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Caracterização e avaliação de sensibilidade a fungicidas e ao herbicida glifosato em isolados de *Colletotrichum* associados à plantas daninhas

Rio Largo
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Tese de Doutorado apresentado ao Programa de Proteção de Plantas da Universidade Federal de Alagoas como requisito para obtenção do título de doutor em Proteção de Plantas.

Orientadora: Profa. Dra.Iraildes Pereira Assunção

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RESUMO

As plantas daninhas podem atuar como hospedeiras alternativas de diversos patógenos, entre eles os fungos do gênero *Colletotrichum*. Nos campos de produção, o uso de produtos químicos, sejam fungicidas ou herbicidas é bastante elevado, principalmente no controle de plantas daninhas, que podem atuar como fonte de inóculos de patógenos causadores de doenças em plantas cultivadas. Devida a falta de informações sobre patógenos associados às plantas daninhas no nordeste brasileiro, o presente trabalho teve como objetivo geral a caracterização e identificação das principais espécies de *Colletotrichum* associadas a três diferentes espécies vegetais: *Euphorbia hirta, Spigelia anthelmia* e *Sida* sp.; e verificar a sensibilidade a três fungicidas e ao herbicida glifosato. No primeiro capítulo foi realizada a identificação e caracterização de espécies do complexo *C. gloeosporioides* associados à *S. anthelmia* e a sensibilidade dos isolados a três fungicidas: tebuconazole, difenoconazole e uma mistura de tebuconazole com trifloxistrobina, em quatro dosagens diferentes, bem como ao herbicida glifosato. Foram identificadas seis espécies através das análises morfoculturais e filogenética multilocus utilizando os genes DNA lyase, gliceraldeído-3-fosfato dehidrogenase (GAPDH), tubulina e a região ApMat: *C. fragariae, C. fructicola, C. queenslandicum, C. siamense* e *C. theobromicola*. Todas as espécies foram patogênicas ao hospedeiro e os três fungicidas apresentaram variados graus de inibição do crescimento micelial, sendo o difenoconazole o mais eficiente. O herbicida glifosato não apresentou nenhum efeito inibitório para as espécies avaliadas. Esse o primeiro relato dessas espécies causando antracnose em *S. anthelmia.* O segundo capítulo identificou e caracterizou *C. truncatum* causando antracnose em três espécies de plantas daninhas. A identificação e caracterização foi realizada através das análises morfoculturais e filogenia multilocus a partir dos genes GAPDH, actina e a região ITS, sendo esse o primeiro relato de *C. truncatum* causando antracnose em *S. anthelmia, Sida* e *E. hirta.*

Palavras-chave: Sensibilidade a Fungicidas; Análise Multilocus; Glifosato

ABSTRACT

Weeds can act as alternative hosts for several pathogens, including fungi of the genus *Colletotrichum*. In the crop fields, the use of chemical products, whether fungicides or herbicides, is quite high, mainly in the control of weeds, which can act as a source of inoculants of disease-causing pathogens in cultivated plants. Due to lack of information on pathogens associated with weeds in northeastern of Brazil, the present study aimed to characterize and identify the main species of *Colletotrichum* associated with three different plant species: *Euphorbia hirta, Spigelia anthelmia* and *Sida* sp. And check the components of adaptability and sensitivity to three fungicides and the herbicide glyphosate. In the first chapter, the identification and characterization of species of the *C. gloeosporioides* complex associated with *S. anthelmia* and the sensitivity of the isolates to three fungicides were carried out: tebuconazol, difenoconazol and a mixture of tebuconazol with trifloxystrobin, in four different dosages, as well as the herbicide. glyphosate. Six species were identified through morphocultural and multilocus phylogenetic analyzes using the genes DNA lyase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), tubulin and the ApMat region: *C. fragariae, C. fructicola, C. queenslandicum, C. siamense* and *C theobromicola*. All species were pathogenic to the host and the three fungicides showed varying degrees of inhibition of mycelial growth, with diphenoconazole being the most efficient. The herbicide glyphosate did not present any inhibitory effect for the species evaluated. This being the first report of these species causing anthracnose in *S. anthelmia*. The second chapter identified and characterized *C. truncatum* causing anthracnose in three weed species. The identification and characterization was performed through morphocultural analysis and multilocus phylogeny from the GAPDH, actin and ITS region, this being the first report of *C. truncatum* causing anthracnose in *S. anthelmia, Sida* and *E. hirta*.

Keywords: Sensibility to Fungicides; Multilocus Analysis; Glyphosate

SUMÁRIO

1. INTRODUÇÃO

As plantas daninhas são aquelas que crescem em locais indesejados e interferem nas atividades humanas causando prejuízos quando não são adequadamente controladas (FOELKEL, 2008). Possuem como uma de suas principais características o rápido crescimento e alta capacidade de produção de sementes, atuando como pioneiras na colonização de áreas cultivadas ou degradadas, além dessas características, elas podem servir como fonte de inóculo para diversos patógenos, como apontam diversos estudos (OLIVEIRA JUNIOR; CONSTANTIN; INOUE, 2011; SALES JÚNIOR et al., 2012; FERRO et al., 2017; GOBATTO et al., 2019; WEBSTER et al., 2019).

Dentre os patógenos encontrados utilizando as plantas daninhas como hospedeiras podemos citar o gênero *Colletotrichum*, considerado um dos dez gêneros fúngicos fitopatogênicos de maior importância para as atividades humanas (DEAN et al., 2012). A principal doença causada em plantas é denominada antracnose, cuja característica principal é sua sintomatologia típica, com pouca variação entre os hospedeiros e caracterizada por uma lesão de coloração que varia do castanho escuro a negra com centro deprimido e, em condições ambientais favoráveis pode ocorrer a formação de uma massa de esporos de coloração alaranjada na superfície da lesão. Sendo o uso de fungicidas, uma das formas mais eficientes de controle dessa doença (AMORIM; REZENDE; BERGAMIN FILHO, 2016).

O uso de fungicidas como forma de controle de doenças é bastante difundido no mundo, e com o avanço da tecnologia e das pesquisas, diversos produtos estão disponíveis no mercado com variados mecanismos de ação. A FRAC (Comitê de Ação de Resistência a Fungicidas) classifica os fungicidas em 13 grandes grupos de acordo com o seu mecanismo de ação. Entre os diversos mecanismos de ação catalogados, dois grupos representam mais de 50% dos fungicidas comercializados: os Inibidores da Quinona Externa (QoI) e os Inibidores da Biossíntese do Ergosterol (SBI). Entre os grupos químicos que compõem esses dois mecanismos de ação, podemos citar os triazóis e as estrobilurinas (HERMANN e STENZEL, 2019).

Outro grupo químico muito utilizado nos campos de cultivos e também em controle de plantas daninhas em jardins residenciais são os herbicidas. Os herbicidas são produtos químicos utilizados para controlar plantas daninhas e entre eles, um dos mais vendidos e conhecidos é o

glifosato. O uso indiscriminado desses produtos pode afetar o processo da doença nas plantas, tanto aumentando a severidade da doença por meio do aumento da suscetibilidade da planta ao patógeno, como pode também exercer um certo grau de controle do crescimento de alguns patógenos (EL-SHANSHOURY et al., 1995; KAO; CHUNG; HUANG, 2019).

Dessa forma, os objetivos do trabalho foram identificar as espécies de *Colletotrichum* causando antracnose em plantas daninhas no nordeste brasileiro e verificar os efeitos de três fungicidas e do herbicida glifosato no crescimento micelial dessas espécies.

2. REVISÃO DE LITERATURA

2.1. Plantas Daninhas

O termo Planta Daninha é utilizado para definir as plantas que crescem em local onde são indesejadas e acabam interferindo nos objetivos das atividades antrópicas realizadas na área, além de poderem causar danos e prejuízos se não forem controladas corretamente (FOELKEL, 2008). Também podem ser chamadas de plantas espontâneas, plantas invasoras, plantas não cultivadas dependendo da área de abordagem utilizada, porém o termo adotado pela SBCPD (Sociedade Brasileira da Ciência das Plantas Daninhas) continua sendo Plantas Daninhas.

As Plantas Daninhas interferem na cultura de forma direta ou indireta. A interferência direta ocorre através da competição, parasitismo e alelopatia e a indireta quando servem como hospedeiro alternativo de pragas e doenças, dificulta a colheita e os tratos culturais ou deprecia a qualidade do produto (PITELLI; MARCHI, 1991).

Algumas características adaptativas levam ao sucesso das Plantas Daninhas na colonização de áreas, bem como dificultam o controle desses indivíduos, sendo as mais relevantes a sua habilidade competitiva na captação de recursos disponibilizados no ambiente, como água, nutrientes e luz; capacidade de alta produção de propágulos; desuniformidade da germinação; capacidade de germinar e emergir a grandes profundidades; mecanismos alternativos de reprodução; alta viabilidade dos propágulos em condições adversas; facilidade de disseminação de suas estruturas reprodutivas; e o rápido crescimento e desenvolvimento inicial (OLIVEIRA JUNIOR; CONSTANTIN; INOUE, 2011).

Esse grupo de plantas apresentam aspectos positivos e negativos (CARVALHO, 2013). Entre os aspectos positivos podemos verificar os efeitos benéficos ao solo pela sua cobertura vegetal; utilização como planta ornamental ou na farmacologia, como os usos antimicrobianos de *Euphorbia hirta* L. e da *Spigelia anthelmia* L., a utilização como alimentação humana e animal. Além desses efeitos, algumas plantas consideradas daninhas também podem ser usadas na obtenção de óleos essenciais para perfumaria, atuarem como flora apícola e controle de pragas domésticas e agrícolas (OLIVEIRA JUNIOR; CONSTANTIN; INOUE, 2011).

Entre os principais aspectos negativos das plantas daninhas destacam-se: a redução na produtividade e no valor da terra, associadas sobretudo à espécie dominante e a sua densidade populacional no local; a perda na qualidade do produto agrícola, pois os restos vegetais tendem a causar prejuízos na colheita e beneficiamento de alguns produtos; dificuldade e aumento do custo de manejo agrícola; problemas com manejo e perdas de água; acarretar danos à vida e à saúde do ser humano e animal, pois algumas plantas podem apresentar substâncias tóxicas ao ser humano; além de servirem como fonte de inóculo para doenças e pragas (CARVALHO, 2013).

