G A I I  L V A M M G I G F L  G Y V L P Y G Q M S  L * G A T V I
89.13   M Y Y G S Y R A P R  T L V * V I G A I I  L V A M M G I G F L  G Y V L P Y G Q M S  L * G A T V I
895     M Y Y G S Y R A P R  T L V * V I G A I I  L V A M M G I G F L  G Y V L P Y G Q M S  L * G A T V I
929     M Y Y G S Y R A P R  T L V * V I G A I I  L V A M M G I G F L  G Y V L P Y G Q M S  L * A A T V I
938     M Y Y G S Y R A P R  T L V * V I G A I I  L V A M M G I G F L  G Y V L P Y G Q M S  L * G A T V I
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Figure S4.6.1. Sanger sequencing in *cyt b* gene of *Colletotrichum truncatum* from soybean.

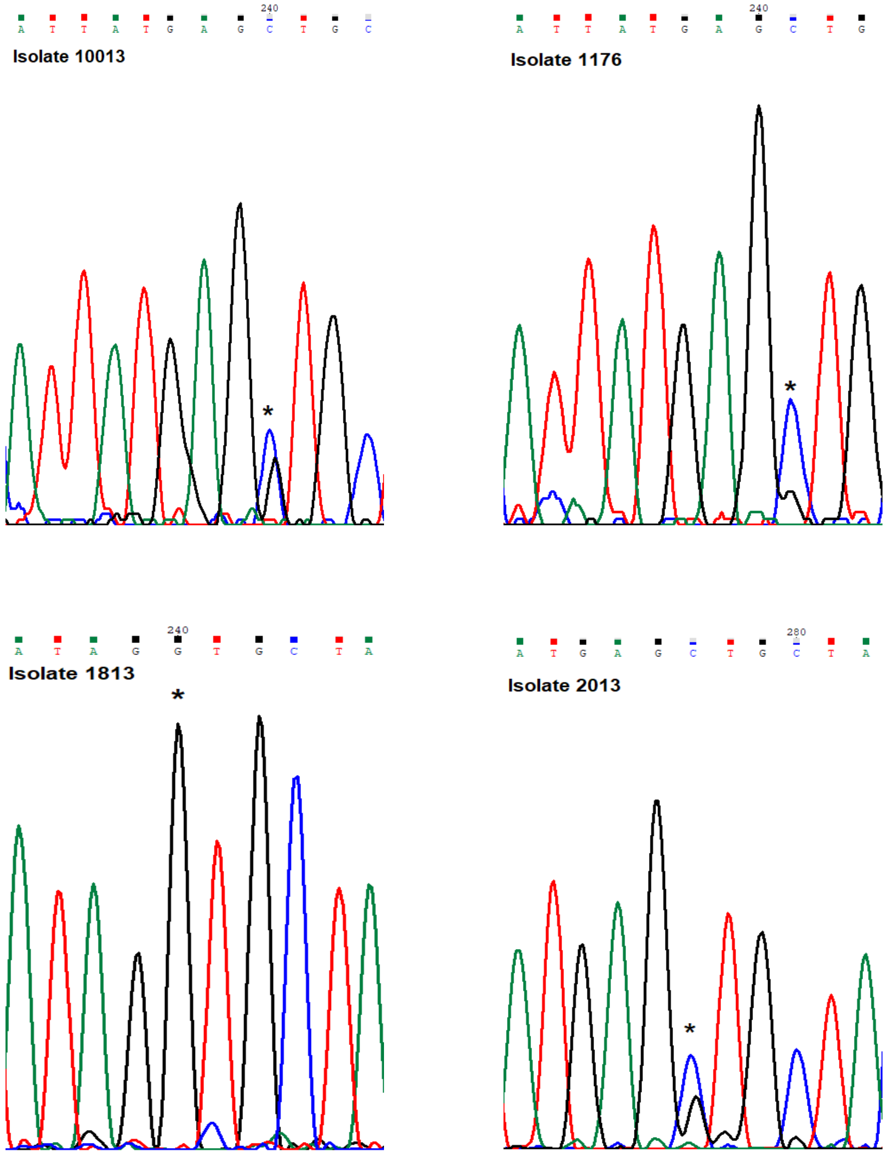

Figure S4.6.2. Nucleotide chromatogram sequencing mutation from GGT in sensitive isolates to GCT in resistant *Colletotrichum truncatum* in *cyt b* gene.

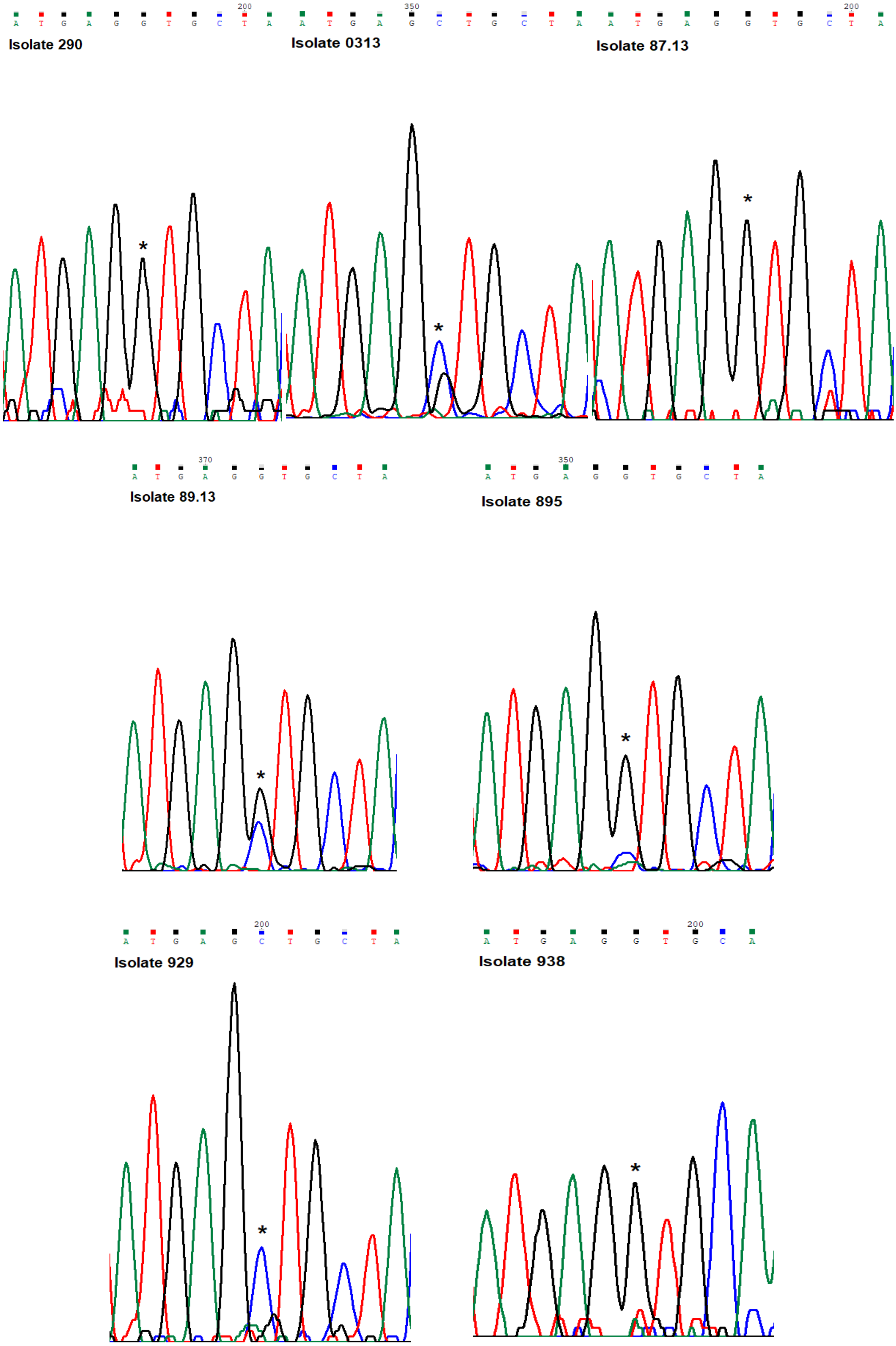

Figure S4.6.2. Nucleotide chromatogram sequencing mutation from GGT in sensitive isolates to GCT in resistant *Colletotrichum truncatum* in *cyt b* gene.

5. ARTIGO C: “Mechanisms of fungicide resistance and genetic diversity of *Corynespora cassiicola* isolates from soybean based on the polymorphisms of *cyp51*, β-tubulin, *cyt b*, SdhB, SdhC and SdhD target genes”

ABSTRACT

Target spot, caused by the fungus *Corynespora cassiicola* is controled by fungicide application of demethlylation inhibitors (DMI), quinone outside inhibitors (QoI), methyl benzimidazole carbamate (MBC) and succinate dehydrogenase inhibitor (SDHI). However, excessive sprays have contributed to development of less sensitivity populations, in Brazil. Here, we phenotype *C. cassiicola* populations and tested the cross-resistance of MBC (carbendazim and thiophanate-methyl) and QoI (azoxystrobin, picoxystrobin and pyraclostrobin) by radial growth assay in amended media using discriminatory dose at 10 µg/mL. We also determined the effective concentration to inhibit 50% of fungal spore germination (EC50) to DMI, QoI and MBC characterized the possible polymorphisms in the target genes *cyp51*, β-tubulin, *cyt b* and Sdh (subunits b, c, d) and genetic population diversity in sensitive and resistant isolates from the variations obtained in the different genes analyzed. Fungi were isolated from several soybean fields sampled in different crop seasons. The EC50 was determined using microtiter method. Genes of fungicide resistance were sequenced using next generation sequencing approach. There is a high positive cross-resistance correlation among active ingredients inside MBC and QoI chemical groups. The EC50 for the three fungicides ranging from 0 to 100 µg/mL. For *cyp51*, it was detected substitutions at S279N and I299V positions of isolates collected in 2016 year. Molecular characterization for MBC revealed S168Y, I189V, E198A, F200Y and S275N mutations. Molecular analysis detected heterozygous variation for all isolates, change from glycine to alanine at position 143 (G143A) for QoI target gene while other mutations (F129L and G137R) were not found. Substitutions for Sdh (subunits A, B, C, D) were found at positions 318, 454, 461, 556, (A-E318D, I454V, A461S, Y556F), 34, 39, 260 (B-A34V, V39I, K260R), 37, 61, 85, 163, (C-Q37H, S61T, G85S, I163V) and 126 (D-V126I), respectively. Homozygous and heterozygous variants were found for *cyp51*, β-tubulin, *cyt b*, SdhB, SdhC and SdhD fungicide resistance genes. High genetic diversity of *C. cassiicola* between sensitive and resistant isolates confirmed that genetic mutations found in this study, are responsible for the high genetic variability of the isolates. Therefore, these results showed that high selection pressure caused by the frequent fungicide applications with the same mode of action selected resistant populations of *C. cassiicola* from soybean to the fungicides MBC and QoI

Keywords: target spot, fungicide resistance, DMI, QoI, MBC, and SDHI.

5.1 INTRODUCTION

Target spot, caused by the fungus *Corynespora cassiicola* [Berk. & M. A. Curtis C. T. (Wei, 1950), was first reported in soybean (*Glycine max* (L.) Merr.) in Brazil in 1976 (ALMEIDA et al., 1976). Since then, the incidence of the disease has been increase and nowadays, it has been report in all Brazilian soybean growing regions (GODOY et al., 2017). The fungus infects leaves, petioles, pods, seeds, stem, hypocotyl and roots (HARTMAN; SINCLAIR; RUPE, 1999). The typical symptoms of the target spot are brown necrosis in the center of the lesion surrounded by a yellow margin (ALMEIDA et al., 2005). The rapid expansion of the lesion lead to premature defoliation, necrotic lesions on petioles and stems, exposition and germination of the grains in the pods (SINCLAIR; BACKMAN, 1989). The management strategies include seed treatment, crop rotation, resistant cultivars and chemical control (KLINGELFUSS; YORINORI, 2001). Yield losses can reach up to 50% in susceptible cultivars (GODOY et al., 2017). For years, application of methyl benzimidazole carbamate (MBC), quinone outside inhibitor (QoI), demethlylation inhibitors (DMI) and recently succinate dehydrogenase inhibitor (SDHI) fungicides groups have been used to managing this pathogen.

DMI commercial compounds, imidazoles and triazoles, have been the most important DMI fungicides in agriculture. Since 1970, more than 40 molecules have been used to control a large number of pathogens (ZIOGAS; MALANDRAKIS, 2015). The mode of action of the DMI fungicides occurs in the cell membrane, inhibiting the demethylation of C-14 during the formation of the sterol in the target site cytochrome P450 14 α-sterol demethylase (*cyp51*) gene (ZIOGAS; MALANDRAKIS, 2015). The genetic expression for DMI resistance is polygenic for several plant pathogens, and high resistance levels are observed only after a gradual adaptation (GISI et al., 2000). The DMI resistance mechanisms are variable acting individually or combined (ZIOGAS; MALANDRAKIS, 2015). The resistance mechanisms involve one or more point mutations, super expression of *cyp51* gene, during esters formation, fungicide intracellular reduction and accumulation mediated by regulation energy increase and efflux transporters (BRENT, 2011). The most frequent mutation observed is the substitution from tyrosine (Y) at codon 134, 136 and 137 by phenylalanine (F) (BECHER; WIRSEL, 2012). In Brazil, the excessive use of fungicides on soybean crops led to less sensitive population selection to DMI in *Phakopsora pachyrhizi*, causal agent of Asian soybean rust. Two mechanism of resistance were reported in this population, point mutations in Y131F, Y131H, K142R, F120L, I145F and I475T and over-expression of *cyp51* gene (SCHMITZ et al., 2014).

MBC fungicides represent the initial step of fungicide resistance problems (DELP, 1981). MBC fungicides were placed on the market in 1960 and, thereafter, at least 130 fungi species developed some kind of resistance (FRAC, 2013). MBC act inhibiting microtubule assembly during nuclear division by binding to the β–tubulin protein, and consequently inhibiting septal formation and germ tube growth (SCHROEDER; PROVVIDENTI, 1969; GEORGOPOULOS; DOVAS, 1973; DAVIDSE 1986; DAVIDSE; ISHII, 1995). Point mutations have been observed in several codons such as 6, 50, 167, 198, 200 and 240. The E198A / G / K / Q and F200Y mutations are the most frequent (KOENRAADT; JONES, 1992; MA; MICHAILIDES, 2005). Benzimidazole fungicides are classified as having a high risk of developing resistance (FRAC 2013). On soybean crops, the main class of fungicide used are carbendazim and thiophanate-methyl. Resistance of *C. cassiicola* fungus, isolated from soybean leaves, has been reported in the last years in Brazil (XAVIER et al., 2013; AVOZANI; REIS; TONIN, 2014; TERAMOTO et al.; 2017) and Japan (DATE et al; 2004; HUANG et al.; 2013) in different productive regions. In addition, in MBC benzimidazole-resistant isolates, positive cross-resistance usually occurs between different benzimidazole compounds, and thus, resistance to one compounds results in loss of efficacy for all other classes of benzimidazoles (DELP, 1987).

Azoxystrobin (QoI) was first used on soybean in Brazil to control late season diseases in 2000 (OLIVEIRA, 2000). The specific action of QoI fungicide is in mitochondrial respiration by the inhibition of electron transfer at Qo site of the cytochrome b (*cyt b*) gene at complex III (BECKER et al., 1981). Thus, a blockage in the electron flow occurs, reducing ATP production in the fungus cells (BECKER et al., 1981) which affects spore germination and hyphal growth (BARTLETT et al., 2002). Some mutations in the target gene are mediated by overexpression of alternative oxidase (AOX) bypassing the *cyt b*. These mutations do not have a strong effect on resistance (SIEROTZKI, 2015) and do not produce stable target site mutation in the field (JOSEPH-HORNE; HOLLOMON, 2000).

Three point mutations mediated field resistance to QoI. Mutations with amino acid substitutions from glycine to alanine at position 143 (G143A), from phenylalanine to leucine at position 129 (F129L), and from glycine to arginine at position 137 (G137R). The G143A has the strongest effect on the resistance and is the most widely spread in number and frequency among the mutations reported in pathogens species (GISI et al., 2002; GRASSO et al., 2006; SIEROTZKI, 2015). Resistance levels in G143A mutation are high and have a significant impact on disease control (SIEROTZKI, 2015). Mutation at position G143A of *C. cassiicola* isolates in cucumber was reported in Japan (ISHII et al., 2007). Regardless of the amino acid position mutation present, cross-resistance among QoI fungicide have been reported in different fungi species (SIEROTZKI, 2015). The G143A mutation causes a high level of cross-resistance to azoxystrobin and tryfloxystrobin (SIEROTZKI, 2015). In addition, G143A mutation confers cross-resistance between different QoIs, whereas F129L does not (KIM et al., 2003). The G143A mutation in *C. cassiicola* was reported in Brazil by FRAC in 2016 (FRAC, 2018).

SDHI fungicide group started to be applied in soybean fields in Brazil, in mixtures with QoI fungicides groups in 2013 to control *P. pachyrhizi*, (GODOY; MEYER, 2014). The SDHI fungicides group acts inhibiting the production of the target enzyme SDH, which acts in complex II of the electron transport chain in the mitochondria of the fungus, by blocking the binding site of ubiquinone (Q-site) (YANKOVSKAYA et al., 2003; HUANG et al., 2006). SDH are composed of four subunits (A, B, C and D) and is highly conserved throughout a range of organisms (HORSEFIELD; IWATA; BYRNE, 2004). The binding site of SDH inhibitors occurs in B, C and D subunits (KEON; WHITE; HARGREAVES, 1991; BROOMFIELD; HARGREAVES, 1992; MATSSON et al., 1998; SKINNER et al., 1998; MATSSON; HEDERSTEDT, 2001; ITO et al., 2004; LI et al., 2006). The molecular mechanism of resistance in *C. cassiicola* to boscalid in SdhB occur due to different amino acid substitutions resulting in different sensitivities to the fungicide (MIYAMOTO et al., 2010). Mutations in the SdhB gene leading to the substitution of histidine with tyrosine at amino acid position 278 (H278Y) for very high resistance isolates, while at the same position, the substitution to arginine conferred by a mutation to CGC (H278R) was detected in high resistance isolates. Additionally, other mutations showed a substitution from serine to proline (S73P) in SdhC and from serine to proline (S89P) and glycine to valine (G109V) in SdhD have been detected in not all but, some of the moderately boscalid resistant isolates (MIYAMOTO et al., 2010). Field resistant populations of *C. kikuchii* to SDHI fungicides were already reported in several documented cases (MIYAMOTO et al., 2008; ISHII et al., 2008; MIYAMOTO et al., 2009; MIYAMOTO et al., 2010).

In this study, we defined the sensitivity or resistance levels to the fungicides MBC (carbendazim and thiophanate methyl) and QoI (azoxystrobin, picoxystrobin and pyraclostrobin) and, in the same experiment, we analysed the cross-resitance into QoI and MBC active ingredients. We also defined the effective concentration to inhibition 50% (EC50) of spore germination of *C. cassiicola* to the fungicides prothioconazole (DMI), carbendazim (MBC) and pyraclostrobin (QoI) in addition we characterize polymorphisms in the target genes *cyp51*, β-tubulin, *cyt b* and their correlation with resistance. These data allow us identify possible *C. cassicola* isolates resitant to DMI, MBC and QoI fungicides in soybean and to test the hypothesis that resistant isolates have impact on the genetic diversity.

5.2 MATERIALS AND METHODS

5.2.1 Fungal isolates

Soybean leaflets with symptoms of target spot, were collected in different soybean regions and growing seasons. A total of 59 isolates of *C. cassiicola* from the Brazilian states: Paraná [(310-Sarandi), (1412-Tamarana), (1415-Mauá da Serra), (1443-Cruzmaltina), (1449-Paiquerê), (1469-Arapuã), (1663-Alvorada do sul), (1664; 1665; 1667-Cambé), (1671-Cascavel), (1675; 1676; 1677; 1678; 1679-Engenheiro Beltrão), (1683; 1684;1685-Fênix), (1686; 1688, 1689; 1690; 1691-Floresta), (1707-Londrina), (1708; 1710; 1711-Malu), (1714-Marilândia do sul), (1715-Palotina), (1726; 1727; 1728; 8216-Primeiro de Maio), (1737, 1738-Toledo)], Mato Grosso [(313; 1219-Nova Mutum), (1474- Sorriso); (1668-Campo Novo Parecis), (1672; 1673; 1681-Cláudia), (1478, 1734-Sinop), (1699; 1700;-Juara), (1723; 1724; 1725-Porto dos Gaúchos), (1730; 1733-Sapezal), (1735; 1736-Tangará), Mato Grosso do Sul [(1696-Itaporã) (1722-Ponta Porã) (1674-Dourados) and two samples from Paraguay (1719; 1720) (Figure 1).

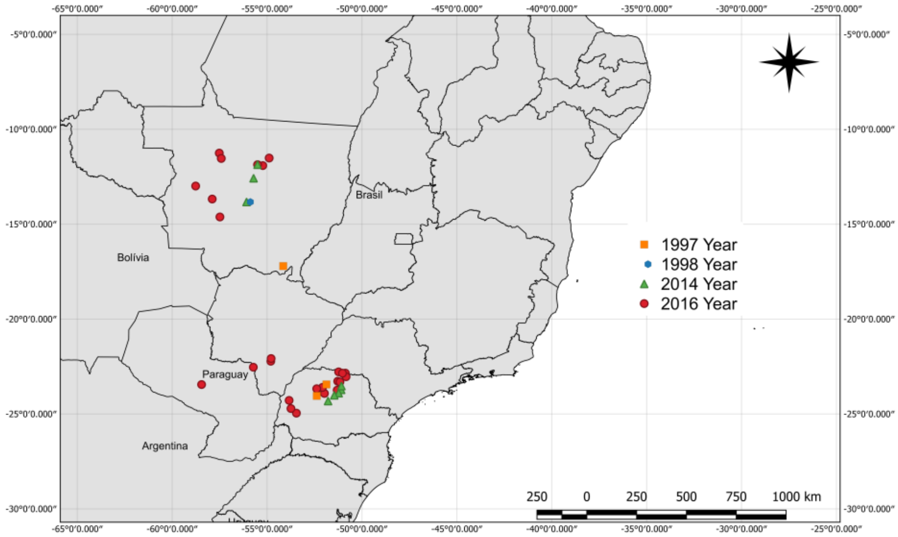

Figure 5.1 Geographic location and year of collection of *Corynespora cassiicola* isolates from soybean.

Fungal isolates were obtained by indirect isolation (ALFENAS et al., 2007) and cultured in Petri dishes containing potato-dextrose-agar (PDA) culture medium (4 g L^{-1} potato extract, 20 g L^{-1} dextrose, 15 g L^{-1} agar, pH 5.6 ± 0.2). The identification of the species occurred by comparing the morphological characteristics of spores of *C. cassiicola* (ELLIS, 1971).

The oldest isolates 310 and 313, belongs to Brazilian agricultural research corporation - Embrapa soybean (National soybean research center - Embrapa soybean) Mycological collection. These were isolated before the intensive use of fungicides, therefore, they were already being cultivated in Petri dishes with PDA culture medium at 25 ± 2°C.

In order to ensure genetic uniformity, monosporic isolates of *C. cassiicola* were used. To develop monosporic isolates, Petri dishes with purified isolates were remained under continuous light during 7 days. The aerial mycelium formed in the colonies, was transferred to microcentrifuge tubes containing autoclaved distilled water. Aliquots of 50 µL up to 100 µL of the diluted conidia suspension in sterile destilated water were pipetted and plated in water-agar (20 g L^{-1} agar) culture medium and incubated at 25 ± 2°C and 12h:12h photoperiod during 6 hours. Then, using a stereomicroscope, one germinated spore was transferred individually to Petri dishes with PDA culture medium.

5.2.2 Cross-resistance *in vitro*

In order to classify sensitive and resistant isolates, the methodology of inhibition of mycelial growth in amended medium, with a discriminatory dose was used. The isolates were cultivated in PDA culture medium at 24 ºC under 12 h / 12 h photoperiod for 7 days. Petri dishes with 15 mm diameter were equally divided into four subunits containing two isolates with two replicates. Each isolate was composed of four replicates. Mycelial discs with 4 mm diameter from the colony border were transferred to Petri dishes containing PDA medium and fungicides.

The fungicides were diluted in autoclaved distilled water and mixed with the PDA culture medium. The final concentration of active ingredients (a. i.) was 10 µg / mL. The following commercial formulations were used: and MBC: [carbendazim (50% a.i.; Bendazol®, Adama Ltd.), thiophanate--methyl (50% a.i.), Cercobin 500 SC®, Iharabras S. A. Industries Chemicals)] and QoI [azoxystrobin (25% a.i. Priori®, Syngenta Crop Protection Ltd.), picoxystrobin (25% a.i. Oranis®, DuPont S.A.); and pyraclostrobin (25% a.i. Comet®, Basf SA)]. Petri dishes with PDA culture medium and without fungicides treatments were used as control. The experiment was repeated twice.

Five days after incubation at 24 °C ± 2 °C and 12 h: 12 h photoperiod was determined the percentage of control of each fungicide by mycelial growth (mean diameter of two diametrically opposite measures). Cross-resistance was analyzed comparing the percentage of control between fungicides with the same mode of actions.

5.2.3 Baseline sensitivity

C. cassiicola isolates were collected in different Brazilian states and one isolate from Paraguay during the seasons of 1996 to 2016. These isolates were exposed to different concentrations of MBC, QoI and DMI fungicides to establish the effective concentration (EC50) to inhibit 50% of fungal sporulation. The oldest isolates, 310, 312 and 313, from 1992, 1997 and 1998, respectively, collected prior to the intensive use of fungicides were used for possible levels of comparison between susceptible and resistant isolates.

Spores of *C. cassiicola* were exposed to the commercial formulations: MBC [(carbendazim (50% a.i. Bendazol®, Nortox)], QoI [(25% a.i. Pyraclostrobin Comet®; Basf)] and DMI [prothioconazole (25% de a.i Proline®; Bayer CropScience)].

A stock solution of 600 µg / mL was prepared to obtain the following concentrations of the fungicides: 0; 0.0125; 0.05; 0.20; 4.0; 20.0; 100.0 µg / mL. The experimental design was completely randomized.

An aliquot of 50 µL of the conidia suspension at 10^5 conidia / mL^{-1} and other of 50 µL of the different doses of the fungicides were mixed in each 96-well polystyrene microplate. In each microplate a "standard sample control" was added, which was amended with YBS medium (5 g yeast extract L^{-1}, 5 g bacto peptone L^{-1}, 10 g sodium acetate L^{-1}) + 50 µL of the different concentrations of the commercial formulations that was used to calculate the difference between final absorbance value of the "standard sample control" and the absorbance of the different doses of the fungicides + spores. For each isolate and fungicide concentration, four replicate wells were used. The experiment was performed once.

The microplates were sealed with adhesive film and incubated at 180 rpm in shaker for 40 minutes. After that, the microplates were conditioned in a growth chamber at 25 ºC and 12 h/12 h photoperiod for 7 days. The absorbance of each sample was measured on a microplate reader (ASYS, Eugendorf, Austria) with a wavelength of 540 nm. The linear regression model values were quantified following the methodology described by Xavier (2017).

Based on the sensitivity differences of the isolates to the fungicides tested, the isolates were classified into three phenotypes: highly resistant (HR), EC50 ≥50 µg mL^{-1}; moderately resistant (MR), 50 µg mL^{-1} <EC50 <1.0 µg mL^{1}; and sensitive (S), EC50 ≤1.0 µg mL^{-1} (EDGINGTON; KHEW; BARRON, 1971).

5.2.4 Correlation EC50 X M.G.I. methods

The categorical variables were used to measure Cohen's kappa

(COHEN, 1960) coefficient. The classification of phenotype categories were different between the methods. Therefore, to standardize these analysis, values 0 were assigned for the isolates classified as sensitive and value 1 for the isolates classified as moderately resistant and resistant. For this analysis, the isolates that presented the same phenotype classification were quantified in order to determine the percentage of agreement between the methods.

5.2.5 DNA extration

After the EC_{50} establishment, sensitive and resistant to fungicide monosporic isolates, were grown in potato and dextrose liquid medium for mycelial filtration. In the sequence, sample DNA were extracted for all samples.

Three mycelium discs of 7 mm were added into potato-dextrose (PD) liquid medium and grown (4 g L^{-1} potato extract, 20 g L^{-1} dextrose) during 7 to 12 days. Thus, the mycelial mass was filtered through vacuum filtration. The dried mycelium were frozen in liquid nitrogen, macerated and stored in a 1.5 mL microcentrifuge tube at -80 ºC. The DNA was extracted following the modified CTAB-DNA extraction.

The macerated tissue was homogenized with 1000 µL of extraction buffer (1M Tris HLC (pH 8.0), 0.5M EDTA (pH 8.0), 5M NaCl, 0.1% β-mercaptoethanol, 200 µL CTAB to 5%) and incubated in a warm bath at 65 ° C for 60 minutes, with shaking and inversion of the microcentrifuge tubes every 15 minutes. The samples were centrifuged at 6000 rpm for 10 minutes. 700 µL of the supernatant was collected and transferred to a new 1.5 mL microcentrifuge tube. Then 700 µL of phenol: chloroform solution: iso-amyl alcohol (24: 24: 1) was added. After further centrifugation at 10,000 rpm for 5 minutes, 700 µL of the supernatant was transferred to a new microcentrifuge tube. For DNA precipitation, 500 µl of isopropanol was added and then the samples were homogenized in vortex and incubated at -20 ° C for 30 minutes. The DNA pellet was washed with 70% ethanol and resuspended in 60 µl of mili-q water + 6 µl of sodium acetate (3M) and 120 µl of 100% ethanol and was stored at 4 °C. Thus, the samples were centrifuged at a speed of 14,000 rpm for 5 minutes. The supernatant was discarded and pellet was washed again with 70% ethanol and dried. Samples were resuspended in 80 µL of mili-q + RNAse water at the final concentration of 80 µg / mL. DNA quality was assessed by electrophoresis on 1% agarose gel.

5.2.6 Primers design, PCR and fragments purification

The primers pairs for the genes, β-tubulin (Genbank / EMBL access JQ965175.1), *cyp51*, SdhB (Genbank / EMBL access AB548738.1), SdhC (JGI Genome Portal> Corca1 | scaffold_1 JGI Fungi) and SdhD (JGI Genome Portal> Corca1|233004|CE232865_6976) were designed using sequences of *C. cassiicola*, deposited at database of the National Center of Biotechnology Information (NCBI). The primers pair for *cyt b* gene was described previously by Ishii et al., 2007.

The amplicons length range from 730 to 1449 and were estimated by identification of the main mutations reported previously at literature. To achieve this, the primers forward and reverse were design to cover the maximum size of each the gene analysed.

The primers were designed using the Primer 3 plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).The specificity of the primers was compared by alignment with other sequences by BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Primer pairs forward (F) and reverse (R) were described as follows: MBC [(primer MBC_F) 5'-AGGCTTCCGGTAACAAGTTC-3'; (primer MBC_R) 5'-TGCCGACAAAGGTAGACGAC-3']; QoI [(primer QoI_F) 5'-TATTATGAGAGATGTAAATAATGG-3'; (primer QoI_R) 5'-AACAATATCTTGTCCAATTCATGG-3']; DMI [(primer DMI_F), 5'-ATCATCGGCAGCACCATC-3' (primer DMI_R) 5'- TTACTTCTTTTCCCGCCTCTC-3']; S*dh* subunit B [(primer *Sdh*B_F) 5'-TACGTGCACTACACACCCATT-3'; (primer *Sdh*B_R) 5'- AGCAGTTGAGAATCGTGTGG-3']; S*dh* subunit C [(primer *Sdh*C_F) 5'-CGAGACGACGAGCAGAATG-3'; (primer *Sdh*C_R)] 5'-AAGGTCTAGCGGGCTTACATC-3'; Sdh subunit D [(primer *Sdh*D_F) 5'-AGAAGATTGAGGGCACCTTG-3'; (primer *Sdh*D_R) 5'-GCATCTTTAACACAGGAACTTCTC-3'.

