UNIVERSIDADE ESTADUAL DE LONDRINA

JANAINA EMILIANO

AVALIAÇÃO DA ATIVIDADE ANTIFÚNGICA DE PRODUTOS DO METABOLISMO SECUNDÁRIO DE *PSEUDOMONAS AERUGINOSA* E *BURKHOLDERIA METALLICA* SOBRE A GERMINAÇÃO CARPOGÊNICA E INFECÇÃO EM FEIJOEIRO POR *SCLEROTINIA SCLEROTIORUM*

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Tese apresentada ao Programa de Pós-Graduação em Microbiologia da Universidade Estadual de Londrina como parte dos requisitos para a obtenção do título de Doutora em Microbiologia

Orientador: Prof. Dr. Galdino Andrade Filho

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“A menos que modifiquemos à nossa maneira de pensar, não seremos capazes de resolver os problemas causados pela forma como nos acostumamos a ver o mundo”.

(Albert Einstein)

EMILIANO, J. **Avaliação da atividade antifúngica de produtos do metabolismo secundário de *Pseudomonas aeruginosa* e *Burkholderia metallica* sobre a germinação carpogênica e infecção em feijoeiro por *Sclerotinia sclerotiorum*.** 2020. 92 f. Tese (Doutorado em Microbiologia) – Universidade Estadual de Londrina, Londrina, 2020

RESUMO

Sclerotinia sclerotiorum (Lib.) De Bary é um fungo fitopatogênico filamentoso conhecido como mofo branco. O fungo produz estruturas de resistência chamadas escleródios, que podem permanecer viáveis no solo por anos. O manejo da doença é baseado na rotação de culturas e no uso de fungicidas, os quais contaminam o meio ambiente e selecionam isolados resistentes. Portanto, o desenvolvimento de novos produtos de origem natural para o controle desse fitopatógeno é importante. Nesse contexto, o presente estudo teve como objetivo avaliar o efeito antifúngico de moléculas bioativas produzidas por *Pseudomonas aeruginosa* cepa LV e *Burkholderia metallica* cepa RV7S3 sobre a germinação carpogênica de escleródios de *Sclerotinia sclerotiorum* e seu potencial para o controle da infecção em feijoeiro. A fração com atividade antifúngica produzida pela cepa LV foi denominada F4A, e as frações produzidas pela cepa RV7S3 foram denominadas FD e FRV7. Esta última é uma fração semi-purificada a partir de FD, e apesar de ter efeito antifúngico com baixas concentrações em testes *in vitro* de ágar difusão, apresenta baixo rendimento, portanto não foi utilizada em testes com gerbox e casa de vegetação. Escleródios produzidos em substrato à base de cenoura foram inoculados em gerbox e tratados com F4A e FD separadamente. Ao final de 55 dias de incubação, foi possível observar inibição crescente da germinação carpogênica dos escleródios, com 100% de inibição nos gerbox tratados com as frações. Folhas destacadas de feijoeiro foram infectadas com *S. sclerotiorum* e tratadas com as moléculas bioativas. Ambas as frações controlaram o desenvolvimento do fungo, no entanto, F4A a pesar de impedir a infecção fúngica, apresentou fitotoxicidade nas folhas tratadas, portanto os testes em casa de vegetação foram realizados apenas com a fração FD produzida por *B. metallica* cepa RV7S3. Plantas de feijão foram infectadas com mofo branco e realizado tratamento preventivo+curativo com a aplicação de FD, mantendo 40% das plantas viáveis sem causar fitotoxicidade e impedindo o desenvolvimento fúngico. A fração F4A apresenta potencial para o controle de *S. sclerotiorum*, no entanto novas estratégias para diminuir a fitotoxicidade devem ser consideradas. O tratamento com os metabólitos secundários produzidos pela cepa RV7S3 apresentaram efeito antifúngico sem causar danos na planta, no entanto estudos futuros precisam ser realizados para otimizar a produção e aumentar o rendimento da fração FRV7, pois esta possivelmente apresentaria maior porcentagem de sobrevivência de plantas em concentrações menores de produto.

Palavras-chave: fitopatógeno**;** mofo branco; germinação carpogênica; antimicrobianos; feijão comum; microscopia eletrônica de varredura.