2.2 Plantas Daninhas como Fonte de Inóculo

Existem evidências de que plantas daninhas associadas a campos de cultivo servem como hospedeiros alternativos para patógenos e podem desempenhar um papel importante na epidemiologia de algumas doenças (SALES JÚNIOR et al., 2012), como por exemplo as ferrugens que sobrevivem no campo através da colonização de plantas voluntárias após a colheita, e voltam ao seu hospedeiro principal quando do replantio (AMORIM; REZENDE; BERGAMIN FILHO, 2018).

Há também o caso do capim arroz (*Echinochloa crusgalli* (L.) P. Beau.) que atua como hospedeiro alternativo de *Pyricularia oryzae* o agente causal do brusone do arroz, além de algumas espécies de vírus causadores de mosaico, como por exemplo: *Sida yellow mosaic virus* (SiYMV) e o *Euphorbia yellow mosaic virus* (EuYMV) *(*OLIVEIRA JUNIOR; CONSTANTIN; INOUE, 2011; FERRO et al., 2017*).*

Pesquisas realizadas desde a década de 1980 já atestam esse fato, como o trabalho de Batista (1984) que verificou a presença de *Colletotrichum guaranicola* em plantas daninhas associadas a campos de cultivo de guaraná. Raid e Pennypacker (1987) que verificaram que *Colletotrichum coccodes*, patógeno associado à antracnose em tomateiro (*Solanum lycopersicum* L.) estava também associado com plantas daninhas presentes nos campos de cultivo, e que atuavam como fonte de inóculo para a cultura.

Alguns trabalhos relataram a ocorrência de *C. gloeosporioides* causando antracnose em plantas daninhas com potencial para causar doenças em plantas cultivadas (MORIN et al., 1996; ZHU et al., 2018).

Trabalhos realizados por BERRIE e BURGESS (2001) e PARIKKA e LEMMETTY (2009) constataram a capacidade de plantas daninhas atuarem como fonte de inóculo e hospedeiras alternativas de *C. acutatum* associado à cultura do morango, sendo que Freeman, Horowitz e Sharon (2001) verificaram que essa mesma espécie fúngica foi isolada de plantas assintomáticas e esses isolados eram patogênicos ao morango.

Outros trabalhos em campos de cultivo de morango constataram outras espécies de *Colletotrichum* associadas às plantas daninhas como hospedeiras alternativas, como *C. nymphaeae* (KARIMI; ARZANLOU e PERTOT, 2019) e *C. fructicola* (HIRAYAMA et al., 2018). Plantas Daninhas também podem servir de fonte de inóculo para outros agentes fitopatogênicos como vírus, viróides, bactérias e nematoides, como atestam os trabalhos de SMAGGHE e DE JONGHE (2015), FERNÁNDEZ-SANZ, RODICIO e GONZÁLEZ (2016), AGUIAR et al. (2018), OCIMATI et al. (2018), VAN BOGAERT, FAYETTE et al. (2018), GOBATTO et al. (2019) e WEBSTER et al. (2019).

2.3 Gênero *Colletotrichum*

O gênero *Colletotrichum* inclui uma variedade elevada de espécies que atuam como patógenos de plantas com grande importância econômica e social, causando doenças em várias espécies vegetais. Encontra-se distribuído globalmente, sobretudo nas regiões tropicais e subtropicais, sendo considerado o oitavo gênero fúngico fitopatogênico de maior importância no mundo (DEAN et al., 2012; CANNON et al., 2012). Foi catalogado inicialmente em 1851 por Corda, porém a classificação foi controversa com relação a outro gênero existente e identificado em 1790 por Toda, o *Vermicularia.*

Com a classificação saccardiana proposta em 1884 o gênero *Vermicularia* foi considerado como um estágio de desenvolvimento de *Colletotrichum*. Na década de 1950, Von Arx unificou os dois gêneros em um e adotou o nome *Colletotrichum*, reduzindo assim o número de espécies para 11 (MENEZES, 2006). Com a classificação proposta por Sutton em 1992 o número de espécies foi alterado para 40 e em 2009, Hyde propôs 66 espécies pertencentes ao gênero.

Atualmente o gênero possui aproximadamente 200 espécies (MONGKOLPORN; TAYLOR, 2018) divididas em 14 complexos de espécies, sendo eles: *C. acutatum, C. boninense,*

C. caudatum, C. dracaenophilum, C. dematium, C. destructivum, C. gigasporum, C. gloeosporioides, C. graminicola, C. magnum, C. orbiculare, C. orchidearum, C. spaethianum, C. truncatum (CANNON et al., 2012; DAMM et al., 2019).

A principal característica desses fungos é a formação de acérvulos no tecido do hospedeiro, local onde os conídios são formados, produzem esporos unicelulares, hialinos e recobertos por uma matriz gelatinosa que auxilia na germinação e penetração no tecido hospedeiro. Algumas espécies podem produzir setas (MENEZES, 2006).

As espécies de *Colletotrichum* adotam diversas estratégias para colonizar o tecido vegetal, como a hemibiotrofia intracelular e a necrotrofia subcuticular (VARGAS et al., 2012). Durante sua fase patogênica ocorre o desenvolvimento do apressório que, auxilia na colonização do hospedeiro através da liberação de substâncias químicas e enzimáticas que degradam a cutícula e posteriormente causam necrose do tecido afetado (O'CONELL et al., 2012). Essa ação é auxiliada pela produção de metabólitos fitotóxicos que variam de acordo com a planta hospedeira e o patógeno, entre as substâncias já identificadas existem o colletotrichin, colletodiol, colletol e coletalol (JOSHI, 2018).

A grande maioria das plantas cultivadas é suscetível a pelo menos uma espécie de *Colletotrichum* causando perdas econômicas significativas afetando tanto a fase de desenvolvimento vegetativo como na pós-colheita (DEAN et al., 2012). Entre as principais culturas afetadas podemos citar a banana, mamão, cacau, flores tropicais, anonáceas, pimentas, pimentão, feijão, milho, entre outras (RANANTHUNGE; SANDANI, 2016; RAMDIAL, DE ABREU, RAMPERSAD, 2017; SILVA et al., 2017; HERATH, MANAMGODA, UDAYANGA, 2019; VIEIRA et al., 2020). Da mesma forma, as plantas daninhas podem ser afetadas pela presença do fungo em seus tecidos vegetais (RAID; PENNYPACKER, 1987; HIRAYAMA et al., 2018).

A principal doença causada por *Colletotrichum* nas plantas hospedeiras é a antracnose, porém a depender da espécie e do hospedeiro a doença causada pode receber outra nomenclatura, como é o caso da podridão vermelha causada por *C. falcatum* em cana-de-açúcar. A antracnose possui sintomatologia típica na maioria dos hospedeiros sendo caracterizada por manchas de coloração castanho escuro a negra com centro deprimido e, em condições ambientais favoráveis pode ocorrer a formação de uma massa de esporos de coloração alaranjada na superfície da lesão

(AMORIM; REZENDE; BERGAMIN FILHO, 2016). Dentre as principais formas de controle para a doença, o controle químico é um dos principais utilizados nos campos de produção.

2.4 O uso de agrotóxicos no controle de doenças

Entre as diversas ferramentas disponíveis para o manejo de doenças encontra-se o uso de fungicidas ou outros produtos químicos equivalentes. Os fungicidas podem ser definidos como compostos químicos empregados no controle de doenças ou capazes de prevenir a infecção dos tecidos vegetais causadas por fungos, bactérias e algas (GARCIA, 1999).

Produtos com propriedades fungicidas são usados na agricultura há muito tempo, porém de forma bastante empírica e, apenas no século XIX houve uma utilização de forma mais comercial e científica dos fungicidas, com o surgimento da calda bordalesa e, posteriormente dos fungicidas orgânicos e sintéticos (LEADBEATER, 2015).

Dentre as principais vantagens para a utilização de fungicidas podemos destacar a facilidade de aplicação, agilidade na resposta do uso, além do menor custo de aplicação a longo prazo. Esses fatores contribuem para a expansão da escolha de fungicidas para controle de doenças no campo (GHINI e KIMATI, 2000).

O Comitê de Ação de Resistência a Fungicidas (FRAC) classifica os fungicidas de acordo com o seu modo de ação, dividindo-os em 56 modos de ação diferentes agrupados em 13 grandes grupos de mecanismo de ação. No ano de 2015 o mercado mundial de fungicidas movimentou aproximadamente 12,1 bilhões de euros, sendo os fungicidas dos grupos dos Inibidores da Biossíntese do Esterol (G) e dos Inibidores da Quinona Externa, mais precisamente os inibidores do Complexo III (C3) os mais comercializados com 50,2% de participação no mercado (HERMANN e STENZEL, 2019).

Os fungicidas inibidores da respiração fúngica possuem amplo espectro de ação, sendo eficientes no controle de Oomicetos e Eumycota (representantes do Reino Fungi). Dentro do grupo dos inibidores da respiração (C), estão inseridos os inibidores do complexo III (citocromo *bc1*-ubiquinol oxidase), entre esses fungicidas estão as estrobilurinas (KUMAR e GUPTA, 2012).

As estrobilurinas possuem grande força comercial mundial e ainda contam com grande potencial de desenvolvimento. São compostos análogos à estrobilurina-A, obtida a partir do cogumelo selvagem *Strobilurus tenacellus* e sua síntese foi iniciada na década de 1990 pela BASF e Syngenta, pois esse metabólito, além de fungitóxico apresenta boa estabilidade à luz e propriedades sistêmicas não fitotóxicas (KUMAR e GUPTA, 2012). Existem mais de dez produtos no mercado, entre eles a Trifloxistrobina.