PCR reactions for the target genes were prepared to a final volume of 50 µL containing 100 ng DNA from each sample, 1 µM of each primer, 10 µM dNTPs (Thermo Fischer Scientific), 5 X Phusion HF Buffer (Thermo Fischer

Scientific) and 0.5 U Phusion DNA Polymerase (Thermo Fischer Scientific). The optimum annealing temperature was determined for each primer (Supplementary table S5.1). Annealing temperature ranged from 57ºC to 63ºC, followed by extension time which ranged from 30 to 60s and 26 to 35 cycles for amplification. All PCR reactions were performed on the MWG Biotech Inc Primus 96 thermal cycler. The PCR products were separated by electrophoresis using a 1% agarose gel and visualized under UV 360 nm light.

The PCR amplicons for all samples for each gene were mixed and then purified using Nucleospin gel and Clean-up PCR (Macherey-Nagel) following the manufacturer's recommendations. The amplicon pools were quantified with the Qubit® (Thermo Fischer Scientific) for a final concentration of 3 ng / µL. After, the amplicon sequences were sent to Institute of Clinical Molecular Biology (IKMB) for processing.

5.2.7 NGS library preparation and genome association analysis

The Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) was used to prepare the amplicon library following the manufacture’s instructions. The final 59 libraries were distributed into four lanes on a flow cell for sequencing on an Illumina HiSeq 2500 system, utilizing a 150 bp paired-end reads length with an expected coverage of 30X.

The initial base calling, barcodes/adaptors trimming, quality filtering of the reads generated with the Illumina analysis pipeline (Fastq format) were performed using Trimmomatic 0.36 software (BOLGER; LOHSE; USABEL, 2014) and checked by FastQC software (ANDREWS et al., 2010). High-quality reads generated from all *C. cassiicola* genes sequenced from isolates were concatenated and *de novo* assembled with Trinity software (HAAS et al., 2013). The *C. cassicola* genes of interest were identified by a blast2seq analyse (TATUSOVA; MADDEN, 1999)*,* applying *cpy51*, *cyt b*, β-tubulin, *S*dhA, *S*dhB, *S*dhC*,* and *S*dhD sequences from close related fungi species. The seven *C. cassicola* genes sequences were used, together with available sequence information from NCBI and MycoCosm - JGI database (https://genome.jgi.doe.gov/programs/fungi/index.jsf), to build the complete reference sequence for these genes (Supplementary table S5.1). *C. cassicola cpy51*, *cyt* b, β-tubulin, SdhA, SdhB, SdhC*,* and SdhD gene structure were predicted using

Softberry software (SOFTBERRY Inc., NY). The summary in silico method workflow is available in figure 5.3.

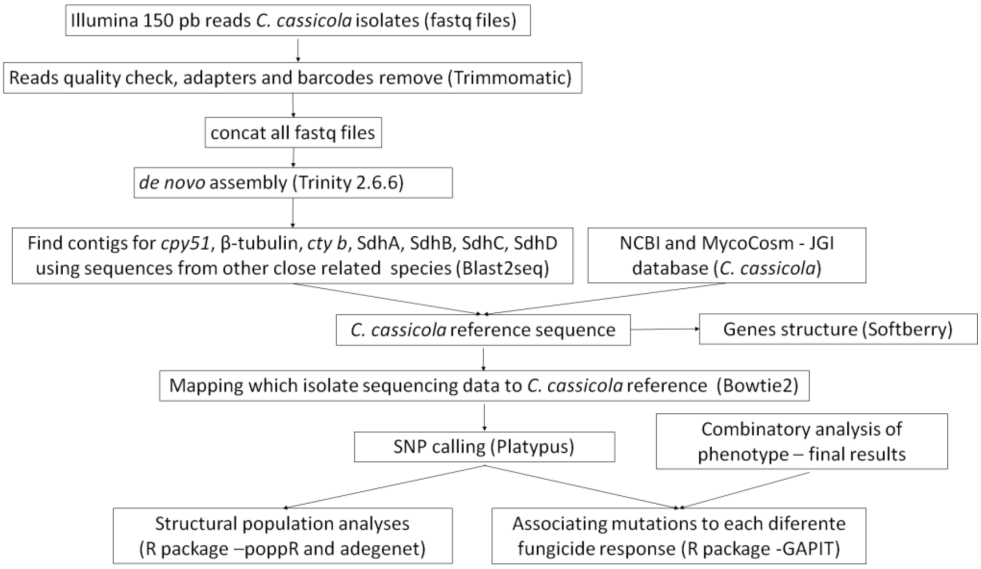

Figure 5.3 *in silico* analysis summary workflow for genetic characterization of *cpy51*, *β-tubulin*, *cty b*, SdhA, SdhB, SdhC, and SdhD genes from *C. cassicola* isolates.

High-quality reads were then aligned, by isolate, to the obtained *C. cassicola* reference sequence for the seven genes using the software bowtie2 (LANGMEAD; SALZBERG, 2012). The mapped reads were processed using Picard tools version 2.11 (Java, http://broadinstitute.github.io/picard/) to remove duplicate values, and a binary file of the extension bam representing the assembled genome of each sequenced isolate was generated. For SNP/InDel calling, we used Platypus version 0.8.1 (RIMMER et al., 2014) with minimum for two reads coverage. InDels were removed and >20% of missing data were removed from variant calling file (vcf) sing Vcftools version 0.1.12b (DANECEK et al. 2011) and remaining missing data were imputed with software Beagle 4 (AYRES et al., 2011).

5.2.8 QoI Sanger sequencing for validation

To validate NGS methodology 29 *C. cassiicola* isolates were randomly selected and sequenced using the primers described for *cyt b* gene. PCR reactions were prepared to a final volume of 50 µL containing 100 ng DNA from each sample, 1 µM of each primer, 10 µM dNTPs (Thermo Fischer Scientific), 5 X Phusion HF Buffer (Thermo Fischer Scientific) and 0.5 U Phusion DNA Polymerase (Thermo

Fischer Scientific). PCR cycles were 35 cycles of initial denaturation at 98 °C for 10s, annealing at 57 °C for 60s, elongation at 72 °C for 30s and final extension for 8 minutes at 72 °C.

PCR products were adjusted to 25 ng / µL in the Nanodrop TM OneC spectrophotometer and were purified using Nucleospin gel and Clean-up PCR (Macherey-Nagel) following the manufacturer's recommendations. All PCR reactions were performed on the MWG Biotech Inc Primus 96 thermal cycler.

Sanger PCR reaction consisted of 0.6 µL of alkaline phosphatase (FAP) (Thermo Fisher Scientific) (1 U/ mL) enzyme, Exonuclease I (Thermo Fisher Scientific) (20 U / µL) 0.15 µL, 8 µL PCR products and 1.25 µL of dH2O (ChemSolute). The final volume was performed at 10 µL. The digestion reaction consisted of 10 min of purification at 37 ° C and 15 min inactivation of the enzymes at 75 ° C. The reaction consisted of 0.7 µL of Big Dye Terminator v3.1 (Applied Biosystems), 1.5 µL of Big Dye Terminator v3.1, 1.5 µL 5X sequencing buffer (Applied Biosystems), 1 µL (3,2 µM) of each primer pairs and 4.8 µL of dH_2O (ChemSolute). Sequencing amplifications program were carried out under following conditions: 1 min of initial denaturation at 96 °C; 25 cycles of denaturation of 10 s at 96 °C, annealing of 5 s at 50 °C and extension of 4 min at 60 °C; followed by a final extension at 10 °C. The sequencing was performed using 3730xl DNA Analyzer (Applied Biosystems, Foster, CA).

The nucleotide sequences were aligned with the reference sequence (NCBI/ access AB262969.1) of *Botryotinia fuckeliana* mitochondrial *cyt b* gene and translated into amino acid sequences using Bioedit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

5.2.9 Genetic analysis

The genome association and prediction integrated tool (GAPIT) package in R (LIPKA, et al., 2012) was used to analyse phenotype and genotype association. GAPIT analyses were performed using a mixed linear model (MLM) statistical method.

Population genetic analyses were performed using ‘poppr’ (KAMVAR et al. 2014) and “adegenet” R packages (JOMBART, 2008). Minimum spanning network was applied to visualize population structure by haplotypes according to their origin location using the bitwise's genetic distance. Minimum spanning network relationship was analysed for all seven genes and also separated per gene. An UPGMA (SCHLIEP, 2010) distance tree was build to identify similarity into *C. cassiicola* groups, genetically related using poppr package (KAMVAR et al. 2014).

5.3 RESULTS

5.3.1 Cross-resistance in MBC and QoI

The cross-resistance among azoxystrobin, picoxystrobin and pyraclostrobin (QoI) and between carbendazim and thiophanate-methyl (MBC) fungicides was confirmed by positive linear correlations. The highest correlation was observed for carbenzadim and thiophanate-methyl (R=0.91), while the lowest correlation was between pyraclostrobin x picoxystrobin (R=0.6) fungicides (Figure 5.2).

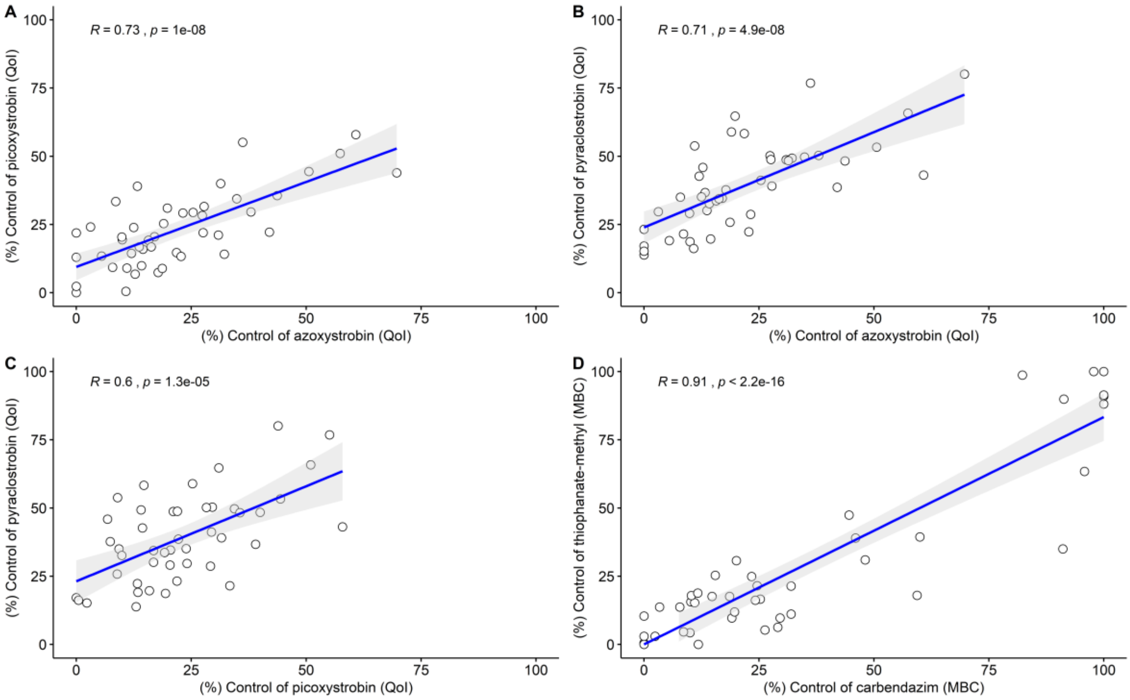

Figure 5.2. Cross-resistance among azoxystrobin, picoxystrobin and pyraclostrobin (QoI) and carbendazim and thiophanate-methyl (MBC) fungicides of *Corynespora cassiicola* isolates from soybean fields in Brazil.

5.3.2 EC50 of spore germination to DMI, MBC and QoI fungicides

EC50 values of the three fungicides groups ranged between 0 and 100 µg/mL. The EC50 values of the isolates collected in 1997 and 1998 (310 and 313, respectively), before the intensive use of fungicides ranged from 0 to 80.8 µg / mL a.i. for prothioconazole (DMI) (Supplementary figure S5.1), 0.2 to 9.5 µg / mL a.i. for carbendazim (MBC) (Supplementary figure S5.2), and 0 to 1.7 µg / mL a.i. for pyraclostrobin (QoI) (Supplementary figure S5.3). Among the resistant isolates, the isolates 1478, 1668 and 1708 were classified as highly resistant simultaneously to three fungicides groups with EC50 higher than 50 (Figure 5.3). Additionally, isolates with high resistance to more than one chemical group were also observed for pyraclostrobin and prothioconazole (1683, 1711, 1719), pyraclostrobin and carbendazim (1415, 1449, 1684) and prothioconazole and carbendazim (1671, 1707) (Supplementary figure S5.1, S5.2, S5.3).

Spore germination was suppressed in 17.5% treated with carbendazim, 31.7% isolates treated with prothioconazole and 33.3 % treated with pyraclostrobin fungicides. However, the majority of carbendazim (74.6%), prothioconazole (57.1%) and pyraclostrobin (44.5%) and fungicides had being classified as moderately and highly resistant. Additionally, 7.9 % of carbendazim, 11.1% of prothioconazole and 22.2% of pyraclostrobin have not EC50 estimated, due to the inhibition spore germination occurred above the highest dose (100 µg / mL) (Supplementary figure S5.4).

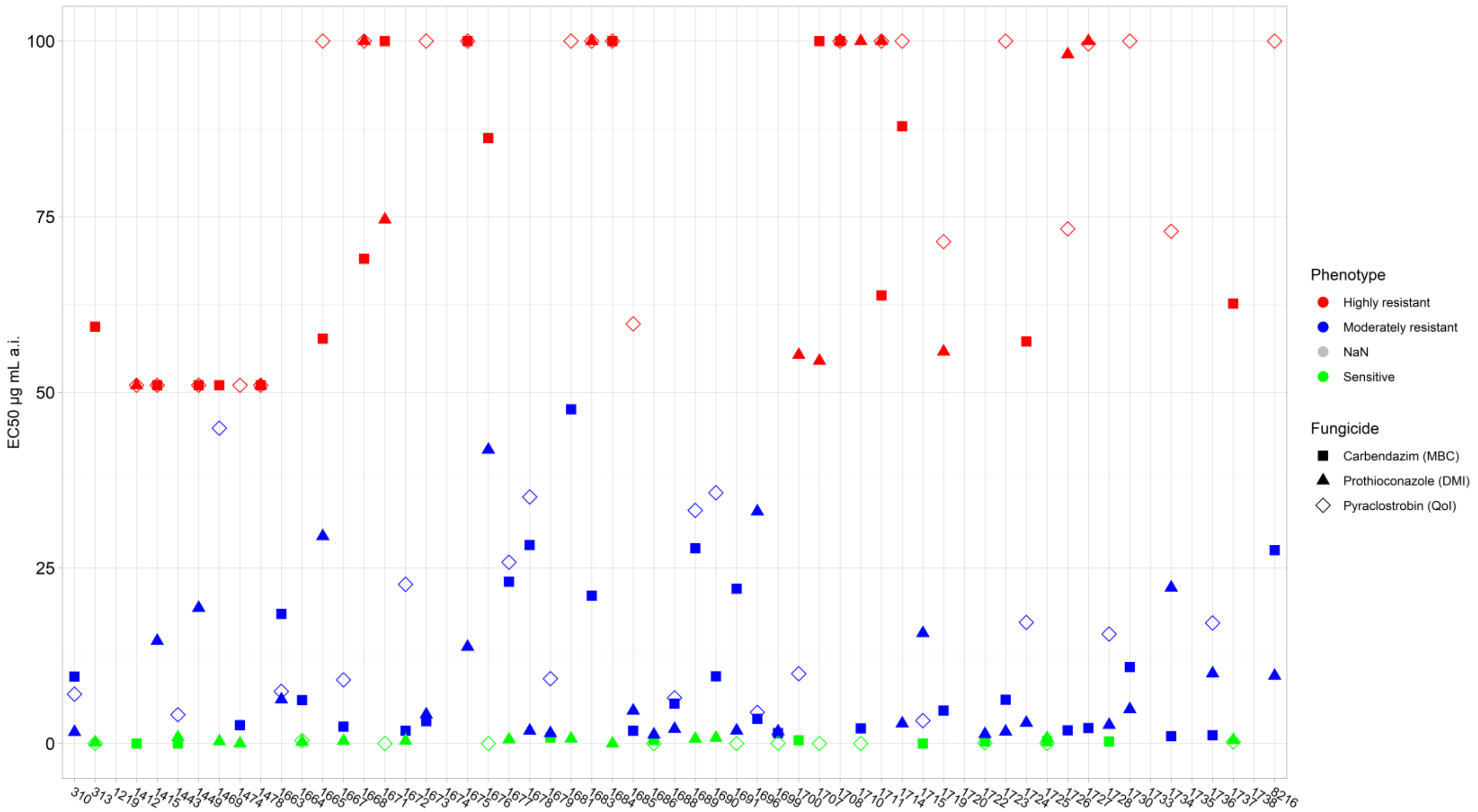

Figure 5.3 Variation of EC50 of *Corynespora cassiicola* isolates from soybean. Colors scale represents isolate classification into different phenotype. Different geometric shapes represents the fungicides carbendazim (MBC), prothioconazole (DMI) and pyraclostobin (QoI).

5.3.3 Correlation of M.G.I. and EC50 methods

To identify correlations between M.G.I. and EC50 phenotype methods, were used combinatorial analysis. Isolates that presented missing data were discarded. High agreement was observed for both methods, with Cohen's Kappa coeficiente (k) igual 0.73 for QoI fungicides and 0.82 for MBC fungicides (Table 1).

Table 1. Correlation between E50 and M.G.I. phenotype methods used to discriminate fungicide resistance in *Corynepora cassiicola* isolates to QoI and MBC fungicide groups.

QoI	**K***	**Total of isolates**
EC50 (pyraclostrobin) x pyraclostrobin	0.73	45
MBC		
EC50 (carbendazim) x M.G.I. carbendazim	0.82	44

*Cohen's Kappa coefficient

5.3.4 Molecular characterization of *cyp51,* β-tubulin, *cyt b* and Sdh (subunits a, b, c, d) fungicide target genes

Molecular characterization of *C. cassiicola* to fungicide resistance target genes resulted in a total of 132 synonymous and nonsynonymous mutations, of which 11 mutations were found for the *cyp51* gene, 12 mutations for the β-tubulin gene, 21 for the *cyt b* gene, 52 for SdhA, 16 for SdhB, 15 for SdhC genes and 5 mutations for the SdhD gene (Supplementary Table S.5.2). The genes structure of *C. cassiicola* in *cyp51*, β-tubulin and *cyt b,* SdhA, SdhB, SdhC and SdhD fungicide resistance target genes resulted in a 2187 bp, 3856 bp, 1161 bp, 2510 bp. 1513 bp, 989 bp and 1571 fragment length, respectively (Figure 5.4).

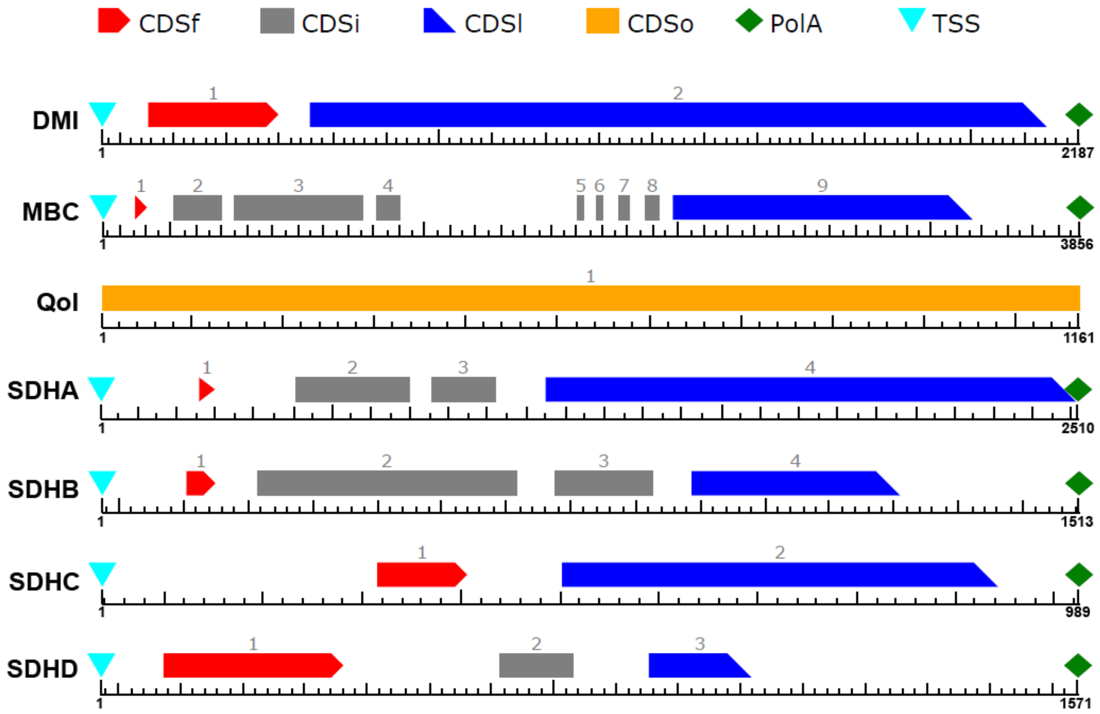

Figure 5.4 Prediction of *cyp51* (DMI), β-tubulin (MBC), *cyt b* (QoI) and Sdh*A, Sdh*B, *S*dhC and *S*dhD genes using genomic DNA of *Corynespora cassiicola* isolates. CDSf - First (Starting with Start codon); CDSi - internal (internal exon), CDSl - last coding segment, ending with stop codon); CDSo: coding sequence, solo - predicted gene with a single exon; PolA: polyadenylation signal sequence (AATAAA) and TSS - Position of transcription start (TATA-box position and score).

Nucleotide sequencing for *cyp51* target site revelead heterozygous variant at codon S279N and I299V (Table 2). The S279N mutation was detected only for isolate 1715 collected from Palotina-Paraná Brazilian state, whereas I299V mutation was found for the isolates 1715, 1722 and 1726. The mutation S279N led a substitutions from (AGT/Serine to AAT/Asparagine at codon 279), while for I299V mutation led a nucleotide substitution from (ATT/Isoleucine to GTT/Valine at codon 299) (Table 3, Supplementary Figure S5.3). In addition, nine synonymous mutations were identified (Supplementary table S5.3).

In the β-tubulin target was found mutations at codon S168Y, I189V, E198A, F200Y and S275N. Mutant isolates had nucleotide sequencing substitutions from (TTC/Serine to TAC/ Tyrosine at codon 168), (ATC/Isoleucine to GTC/Valine at codon 189), (GAG/Glutamic acid to GCG/Alanine at position 198), (TTC/Phenylalanine to TAC/ Tyrosine at codon 200) and (AGC/Serine to AAC/Asparagine at codon 275). The S168Y mutation was detected in one isolate from Parana Brazilian state. The I189V was reported for the two oldest isolates collected in 1997 and 1998. The E198A mutation was detected in 24 isolates. Of these, 18 isolates presented high frequency of the alter allele “A” and six isolates (310, 313, 1674, 1722, 1728 and 1735) showed heterozygous variant (alelles A and C) at codon 198 (Supplementary Figure S5.4).

The heterozygous variation in F200Y mutation was also observed for isolates 1674 and 1735. In total, 13 isolates showed the substitution of the thymine to alanine resulting the F200Y mutation. All isolates that showed the F200Y mutation also had the E198A mutation. The homozygous variant mutation, S275N, occurred only for the isolate 1720 from Paraguay (Figure S5.4, table 3). In addition to these mutations, eight synonymous mutations were identified (Supplementary table S5.3).

The heterozygose variant mutation in *cyt b* gene, at position 78 occurred only for the isolate 1722 from Mato Grosso do Sul Brazilian state. This mutation alter from (ATA/Isoleucine to GTA/Valine at codon 78). Heterozygous variation was also found at position 143 and was detected for all isolates. In this mutation, the frequency of alter allele for each isolate was quantified and the oldest isolates had the lowest allele frequencies 20% (310) and 23% (313) among all the isolates analyzed (Table 3). In this mutation, were replaced in the nucleotide sequence of GGT (sensitive) to GCT (resistant) at amino acid position 143 (Table 3). No isolates contained the F129L or G137R mutation (Figure 3). In addition to these mutations, nineteen synonymous mutations were identified (Supplementary table S5.3).

In our molecular analysis, we also analyzed the mutations in the s*dha* gene. We were found the mutations E318D, I454V, A461S and Y556F. Heterozygous variation of alleles G|C and G|T that caused nucleotide substitutions from (GGA/Glutamate to GAC|GAT/Aspartate at codon 318) alleles were found for isolates 1676 from Paraná and 1722 from Mato Grosso do Sul, respectively. The mutation at codon 454 was observed for isolates 1676, 1722 and also for the oldest isolates 310 and 313. The isolates that showed nucleotide sequencing substitutions from (ATC / Isoleucine to GTC / Valine at codon 454). Isolates 1674 and 1722 also showed variations in the frequency of the G and T alleles at codon 461 showing substitution from (GCC / Glutamic acid to TCC / Alanine at position 461). Whereas, for isolates 1676, 1722 and 1735, had mutations in the allelic frequency from A to T at codon 556 (TAC / Tyrosine to TTC / Phenilalanine at codon 556) (Table 3, Supplementary Figure S5.5). In addition to these mutations, forty-four synonymous mutations were identified (Supplementary table 2).

For gene Sdh subunit B the nucleotide sequencing revelead heterozygous variant at codon 34, 39 and 260 for the isolate 310. These mutations alter the amino acids from (GCC/Alanine to GTC/Valine at position 34), (GTC/Valine to ATC/Isoleucine at position 39) and (AAG/Lysine to AGG/Arginine at position 260). The A34V and V39I mutations were also detected for the isolate 313, whereas K260R mutation was found for 15 isolates (Table 3, Supplementary Figure S5.5). In addition to these mutations, twelve synonymous mutations were identified (Supplementary table 2).

The SdhC genes of *C. cassiicola* isolates showed amino acid substitution at positions 37, 61, 85 and 163. In the oldest isolates ,310 and 313, CAG was replaced by CAC, resulting in the substitution of glutamine by histidine at position 37, TCC was replaced by ACC resulting in the substitution of Serine by threonine at position 61, GGC was replaced by AGC resulting in the substitution of glycine by serine at position 85 and ATT was replaced by GTT resulting tin substitution isoleucine by valine. Isolates 1710 and 1722 present mutations Q37H and I163V, respectively (Table 3, Supplementary Figure S5.5). In addition to these mutations, eleven synonymous mutations were identified (Supplementary table 2).

In the SdhD gene was found mutations at codon V126I. The isolates had nucleotide sequencing substitutions from (GTC/valine to ATC/ isoleucine at codon 126). The isolates 1474 and 1636 presented high frequency of the alter allele “A” and the isolates 310, 313 and 1735 presented heterozygous variant, showed both alelles A and G at codon 126 (Table 3, Supplementary Figure S5.5). In addition to these mutations, two synonymous mutations were identified (Supplementary table 2).

Molecular characterization of *C. cassiicola* isolates could found multiple mutations among fungicide resistance target genes. Triple mutation in *cyp51*, β-tubulin and *cyt b* target genes, was related for two isolates 1715 from Paraná Brazilian state and 1722 from Mato Grosso do Sul Brazilian state. From 59 isolates analyzed 41% showed mutation for the target position E198A + G143A and 22% of isolates mutated at codon position F200Y + G143A, resulted in resistant isolates for MBC and QoI fungicides, respectively (Table 3, Supplementary Figure S5.5). In addition, triple mutation at positions G143A + E198A + K260R for the *cytb*, β-tubulin and SdhB genes also were observed for the isolates 310, 313, 1700, 1707 and 1736. Multiple mutations in G143A + E198A + V126I for the *cytb*, β-tubulin and

SdhD genes also was reported for three isolates (1474, 1735 and 1736) all collected in from Mato Grosso do Sul Brazilian state.

5.3.5 QoI sequencing validation using Sanger sequencing method

Sanger sequencing was performed for 29 *C. cassiicola* isolates randomly selected for *cyt b* gene. Among those, six isolates were considered wild (Supplementary figure S.5.6). The heterozygous variations observed in the NGS were also observed at nucleotide amino acid chromatogram files (Supplementary figure S5.7). Analyzing chromatographic peaks in the region of position 143 it was possible to verify the occurrence of simultaneous peaks indicating the presence of the C alleles and G at codon 428 for all isolates analyzed (Supplementary figure S5.7). Divergent results were verified between the metologies analyzed. Chromatographic waves of Sanger sequencing showed the presence of noise and heterozygosis at codon G143A, which was clearly identified by the NGS**.**

Table 2. Codon position, reference and altered alleles of *Corynespora cassiicola* isolates for the fungicides DMI, MBC, QOI and SDHA, SDHB, SDHC and SDHD divided by geographic origin.

