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Sensibilidade e adaptabilidade de *Colletotrichum musae* a fungicidas utilizados em pós-colheita no Brasil

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SENSIBILIDADE E ADAPTABILIDADE DE *Colletotrichum musae* A FUNGICIDAS UTILIZADOS EM PÓS-COLHEITA NO BRASIL

Dissertação apresentada ao Programa de Pós-Graduação em Fitopatologia da Universidade Federal Rural de Pernambuco, como parte dos requisitos para obtenção do título de Mestre em Fitopatologia.

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SUMÁRIO

RESUMO GERAL

A antracnose, causada por *Colletotrichum musae*, é uma das principais doenças em pós-colheita na cultura da banana no Brasil. Atualmente, apenas dois produtos são registrados para seu controle, cujos ingredientes ativos são imazalil e tiabendazol. A exposição constante de populações de *C. musae* a fungicidas pode levar à pressão de seleção, resultando na redução da sensibilidade de isolados e, consequentemente, na ineficácia de controle. Neste estudo, foi investigada a sensibilidade de 218 isolados de *C. musae*, obtidos de diversas regiões brasileiras, aos fungicidas imazalil e tiabendazol. A estimativa da concentração efetiva capaz de inibir 50% do crescimento micelial (CE_{50}) foi utilizada para selecionar quatro isolados com os menores valores (isolado sensível - S) e quatro com os maiores valores (isolado menos sensível - MS) para ambos os fungicidas. Estes isolados foram avaliados quanto à estabilidade da sensibilidade, eficácia no controle da antracnose em frutos de banana, variáveis relacionadas à aptidão (crescimento micelial, produção e germinação de conídios, e virulência) e habilidade competitiva. Os isolados apresentaram sensibilidade diferencial aos fungicidas, com valores de CE_{50} variando de 0,03 a 1,29 $\mu g.mL^{-1}$ para imazalil e de 0,10 a 80,35 $\mu g.mL^{-1}$ para tiabendazol. Não houve alteração na sensibilidade dos isolados S e MS após dez gerações em meio BDA sem fungicida ($P < 0,05$). Foi verificada uma correlação positiva entre a sensibilidade aos dois fungicidas ($r = 0,59$; $P < 0,05$). Embora ambos tenham sido capazes de controlar a antracnose causada por isolados S em frutos destacados de banana, apenas imazalil foi eficiente no controle da doença causada por isolados MS. A análise de variáveis relacionadas à aptidão mostrou que não houve diferença entre os grupos de isolados S e MS para ambos os fungicidas, embora tenha sido verificada uma grande variação dentro de cada grupo. De modo geral, os isolados MS apresentaram boa capacidade de competição. Isso foi verificado, inclusive, para um isolado MS a tiabendazol, no qual foi detectada a presença de mutação no códon 200 do gene β-tubulina, indicando que a mutação não resultou em penalidades de aptidão. Os resultados permitem um melhor entendimento sobre a sensibilidade e aptidão de isolados de *C. musae* provenientes de áreas de cultivo de banana do Brasil. Além disso, demonstram a importância do monitoramento periódico das populações do fungo, para verificar a frequência de isolados com sensibilidade reduzida aos fungicidas, visando ao manejo mais eficiente da antracnose da bananeira.

Palavras-chave: *Musa* spp., antracnose, benzimidazóis, habilidade competitiva, inibidores da biossíntese de ergosterol, resistência.

GENERAL ABSTRACT

Anthracnose, caused by *Colletotrichum musae,* is one of the main postharvest diseases in banana in Brazil. Currently, only two products are registered for its control, whose active ingredients are imazalil and thiabendazole. The constant exposure of populations of *C. musae* to fungicides may lead to selection pressure, resulting in reduced sensitivity of isolates and, consequently, in ineffective control. In this study, the sensitivity of 218 isolates of *C. musae* obtained from several Brazilian regions to the fungicides imazalil and thiabendazole was investigated. The estimative of the effective concentration able to inhibit 50% of mycelial growth (EC_{50}) was used to select four isolates with the lowest values (S - sensitive isolate) and four with the highest values (less sensitive isolate - LS) for both fungicides. These isolates were evaluated for stability of sensitivity, effectiveness to control the anthracnose in banana fruit, fitness-related variables (mycelial growth, conidial production and germination, and virulence) and competitive ability. The isolates showed differential sensitivity to the fungicides, with EC_{50} values ranging from 0.03 to 1.29 $\mu g.mL^{-1}$ for imazalil, and from 0.10 to 80.35 $\mu g.mL^{-1}$ for thiabendazole. There was no change in the sensitivity of S and LS isolates after ten generations in fungicide-free PDA medium ($P < 0.05$). There was a positive correlation between sensitivity to both fungicides ($r = 0.59$, $P < 0.05$). Although both fungicides were able to control the anthracnose caused by S isolates in banana detached fruit, only imazalil was efficient in controlling the disease caused by LS isolates. The analysis of fitness-related variables showed no difference between the groups of S and LS isolates for both fungicides, although a great variation within each group has been verified. In general, LS isolates had a good competitive hability. This was also found for one LS isolate to thiabendazole, in which the presence of mutation was detected at codon 200 of the β-tubulin gene, indicating that the mutation did not result in fitness penalties. Our results allow a better understanding of the sensitivity and fitness of isolates of *C. musae* from banana growing areas in Brazil. In addition, the findings demonstrate the importance of periodic monitoring of fungal populations, in order to verify the frequency of isolates with reduced sensitivity to fungicides, aiming at a more efficient management of anthracnose in banana.

Keywords: *Musa* spp., anthracnose, benzimidazole, competitive ability, ergosterol biosynthesis inhibitor, resistance.

CAPÍTULO I

Introdução Geral

SENSIBILIDADE E ADAPTABILIDADE DE *Colletotrichum musae* A FUNGICIDAS UTILIZADOS EM PÓS-COLHEITA NO BRASIL

INTRODUÇÃO GERAL

1. A cultura da bananeira e seu cultivo no Brasil

A bananeira (*Musa* spp.), pertencente à família Musaceae, é originária do Continente Asiático, e tem sido cultivada em todo o mundo, principalmente em regiões tropicais. A cultura está presente em mais de 80 países, ocupando área superior a 4 milhões de hectares, com uma produção que ultrapassa 100 milhões de toneladas (FAOSTAT, 2017). Está entre as dez principais culturas produzidas em todo o mundo, estando concentrada em países como Índia (30 milhões de toneladas), China (11 milhões de toneladas), Indonésia, Brasil e Equador, correspondendo a 55,2% da produção mundial (FAOSTAT, 2016). É considerada, em alguns países, como uma das principais fontes de arrecadação e geração de emprego e renda para uma parte expressiva da população, contribuindo para o desenvolvimento das regiões envolvidas em sua produção (FAO, 2016; FAO, 2011; VIEIRA, 2011).

O Brasil é o quarto maior produtor mundial, produzindo cerca de 6,8 milhões de toneladas, em uma área cultivada de 469 mil hectares, o que corresponde a 25% do volume total da fruticultura brasileira. Dentre as regiões de destaque no cultivo, estão o Nordeste (34%) e o Sudeste (34%), com produção de 2.285.796 e 2.268.400 toneladas, respectivamente. Em seguida, estão as regiões Sul (15%), Norte (13%) e Centro-Oeste (4%), com 1.035.695, 883.184 e 291.249 toneladas, respectivamente (FAOSTAT, 2017). A região Nordeste se destaca na produção de banana no Brasil, graças ao estado da Bahia, considerado o segundo maior estado produtor, perdendo apenas para o estado de São Paulo, com 1.084.548 toneladas, em uma área cultivada de 72,7 hectares. Outros estados de destaque são Minas Gerais (11%), Santa Catarina (11%) e Pará (9%), com 773.197, 721.579 e 504.907 toneladas, respectivamente (IBGE, 2017).

Embora o Brasil tenha cerca de 6% de participação na produção mundial de banana, o País é responsável por apenas 2% das exportações mundiais do produto. Em 2017, as exportações de banana brasileira atingiram 41,4 mil toneladas, representando cerca de 0,6% das 7,185 milhões de toneladas produzidas. Os principais países de exportação são Uruguai (21,5 mil toneladas), Argentina (16,5 mil toneladas) e Polônia (2 mil toneladas) (EMBRAPA, 2017).

A bananeira é uma planta monocotiledônea, herbácea, perene, com porte de dois a cinco metros, sendo que algumas variedades podem atingir quase oito metros de altura (FARBER *et al.*, 2014). Na evolução das bananeiras comestíveis, cruzamentos interespecíficos entre as espécies selvagens diploides *Musa acuminata* (genoma AA) e *Musa balbisiana* (genoma BB) originaram a maioria dos genótipos de bananeiras (SIMMONDS; SHEPHERD, 1955). Cada cultivar deve conter combinações variadas de genomas dessas espécies parentais, cujas combinações resultam os grupos diplóides (AA, BB e AB), triplóides (AAA, AAB e ABB) e tetraplóides (AAAA, AAAB, AABB e ABBB) (COSTA, 2008). As principais variedades cultivadas do grupo AAA são Prata, Pacovan, Prata Anã, Nanica, Maçã, Mysore e Cavendish (Banana D'Água ou Caturra), e do grupo AAB, Terra e D'angola (CARVALHO *et al.*, 2011).

Atualmente, existem cerca de 180 variedades de bananas, distribuídas mundialmente. No Brasil, são produzidas 35 variedades, sendo a maior parte da produção destinada ao consumo *in natura*, embora seja também destinada ao processamento industrial, nos setores alimentício, farmacêutico e de cosméticos. Para o consumo *in natura*, as bananeiras do tipo Prata, Prata Anã e Pacovan são as mais plantadas (63%), seguidas por Nanica (24%) e Maçã (3%). Já as bananeiras do tipo Terra (plátanos) são consumidas fritas, cozidas ou assadas, e representam 9% da produção no Brasil. O restante (1%) corresponde a outros tipos de banana (EMPRAPA, 2015). A produção nordestina concentra-se nas variedades Prata e Pacovan, sendo que a Pacovan apresenta maior destaque nos estados do Ceará e Pernambuco, os quais atendem principalmente às capitais nordestinas. No sul da Bahia, encontra-se um polo forte de produção de Prata Anã, e o estado do Rio Grande do Norte, com a produção de Nanica e Grand Naine voltada para a exportação, principalmente para o mercado europeu (SENA, 2011)

Uma vez que 98% da produção brasileira de bananas é totalmente dirigida ao mercado interno, devido à grande população e ao elevado consumo per capita, o país não desenvolveu boas práticas de manejo e conservação pós-colheita exigidas para transporte ao mercado externo, como fizeram os países tradicionalmente exportadores do produto (ANÚARIO BRASILEIRO DE FRUTICULTURA, 2018). Além disso, o desempenho econômico da bananicultura depende de vários fatores, que incluem a variedade escolhida, as condições edafoclimáticas, os tratos culturais e fitossanitários, a incidência de pragas e doenças, o custo de aquisição de insumos, a comercialização e os preços pagos aos produtores (SANTANA; ALMEIDA; SOUZA, 2004).