A sua atividade fungicida resulta da inibição do transporte de elétrons mitocondrial, interrompendo assim o ciclo energético do fungo paralisando a produção de ATP. A trifloxistrobina apresenta movimentação translaminar na planta e, entre seus efeitos pode ser observado a senescência tardia, alteração no ponto de compensação de CO_2, redução da abertura estomática e do consumo de água, bem como melhoria na tolerância do estresse oxidativo (BARTLET et al, 2002).

Os inibidores da biossíntese do ergosterol (SBI) existem no mercado desde a década de 1980. Um dos principais motivos do sucesso da sua utilização como fungicida está na diferença entre os esteróis de fungos e plantas, pois essas moléculas conseguem atingir uma gama de patógenos sem, contudo, afetar as plantas (YANG et al., 2011), sendo o ergosterol, o esterol dominante nos Ascomicetos e Basidiomicetos.

Esse grupo de fungicidas possuem características que o tornam mais ativos e seguros ao ambiente, além de possuir uma parcela considerável de produtos com atividades curativas ou erradicantes eficientes e podem apresentar um risco de desenvolvimento de resistência relativamente baixo (NABI et al., 2017). São divididos em quatro categorias de acordo com o seu sítio de ação, entre eles os inibidores da isomerização, da redução, da transmetilação e da desmetilação do C_{14}, onde estão inseridos o difenoconazole e tebuconazole (FRAC).

Os fungicidas inibidores da desmetilação do C14 (DMI) formam um dos principais grupos de SBI. Atuam inibindo a desmetilação catalisada pelo citocromo P-450 (enzima esterol C-14 desmetilase) afetando diretamente a produção de ergosterol, o principal lipídio componente da membrana celular fúngica (RODRIGUES, 2006).

Alguns estudos sugerem que alguns herbicidas podem atuar como fungicidas ou potencializar os efeitos danosos dos patógenos, a depender do organismo alvo, dosagens e características, entre esses herbicidas avaliados com atividades fungicidas podemos citar o glifosato (EL-SHANSHOURY et al., 1995).

2.5 Herbicidas e suas influências no processo doença

O uso de produtos químicos na agricultura representa um dos principais custos de produção de alimentos e dentre esses produtos temos uma grande utilização de herbicidas para o controle de plantas daninhas. O uso inadequado desses produtos pode afetar, entre outros processos, a dinâmica das doenças nas plantas.

Todos os efeitos dos herbicidas sobre a população microbiana ainda não estão totalmente esclarecidos, embora de maneira geral essa interação pode ser benéfica para o produtor, quando o herbicida atua como fungicida e auxiliando o controle da doença, ou benéfica para o patógeno, aumentando a severidade ou incidência da doença (EL-SHANSHOURY et al., 1995).

Alguns estudos mostram que os herbicidas podem aumentar a colonização de patógenos e a incidência ou severidade da doença e, quando isso ocorre, recebe o nome de doença iatrogênica (KORTEMKAMP, 2011; KAO, CHUNG e HUANG, 2019). Um dos grandes problemas com relação a esses casos é que os herbicidas foram formulados para controle de plantas daninhas e não para afetar patógenos (KAO, CHUNG e HUANG, 2019).

Trabalhos como os realizados por Altman e Campbell (1977), Caulder et al. (1987), Ahmad e Malloch (1995), Johal e Huber (2009) demonstram que após aplicação de herbicidas houve um aumento da severidade da doença causada por alguns patógenos, como antracnose causada por *Colletotrichum gloeosporioides* que teve sua severidade aumentada pelo uso posterior do glifosato (KAO, CHUNG e HUANG, 2019).

O glifosato é um herbicida que atua na rota do ácido chiquímico, um percussor de metabólitos de defesa de plantas e que também está presente nos fungos. Dessa forma, esse herbicida pode afetar tanto a resposta de defesa das plantas, aumentando sua suscetibilidade aos patógenos, como inibir o desenvolvimento fúngico (HAMMERSCHMIDT, 2018).

Alguns estudos sugerem que o glifosato tende a atuar como fungicida, principalmente em doenças de parte aérea, como as ferrugens, e aumentar a severidade de doenças que afetam as raízes ou são disseminadas pelo solo (HAMMERSCHMIDT, 2018). Há também um efeito indesejado pelo uso do herbicida que pode aumentar a produção de inóculo, como verificado por Hirayama et al (2018) em campos de cultivo de morango no Japão. Dessa forma é importante verificar os efeitos do glifosato sobre os patógenos, pois as respostas do produto variam caso a caso.

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CAPÍTULO 1

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Identification and characterization of *Colletotrichum* species associated with *Spigelia anthelmia* in northeastern Brazil

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Abstract: The aim of this study was to identify and characterize *Colletotrichum* species associated with *Spigelia anthelmia* in northeastern Brazil, as well as to verify the sensitivity of these species to three fungicides and the herbicide glyphosat. The isolates were collected in two states in the Northeast of Brazil, with three cities in Alagoas and one city in Sergipe. Through morphological and phylogenetic multilocus analyzes, six species of the *C. gloeosporioides* complex were identified: *C. fragariae, C. fructicola, C. queenslandicum, C. siamense, C. theobromicola* and *C. tropicale*. In pathogenicity tests, all tested isolates caused considerable damage when there was previous injury to the host tissue. The fungicides selected for the sensitivity test were: difenoconazol, tebuconazol and a mixture of tebuconazole + trifloxystrobin in four different dosages: 0.5, 1, 5 and 10 µg a. i. ml^{-1}. All fungicides tested were efficient in controlling fungi *in vitro*, with difenoconazol having the highest percentage of inhibition. Glyphosate was tested at the same concentrations as fungicides, but no inhibitory effect was found in the evaluated isolates. This is the first report of *C. siamense*, *C. queenslandicum*, *C. fragariae*, *C. theobromicola*, *C. fructicola* and *C. tropicale* causing anthracnose in plants of *S. anthelmia*.

Keywords: ApMat region; fungicides sensitivity; Weeds disease; Glyphosate

INTRODUCTION

Spigelia anthelmia L. (Longoniaceae) is an annual herbaceous species native to South America and widely distributed in tropical regions of the world (Hedge, Hebbar and Hedge 2018). It is a species found in humid and deforested areas, being also considered a weed associated with some important crops such as banana (Almeida et al. 2019), corn and rice (Ribeiro, Alves and Pereira 2019), acerola (Sousa et al. 2020), sweet potato (Silva et al. 2017), cassava (Quee et al. 2016) and other crops. For being considered a weed can also serve as a host of pathogens, as *Colletotrichum* (Sousa et al. 2021).

Pathogenic fungi may use weeds as alternative hosts and therefore weeds serve as inoculum source in the field. Among these fungi, the genus *Colletotrichum* has several reports causing anthracnose in several species of weeds, as observed since 1980's by Batista (1984), Raid and Pennypacker (1987), Hirayama et al. (2018) and Karimi, Arzalou and Pertot (2019). Due to the possibility of pathogenic fungi surviving on weeds, knowledge about fungal pathogenic species associated with these plants is important to assist control management in the field. The use of fungicides the main method of controlling the disease.

Using fungicides to control *Colletotrichum* is quite common in the world (Ishii et al. 2016), but resistance to these products can be detrimental to the control of the disease in the field, with cases of resistance in *Colletotrichum* against fugicides being already reported. Chemical groups, such as benzimidazoles (Chung et al. 2006), quinone outside inhibitor (QoI) (Hu et al. 2015) and sterol demethylation-inhibiting (DMI) (Wong and Midland 2007) are reported, although they are still widely used in the management of anthracnose.

Therefore the aim of the present study was to identify and characterize *Colletotrichum* species associated with *S. anthelmia* in the northeastern region in Brazil and to verify the sensitivity of these isolates to three fungicides and the herbicide glyphosate.

MATERIALS AND METHODS

Fungal Isolates and Morphological Studies

From March to December 2017 plants of *Spigelia anthelmia* with symptoms of foliar anthracnose were observed and collected in four cities in two northeastern states: Aracaju (Sergipe) and Rio Largo, Coruripe and Maceio (Alagoas) from experimental crop fields and degraded areas. Symptomatic leaves were collected, and fragments of approximately 5 mm in length were obtained from the transition area between diseased and healthy tissue, fragments were surface sterilized in 70% C2H5OH for 30 seconds and then in 2% NaOCl for 1 min before rinsed two times in sterile distilled water. The tissue fragments dried and four fragments were transferred to Petri dishes containing potato dextrose agar (PDA). The cultures were incubated for seven days at 25°C under 12- h photoperiod.

Single monosporic isolates were obtained from each culture, then stored on PDA medium (Sigma-Aldrich) at 25 °C for further analysis. The isolates were stored in the Culture Collection of UFAL (COUFAL).

Preliminary identification of the isolated fungi was based on morphological characteristics (Sutton 1992). Culture diameter and appearance were analyzed in triplicate after 10 days fungal growth at 25 °C and photoperiod of 12 h. The morphology of the conidia and formation of conidiomata, PDA medium was used, and the cultures were incubated for seven days at 25°C with a photoperiod of 12 hr.

DNA extraction and molecular analysis

To confirm the identification, genomic DNA was extracted from pure cultures grown on PDA. Mycelium was harvested and ground in liquid nitrogen, then submitted to the DNA extraction protocol of Doyle and Doyle (1987). Initially, sequencing the intergenic spacer between the 3' end of the DNA lyase and the mating type locus MAT1-2-1 (APN2/MAT-IGS) amplified and sequenced using primers CgDL_F6 and CgMAT1_F2 (Rojas et al. 2010) for all selected isolates. This marker was used to identify the isolates belonging to the C. gloeosporioides species complex, and isolates representing the range of genetic diversity based on this analysis were selected to amplify an additional three loci: DNA lyase (APN2), β-tubulin (TUB2), and

glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The choice of genes was based on the analysis described by Vieira et al. (2017).