Ref. Pos.	**571**	**630**	**338**	**400**	**428**	**434**	**659**	**23**	**219**		**143**	**549**	**570**	**856**	**53**	**67**	**731**	**111**	**181**	**253**	**487**	**163**
Ref. Allele[1]	**G**	**A**	**C**	**A**	**C**	**T**	**G**	**G**	**G**		**G**	**A**	**G**	**A**	**C**	**G**	**A**	**G**	**T**	**G**	**A**	**G**
Alt. Allele	**A**	**G**	**A**	**G**	**A**	**A**	**A**	**A**	**C**		**C,T**	**G**	**T**	**T**	**T**	**A**	**G**	**C**	**A**	**A**	**G**	**A**
	DMI		**MBC**					**QoI**			**SDHA**				**SDHB**			**SDHC**				**SDHD**
Code	S279N	I299V	S168Y	I189V	E198A	F200Y	S275N	V78I	G143A	(%)[2]	E318D	I454V	A461S	Y556F	A34V	V39I	K260R	Q37H	S61T	G85S	I163V	V126I
1685	*	*	*	*	A	*	*	*	G|C	56	*	*	*	*	*	*	*	*	*	*	*	*
1686	*	*	*	*	A	*	*	*	G|C	64	*	*	*	*	*	*	*	*	*	*	*	*
1728	*	*	*	*	C|A	*	*	*	G|C	62	*	*	*	*	*	*	*	*	*	*	*	*
310	*	*	*	G	C|A	*	*	*	G|C	20	*	G	*	*	C|T	G|A	G	C	A	A	G	G|A
1412	*	*	*	*	*	*	*	*	G|C	62	*	*	*	*	*	*	*	*	*	*	*	*
1415	*	*	*	*	*	*	*	*	G|C	82	*	*	*	*	*	*	A|G	*	*	*	*	*
1443	*	*	*	*	*	*	*	*	G|C	56	*	*	*	*	*	*	*	*	*	*	*	*
1449	*	*	*	*	A	A	*	*	G|C	78	*	*	*	*	*	*	*	*	*	*	*	*
1469	*	*	*	*	*	*	*	*	G|C	56	*	*	*	*	*	*	*	*	*	*	*	*
1663	*	*	*	*	*	*	*	*	G|C	53	*	*	*	*	*	*	*	*	*	*	*	*
1664	*	*	*	*	*	*	*	*	G|C	87	*	*	*	*	*	*	*	*	*	*	*	*
1665	*	*	*	*	*	*	*	*	G|C	53	*	*	*	*	*	*	*	*	*	*	*	*
1667	*	*	*	*	*	*	*	*	G|C	58	*	*	*	*	*	*	*	*	*	*	*	*
1671	*	*	*	*	*	*	*	*	G|C	66	*	*	*	*	*	*	A|G	*	*	*	*	*
1675	*	*	*	*	*	*	*	*	G|C	40	*	*	*	*	*	*	*	*	*	*	*	*
1676	*	*	A	*	*	*	*	*	G|C	50	G|C	*	*	A|T	*	*	*	*	*	*	*	*
1677	*	*	*	*	A	A	*	*	G|C	83	*	*	*	*	*	*	*	*	*	*	*	*

Ref. Pos.	571	630	338	400	428	434	659	23	219		143	549	570	856	53	67	731	111	181	253	487	163
Ref. Allele[1]	G	A	C	A	C	T	G	G	G		G	A	G	A	C	G	A	G	T	G	A	G
Alt. Allele	A	G	A	G	A	A	A	A	C		C,T	G	T	T	T	A	G	C	A	A	G	A
	DMI		MBC					QoI			SDHA				SDHB			SDHC				SDHD
Code	S279N	I299V	S168Y	I189V	E198A	F200Y	S275N	V78I	G143A	(%)[2]	E318D	I454V	A461S	Y556F	A34V	V39I	K260R	Q37H	S61T	G85S	I163V	V126I
1678	*	*	*	*	A	A	*	*	G|C	71	*	*	*	*	*	*	*	*	*	*	*	*
1679	*	*	*	*	*	*	*	*	G|C	82	*	*	*	*	*	*	A|G	*	*	*	*	*
1683	*	*	*	*	*	*	*	*	G|C	53	*	*	*	*	*	*	*	*	*	*	*	*
1684	*	*	*	*	*	*	*	*	G|C	77	*	*	*	*	*	*	A|G	*	*	*	*	*
1688	*	*	*	*	A	A	*	*	G|C	71	*	*	*	*	*	*	*	*	*	*	*	*
1689	*	*	*	*	*	*	*	*	G|C	52	*	*	*	*	*	*	*	*	*	*	*	*
1690	*	*	*	*	A	A	*	*	G|C	49	*	*	*	*	*	*	*	*	*	*	*	*
1691	*	*	*	*	A	A	*	*	G|C	66	*	*	*	*	*	*	*	*	*	*	*	*
1707	*	*	*	*	A	*	*	*	G|C	75	*	*	*	*	*	*	A|G	*	*	*	*	*
1708	*	*	*	*	A	A	*	*	G|C	80	*	*	*	*	*	*	*	*	*	*	*	*
1710	*	*	*	*	*	*	*	*	G|C	61	*	*	*	*	*	*	*	G|C	*	*	*	*
1711	*	*	*	*	*	*	*	*	G|C	44	*	*	*	*	*	*	*	*	*	*	*	*
1714	*	*	*	*	*	*	*	*	G|C	81	*	*	*	*	*	*	*	*	*	*	*	*
1715	G|A	A|G	*	*	A	A	*	*	G|C	86	*	*	*	*	*	*	*	*	*	*	*	*
1726	*	A|G	*	*	*	*	*	*	G|C	59	*	*	*	*	*	*	*	*	*	*	*	*
1727	*	*	*	*	*	*	*	*	G|C	73	*	*	*	*	*	*	A|G	*	*	*	*	*
1733	*	*	*	*	A	A	*	*	G|C	58	*	*	*	*	*	*	*	*	*	*	*	*
1737	*	*	*	*	*	*	*	*	G|C	83	*	*	*	*	*	*	A|G	*	*	*	*	*
1738	*	*	*	*	*	*	*	*	G|C	38	*	*	*	*	*	*	*	*	*	*	*	*
8216	*	*	*	*	*	*	*	*	G|C	19	*	*	*	*	*	*	A|G	*	*	*	*	*
1719	*	*	*	*	A	*	*	*	G|C	59	*	*	*	*	*	*	*	*	*	*	*	*

Ref. Pos.	571	630	338	400	428	434	659	23	219		143	549	570	856	53	67	731	111	181	253	487	163
Ref. allele [1]	G	A	C	A	C	T	G	G	G		G	A	G	A	C	G	A	G	T	G	A	G
Alt. Allele	A	G	A	G	A	A	A	A	C		C,T	G	T	T	T	A	G	C	A	A	G	A
	DMI		MBC					QoI			SDHA				SDHB			SDHC				SDHD
Code	S279N	I299V	S168Y	I189V	E198A	F200Y	S275N	V78I	G143A	(%)^2	E318D	I454V	A461S	Y556F	A34V	V39I	K260R	Q37H	S61T	G85S	I163V	V126I
1720	*	*	*	*	*	*	G|A	*	G|C	53	*	*	*	*	*	*	*	*	*	*	*	*
1699	*	*	*	*	A	*	*	*	G|C	68	*	*	*	*	*	*	*	*	*	*	*	*
1700	*	*	*	*	A	*	*	*	G|C	44	*	*	*	*	*	*	A|G	*	*	*	*	*
313	*	*	*	G	C|A	*	*	*	G|C	23	*	G	*	*	T	A	G	C	A	A	G	G|A
1219	*	*	*	*	*	*	*	*	G|C	73	*	*	*	*	*	*	*	*	*	*	*	*
1474	*	*	*	*	*	A	*	*	G|C	50	*	*	*	*	*	*	*	*	*	*	*	A
1478	*	*	*	*	A	*	*	*	G|C	51	*	*	*	*	*	*	A|G	*	*	*	*	*
1668	*	*	*	*	*	*	*	*	G|C	57	*	*	*	*	*	*	*	*	*	*	*	*
1672	*	*	*	*	*	*	*	*	G|C	63	*	*	*	*	*	*	*	*	*	*	*	*
1673	*	*	*	*	C|A	*	*	*	G|C	58	*	*	*	*	*	*	*	*	*	*	*	*
1681	*	*	*	*	*	*	*	*	G|C	63	*	*	*	*	*	*	*	*	*	*	*	*
1723	*	*	*	*	*	*	*	*	G|C	60	*	*	*	*	*	*	*	*	*	*	*	*
1724	*	*	*	*	*	*	*	*	G|C	46	*	*	*	*	*	*	A|G	*	*	*	*	*
1725	*	*	*	*	*	*	*	*	G|C	88	*	*	*	*	*	*	*	*	*	*	*	*
1730	*	*	*	*	*	*	*	*	G|C	58	*	*	*	*	*	*	A|G	*	*	*	*	*
1734	*	*	*	*	*	*	*	*	G|C	54	*	*	*	*	*	*	*	*	*	*	*	*
1735	*	*	*	*	C|A	T|A	*	*	G|C	59	*	*	*	T	*	*	*	*	*	*	*	G|A
1736	*	*	*	*	A	A	*	*	G|C	38	*	*	*	*	*	*	A|G	*	*	*	*	A
1696	*	*	*	*	A	*	*	*	G|C	21	*	*	*	*	*	*	*	*	*	*	*	*
1674	*	*	*	*	*	T|A	*	*	G|C	44	*	A|G	G|T	*	*	*	*	*	*	*	*	*
1722	*	A|G	*	*	C|A	*	*	A	G|C	60	G|T	A|G	G|T	A|T	*	*	*	*	*	*	A|G	*

Local[3]	**MT**	**MS**	**PR**	**PY**

[1] The symbol (*) represents the same allele of the reference sequence;

[2] Percentage of allelic frequency for the *cyt b* gene in the G143A mutation;

[3] Local of origin: MT (Mato Grosso), MS (Mato Grosso do Sul), PR (Paraná) and PY (Paraguay);

5.3.6 Phenotype association of *cyp51* fungicide target gene and mutations

In order to identify the association of the β-tubulin phenotype with the genotype, genome association was performed using only data considered significant from the combinatorial analysis between M.G.I. and EC50 methods.

We observed two associations above the Bonferroni threshold adjusted for α = 0.05 (Figure 5.4). Two of these SNPs were nonsynonymous substitutions for the genes, β-tubulin E198A (p-value 0.027) and F200Y (p-value 0.037). The other two mutations were observed for *cyt b* gene Threonine (p-value 0.029) and SdhC Alanine (p-value 0.029), both of which are synonymous mutations and showed high associations between phenotype and genotype (Figure 5.5).

For the *cyt b* gene, genome association was not efficient for the correlation of mutations, possibly due to the low number of sensitive isolates presented in the combinatorial analysis.

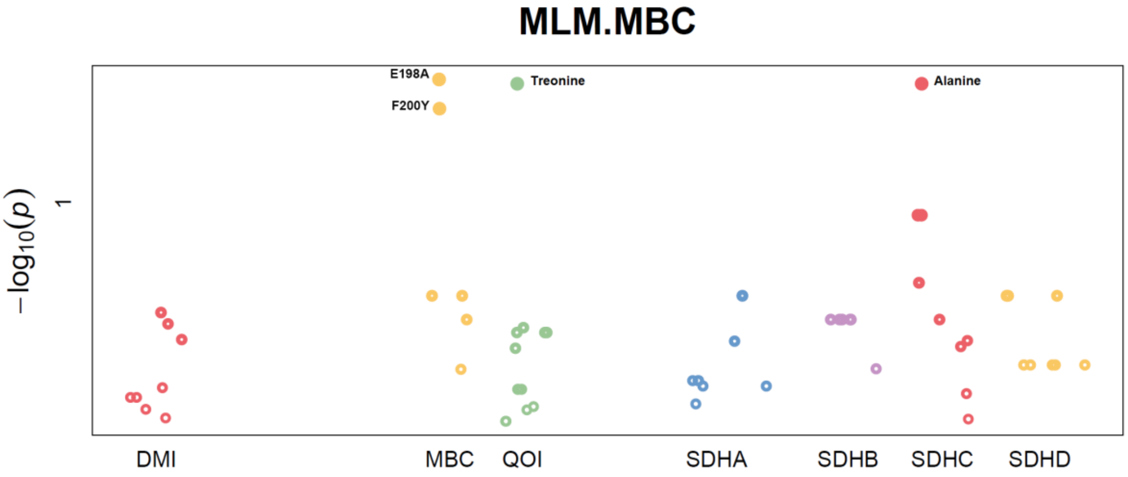

Figure 5.5. Manhattan plot of the genome association (GA) of *Corynespora cassiicola* isolates for β-tubulin fungicide resistance target gene. GA mapping was performed using a mixed linear model (MLM). Significant associations were identified for the mutations E198A and F200Y.

5.3.7 Genetic relatedness of *Corynespora cassiicola* from soybean based on the pholymorfims on the fungicide target genes *cyp51*, β-tubulin, *cyt b*, SdhA, SdhB, SdhC, SdhD

Data from the bitwise gene length matrices per gene were analyzed using a UPGMA distance tree. The number of clusters (K) ranged from 5 for genes (*sdhb*, *sdhc*) and 6 for genes (*cyp51*, β-tubulin, *cyt b*, *S*dhA, *S*dhD), and the number of clusters, which is the best K value, was determined according to the Bayesian Information Criterion (BIC).

Cluster analysis for the *cyp51* gene revelead that mutant isolates were more similar to non-mutated isolate. Cluster 2 (orange) was composed by the isolate 1715 which was detected the mutations S279N and I299V. In addiction cluster 5 (light green) separeted the isolates 1722 and 1726 that showed the mutations S279N (Figure 5.6).

The genetic diversity for β-tubulin (MBC) gene was divided into 6 clusters. Mutant isolates at positions E198A and F200Y were distributed into clusters 1 (dark green), cluster 4 (pink) and cluster 2 (orange). Cluster 6 (yellow) was composed of 18 non-mutant isolates and for the isolates 1735 and 1728 that showed the E198A + F200Y mutation (Figure 5.7).

For QoI fungicides, the cluster 6 (yellow) showed a mutation at position I78V for the isolate 1722. The others cluster shared the mutation at position 428, which resulting in mutation at position G143A. The six cluster indicating that there have been some changes at *cyt b* loci according to geographic origins (Figure 5.8).

The genetic diversity for SdhA into mutant and non mutant isolates were distributed between six clusters. The isolates that showed mutations were separated in thee cluster. Cluster 2 (orange) wich separed the isolate 1674 mutation at I454V and A461S, cluster 4 (isolate 1722 mutations at E318D, I454V, A461S and Y556F, 1735 (mutation Y556F), 1676 (mutation at E318D) and Cluster 6 (yellow), composed by 310 and 313 which resulting mutations at I454V (Figure 5.9).

The genetic diversity for SdhB into sensitive and resistant isolates were distributed between five clusters. Cluster 1 (dark green) separates the isolates 310 and 313 which represents samples collected before the intensive use of fungicide and mutations at positions A34V, V39I and K260R. The most frequent cluster 4 (pink) with 51 isolates (Figure 5.10).

Three cluster were three clusters were generated for the SdhC gene. The Cluster 4 (pink) represents samples collected before the intensive use of fungicide (310 and 313) and revealed mutations F61N and G85S. The most frequent haplotype is cluster 3 (blue) (n = 30), which did not showed any substitution or mutations sites in any region (Figure 5.11).

Genetic structure of SdhD was separated into five clusters. Mutations were found at V126I in samples 310, 313 and 1735 separated into cluster 3 (blue). The most frequent cluster 4 (n = 48), which did not showed any substitution or mutations sites in any region. In addition the samples (1474) from Sorriso-Mato Grosso Brazilian state and (1736) from Tangará-Mato Grosso Brazilian state, collected in 2014 and 2016, respectively, also presented V126I mutations and were separeted into cluster 1 (dark green) (Figure 5.12).

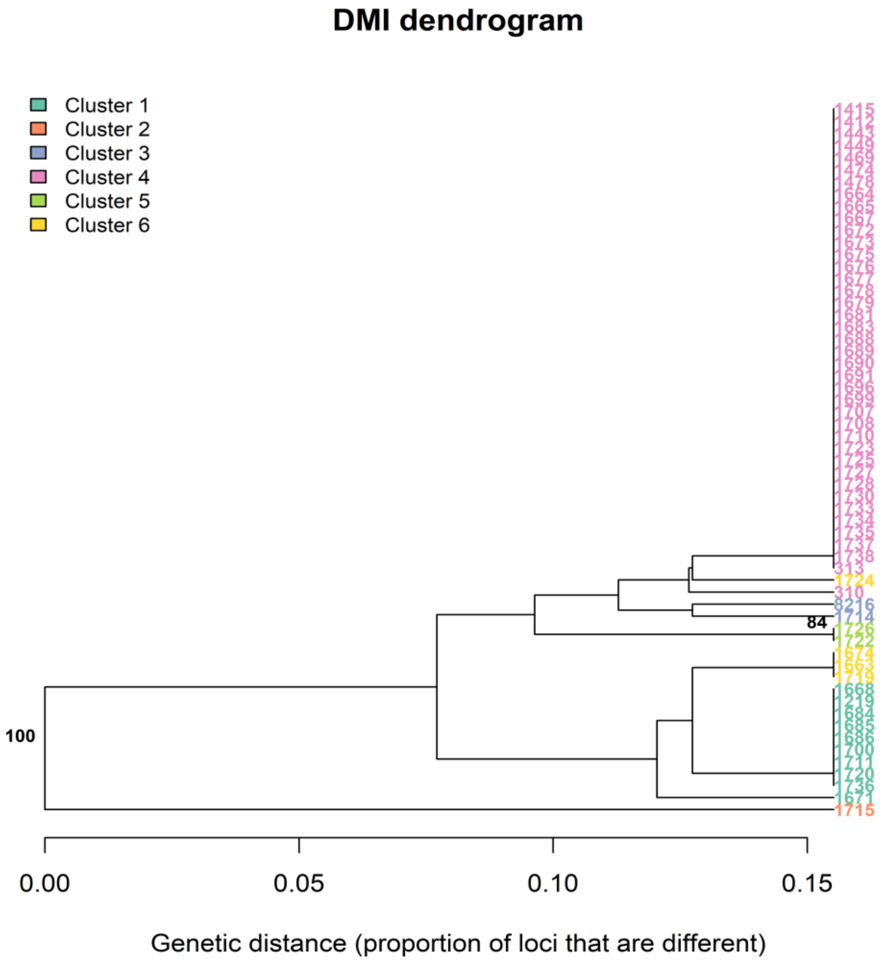

Figure 5.6. UPGMA clustering dendrogram of *C. cassiicola* isolates for *cyp51* fungicide resistance target gene. Distance is based on genetic similarity.

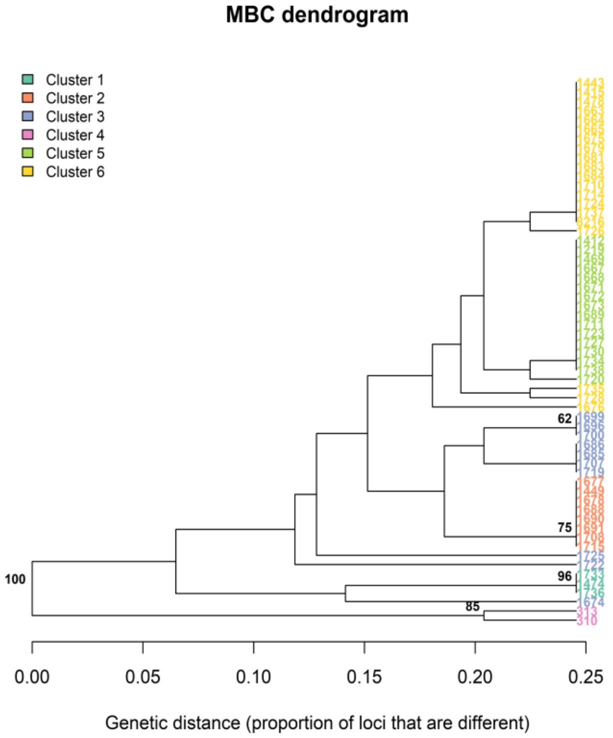

Figure 5.7. UPGMA clustering dendrogram of *C. cassiicola* isolates for β-tubulin fungicide resistance target gene. Distance is based on genetic similarity.

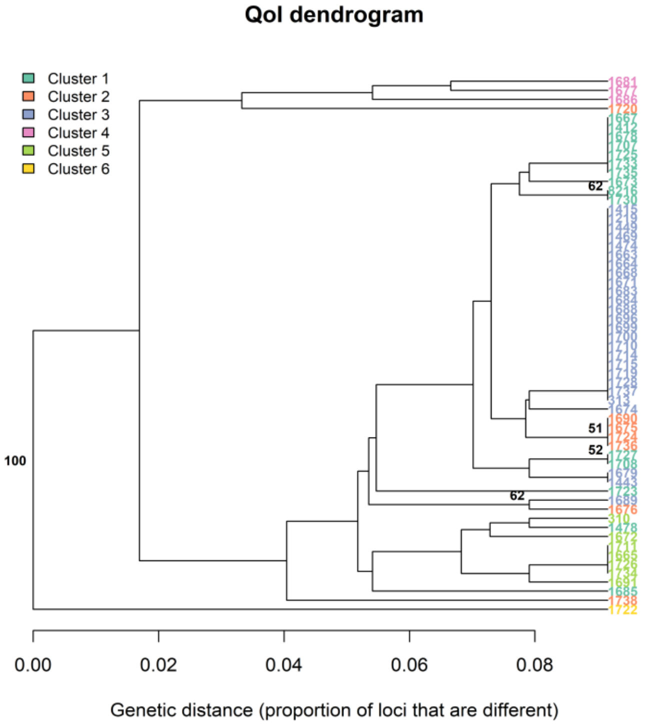

Figure 5.8. UPGMA clustering dendrogram of *C. cassiicola* isolates for *cyt b* fungicide resistance target gene. Distance is based on genetic similarity.

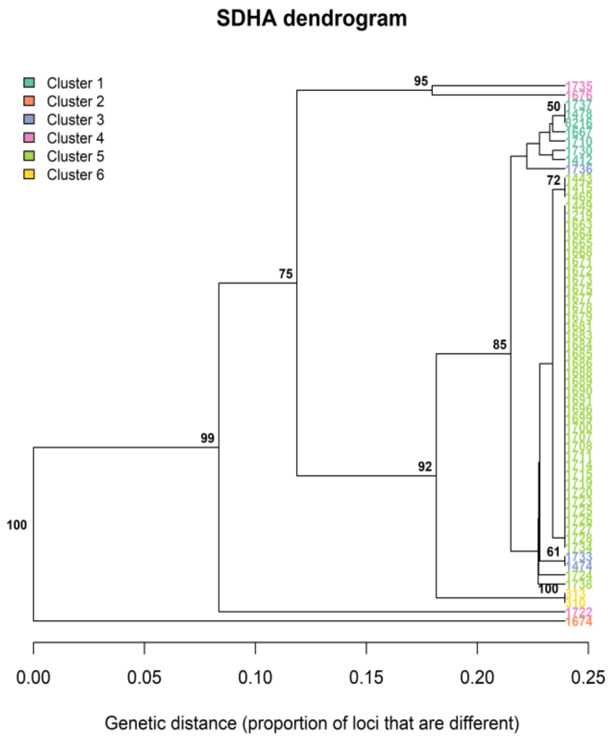

Figure 5.9. UPGMA clustering dendrogram of *C. cassiicola* isolates for SdhA fungicide resistance target gene. Distance is based on genetic similarity.

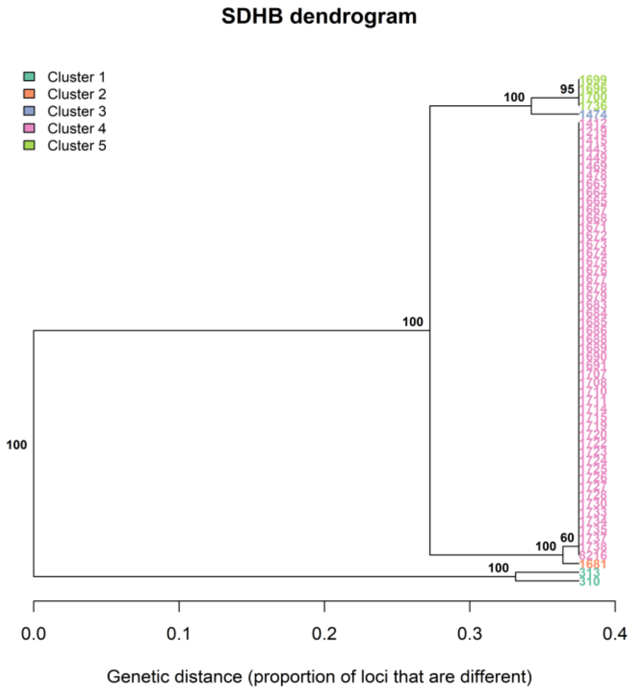

Figure 5.10. UPGMA clustering dendrogram of *C. cassiicola* isolates for SdhB fungicide resistance target gene. Distance is based on genetic similarity.

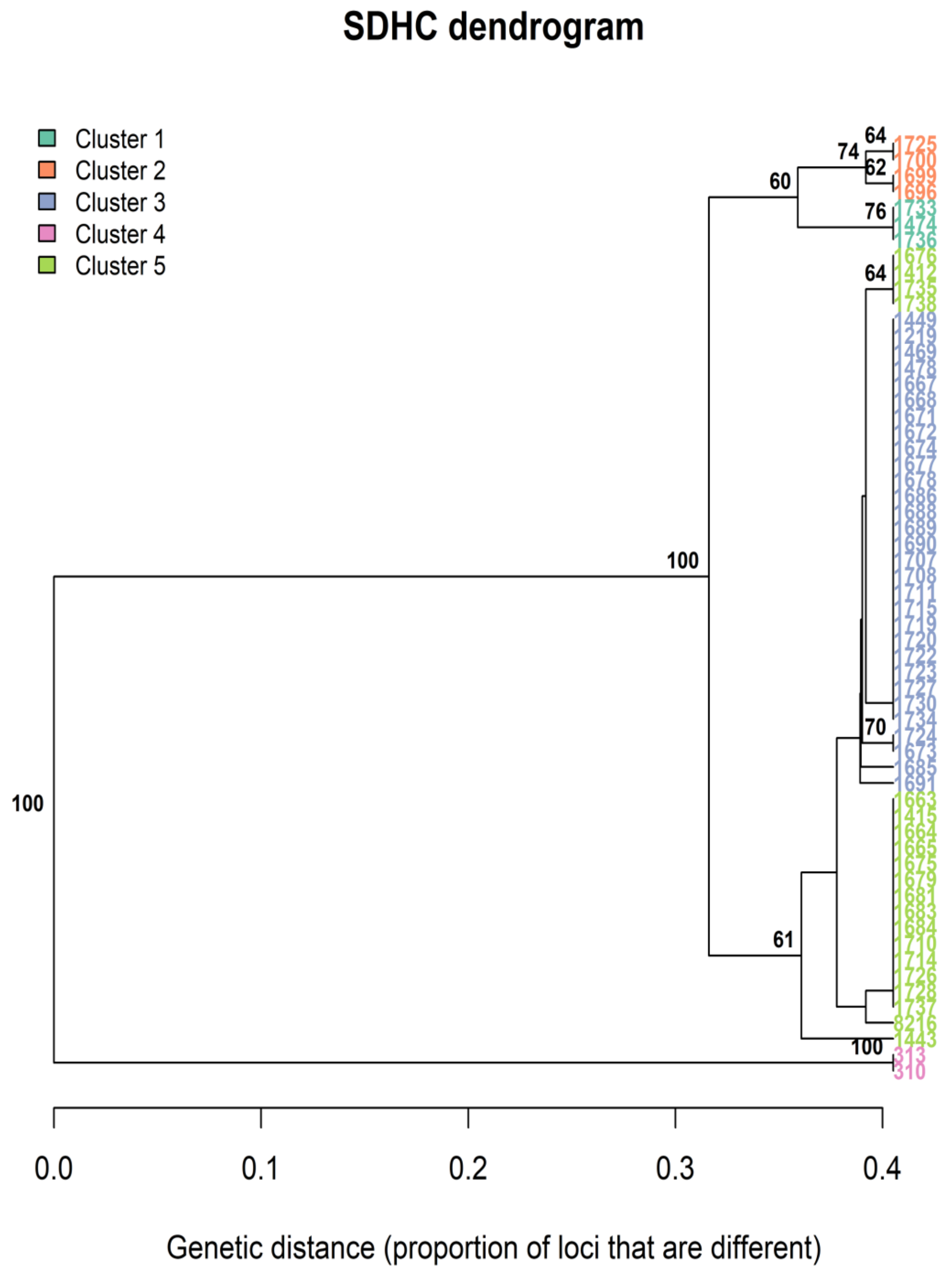

Figure 5.11. UPGMA clustering dendrogram of *C. cassiicola* isolates for SdhC fungicide resistance target gene. Distance is based on genetic similarity.

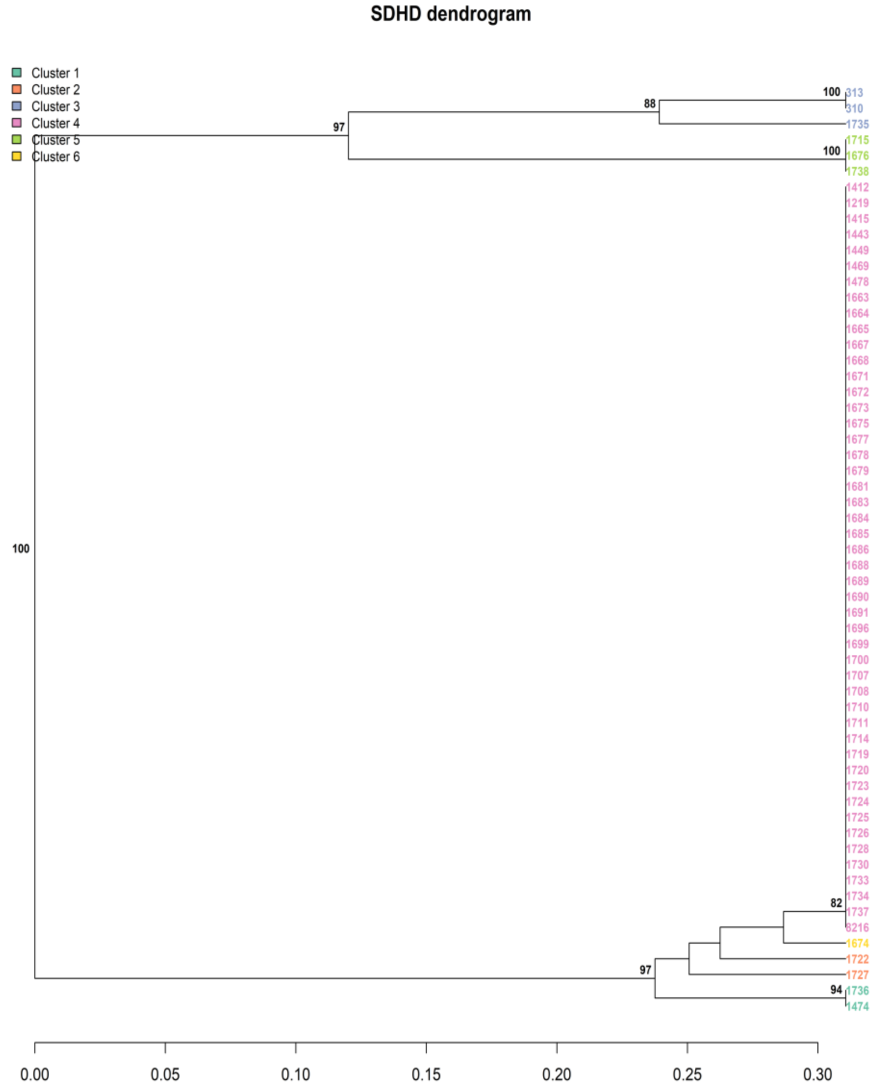

Figure 5.12. UPGMA clustering dendrogram of *C. cassiicola* isolates for SdhD fungicide resistance target gene. Distance is based on genetic similarity

5.3.8 Relationship among *C. cassiicola* genotypes

The minimum spanning network of *C. cassiicola* distinguished the most frequent genectic group and were placed close to one another (Figure 5.13). The isolates from PR and MT 1734 + 1711, 1683 + 1663 and 1679 + 1415 share the same haplotypes. The global network showed from Mato Grosso do Sul (MS), Mato Grosso (MT) and Paraguay (PY) and Paraná (PR) were distributed all across the network and were clustered around the most frequent genotype. Bitwise genetic distance in the minimum spanning network varied from 0.004 to 0.287 (Figure 5.13).