A ocorrência de doenças em pós-colheita da banana é um dos problemas que mais prejudica a qualidade do fruto, com perdas que podem atingir de 40 a 60% da produção, principalmente em casos de infecções quiescentes, invibializando o transporte por períodos mais longos e a aceitação do produto no destino final (NEGREIROS *et al*., 2013; PRUSKY *et al*., 2013). Em um estudo avaliando a presença de doenças em pós-colheita na cultura, foram identificados diferentes espécies de fungos tanto em frutos maduros quanto verdes, incluindo *Colletotrichum musae*, *Colletotrichum acutatum*, *Cladosporium musae*, *Penicilium* sp. e *Alternaria sp*. (MORAES; ZAMBOLIM; LIMA, 2006). Porém, *Colletotrichum musae* foi relatado como sendo o agente primário causador de podridões nas frutas, mostrando que a antracnose é umas das mais importantes doenças em pós-colheita na cultura.

2. Antracnose na banana

A antracnose na banana é causada por diferentes espécies do gênero *Colletotrichum*, incluindo *C. musae* (VIEIRA *et al*., 2017), *Colletotrichum siamense* (KUMAR *et al*., 2017; VIEIRA *et al*., 2017), *Colletotrichum scovillei* (VIEIRA *et al*., 2017; ZHOU *et al*., 2017), *Colletotrichum gloeosporioides, Colletotrichum tropicale, Colletotrichum chrysophilum, Colletotrichum theobromicola*, (VIEIRA *et al*., 2017; ZAKARIA *et al*., 2009; PÉREZ *et al*., 2001), *Colletotrichum karstii* (DAMM *et al*., 2012a) e *Colletotrichum paxtonii* (SHERRIFF *et al*., 1994; JOHNSTON; JONES 1997; DAMM *et al*.,2012b). No entanto, *C. musae* é a mais adaptada e predomina como o agente etiológico desta doença (PLOETZ; THOMAS; SLABAUGH, 2003).

A espécie *C. musae* foi primeiramente classificada como *Myxosporium musae* Berk. & M.A. Curtis (BERKELEY, 1874), sendo posteriormente transferida para o gênero *Gloeosporium* Desm. & Montag. (MONTAGNE, 1849), que se caracteriza por não apresentar setas no acérvulo, passando a ser chamada de *Gloeosporium musarum* Cooke & Massee (COOKE, 1887; BAXTER; WESTHUIZEN; EICKER, 1985). Por fim, foi reclassificada por von Arx (1957a, b) no gênero *Colletotrichum* e foi epitipificado por Su *et al*., 2011.

A espécie *C. musae* apresenta, frequentemente, colônias com micélio aéreo abundante e de tonalidade esbranquiçada, que se tornam acinzentadas ou rosadas com o passar do tempo, além de considerável massa de conídios de coloração alaranjada, geralmente coalescente. As colônias variam, também, quanto à formação de micélio aéreo, desde flocoso sem conídios aparentes até micélio escasso, submerso e bem esporulado. A literatura não relata a presença de microescleródios. Os conídios são geralmente hialinos, retos, cilíndricos, obtusos nos

ápices, unicelulares, possuindo de 10,0-18,0 µm de comprimento x 3,0-6,5 µm de largura (COUTO; MENEZES, 2004; PLOETZ; THOMAS; SLABAUGH, 2003; SANTOS *et al.*, 2015.). Os apressórios são bastante comuns, médios e de coloração castanho-escura, apresentando forma irregular, muitas vezes, com lóbulos grandes ou profundos, os quais medem 9,0-13,0 x 9,0- 11,5 µm (SUTTON, 1980).

A antracnose pode causar perdas de produção de até 40%, afetando a qualidade dos frutos comercializáveis (PESSOA *et al*., 2006). A importância dessa doença está relacionada à frequência com que aparece, principalmente, nas condições do comércio interno, nas quais pouco cuidado é realizado (COELHO *et al*., 2010).

O principal impacto da doença se dá pela capacidade do fungo infectar os frutos ainda verdes, antes da colheita, permanecendo latentes ou quiescentes até o início da maturação. A infecção quiescente ocorre quando o patógeno infecta a planta, porém mantém seu nível metabólico baixo, não ocorrendo o surgimento dos sintomas da doença. Entretanto, algumas condições, como mudança ambiental, estresse nutricional ou estádio de maturação da planta, podem ativar os fatores de patogenicidade, resultando em parasitismo ativo nos tecidos do hospedeiro (PRUSKY *et al*., 2013).

Os sintomas da doença são caracterizados pela formação de lesões deprimidas, com formato irregular e coloração escura, as quais coalescem com o avanço da doença. Sob condições de alta umidade, é possível observar sobre as lesões uma massa mucilaginosa de coloração alaranjada formada sobre os acérvulos, onde são encontrados os conídios. Geralmente, as lesões são superficiais, mas podem, em casos severos, com o amadurecimento da fruta, atingir a polpa (CORDEIRO; MATOS; KIMATI, 2016).

Em regiões tropicais e subtropicais, a predominância de altas temperaturas, precipitações bem distribuídas e elevada umidade relativa do ar, favorecem o cultivo da bananeira, em função de ser adaptada ao clima dessas regiões. Entretanto, essas condições também propiciam o desenvolvimento do fungo *C. musae*. As condições ideais de temperatura e umidade para a ocorrência da antracnose estão em torno de 25-30 ºC e 90 ± 5%, respectivamente. A germinação dos conídios ocorre na superfície de frutos verdes ou maduros, na presença de um filme de água, formando o apressório no período de quatro horas. A penetração se dá após 24-72 horas, e os sintomas só aparecem na época de maturação dos frutos (AGRIOS, 2005; CORDEIRO; MATOS; KIMATI, 2016).

A antracnose, geralmente, mantém-se restrita ao pericarpo da fruta, raramente afetando a polpa. Porém, em condições de alta temperatura ou quando as frutas encontram-se em

estádio avançado de maturação, o fruto pode se tornar impróprio para o consumo, inviabilizando a exportação, transporte, embalagem e comercialização (OLIVEIRA *et al*., 2013).

O pH é outro fator ambiental importante para espécies do gênero *Colletotrichum*. As espécies *C. gloeosporioides* e *C. musae* caracterizam-se por crescer bem em substrato ácido até a neutralidade, desenvolvendo-se numa faixa de pH de 4,0 a 7,0 (GRIFFIN, 1994). A acidez pode afetar os atributos sensoriais das frutas, como aroma, sabor, textura e cor (SOTO BALLESTERO, 1992; MATSUURA *et al*., 2002).

A sobrevivência do fungo ocorre, geralmente, nos restos vegetais das plantas, como pecíolos e folhas velhas, e nos vestígios florais dos cachos e brácteas. Os conídios são disseminados por respingos da água da chuva ou da irrigação por aspersão e pelo vento, para as flores e frutos novos (CORDEIRO; MATOS; KIMATI, 2016).

Para o controle de *C. musae*, devem ser considerados aspectos relacionados ao manejo adequado na pré-colheita, colheita e pós-colheita. Os cuidados devem ter início no campo de produção, com a adoção de práticas culturais, como a eliminação e remoção de folhas velhas, brácteas e restos florais das plantas (PLOETZ; THOMAS; SLABAUGH, 2003; VENTURA; HINZ, 2002). Nas fases de colheita e pós-colheita é fundamental evitar ferimentos nos frutos. As práticas, incluindo o processo de retirada do cacho e lavagem dos frutos, requerem um manuseio extremamente cauteloso dos frutos, além de medidas rigorosas de assepsia, a fim de controlar o micélio quiescente, evitando infecções secundárias durante o armazenamento e transporte. Outro cuidado é na concentração de etileno, responsável pelo amadurecimento do fruto climatérico, uma vez que esse fitohormônio é produzido tanto pelo hospedeiro quanto pelo fitopatógeno (PLOETZ; THOMAS; SLABAUGH, 2003).

A utilização de cultivares resistentes à doença é, para o produtor, a forma mais prática e econômica de controle. Porém, devido à variabilidade apresentada pela espécie, e a ocorrência de duas ou mais espécies de *Colletotrichum* parasitando um mesmo hospedeiro, a obtenção de cultivares resistentes tem sido um grande desafio para os melhoristas, não havendo registro de cultivares resistentes para a doença (CORDEIRO; MATOS; KIMATI, 2016).

Atualmente, os fungos em pós-colheita são controlados, principalmente, pela aplicação de fungicidas, por imersão ou por atomização dos frutos. Estes tratamentos atuam sobre patógenos em ferimentos ou sobre aqueles de infecção quiescente e possuem a grande vantagem de seu efeito residual (SENHOR *et al*., 2009). Os sistemas de embalagem e transporte em condições refrigeradas também têm contribuído para a redução dos problemas

com *C. musae* (CORDEIRO; MATOS; KIMATI, 2016).

Embora não existam fungicidas registrados para aplicação em campo para controle da antracnose no Brasil, já foi verificada a ação de benzimidazóis sob *C. musae* (VIEIRA *et al*., 2017). Isso pode ter sido ocasionado pela aplicação em campo para outras doenças na cultura, como o tiofanato-metílico utilizado para controlar a sigatoka amarela (MAPA 2019).

Para o controle da antracnose na banana em pós-colheita, até o momento, apenas dois produtos são registrados no Ministério da Agricultura, Pecuária e Abastecimento (MAPA). Os ingredientes ativos são imazalil e tiabendazol, pertencentes aos grupos químicos dos imidazóis e benzimidazóis, respectivamente (MAPA, 2019). O fato de ser o principal método de manejo tem preocupado, devido à baixa efetividade, principalmente do tiabendazol, no controle da doença (JOHANSON; BLAZQUEZ, 1992).