The primers, reagents and PCR amplification conditions for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are detailed in Templeton et al. (1992), APN2/MAT-IGS detailed in Vieira et al. (2017), β-tubulin (TUB2) detailed in O'Donnell and Cigelnik (1997) and DNA lyase detailed by Rojas et al. (2010). PCR products were purified and sequenced by Macrogen (Macrogen, Inc.). Sequences were analyzed visually using the software Staden Package 2.0, and the edited sequences were compared against the GenBank database, National Center for Biotechnological Information (NCBI) (Table 3), using the BLASTn tool. The sequences obtained were aligned with additional sequences retrieved from GenBank using the multiple sequence alignment software Mafft version 7 online and and the alignments were treated using Gblocks Server (version 0.91b).

Bayesian inference concatenated analyses were performed as described by Sousa et al. (2018). The best nucleotide substitution models were selected according to the Akaike information criterion (AIC) and were HKY+G for TUB2, HKY for GAPDH, HKY+I for APN2 and K80+G for APN2/MAT-IGS.

Pathogenicity assays

To confirm pathogenicity isolates representatives of each *Colletotrichum* species was used. The isolates were cultured on PDA for 10 days at 25°C under a 12 h photoperiod to induce sporulation. The inoculum was a conidial water suspension at a concentration of 1 × 106 spores/mL and 20 µl of this suspension were deposited on free of disease leaf surfaces of *S. anthelmia*. Some leaves were previously injured in order to compare the symptoms in leaves with and without injury. After inoculation, leaves were maintained in a moist chamber for 48 h using plastic bags. These bags were then removed, and the leaves were kept at a temperature of 25°C. The experiment was arranged in a completely randomized design with three replicates per isolate. Disease development was observed until seven days after inoculation. Control treatments were constituted of leaves inoculated with 20 µl of sterile distilled water.

Sensitivity to fungicides and glyphosate herbicide

The commercial formulation of difenoconazol (Score, 25% a.i., Syngenta Crop Protection, Paulinia, SP), tebuconazol (Nortox, 20% a.i., Nortox S/A, Arapongas, PR) and a mixture of trifloxistrobin + tebuconazol (Nativo, 10% and 20% a.i., Bayer S.A., São Paulo, SP) were used in the sensitivity to fungicides tests. The fungicides were diluted in DMSO (dimethil sulfoxide) to prepare a stock suspension at a concentration of 10 µg a.i./mL. From this solution, the following concentrations were tested: 0.5; 1. 5 and 10 µg a.i./mL. To test glyphosate (Roundup Original DI, 44.5 and 37% a.i., Monsanto do Brasil LTDA., São Jose dos Campos, SP) we used the concentrations and methodology used in fungicides' tests.

Mycelial plugs (6 mm in diameter) from isolates were removed using a sterile plastic straw from 7-day-old cultures grown on potato dextrose agar (PDA) in Petri dishes at 25 ºC temperature under continuous darkness. Each PDA plate received one mycelial plug that was placed upside down so that fungal mycelia had direct contact with the surface of the medium. Plates were sealed and incubated at 25 ºC temperature under continuous darkness for 7 days. Two radial measurements of mycelial growth were taken from each colony. Three plates per isolate were used.

The evaluation of fungitoxicity in vitro was performed through the percentage of mycelial growth inhibition (PMGI), comparing the average diameter, in cm, between the colonies in treatments with fungicide and the control, after seven days of incubation as proposed by Edgington et al. (1971). The resulting graphic was designed using the software Sigmaplot version 11.0. and the statistical analysis using the software Sisvar version 5.6.

RESULTS AND DISCUSSION

A total of 25 isolates were collected from *S. anthelmia* with symptoms of foliar anthracnose. Some representative isolates were selected for sequencing of the ApMat region (APN2/MAT-IGS) for a preliminary assessment of the species, once it is a highly reliable marker for determining the species of *C. gloeosporioides* complex. From the previous analysis, the following species were identified: *C. fragariae, C. queenslandicum, C. fructicola, C. tropicale, C. theobromicola* and *C. siamense* (Table 1). The final identification of isolates was confirmed by phylogenetic analysis based on the concatenation of DNA lyase, β-tubulin, glyceraldehyde-3-phosphate dehydrogenase and the ApMat.

Table 1. Origin and distribution of collected isolates

The choice of the ApMat region for prior identification of the isolates of the *C. gloeosporioides* complex is supported by studies such as the one performed by Vieira et al. (2017) and Vieira et al. (2020), which proved the support for species differentiation provided by this region. Sharma, Pinnaka and Shenoy (2015), argue that the use of this marker is indicated to differentiate species of the *C. gloeosporioides* complex. The other markers chosen showed the best phylogenetic signals for this complex (Figure 1).

Fig 1 Phylogenetic tree based on alignment of nucleotides sequences of genes DNA lyase, TUB2, GAPDH and ApMat region using Bayesian analysis.

Colonies growing on PDA, after 10 days, showed characteristics consistent with previous descriptions by other authors (Figure 2). The morphological and cultural characteristics of the isolates are showed in Table 2.

Fig 2 Colonies of *Colletotrichum* isolates on PDA, 15-day-old (a) *C. fragariae* from above; (b) *C. fragariae* from below; (c) *C. queenslandicum* from above; (d) *C. queenslandicum* from below; (e) *C. siamense* from above; (f) *C. siamense* from below; (g) *C. fructicola* from above; (h) *C. fructicola* from below.

Table 2 Morphological and cultural characteristics of *C. gloeosporioides* complex isolates.

The *C. gloeosporioides* complex has already been identified causing diseases in several cultures and contains approximately 22 species. Among these species: is *C. fragariae*, initially associated with anthracnose in strawberries and considered, in some studies, to be synonymous with *C. theobromicola* (Weir, Johnson and Damm 2012). Due to the support observed by the phylogenetic tree and its differentiation, in this work we considered two distinct species. *C. theobromicola* showed a light brown color on the back and a thick white aerial mycelium on the upper part, with an orange spore mass formation (Weir, Johnson and Damm 2012), having already been reported in northeastern Brazil in *Annona* spp. (Costa et al. 2019), carnauba palm (Araujo et al. 2018), and cultivated and wild cassava (Oliveira et al. 2018). *C. fructicola* initially reported causing anthracnose in coffee in Thailand and quite diverse (Weir, Johnson and Damm 2012). In Brazil, it has been observed in *Annona* (Costa et al. 2019), *Licania tomentosa* (Lisboa et al. 2018), cactos prickly pear (Oliveira et al. 2018), lima bean (Sousa et al. 2018) and mango (Lima et al. 2015).

The same morphological characteristics obtained by Rojas et al. (2010) were observed in this work for *C. tropicale*, being a species widely observed in tropical forests and affecting crops such as mango, where it has been reported causing anthracnose (Lima et al. 2015) and as endophytic (Vieira et al. 2014), also causing disease in *Capsicum* spp. (Silva et al. 2017), *Annona* (Costa et al. 2019), and carnauba palm (Araujo et al. 2018) in northeastern Brazil. *C. queenslandicum* received this name due to the place of collection of its isolates, Queensland, Australia. The morphological characteristics of the culture were compatible with those observed by Weir, Johsnon and Damm (2012), with the formation of an orange mass of hyaline, cylindrical and rounded ends. They have been reported in several cultures in Australia (James et al. 2014; Silva et al. 2017; Shivas et al.

2018), having reports in Brazil causing anthracnose in tree species such as *Licania tomentosa* (Lisboa et al. 2018), cashew (Veloso et al. 2018) and other trees.

Found in different regions of the planet and associated with a wide range of hosts, *C. siamense* is considered geographically and biologically diverse (Weir, Johnson and Damm 2012), some of the reported hosts for this species are strawberry (Capobiango et al. 2016), *Capsicum* (Silva et al. 2017), cactos prickly pear (Oliveira et al. 2018), and cassava (Oliveira et al. 2018). In the pathogenicity test, all isolates were pathogenic only when the leaves had previous injuries. In treatments performed without injury, no isolate was able to exert pathogenicity. This indicates that these fungal isolates are not able to actively penetrate the host's tissue.

Regarding the sensitivity of the fungicides to the selected isolates (Figure 3), *C. queenslandicum* was inhibited by the three fungicides at all concentrations evaluated, with inhibition rates above 50%. The fungicide difeconazole was the most efficient to control *C. fructicola* and *C. fragariae*, while in *C. siamense* difenoconazol only differed from the others at a concentration of 10 µg a. i. mL-1. Despite these differences between the tested products we can confirm that the selected fungicides inhibit the growth of these *Colletotrichum* isolates obtained from *S. anthelmia*.

Fig. 3 Graph showing the results of the sensitivity of *Colletotrichum* isolates to different fungicides and different dosages. Where TB - tebuconazole, DF - difenoconazil, TX + TB - trifluoxystrobin + tebuconazole and the numbers next to the fungicide acronyms represent the dosages

Difenoconazole and Tebuconazole are fungicides that inhibit ergosterol biosynthesis, due to this characteristic they are more active and safer to the environment, in addition to low risk of developing resistance (Nabi et al. 2017). Although sensitivity levels may vary between the two fungicides, as observed by Wang et al. (2019), when *Colletotrichum* isolates obtained from nuts were more sensitive to difeconazole when compared to tebuconazole and by He et al. (2019), with isolates obtained from strawberry, these results are similar to those obtained in the present study for some of the species studied.

Strobilurins, such as trifloxystrobin are compounds similar to strobilurin-A, obtained from the wild mushroom *Strobilurus tenacellus*, and in addition to fungitoxics, they have good light stability and non-phytotoxic systemic properties (Kumar and Gupta 2012). Despite its relative efficiency in controlling *Colletotrichum*, it was less efficient than the fungicides tebuconazol and difenoconazol.

The herbicide glyphosate did not have any negative effect on the evaluated isolates, the growth rates were similar to the controls for all concentrations tested. However, there the formation of spore masses occured in several isolates. The effects of glyphosate in *Colletotrichum* isolates are varied. It is expected that fungi will be inhibited due to the mode of action of this herbicide, although it was not observed in this study. Hirayama et al. (2018), observed an increase in the production of inoculants and a relationship between the presence of infected weeds and the presence of the disease in the next production cycle. The increase in production of *Colletotrichum* inocula caused by the use of glyphosate was also observed by Kao, Chung and Huang (2019).