EMILIANO, J. **Evaluation of antifungal activity of secondary metabolism products of *Pseudomonas aeruginosa* and *Burkholderia metallica* on carpogenic germination and infection in common bean by *Sclerotinia sclerotiorum***. 2020. 92 p. Tese (Doutorado em Microbiologia) – Universidade Estadual de Londrina, Londrina, 2020

ABSTRACT

Sclerotinia sclerotiorum (Lib.) De Bary is a filamentous phytopathogenic fungus known as white mold. The fungus produces resistance structures called sclerotia, which can remain viable in the soil for years. The management of the disease is based on crop rotation and the use of fungicides, which contaminate the environment and select resistant isolates. Therefore, the development of new products of natural origin to control this phytopathogen is important. In this context, the present study had as objective to evaluate the antifungal effect of bioactive molecules produced by *Pseudomonas aeruginosa* strain LV and *Burkholderia metallica* strain RV7S3 on the carpogenic germination of *Sclerotinia sclerotiorum* sclerotia and its potential for controlling infection in bean plants. The fraction with antifungal activity produced by the LV strain was called F4A, and the fractions produced by the RV7S3 strain were called DP and FRV7. The latter is a semi-purified fraction from DP, and despite having an antifungal effect with low concentrations *in vitro* diffusion agar tests, it has low yield, therefore it was not used in tests with gerbox and greenhouse. Sclerotia produced on carrot-based substrate were inoculated in gerbox and treated with F4A and DP separately. At the end of the 55 days of incubation, it was possible to observe increasing inhibition of carpogenic germination of sclerotia, with 100% inhibition in the gerbox treated with the fractions. Detached bean plants leaves were infected with *S. sclerotiorum* and treated with bioactive molecules. Both fractions controlled the development of the fungus, however, F4A despite preventing fungal infection, showed phytotoxicity in the treated leaves, so the tests in the greenhouse were performed only with the DP fraction produced by *B. metallica* strain RV7S3. Bean plants were infected with white mold and performed preventive+curative treatment with DP application, maintaining 40% of viable plants without causing phytotoxicity and preventing fungal development. The F4A fraction has the potential to control of *S. sclerotiorum*, however new strategies to decrease phytotoxicity must be considered. The treatment with secondary metabolites produced by the RV7S3 strain showed antifungal effect without causing damage to the plant, however future studies need to be carried out to optimize the production and increase the yield of the FRV7 fraction, as this would possibly present a better percentage of plant survival in lower concentrations of product.

Keywords: phytopathogen; white mold; carpogenic germination; antimicrobials; common beans; scanning electron microscopy.

LISTA DE ILUSTRAÇÕES

REVISÃO BIBLIOGRÁFICA

ARTIGO I

ARTIGO II

LISTA DE ABREVIATURAS E SIGLAS

AEP	Amorphous Extracellular Polysaccharides
AO	Ácido Oxálico
bh	Branched Hyphae
CFU	Colony Forming Unit
DMSO	Dimethylsulfoxide
DP	Dichloromethane Phase
FC	Flash Chromatography
FD	Fração Diclorometano
ih	Infection Hyphae
IND	Indolinona
lr	**L**eaf Ribs
MFC	Minimum Fungicidal Concentration
NA	Nutrient Agar
NaCl	Cloreto de Sódio
NB	Nutrient Broth
PCA	Fenazina Ácido Carboxílica
PCN	Fenazina Carboxiamida
PDA	Potato Dextrose Agar
RBD	Randomized Block Design
Rf	Retention Factor
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy
TLC	Thin Layer Chromatography
tr	Trichomes
VLC	Vacuum Liquid Chromatography

SUMÁRIO

1. INTRODUÇÃO

Sclerotinia sclerotiorum (Lib) de Bary é um fungo filamentoso cosmopolita e necrotrófico, causador da doença conhecida como mofo branco ou podridão branca, que é veiculada pelo solo. Possui pouca especificidade, com uma ampla gama de hospedeiros com mais 400 espécies de plantas, responsável por sérias perdas anuais de muitas culturas economicamente importantes, incluindo a soja, o feijão, a batata e a canola (KABBAGE et al., 2015; BOLTON et al., 2006; BOLAND e HALL, 1994).