3. Resistência a fungicidas dos grupos Metil Benzimidazol Carbamato (MBC) e Inibidores da Biossíntese de Ergosterol (DMI)

A introdução dos fungicidas com modo de ação sítio-específico ocorreu no final dos anos 70. Dentre os primeiros fungicidas específicos estão as carboxamidas, os benzimidazóis e os primeiros inibidores da biossíntese de esterol, como os triazóis (BRENT; HOLLOMON, 2007).

Os fungicidas MBCs, conhecidos como grupo químico dos benzimidazóis (benomil, carbendazim, tiabendazol e tiofanato-metílico) possuem alta afinidade por proteínas tubulinas (α e β-tubulina), atuando na inibição da polimerização dos microtúbulos e, consequentemente, na interrupção da mitose, ocorrendo uma falha na separação do novo núcleo, o que acarretará na morte celular (DAVIDSON *et al*., 2006; DELEN; TOSUN, 2004; RODRIGUES, 2006). Os fungicidas DMIs, incluindo o grupo dos imidazóis (como imazalil e procloraz) atuam na enzima C-14-demetilase, inibindo a desmetilação oxidativa dependente de citocromo-P450 na via biossintética do ergosterol, levando ao rompimento da membrana e ao vazamento de eletrólitos, responsável pelo crescimento de muitos fungos fitopatogênicos (ZIOGAS; MALANDRAKIS, 2015).

Desde os anos 70, o número de casos de fungos fitopatogênicos resistentes a fungicidas vem aumentando consideravelmente na agricultura. A especificidade é um dos fatores que levam ao alto risco da resistência adquirida pelo patógeno, sendo considerado o principal fator da pressão de seleção da população do fungo, devido ao uso intensivo de fungicidas (ESTEP

et al., 2015; JULIATTI *et al*., 2015). O primeiro relato de resistência aos fungicidas MBC = ocorreu dois anos após a sua introdução no mercado na cultura do pepino ao fungicida benomyl nos Estados Unidos (SCHROEDER; PROVVIDENTI, 1969; BRENT; HOLLOMON, 2007; KUCK; GISI, 2007). A resistência aumentou consideravelmente durante 20 anos, e sinais de alerta para a seleção de populações de patógenos resistentes logo se tornou uma realidade, após o seu uso em larga escala. Consequentemente, o número de patógenos previamente controlados por esse grupo de fungicidas foi reduzido (DELEN; TOSUN, 2004). Para os fungicidas DMIs, o risco de resistência é relativamente baixo quando comparado aos benzimidazóis, acredita-se que os baixos níveis de resistência, estejam associados a um único evento molecular envolvido, resultando na redução da penetração do produto pela membrana ou devido a um ativo sistema efluxo (GUINI; KIMATI, 2002; FRAC, 2018).

Quanto as características da resistência, para fungicidas pertencentes a um mesmo grupo químico pode haver a resistência cruzada, na qual um isolado resistente a um fungicida pode apresentar resistência a outros fungicidas com o mesmo modo de ação (GHINI; KIMATI, 2002). Por outro lado, a resistência múltipla, que é conferida por mais de um fator de ordem genética, ocorre quando um isolado se torna resistente a fungicidas de grupos químicos diferentes (ZAMBOLIM; VENÂNCIO; OLIVEIRA, 2007).

Diversos estudos têm demonstrado a resistência a fungicidas MBC em fungos fitopatogênicos, associada a mutações nos códons 6, 50, 167, 198, 200 e 240 do gene da β-tubulina (MA; MICHAILIDES, 2005). As mutações mais comumente encontradas ocorrem nos códons 198 e 200, para os quais foram verificados níveis diferenciados de resistência, sendo que isolados com a mutação no códon 198 foram altamente resistentes, enquanto a mutação no códon 200 resultou em uma resistência intermediária (BANNO *et al.,* 2008; BARALDI *et al*., 2003; KOENRAADT *et al*., 1992; MA *et al*., 2003; MA *et al*., 2005; MCKAY *et al*., 1998). Em isolados de *C. gloesporioides* e *C. cereale*, foram detectadas mutações pontuais no códon 198 (substituição da cadeia de – nucleotídeos - GAG por GCG), gerando isolados resistentes e altamente resistentes, respectivamente, e no códon 200 (substituição de TTC por TAC), gerando isolados moderadamente resistentes (CHUNG *et al.,* 2010; PERES *et al.,* 2004; YOUNG *et al.,* 2010). Já em isolados de *C. musae*, observou-se a mutação no códon 200, convertendo o aminoácido fenilalanina em tirosina na região TUB2 (GRIFFEE, 1973; VIEIRA *et al*., 2017).

Para os DMIs, foram descritas mutações pontuais associadas aos genes *Cyp51A* e

Cyp51B (MAIR *et al*., 2016; MELLADO *et al*., 2001). Essas mutações podem ocorrer por meio de diferentes mecanismos de resistência: i) alteração da enzima CYP51; ii) superexpressão do gene *CYP51*; iii) efluxo ativo melhorado dos DMIs; iv) modificação da biossíntese de esteróis (COOLS; FRAAIJE., 2012). Por exemplo, em populações de *Monilinia fructicola*, isolados resistentes apresentaram a substituição do aminoácido tirosina por fenilalanina no códon 136 (Y136F) do gene *Cyp51B* (LUO *et al.*, 2008; CHEN *et al.*, 2012); em *Blumeria graminis*, a mutação no códon 147 levou à substituição de lisina por glutamina (K147Q), (WYAND; BROWN, 2005). Com relação ao gênero *Colletrotrichum*, alguns estudos têm sido realizados, avaliando a resistência aos DMIs, como é o caso de *C. gloeosporioides* em videira e morangueiro (XU *et al.*, 2014) e *C. gloeosporioides* e *C. acutatum* em citros (GAMA, 2017). No entanto, até o momento, não foi verificada a ocorrência de mutações nos códons 136, 147 e 175 para o gênero *Colletotrichum*, como relatado em outros estudos envolvendo estes fungicidas. Portanto, são necessários mais estudos para a verificação dos genes envolvidos na indução da resistência aos DMIs.

A ocorrência de mutação em um ou poucos genes que conferem características importantes pode causar alterações na adaptabilidade do isolado resistente, ou seja, na sua habilidade de se desenvolver, reproduzir e sobreviver (BERGAMIN FILHO; AMORIM, 2001). Em muitos casos, isolados resistentes podem ter menor aptidão que isolados sensíveis, comprometendo sua dominância na população na ausência da pressão de seleção do fungicida. Alternativamente, isolados resistentes podem apresentar características semelhantes às de isolados sensíveis e persistirem durante longo período, mesmo sem utilização dos fungicidas (ZHONGHUA; MICHAILIDES, 2005).

De modo geral, os níveis de resistência a fungicidas e a mudança na sensibilidade podem ser avaliados em laboratório, pela exposição de isolados fitopatogênicos provenientes de uma população do campo ao fungicida. Essa resposta é geralmente mensurada pela inibição do crescimento micelial, porcentagem de germinação do esporo, ou infecção na planta para fungos biotróficos. Com os dados obtidos, pode-se estimar a concentração efetiva de fungicida capaz de inibir 50% do crescimento micelial (CE_{50}), para cada amostra individual (DAMICONE; SMITH, 2012).

A adaptabilidade dos isolados pode ser estimada por meio da avaliação de características epidemiológicas, tanto *in vitro* quanto *in vivo*. As variáveis incluem taxa de crescimento micelial, temperatura ótima de crescimento, produção e germinação de esporos, sensibilidade osmótica, virulência (KIM; XIAO, 2011; OLIVER; HEWIT, 2014; RALLOS *et*

al., 2014).

Tem sido demonstrado que isolados insensíveis a MBC apresentam estabilidade e capacidade de persistir na população, mesmo com o uso descontinuado dos fungicidas (ISHII, 2015; WALKER *et al*., 2013). Isolados de *C. musae* com reduzida sensibilidade a tiofanato-metilico apresentaram características relacionadas à aptidão (crescimento micelial, germinação e produção de esporos, sensibilidade osmótica e virulência) semelhantes às de isolados sensíveis (VIEIRA *et al*., 2017). Por outro lado, isolados de *M. fructicola* com baixa sensibilidade a DMI apresentaram desvantagens quanto à capacidade de esporulação, virulência e período de incubação, quando comparado aos isolados sensíveis (LICHTEMBERG, 2015)

Informações sobre a sensibilidade e adaptabilidade de isolados resistentes a fungicidas são essenciais para a avaliação, implementação e redirecionamento de estratégias de manejo de doenças de plantas, além de possibilitarem o melhor entendimento da estrutura populacional do patógeno e dos processos de dispersão do inóculo (BROWN, 2006). Desta forma, este trabalho teve como objetivo avaliar a sensibilidade e a adaptabilidade de isolados de *C. musae* provenientes de áreas de cultivo de bananeira de diversas regiões brasileiras aos fungicidas imazalil e tiabendazol.

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CAPÍTULO II

Analysis of *Colletotrichum musae* populations from Brazil reveal presence of isolates with reduced sensitivity to fungicides used in postharvest and with high competitive ability

Submissão: Plant Pathology

Qualis CAPES (Ciências Agrárias I): A1

Analysis of *Colletotrichum musae* populations from Brazil reveal presence of isolates with reduced sensitivity to fungicides used in postharvest and with high competitive ability

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Running head: Resistance of *C. musae* to fungicides

Keywords: *Musa* spp., anthracnose, chemical control, competitive ability

Abstract

Anthracnose, caused by *Colletotrichum musae*, is the main disease in postharvest of banana in Brazil. The disease management is based on the use of fungicides, but only two active ingredients (a.i.), imazalil and thiabendazole, are registered to control the disease. The constant exposure of *C. musae* populations to fungicides may lead to reduced sensitivity of isolates, resulting in ineffective control. In this study, the sensitivity of 218 isolates of *C. musae* to imazalil and thiabendazole was evaluated, as well the fitness and competitive ability of less sensitive isolates. In general, the isolates showed a differential sensitivity to both fungicides. Although a positive correlation between the sensitivity to imazalil and thiabendazole has been verified, the isolates were more sensitive to imazalil. The estimated effective concentration of the fungicide able to inhibit mycelial growth by 50% (EC_{50}) was used to select four isolates with the lowest and the highest values for both fungicides, which were considered as sensitive (S) and less sensitive (LS), respectively. These isolates were able to maintain the level of sensitivity to both fungicides after ten successive transfers on free-fungicide medium. The analysis of control effectiveness revealed that both fungicides were efficient to control the disease caused by S isolates of *C. musae* in banana detached fruit, but only imazalil was able to control the disease caused by LS isolates. In general, the analysis of fitness-related variables (mycelial growth, sporulation, germination, and virulence) showed no difference between the groups of S and LS isolates for both fungicides, but a large variation was observed within the group. The high competitive ability of LS isolates to thiabendazole indicates that the resistance-conferring mutation did not result in fitness penalties. Our results allow a better understanding on the sensitivity and fitness of isolates of *C. musae* from Brazil, and demonstrate the importance of periodic monitoring to verify the frequency of less

sensitive isolates in populations, aiming at a more effective management of anthracnose in banana orchards in Brazil.