To our knowledge, this is the first report of *C. siamense, C. queenslandicum, C. fragariae, C. theobromicola, C. fructicola* and *C. tropicale* causing anthracnose in plants of S. anthelmia. The herbicide glyphosate had negative effect on these isolates and all tested fungicides were effective in controlling the mycelial growth, indicating the use of these products may be useful to control these pathogens in cultures that can serve as hosts.

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Table 1 Origin and distribution of collected isolates

Specie	Isolates Collected	Origin
C. fragariae	1	Rio Largo, AL
C. fructicola	1	Aracaju, SE
C. theobromicola	1	Rio Largo, AL
C. siamense	2	Aracaju, SE
	7	Rio Largo, AL
C. queenslandicum	3	Aracaju, SE
	2	Coruripe, AL
C. tropicale	6	Aracaju, SE
	2	Maceió, AL

Table 2 The morphological and cultural characteristics of the isolates of *C. gloeosporioides* complex

Specie	Colony Color	Mycelial Growth Rate (mm/day)	Conidia Format	Conidia Size (μm)
C. fragariae	gray on the top and white on the bottom	9.26	tapered conidium with rounded tips	5.54 (3.72 – 8.59) x 16.28 (11.92 – 25.18)
C. fructicola	non-cotton white with gray on both sides	8.45	rod-shaped with a tapered and a rounded end	5.08 (4.00 – 6.21) x 18.60 (15.32 – 21.97)
C. theobromicola	white cottony aerial hyphae that later became brown	4.4	straight to cylindrical	4.05 (3.5 – 6.5) x 15.50 (7.5 – 25.5)
C. queenslandicum	light gray on the top with orange mucilage and white on the obverse	8.60	Rod-shaped	6.50 (3.26-7.57) x 17.28 (11.46 – 20.80)
C. siamense	white with cottony aerial mycelium on the upper edges and gray on the lower	9.08	Rod-shaped	5.05 (3.61 – 7.27) x 18.21 (14.84 – 20.31)
C. tropicale	White cottony aerial hyphae that later became gray	7,50	Cylindrical	5,01 (4,78 – 6,05) x 17,54 (14,85 – 21,12)

Table 3 Isolates of Colletotrichum spp. used in this study with GenBank accession numbers.

Species	Culture[1]	Host	Country	GenBank accession number[2]			
				APN2	APN2/MAT-IGS	GAPDH	TUB2
C. aenigma	ICMP 18608*	*Persea americana*	Israel	-	KM360143	JX010044	JX010389
C. aeschynomenes	ICMP 17673, ATCC 201874*	*Aeschynomene virginica*	USA	-	KM360145	JX009930	JX010392
C. alienum	ICMP 12071*	*Malus domestica*	New Zealand	-	KC888927	JX010028	JX010411
C. asianum	ICMP 18580*, B26CBS 130418	*Coffea arabica*	Thailand	-	FR718814	JX010053	JX010406
C. chrysophilum	CMM 4268*, URM 7362	*Musa* sp.	Brazil	KX094018	KX094325	KX094183	KX094285
C. chrysophilum (*C. ignotum*)	8395	*Theobroma cacao*	Panama	GU994415	GU994444	KX094176	GU994473
C. chrysophilum (*C. ignotum*)	E183	*Genipa americana*	Panama	GU994414	GU994443	KX094178	GU994472
C. communis	GO01	*Citrus* sp.	India	-	KC790720	KF452016	KF452029
C. communis	MU1	*Mangifera indica*	India	-	JQ894582	JQ894632	JQ894602
C. dianesei	CMM 4083	*Mangifera indica*	Brazil	KX093995	KX094304	KX094156	KX094268
C. dianesei	CMM 4084	*Mangifera indica*	Brazil	KX093996	KX094305	KX094157	KX094269
C. dianesei	CMM 4085*	*Mangifera indica*	Brazil	KX093997	KX094306	KX094158	KX094270
C. endomangiferae	CMM 3740	*Mangifera indica*	Brazil	-	KJ155452	KC702954	KM404169
C. endomangiferae	CMM 3814*	*Mangifera indica*	Brazil	-	KJ155453	KC702955	KM404170
C. fragariae	Bra5	*Coffea* sp.	Brazil	-	FR718801	-	FR719885
C. fragariae	Bra8	*Coffea* sp.	Brazil	-	FR718802	-	FR719886
C. fragariae	MTCC 10325				JQ807844		JQ071917
C. fructicola	1087	*Theobroma cacao*	Panama	GU994409	GU994438	KX094174	KX094279
C. fructicola	3589	*Theobroma cacao*	Panama	GU994411	GU994440	KX094175	KX094280
C. fructicola	Coll1126	*Vaccinium macrocarpon*	USA	JX145239	JX145315	-	JX145187
C. fructicola	Coll919	*Rhexia virginica*	USA	JX145269	JX145324	-	JX145219
C. ignotum	E886	*Vaccinium corymbosum*	USA	GU994412	GU994441	-	GU994470
C. fructicola	GM567	*Mangifera indica*	India	-	JQ894576	JQ894630	JQ894600

C. fructicola	ICMP 18581*, CBS 130416, LC0033	*Coffea arabica*	Thailand	-	JQ807838	JX010033	JX010405
C. gloeosporioides	IMI 356878*, ICMP 17821, CBS 112999, MTCC 10323	*Citrus sinensi*	Italy	GU994416	JQ807843	JX010056	JX010445
C. gloeosporioides	LF318	*Camelia sinensis*	China		KJ954541	KJ954828	KJ955275
C. gloeosporioides	LF534	*Camelia sinensis*	China		KJ954569	KJ954859	KJ955305
C. horii	ICMP 10492*	*Diospyros kaki*	Japan	-	JQ807840	GQ329681	JX010450
C. hymenocallidis	MTCC 10286	*Hymenocallis americana*	China	-	JQ807842	JX010019	JX010410
C. jasmini-sambac	ICMP 19118*	*Jasminum sambac*	Vietnam	-	JQ807841	HM131497	JX010415
C. melanocaulon	Coll126	*Vaccinium macrocarpon*	USA	JX145245	JX145309	KX094186	KX094289
C. melanocaulon	CBS133251 Coll131	*Vaccinium macrocarpon*	USA	JX145247	JX145313	KX094187	KX094290
C. musae	CBS 116870*, ICMP 19119	*Musa* sp.	USA	-	KC888926	JX010050	HQ596280
C. musae	CMM 4423	*Musa* sp.	Brazil	KX094010	KX094328	KX094195	KX094294
C. nupharicola	CBS 470.96*, ICMP 18187	*Nuphar lutea subsp. polysepala*	USA	JX145275	JX145319	JX009936	JX010397
C. nupharicola	CBS 472.96, ICMP 17940	*Nymphaea odorata*	USA	JX145276	JX145320	JX010031	JX010399
C. queenslandicum	ICMP 1778*	*Carica papaya*	Australia	-	KC888928	JX009934	JX010414
C. salsolae	ICMP 19051*	*Salsola tragus*	Hungary	-	KC888925	JX009916	JX010403
C. siamense	ICMP 18578*, CBS 130417, LC0034	*Coffea arabica*	Thailand	-	JQ899289	JX009924	JX010404
C. theobromicola	MTCC 11350 ICMP 18649	*Theobroma cacao*	Panama	GU994419	KC790726	JX010006	JX010447
C. theobromicola	GJS 08.43	*Theobroma cacao*	Panama	GU994418	GU994447	-	GU994476
C. theobromicola	GJS 08.48	*Theobroma cacao*	Panama	GU994417	GU994446		GU994475
C. tropicale	CBS 124949*, ICMP 18653, 5101	*Theobroma cacao*	Panama	GU994396	GU994425	JX010007	GU994454
C. tropicale	CMM 3767	*Mangifera indica*	Brazil	-	KJ155464	KC702960	KC992345

C. tropicale	CMM 3780	*Mangifera indica*	Brazil	-	KJ155467	KC702961	KC992343
C. tropicale	Coll B80918	*Terpsichore taxifolia*	Puerto Rico	JX145264	JX145307	-	JX145214
Colletotrichum sp. (*C. siamense s. l.*)	7767	*Theobroma cacao*	Panama	GU994403	GU994432	-	GU994461
Colletotrichum sp. (*C. siamense s. l.*)	CMM 4247	*Musa sp.*	Brazil	KX094009	KX094301	KX094155	KX094261
Colletotrichum sp. (*C. siamense s. l.*)	GJS 0852	*Theobroma cacao*	Panama	GU994404	GU994433	-	GU994462
Colletotrichum sp. (*C. siamense s. l.*)	GN00	*Azadiractha indica*	India	-	KC790673	KC790735	KC790868
Colletotrichum grevillae	CBS 132879	*Grevillae* sp.	Italy	-	-	KC297010	KC297102
C. tropicale	COUFAL 0284	*Spigelia anthelmia*	Brazil				
C. tropicale	COUFAL 0285	*Spigelia anthelmia*	Brazil				
C. tropicale	COUFAL 0286	*Spigelia anthelmia*	Brazil				
C. siamense	COUFAL 0287	*Spigelia anthelmia*	Brazil				
C. siamense	COUFAL 0288	*Spigelia anthelmia*	Brazil				
C. siamense	COUFAL 0289	*Spigelia anthelmia*	Brazil				
C. queenslandicum	COUFAL 0290	*Spigelia anthelmia*	Brazil				
C. queenslandicum	COUFAL 0291	*Spigelia anthelmia*	Brazil				
C. queenslandicum	COUFAL 0292	*Spigelia anthelmia*	Brazil				
C. queenslandicum	COUFAL 0293	*Spigelia anthelmia*	Brazil				
C. fructicola	COUFAL 0294	*Spigelia anthelmia*	Brazil				
C. fragariae	COUFAL 0295	*Spigelia anthelmia*	Brazil				
C. theobromicola	COUFAL 0296	*Spigelia anthelmia*	Brazil				

Fig. 1 Phylogenetic tree based on alignment of nucleotides sequences of genes DNA lyase, TUB2, GAPDH and ApMat region using Bayesian analysis.