As características da doença são o desenvolvimento de micélio branco, em caule e frutos, e a formação de escleródios (agregados de hifas com camada exterior contendo melanina, uma forma de resistência do fungo) em tecidos infectados da planta hospedeira ou no solo. Os escleródios podem continuar viáveis no solo, por até 8 anos, ou até o momento em que encontra condições adequadas (alta umidade, temperatura amena, 18 a 22ºC, e luminosidade moderada) para a germinação. Esta pode ser miceliogênica, na qual serão produzidas hifas, infectando diretamente os tecidos da planta, ou carpogênica, com a produção de apotécios, os quais liberam ascósporos infecciosos. O maciço potencial reprodutivo, juntamente com capacidade de sobrevivência a longo prazo, faz dos escleródios componentes centrais na epidemiologia da doença (ONARAN e YANAR, 2011; BOLTON et al., 2006).

Uma das principais doenças que ocorrem no feijoeiro é o mofo branco, causando prejuízos a produção devido a redução da produtividade e queda da qualidade da semente, levando a perdas econômicas que chegaram a 36 milhões de reais em 2007 no estado de Goiás (RICARDO et al. 2009). A principal forma de controle do fungo são a rotação de culturas agrícolas e a pulverização de fungicidas. A rotação de culturas não resolve o problema, devido à persistência dos escleródios no solo (FERNANDO et al., 2007). Já o uso intensivo de fungicidas tem causado sérios problemas ambientais e aumentado a frequência e distribuição de isolados fúngicos resistentes, portanto, é importante o desenvolvimento de novos produtos para o controle de fitopatógenos (LI et al., 2006).

Os antimicrobianos naturais são compostos com capacidade para inibir o crescimento de microrganismos. Muitas bactérias utilizam-se do seu metabolismo secundário para produzirem compostos bioativos como forma de reduzir a competição por nutrientes no ambiente natural. Esses metabólitos secundários como são chamados, não são essenciais para o desenvolvimento do microrganismo produtor, são moléculas de peso molecular relativamente baixo, biossintetizados em vias especializadas a partir de metabólitos primários, exibem uma vasta gama de atividades biológicas e desempenham um papel importante na regulação de interações entre organismos (MADIGAN et al, 2010).

Dentre os principais organismos produtores de antimicrobianos com atividade antifúngica, destacam-se os gêneros*, Pseudomonas* e *Burkholderia* (XIE et al., 2003). Pseudomonas spp. produzem uma grande variedade de metabólitos secundários, incluindo antimicrobianos, antimitóticos, herbicidas, anti-helmínticos e nematicidas. Dentre os compostos produzidos se destacam os pertencentes a família dos sideróforos, fenazinas, pirroles e indolinonas (NAVARRO et al., 2019). Bactérias do gênero *Burkholderia* apresentam versatilidade na capacidade para produção de metabólitos secundários entre os quais estão a pirrolnitrina (EL-BANNA e 5 WINKELMANN, 1998), fenazinas (PIERSON e PIERSON, 2010), sideróforos (DARLING *et al*., 1998); bem como peptídeos antimicrobianos, como xylocandinas (MEYERS *et al*., 1987), occidiofunginas (LU *et al*., 2009) e burkholdinas, muitos com propriedades antifúngicas, antibacterianas, herbicidas ou inseticidas.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar a ação antifúngica de moléculas bioativas produzidas por *Pseudomonas aeruginosa* cepa LV e *Burkholderia metallica* cepa RV7S3 sobre a germinação carpogênica de escleródios de *Sclerotinia sclerotiorum* e seu potencial para o controle da infecção em feijoeiro.

2.2 OBJETIVOS ESPECÍFICOS

1. Padronizar condições de incubação *in vitro* de *S. sclerotiorum* para otimizar a produção de escleródios e sua germinação carpogênica*;*
2. Produzir e purificar as moléculas bioativas, das cepas LV e RV7S3, com atividade antifúngica sobre *S. sclerotiorum*;
3. Avaliar o efeito de diferentes concentrações das moléculas bioativas sobre a germinação carpogênica dos escleródios*;*
4. Avaliar o potencial controle da infecção do mofo branco na infecção de folhas destacadas e mudas de feijoeiro;
5. Verificar alterações estruturais em folhas de feijão infectadas e tratados com moléculas bioativas.