Introduction

Among of diseases in postharvest affecting the production of banana (*Musa* spp. L.) in Brazil, anthracnose caused by *Colletotrichum musae* (Berk& Curtis) von Arx, is considered the most important disease in the crop, being present in all production areas and limiting the Brazilian - international trade (Rani & Thammaiah, 2014).

The major challenge in the disease management is related to the life style of the pathogen, defined as quiescent, in which the pathogen stays dormant within the host tissues for a long time. During the fruit ripening process, physiological and biochemical changes activate different signal-transduction pathways related to both host and pathogen responses, which are important in maintaining or facilitating the transition from the quiescent to the necrotrophic lifestyle (Prusky *et al*., 2013).

The main strategy to manage the disease is the chemical control, by immersion of banana fruit in fungicide solution (Senhor *et al*., 2009). However, to date, only two products are registered in the Ministério da Agricultura, Pecuária e Abastecimento (MAPA) to control anthracnose on banana in Brazil (MAPA, 2019). The active ingredients (a.i.) are imazalil and thiabendazole, which belong to Demethylation Inhibitor (DMI) and Methyl Benzimidazole Carbamato (MBC) groups, respectively. Both systemic fungicides are highly selective, acting in a specific target site (Brent & Hollomon, 2007). This characteristic is one of the main factors involved with the development of resistance, , which can result in a considerable reduction in the sensitivity of the pathogen to the chemical compound (Capote *et al*., 2012).

Fungicides belonging to MBC group have a high risk of resistance acquired by the pathogen (FRAC, 2018). They are highly selective and have high affinity for tubulin proteins (α- and β-tubulin). Their action mode consists in binding to these proteins, preventing the formation of microtubules and consequently the cell mitotic division (FRAC, 2016;

Rodrigues, 2006). Resistance to benzimidazoles has been detected in many fungal species, and it has been associated with point mutations in the β-tubulin gene, resulting in a change in the amino acid sequence at the binding site (Ma & Michaelides, 2005; Young, 2015). In *Colletotrichum*, point mutations have been detected at codon 198 (substitution of the amino acid chain GAG by GCG), and at codon 200 (substitution of TTC by TAC), leading to resistant and moderately resistant isolates, respectively (Chung *et al*., 2010, Griffee, 1973, Peres *et al.*, 2004, Vieira *et al*., 2017, Young *et al*., 2010).

The DMI fungicides have a relatively lower risk of resistance than MBCs (FRAC, 2018). They act by inhibiting the enzyme 14α-demethylase (P450), which is important in the ergosterol biosynthesis pathway (Ziogas & Malandrakis, 2015). Among the mechanisms of resistance to DMIs described for several phytopathogenic fungi, mutation in the cytochrome P450 14α-demethylase (CYP51) gene, which codes for the target enzyme of azole fungicides, is the most commonly found (Luo *et al.*, 2008; Ziogas & Malandrakis, 2015). Although some reports have demonstrated point mutations in the Cyp51A and Cyp51B genes for some pathogens (Mair *et al*., 2016), in *Colletrotrichum* genus the occurrence of mutation in specific codons has not been verified (Xu *et al*., 2014).

The emergence of fungal populations with resistance to fungicides is a great challenge, since it leads to the loss of control effectiveness. For this reason, growers commonly increase the number of fungicide applications to achieve an effective control of the disease (Zambolim *et al*., 2007). This practice may lead to a selection pressure, increasing the resistant isolates in the population, which results in an ineffective control. However, it is important to consider that resistance may have a fitness cost related to the ability of the isolate to develop, reproduce, survive, and cause disease. Thus, in the absence or at low doses of fungicide, resistant individuals can be less competitive than sensitive ones (Ma & Michailides 2005). On the other hand, some resistant isolates may be able to persist in the population for many years,

even with the discontinued use of the fungicide. In addition, the presence or absence of fitness penalties can also be used to infer whether isolates containing mutations associated with resistance decrease or persist between growth seasons. (Hawkins & Fraaije, 2018).

To our knowledge, studies regarding the sensitivity of Brazilian *C. musae* populations from banana to the fungicides imazalil and thiabendazole are absent. Thus, the objectives of this study were: (i) to analyze the sensitivity to imazalil and thiabendazole of *C. musae* isolates obtained from different banana orchards in Brazil; (ii) to verify the occurrence of multiple sensitivity between imazalil and thiabendazole; (iii) to evaluate the stability of sensitivity in sensitive (S) and less sensitive (LS) isolates; (iv) to assess the fungicide effectiveness to control S and LS isolates in detached banana fruit; (v) to verify the relationship between fungicide sensitivity and fitness-related variables; (vi) to verify the competitive ability of LS isolates; and (vii) to investigate the occurrence of mutation associated with the reduction of sensitivity.

Material and method

Fungal isolates

A total of 218 isolates of *C. musae* were obtained from the Culture Collection of the Micology Laboratory at the Universidade Federal Rural de Pernambuco (Recife, Pernambuco, Brazil). The isolates were obtained from banana orchards in the Brazilian states of Bahia, Distrito Federal, Espírito Santo, Goiás, Minas Gerais, Pará, Paraná, Pernambuco, Santa Catarina, and São Paulo (Fig. 1), and were previously identified by phylogenetic inference (Vieira *et al*., 2017).

In vitro fungicide sensitivity assay

The sensitivity of *C. musae* isolates to the fungicides imazalil and thiabendazole was evaluated by a mycelial growth assay, using the commercial formulations Magnate 500 EC WP (500 $g.kg^{-1}$ a.i., Adama Brazil), and Tecto 485 SC (485 $g.kg^{-1}$ a.i., Syngenta Crop Protection), respectively. The fungicides were solubilized in sterile distilled water and added to molten (45 °C) potato dextrose agar (PDA) medium at different concentrations: 0.05, 0.1, 0.5, 1.0, and 5.0 μg a.i. mL^{-1} for imazalil, and 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 50.0, and 100.0 μg a.i. mL^{-1} for thiabendazole. Five-mm-diameter plugs were obtained from the edge of a four-day-old colony of each isolate and transferred to PDA medium amended with the fungicides at different concentrations. Fungicide-free PDA medium was used as control. Three replicates were used to evaluate each combination of isolate - fungicide concentration. The cultures were incubated at 25 °C in the dark. The colony diameter was measured in two perpendicular directions at four days after incubation. The percentage of mycelial growth (PMG) was obtained by using the formula: PMG = (100*F)/C, where F corresponds to mycelial growth in PDA medium containing fungicide, and C is the control (mycelial growth in fungicide-free PDA medium). The value was subtracted from 100 to yield the percentage of growth inhibition at each fungicide concentration. The effective concentration of the fungicide that was able to inhibit mycelial growth by 50% (EC_{50}) was calculated for each isolate by linear regression of the mycelial growth inhibition versus the log10 transformation of the fungicide concentration for all isolate-fungicide combinations. For each fungicide, four isolates with the lowest and the highest EC_{50} values were selected, and considered as sensitive (S) and less sensitive (LS), respectively (Table 2). These isolates were used to evaluate the stability of sensitivity, the effectiveness of fungicides to control anthracnose in banana fruit, the fitness-

145 related variables (mycelial growth rate, germination, conidial production, and virulence), the
146 competitive ability of conidia, and the presence of mutations associated with the insensitivity.
147
148 **Stability of the sensitivity to imazalil and thiabendazole**
149
150 The stability of the sensitivity of S and LS isolates to imazalil and thiabendazole was
151 evaluated by assessing the mycelial growth after ten sequential transfers. Five-mm-diameter
152 plugs were transferred to fungicide-free PDA medium every three days, and EC_{50} was
153 calculated before the first transfer (T_0) and after ten transfers (T_{10}). The EC_{50} was calculated as
154 described above.
155
156 **Fungicide assay in fruit**
157 To evaluate the in vivo effectiveness of imazalil and thiabendazole to control S and LS
158 isolates of *C. musae*, banana fruit were treated with the commercial formulation of both
159 fungicides prior to inoculation. First, banana fruit (cv. Prata) at maturation stage two (Von
160 Loesecke, 1950) were surface disinfested using detergent, rinsed with distilled water,
161 immersed for 5 min in 1% NaOCl, rinsed two times with distilled water and kept on a clean
162 surface until dry. Two 5 × 2mm (diameter x depth) plugs were removed one on each side of
163 the fruit with the aid of a cork borer. The manufacturer-recommended doses of fungicides to
164 control anthracnose in the field (Magnate 500 EC - 0.4 mL. mL^{-1}, and Tecto 485 SC - 0.18
165 mL. mL^{-1}) were prepared and sprayed onto the fruit using a spray bottle. Fruit sprayed with
166 sterile distilled water were used as control. After three hours, 5 × 2 mm (diameter x depth)
167 mycelial plugs obtained from four-day-old cultures of S and LS isolates were inserted where
168 the fruit plugs were removed. Four replicates containing three fruit were used. The fruit were
169 kept in a moist chamber at 30 °C in the dark for 24 h the moist chamber was removed. After

this period, the fruit were kept at the same temperature. The lesion diameter (LD; mm) was measured in two perpendicular directions at three days after inoculation for all replicates.