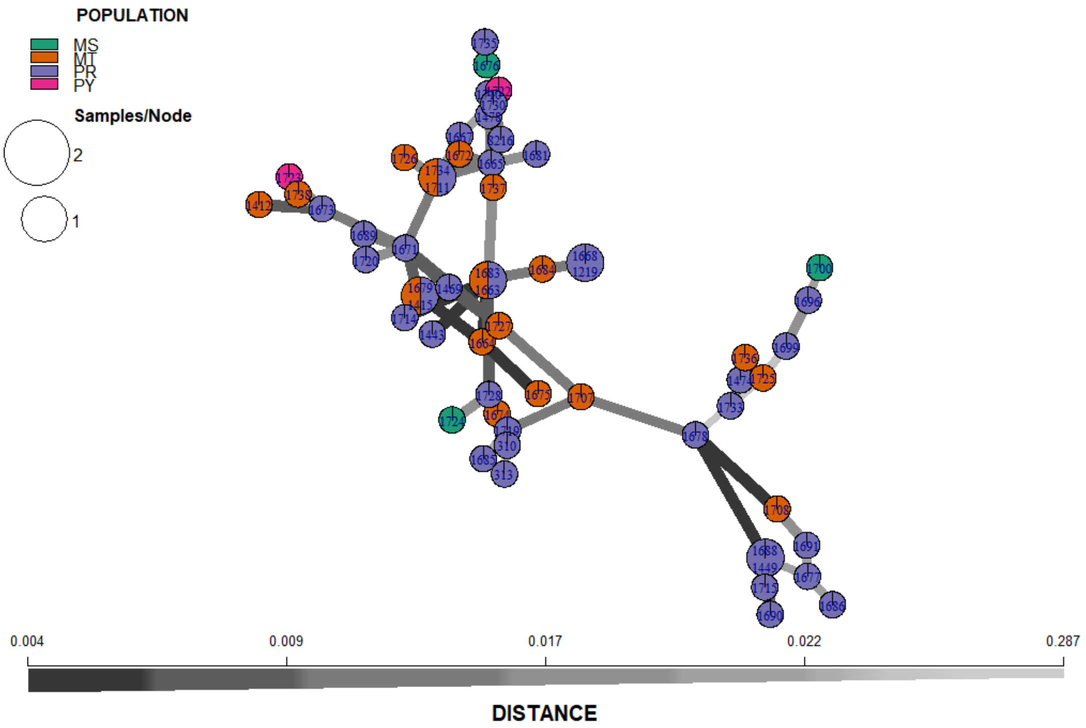

Figure 5.13. Median spanning network of *Corynespora cassiicola* from soybean for fungicide resistance target genes *cyp51*, β-tubulin, *cyt b*, SdhA, SdhB, SdhC, SdhD. Each circle represents a haplotype. Node sizes are proportional to the number of isolates origin. Coloured slices represent different geographic origin.

5.3.9 Relationship among *C. cassiicola* genotypes separete per gene to fungicide resistance target genes

The genetic diversity for *cyp51* sensitive and resistant isolates was distributed into 10 haplotypes groups. The haplotypes with substitution at codon position I299V and S275N showed lower genetic distance compared to others haplotypes (Figure 5.13). The isolates 1722 is locate in one haplotype and showed mutations S275N + I299V (Figure 5.14).

The geographical distribution for β-tubulin gene was divided into 16 haplotypes. Point genetic variations conferring resistance to the MBC fungicide were observed in three haplotypes E198A+ F200Y [(1449, 1677, 1678, 1688, 1690, 1691, 1708 and 1715 and 1449, 1677, 1678, 1688, 1690, 1691, 1708 and 1715)] and E198A [(1685, 1686, 1707 and 1719)]. The most frequent haplotypes are (n = 15 and n=16) did not show any mutation which resulted in MBC resistance (Figure 5.15). The isolates 310 and 313 both showed substitutions I189V and each was divide into one haplotype. The isolate ESMC 1676 isolate shared one haplotype and exhibited a modification at position S168Y. Also the isolate 1720 share one haplotype and showed mutation S275N (Figure 5.15).

For QoI fungicides all isolates analysed shared the mutation at position 428, which resulting in mutation at position G143A position. The relationship into the haplotypes were separeted by different due the indicating that there have been some changes at *cyt b* loci according to geographic origins (Figure 5.16).

The genetic diversity for Sdh genes (A, B, C and D) into sensitive and resistant isolates were distributed between 16, 6, 12 and 8 haplotypes groups. The minimum spanning network for SdhB separates in different haplotype the samples 310 and 313 which exhibit the mutation A34V, V39I and K260R. The most frequent haplotype (n = 51), which showed mutation K260R and not mutated isolates (Figure 5.17).

In total 12 haplotypes were found for SdhC. One haplotype represents samples collected before the intensive use of fungicide (310 and 313). The most frequent haplotype (n = 26), which did not showed any substitution or mutations sites in any region. Mutation I163V was found for isolates 1722 which was separated into second most frequent haplotype (n=14) (Figure 5.18).

The minimum spanning network separates SdhD into eight haplotypes groups. The most frequent haplotype is H2 (n = 48), which did not showed any substitution or mutations sites in any region. The samples (1474) from Sorriso-Mato Grosso Brazilian state, 310 from Sarandi-Paraná Brazilian state, 313 from Nova Mutum-Mato Grosso Brazilian state and (ESMC 1735 and 1736) from Tangará-Mato Grosso Brazilian state, present V126I mutations and which is possible mutation selection for resistance to SdhD fungicide (Figure 14) (Figure 5.19).

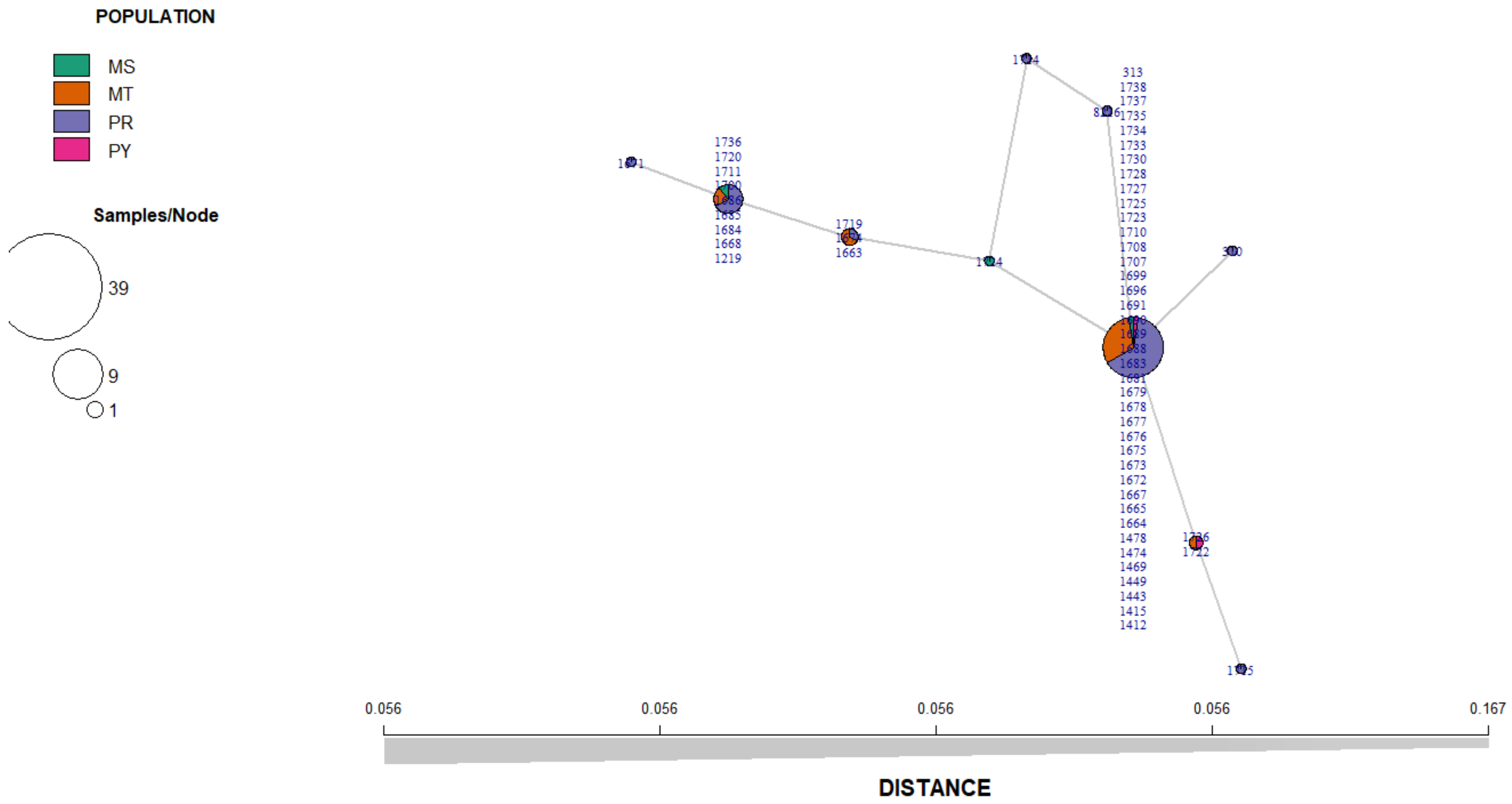

Figure 5.14. Median joining network of *Corynespora cassiicola* from soybean for fungicide resistance target genes *cyp51.* Each circle represents a haplotype. Cicle size is proportional to the number of isolates origin into the haplotypes. Coloured slices represent different geographic origin.

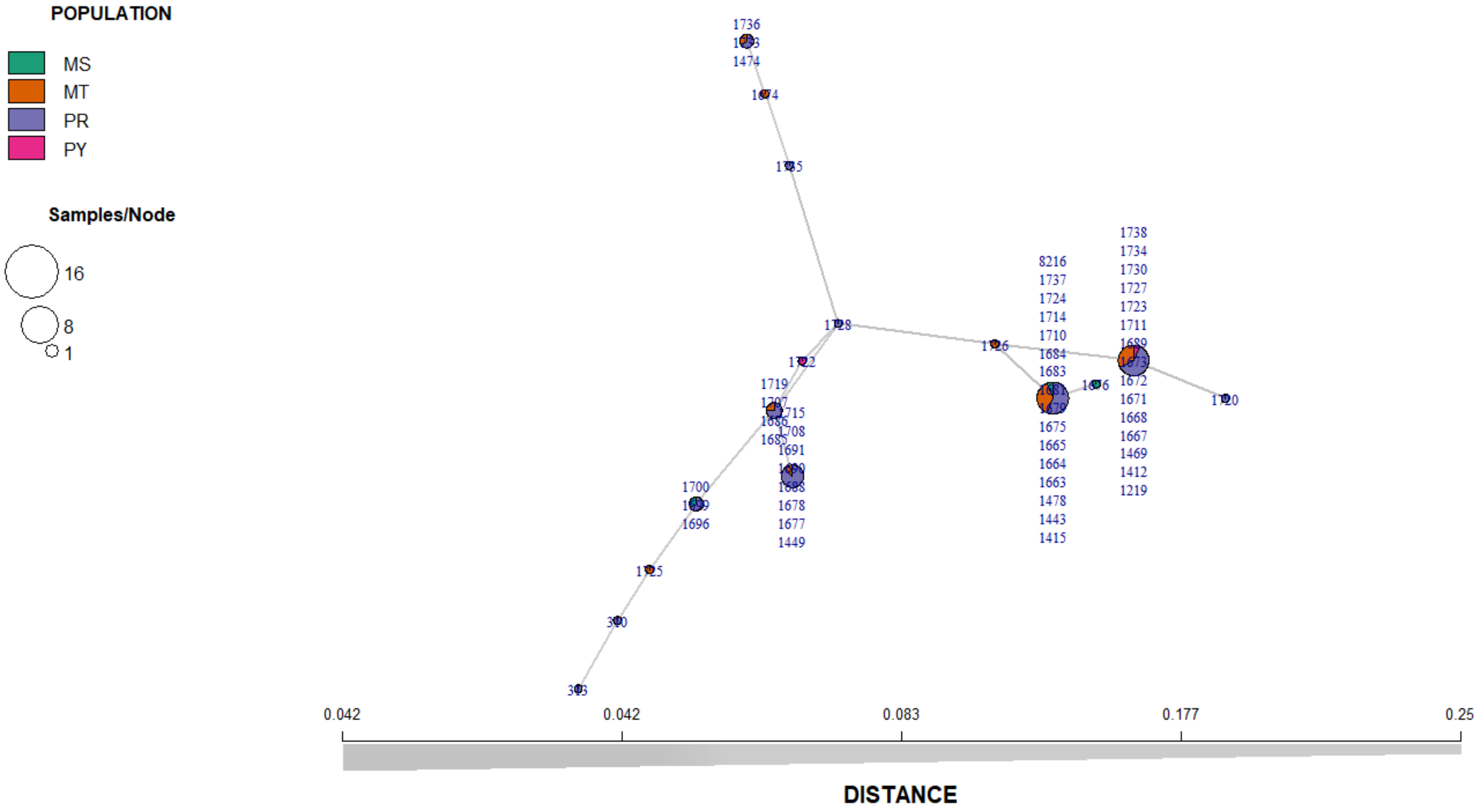

Figure 5.15 Median joining network of *Corynespora cassiicola* from soybean for fungicide resistance target genes β-tubulin. Each circle represents a haplotype. Cicle size is proportional to the number of isolates origin into the haplotypes. Coloured slices represent different geographic origin.

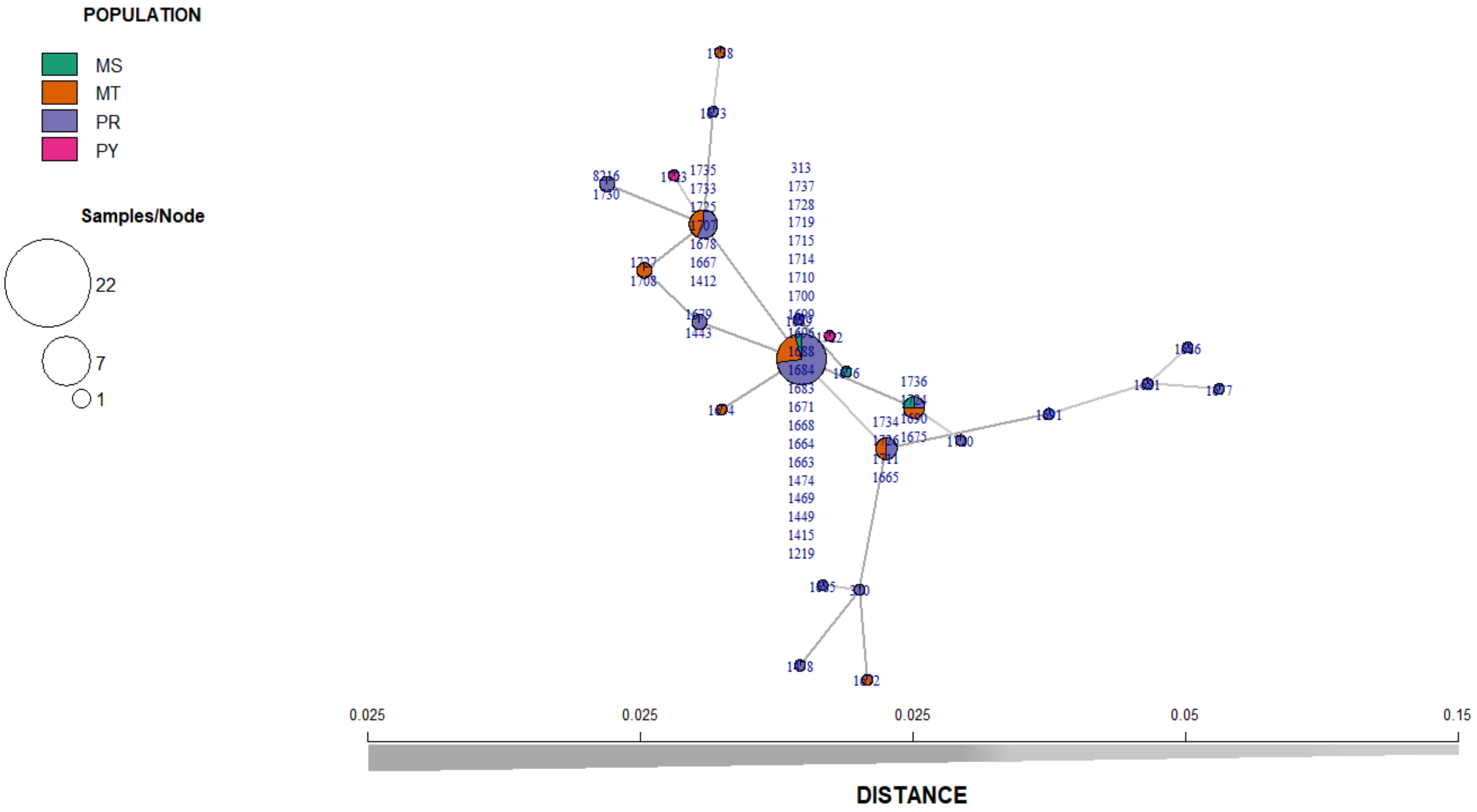

Figure 5.16. Median joining network of *Corynespora cassiicola* from soybean for fungicide resistance target genes *cyt b*. Each circle represents a haplotype. Cicle size is proportional to the number of isolates origin into the haplotypes. Coloured slices represent different geographic origin.

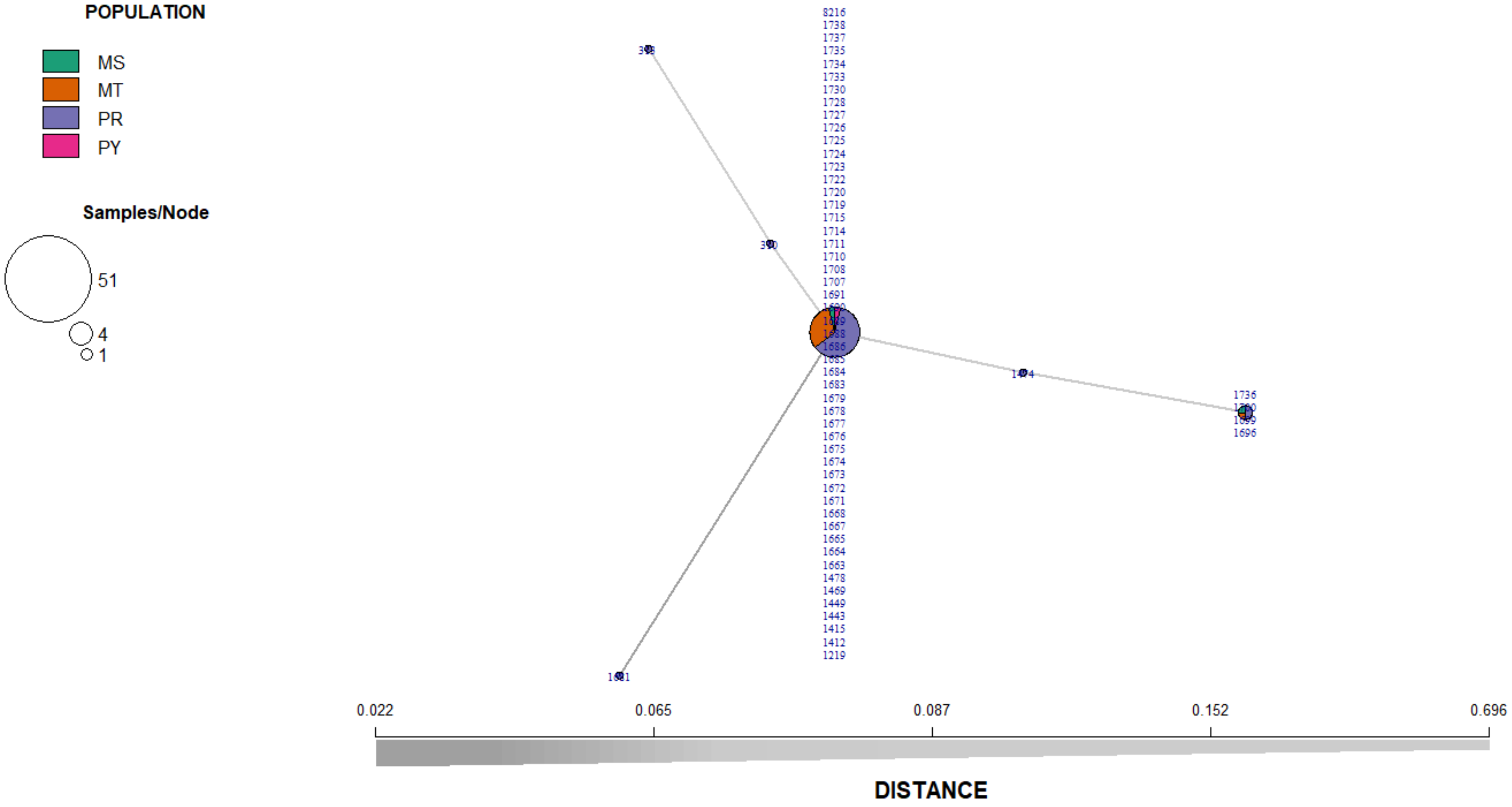

Figure 5.17. Median joining network of *Corynespora cassiicola* from soybean for fungicide resistance target genes SdhB. Each circle represents a haplotype. Cicle size is proportional to the number of isolates origin into the haplotypes. Coloured slices represent different geographic origin.

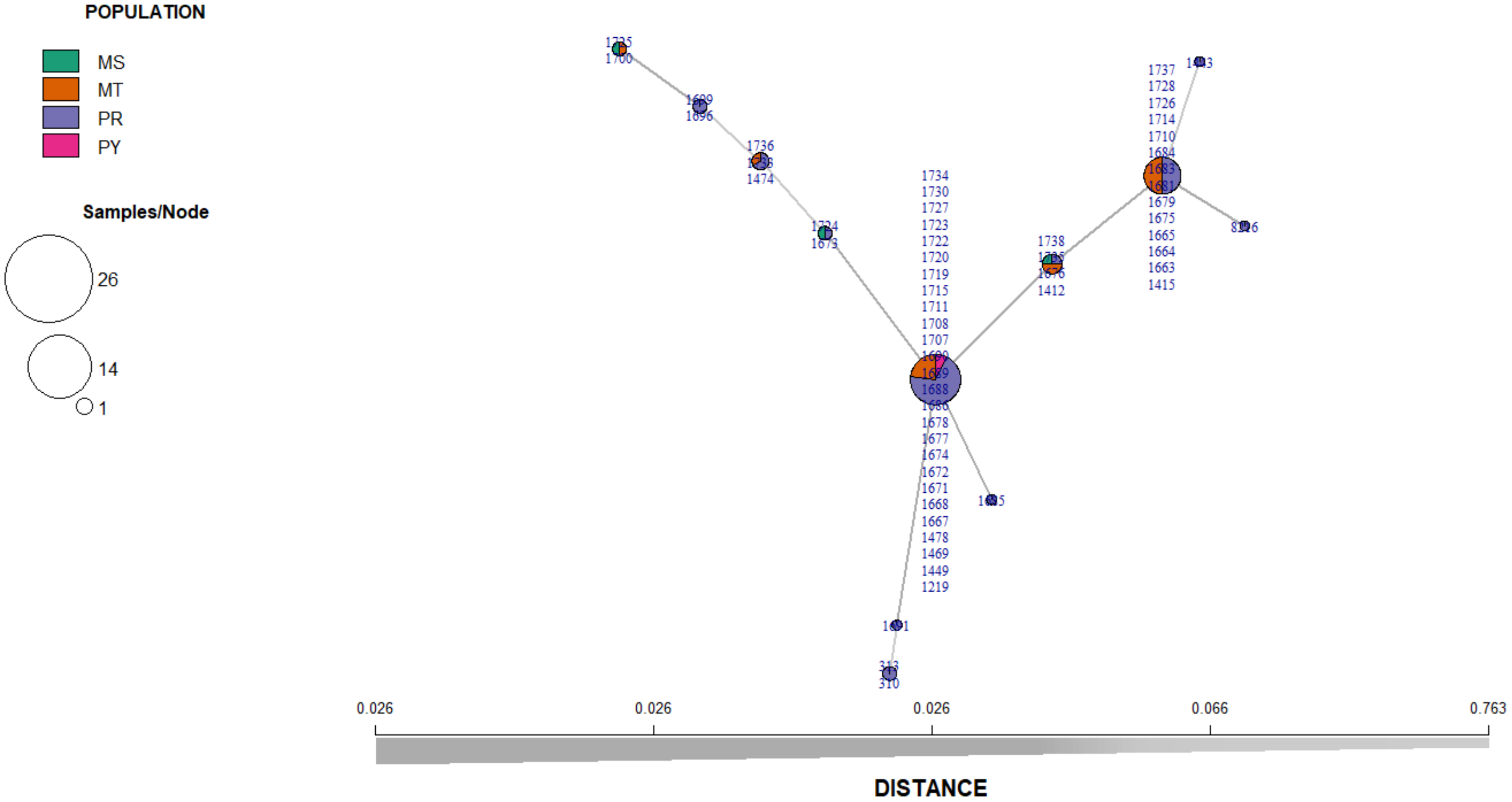

Figure 5.18. Median joining network of *Corynespora cassiicola* from soybean for fungicide resistance target genes SdhC. Each circle represents a haplotype. Cicle size is proportional to the number of isolates origin into the haplotypes. Coloured slices represent different geographic origin.

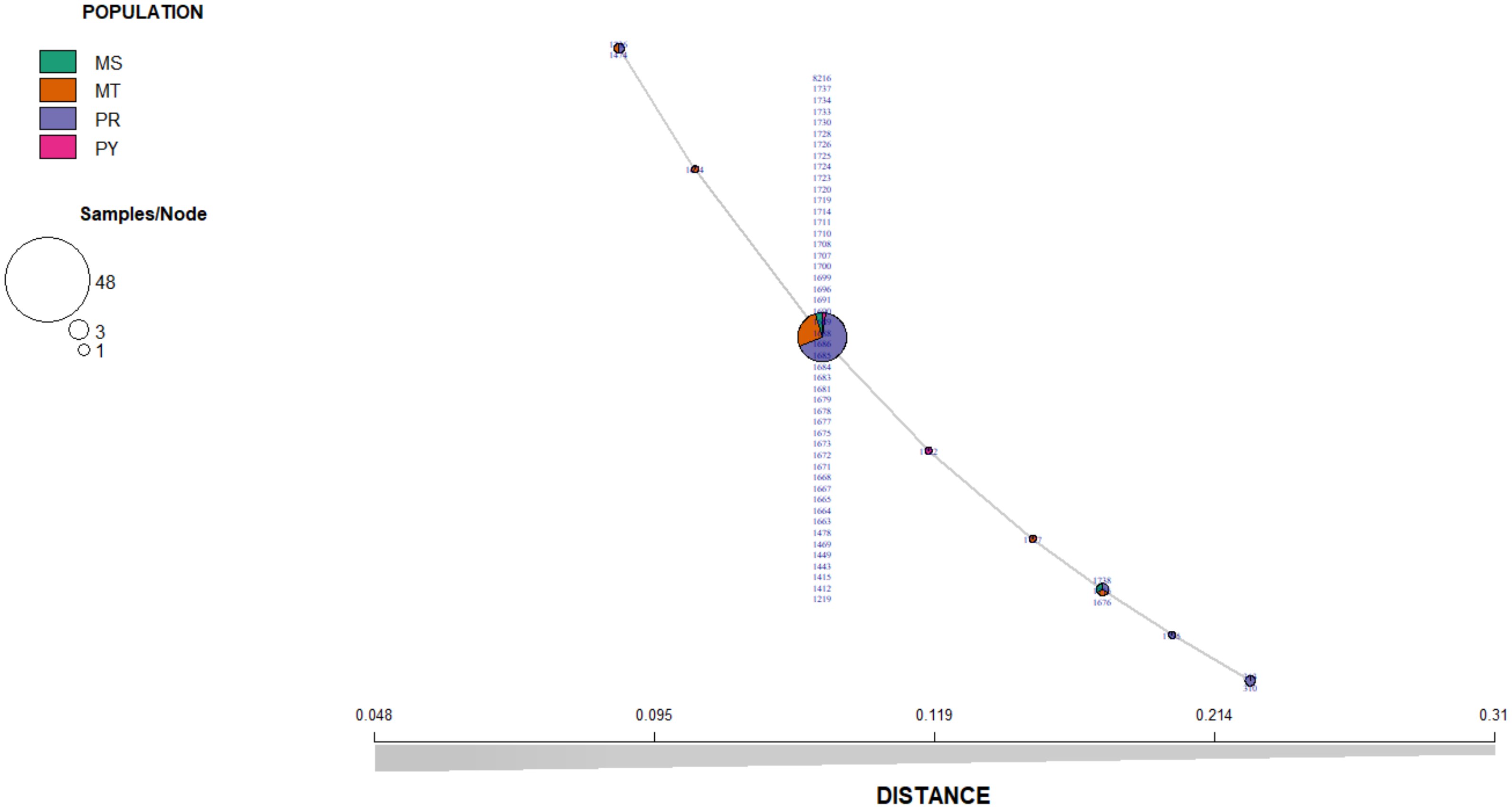

Figure 5.19. Median joining network of *Corynespora cassiicola* from soybean for fungicide resistance target genes SdhD. Each circle represents a haplotype. Cicle size is proportional to the number of isolates origin into the haplotypes. Coloured slices represent different geographic origin.

5.4 DISCUSSION

In this research, we analyzed the baseline sensitivity and cross-resistance for *C. cassiicola* isolates from soybean to DMI, QoI and MBC fungicides. This is the first next generation sequencing study in soybean to identify fungicide resistance in *C. cassicola* to fungicide target genes for *cyp51,* β-tubulin, *cytb* and Sdh (subunits b, c, d). Using genome associated prediction integrated tool we showed the most significant mutation in β-tubulin gene correlated with EC50 and M.G.I. phenotype methods. Clustering analysis showed that resistant and sensitive populations to fungicide target genes *cyp51,* β-tubulin, *cyt b* and Sdh (subunits b, c, d) were genetically distinct. Using next generation sequencing we could confirm heteroplasmid is occoring in *C. cassiicola* in *cyp51*, β-tubulin, *cyt b,* SdhB*,* SdhC and SdhD genes. In addition we also characterized sequencing variability of *C. cassicola* to fungicide target genes.

Sensitive and resistant phenotypes of *C. cassiicola* were distinguished using mycelial growth inhibition test in amended-medium with MBC and QoI fungicides discriminatory dose at 10 μL/mL^{-1}. Results from *in vitro* bioassay using different fungi pathogens for QoI fungicides, showed that the resistance is related with a bypass by an alternative respiration, which is inhibited by salicylhydroxamic acid (SHAM) or propyl gallate (PG) (MIGUEZ et al., 2004; ISHII et al., 2009). Studies evaluating spore germination in QoI fungicides of *C. cassiicola* from soybean have shown there was no significant effect of SHAM in increasing the sensitivity of *C. cassiicola* isolates, suggesting that *C. cassicola* has no alternative respiration (TERAMOTO et al., 2017).

Results from cross-resistance obtained by *in vitro* in mycelial growth test, using discriminatory doses, showed strong positive correlation of *C. cassiicola* isolates exposed to QoI (azoxystrobin x picoxystrobin, picoxystrobin x pyraclostrobin, azoxystrobin x pyraclostrobin) and MBC (carbendazim x thiophanate-methyl) fungicide groups. Similar results were found *Pyricularia grisea* (KIM et al; 2003); *Pyrenophora teres* (SIEROTZKI et al., 2007), *Cercospora kikuchii* (PRICE et al., 2015) and recently, in *C. cassicola* from cucumber in China (DUAN et al., 2019).

We identified three *C. cassiicola* isolates classified as resitant for the DMI, QoI and MBC fungicides group. In addition, the EC50 for 59 isolates, 5%, 14% and 8% of carbendazim, pyraclostrobin and prothioconazole, respectively, show EC_{50}

greater than 100 µg/mL. Resistant isolates of *C. cassiicola* from soybean in Brazil have been previously reported (XAVIER et al., 2013; TERAMOTO et al., 2017).

For the DMI fungicide, the highly resistant phenotype was detected in 32% of the isolates. The sequencing of *cyp51* gene demonstrated a heterozigotic variation for both mutations S279N and I299V. DMI mutations were identified for isolate 1715 in S279N and 1715, 1722 and 1726 for I299V mutations. The lowest sensitivity of *C. cassiicola* populations from soybean in Brazil to DMI was reported by Xavier et al. (2013) and Teramoto et al. (2017) and also in *Phakopsora pachyrhizi* by Schmitz et al. (2014) and Klosowki et al. (2016).