3. REVISÃO BIBLIOGRÁFICA

3.1 Características gerais do mofo branco

Sclerotinia sclerotiorum (Lib.) de Bary é um fungo fitopatogênico do filo Ascomycota, subfilo Pezizomycota, Classe Leotiomycetes, ordem Helotiales e família Sclerotineaceae.. A família Sclerotiniaceae inclui espécies que produzem ascos a partir de apotécios castanhos, que surgem de um escleródio dentro ou associado a uma planta hospedeira (HOLST-JENSEN *et al*., 1997b; WHETZEL, 1945). O fungo foi descrito pela primeira vez em 1837 como *Peziza sclerotiorum* (LIBERT, 1837), até que a espécie foi transferida para um novo gênero e renomeada *Sclerotinia libertania* Fuckel em homenagem a Libert (FUCKEL, 1870), o nome foi aceito por alguns autores, no entanto após vários conflitos (WAKEFIELD, 1924; PURDY, 1979) aceitou-se que a nomenclatura correta foi citada por de BARY (1884).

Também conhecido como mofo branco, *S. sclerotiorum* tem distribuição mundial e potencial de causar doenças em mais de 400 espécies de plantas pertencentes a 75 famílias dentre as quais estão culturas economicamente importantes, como o feijão *(Phaseolus vulgaris* L.), soja (*Glycine max* L.), canola *(Brassica rapa* L., *Brassica campestris* L.), girassol *(Helianthus* spp) entre outras (BOLAND e HALL 1994; BOLTON et al., 2006; SAHARAN e MEHTA, 2008).

O fungo infecta folhas, flores, frutos e caule das plantas hospedeiras formando lesões encharcadas que progridem rapidamente ao redor da área infectada atingindo o interior do caule. As lesões se desenvolvem em tecidos necrosados, seguidos de podridão ou murcha com a formação de micélio branco fofo (Fig. 1) e escleródios dentro dos tecidos e vagens, mas podem se formar na superfície dos tecidos durante condições de alta umidade (BOLTON et al., 2006; PELTIER et al., 2012)

Figura 1 – Folhas e mudas de feijoeiro infectadas com *S. sclerotiorum*. Fonte: Próprio autor.

A degradação da parede celular da planta ocorre pela ação do ácido oxálico (CESSNA et al., 2000), enzimas líticas extracelulares, incluindo celulases, hemicelulases e pectinases (RIOU et al., 1991), aspartil protease (POUSSEREAU et al., 2001), endo-poligalacturonases (COTTON et al., 2002) e protease ácida (GIRARD et al., 2004). Sob as condições ácidas fornecidas pelo ácido oxálico, tais enzimas levam à degradação da parede celular da planta. O ácido oxálico (AO) exerce um efeito tóxico no tecido do hospedeiro acidificando o ambiente e sequestrando o cálcio nas lamelas médias, levando à perda da integridade do tecido vegetal (BATEMAN e BEER, 1965; GODOY et al., 1990). Ele também limita diretamente os compostos de defesa do hospedeiro, suprimindo a ação de enzimas antioxidantes. Em conjunto, as enzimas que degradam a parede celular da planta, causam maceração dos tecidos vegetais e necrose seguida pela morte da planta (COLLMER e KEEN, 1986).

3.1.1 Estrutura de resistência e reprodução do mofo branco

O escleródio é a estrutura primária de sobrevivência de *S. sclerotiorum* podendo manter-se viável no solo de 3 a 10 anos, pois são resistentes às condições químicas e fisicamente adversas, bem como à degradação biológica. No entanto, sua sobrevivência depende de inúmeros fatores, incluindo tamanho e forma esclerotial, tipo e química do solo, populações microbianas do solo, culturas anteriores plantadas e condições climáticas (MERRIMAN 1976; WU et al. 2008; ADAMS e AYERS, 1979).

O escleródio é uma estrutura rígida de repouso que consiste em um interior de cor clara, denominado medula, e um exterior preto protetor que cobre a casca (Fig. 2). O anel externo é composto por células cujas paredes contêm melanina (BUTLER et al. 2009). Trata-se de uma macromolécula composta por vários tipos de monômeros fenólicos ou indólicos que protegem fungos de condições ambientais tais como luz visível ou ultravioleta, metais tóxicos, enzimas líticas, microrganismos antagônicos e à degradação química (MORDOMO e DIA 1998). A parte interna do escleródio, a medula, está inserida em uma matriz fibrilar composta de carboidratos e proteínas (LE TOURNEAU 1979).