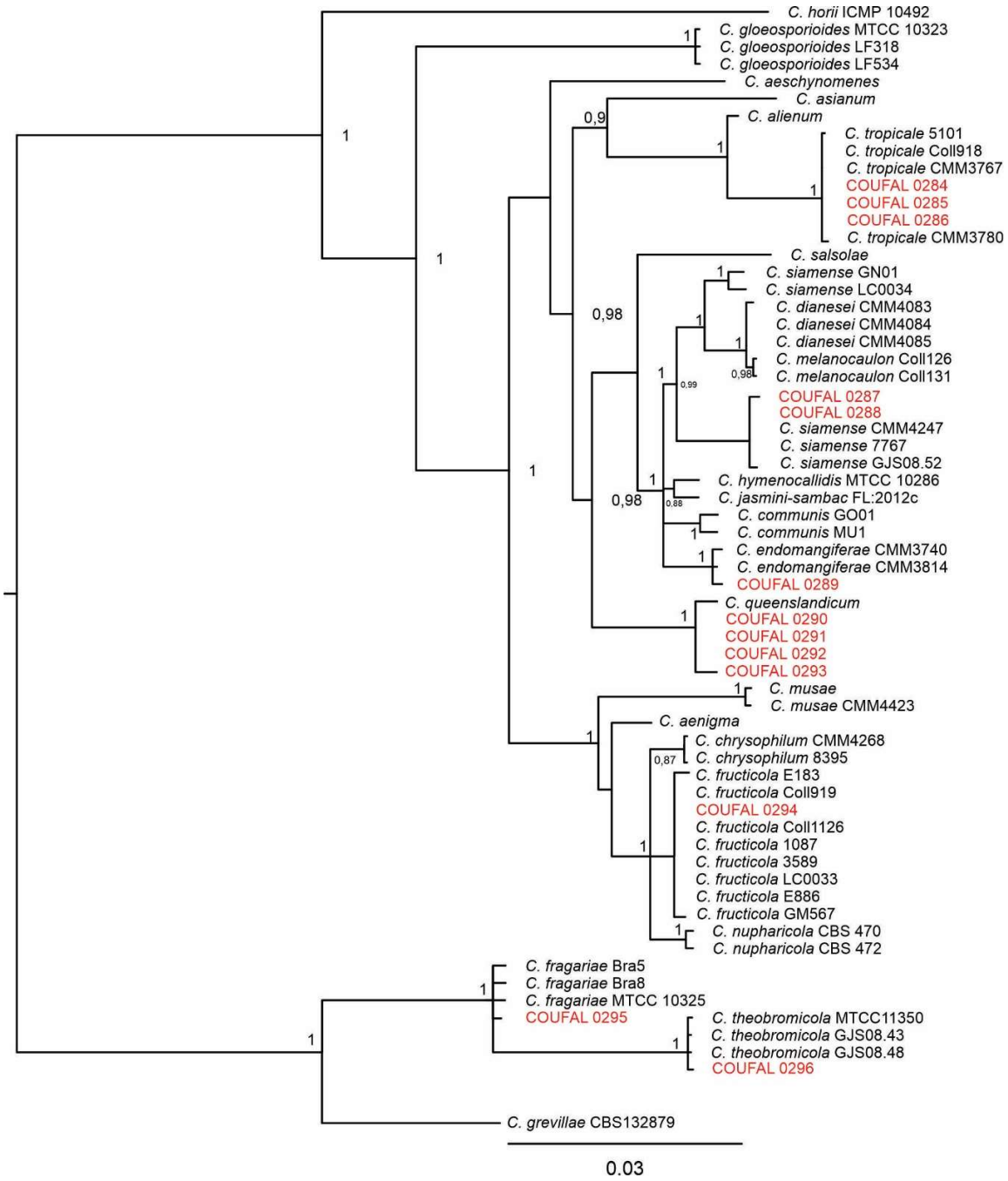


Fig. 2 Colonies of *Colletotrichum* isolates on PDA, 15-day-old (a) *C. fragariae* from above; (b) *C. fragariae* from below; (c) *C. queenslandicum* from above; (d) *C. queenslandicum* from below; (e) *C. siamense* from above; (f) *C. siamense* from below; (g) *C. fructicola* from above; (h) *C. fructicola* from below.

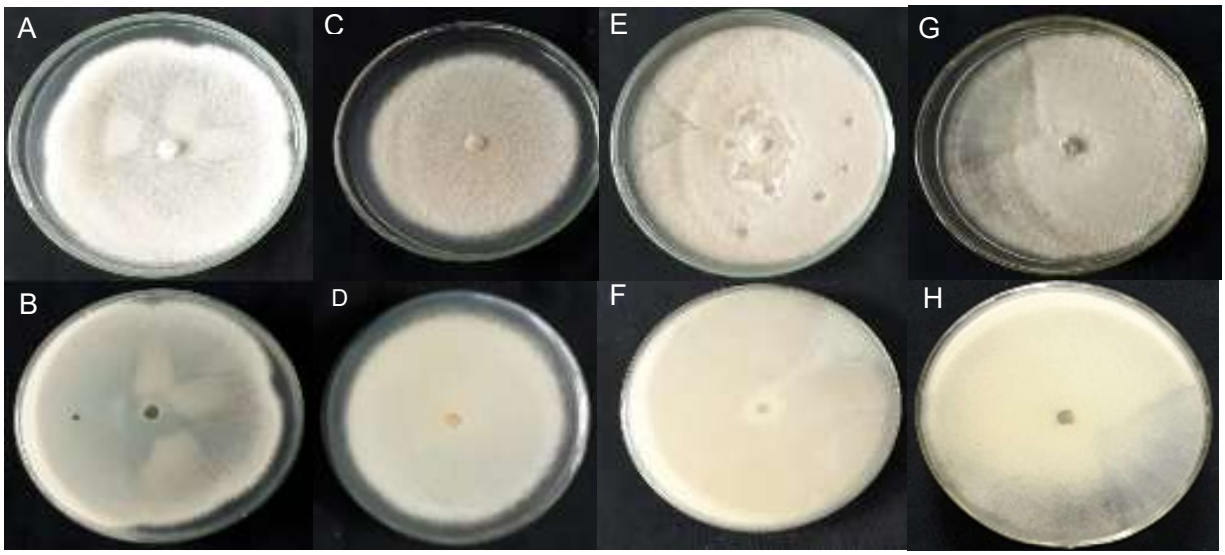
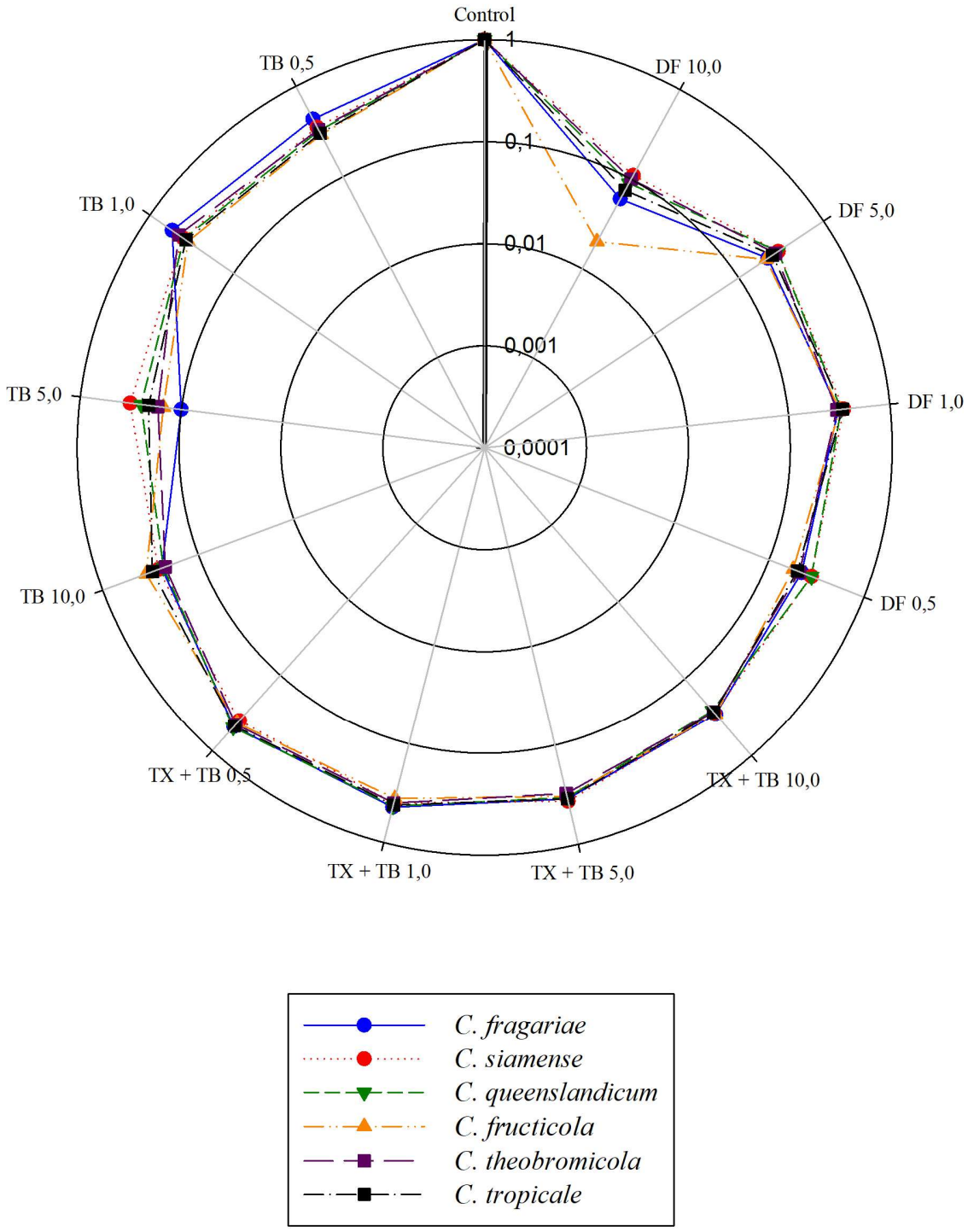


Fig. 3 Graph showing the results of the sensitivity of *Colletotrichum* isolates to different fungicides and different dosages. Where TB - tebuconazole, DF - difenoconazil, TX + TB - trifluoxystrobin + tebuconazole and the numbers next to the fungicide acronyms represent the dosages



CAPÍTULO 2

Revista: Crop Protection

Colletotrichum truncatum causing anthracnose in weeds in Brazil

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Abstract

Weeds can serve as alternative hosts for several plant pathogens, including the *Colletotrichum* genus. The aim of this study was to identify, based on the morphology and multilocus analysis isolated from *C. truncatum* associated with weeds. Leaves with typical symptoms of anthracnose were collected from three plant species: *Euphorbia hirta* L., *Sida* sp. and *Spigelia anthelmia* L. in experimental plantations in Rio Largo, Brazil. After the morphological characterization of the isolates, a multilocus analysis was performed using the genes Actin (ACT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the ITS region (ITS) in order to confirm the species of the selected isolates. The nine isolates selected for the study had curved conidia and the characteristic colonies presented by *C. truncatum*, and later identification was confirmed through phylogenetic analysis. All isolates were pathogenic to their original hosts, with tissue previous injury. To our knowledge, this is the first report of *C. truncatum* causing anthracnose in *Spigelia anthelmia* and *Sida* sp. at the world and the first observation of this pathogen causing disease in *E. hirta* in Brazil.