The low sensitivity of *C. cassiicola* due to de high selection pressure in Brazilian soybean growing regions to MBC active ingredient was evidenced in a network study of fungicide trials from data collected during 2012-2016 harvest. It was observed carbendazim had the lowest efficacy control (32.4%) among fungicides tested azoxystrobin + benzovindiflupyr, fluxapyroxad + pyraclostrobin, epoxiconazole + fluxapyroxad + pyraclostrobin, mancozeb and prothioconazole + trifloxystrobin (EDWARDS MOLINA et al., 2018).

In this study, genotype for β-tubulin gene, showed two double mutation at positions I189V + E198A and E198A + F200Y. Single mutations were identified for isolate 1676 from Paraná Brazilian state at position S168Y and S275N mutation for isolate 1720 from Paraguay. Recently, three double mutations E198A + M163I, E198A + F167Y and E198A + F200S were also detected in *C. cassiicola* populations from cucumber in China (DUAN et al., 2019). Different point mutation of β-tubulin gene cause different resistant level to benzimidazoles fungicides, e.g., F167Y or F200Y in *Fusarium graminearum* conferring moderate-level of resistance, while, E198A substitutions in *F. graminearum* resulte in high-level of resistance (DUAN et al., 2014, 2016). Therefore, using genome association analysis, we correlated MG.I and EC50 fenotypes and genotype of β-tubulin gene and we found four SNPs above the Bonferroni threshold. Two of these SNPs are synonumous mutations and express correlation for *cyt b* (Threonime) and SdhC (Alanine) genes. The strongest association for β-tubulin gene were found for the mutations E198A and F200Y.

High frequency of highly resistant isolates to QoI fungicides was observed for isolates collected in 2014 and 2016. A substitutions of I78V was found for the isolate 1722 collected in Ponta Porã-Mato Grosso do Sul Brazilian state.

Addictionally, heterozygous variant of *C. cassiicola* isolates with mutations at positions G143A was observed for all isolates and was confirmed by Sanger sequencing cromatography. The mutations F129L and G137R were not found. Ishii et al., (2007; 2009) reported the heteroplasmic sequences in *C. cassiicola* from cucumber grown in PDA in the presence and absence of QoI fungicide. However, not all *C. cassiicola* isolates showing moderate and high EC50 resistance presented G143A mutation, indicating a low correlation between sequence and colorimetric microtiter methodology. This distinct nature between QoI phenotyping and *cyt b* genotype could be explained by the dynamic nature of fungi mitochondria, which often repeat division and fusion independently of nuclear division, influencing the balance and instability of the mutated sequence in *cyt b* gene (ISHII et al., 2009).

Analysing the mechanisms of resistance in SDHI genes we found substitution in the SdhA, SdhB, SdhC and SdhD genes at positions 34, 39 and 318, 454, 461, 556, (A-E318D, I454V, A461S, Y556F), 34, 39, 260. B-A34V, V39I, K260R), 37, 61, 85, 163, (C-Q37H, S61T, G85S, I163V) and 126 (D-V126I), respectively. The most frequent mutation was in K260R in the SdhB gene, which were found 15 isolates, followed by the V126I substitution, found in 5 isolates. For all mutations in SDH genes, homozygous and heterozygous variations were observed. This genomic characterization has also been reported in *Rizoctonia solani* (MU et al., 2013) and in *R. cerealis* (SUN et al., 2017).

The comparison between sensitive and resistant haplotypes resulted in variability among populations to DMI, MBC, QoI and SDHI confirming that genetic mutations of fungicides target genes are responsible for the genetic variability among the populations. In this study, genetic variation was caused by frequent sprays of fungicides with the same mode of action, resulting in a local directional selection pressure.

This research revealed that *C. cassiicola* resistant isolates to QoI, MBC and DMI is widely spread in soybean fields, regardless of the location and date of sample collection, based on the high frequency of the mutations and resistance observed. In addition, cross-resistance was demonstrated inside active ingredients of MBC and QoI chemical groups. This research also shows possible point mutations in *cyp51* gene at S279N and I299V, double mutations in β-tubulin gene at codons I189V + E198A and E198A and single mutations at codon position S168Y, I189V, S275N. We also found in QoI fungicides G143A mutation in *cytb* gene and the occurrence of substitutions for I78V. Multiple resistance was confirmed to MBC and QoI fungicides. These results suggest that isolates of *C. cassiicola* to MBC, QoI and DMI in present at Brazilian soybean field and this increasing the risk for the selection pressure for resistant isolates. Despite, sdha gene does not confer fungicide resistance substitutions was related at position 318, 454, 461, 556 (A-E318D, I454V, A461S, Y556F). In addition, amino acid substitution in SdhB, SdhC and SdhD genes at positions 34, 39 and 260 (B-A34V, V39I, K260R), 37, 61, 85, 163 (C-Q37H, S61T, G85S, I163V) and 126 (D-V126I), respectively could be associated with possible potential mutations, which confer SDHI fungicide resistance. In addition, heterozygous variants were confirmed for all genes analyzed. Therefore, anti-resistance strategies have to be implemented for efficiency recovery of MBC, QoI and DMI for management target spot in soybean.

Despite the large number of new mutations observed in genes that confer fungicide resistance, it was not possible to correlate the new mutations with the phenotypic results of the microtiter method. For this case, two possible hypotheses can be pointed out. The first is that point mutations may be correlated with the genetic variability of the isolate, the second is the methods and the different multiplications of the pathogen may be at different times. For this last hypothesis new studies must be analyzed.

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5.5 REFERENCES

ALFENAS, A. C.; FERREIRA, F. A.; MAFIA, R. G.; GONÇALVES, R. C. **Isolamento de fungos fitopatogênicos**. Métodos em fitopatologia. Viçosa: Ed. da UFV, p. 54, 2007.

ALMEIDA, A. M. R.; FERREIRA, L. P.; YORINORI, J. T.; SILVA, J. F. V.; HENNING, A. A.; GODOY, C. V.; COSTAMILAN, L. M.; MEYER, M. C. Doenças da soja. In: KIMATI, H.; AMORIM, L.; REZENDE, J. A. M.; BERGAMIN FILHO, A.; CAMARGO, L. E. A. **Manual de Fitopatologia: doenças de plantas Cultivadas**. 4. ed. vol. 2. São Paulo: Editora Agronômica Ceres. 570-588 p, 2005.

ALMEIDA, A. M. R.; MACHADO, C. C.; FERREIRA, L. P.; LEHMAN, P. S.; ANTONIO, H. Ocorrência de *Corynespora cassiicola* (Berk. & Curt.) Wei no Estado de São Paulo. **Fitopatologia Brasileira**, v.1, p.111-112, 1976.

ANDREWS, S. **FastQC: a quality control tool for high throughput sequence data** (2010). Disponível em: http://www.bioinformatics.babraham.ac.uk/projects/fastqc. Accessed in: March. 2018.

AVOZANI, A.; REIS, E. M.; TONIN, R. B. Sensitivity loss by *Corynespora cassiicola*, isolated from soybean, to the fungicide carbendazim. **Summa Phytopathologica**, v. 40, n.3, p. 273-276, 2014.

AYRES, D. L.; DARLING, A., ZWICKL, D. J.; BEERLI, P.; HOLDER, M. T.; LEWIS, P. O.; RAMBAUT, A. BEAGLE: an application programming interface and high-performance computing library for statistical phylogenetics. **Systematic biology**, v. 61, n. 1, p. 170-173, 2011.

BARTLETT, D. W.; GODWIN, J. R.; CLOUGH, J. M.; HALL, A. A.; HAMER, M.; PARR-DOBRZANSKI, B. The strobilurin fungicides. **Pest Management Science.** 58:649–662, 2002.

BECHER R.; WIRSEL, S.G.R. Fungal cytochrome P450 sterol 14α-demethylase (CYP51) and azole resistance in plant and human pathogens. **Applied Microbiology Biotechnology,** v. 95, p. 825–840, 2012.

BECKER, W. F.; VON, JAGOW, G.; ANKE, T.; STEGLICH, W. Oudemansin, strobilurin A, strobilurin B and myxothiazol: new inhibitors of the bc1 segment of the respiratory chain with an E-ßmethoxyacrylate system as common structural element. **FEBS Lett**, v. 132, p. 329–333, 1981.

BOLGER, A.M.; LOHSE, M.; USADEL, B. Trimmomatic: a flexible trimmer for Illumina sequence data**. Bioinformatics**, v. 30, n. 15, p. 2114-2120, 2014.

BRENT, K. J. **Historical perspective of fungicide resistance**. In: THIND, S.T. (ed.) Fungicide Resistance in Crop Protection. **Punjab Agricultural University**, India, pp. 3-18, 2011.

BROOMFIELD, P. E.; HARGREAVES, J. A. A single amino-acid change in the iron-sulphur protein subunit of succinate dehydrogenase confers resistance to carboxin in *Ustilago maydis*. **Current genetics**, v. 22; n. 2, p. 117-121, 1992.

COHEN, J. A coefficient of agreement for nominal scales. **Educational and psychological measurement**, v. 20, n. 1, p. 37-46, 1960.

DANECEK, P., AUTON, A., ABECASIS, G., ALBERS, C. A., BANKS, E., DEPRISTO, M. A.; MCVEAN, G. The Variant Call Format and VCFtools. **Bioinformatics**, v. 27, n. 15, p. 2156-2158, 2011.

DATE, H.; KATAOKA, E.; TANINA, K.; SASAKI, S.; INOUE, K.; NASU, H.; KASUYAMA, S. Sensitivity of *Corynespora cassicola*, causal agent of *Corynespora* target spot of tomato, to thiophanate methyl and diethofencarb. **Japanese Journal of Phytopathology**, v. 70, p. 7-9, 2004.

DAVIDSE, L. C. Benzimidazole fungicides: mechanism of action and biological impact. **Annual Review of Phytopathology**, v.24, p.43–65, 1986.

DAVIDSE, L.; ISHII, H. Biochemical and molecular aspects of benzimidazoles, N -phenylcarbamates and N -phenylformamidoximes and the mechanisms of resistance to these compounds. **Modern selective fungicides**, p. 305–322, 1995.

DELP, C. J. Benzimidazole and related fungicides. **Modern selective fungicides: properties, applications, mechanisms of action**. Edi, H. Lyr in cooperation with H. Buchenhauer, 1987.

DELP, C. J. Resistance to plant disease control agents: how to cope with it. In: **Proceedings of symposia: IX International Congress of Plant Protection**, Washington, DC, USA, August 5-11, 1979. Minneapolis, Minn. (USA). Published for the Congress by Burgess Pub. 1981. p. 253-261, 1981.

DUAN, Y.; DUAN, Y., ZHANG, X., GE, C., WANG, Y., CAO, J., JIA, X; ZHOU, M. Development and application of loop-mediated isothermal amplification for detection of the F167Y mutation of carbendazim-resistant isolates in Fusarium graminearum. **Scientific reports**, v. 4, p. 7094, 2014.

DUAN, Y.; YANG, Y.; LI, T.; ZHAO, D.; CAO, J.; SHI, Y.; ZHOU, M. Development of a rapid and high-throughput molecular method for detecting the F200Y mutant genotype in benzimidazole-resistant isolates of Fusarium asiaticum. **Pest management science**, v. 72, n. 11, p. 2128-2135, 2016.

DUAN, Y; Xin, W.; Lu, F.; Li, T.; Li, M.; Wu, J.; Zhou, M. Benzimidazole-and QoI-resistance in Corynespora cassiicola populations from greenhouse-cultivated cucumber: An emerging problem in China. **Pesticide biochemistry and physiology**, v. 153, p. 95-105, 2019.

EDGINGTON, L. V.; KHEW, K. L.; BARRON, G. L. Fungitoxic spectrum of benzimidazole compounds. **Phytopathology**. v. 61, p. 42-44, 1971.

EDWARDS MOLINA, J. P.; PAUL P. A.; AMORIM, L.; DA SILVA, L. H. C. P.; SIQUERI, F. V.; BORGES, E. P.; NUNES JUNIOR, J.; MEYER, M. C.; MARTINS, M. C.; BALARDIN, R. S.; CARLIN, V. J.; GRIGOLLI, J. F. J.; DE BELUFI, R. M.; GODOY, C. V. Meta-analysis of fungicides efficacy on soybean target spot and cost-benefit assessment. **Plant Pathology**, 2018.

ELLIS, M. B. Dematiaceous hyphomycetes. **Commonwealth Mycological Institute**, Kew, Surrey, United Kingdom, p. 608 (1971).

FRAC- Fungicide Resistance Action Committee 2013. **List of plant pathogenic organisms resistant to disease control agents**. Available in: http://www.frac.info/publication/anhang/List-of-resistant plantpathogens_2013.pdf>. Accessed on August 24, 2015.

FRAC Fungicide Resistance Action Committee. QoI working group of FRAC. Online publication, available from http://www.frac.info/docs/default-source/qoi-wg/qoi-meeting-minutes/minutes-of-the-2017-qoi-meeting-updated-in-may-2018 recommendations-for-2018.pdf?sfvrsn=a0764b9a_2. Accessed on May 24, 2018

GEORGOPOULOS, S. G.; DOVAS, C. Occurrence of *Cercospora beticola* strains resistant to benzimidazole fungicides in northern Greece. **Plant Disease**, v. 57, p. 321–324, 1973.

GISI, U.; CHIN, K. M.; KNAPOVA, G.; KÜNG FÄRBER, R.; MOHR, U.; PARISI, S.; SIEROTZKI, H.; STEINFELD, U. Recent developments in elucidating modes of resistance to phenylamides, DMI and strobilurin fungicides. **Crop Protection**. v. 38, p.1328-1349, 2000.

GISI, U.; SIEROTZKI, H.; COOK, A.; MCCAFFERY, A. Mechanisms influencing the evolution of resistance to Qo inibitor fungicides. **Pest Management Science**. West Sussex, v.58, p.859-867, 2002.

GODOY, C. V.; MEYER, M. C. Informativo fundação meridional. 2014. Available at: http://fundacaomeridional.com.br/artigos/2014/11/10/resistencia-a-fungicidas-na-cultura-da-soja. Accessed on September 01, 2015.

GODOY, C. V.; UTIMADA, C. M.; MEYER, M. C.; CAMPOS, H. D; PIMENTA, C. B.; MIGUEL-WRUCK, D. S. Eficiência de fungicidas para o controle da mancha-alvo, *Corynespora cassiicola*, na safra 2016/17: resultados sumarizados dos ensaios cooperativos. **Circular técnica**. Londrina, PR, Julho, 2017.

GRASSO, V.; PALERMO, S.; SIEROTZKI, H.; GARIBALDI, A.; GISI, U. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. **Pest Management Science,** v. 62, p. 465–472, 2006.

HAAS, B. J., PAPANICOLAOU, A., YASSOUR, M., GRABHERR, M., BLOOD, P. D., BOWDEN, J.; MACMANES, M. D. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. **Nature protocols**, v. 8, n. 8, p. 1494, 2013.

HARTMAN, G. L.; SINCLAIR, J. B.; RUPE, J. C. Compendium of soybean diseases, IV edition. **The American Phytopathological society**. St. Paul. Min., USA, 1999.

HORSEFIELD R.; IWATA S.; BYRNE B. Complex II from a structural perspective. **Current Protein & Peptide Science**, v.5, p.107–118, 2004.

HUANG, D.; SHI, Y.; XIE, X.; WANG, W.; LI, B. Sensitivity of *Corynespora cassiicola* to Carbendazim and Diethofencarb. **Journal of Pure and Applied Microbiology**, v.*7*, n.2, p.891-898, 2013.

HUANG, L. S., SUN, G., COBESSI, D., WANG, A. C., SHEN, J. T., TUNG, E. Y.; BERRY, E. A. 3-nitropropionic acid is a suicide inhibitor of mitochondrial respiration that, upon oxidation by complex II, forms a covalent adduct with a catalytic base arginine in the active site of the enzyme. **Journal of Biological Chemistry**, v. 281, n.9, p.5965-5972, 2006.

ISHII, H.; FOUNTAINE, J.; CHUNG, W. H.; KANSAKO, M.; NISHIMURA, K.; TAKAHASHI, K.; OSHIMA, M. Characterisation of QoI-resistant field isolates of *Botrytis cinerea* from citrus and strawberry. **Pest management science**, v. 65, n.8, p.916-922, 2009.

ISHII, H.; FOUNTAINE, J.; MIYAMOTO, T.; NISHIMURA, K.; TOMITA, Y. Occurrence of a mutation in the succinate dehydrogenase gene found in some isolates of cucumber *Corynespora* leaf spot fungus resistant to boscalid. **Japanese Journal of Phytopathology**, v. 74, p. 38–39, 2008.

ISHII, H.; YANO, K.; DATE, H.; FURUTA, A.; SAGEHASHI, Y.; YAMAGUCHI, T.; SUGIYAMA, T. T.; NISHIMURA, K.; HASAMA, W. W. Molecular Characterization and Diagnosis of QoI Resistance in Cucumber and Eggplant Fungal Pathogens. **Phytopathology**, v.97, n.11, p.1458–66, 2007.

ITO, Y.; MURAGUCHI, H.; SESHIME, Y.; OITA, S.; YANAGI, S. O. Flutolanil and carboxin resistance in *Coprinus cinereus* conferred by a mutation in the cytochrome b560 subunit of succinate dehydrogenase complex (Complex II). **Molecular Genetics and Genomics**, v.272, n.3, p. 328–335, 2004.

JOMBART, Thibaut. adegenet: a R package for the multivariate analysis of genetic markers. **Bioinformatics**, v. 24, n. 11, p. 1403-1405, 2008.

JOSEPH-HORNE, T.; HOLLOMON, D. W. Functional diversity within the mitochondrial electron transport chain of plant pathogenic fungi. **Pest Management Science**, v.56, n. 1, p. 24-30, 2000.

KAMVAR, Zhian N.; TABIMA, Javier F.; GRÜNWALD, Niklaus J. Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. **PeerJ**, v. 2, p. e281, 2014.

KEON, J. P.; WHITE, G. A.; HARGREAVES, J. A. Isolation, characterization and sequence of a gene conferring resistance to the systemic fungicide carboxin from the maize smut pathogen, *Ustilago nuda*. **Current Genetics**, v.19, n.6, 475–481, 1991.

KIM, Y. S.; DIXON, E.; W.; VINCELLI, P.; FARMAN, M. L. Field resistance to strobilurin (QoI) fungicides in *Pyricularia grisea* caused by mutations in the mitochondrial cytochrome b gene. **Phytopathology**, v.*93*, n.7, p. 891-900, 2003.

KLINGELFUSS, L. H.; YORINORI, J. T. Latent infection by *Colletotrichum truncatum* and *Cercospora kikuchii* in soybean. **Fitopatologia Brasileira**, v. 26, n.2, p.158-164, 2001.

KLOSOWSKI, A. C.; BRAHM, L.; STAMMLER, G.; DE MIO, L. L. M. Competitive Fitness of *Phakopsora pachyrhizi* Isolates with Mutations in the CYP51 and CYTB Genes. **Phytopathology**, v. 106, n. 11, 1278-1284, 2016.

KOENRAADT, H.; JONES, A. L. The use of allele-specific oligonucleotide probes to characterize resistance to benomyl in field strains of *Venturia inaequalis*. **Phytopathology**, v. 82, p.1354–1358, 1992.

LANGMEAD, Ben; SALZBERG, Steven L. Fast gapped-read alignment with Bowtie 2. **Nature methods**, v. 9, n. 4, p. 357, 2012.

LI, J.; ZHOU, M.; LI, H.; CHEN, C.; WANG, J.; ZHANG, Y. A study on the molecular mechanism of resistance to amicarthiazol in *Xanthomonas campestris* pv. *citri.* **Pest Management Science**, v. 62, p. 440–445, 2006.

LIPKA, A. E., TIAN, F., WANG, Q., PEIFFER, J., LI, M., BRADBURY, P. J.; ZHANG, Z. GAPIT: genome association and prediction integrated tool. **Bioinformatics**, v. 28, n. 18, p. 2397-2399, 2012.

MA, Z.; MICHAILIDES, T. J. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. **Crop Protection**, v. 24, p. 853–863, 2005.

MATSSON, M.; ACKRELL, B. A.; COCHRAN, B.; HEDERSTEDT, L. Carboxin resistance in *Paracoccus denitrificans* conferred by a mutation in the membrane-anchor domain of succinate: quinone reductase (complex II). **Archives of Microbiology**, v. 170, n. 1, p. 27-37, 1998.

MATSSON, M.; HEDERSTEDT, L. The carboxin-binding site on Paracoccus denitrificans succinate: quinone reductase identified by mutations. **Journal of bioenergetics and biomembranes**, v.33, n. 2, p. 99-105, 2001.

MIGUEZ, M.; REEVE, C.; WOOD, P. M.; HOLLOMON, D. W. Alternative oxidase reduces the sensitivity of *Mycosphaerella graminicola* to QOI fungicides. Pest Management Science, Hoboken, v.60, n.1, p.3-7, 2004.

MIYAMOTO, T.; ISHII, H.; SEKO, T.; KOBORI, S.; TOMITA, Y. Occurrence of *Corynespora cassiicola* isolates resistant to boscalid on cucumber in Ibaraki Prefecture, Japan. **Plant Pathology**, v.58, n. 6, p. 1144-1151, 2009.

MIYAMOTO, T.; ISHII, H.; SEKO, T.; TOMITA, Y.; KOBORI, S.; OGAWARA, T. Occurrence of boscalid-resistant isolates of cucumber *Corynespora* leaf spot fungus (*C. cassiicola*). **Japanese Journal of Phytopathology**, 74, 37, 2008.

MIYAMOTO, T.; ISHII, H.; STAMMLER, G.; KOCH, A.; OGAWARA, T.; TOMITA, Y.; KOBORI, S. Distribution and molecular characterization of *Corynespora cassiicola* isolates resistant to boscalid. **Plant Pathology**, v. 59, n. 5, p. 873-881, 2010.

MU, W.; LI, B.; CHEN, C.; LIU, X.; HAO, J. Molecular mechanisms of thifluzamide resistance in *Rhizoctonia solani* (Abstr.). ***Phytopathology*** 104(Suppl. 1):S1.4, 2013.

OLIVEIRA, W. F. D.; CAETANO, F. V.; DIAS, E. M.; BATISTA, R. G.; NONATO, A. R. Eficiência de produtos fitossanitários pulverizados na cultura da soja (*Glycine max* L.), no controle da mancha parda (*Septoria glycines*) e crestamento foliar-mancha púrpura dos grãos (*Cercospora kikuchii*), 2000.

PRICE III, P. P.; PURVIS, M. A.; CAI, G.; PADGETT, G. B.; ROBERTSON, C. L.; SCHNEIDER, R. W.; ALBU, S. Fungicide resistance in *Cercospora kikuchii*, a soybean pathogen. **Plant disease**, v. 99, n. 11, p. 1596-1603, 2015.

RIMMER, A., PHAN, H., MATHIESON, I., IQBAL, Z., TWIGG, S. R., WILKIE, A. O.Integrating mapping-, assembly-and haplotype-based approaches for calling variants in clinical sequencing applications. **Nature genetics**, v. 46, n. 8, p. 912, 2014.

SCHMITZ, H. K.; MEDEIROS, C. A.; CRAIG, I. R.; STAMMLER, G. Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. **Pest management science**, v. 70, n. 3, p. 378-388, 2014.

SCHROEDER, W. T.; PROVVIDENTI, R. Resistance to benomyl in powdery mildew of cucurbits. **Plant Disease Report**, v. 53, p. 271-275, 1969.

SCHLIEP, K.P. phangorn: phylogenetic analysis in R. **Bioinformatics**, v. 27, n. 4, p. 592-593, 2010.

SIEROTZKI, H. Respiration Inhibitors: Complex III. In: Ishii H, Hollomon D, editors. **Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management**. Japan: Springer, p. 119-143, 2015.

SIEROTZKI, H.; FREY, R.; WULLSCHLEGER, J.; PALERMO, S.; KARLIN, S.; GODWIN, J.; GISI, U. Cytochrome b gene sequence and structure of Pyrenophora teres and P. tritici-repentis and implications for QoI resistance. **Pest Management Science: formerly Pesticide Science**, v. 63, n. 3, p. 225-233, 2007.

SINCLAIR, J. B.; BACKMAN, P. A. Compendium of soybean Disease. 3 ed. Edition St Paul, Minnesota. **American Phytopathological Society**, p. 106. 1989.

SKINNER, W.; BAILEY, A.; RENWICK, A.; KEON, J.; GURR, S.; HARGREAVES, J. A single amino-acid substitution in the iron-sulphur protein subunit of succinate dehydrogenase determines resistance to carboxin in *Mycosphaerella graminicola*. **Current Genetics**, v. 34*,* n. 5, p. 393-398, 1998.

TATUSOVA, T. A.; MADDEN, T. L. BLAST 2 Sequences, a new tool for comparing protein and nucleotide sequences. **FEMS microbiology letters**, v. 174, n. 2, p. 247-250, 1999.

SUN, H. Y.; LU, C. Q.; LI, W.; DENG, Y. Y.; CHEN, H. Homozygous and heterozygous point mutations in succinate dehydrogenase subunits b, c and d of *Rhizoctonia cerealis* conferring resistance to thifluzamide. **Pest management science**, v. 73, n. 5, p. 896-903, 2017.

TERAMOTO, A.; MEYER, M. C.; SUASSUNA, N. D.; CUNHA, M. G. *In vitro* sensitivity of *Corynespora cassiicola* isolated from soybean to fungicides and field chemical control of target spot. **Summa Phytopathologica**, v. 43, n. 4, p. 281-289, 2017.

XAVIER, S. A. **Microtitulação colorimétrica para avaliar a sensibilidade de *Corynespora cassiicola* e *Colletotrichum* spp. a fungicidas e caracterização de isolados de *Colletotrichum* spp**. Tese (Doutorado em Agronomia) - Universidade Estadual de Londrina, Londrina. 2017.

XAVIER, S. A., CANTERI, M. G., BARROS, D. C. M., GODOY, C. V. Sensitivity of *Corynespora cassiicola* from soybean to carbendazim and prothioconazole. **Tropical Plant Pathology**, v. 38, p. 431-435, 2013.

YANKOVSKAYA, V.; HORSEFIELD, R.; TÖRNROTH, S.; LUNA-CHAVEZ, C.; MIYOSHI, H.; LÉGER, C.; IWATA, S. Architecture of succinate dehydrogenase and reactive oxygen species generation. **Science**, v. 299, n. 5607, p. 700-704, 2003.

ZIOGAS, B. N.; MALANDRAKIS, A. A. Sterol Biosynthesis Inhibitors: C14 Demethylation (DMIs). In: Ishii H, Hollomon D, editors. **Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management**. Japan: Springer, p. 201-216, 2015.

5.6 SUPPLEMENTARY INFORMATION

Table S5.6.1. Primer pairs sequence and PCR conditions used to identification of point mutation in *Corynespora cassiicola* isolates from soybean in *cyp51,* β-tubulin, *cyt b*, Sdh (subunits b, c and d) to the fungicide resistance target genes.

Gene	**Primer_F sequencing**	**Annealing position**	**Primer_R sequencing**	**Annealing position**	**Annealing temperature (ºC)**	**Amplicon size (bp)**
cyp51 (DMI)	ATCATCGGCAGCACCATC	267	TTACTTCTTTTCCCGCCTCTC	729	63	462
β-tubulin (MBC)	AGGCTTCCGGTAACAAGTTC	2246	TGCCGACAAAGGTAGACGAC	3196	63	950
Cytb (QoI)[1]	TATTATGAGAGATGTAAATAATGG	201	AACAATATCTTGTCCAATTCATGG	486	57	285
Sdh*A* (SDHA)[2]	CTCGTGGTGAGGGTGGTTACCT	1489	CGCTTGAAAGGTGGAACAGC	2494	63	1005
Sdh*B* (SDHB)	TACGTGCACTACACACCCATT	192	AGCAGTTGAGAATCGTGTGG	1162	63	970
Sdh*C* (SDHC)	CGAGACGACGAGCAGAATG	263	AAGGTCTAGCGGGCTTACATC	797	63	534
Sdh*D* (SDHD)	AGAAGATTGAGGGCACCTTG	306	GCATCTTTAACACAGGAACTTCTC	1295	63	989

[1] Ishii et al., 2007;

[2] Miyamoto et al., 2010.

Supplementary table S5.6.2. Identification code, year, location, phenotype characterization based in the EC$_{50}$ values of isolates of *Corynespora cassiicola* from soybean fields in Brazil, to carbendazim (MBC), pyraclostrobin (QoI) and prothioconazole (DMI) and mycelial growth inhibition in amended-medium at 10 µL/mL^{-1} to QoI (azoxystrobin, picoxystrobin and pyraclostrobin) and MBC (carbendazim and thiophanate-methyl).