Figura 2 – Escleródios de *S. sclerotiorum*. Fonte: Próprio autor

O desenvolvimento esclerótico pode ser dividido em três estágios distintos: (i) iniciação, o aparecimento de pequenas formas iniciais distintas de hifas entrelaçadas, que se desenvolvem terminalmente por ramificação repetida de hifas primárias longas e aéreas; ii) desenvolvimento, aumento de tamanho; e iii) maturação, caracterizada por delimitação de superfície, consolidação interna e melanização, frequentemente associada à secreção de exudatos (TOWNSEND e WILLETTS, 1954; CHET e HENIS, 1975; LE TOURNEAU, 1979; WILLETTS e BULLOCK, 1992; WILLETTS e WONG, 1980).

Possui dois mecanismos de germinação que iniciam os processos de infecção vegetal: germinação miceliogênica e germinação carpogênica. Esses processos permitem que o patógeno atue como um patógeno transmitido pelo ar e pelo solo, respectivamente.

3.1.2 Germinação miceliogênica

Na germinação miceliogênica, o escleródio produz hifas e micélio. A infecção de plantas hospedeiras por micélio frequentemente ocorre na linha do solo ou abaixo dela. Os escleródios germinam na presença de nutrientes exógenos e produzem hifas que invadem a matéria orgânica inanimada, formando micélio que infecta os tecidos vivos do hospedeiro, resultando em podridões basais (SAHARAN e MEHTA, 2008; STEYN, 2015).

A regulação da germinação miceliogênica ocorre devido da melanização esclerotial. Escleródios melanizados incompletamente são propensos à redução da sobrevida como resultado do aumento da degradação microbiana, sensibilidade a condições adversas e ausência de dormência. A germinação miceliogênica ocorre quando temperaturas moderadas (20-25 °C) e alta umidade (>80%) mantem-se por no mínimo 12 horas. Flutuações de umidade do solo entre 30% e 100% da capacidade de campo promovem a germinação miceliogênica e a infecção do hospedeiro. (HUANG e DUECK, 1980; HUANG, 1985).

3.1.2 Germinação carpogênica

A germinação carpogênica começa com o crescimento do fungo ativo nas regiões do córtex ou medula do escleródio. As células fúngicas em crescimento formam primórdios densos que rompem a casca do escleródio e continuam crescendo como hastes em forma de tubo chamadas estipes, que após emergir do solo e expostos à luz ultravioleta (<390 nm) se diferenciam em apotécios (Fig. 3) (SAITO, 1973; JONES, 1974).

Figura 3 – Germinação carpogênica de escleródios de *S. sclerotiorum,* formação de apotécios. Fonte: Próprio autor.

Na formação de um apotécio, a ponta da estipe se expande para formar uma superfície superior composta de himênio e hipotécio, camadas que são sustentadas pelos tecidos afilados da antiga estipe, agora denominado excípulo, dando à estrutura geral a aparência de um sino de trompete de cor bege a amarelo (Fig 4). No himênio nascem numerosos ascos e estruturas semelhantes a cabelos, as paráfises. No asco, ocorre recombinação genética e os produtos são oito ascósporos, bem alinhados perto da ponta de cada asco. Um vacúolo, que é responsável por aumentar a pressão hidrostática dentro do asco, se forma abaixo da cadeia de ascósporos. Conforme aumenta a pressão hidrostática, o asco se expande, mas sua expansão lateral é restringida pelas paráfises e asco vizinhos. Portanto, a maior parte da expansão está perto da ponta, até que cada asco se projete além das paráfises. Em algum ponto, a pressão excede, a parede suportável se estica e o asco explode (KOSASIH e WILLETTS, 1975; JAYACHANDRAN *et al*. 1987).