Molecular analysis of the insensitivity to imazalil and thiabendazole

To investigate the molecular basis of insensitivity to thiabendazole, three LS isolates (LN4, RP3, and RP7) and one S isolate (59) were analyzed by sequencing a partial sequence of β-tubulin gene. For imazalil, two LS isolates (IN5, and SV5) and one S isolate (59) were analyzed by sequencing a partial sequence of Cyp51B gene. Isolates were grown on PDA at 25 °C in the dark for seven days. The mycelium was scraped from the culture medium and transferred to 1.5 mL microtubes. The tubes were frozen, and the mycelium was macerated with a pestle until a fine powder was obtained. Genomic DNA was extracted using the Wizard™ Genomic DNA Purification Kit (Promega) following the manufacturer's instructions. The DNA was used as a template to amplify the gene fragments, using the primers T1 (5'-AACATGCGTGAGATTGTAAGT-3') and T22 (5'-TCTGGATGTTGTTGGGAATCC-3') (O'donnell & Cigelnik, 1997) for β-tubulin gene, and the primers Cyp51BF (5'-ATATTCGTCTTGTGCGTGCG-3') and Cyp51BR (5'-GCAGACTAGACGGTCACCA-3') (Martel *et al.,* 2010) for Cyp51B gene. PCR was performed using the Kit GoTaq™ G2 Colorless Master Mix (Promega) following the manufacturer's recommendations. For β-tubulin, the amplification parameters consisted of an initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 30s, 53 °C for 30s, and 72 °C for 1 min, and a final elongation step of 72 °C for 10 min. For Cyp51BR, the amplification parameters were an initial denaturation at 95 °C for 2 min followed by 35 cycles of 95 °C for 30s, 56 °C for 30s, and 72 °C for 3 min, and a final elongation step of 72 °C for 5 min. Unincorporated primers and remaining dNTPs were removed from PCR products using

ExoSAP-IT™ (GE Healthcare) according to the manufacturer's instructions. Sequencing was conducted on a LABCEN/CCB sequencing platform at the Universidade Federal de Pernambuco (UFPE, Recife, Brazil). The nucleotide sequences of S and LS isolates were assembled into contigs and edited in DNA Baser Assembler software version 5.0, and aligned and analyzed with the Molecular Evolutionary Genetics Analysis (MEGA) software version 7.0 (MEGA, Pennsylvania, USA). Sequences of resistant isolates of *Colletotrichum cereale* (GenBank code FJ476048.1) and *C. musae* (GenBank code KY711365.1), showing a mutation at codon 198 and 200, respectively, were included in the alignment. One sequence of a wild-type *Colletotrichum gloeosporioides* f. sp. *aeschynomenes* (GenBank code U14138.1) was used as a reference for the assignment of codon position to allow the detection of point mutations.

Analysis of fitness-related variables

Four fitness-related variables were analyzed for S and LS isolates to imazalil and thiabendazole: mycelial growth rate, conidial germination, conidial production, and virulence.

To evaluate the mycelial growth rate (MGR), a plug (five-mm in diameter) was removed from the edge of a four-day-old culture of each selected isolate and transferred to the center of a Petri dish containing fungicide-free PDA. The plates were incubated in the dark at 30 °C. Five replicates per isolate were used. The colony diameter was measured at 72 h after incubation in two perpendicular directions, and the average was used to calculate the MGR (mm).

The germination of conidia was evaluated by spreading 40 μL of a conidial suspension ($1x10^5$ conidia. mL^{-1}) on Petri dishes containing fungicide-free 2% agar medium. The dishes were kept at 25 °C in the dark for 24 h. Three replicates per isolate were used. The percentage

of germination was evaluated with the aid of an optical microscope, by analyzing 100 conidia per plate. Conidia presenting the germ tube larger than the total length were considered to have germinated. The percentage of germination was obtained by the average of three replicates.

For conidial production, isolates were cultured in PDA medium at 25 °C in the dark for seven days. Four mycelial plugs (five-mm in diameter) were removed at 1 cm from the center of the culture, and placed in a flask containing 1 mL of sterile distilled water. Three flasks were used per isolate. To release the conidia from the plug, the flasks were vortexed for 10 s. The conidial concentration (conidia. mL^{-1}) was estimated using a hemocytometer and the result was the average of three replicates.

The virulence of S and LS isolates was evaluated by inoculating mycelial plugs from five-day-old cultures in banana fruit (cv. Prata). Four replicates containing three fruit were used. The fruit were kept in a moist chamber at 25 °C in the dark for 24 h. After this period, the fruit were kept at the same temperature. The lesion diameter (LD - mm) was measured in two perpendicular directions at five days after inoculation for all replicates.

Competitive ability of the isolates

To analyze the competitive ability of the isolates, two experiments (in vitro and in vivo) were performed in the absence of fungicide. The isolates were selected according to the EC_{50} value. For imazalil, two mixtures were prepared: one mixture (M1) contained the isolates RM12 (S) and UP14 (LS), and the other mixture (M2) contained the isolates RM12 (S) and SV5 (LS). For thiabendazole, the mixture (M3) was prepared with the isolates 59 (S) and RP3 (LS). A conidial suspension of each isolate was prepared in sterile distilled water and used to obtain the mixed conidial suspension containing S and LS isolates. The concentration

of conidial suspensions used in the mixture was $1x10^5$ conidia. mL^{-1} for M1 and M3, and $1x10^4$ conidia. mL^{-1} for M2. Each mixture was used to obtain three different suspensions with the proportions (% LS:S) of 70%:30%, 50%:50%, and 30%:70%.

For the in vitro experiment, 100 µL of mixed conidial suspension at different proportions were spread on a Petri dish containing PDA medium. The plates were kept at 25 °C with 12-h photoperiod for five days. After this period, conidia from the colony were collected in 5 mL of sterile distilled water, and 100 µL of this suspension were used for the subsequent transfer cycle. Five successive transfers were performed, following the same procedure.

The in vivo experiment was performed by inoculating banana fruit (cv. Prata) with 20 µL of each mixture M1 and M2, in the same proportions described above. The fruit were placed in plastic containers and incubated for three days at 25 °C. Conidia were removed from the center of the lesion, suspended in 2 mL of distilled water, and considered as a new source of inoculum. Subsequent inoculations were done following the same procedure described above. Five successive inoculation cycles were performed prior to evaluation.

For both in vitro and in vivo assays, three replicates were used for each combination of mixture (M1, M2, and M3) and LS:S proportion (70%:30%, 50%:50%, and 30%:70%). The experiments were performed twice. For each combination, 15 individual conidia obtained from an experimental unit were used for the conidial competition assay, totalizing 90 conidia. Single conidium was grown on fresh PDA medium amended with the fungicides imazalil or thiabendazole at the discriminatory dose of 0.05 and 0.1 µg a.i. mL^{-1}, respectively. The discriminatory dose was calculated according to the criteria proposed by Lehner *et al.* (2015). The conidia were incubated at 25 °C and the relative growth was evaluated at four days after incubation. For each colony from a single conidium, three replicates (Petri dishes) were prepared. The LS isolates corresponded to those which mycelial growth was greater than 50%

of the control (without fungicide). Pure isolates were used as absolute control. The final proportion of LS isolates after five cycles was compared to the initial proportion.

Statistical analysis

Data obtained in the experiments of in vitro sensitivity, stability of sensitivity, effectiveness of fungicides in infected banana fruit, and analysis of fitness-related variables were evaluated by Student's t-test (for analysis between groups) at 5% of significance. The correlation between the sensitivity to imazalil and thiabendazole was analyzed by Pearson's correlation. All statistical analyses were performed using the software R package (R core team 2017). Graphical representations were performed using SigmaPlot (Scientific Graphing software 2008).

Results

In vitro fungicide sensitivity assay

The isolates of *C. musae* showed differences in sensitivity to both fungicides. For imazalil, the EC_{50} values were low, ranging from 0.03 to 1.29 $\mu g.mL^{-1}$ (0.29 $\mu g.mL^{-1}$ on average), whereas for thiabendazole these values were higher, from 0.10 to 80.35 $\mu g.mL^{-1}$ (1.98 $\mu g.mL^{-1}$ on average). For imazalil, the frequency of isolates with EC_{50} values lower than 0.11 $\mu g.mL^{-1}$ was 19.7%, whereas 65.6% ranged from 0.11 to 0.50 $\mu g.mL^{-1}$, and 14.7% was from 0.51 to 1.50 $\mu g.mL^{-1}$ (Fig. 2a). For thiabendazole, the estimated EC_{50} values were lower than 0.11 $\mu g.mL^{-1}$ in 0.9% of the isolates, from 0.11 to 0.50 $\mu g.mL^{-1}$ in 86.7%, from 0.51 to 1.50 $\mu g.mL^{-1}$ in 7.3%, and greater than 1.50 $\mu g.mL^{-1}$ in 5.1% of the isolates (Fig. 2b). A

positive correlation ($r=0.59$; $P<0.05$) was verified between the sensitivity to imazalil and thiabendazole (Fig. 3).

For each fungicide, the isolates were grouped according to the extremes of sensitivity. Four isolates with the lowest EC_{50} values were selected and considered the most sensitive (S) and four isolates with the highest EC_{50} values were considered the least sensitive (LS). As shown in the Table 1, for imazalil the EC_{50} value was 0.03 $\mu g.mL^{-1}$ for all selected S isolates, and these values were lower than the EC_{50} of the four LS isolates, which ranged from 1.16 to 1.29 $\mu g.mL^{-1}$ (1.24 $\mu g.mL^{-1}$ on average). For thiabendazole, the EC_{50} values of the S isolates ranged from 0.10 to 0.15 $\mu g.mL^{-1}$ (0.12 $\mu g.mL^{-1}$ on average) and were lower than the values obtained for LS isolates, which ranged from 47.31 to 80.35 $\mu g.mL^{-1}$ (58.23 $\mu g.mL^{-1}$ on average).

The evaluation of EC_{50} values by the distribution of isolates per state revealed that the isolates from São Paulo had the highest values, with an average of 0.50 and 8.42 $\mu g.mL^{-1}$ for imazalil and thiabendazole, respectively. These values were higher than that verified for isolates from other states, which averages did not exceed 0.36 and 0.51 $\mu g.mL^{-1}$, respectively (Table 2).

Stability of the sensitivity to imazalil and thiabendazole

No significant alteration was verified in the sensitivity of S and LS isolates after ten successive transfers to fungicide-free PDA medium ($P>0.05$) (Table 3). The EC_{50} value for imazalil and thiabendazole of all isolates did not differ after successive cycles, indicating that the level of sensitivity to both fungicides was maintained.

Fungicide assay in fruit

The treatment of banana fruit with imazalil prior to inoculation with S and LS isolates of *C. musae* led to a significant ($P \leq 0.05$) reduction of the disease severity (Table 4). The lesion diameter (LD) decreased by 57.1% and 64.9% in fruit inoculated with S and LS isolates, respectively, compared to untreated fruit. On the other hand, thiabendazole was effective only to control S isolates, decreasing the LD in 60.3%.