Code	**Year**	**State**	**EC$_{50}$ DMI**[d,e]		**MGI (%) at 10 µL/ mL and phenotype**[f]						**EC$_{50}$ QoI**[d,e]		**MGI (%) at 10 µL/ mL^{-} and phenotypes**[f]				**EC$_{50}$ MBC**[d,e]	
					azoxystrobin		**picoxystrobin**		**pyraclostrobin**				**carbendazim**		**thiophanate-methyl**			
313	1998	MT	0.0	S	60.8	S	57.9	S	43.1	R	0.0	S	100.0	S	88.1	S	1.5	MR
1663	2016	PR	6.3	MR	14.5	R	15.9	R	19.7	R	7.4	MR	3.4	R	13.7	R	18.4	MR
1664	2016	PR	0.2	S	7.9	R	9.3	R	35.0	R	0.4	S	10.0	R	4.3	R	6.2	MR
1665	2016	PR	29.5	MR	34.9	R	34.4	R	49.7	R	>100	HR	24.6	R	21.5	R	57.6	HR
1667	2016	PR	0.4	S	0.0	R	21.9	R	23.2	R	9.0	MR	2.4	R	3.0	R	2.4	MR
1668	2016	MT	>100	HR	38.0	R	29.6	R	50.3	S	>100	HR	20.1	R	30.7	R	69.0	HR
1671	2016	PR	74.6	HR	13.7	R	16.8	R	30.1	R	0.0	S	0.0	R	0.5	R	>100	HR
1672	2016	MT	0.4	S	25.4	R	29.4	R	41.2	R	22.5	MR	29.6	R	9.7	R	1.8	MR
1673	2016	MT	4.1	MR	8.6	R	33.4	R	21.5	R	>100	HR	0.0	R	10.4	R	3.2	MR
1675	2016	PR	13.8	MR	32.2	R	14.1	R	49.3	R	>100	HR	19.1	R	9.7	R	>100	HR
1677	2016	PR	0.6	S	0.0	R	0.0	R	17.1	R	25.8	MR	11.0	R	15.3	R	23.0	MR
1678	2016	PR	1.8	MR	13.3	R	39.0	R	36.7	R	35.1	MR	23.4	R	24.9	R	28.2	MR

Code	Year	State	EC$_{50}$ DMI^{d,e}		MGI (%) at 10 µL/ mL and phenotype^{f}						EC$_{50}$ QoI^{d,e}		MGI (%) at 10 µL/ mL^{-} and phenotypes^{f}				EC$_{50}$ MBC^{d,e}	
					azoxystrobin		picoxystrobin		pyraclostrobin				carbendazim		thiophanate-methyl			
1679	2016	PR	1.5	MR	30.9	R	21.1	R	48.7	R	9.2	MR	8.6	R	4.6	R	0.8	S
1681	2016	MT	0.7	S	14.2	R	9.9	R	32.6	R	>100	HR	0.0	R	3.0	R	47.6	MR
1683	2016	PR	>100	HR	57.4	S	51.0	S	65.8	S	>100	HR	60.0	S	39.4	R	21.0	MR
1684	2016	PR	0.0	S	43.7	R	35.6	R	48.3	R	>100	HR	15.5	R	25.3	R	>100	HR
1685	2016	PR	4.7	MR	0.0	R	13.0	R	13.8	R	59.7	HR	100.4	S	58.5	S	1.8	MR
1686	2016	PR	1.3	MR	12.5	R	23.9	R	35.1	R	0.0	S	95.8	S	63.4	S	0.5	S
1688	2016	PR	2.1	MR	19.0	R	25.3	R	58.9	S	6.5	MR	48.1	R	31.0	R	5.7	MR
1689	2016	PR	3.2	MR	0.0	R	2.3	R	15.2	R	33.1	MR	44.6	R	47.4	R	27.8	MR
1690	2016	PR	0.8	S	5.5	R	13.4	R	19.1	R	35.7	MR	11.8	R	0.0	R	9.6	MR
1691	2016	PR	1.8	MR	17.0	R	20.5	R	34.6	R	0.0	S	32.0	R	11.1	R	22.0	MR
1696	2016	MS	33.0	MR	27.4	R	28.3	R	50.2	S	4.4	MR	100.0	S	90.9	S	3.5	MR
1699	2016	MT	1.7	MR	36.2	R	55.1	S	76.8	S	0.1	S	91.3	S	89.9	S	1.5	MR
1700	2016	MT	0.6	S	31.4	R	40.0	R	48.4	R	3.7	MR	97.8	S	100.0	S	29.9	MR
1707	2016	PR	54.5	HR	15.7	R	19.2	R	33.7	R	0.0	S	100.0	S	91.4	S	>100	HR
1708	2016	PR	>100	HR	27.8	R	31.6	R	39.1	R	>100	HR	59.4	S	18.0	R	>100	HR
1710	2016	PR	>100	HR	12.0	R	14.4	R	42.7	R	0.0	S	29.1	R	6.3	R	2.1	MR

Code	Year	State	EC$_{50}$ DMI^{d,e}		MGI (%) at 10 µL/ mL and phenotype^{f}						EC$_{50}$ QoI^{d,e}		MGI (%) at 10 µL/ mL^{-} and phenotypes^{f}				EC$_{50}$ MBC^{d,e}	
					azoxystrobin		picoxystrobin		pyraclostrobin				carbendazim		thiophanate-methyl			
1711	2016	PR	>100	HR	21.8	R	14.7	R	58.3	S	>100	HR	10.3	R	17.9	R	63.8	HR
1714	2016	PR	2.8	MR	27.6	R	22.0	R	48.8	R	>100	HR	0.0	R	0.0	R	87.9	HR
1715	2016	PR	15.7	MR	3.1	R	24.1	R	29.7	R	3.2	MR	26.3	R	5.3	R	0.0	S
1719	2016	PY^{c}	55.8	HR	11.0	R	9.0	R	53.8	S	71.4	HR	82.3	S	98.7	S	4.6	MR
1722	2016	MS	1.3	MR	50.6	S	44.4	R	53.3	S	0.0	S	91.1	S	35.0	R	0.3	S
1723	2016	MT	1.7	MR	16.3	R	16.8	R	34.4	R	>100	HR	18.6	R	17.6	R	6.2	MR
1725	2016	MT	0.8	S	10.0	R	19.4	R	18.7	R	0.0	S	7.8	R	13.7	R	0.3	S
1726	2016	PR	98.1	HR	19.8	R	31.0	R	64.7	S	73.3	HR	46.0	R	39.0	R	1.8	MR
1727	2016	PR	0.0	S	22.8	R	13.3	R	22.3	R	0.0	S	19.7	R	11.9	R	32.6	MR
1728	2016	PR	2.6	MR	12.8	R	6.8	R	45.9	R	15.6	MR	100.0	S	100.0	S	0.2	S
1730	2016	MT	4.9	MR	17.8	R	7.4	R	37.7	R	>100	HR	11.7	R	18.8	R	10.9	MR
1734	2016	MT	22.1	MR	23.2	R	29.2	R	28.7	R	72.9	HR	25.3	R	16.6	R	1.0	MR
1736	2016	MT	9.9	MR	9.9	R	20.4	R	29.1	R	17.1	MR	32.0	R	21.4	R	1.1	MR
1737	2016	PR	0.5	S	18.7	R	8.9	R	25.8	R	0.2	S	0.0	R	0.0	R	62.6	HR
8216	2016	PR	9.6	MR	10.8	R	0.5	R	16.2	R	>100	HR	24.2	R	16.2	R	27.5	MR

[a]Embrapa soybean Mycological Collection; [b]EC_{50} data source by Xavier 2017; [c](PY)-Paraguay, samples collected from Paraguay; [d] NT- (Not tested); [e]Classification of EC_{50} effective concentration to inhibit 50% of conidia germination for the classes of fungicides, carbendazim (MBC) pyraclostrobin (QoI) and prothioconazole (DMI) classification of the phenotypes in the categories, (S-Sensitive) $EC_{50} \leq 1$ µg / mL^{-1}; (MR- Moderately resistant) EC_{50} 1 µg $mL^{-1} < EC_{50} < 50$ µg / mL^{-1}; (HR-Highly resistant) $EC_{50} \geq 50$ µg / mL^{-1}; [f](-), absence of mutation.

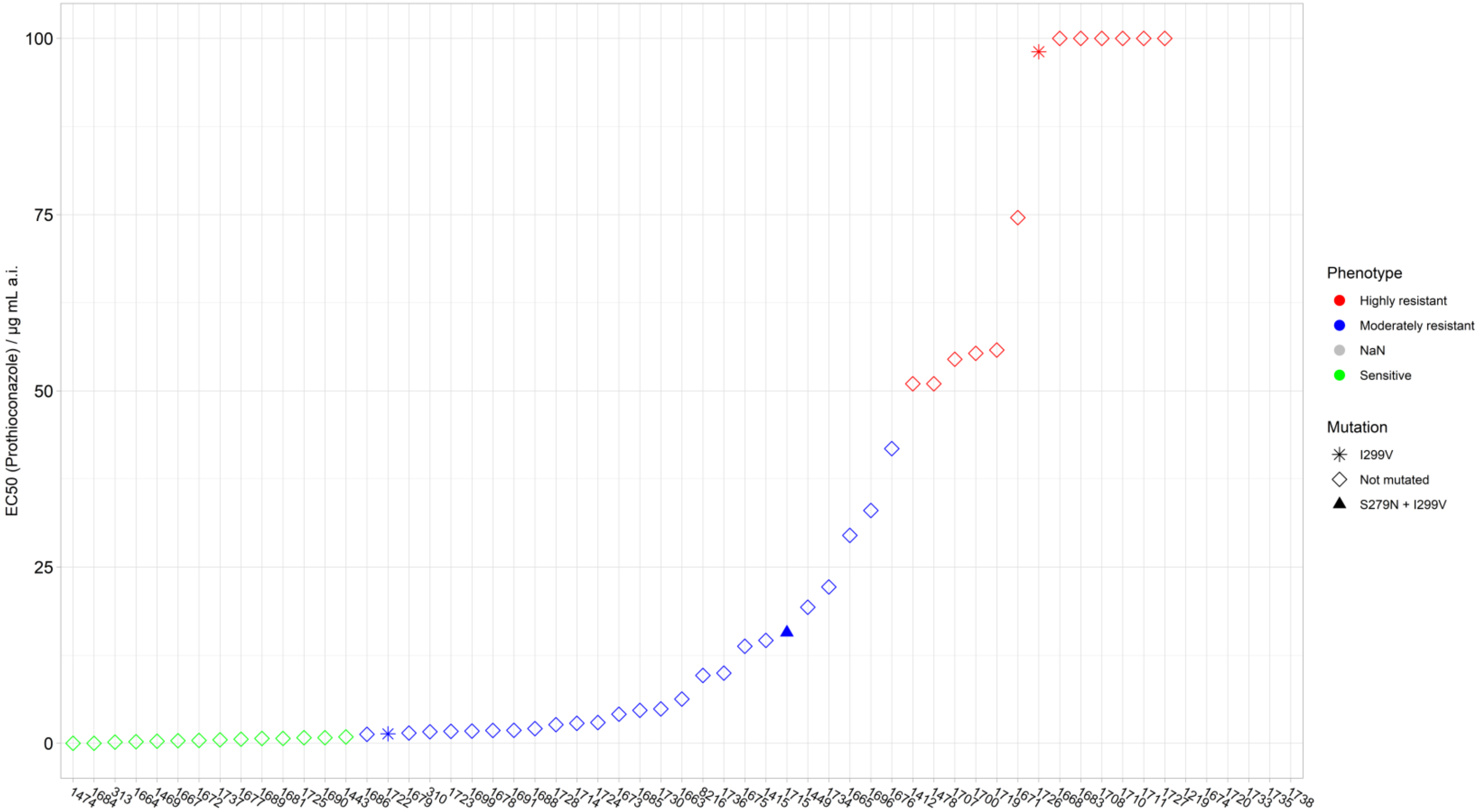


Figure S5.6.1 Variation of EC50 of *Corynespora cassiicola* isolates from soybean. Colors scale represents isolate classification into different phenotype. Different geometric shapes represent the nonsynonymous mutation to the fungicide prothioconazole (DMI).

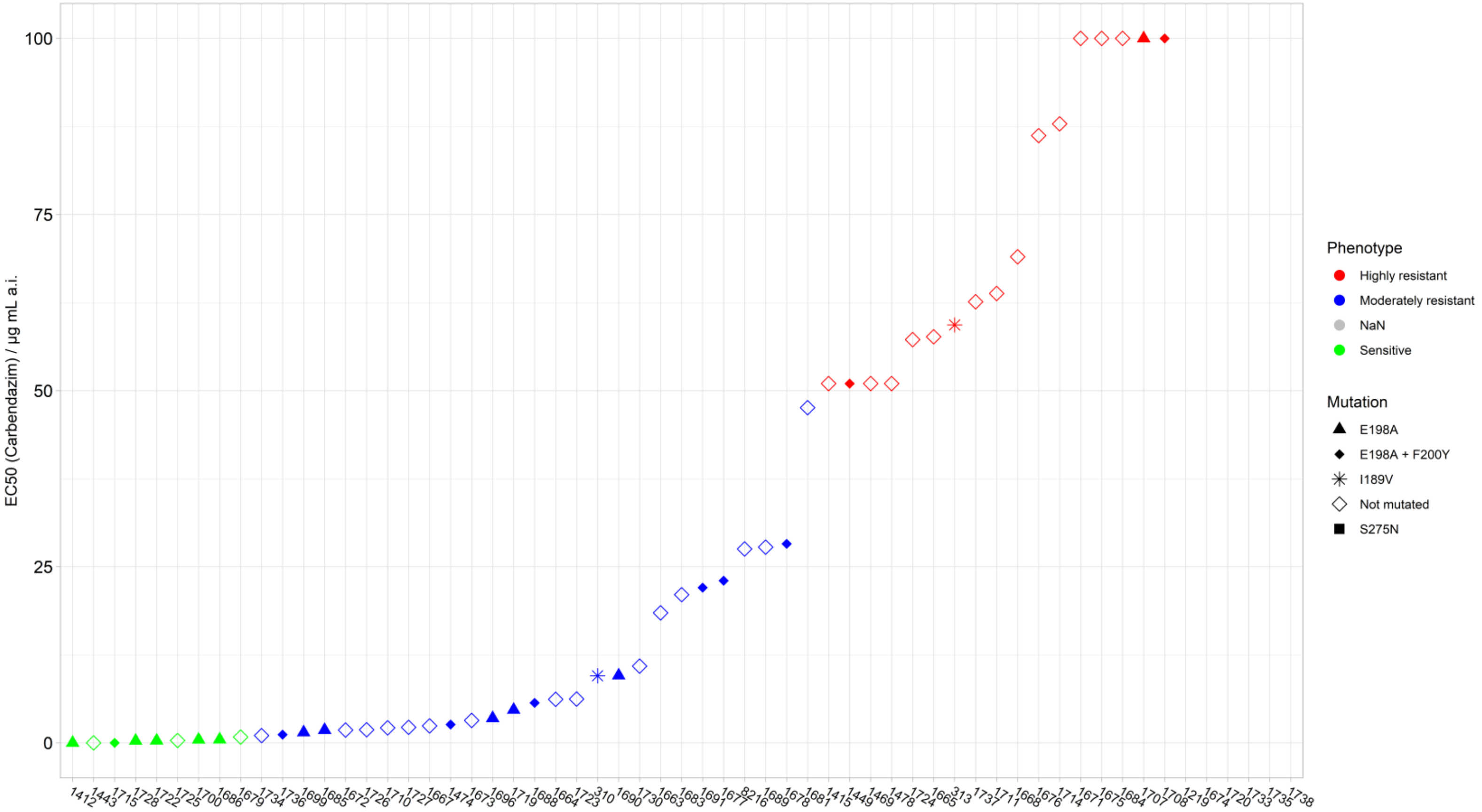


Figure 5.2 Variation of EC50 of *Corynespora cassiicola* isolates from soybean. Colors scale represents isolate classification into different phenotype. Different geometric shapes represent the nonsynonymous mutation to the fungicide carbendazim (MBC).

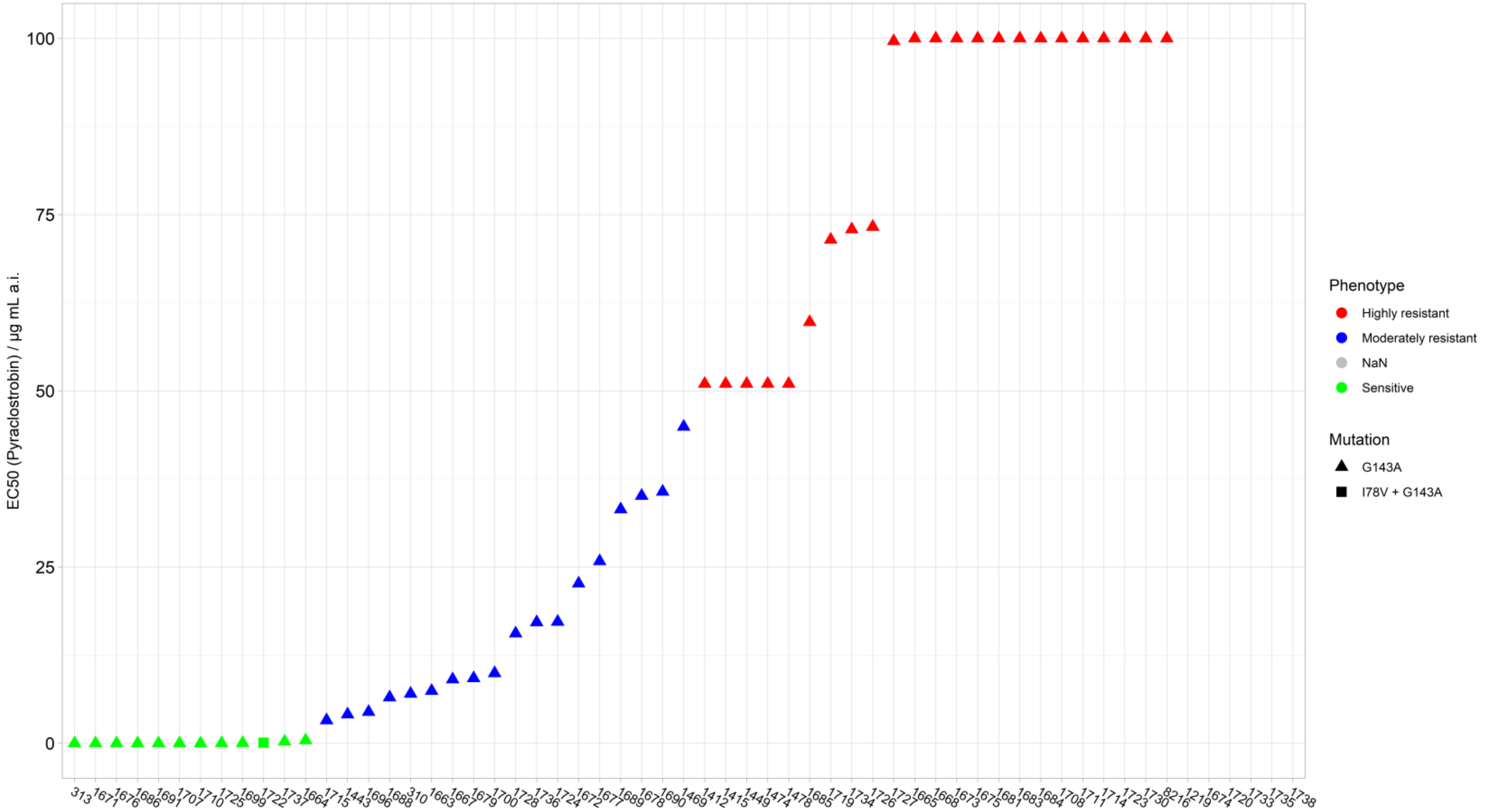


Figure 5.3 Variation of EC50 of *Corynespora cassiicola* isolates from soybean. Colors scale represents isolate classification into different phenotype. Different geometric shapes represent the nonsynonymous mutation to the fungicide pyraclostrobin (QoI).

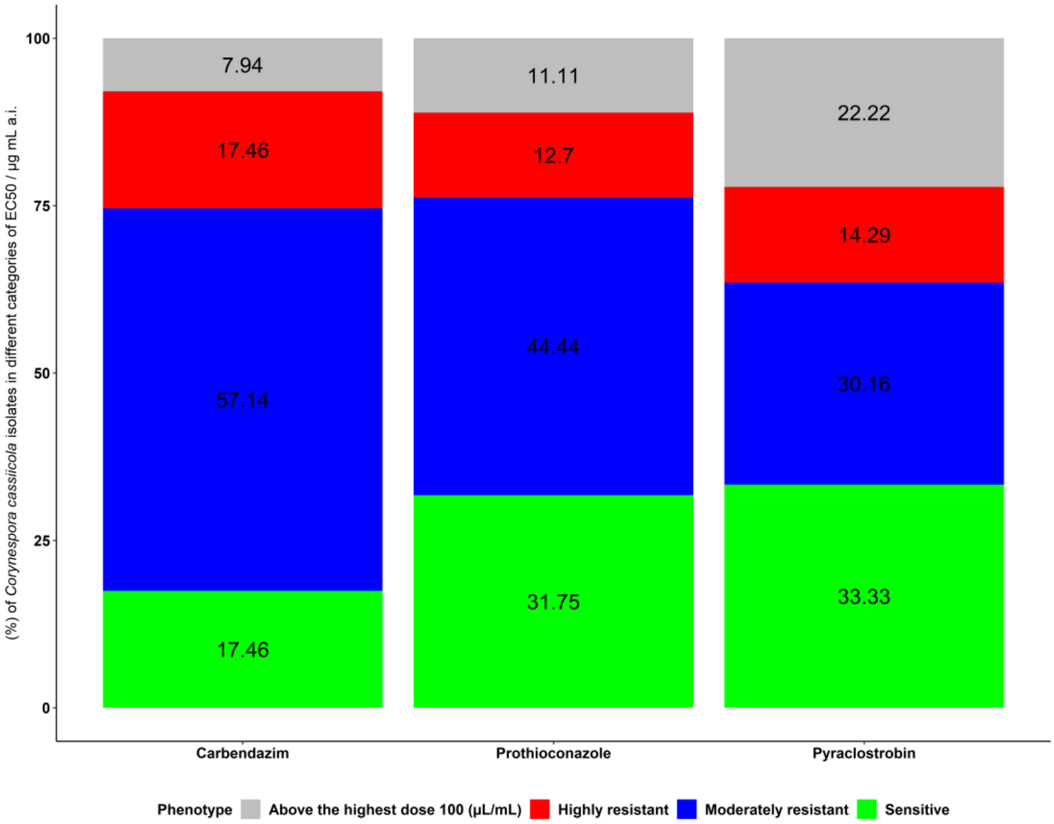


Figure S5.4. Percentage of *Corynespora cassiicola* isolates using EC50 (effective concentration to inhibit 50% of conidia germination) for the fungicides carbendazim-MBC, pyraclostrobin-QoI and prothioconazole-DMI in the categories, (Sensitive) $EC_{50} \leq 1$ µg / mL^{-1}; (Moderately resistant) EC50 1 µg $mL^{-1} < EC_{50} < 50$ µg / mL^{-1}; (Highly resistant) $EC_{50} \geq 50$ µg / mL^{-1}. EC50 was not estimated for some isolates due estimated due to 50% inhibition of spore germination occurs above the highest dose evaluated (100 µL/mL).

Supplementary table S5.3. Codon position, amino acid change and nonsynonymous and synonymous mutations for *cyp51* (DMI), β-tubulin (MBC), *cyt b* (QoI) and Sdh (subunits a, b, c, d) fungicide target genes of *Corynespora cassiicola* isolates.

DMI				**MBC**				**QoI**				**QoI**			
Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations		Codon position	Amino acid change	Mutations	
		non-syn	syn			non-syn	syn			non-syn	syn			non-syn	syn
831	TCG/TCA	S		503	TCC/TAC	-	S168Y	232	ATA/GTA	-	I78V	531	AAT/AAC	N	
836	AGT/AAT	-	S279N	519	CCT/CCC	P		255	ACT/ACA	T		537	TTT/TTC	F	
843	GAA/GAG	E		565	ATC/GTC	-	I189V	273	TTT/TTC	F		573	GCT/GCC	A	
849	GAC/GAT	D		593	GAG/ GCG	-	E198A	282	TAT/TAC	Y		591	CAC/CAT	H	
895	ATT/GTT	-	I299V	599	TTC/TAC	-	F200Y	288	CAT/CAC	H		627	AAC/AAT	N	
990	CTT/CTG	L		645	CTT/CTC	L		405	CCT/CCA	P		696	ATA/ATT	I	
1149	CCC/CCG	P		714	ACG/ACC	T		411	GGA/GGT	G		813	GTA/GTT	V	
1164	CTC/CTT	L		738	CTC/CTG	L		420	TCT/ TCA/ TCC	S		834	CCT/CCA	P	
1197	GGC/GGG	G		822	ACC/ACT	T		428	GGT/GCT	-	G143A				
1221	ACT/ACC/ACG	T		824	AGC/AAC	-	S275N	435	ACT/ACA	T					
1365	GTC/GTG/GTT	V		834	GCC/GCT	A		507	TTT/TTC	F					
				882	TTT/TTC	F		522	GCA/GCT	A					
				1053	ACT/ACC	T		525	ACA/ACT	T					

SdhA				SdhA				SdhA				SdhB			
Codon position	Amino acid change	Mutations non-syn	Mutations syn	Codon position	Amino acid change	Mutations non-syn	Mutations syn	Codon position	Amino acid change	Mutations non-syn	Mutations syn	Codon position	Amino acid change	Mutations non-syn	Mutations syn
26	TCT/TCC	S		332	ACC/ACT	T	357	893	ACG/ACC/ACT	T		101	GCC/GTC	-	A34V
35	GAG/GAA	E		338	GAC/GAT	D	376	914	CCT/CCA/CCC	P		115	GTC/ATC	-	V39I
50	CGT/CGC	R		362	GTC/GTT	V	400					129	GCC/GCT	A	
62	AAC/AAT	N		380	TGC/TGT	C						165	GTT/GTC	V	
77	GCT/GCC	A		389	GCT/GCC	A						174	AGT/AGC	S	
83	CGC/CGT	R		392	GCC/GCT	A						180	ACC/ACT	T	
89	GTT/GTC	V		413	GCC/GCT	A						186	ACT/ACC	T	
101	TCC/TCG	S		476	ACC/ACT	T						297	GAC/GAT	D	
107	ACC/ACG	T		482	CGC/CGT	R						360	CCG/CCA	P	
119	CGC/CGT	R		521	TCG/TCC	S						471	ACC/ACT	T	
122	GAG/GAA	E		539	GAG/GAA	E						510	ATT/ATC	I	
137	GGA/GGC/GGT	G		545	ATC/ATT	I						537	ACG/ACA	T	
143	GAG/GAC/GAT	-	E318D	549	ATC/GTC	-	I454V					765	GAT/GAC	D	
174	TTG/CTG	L		570	GCC/TCC	-	A461S					779	AAG/AGG	-	K260R
182	CCT/CCC	P		590	ACT/ACC	T						798	GAT/GAC	D	
215	TCC/TCG/TCT	S		614	CAA/CAG	Q									
221	ACC/ACG/ACT	T		695	GAT/GAC	D									
230	ATT/ATC	I		755	GTT/GTC	V									
254	AAG/AAA	K		764	CTC/CTT	L									
296	GGT/GGC/GGG	G		794	GTG/GTC	V									
305	CCC/CCA/CCT	P		827	GAA/GAG	E									
317	ACC/ACT	T		839	GCG/GCC	A									
323	GAG/GAA	E		856	TAC/TTC	-	Y556F								

SdhC				**SdhD**			
Codon position	Amino acid change	Mutations		Codon position	Amino acid change non-syn	Mutations	
		non-syn	syn			non-syn	syn
		A					
99	GCT/GCC	A		CCT/CCC	P		
102	GCT/GCC	-	Q37H	GTC/ATC			
111	CAG/CAC	-	S61T	CTG/TTG	L		
181	TCC/ACC	Y					
210	TAC/TAT	A					
243	GCC/GCT	-	G85S				
253	GGC/AGC	L					
258	CTT/CTC	L					
282	CTG/CTC	A					
288	GCC/GCT	P					
291	CCC/CCT	G					
414	GGT/GGC	I					
438	ATT/ATC	-	I163V				
487	ATT/GTT	T					

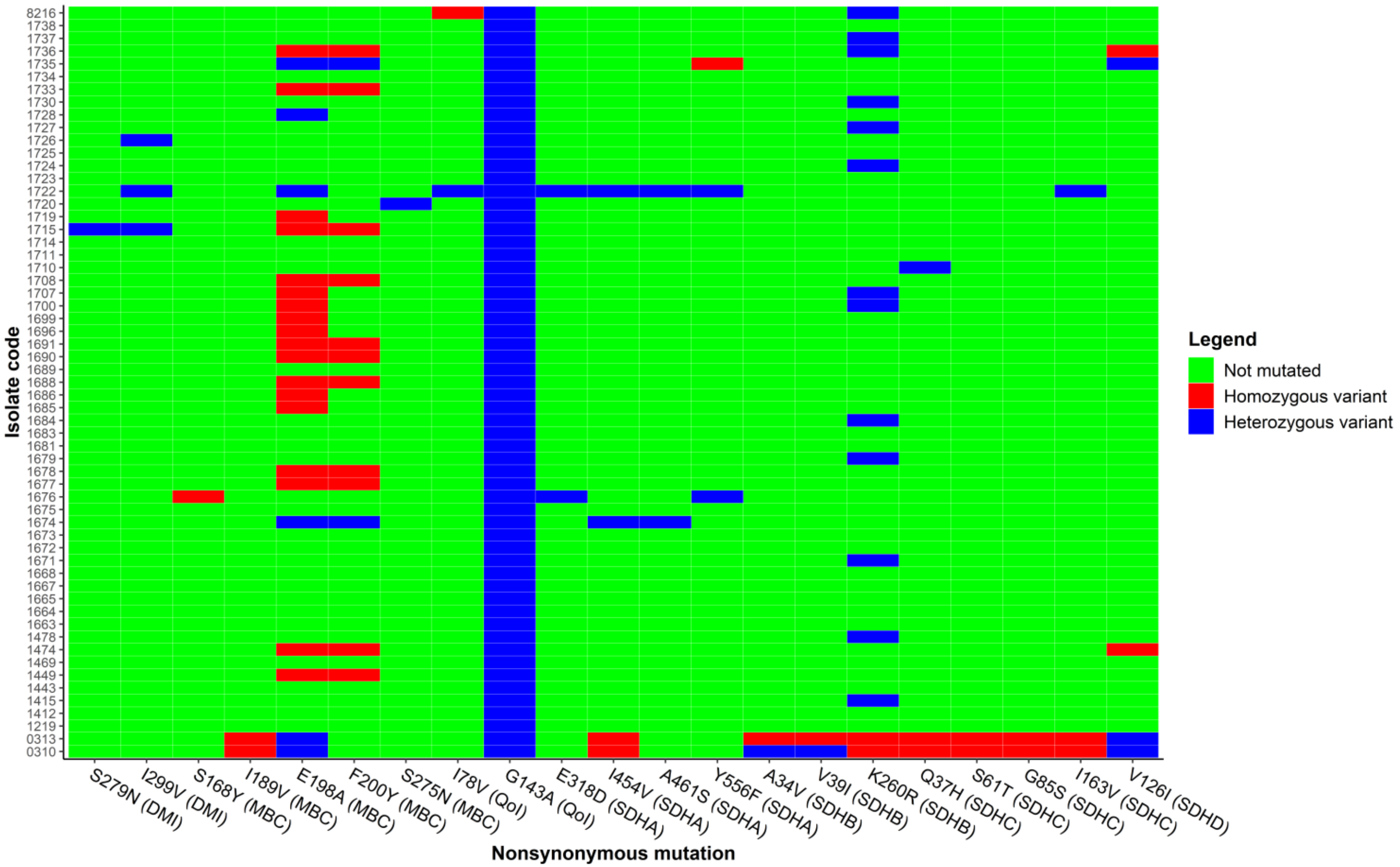
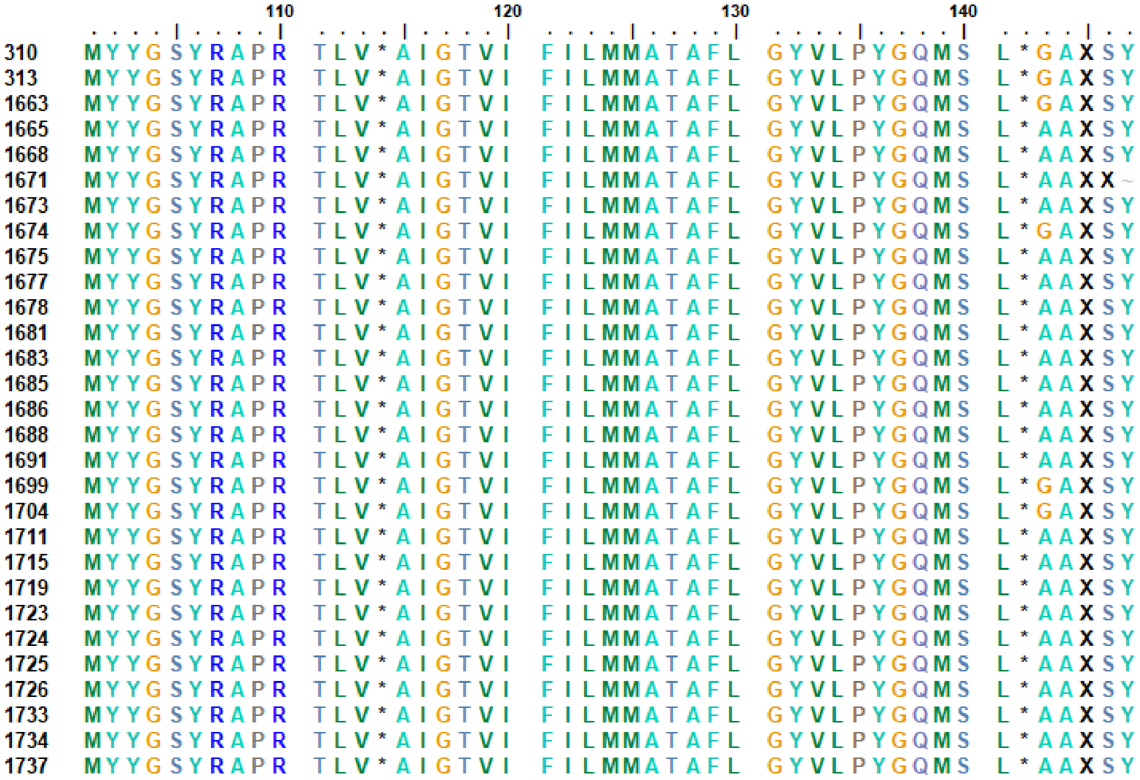


Figure S5.5. Heatmap of nonsynonymous mutations *cyp51*, β-tubulin, *cyt b* and SdhA, SdhB, SdhC and SdhD genes, for fungicide target site resistance in *Corynespora cassiicola* isolates. Colors on the map represent genetic variations for each isolate.