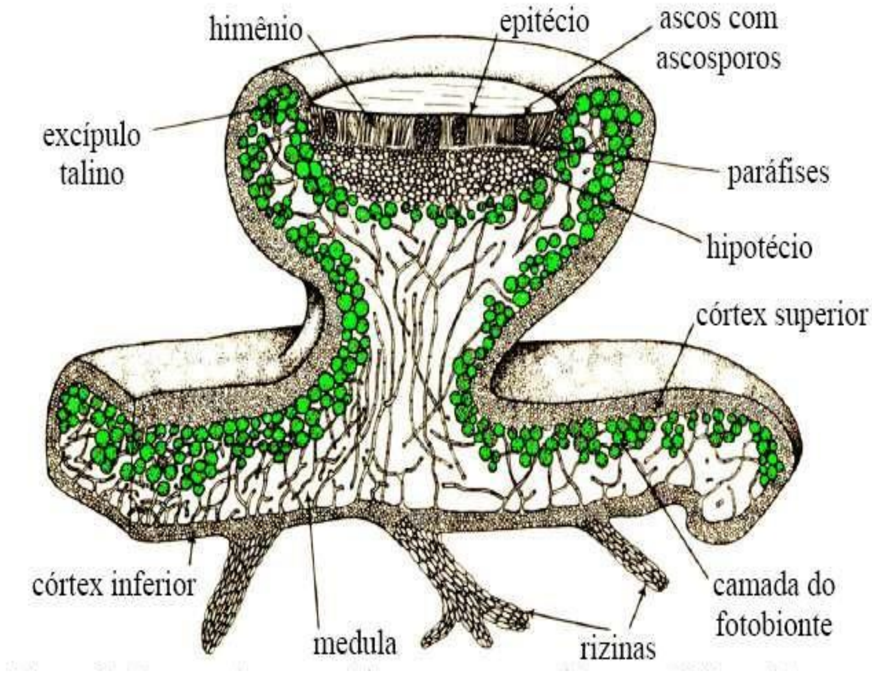

Figura 4 – Componentes de um apotécio. Fonte: THOMSON, 1962.

A produção de apotécios requer o amadurecimento e o pré-condicionamento de escleródios por pelo menos duas semanas a 10-20 ºC em solo úmido com capacidade de campo superior a 80%, e com base alimentar não viva na rizosfera dentro dos primeiros 2 cm da superfície do solo (ABAWI e GROGAN, 1979). A necessidade de umidade e temperaturas relativamente baixas sob o dossel da planta para a germinação carpogênica são as razões pelas quais os períodos chuvosos ou campos irrigados estão associados a surtos de doenças na maioria das lavouras (SAHARAN e MEHTA, 2008; CLARKSON et al., 2004).

3.1.3 Ascósporos

A liberação de ascósporos e sua sobrevivência são fatores importantes para o desenvolvimento da doença e no ciclo de vida desse patógeno. Cada apotécio pode produzir de 2 a 30 milhões de ascósporos em um período de vários dias. Os ascósporos são cobertos por mucilagem pegajosa que pode ser resíduo do líquido do asco ou parte da parede celular. A mucilagem não apenas cimenta o esporo em qualquer objeto com o qual entre em contato, mas também os agrupam. Esporos recém produzidos geralmente sobrevivem de 5 a 21 dias, alta umidade relativa e luz ultravioleta são prejudiciais à sua sobrevivência (WU, 1988; CLARKSON et *al*., 2004).

Ascósporos de *Sclerotinia* são fisicamente disparados da superfície do himênio para cima. A exibição é muito espetacular pois dezenas de milhares de ascos disparam seus ascósporos quase simultaneamente, produzindo uma grande nuvem de esporos (Fig. 5). O disparo costuma ser forte o suficiente para sacudir todo o apotécio e os esporos são impelidos vários centímetros acima do himênio. Estipes são fototrópicas, pois alinham o himênio com a fonte de luz mais forte. As pontas do asco também são fototrópicas, o que ajuda a ajustar a trajetória dos esporos para o potencial máximo de dispersão. O sol aquece o solo, criando turbulência térmica do ar que ajuda a transportar os esporos para fora do dossel. A mudança na temperatura também acarreta uma mudança na umidade relativa que pode provocar a liberação dos ascósporos (NEWTON e SEQUEIRA, 1972; WEGULO *et al*., 2000; LI et *al*., 1994).

Figura 5 – Apotécios maduros e a liberação de ascósporos. Fonte: PETHYBRIDGE, 2004.

A maioria dos ascósporos cai sobre plantas suscetíveis nas imediações do apotécio, mas alguns podem viajar longas distâncias pelo vento (Fig. 6). Ascosporos podem germinar na superfície do tecido saudável, mas não podem infectar plantas sem uma fonte de nutrientes exógeno (BOLTON et al. 2006), que muitas vezes é fornecida por folhas senescentes, pecíolos ou caules de plantas danificadas (KORA et al. 2003). Assim, a floração é um momento particular porque as flores senescentes servem como fonte de nutrientes para o patógeno (TURKINGTON e MORRALL 1993; ALMQUIST e WALLENHAMMAR 2015).