Identification of point mutation associated with the insensitivity

The analysis of a partial sequence of the β-tubulin gene revealed the presence of a point mutation in the isolates RP7 and LN4, less sensitive to thiabendazole (Table 5). This point mutation is a transversion from thymine (T) to adenine (A) at the second position of codon 200 that results in an amino acid change from phenylalanine (codon TTC) to tyrosine (TAC). The sensitive isolate (59) did not show this mutation.

The amplification of the Cyp51B region was unsuccessful, and other sets of primers should be evaluated to amplify the target gene.

Analysis of fitness-related variables

The mycelial growth on fungicide-free medium and the virulence in banana fruit did not differ ($P>0.05$) between S and LS isolates to both fungicides (Fig. 4). The mycelial growth (in mm) of S and LS isolates was on average 53.1 and 52.1 for imazalil (Fig. 4a), and 51.6 and 59.6 for thiabendazole (Fig. 4e), respectively. The lesion diameter (in mm) for S and LS isolates had an average of 22.8 and 19.5 for imazalil (Fig. 4d), and 23.3 and 19.9 for thiabendazole (Fig. 4h), respectively.

The conidial production (conidia. mL^{-1}) showed a variation within the group for both fungicides (Fig. 4b, f). The mean sporulation for S and LS isolates was $8.7x10^5$ and $2.9x10^5$ for imazalil (Fig. 4b), and $8.9x10^5$ and $12.8x10^5$ for thiabendazole (Fig. 4f), respectively.

The percentage of germination for S and LS isolates to imazalil was 88.6% and 67.8% on average, respectively. However, there was a large variation within the LS group, ranging from 0 to 92.7% (Fig. 4c). For thiabendazole, no difference was found between S and LS isolates, with a mean germination of 86.7 to 89.1%, respectively (Fig. 4g).

One S and one LS isolate to thiabendazole were selected for the competition assay. For imazalil, two LS isolates were selected, which had great differences in fitness variables, to compete with the same S isolate (Table 6).

Competitive ability of the isolates

The competitive ability assays showed different results for the different mixtures of S and LS isolates (Fig. 5).

For imazalil, in the mixture M1 (UP14 + RM12, LS and S), the isolate UP14 (LS) predominated over the isolate RM12 (S) after five successive transfers, achieving a frequency of 100% for all proportions initially used, in both assays (Fig. 5a, d). The mixture M2 (SV5 + RM12, LS and S) had different results in vitro and in vivo (Fig. 5b, e). In the in vitro assay, the frequency of the isolate SV5 (LS) remained constant for all three initial proportions (Fig. 5b). However, in the in vivo assay, the proportion (%) of the isolate SV5 (LS) reduced 49% on average for the three proportions, decreasing from 30 to 13.3, from 50 to 26.7, and from 70 to 40% (Fig. 5e).

For thiabendazole, different results were also obtained in vitro and in vivo with the mixture M3 (RP3 + 59, LS and S) (Fig. 5c, f). In the in vitro assay, the frequency of the

370 isolate RP3 (LS) remained constant for all three initial proportions (Fig. 5c). On the other
371 hand, in the in vivo assay, the isolate RP3 (LS) predominated over the isolate 59 (S) for all
372 proportions evaluated, reaching 86,6% for the proportion of 30:70 and 100% for the other two
373 proportions (50:50, and 70:30) (Fig. 5f).
374

375 ## Discussion

376

377 This is the first large study on the sensitivity of Brazilian *C. musae* population to
378 imazalil and thiabendazole. The isolates showed a differential sensitivity to both fungicides.
379 In general, they were more sensitive to imazalil, as verified by the lower EC_{50} values
380 compared to thiabendazole. This was expected, since imazalil belongs to DMI group, which
381 resistance risk is lower than the MBCs (FRAC, 2018). The maximum EC_{50} values were 1.29
382 $\mu g.mL^{-1}$ and 80.36 $\mu g.mL^{-1}$ for imazalil and thiabendazole, respectively. These values are
383 considered high if compared with other studies involving these groups of fungicides and
384 *Colletotrichum* species. Tavares & Souza (2004), evaluating the control effectiveness of
385 fungicides in papaya, verified that isolates of *C. gloeosporioides* were highly sensitive to
386 imazalil and moderately sensitive to thiabendazole, with estimated EC_{50} less than 1.0 $\mu g.mL^{-1}$
387 and around 33.3 $\mu g.mL^{-1}$, respectively. Recently, the sensitivity of *C. musae* isolates to
388 thiophanate-methyl was evaluated and the estimated EC_{50} was 48.73 $\mu g.mL^{-1}$, indicating a
389 moderate sensitivity (Vieira *et al*., 2017). The differential sensitivity in populations of *C.*
390 *musae* had already been reported, with estimated EC_{50} values less than 1.0 $\mu g.mL^{-1}$ to imazalil
391 and greater than 10 $\mu g.mL^{-1}$ for thiabendazole (Johanson & Blazquez, 1992).

392 In this study, a positive correlation between the insensitivity to imazalil and
393 thiabendazole was verified. Although these fungicides act in different target sites (FRAC,

2018), the multiple resistance between DMIs and MBCs fungicides has already been described to *Monilinia fructicola*, to fungicides propiconazole and methyl thiophanate (Chen *et al*., 2013a). However, it is still little studied, mainly related to the genus *Colletotrichum*. Based on our results, the positive correlation between the insensitivity to both fungicides is probably be associated with the selection pressure in areas with historic of fungicide application. For example, isolates from the state of São Paulo had the lowest sensitivity (highest EC_{50} values) for both fungicides. This is probably related to high productivity in these areas, requiring many applications of fungicides, which leads to a selection of less sensitive isolates (data not shown).

All isolates (S and LS) were able to maintain the level of sensitivity to imazalil and thiabendazole after ten successive transfers on free-fungicide medium. This fact has been also demonstrated in studies with DMIs and MBCs, with no reduction in sensitivity after subcultures of resistant or less sensitive isolates in the absence of fungicides (Chen *et al*., 2013b; Santos *et al.*, 2019). This stability of sensitivity is a concern, since less sensitive isolates can remain in the population after successive cycles, even in the absence of fungicides.

The fungicides imazalil and thiabendazole have been used successfully to control anthracnose in banana (Coelho *et al*., 2010; Khan *et al*., 2001). Indeed, when we evaluated their effectiveness to control the disease caused by sensitive isolates of *C. musae* in banana detached fruit, both fungicides were efficient. However, for less sensitive isolates, only imazalil was able to control the disease. Thus, our results show that the control effectiveness of thiabendazole may be compromised due to the presence of less sensitive isolates, which may lead to control failure in the field.

The analysis of characteristics related to fitness (mycelial growth, sporulation, germination, and virulence) showed no difference between the groups of S and LS isolates for

both fungicides, except for sporulation of S and LS isolates to imazalil. However, a large variation was observed within the group for all variables and for both fungicides. This result shows that, when considering groups to evaluate the variables, individual responses of the isolates are ignored and, consequently, the fitness penalties can be neglected. In addition, it is important to consider that a change in a fitness-related variable not necessarily leads to a penalty that disfavors the isolate. Thus, an analysis that takes into account all variables simultaneously is a more appropriate approach to inferring about the fitness penalties in LS isolates. In this study, a competitive ability assay both in vitro and in vivo was performed with S and LS isolates to imazalil and thiabendazole. In general, LS isolates that showed no change in the fitness-related variables predominated over the S isolate after five successive transfers. However, one LS isolate showing low sporulation, percentage of germination and virulence had its frequency reduced after five transfers in vivo, indicating that the fitness penalties affected its ability to compete with the S isolate. The extent to which such fitness penalties are present has important implications for resistance management strategies.

The isolates with the highest EC_{50} values for thiabendazole showed a mutation at codon 200 of the β-tubulin gene. This mutation has been reported in *C. musae* isolates moderately resistant to thiophanate-methyl (Vieira *et al*., 2017). Isolates of *C. gloeosporioides* from mango and strawberry showing this mutation at codon 200 are moderately resistant (EC_{50} values from 10 to 100 $\mu g.mL^{-1}$) to benomyl, carbendazim, and thiabendazole. On the other hand, mutations at codon 198 have been found in isolates with high EC_{50} values, ranging from 100 to 500 $\mu g.mL^{-1}$ (resistant) or greater than 500 $\mu g.mL^{-1}$ (highly resistant) (Chung *et al*., 2010). But this information should be analyzed with caution because the level of sensitivity can be variable among the MBC fungicides.

In this study, we demonstrated that the sensitivity of isolates of *C. musae* from banana growing areas in Brazil to the fungicides imazalil and thiabendazole is variable and stable. For

444 both fungicides, isolates with reduced sensitivity were found, mainly in the state of São Paulo.
445 However, imazalil was still efficient in controlling the disease, while thiabendazole showed
446 control failure. The high competitive ability of LS isolates to thiabendazole indicates that the
447 resistance-conferring mutation did not result in fitness penalties, and that these variants may
448 increase in frequency in the population, even with the discontinued use of the fungicide.

449 Taken together, our results allow a better understanding on the sensitivity and fitness
450 of isolates of *C. musae* from Brazil, and demonstrate the importance of periodic monitoring to
451 verify the frequency of LS isolates in populations from different producing regions. Such
452 monitoring is important for the conscious implementation of measures for a more effective
453 management of anthracnose in banana orchards in Brazil.

454

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456

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459

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561

562 **Figure legends**

563

564 **Figure 1** Collection sites of isolates of *Colletotrichum musae* from Brazilian banana orchards
565 located in the states of Bahia (BA), Distrito Federal (DF), Espírito Santo (ES), Goiás (GO),
566 Minas Gerais (MG), Pará (PA), Paraná (PR), Pernambuco (PE), Santa Catarina (SC), and São
567 Paulo (SP).

568

569 **Figure 2** Distribution of the frequency of *Colletotrichum musae* isolates according to the
570 effective concentration required to inhibit 50% of the mycelial growth (EC_{50}) for the
571 fungicides imazalil (a) and thiabendazole (b).

572

573 **Figure 3** Correlation between the sensitivity to imazalil and thiabendazole based on the
574 values of effective concentration required to inhibit 50% of the mycelial growth (EC_{50}) of
575 isolates of *Colletotrichum musae*. Each point represents one isolate (n= 218).