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                        110                 120                 130                 140
        ....|....|  ....|....|  ....|....|  ....|....|  ....|..
310     MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*GAXSY
313     MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*GAXSY
1663    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*GAXSY
1665    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1668    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1671    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXX~
1673    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1674    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*GAXSY
1675    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1677    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1678    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1681    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1683    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1685    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1686    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1688    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1691    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1699    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*GAXSY
1704    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*GAXSY
1711    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1715    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1719    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1723    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1724    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1725    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1726    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1733    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1734    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
1737    MYYGSYRAPR  TLV*AIGTVI  FILMMATAFL  GYVLPYGQMS  L*AAXSY
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Figure S5.6. Sanger sequencing in *cyt b* gene of *Corynespora cassiicola* from soybean.

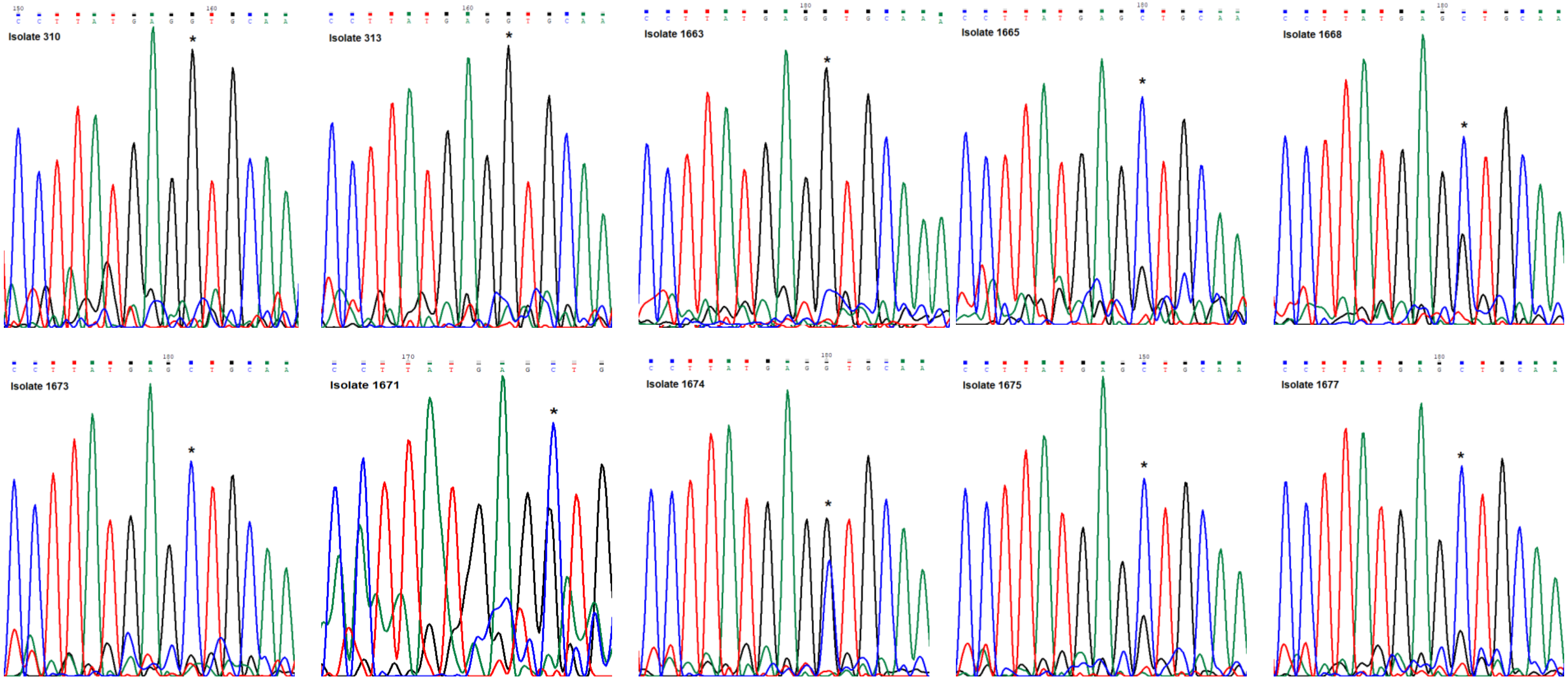

Figure S5.7. Nucleotide chromatogram sequencing mutation from GGT in sensitive isolates to GCT in resistant *Corynespora cassiicola* isolates in cytochrome b gene.

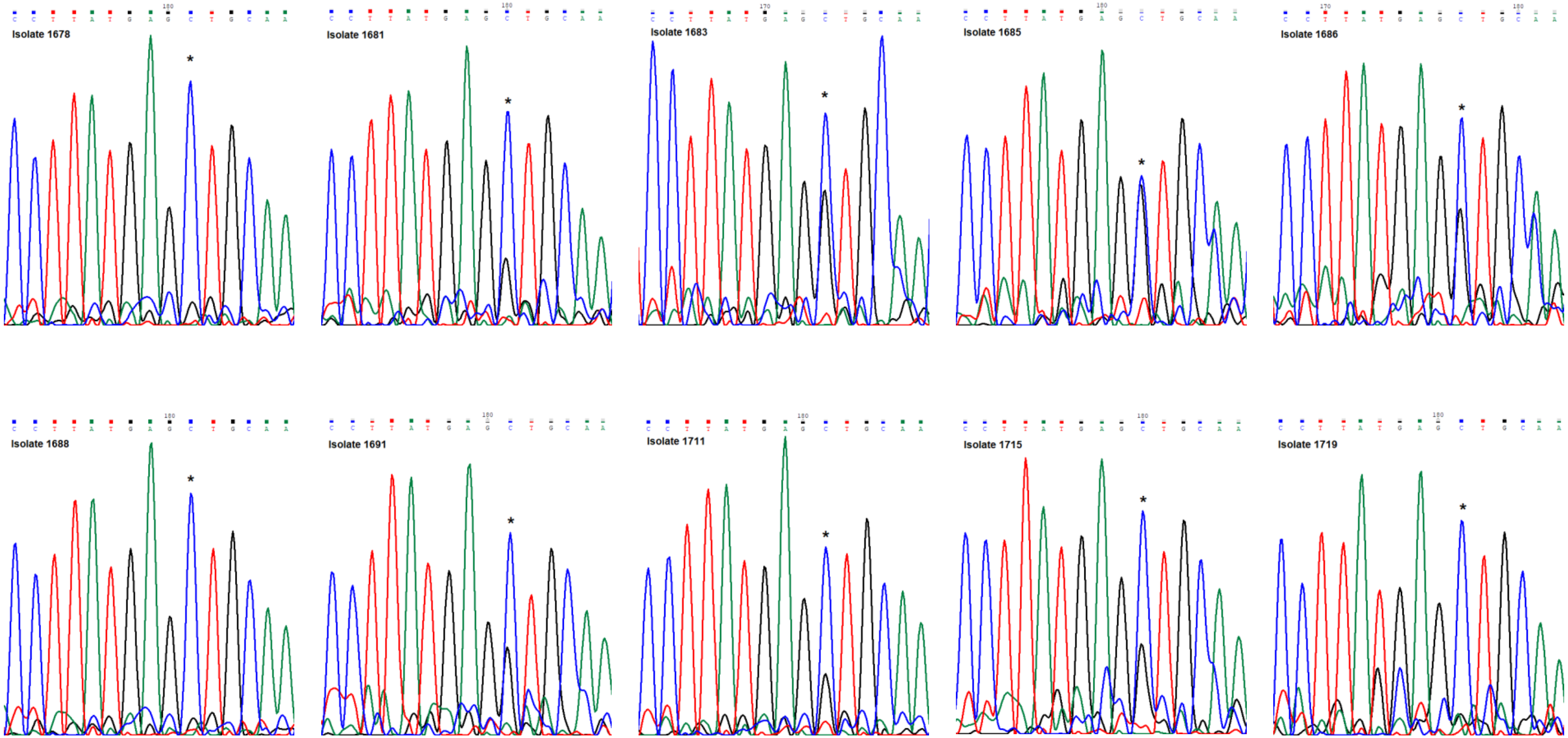

Figure S5.7. Nucleotide chromatogram sequencing mutation from GGT in sensitive isolates to GCT in resistant *Corynespora cassiicola* isolates in cytochrome b gene.

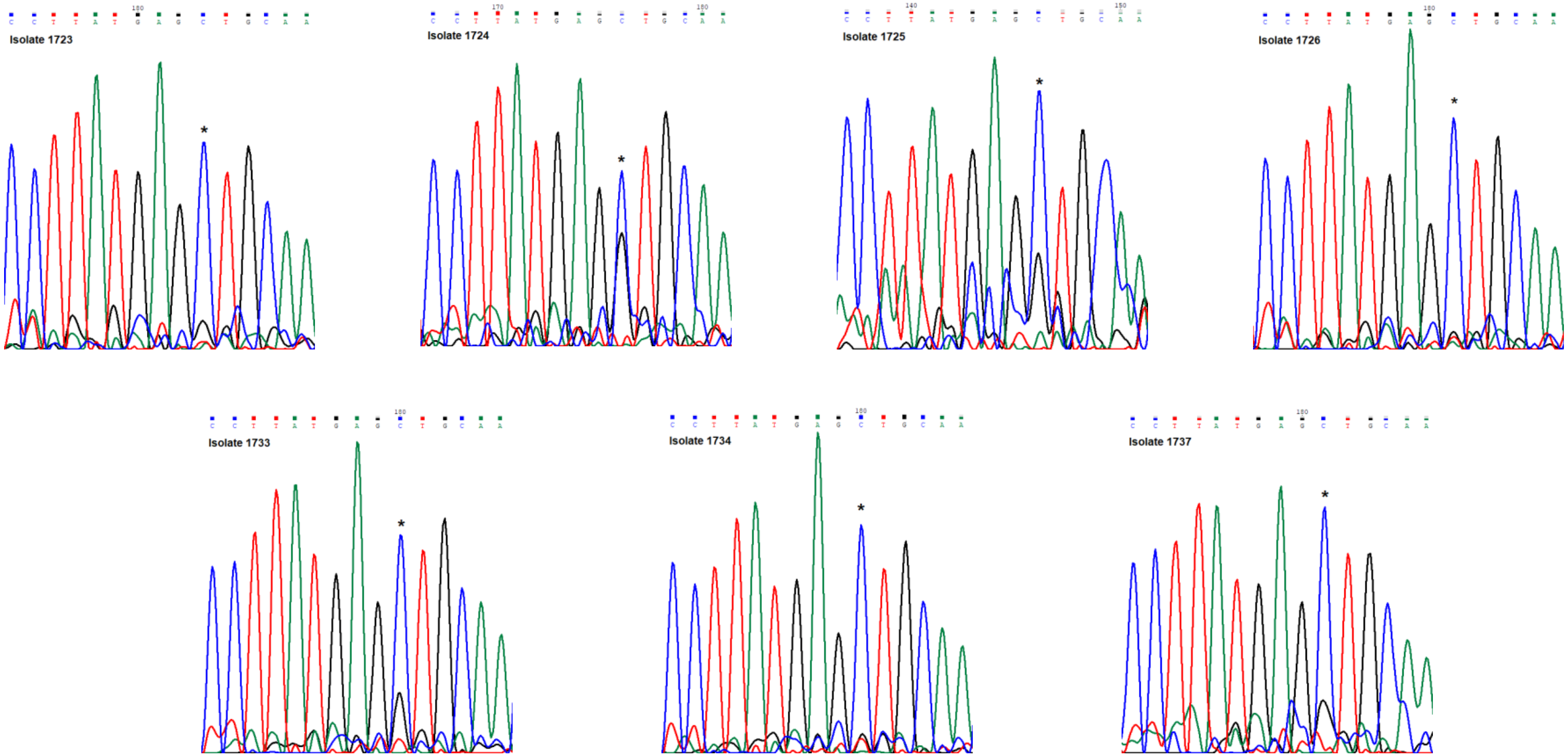

Figure S5.7. Nucleotide chromatogram sequencing mutation from GGT in sensitive isolates to GCT in resistant *Corynespora cassiicola* isolates in cytochrome b gene.

6 CONSIDERAÇÕES GERAIS

Nos ensaios *in vitro* de crescimento micelial, os ingredientes ativos dos grupos químicos MBC e IQe apresentaram baixa fungitoxicidade para o controle de *C. kikuchii* e *C. cassicola* ademais, mais de 69 % dos isolados de ambas as espécies, foram classificados como resistentes. Adicionalmente, constatou-se alta resistência cruzada entres os fungicidas que atuam com os mesmos modos de ação.

Os isolados de *C. cassiicola*, mostraram baixa sensibilidade aos fungicidas DMI, MBC e IQe. Entre os isolados resistentes, três foram classificados como altamente resistentes simultaneamente a MBC, IQe e DMI. Para alguns isolados, a EC50 foi acima da maior dose testada (100 µg/mL).

Para o gene *cyp51* foram encontradas mutações pontuais diferentes para as espécies analisadas. Para *C. kikuchii* foram encontradas 13 mutações pontuais (Q161H, N178D, P180S, P200L, K217R, E274D, S279N, E281K, T290S, I299V, S363N, K442Q e L503V*).* Para *C. truncatum* fora observadas 44 mutações não sinônimas para esse gene, enquanto para *C. cassiicola* foram observadas as mutações S279N e I299V em isolados coletados no ano de 2016.

A mutação E198A e F200Y foram as mais frequente no gene β-tubulina para *C. cassiicola.* A mutação S168Y, I189V, S275N foi relatada pela primeira vez. A mutação A244E foi relatada para *C. truncatum*. Para *C. kikuchii* foram encontradas apenas mutações sinônimas.

A mutação G143A que confere menor sensibilidade ao fungicida IQe foi observada em para acima de 69% dos isolados nas três espécies analisadas. As mutações F129L e G137R não foram detectadas em nenhuma das três espécies analisadas.

Mutações múltiplas aos fungicidas MBC e IQe foi observada os isolados de *C. cassiicola*, sendo as mutações G143A e E198A as mais frequentes. A mutação tripla nos genes alvo *cyp51*, β-tubulina e *cyt b*, foi relacionada a dois isolados 1715 provenientes do estado do Paraná e 1722 provenientes do estado de Mato Grosso do Sul. De 59 isolados analisados, 41% apresentaram mutação para a posição alvo E198A + G143A e 22% dos isolados mutaram na posição F200Y +

G143A, resultaram em isolados resistentes para os fungicidas MBC e QoI, respectivamente.

Para os genes Sdh (subunidades a, b, c, d) foram encontrados 23 pontos de mutação para o gene SdhA e 25 mutações para o gene SdhB em *C. truncatum*. Para *C. kikuchii* foram observadas mutações sinônimas para SdhA e 14 substituições para o gene SdhB: H17L, G27A, T167M, P192L, E200D, N290K, I320N, I434V, A436V, D447N, H448R, E454Q, N513S e Q569K. Para *C. cassiicola* foram identificadas mutações nas posições 318, 454, 461, 556, (A-E318D, I454V, A461S, Y556F), 34, 39, 260 (B-A34V, V39I, K260R), 37, 61, 85, 163, (C-Q37H, S61T, G85S, I163V) e 126 (D-V126I), respectivamente. Variantes homozigóticas e heterozigóticas foram encontradas em todas as espécies e em todos os genes analisados.

Alta diversidade genética de *C. kikuchii* e *C. cassiicola* entre isolados sensíveis e resistentes confirmou que as mutações encontradas dentro das populações de *C. cassiicola* para resistência aos fungicidas DMI, MBC, IQe e SDHI, são responsáveis pela alta variabilidade genética dos isolados.

Estes resultados apontam para a menor eficiência de controle de populações de *C. kikuchii*, *C. truncatum* e *C. cassiicola* a campo, devido à alta pressão de seleção exercida pelas consecutiva aplicações de fungicidas e à baixa variação dos mecanismos de ação dos fungicidas existentes no mercado.

7 REFERÊNCIAS BIBLIOGRÁFICAS GERAIS

ABAG-Associação Brasileira do Agronegócio. O Futuro da soja nacional-Impactos socioeconômicos da Ferrugem Asiática na cadeia da soja nos próximos dez anos. 2015. Disponível em: <http://www.abag.com.br/media/images/0-futuro-da-soja-nacional---ieag---abag.pdf> Acesso em 01/09/2015.

AGRIOS, G. N. **Plant pathology**, 5 ed. Burlington: Elsevier Academic, 922 p, 2005.

AGROFIT 2018. Sistema de agrotóxicos fitossanitários. Available at: http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. Accessed on March, 25, 2018.

ALMEIDA, A. M. R.; FERREIRA, L. P.; YORINORI, J. T.; SILVA, J. F. V.; HENNING, A. A. In: KIMATI, H.; AMORIM, L.; REZENDE, J. A. M.; BERGAMIN FILHO, A.; CAMARGO, L. E. A. **Manual de Fitopatologia: doenças de plantas Cultivadas**. 4. ed. vol. 2. São Paulo: Editora Agronômica Ceres. 570-588 p, 2005.

ALMEIDA, A. M. R.; MACHADO, C. C.; FERREIRA, L. P.; LEHMAN, P. S.; ANTONIO, H. Ocorrência de *Corynespora cassiicola* (Berk. & Curt.) Wei no Estado de São Paulo. **Fitopatologia Brasileira**, v.1, p.111-112, 1976.

ANDRUS, C. F.; MOORE, W. D. *Colletotrichum truncatum* (Schw.), n. comb., on garden and lima beans. **Phytopathology**, v. 25, p.121-125, 1935.

ANGELINI, R. M. de M.; POLLASTRO, S.; FARESTA, F. Genetics of fungicide resistance. In: ISHII, H.; HOLLOMON, D., editors. **Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management**. Japan: Springer.13-34 p, 2015.

ANKE, T. The antifungal strobilurins and their possible ecological role. **Canadian journal of botany**, v. 73, n. S1, p. 940-945, 1995.

ARAÚJO, A. G.; CAFÉ-FILHO, A. C.; CUPERTINO, F. P. Antracnose da soja na região geoeconômica do Distrito Federal. **Fitopatologia Brasileira**, Brasília, v. 13, p. 130, 1988.

ATHOW, K. L. Fungal diseases. In: Caldwell, B.E. (Ed.) Soybeans: Improvement, production and uses. **American Soc. Agronomy**, Madison, USA, p. 459-489, 1973.

AVENOT, H. F.; MICHAILIDES, T. J. Progress in understanding molecular mechanisms and evolution of resistance to succinate dehydrogenase inhibiting (SDHI) fungicides in phytopathogenic fungi. **Crop Protection**, v. 29, n. 7, 643-651, 2010.

AVOZANI, A. **Sensibilidade de *Corynespora cassiicola*, isolados da soja, a fungicidas *in vitro***. Dissertação (Mestrado em Agronomia) - Faculdade de Agronomia e medicina Veterinária programa de Pós-graduação em Agronomia. Passo Fundo, 2011.

AVOZANI, A.; REIS, E. M.; TONIN, R. B. Sensitivity loss by *Corynespora cassiicola*, isolated from soybean, to the fungicide carbendazim. **Summa Phytopathologica**, v. 40, n.3, p. 273-276, 2014.

BAIRD, R. E., MULLINIX, B. G., PEERY, A. B.; LANG, M. L. Diversity and longevity of the soybean debris mycobiota in a no-tillage system. **Plant Disease**, v. 81, n. 5, 530-534, 1997.

BARROS, G. S. A. C.; OSAKI, M.; ALVES, L. R. A.; LIMA, A. F. F.; RIBEIRO, R. G.; CAPELLO, F. P. Custo trimestrais de grãos. **CEPEA/ESALQ**, ano 10, edição 21, 2017.

BECHER R.; WIRSEL, S.G.R. Fungal cytochrome P450 sterol 14α-demethylase (CYP51) and azole resistance in plant and human pathogens. **Applied Microbiology Biotechnology,** v. 95, p. 825–840, 2012.

BECKER, W. F.; VON JAGOW, G.; ANKE, T.; STEGLICH, W. Oudemansin, strobilurin A, strobilurin B and myxothiazol: new inhibitors of the bc1 segment of the respiratory chain with an E-ßmethoxyacrylate system as common structural element. **FEBS Lett**, v.132, p. 329–333, 1981.

BHADAURIA, V.; BANNIZA, S.; VANDENBERG, A.; SELVARAJ, G.; WEI, Y. Overexpression of a novel biotrophy-specific *Colletotrichum truncatum* effector, CtNUDIX, in hemibiotrophic fungal phytopathogens causes incompatibility with their host plants. **Eukaryotic cell**, v. 12, n. 1, p. 2-11, 2013.

BIRLA, K.; RIVERA-VARAS, V.; SECOR, G. A.; KHAN, M. F.; BOLTON, M. D. Characterization of cytochrome b from European field isolates of *Cercospora beticola* with quinone outside inhibitor resistance. **European journal of plant pathology**, v.134, n.3, p. 475-488, 2012.

BLACK, R. J. Complexo soja: fundamentos, situação atual e perspectiva. **Soja: tecnologia de produção II. Piracicaba: ESALQ**, p. 1-18, 2000.

BOLTON, M. D.; RIVERA, V.; SECOR, G. Identification of the G143A mutation associated with QoI resistance in *Cercospora beticola* field isolates from Michigan, United States. **Pest management science**, v. 69, n.1, p. 35-39, 2013.

BORCK, K.; BRAYMER, H. D. The genetic analysis of resistance to benomyl in Neurospora crassa. **Microbiology**, v. 85, n. 1, p. 51-56, 1974.

BRASSEUR, G.; SARIBAŞ, A. S.; DALDAL, F. A compilation of mutations located in the cytochrome b subunit of the bacterial and mitochondrial bc1 complex. **Biochimica et Biophysica Acta (BBA)-Bioenergetics**, v. 1275, n. 1-2, p. 61-69, 1996.

BRENT, K. J. Historical perspective of fungicide resistance. In: THIND, S.T. (ed.) Fungicide Resistance in Crop Protection. **Punjab Agricultural University**, India, pp. 3-18, 2011.

BRENT, K. J. Resistência a fungicidas em patógenos de plantas cultivadas: como manejá-la. **Brussels-Belgium: GPCF (FRAC Monograph No. 1)**, 1995.

BRENT, K. J.; HOLLOMON, D. W. Fungicide resistance in crop pathogens: how can it be managed? FRAC monograph no. 1. **Global Crop Protection Federation**, Brussels, 2007a.

BRENT, K. J.; HOLLOMON, D. W. Fungicide resistance: the assessment of risk. FRAC monograph no. 2. **Global Crop Protection Federation**, Brussels, 2007b.

CAÑAS-GUTIÉRREZ, G. P.; ANGARITA-VELÁSQUEZ, M. J.; RESTREPO-FLÓREZ, J. M.; RODRÍGUEZ, P.; MORENO, C. X.; ARANGO, R. Analysis of the CYP51 gene and encoded protein in propiconazole-resistant isolates of *Mycosphaerella fijiensis*. **Pest management science**, n. 65, v. 8, p. 892-899, 2009.

CHEN, M. D.; LYDA, S. D.; HALLIWELL, R. S. Infection of soybeans with conidia of *Cercospora kikuchii*. **Mycologia**, p. 1158-1165, 1979.

CHONG, P.; ARANGO, R.; STERGIOPOULOS, I.; GUZMÁN, M.; CROUS, P. W.; DA SILVA, G. F.; KEMA, G. H. J. Analysis of azole fungicide resistance in *Mycosphaerella fijiensis*, causal agent of black Sigatoka. In: **Modern fungicides and antifungal compounds VI. 16th International Reinhardsbrunn Symposium, Friedrichroda, Germany, April 25-29, 2010**. Deutsche Phytomedizinische Gesellschaft eV Selbstverlag, 2011. p. 217-222.

CHUPP, C. A monograph of the fungus genus Cercospoia. **A monograph of the fungus genus *Cercospora***, 1954.

CONAB, Companhia Nacional de Abastecimento. Acompanhamento da safra brasileira de grãos, v.5-Safra 2017/2018, n.6 - **Sexto levantamento**, Brasília, p.1-129, março 2018.

COOLS, H. J.; FRAAIJE, B. A. Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. **Pest management science**, v. 69, n. 2, p. 150-155, 2013.

CORLETT, M. An annotated list of the published names in *Mycosphaerella* and *Sphaerella.* 1991.

DA SILVA LOPES, L. N.; SILVA, A. S.; DE OLIVEIRA PEREIRA, C. C.; DE MENEZES, I. P. P.; MALAFAIA, G.; DA PAZ-LIMA, M. L. Sensibilidade de Isolados de *Colletotrichum gloeosporioides* a Fungicidas. **Multi-Science Journal**, v.1, n.1, p. 106-114, 2018.

DA SILVA PESQUEIRA, A.; BACCHI, L. M. A.; GAVASSONI, W. L. Associação de fungicidas no controle da antracnose da soja no Mato Grosso do Sul. **Revista Ciência Agronômica**, v. 47, n. 1, p. 203-212, 2016.

DAMM, U.; WOUDENBERG, J. H. C.; CANNON, P. F.; CROUS, P. W. *Colletotrichum* species with curved conidia from herbaceous hosts. **Fungal Diversity**, v. 39, n. 45, 2009.

DATE, H.; KATAOKA, E.; TANINA, K.; SASAKI, S.; INOUE, K.; NASU, H.; KASUYAMA, S. Sensitivity of *Corynespora cassicola*, causal agent of *Corynespora* target spot of tomato, to thiophanate methyl and diethofencarb. **Japanese Journal of Phytopathology**, v. 70, p. 7-9, 2004.

DAUB, M. E. Peroxidation of tobacco membrane lipids by the photosensitizing toxin, cercosporin. **Plant Physiology**, v. 69, n. 6, p. 1361-1364, 1982.

DAUB, M. E.; BRIGGS, S. P. Changes in tobacco cell membrane composition and structure caused by cercosporin. **Plant Physiology**, v. 71, n. 4, p. 763-766, 1983.

DAVIDSE, L. C. Benzimidazole fungicides: mechanism of action and biological impact. **Annual Review of Phytopathology**, v.24, p.43–65, 1986.

DAVIDSE, L.; ISHII, H. Biochemical and molecular aspects of benzimidazoles, N -phenylcarbamates and N -phenylformamidoximes and the mechanisms of resistance to these compounds. **Modern selective fungicides**, p. 305–322, 1995.

DEKKER, J. Acquired resistance to fungicides. **Annual Review of Phytopathology**, v. 14, n. 1, p. 405-428, 1976.

DELP, C. J. Benzimidazole and related fungicides. Modern selective fungicides: properties, applications, mechanisms of action. Edi, H. Lyr in cooperation with H. Buchenhauer, 1987.

DELP, C. J. Resistance to plant disease control agents: how to cope with it. In: **Proceedings of symposia: IX International Congress of Plant Protection**, Washington, DC, USA, August 5-11, 1979. Minneapolis, Minn.(USA). Published for the Congress by Burgess Pub. 1981. p. 253-261, 1981.

DELP, C. J.; DEKKER, J. Fungicide resistance: definitions and use of terms. **EPPO Bulletin**, v. 15, n. 3, p. 333-335, 1985.

DELYE, C.; LAIGRET, F.; CORIO-COSTET, M. F. A mutation in the 14 alpha-demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. **Applied and Environmental Microbiology**, v. 63, n. 8, p. 2966-2970, 1997.

DIAS, M. D. **Etiologia, diversidade do agente causal e controle químico da antracnose da soja**. 2014. Tese (Doutorado em Agronomia) - Universidade Federal de Brasília, Brasília. 2014.

DIAS, M. D.; FONSECA, M. E. N.; DIAS-NETO, J. J.; SANTOS, M. D.; PANDOLFO, G. M.; BOITEUX, L. S.; CAFÉ-FILHO, A. C. Biology, pathogenicity, and haplotype analyses of *Colletotrichum cliviae*: a novel soybean anthracnose agent in warm tropical areas. **Tropical Plant Pathology**, p. 1-13, 2018.

DIAS M. D.; PINHEIRO, V. F.; CAFÉ-FILHO A. C. Impact of anthracnose on the yield of soybean subjected to chemical control in the north region of Brazil. **Summa Phytopathologica**, v. 41, p. 18–23, 2016.

EL-GHOLL, N. E.; ALFIERI JR, S. A.; RIDINGS, W. H.; SCHOULTIES, C. L. Growth and sporulation in vitro of *Cercospora apii*, *Cercospora arachidicola*, *Cercospora kikuchii*, and other species of *Cercospora*. **Canadian Journal of Botany**, v. 60, n. 6, p. 862-868, 1982.

ELLIS, M. B. Dematiaceous hyphomycetes. **Dematiaceous hyphomycetes.**, 1971.

EMBRAPA. Recomendações técnicas para a cultura da soja no Paraná 1997/98. **Londrina: EMBRAPA-CNPSo**. (EMBRAPA-CNPSo. Documento, 105). 1997.

EMBRAPA. Recomendações técnicas para a cultura da soja no Paraná 1999/2000. **Londrina: EMBRAPA-CNPSo**, (Embrapa Soja. Documentos, 131), 1999.

ESSER, L.; QUINN, B.; LI, Y. F.; ZHANG, M.; ELBERRY, M.; YU, L.; XIA; D. Crystallographic studies of quinol oxidation site inhibitors: a modified classification of inhibitors for the cytochrome bc1 complex. **Journal of molecular biology**, v. 341, n. 1, p. 281-302, 2004.

FARR, D. F.; ROSSMAN, A. Y. Fungal databases. National Fungus Collections, ARS, USDA., Disponível em: https://nt.ars-grin.gov/fungaldatabases/ 2018 Acesso em: 27 abril, 2018.

FERNÁNDEZ-ORTUÑO, D.; TORES, J. A.; DE VICENTE, A.; PEREZ-GARCIA, A. Mechanisms of resistance to QoI fungicides in phytopathogenic fungi. **International Microbiology**, v. 11, p. 1-9, 2008.

FORCELINI, C. A. Doenças em soja: entendendo as diferenças entre biotróficos e necrotróficos. **Revista Plantio Direto**, v. 120, p. 7-10, 2010.

FORCELINI, C. A.; REIS, E. M.; GASSEN, F.; YORINORI, J. T.; HOFFMANN, L.; COSTAMILAN, L.; SILVA, O. C.; BALRDIN; R.; CASA, R. T. Importância econômica da soja. In: GASSEN, F.; BORGES, L. D. **Doenças na cultura da soja.** Editado por Erlei Melo Reis, Passo Fundo, editora Aldeia Norte, 178 p. 2004.

FRAC, FUNGICIDE RESISTANCE ACTION COMMITTEE. List of plant pathogenic organisms resistant to disease control agents. Online publication: http://www. frac. info/docs/defaultsource/publications/list-of-resistant-plant-pathogens/list-of-resistant-plant-pathogenic-organismsfebruary 2013, 2013.

FRAC, FUNGICIDE RESISTANCE ACTION COMMITTEE. SDHI Fungicides. Fungicide Action Committee. Online publication available from: http://www.frac.info/working-group/sdhi-fungicides, 2014.