Keywords: Multilocus analysis; Alternative hosts; *Euphorbia hirta*; *Sida*; *Spigelia anthelmia*.

INTRODUCTION

According to Zimdahl (2018), weeds are plants that grow in unwished places and interfere with human activities causing damage when they are not adequately controlled. Among their negative influences we can mention their ability to behave as alternative hosts for plant pathogens. Studies such as those developed by Hirayama et al. (2018), Karimi et al. (2019), Shiragane, Usami and Shishido (2019), Gobatto et al. (2019), Aguiar et al. (2018), Webster et al. (2019), indicate weeds can serve as inoculum source for various pathogens such as fungi, viruses, bacteria and nematodes.

The *Colletotrichum* genus covers a wide number of species, divided into 14 complexes and due to their wide range of reported hosts, was considered one of the 10 most important fungal pathogen species in the world (Damm et al., 2019; Cannon et al., 2012), being the main causal agent of a plant diseases commonly known as anthracnose. The *C. truncatum* is a cosmopolitan species and widely distributed in the world, affecting many agriculturally important crops such as peppers, papaya fruit, *Capsicum*, rubber tree, cowpea, tomato, soybean (Rananthunge and Sandani, 2016; Vieira et al., 2020; Ramdial, De Abreu, Rampersad, 2017; Silva et al., 2017; Herath, Manamgoda e Udayanga, 2019), in addition to being reported in *Euphorbia hirta* as endophytic (Souza et al., 2017).

Due to the importance of weeds serving as a source of inoculum and the wide host range of *C. truncatum*, in this study we aimed to identify based on morphological and phylogenetic approaches, as well as identify its pathogenicity on weeds in northeastern Brazil.

MATERIALS AND METHODS

Fungal Isolates and Morphological Studies

From August 2016 to December 2017 plants of *Euphorbia hirta* L., *Sida* sp. and *Spigelia anthelmia* L. with symptoms of foliar anthracnose were observed in the experimental area of the CECA/UFAL, Rio Largo, Alagoas, Brazil. Symptomatic leaves were collected, and fragments of approximately 5 mm in length were obtained from the transition area between the diseased and healthy tissue, fragments were surface sterilized in 70% ethanol for 30 seconds and in 2% sodium hypochlorite for 1 min before rinsed two times in sterile distilled water. The tissues dried and four fragments were transferred to Petri dishes containing potato dextrose agar (PDA). The cultures were incubated for seven days at 25°C under 12 hr photoperiod.

Single monosporic isolates were obtained from each culture, then stored in PDA medium (Sigma-Aldrich) at 25 °C for further analysis. The isolates were stored in the Culture Collection of UFAL (COUFAL) under registration code COUFAL 0018, COUFAL 0297, COUFAL 0298 and COUFAL 0299.

Preliminary identification of the isolated fungi was based on morphological characteristics (Sutton 1992). Culture diameter and appearance were analyzed in triplicate after 10 days fungal growth at 25 °C and photoperiod of 12 h. The morphology of the conidia and formation of conidiomata, PDA medium was used, and the cultures were incubated for seven days at 25°C with a photoperiod of 12 hr.

DNA extraction and molecular analysis

To confirm the identification, genomic DNA was extracted with the protocol of Doyle and Doyle (1987). The internal transcribed spacer region (ITS) was amplified with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), actin (ACT) with ACT-512F (5′-ATGTGCAAGGCCGGTTTCGC-3′) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with GDF (5′-GCCGTCAACGACCCCTTCATTGA-3′) and GDR (5′-GGGTGGAGT CGTACTTGAGCATGT-3′).

The reagents and PCR amplification conditions for ITS and ACT are detailed in Liu et al. (2015) and GAPDH detailed in Templeton et al. (1992). PCR products were purified and sequenced by Macrogen (Macrogen, Inc.). Sequences were analyzed visually through the software Staden Package 2.0, and the edited sequences were compared against the GenBank database, National Center for Biotechnological Information (NCBI), using the BLASTn tool. The sequences obtained were aligned with additional sequences retrieved from GenBank using the multiple sequence alignment software Mafft version 7.

Bayesian inference individual and concatenated analyses were performed as described by Sousa et al. (2018). The best nucleotide substitution models, selected according to the Akaike information criterion (AIC), were SYM+I+G for ITS and HKY+G for ACT and GAPDH.

Pathogenicity assays

To confirm pathogenicity the isolates was cultured in PDA for 10 days at 25°C under a 12 hr photoperiod to induce sporulation. The inoculum was constituted by conidial suspension in water at a concentration of 1×10^6 spores/mL and 20 µl of this suspension

were deposited on the leaf surface for each weed tested (*Sida*, *Spigelia* and *Euphorbia*) previously injury with no previous symptoms of the disease. After inoculation, the leaves were placed in plastic containers, each container was lined with paper layers wetted in distilled water to maintain humidity for 48h at 25 ºC. Each leave was set on a sterilized Petri dish to avoid direct contact with water. The plastic containers were partially sealed with plastic bags, and leaves were kept at the same temperature, 25 ºC. The experiment was set in a completely randomized design with three replicates per isolate. Disease development was observed until five days after inoculation. The control leaves 20 µl of sterile distilled water were deposited in the leaf surface.

RESULTS

A total of 108 isolates were collected from different weed species. Nine isolates were identified based on morphological characteristics compatible with *C. truncatum* (Table 1). Four isolates representatives from each weed species were used for morphological and molecular analysis.

Table 1. List of isolates collected from *C. truncatum*, hosts and collection site.

The colonies on PDA showed greenish grey felt-like mycelium with white sectors in aerial view and the reverse was greyish sepia to fuscous black with buff sectors (Figure 1) with a grow rate of 3.5 mm/day. The conidia were hyalin, smooth-walled, falcate, with no septa, and had an average size (50 per isolate) of 11.13 to 15.98 µm long and 1.66 to 2.30 µm wide (Figure 1).

Fig 1 Colonies of *C. truncatum* associated with weeds: from *S. anthelmia* (a and b), *Sida* sp. (c and d), *E. hirta* (e and f)

The identification of isolates was confirmed by phylogenetic analysis based on the multilocus analysis of the ITS, ACT and GAPDH. All isolates were grouped with the isolates of *C. truncatum* and all clades showed high support (Bayesian posterior probability > 0.98; Figure 2).

Fig 2 Phylogenetic tree based on alignment of nucleotides sequences of genes ACT, GAPDH and ITS region using Bayesian analysis

All three isolates caused necrotic lesions that appeared seven days after inoculation. The symptoms in all inoculated leaves were consistent with those observed in the naturally infected plants, whereas the control plants remained healthy. The inoculated isolates were reisolated from the lesions, confirming Koch's postulates (Figure 3).

Fig 3 Pathogenicity test in the original hosts. (a) *S. anthelmia* no injury; (b) *S. anthelmia* with injury; (c) *E. hirta* no injury; (d) *E. hirta* with injury

DISCUSSION

The morphological characteristics of *Colletotrichum* may differ depending on the environmental conditions and the culture medium used. The size of conidia obtained in this study were smaller than those observed by Vieira et al. (2020) using PDA and those by Aktaruzzaman et al. (2018) with SNA. conidia showed to have larger sizes when developed in SNA medium than in PDA.

The color of the colonies can also be affected by differences in temperature and brightness. We observed, in a preliminary test, that the color and the mycelial growth rate differed in terms of luminosity. When submitted to 24h of darkness, growth was delayed and the colony would acquire an orange color, while in a 12h photoperiod the color had characteristics similar to those observed by Vieira et al. (2020), when submitted to a temperature of 28 °C and continuous luminosity. The culture medium used also changes color and growth rates, as noted by Aktaruzzaman et al (2018) and Damm et al (2009).

C. truncatum has a high range of hosts, and has already been reported in cultivated plants and in post-harvest fruits such as papaya (Vieira et al., 2020; Aktaruzzaman et al. 2018), pepper (Katoch, Prabhakar and Sharma 2016), Chinese cabbage (He et al. 2016), *Jatropha* (Torrez-Calzada et al. 2018) among other cultures. In addition to being reported as an endophyte in *E. hirta* in Brazil (Souza et al. 2017). The presence of infected weeds in a given area can significantly affect the production cycle, as observed by Hirayama et al. (2018), giving importance to the observation of pathogens in weeds, as they are known to act as alternative hosts or source of inoculum for the cultures around them.

To our knowledge, this is the first report of *C. truncatum* causing anthracnose in *Spigelia anthelmia* and *Sida* sp. in the world and the first observation of this pathogen causing disease in *E. hirta* in Brazil.

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Table 1. List of isolates collected from *C. truncatum*, hosts and collection site.