576

577 **Figure 4** Fitness-related variables of isolates of *Colletotrichum musae* sensitive (S) and less
578 sensitive (LS) to imazalil (a-d) and thiabendazole (e -h). Values represent the mean of four
579 isolates. Points represent each isolate. Bar represents the standard deviation. *Significant
580 (P=0.05).

581

582 **Figure 5** Frequency of less-sensitive (LS) isolates (% of conidia) in a competition assay with
583 sensitive (S) isolates of *Colletotrichum musae*, containing different initial proportions of LS:S
584 (70%:30%, 50%:50%, and 30%:70%). The experiments were performed both in vitro (a, b, c)
585 and in vivo (d, e, f), using mixtures of isolates S and LS to imazalil (a, b, d, e) and
586 thiabendazole (c, f). The frequency was evaluated after five successive transfers (T_5) and
587 compared with the initial frequency for each proportion (T_0). Points represent the proportion
588 of LS of 15 colonies from a single conidium.

Table 1 List of *Colletotrichum musae* isolates from Brazilian banana orchards selected in this study, showing the lowest (sensitive - S) and the highest (less sensitive - LS) EC_{50} values for the fungicides imazalil and thiabendazole

Isolate code[a]	State of origin	EC_{50} (µg a.i. mL^{-1})[b]			
		Imazalil		Thiabendazole	
		S	LS	S	LS
C39	Distrito Federal	-	-	0.10	-
UP14	Distrito Federal	-	1.27	-	-
UP15	Distrito Federal	-	-	0.15	-
DP12	Pernambuco	0.03	-	-	-
SV5	Pernambuco	-	1.29	-	-
SV22	Pernambuco	-	-	0.13	-
16	Santa Catarina	0.03	-	-	-
59	Santa Catarina	0.03	-	0.10	-
IN5	São Paulo	-	1.19	-	-
LN1	São Paulo	-	-	-	47.31
LN4	São Paulo	-	-	-	51.51
LN5	São Paulo	-	1.16	-	-
RM12	São Paulo	0.03	-	-	-
RP3	São Paulo	-	-	-	80.35
RP7	São Paulo	-	-	-	53.74

[a] Code from the Culture Collection of the Laboratório de Micologia at the Universidade Federal Rural de Pernambuco (Recife, Pernambuco, Brazil).

[b] Effective concentration required to inhibit 50% of the mycelial growth (EC_{50}).

Table 2 Values of the effective concentration of fungicide (imazalil and thiabendazole) required to inhibit 50% of the mycelial growth (EC_{50}) of isolates of *Colletotrichum musae*, considering the state of origin

State of origin of isolates	Number of isolates	Mean EC_{50} (µg a.i. mL^{-1})[a]	
		Imazalil	Thiabendazole
Bahia	04	0.22 (0.13)	0.26 (0.08)
Distrito Federal	15	0.36 (0.29)	0.30 (0.09)
Espírito Santo	04	0.26 (0.29)	0.33 (0.13)
Goiás	15	0.31 (0.21)	0.32 (0.14)
Minas Gerais	31	0.16 (0.08)	0.32 (0.10)
Pará	03	0.29 (0.20)	0.25 (0.07)
Paraná	01	0.27 (0.00)	0.36 (0.00)
Pernambuco	80	0.26 (0.19)	0.51 (1.61)
Santa Catarina	22	0.11 (0.06)	0.39 (0.09)
São Paulo	43	0.50 (0.26)	8.42 (18.8)

[a] Values (µg a.i. mL^{-1}) are the mean of EC_{50} of all isolates from each state. Values in the parentheses represent the standard deviation.

Table 3 Stability of sensitivity to imazalil and thiabendazole of sensitive and less sensitive *Colletotrichum musae* isolates based on the comparison between the initial (T_0) effective concentration required to inhibit 50% of the mycelial growth (EC_{50}) and the following ten sequential transfers on fungicide-free PDA medium (T_{10})

Isolate class[a]	EC_{50} (µg a.i. mL^{-1}) [b]			
	Imazalil		Thiabendazole	
	T_0	T_{10}	T_0	T_{10}
Sensitive	0.03 (0.00) a	0.05 (0.01) a	0.12 (0.02) a	0.14 (0.06) a
Less sensitive	1.23 (0.06) a	1.02 (0.61) a	58.23 (14.9) a	62.12 (8.65) a

[a] Each class is composed of four isolates selected by the lowest and the highest EC_{50} values for imazalil and thiabendazole.

[b]Means followed by the same letter in the line for each fungicide do not differ significantly according to Student's t-test (P=0.05). Values in the parentheses represent the standard deviation.

Table 4 Disease severity (lesion diameter) on detached banana fruit treated with imazalil and thiabendazole prior to inoculation with sensitive and less sensitive isolates of *Colletotrichum musae*

Isolate class[a]	**Lesion diameter (mm)**[b]			
	Imazalil		**Thiabendazole**	
	No Fungicide	Fungicide	No Fungicide	Fungicide
Sensitive	17.15 (1.04) a	7.35 (0.81) b	16.06 (0.22) a	6.38 (1.78) b
Less sensitive	16.03 (1.92) a	5.62 (3.25) b	18.04 (2.13) a	16.91 (1.70) a

[a]Each class is composed of four isolates selected by the lowest and the highest EC_{50} values for imazalil and thiabendazole.

[b]Means followed by the same letter in the line for each fungicide do not differ significantly according to Student's t-test (P=0.05). Values in the parentheses represent the standard deviation.

Table 5 Partial nucleotide and amino acid sequence of the β-tubulin gene from *Colletotrichum musae* isolates sensitive and less sensitive to thiabendazole

Isolate class	Isolate code	GenBank code	Partial nucleotide and amino acid sequence								
			198			199			200[d]		
Less sensitive	RP3	KY711365.1[a]	G	A	G	A	C	C	T	**A**	C
	RP7	This study	G	A	G	A	C	C	T	**A**	C
	LN4	This study	G	A	G	A	C	C	T	**A**	C
	Amino acid		E			T			**Y**		
	C. cereale	FJ476048.1[b]	G	**C**	G	A	C	C	T	C	C
	Amino acid		A			T			F		
Sensitive	59	This study	G	A	G	A	C	C	T	T	C
	C. gloeosporioides f. sp. *aeschynomenes*	U14138.1[c]	G	A	G	A	C	C	T	T	C
	Amino acid		E			T			F		

[a] Access number on GenBank of a sequence from one isolate of *C. musae* resistant to thiophanate-methyl; [b] Access number on GenBank of a sequence from one isolate of *C. cereale* resistant to thiophanate-methyl; [c] Access number on GenBank of a sequence from one isolate of *C. gloeosporioides* f. sp. *aeschynomenes* sensitive to benomyl; [d] Codon position at the β-tubulin gene.

Table 6 Fitness-related variables of isolates of *Colletotrichum musae* sensitive (S) and less sensitive (LS) to imazalil and thiabendazole, selected for the ability competitive assay

Isolates	Class of isolates	Fitness variables			
		MGR[a] (mm)	Germination (%)	Sporulation ($x10^5$/mL)	LD[b] (mm)
Imazalil					
UP14	LS	47.7 (2.4)	88.0 (1.7)	4.3 (4.1)	22.4 (1.5)
RM12	S	58.8 (2.5)	87.0 (1.1)	5.7 (1.5)	28.6 (2.6)
SV5	LS	40.6 (1.5)	0.0 (0.0)	0.2 (0.1)	10.1 (0.9)
Thiabendazole					
59	S	55.7 (2.6)	93.0 (2.0)	10.3 (1.1)	22.2 (1.5)
RP3	LS	53.6 (1.1)	85.3 (2.0)	13.7 (6.6)	21.0 (2.4)

[a] Mycelial growth rate in fungicide-free PDA medium; [b] Lesion diameter (virulence) in inoculated banana fruit. Values in the parentheses represent the standard deviation.