FUJITA, Y. Ecology and control of purple seed stain of soybean caused by *Cercospora kikuchii*. **Bulletin of the Tohoku National Agricultural Experiment Station**, n. 81, p. 51-109, 1990.

FUNGORUM, I. (a). Banco de dados para consulta de táxons fúngicos. Disponível em: http://www.indexfungorum.org/names/NamesRecord.asp?RecordID=268620. 2018 b. Acesso em: 27 de abril de 2018.

FUNGORUM, I. (b). Banco de dados para consulta de táxons fúngicos. Disponível em: http://www.indexfungorum.org/names/NamesRecord.asp?RecordID=280780. 2018 c. Acesso em: 27 de abril de 2018.

FUNGORUM, I. (c). Banco de dados para consulta de táxons fúngicos. Disponível em: http://www.indexfungorum.org/names/NamesRecord.asp?RecordID=296024. 2018 a. Acesso em: 27 de abril de 2018.

GALLI, J.A.; PANIZZI, R.C.; VIEIRA, R.D. Avaliação da resistência de variedades de soja a *Colletotrichum dematium* var. *truncata*. In: Congresso Brasileiro de Soja, 4, 2006, Londrina. Resumos. Londrina: Embrapa Soja, 2006. p.101.

GARDNER, M. W. Indiana plant diseases. **Proceeding Indiana Academy Science**, v. 35, p. 237-257, 1925.

GASPAROTTO, L.; PEREIRA, J. C. R. **Doenças da seringueira no Brasil**. **Brasília: EMBRAPA**, v. 2, 2012.

GAWADE, D. B.; SURYAWANSHI, A. P.; PAWAR, A. K.; APET, K. T.; DEVGIRE, S. S. Field evaluation of fungicides, botanicals and bioagents against anthracnose of soybean. **Agricultural Science Digest**, v. 29*,* n. 3, p. 174-177, 2009.

GEORGOPOULOS, S. G. Genetics and population dynamics. 1988.

GEORGOPOULOS, S. G.; DOVAS, C. Occurrence of *Cercospora beticola* strains resistant to benzimidazole fungicides in northern Greece. **Plant Disease**, v. 57, p. 321–324, 1973.

GISI, U.; CHIN, K. M.; KNAPOVA, G.; KÜNG FÄRBER, R.; MOHR, U.; PARISI, S.; SIEROTZKI, H.; STEINFELD, U. Recent developments in elucidating modes of resistance to phenylamides, DMI and strobilurin fungicides. **Crop Protection**. v. 38, p.1328-1349, 2000.

GLAETTLI, A.; STAMMLER, G.; SCHLEHUBER, S. Mutations in the target proteins of succinate-dehydrogenase inhibitors (SDHI) and 14α-demethylase inhibitors (DMI) conferring changes in the sensitivity-structural insights from molecular modelling. In: **Association Française de Protection des Plantes, 9ème conférence international sur les maladies des plantes, Tours, France, 8 et 9 Décembre 2009**. Association Française de Protection des Plantes (AFPP), 2009. p. 670-681.

GODOY, C. V. Risk and management of fungicide resistance in the Asian soybean rust fungus *Phakopsora pachyrhizi*. **Fungicide resistance in crop protection: Risk and management**, p. 87-95, 2012.

GODOY, C. V.; ALMEIDA, A. M. R.; COSTAMILAN, L. M.; MEYER, M. C.; DIAS, W. P.; SEIXAS, C. D. S.; SOARES, R. M.; HENNING, A. A., YORINORI, J. T.; FERREIRA, L. P.; SILVA, J. F. V. Doenças da soja. In: KIMATI H, AMORIM L, REZENDE J. A. M., BERGAMIN FILHO A., CAMARGO L. E. A. (Eds.). **Manual de Fitopatologia - Vol. 2. Doenças de Plantas Cultivadas. 5**. ed. Ouro Fino MG. Editora Agronômica Ceres. p. 657-675. 2016.

GODOY, C. V.; MEYER, M. C. Informativo FUNDAÇÃO MERIDIONAL. 2014 http://fundacaomeridional.com.br/artigos/2014/11/10/resistencia-a-fungicidas-na-cultura-da-soja. Acesso: 01 de setembro de 2015.

GODOY, C. V.; UTIAMADA, C. M.; MEYER, M. C.; CAMPOS, H. D.; PIMENTA, C. B.; BORGES, E. P.; DA SILVA, L. H. C. P. Eficiência de fungicidas para o controle da mancha-alvo, *Corynespora cassiicola*, na safra 2012/13: resultados sumarizados dos ensaios cooperativos. **Embrapa Soja-Circular Técnica (INFOTECA-E)**, 2013.

GODOY, C. V.; UTIMADA, C. M.; MEYER, M. C.; CAMPOS, H. D; PIMENTA, C. B.; MIGUEL-WRUCK, D. S. Eficiência de fungicidas para o controle da mancha-alvo, *Corynespora cassiicola*, na safra 2016/17: resultados sumarizados dos ensaios cooperativos. **Circular técnica**. Londrina, PR, Julho, 2017.

GOULART, A. C. P. **Fungos em sementes de soja: detecção e importância**. EMBRAPA, Empresa Brasileira de Pesquisa Agropecuária, Centro de Pesquisa Agropecuária do Oeste, Ministério da Agricultura e do Abastecimento, 1997.

GRASSO, V.; PALERMO, S.; SIEROTZKI, H.; GARIBALDI, A.; GISI, U. Cytochrome b gene structure and consequences for resistance to Qo inhibitor fungicides in plant pathogens. **Pest Management Science,** v. 62, p. 465–472, 2006.

GRIFFITHS, A. J. F. Mitochondrial inheritance in filamentous fungi. **Journal of Genetics**, v. 75, n. 3, p. 403-414, 1996.

GUDMESTAD, N. C.; ARABIAT, S.; MILLER, J. S.; PASCHE, J. S. Prevalence and impact of SDHI fungicide resistance in *Alternaria solani*. **Plant disease**, v. 97, n. 7, p. 952-960, 2013.

GUPTA, G. K.; SHARMA, S. K.; KUMAR, V.; RAMTEKE, R. Effect of purple seed stain disease on physical and biochemical traits of soybean. **Soybean Research**, v. 12, n. 1, p. 77-84, 2014.

HAMAWAKI, O. T.; JULIATTI, F. C.; GOMES, G. M.; RODRIGUES, F. A.; SANTOS, V. L. M. Avaliação da qualidade fisiológica e sanitária de sementes de genótipos de soja do ciclo precoce/médio em Uberlândia, Minas Gerais. **Fitopatologia brasileira**, v. 27, n. 2, p. 201-205, 2002.

HARTMAN, G. L.; MANANDHAR, J. B.; SINCLAIR, J. B. Incidence of *Colletotrichum* spp. on soybeans and weeds in Illinois and pathogenicity of *Colletotrichum truncatum*. **Plant Disease**, v. 70, n. 8, p. 780-782, 1986.

HARTMAN, G. L.; SINCLAIR, J. B.; RUPE, J. C. Compendium of soybean diseases, IV edition. **The American Phytopathological society**. St. Paul. Min., USA, 1999.

HEICK, T. M.; JUSTESEN, A. F.; JØRGENSEN, L. N. Resistance of wheat pathogen

Zymoseptoria tritici to DMI and QoI fungicides in the Nordic-Baltic region-a status. *European Journal of Plant Pathology*, *149*(3), 669-682, 2017.

HENNING, A. A.; ALMEIDA, A. M. R.; GODOY, C. V.; SEIXAS, C. D. S.; YORINORI, J. T.; COSTAMILAN, L. M.; DIAS, W. P. Manual de identificação de doenças de soja. **Embrapa Soja-Documentos (INFOTECA-E)**, 2005.

HOLLOMON, D. W. **Resistência a fungicidas, definições e conceitos**, 2012. Disponível em: http://www.frac-brasil.org.br/frac/secao.asp?l=10&c=52. Acesso em agosto, 2015.

HUANG, D.; SHI, Y.; XIE, X.; WANG, W.; LI, B. Sensitivity of *Corynespora cassiicola* to Carbendazim and Diethofencarb. **Journal of Pure and Applied Microbiology**, v.*7*, n.2, p.891-898, 2013.

HYDE, K. D.; CAI, L.; MCKENZIE, E. H. C.; YANG, Y. L.; ZHANG, J. Z.; PRIHASTUTI, H. *Colletotrichum*: a catalogue of confusion. **Fungal Diversity**, v. 39, n. 1, 2009.

IMAZAKI, I.; IIZUMI, H.; ISHIKAWA, K.; SASAHARA, M.; YASUDA, N.; KOIZUMI, S. Effects of thiophanate-methyl and azoxystrobin on the composition of *Cercospora kikuchii* populations with thiophanate-methyl-resistant strains. **Journal of General Plant Pathology**, v. 72, n. 5, p. 292-300, 2006.

ISHII, H.; MIYAMOTO, T.; USHIO, S.; KAKISHIMA, M. Lack of cross-resistance to a novel succinate dehydrogenase inhibitor, fluopyram, in highly boscalid-resistant isolates of *Corynespora cassiicola* and *Podosphaera xanthii*. **Pest management science**, v. 67, n. 4, p. 474-482, 2011.

ISHII, H.; YANO, K.; DATE, H.; FURUTA, A.; SAGEHASHI, Y.; YAMAGUCHI, T.; SUGIYAMA, T. T.; NISHIMURA, K.; HASAMA, W. W. Molecular Characterization and

Diagnosis of QoI Resistance in Cucumber and Eggplant Fungal Pathogens. **Phytopathology**, v.97, n.11, p.1458–66, 2007.

KALAMARAKIS, A. E.; DE WAARD, M. A.; ZIOGAS, B. N.; GEORGOPOULOS, S. G. Resistance to fenarimol in *Nectria haematococca* var. *cucurbitae*. **Pesticide Biochemistry and Physiology**, v. 40, n. 3, p. 212-220, 1991.

KILPATRICK, R. A. Longevity of *Cercospora kikuchii* on soybean stems. **Phytopathology**, v. 46, n. 1, p. 58-58, 1956.

KLINGELFUSS, L. H.; YORINORI, J. T. Latent infection by *Colletotrichum truncatum* and *Cercospora kikuchii* in soybean. **Fitopatologia Brasileira**, v. 26, n.2, p.158-164, 2001.

KLOSOWSKI, A. C.; BRAHM, L.; STAMMLER, G.; DE MIO, L. L. M. Competitive Fitness of *Phakopsora pachyrhizi* Isolates with Mutations in the CYP51 and CYTB Genes. **Phytopathology**, v. 106, n. 11, 1278-1284, 2016a.

KLOSOWSKI, A. C.; MAY DE MIO, L. L.; MIESSNER, S.; RODRIGUES, R.; STAMMLER, G. Detection of the F129L mutation in the cytochrome b gene in *Phakopsora pachyrhizi*. **Pest management science**, v. 72, n. 6, p. 1211-1215, 2016b.

KOENRAADT, H.; JONES, A. L. The use of allele-specific oligonucleotide probes to characterize resistance to benomyl in field strains of *Venturia inaequalis*. **Phytopathology**, v. 82, n. 11, p. 1354-1358, 1992.

KÖLLER, W. Antifungal agents with target sites in sterol functions and biosynthesis. **Target sites of fungicide action**, v. 7, p. 119e206, 1992.

KUROZAWA, C.; PAVAN, M. A.; REZENDE, J. A. M. Doenças das Curcubitáceas. In: KIMATI, H.; AMORIM, L. REZENDE, J. A. M.; BERGAMIN FILHO, A.;

CAMARGO, L. E. A. Manual de fitopatologia – Doenças das Plantas Cultivadas. Ed. São Paulo: Editora Agronômica Ceres, 2005, v. 2, p. 293-302. 2005.

KUYAMA, S.; TAMURA, T. Cercosporin. A pigment of *Cercosporina kikuchii* Matsumoto et Tomoyasu. I. Cultivation of fungus, isolation and purification of pigment. **Journal of the American Chemical Society**, v. 79, n. 21, p. 5725-5726, 1957.

LALÈVE, A.; GAMET, S.; WALKER, A. S.; DEBIEU, D.; TOQUIN, V.; FILLINGER, S. Site-directed mutagenesis of the P225, N230 and H272 residues of succinate dehydrogenase subunit B from *Botrytis cinerea* highlights different roles in enzyme activity and inhibitor binding. **Environmental microbiology**, v. 16, n. 7, p. 2253-2266, 2014.

LASSERON-DE FALANDRE, A.; DABOUSSI, M. J.; LEROUX, P. Inheritance of resistance to fenpropimorph and terbinafine, two sterol biosynthesis inhibitors, in *Nectria haematococca*. **Phytopathology**, v. 81, n. 11, p. 1432-1438, 1991.

LATUNDE-DADA, A. O.; LUCAS, J. A. Localized hemibiotrophy in *Colletotrichum*: cytological and molecular taxonomic similarities among *C. destructivum*, *C. linicola* and *C. truncatum*. **Plant Pathology**, v. 56, n. 3, p. 437-447, 2007.

LEHMAN, S. G. Purple stain of soybean seeds. **North Carolina Agriculture Experimental Station Bulletin**, v. 369, p. 1-11. 1950.

LEHMAN, S. G.; WOLF, A. F. Soybean anthracnose. **Journal of Agricultural Research**, v. 33, p. 38-39, 1926.

LEROUX, P.; CHAPELAND, F.; DESBROSSES, D.; GREDT, M. Patterns of cross-resistance to fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*) isolates from French vineyards. **Crop protection**, v. 18, n. 10, p. 687-697, 1999.

LEROUX, P.; WALKER, A. S. Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. **Pest Management Science**, v. 67, n. 1, p. 44-59, 2011.

LIU, S. T. Seed-borne diseases of soybean. **Bot. Bull. Acad. Sinica**, v. 2, n. 1, p. 69-80, 1948.

LOEFFLER, R. S. T.; BUTTERS, J. A.; HOLLOMON, D. W. The lipid content of powdery mildews and their sensitivity to fungicides. In: **British Crop Protection Conference–Pests and Diseases 9A–12**. 1984. p. 911-916.

MA, Y. H. 1984. Development of soybean genetic and breeding research in China, pp. 15-19. In S. Wong (ed.), Proceedings of the 2nd U.S.-China soybean symposium, 28 July-2 August 1984, Changchun, Jilin, China.

MA, Z.; MICHAILIDES, T. J. Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. **Crop Protection**, v. 24, n. 10, p. 853-863, 2005.

MACRI, F.; VIANELLO, A. Photodynamic activity of cercosporin on plant tissues. **Plant, Cell & Environment**, v. 2, n. 3, p. 267-271, 1979.

MANANDHAR J. B., HARTMAN G. L. Anthracnose. In: Hartman GL, Sinclair JB, Rupe JC (eds), Compendium of Soybean Diseases, 4th edn. St. Paul, MN, APS Press, 1999, pp. 13–14.

MANANDHAR, J.B.; HARTMAN, G.L. Anthracnose. In: HARTMAN, G.L.; SINCLAIR, J.B.; RUPE, J.C. (Eds.) Compendium of soybean diseases. 4 ed., Minnesota: APS, p. 13-14, 2008.

MATSUMOTO, T. Beobachtungen ueber Sporenbildungen des Pilzes, *Cercosporina Kikuchii*. **Japanese Journal of Phytopathology**, v. 2, n. 2, p. 65-69, 1928.

MATSUMOTO, T.; TOMOYASU, R. Studies on purple speck of soybean seed. **Japanese Journal of Phytopathology**, v. 1, n. 6, p. 1-14, 1925.

MCGEE, D. C. Treatment of soybean seeds. **Seed Treatment**, n. Edn 2, p. 185-200, 1986.

MCLEAN, K. S.; ROY, K. W. Incidence of *Colletotrichum dematium* on prickly sida, spotted spurge and smooth pigweed and pathogenicity to soybean. **Plant disease**, v. 72, n. 5, p. 390-393, 1988.

MEYER, M. C.; KLEPKER, D. Manejo da Antracnose em soja. **Fitopatologia Brasileira**, v. 32, agosto 2007.

MEYER, M. C.; RODACKI, M. E. Efeito de duas aplicações de fungicidas no controle da ferrugem da soja, DFC e antracnose no Maranhão e Tocantins. In: Reunião de Pesquisa de Soja da Região Central do Brasil, 27, 2005, Cornélio Procópio. **Resumos**. Londrina: Embrapa Soja, 2005, p. 201-202.

MIYAGAWA, N.; FUJI, M. Occurrence of QoI-fungicide-resistant strains of *Magnaporthe oryzae* on rice and fungicidal effective. In: **23rd Symposium of PSJ Research Committee on Fungicide Resistance, Gifu, Japan**. 2013. p. 25-35.

MIYAMOTO, T.; ISHII, H.; SEKO, T.; KOBORI, S.; TOMITA, Y. Occurrence of *Corynespora cassiicola* isolates resistant to boscalid on cucumber in Ibaraki Prefecture, Japan. **Plant Pathology**, v. 58, n. 6, p. 1144-1151, 2009.

MIYASAKA, S. **Contribuição para o Melhoramento da Soja no Estado de São Paulo**. (Dissertação) Piracicaba, ESALQ. 1958.

MOLNA, A.; HORNOK, L.; PESTI, M. The high level of benomyl tolerance in *Fusarium oxysporum* is determined by the synergistic interaction of two genes. **Experimental mycology**, v. 9, n. 4, p. 326-333, 1985.

MURAKISHI, H. H. Purple seed stain of Soybean. **Phytopathology**, v. 41, n. 4, 1951.

NEERGARD, P. Seed Pathology, 2 ed. London. **MacMillan Press**. 300p.1979.

OLIVE, L. S.; BAIN, D. C.; LEFEBVRE, C. L. A leaf spot of Cowpea and Soybean caused by an undescribed species of *Helminthosporium*. **Phytopathology**, v. 35, n. 10, 1945.

ORR, H. A. Fitness and its role in evolutionary genetics. **Nature Reviews Genetics**, v. 10, n. 8, p. 531, 2009.

ORTH, C. E.; SCHUH, W. Resistance of 17 soybean cultivars to foliar, latent, and seed infection by *Cercospora kikuchii.* **Plant disease**, v.78, n. 7, p. 661-664, 1994.

ORTH, C.E.; SCHUH, W. Histological comparison of latent and active infection of soybean foliage by *Cercospora Kikuchii.* **Phytopathology,** v**.** 82, p.1159, 1992.

PASCHE, J. S.; PICHE, L. M.; GUDMESTAD, N. C. Effect of the F129L mutation in *Alternaria solani* on fungicides affecting mitochondrial respiration. **Plant Disease**, v. 89, n. 3, p. 269-278, 2005.

PASCHE, J. S.; WHARAM, C. M.; GUDMESTAD, N. C. Shift in sensitivity of *Alternaria solani* (potato early blight) to strobilurin fungicides. In: **Brighton crop protection conference pests and diseases**. 2002. p. 841-846.

PERES, N. A. R.; SOUZA, N. L.; PEEVER, T. L.; TIMMER, L. W. Benomyl sensitivity of isolates of *Colletotrichum acutatum* and *C. gloeosporioides* from citrus**. Plant Disease**, v.88, n.2, p.125-130, 2004.

PICININI, E. C.; FERNANDES, J. M. Doenças de soja: diagnose, epidemiologia e controle. **Embrapa Trigo-Documentos (INFOTECA-E)**, 2000.

POLLACK, F. G. **An annotated compilation of Cercospora names**. J. Cramer, 1987.

PONTZEN, R.; POPPE, B.; BERG, D. Mode of action of sterol biosynthesis inhibitors in obligate parasites. **Pesticide science**, v. 30, p. 357–360, 1990.

PRICE III, P. P.; PURVIS, M. A.; CAI, G.; PADGETT, G. B.; ROBERTSON, C. L.; SCHNEIDER, R. W.; ALBU, S. Fungicide resistance in *Cercospora kikuchii*, a soybean pathogen. **Plant disease**, v. 99, n. 11, p. 1596-1603, 2015.

REIS, E. M.; REIS, A. C; FORCELINI, C. A. **Manual de fungicidas: guia para o controle de doenças de plantas**. 5. ed. Passo Fundo. (Ed.) Universidade de Passo Fundo - RS, p. 153. 2007.

ROY, K. W. Seedling diseases caused in soybean by species of *Colletotrichum* and *Glomerella*. **Phytopathology**, v. 72, n. 8, p. 1093-1096, 1982.

ROY, K. W.; ABNEY, T. S. Purple seed stain of soybeans. **Phytopathology**, v. 66, n. 1045, p. e1049, 1976.

SAKAI, Y. Effects of sowing dates on the progress of disease caused by purple stain of soybean. **Environment Control in Biology**, v. 38, n. 4, p. 181-187, 2000.

SARTORATO, A. S. " *In vitro*" sensitivity of isolated *Colletotrichum lindemuthianum* to fungicides. **Pesquisa Agropecuária Tropical,** v.*36,* n.3, p. 211, 2006.

SARTORI, J. E. Avaliação da sensibilidade in vitro de isolados de *Colletotrichum lindemuthianum* a fungicidas, 2007. Dissertação (Mestrado em Agronomia)- Universidade Estadual Paulista Julio de Mesquita Filho- Faculdade de Ciências Agronômicas Campus de Botucatu- UNESP, Botucatu, SP.

SCHMITZ, H. K.; MEDEIROS, C. A.; CRAIG, I. R.; STAMMLER, G. Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. **Pest management science**, v. 70, n. 3, p. 378-388, 2014.

SCHROEDER, W. T.; PROVVIDENTI, R. Resistance to benomyl in powdery mildew of cucurbits. **Plant Disease Report**, v. 53, p. 271-275, 1969.

SCHUH, W. *Cercospora* blight, leaf spot, and purple seed stain. **Compendium of soybean diseases**, p. 17-18, 1990.

SCHUH, W. Effect of pod development stage, temperature, and pod wetness duration on the incidence of purple seed stain of soybeans. **Phytopathology**, v. 82, n. 4, p. 446-451, 1992.

SCHUH, W. Influence of temperature and leaf wetness period on conidial germination *in vitro* and infection of *Cercospora kikuchii* on soybean. **Phytopathology**, v. 81, n. 10, p. 1315-1318, 1991.

SEMAR, M.; STROBEL, D.; KOCH, A.; KLAPPACH, K.; STAMMLER, G. Field efficacy of pyraclostrobin against populations of *Pyrenophora teres* containing the F129L mutation in the cytochrome b gene. **Journal of plant diseases and protection**, v. 114, n. 3, p. 117-119, 2007.

SIEROTZKI, H. Respiration Inhibitors: complex III. In: Ishii H, Hollomon D, editors. **Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management.** Japan: Springer, Tokyo, 2015. p 119-143.

SIEROTZKI, H.; FREY, R.; WULLSCHLEGER, J.; PALERMO, S.; KARLIN, S.; GODWIN, J.; GISI, U. Cytochrome b gene sequence and structure of *Pyrenophora teres* and *P. tritici-repentis* and implications for QoI resistance. **Pest management science**, v. 63, n. 3, p. 225-233, 2007.

SILVA, L. H. C. P.; CAMPOS, H.; SILVA, J. Fortalecida e agressiva. **Revista Cultivar–Grandes Culturas. Ano X (114)**, p. 20-22, 2008.

SILVA, W. P. K.; MULTANI, D. S.; DEVERALL, B. J.; LYON, B. R. RFLP and RAPD analyses in the identification and differentiation of isolates of the leaf spot fungus *Corynespora cassiicola*. **Australian Journal of Botany**, v. 43, n. 6, p. 609-618, 1995.

SIMÕES, K.; HAWLIK, A.; REHFUS, A.; GAVA, F.; STAMMLER, G. First detection of a SDH variant with reduced SDHI sensitivity in *Phakopsora pachyrhizi*. **Journal of Plant Diseases and Protection**, v. 125, n. 1, p. 21-26, 2018.

SINCLAIR, J. B. Compedium of soybean diseases 2 nd. ed. **American Phytopathology Society: St. Paul**, p. 104, 1982.

SINCLAIR, J. B. Control of seedborne pathogens and diseases of soybean seeds and seedlings. **Pest Management Science**, v. 37, n. 1, p. 15-19, 1993.

SINCLAIR, J. B.; BACKMAN, P. A. Compendium of soybean Disease. 3 ed. Edition St Paul, Minnesota. **American Phytopathological Society**, p. 106. 1989.

SINDIVEG – Sindicato Nacional da Indústria de Produtos para Defesa Vegetal. 2017. http://sindiveg.org.br/estatisticas-do-setor/. Acesso em: 27 de abril de 2018.

SOARES, A. P. G.; GUILLIN, E. A.; BORGES, L. L.; DA SILVA, A. C.; DE ALMEIDA, Á. M.; GRIJALBA, P. E.; DE OLIVEIRA, L. O. More Cercospora species infect soybeans across the Americas than meets the eye. **PloS one**, v. 10, n. 8, p. e0133495, 2015.

STAMMLER, G.; BRIX, H. D.; GLÄTTLI, A.; SEMAR, M.; SCHOEFL, U. Biological properties of the carboxamide boscalid including recent studies on its mode of action.

In: **Proceedings XVI International Plant Protection Congress, Glascow**. 2007. p. 40-45.

STAMMLER, G.; BRIX, H. D.; NAVE, B.; GOLD, R.; SCHOEFL, U. Studies on the biological performance of boscalid and its mode of action. In **Modern fungicides and antifungal compounds V: 15th International Reinhardsbrunn Symposium, Friedrichroda, Germany, May 6-10, 2007** (pp. 45-51). Deutsche Phytomedizinische Gesellschaft eV Verlag, 2008. p. 45-51.

STAMMLER, G.; WOLF, A.; GLAETTLI, A.; KLAPPACH, K. Respiration Inhibitors: Complex II. In: Ishii H, Hollomon D, editors. **Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management**. Japan: Springer. 2015, p. 105-117.

STANDISH, J. R., TOMASO-PETERSON, M., ALLEN, T. W., SABANADZOVIC, S., & ABOUGHANEM-SABANADZOVIC, N. Occurrence of QoI fungicide resistance in *Cercospora sojina* from Mississippi soybean. **Plant Disease**, v. 99, n. 10, 1347-1352, 2015.

SUZUKI, K. Studies on the cause of “Shihan” of soybean. Chosen Nakaiho, v. 16, p. 24-28, 1921.

TAI, F. L.; CHEO, C. C. Notes on Chinese fungi VIII. **Bull. Chin. bot. Soc. Bull. Chin. bot. Soc.**, v. 3, n. 1, 1937.

TANAKA, M. A. S.; PASSOS, F. A.; BETTI, J. A. Resistência de *Colletotrichum fragariae* e *C. acutatum* ao benomyl na cultura do morango no Estado de São Paulo. **Scientia Agricola**, v. 54, n.3, p.139-146, 1997.

TECNOLOGIAS DE PRODUÇÃO DE SOJA – Região Central do Brasil 2014. Londrina: Embrapa Soja, p. 268. 2013.

TENG, S. C. A Contribution to Our Knowledge of the Higher Fungi of China. **A Contribution to Our Knowledge of the Higher Fungi of China.** 1939.

TERAMOTO, A.; MACHADO, T. A.; NASCIMENTO, L. M.; MEYER, M. C.; CUNHA, M. G. Sensibilidade a fungicidas de isolados de *Corynespora cassiicola* provenientes do Estado de Goiás. In: **Embrapa Soja-Artigo em anais de congresso (ALICE)**. In: CONGRESSO BRASILEIRO DE SOJA, 6., 2012, Cuiabá. Soja: integração nacional e desenvolvimento sustentável: anais. Brasília, DF: Embrapa, 2012, 2012.

TERAMOTO, A.; MEYER, M. C.; SUASSUNA, N. D.; CUNHA, M. G. *In vitro* sensitivity of *Corynespora cassiicola* isolated from soybean to fungicides and field chemical control of target spot. **Summa Phytopathologica**, v. 43, n. 4, p. 281-289, 2017.

TIFFANY, L. H.; GILMAN, J. C. Species of *Colletotrichum* from legumes. **Mycologia**, v. 46, n. 1, p. 52-75, 1954.

TO-ANUN, C.; HIDAYAT, I.; MEEBOON, J. Genus *Cercospora* in Thailand: taxonomy and phylogeny (with a dichotomous key to species). **Plant Pathology & Quarantine**, v. 1, n. 1, p. 11-87, 2011.

UPCHURCH, R. G.; WALKER, D. C.; ROLLINS, J. A.; EHRENSHAFT, M.; DAUB, M. E. Mutants of *Cercospora kikuchii* altered in cercosporin synthesis and pathogenicity. **Applied and environmental microbiology**, v. 57, n. 10, p. 2940-2945, 1991.

USDA United States Department of Agriculture**. Word Agricultural Supply and Demand Estimates.** April 10, 2018.

VALLIÈRES, C.; TROUILLARD, M.; DUJARDIN, G.; MEUNIER, B. Deleterious effect of the Qo inhibitor compound resistance-conferring mutation G143A in the intron-containing cytochrome b gene and mechanisms for bypassing it. **Applied and environmental microbiology**, v. 77, n. 6, p. 2088-2093, 2011.

VATHAKOS, M. G.; WALTERS, H. J. Production of conidia by *Cercospora kikuchii* in culture. **Phytopathology**, v. 69, n. 8, p. 832-833, 1979.

WALTERS, H. J. Cercospora leaf blight of soybeans. **Phytopathology News**, v. 12, p. 165-166, 1978.

WILCOX, J. R.; ABNEY, T. S. Effects of *Cercospora kikuchii* on soybeans. **Phytopathology**, v. 63, n. 6, p. 796-797, 1973.

WRATHER, J. A.; ANDERSON, T. R.; ARSYAD, D. M.; GAI, J.; PLOPER, L. D.; PORTA-PUGLIA, A.; YORINORI, J. T. Soybean disease loss estimates for the top 10 soybean producing countries in 1994. **Plant disease**, v. 81, n. 1, p. 107-110, 1997.

WYAND, R. A.; BROWN, J. K. M. Sequence variation in the CYP51 gene of *Blumeria graminis* associated with resistance to sterol demethylase inhibiting fungicides. **Fungal Genetics and Biology**, v. 42, n. 8, p. 726-735, 2005.

XAVIER, S. A.; CANTERI, M. G., BARROS, D. C. M., GODOY, C. V. Sensitivity of *Corynespora cassiicola* from soybean to carbendazim and prothioconazole. **Tropical Plant Pathology**, v. 38, n. 5, p. 431-435, 2013.

YORINORI, J. T. Levantamento e avaliação da situação de doenças da soja na safra 1987/88. In: Resultados de Pesquisa de soja, 1987/88. Londrina, **EMBRAPA – CNPSo**, p. 158. 1989.

YOUNG, D. H. Anti-tubulin agents. In: Ishii H.; Hollomon D., editors. **Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management.** Japan: Springer. 2015, p 119-143.

ZENG, F.; ARNAO, E.; ZHANG, G.; OLAYA, G.; WULLSCHLEGER, J.; SIEROTZKI, H.; BRADLEY, C. A. Characterization of quinone outside inhibitor fungicide resistance in *Cercospora sojina* and development of diagnostic tools for its identification. **Plant disease**, v. 99, n. 4, p. 544-550, 2015.

ZHANG, G.; PEDERSEN, D. K.; PHILLIPS, D. V.; BRADLEY, C. A. Sensitivity of *Cercospora sojina* isolates to quinone outside inhibitor fungicides. **Crop protection**, v. 40, p. 63-68, 2012.

ZIOGAS, B. N.; MALANDRAKIS, A. A. Sterol Biosynthesis Inhibitors: C14 Demethylation (DMIs). In: Ishii H, Hollomon D, editors. **Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management**. Japan: Springer, Tokyo, 2015, p 201-216.