Host	Number of Isolates	Collection place
Sida sp.	2	Rio Largo, AL
Euphorbia hirta	6	Rio Largo, AL
Spigelia anthelmia	1	Rio Largo, AL

Fig 1 Colonies of *C. truncatum* associated with weeds: from *S. anthelmia* (a and b), *Sida* sp. (c and d), *E. hirta* (e and f)

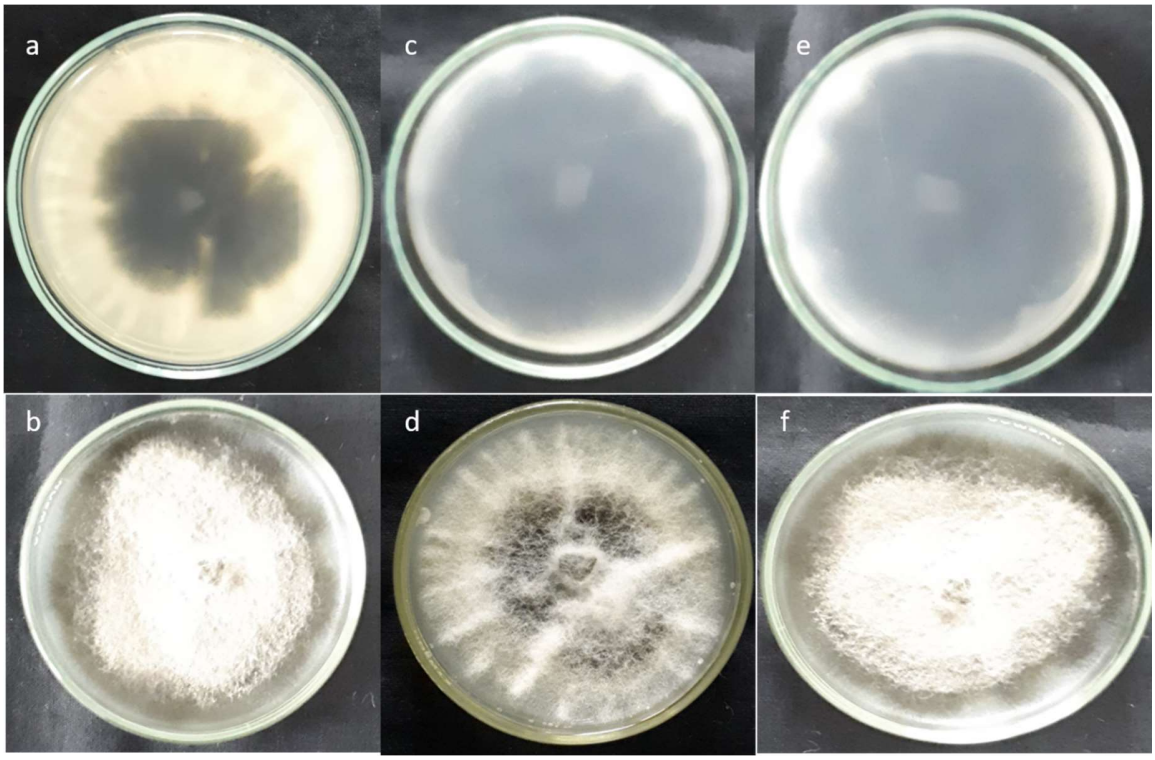

Fig 2 Phylogenetic tree based on alignment of nucleotides sequences of genes ACT, GAPDH and ITS region using Bayesian analysis

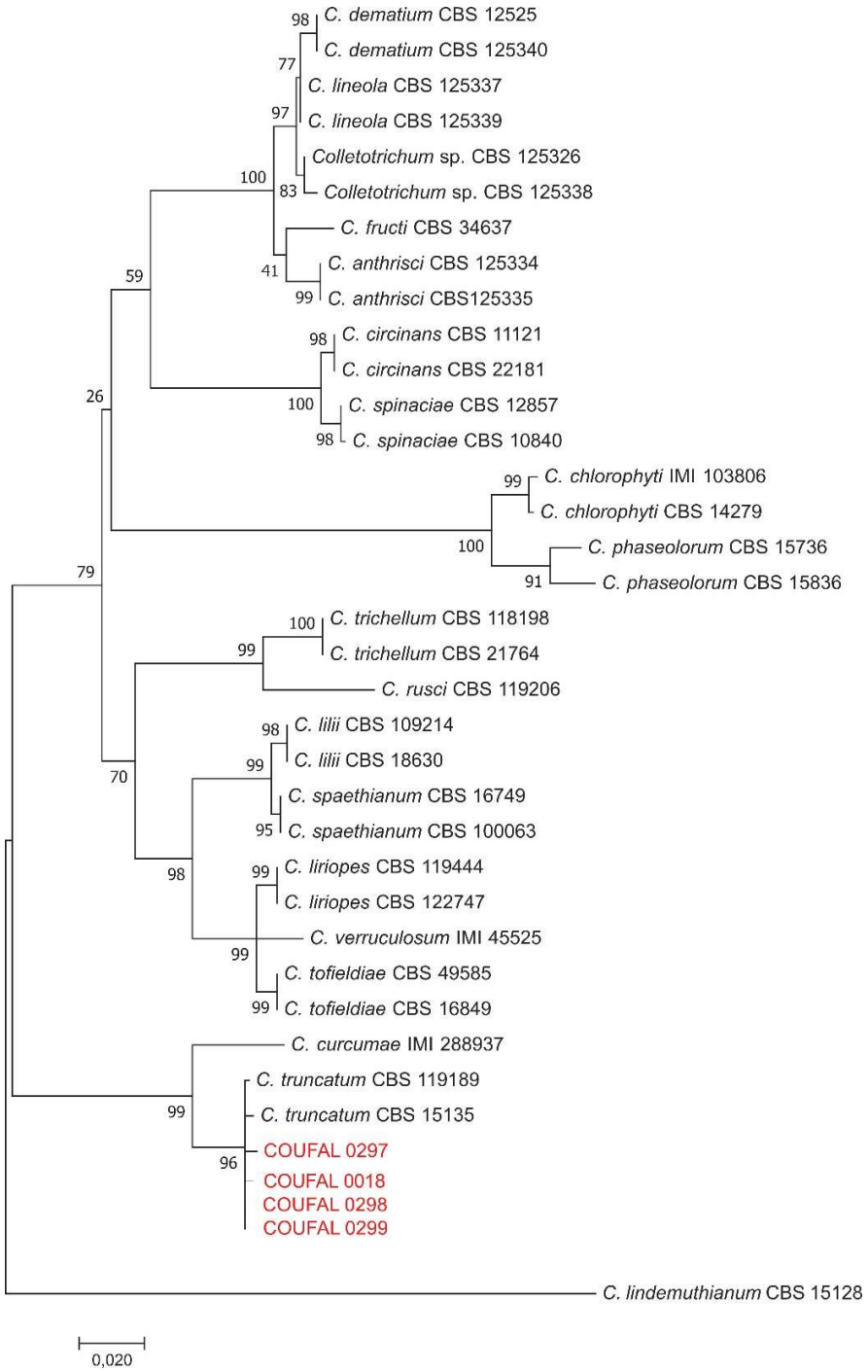

Fig 3 Pathogenicity test in the original hosts. (a) *S. anthelmia* no injury; (b) *S. anthelmia* with injury;

Supply Material. Isolates of *Colletotrichum* spp. used in this study with GenBank accession numbers.

Specie	Strain	Genbank acession numbers		
		ITS	GAPDH	Actin
C. anthrisci	CBS: 125334	GU227845.1	GU228237.1	GU227943

C. anthrisci	CBS: 125335	GU227846.1	GU228238.1	GU227944.1
C. chlorophyti	IMI: 103806	GU227894.1	GU228286.1	GU227992
C. chlorophyti	CBS: 142.79	GU227895.1	GU228287	GU227993
C. circinans	CBS: 111.21	GU227854.1	GU228246.1	GU227952
C. circinans	CBS: 221.81	GU227855.1	GU228247	GU227953
C. curcumae	IMI: 288937	GU227893.1	GU228285.1	GU227991
C. dematium	CBS: 125.25	GU227819.1	GU228211	GU227917
C. dematium	CBS:125340	GU227820	GU228212	GU227918
C. lineola	CBS:125337	GU227829	GU228221	GU227927
C. lineola	CBS:125339	GU227830	GU228222	GU227928
Colletotrichum sp.	CBS:125326	GU227827	GU228219_1_	GU227925
Colletotrichum sp.	CBS:125338	GU227828	GU228220	GU227926
C. spinaciae	CBS:128.57	GU227847	GU228239	GU227945
C. spinaciae	CBS:108.40	GU227848	GU228240	GU227946
C. fructi	CBS:346.37	GU227844	GU228236	GU227942
C. lilii	CBS:109214	GU227810	GU228202	GU227908
C. lilii	CBS:186.30	GU227811	GU228203	GU227909
C. spaethianum	CBS:167.49	GU227807	GU228199	GU227905
C.spaethianum	CBS:100063	GU227808	GU228200	GU227906
C. liriopes	CBS:119444	GU227804	GU228196	GU227902
C. liriopes	CBS:122747	GU227805	GU228197	GU227903
C. verruculosum	IMI:45525	GU227806	GU228198	GU227904
C. tofieldiae	CBS:495.85	GU227801	GU228193	GU227899
C. tofieldiae	CBS:168.49	GU227802	GU228194	GU227900
C. rusci	CBS:119206	GU227818	GU228210	GU227916
C. trichellum	CBS:118198	GU227813	GU228205	GU227911
C. trichellum	CBS:217.64	GU227812	GU228204	GU227910

C. phaseolorum	CBS:157.36	GU227896	GU228288	GU227994
C. phaseolorum	CBS:158.36	GU227897	GU228289	GU227995
C. truncatum	CBS:151.35	GU227862	GU228254	GU227960
C. truncatum	CBS:119189	GU227863	GU228255	GU227961
C. lindemuthianum	CBS:151.28	GU227800	GU228192	GU227898
COUFAL 0297				
COUFAL 0018				
COUFAL 0298				
COUFAL 0299				