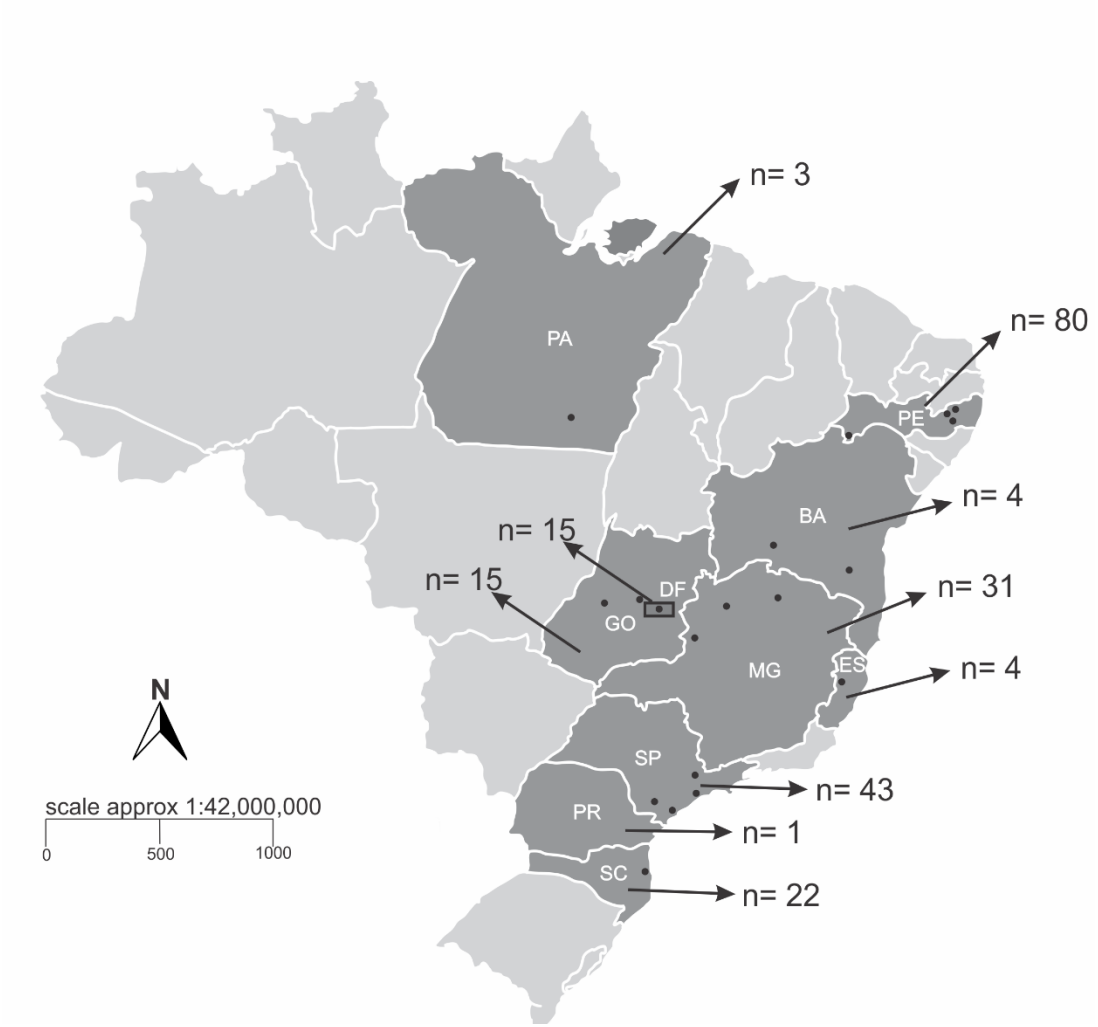

Figure 1

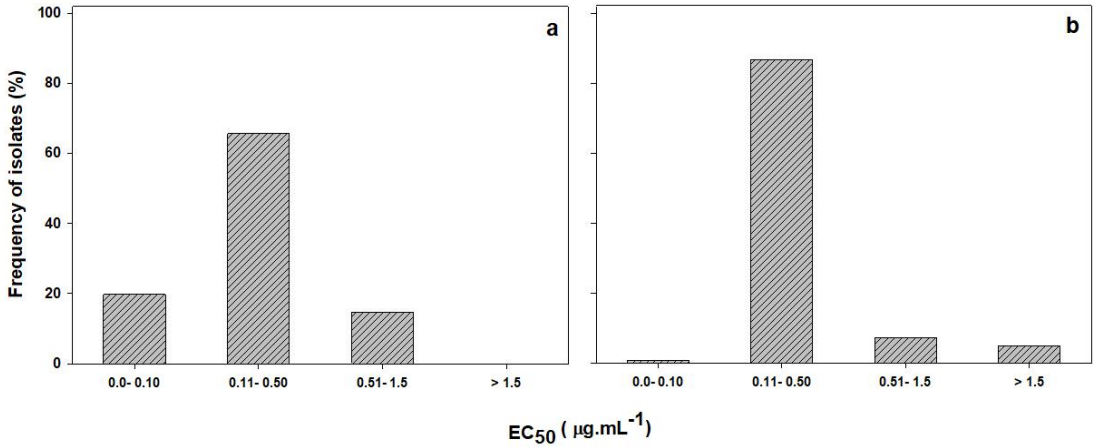

Figure 2

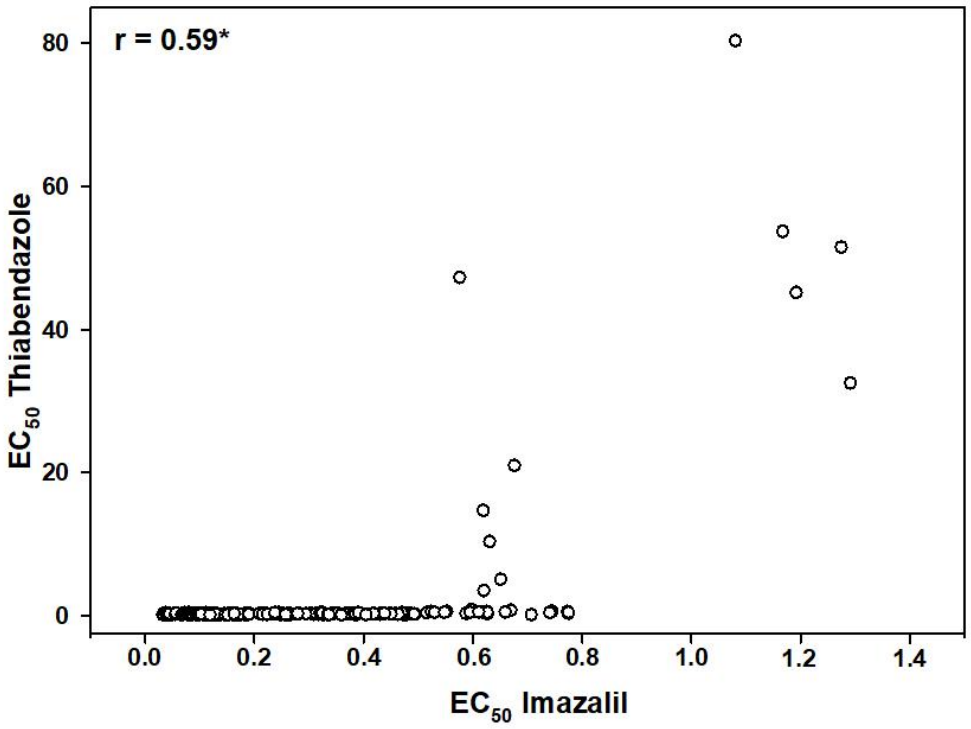

Figure 3

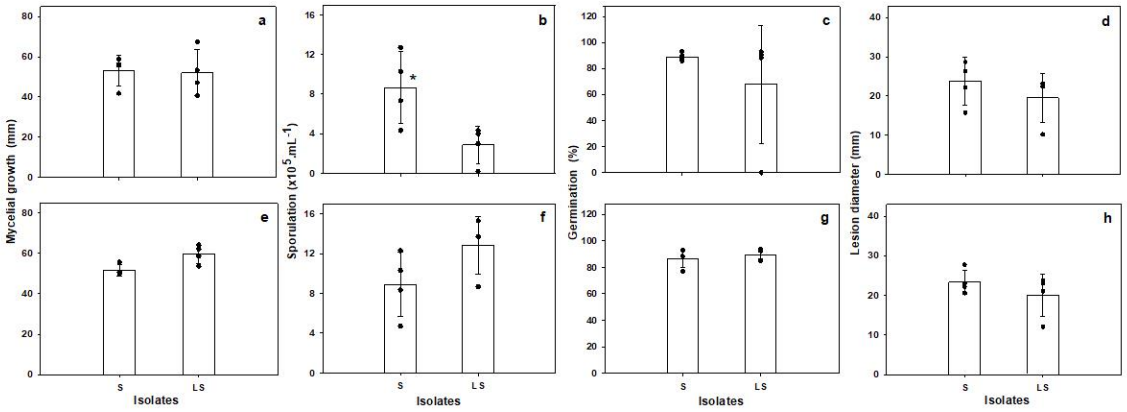

Figure 4

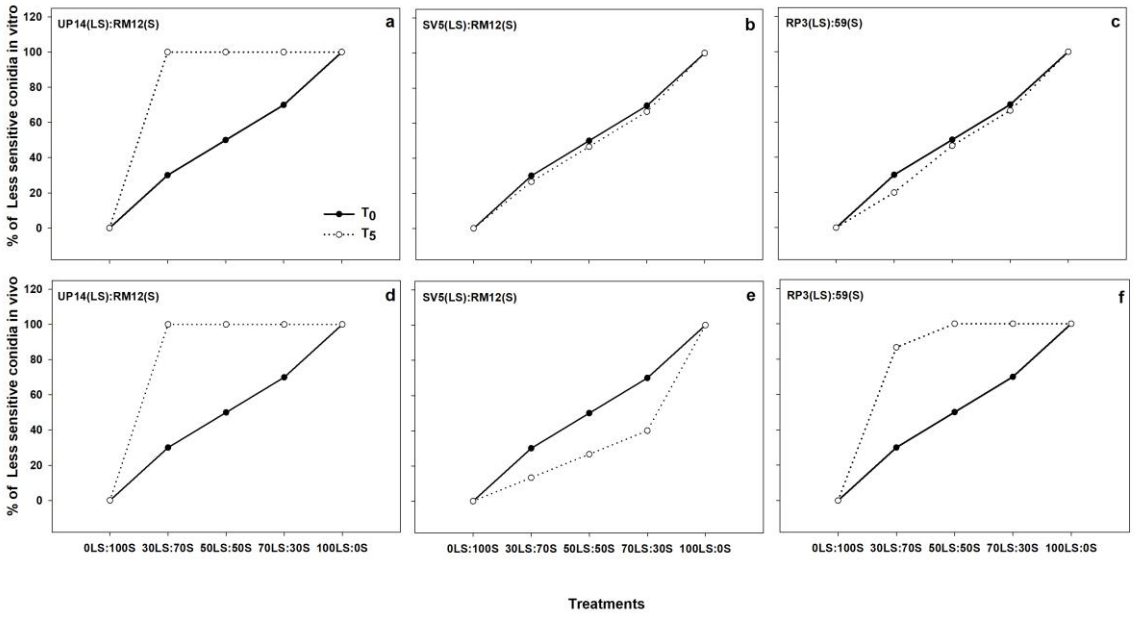

Figure 5

CONCLUSÕES GERAIS

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- De modo geral, os isolados de *Colletotrichum musae* obtidos em áreas de cultivo de bananeira no Brasil apresentaram níveis variados de sensibilidade aos fungicidas imazalil e tiabendazol;
- Os isolados de *C. musae* obtidos do estado de São Paulo apresentaram menor sensibilidade à tiabendazol, quando comparado aos isolados provenientes dos demais estados brasileiros;
- Houve uma correlação positiva entre a sensibilidade dos isolados aos fungicidas imazalil e tiabendazol;
- O fungicida imazalil apresentou boa eficácia de controle da antracnose em frutos de banana inoculados com isolados sensíveis e menos sensíveis. Por outro lado, tiabendazol não foi capaz de controlar a doença causada por isolados menos sensíveis;
- Os isolados de *C. musae* menos sensíveis a ambos os fungicidas apresentaram estabilidade na sensibilidade e boa adaptabilidade. Porém, para alguns isolados menos sensíveis a imazalil, foram verificadas variações nas características adaptativas;
- A sensibilidade reduzida dos isolados de *C. musae* a tiabendazol está relacionada à mutação no códon 200 da região codificante de β-tubulina. No entanto, a presença dessa mutação não resultou em penalidade de aptidão;
- Embora o fungicida imazalil tenha se mostrado eficiente no manejo da antracnose na banana, mesmo para isolados com sensibilidade reduzida, é importante o monitoramento constante das populações do fungo, para avaliar possíveis mudanças na sensibilidade;
- Os resultados apontam a necessidade de adotar estratégias mais eficientes, como a implementação de registro de novas moléculas de fungicidas, visando ao controle efetivo da antracnose na banana.