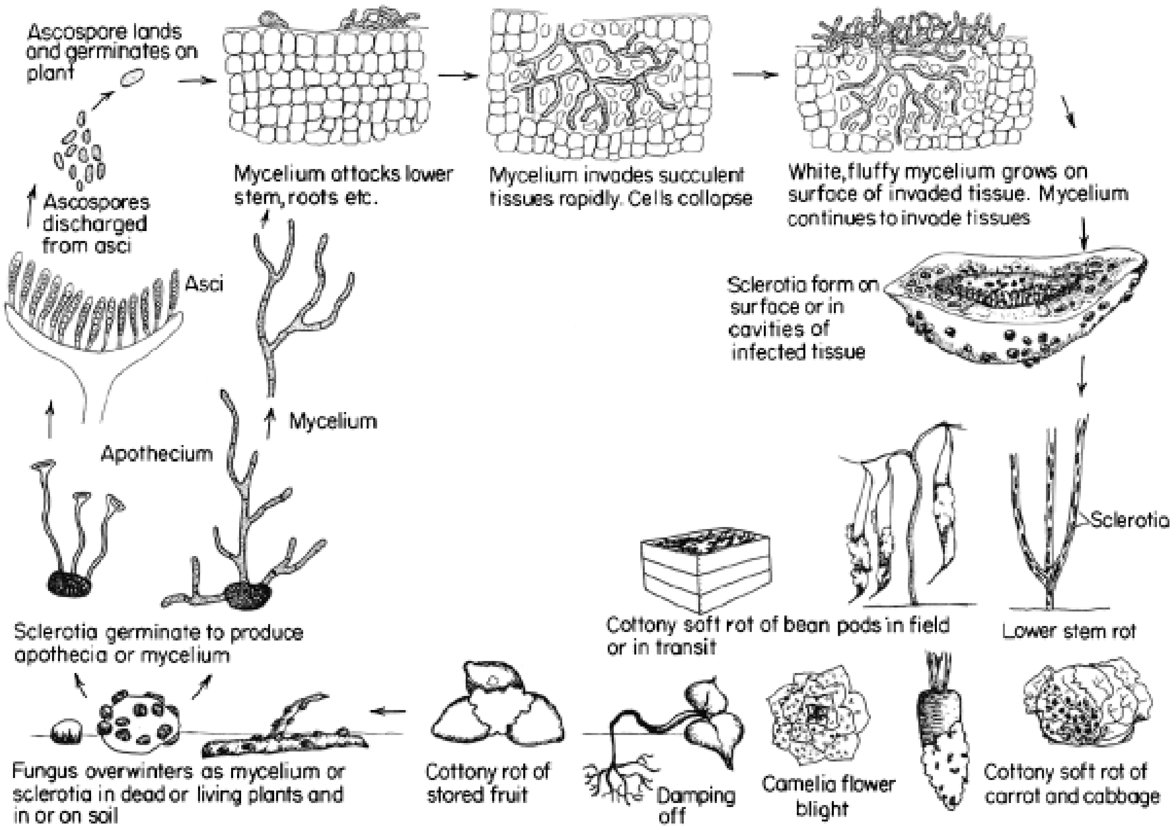

Figura 6 – Ciclo de desenvolvimento/infecção de *S. sclerotiorum*. Fonte: AGRIOS, 1997.

3.2 Cultura do feijoeiro

O feijão comum (*Phaseolus vulgaris* L.) pertence à família Fabaceae que compreende aproximadamente 650 gêneros e 18.000 espécies, distribuídas nas subfamílias Caesalpinioideae, Faboideae e Mimosoideae. Suas espécies são amplamente distribuídas no mundo todo; além de cultivadas nos trópicos, também se desenvolvem em zonas temperadas dos hemisférios Norte e Sul (POLHILL et al., 1981).

O Brasil é o maior consumidor e o maior produtor mundial de feijão, sendo consumidos mais de sete tipos diferentes de feijão no território nacional e produzidas mais de 3.000.000 t/ha. Sua boa aceitação no mercado aliado aos benefícios proporcionados ao ambiente edáfico pelo plantio de leguminosas torna o feijão uma excelente opção de cultivo para pequenos e médios agricultores (ABREU et al., 2004; SILVA et al., 2004; VILELA et al., 2009).

A cultura do feijoeiro é suscetível a inúmeras espécies de patógenos, levando à acentuadas perdas na produção. Tais patógenos causam doenças que limitam a produção de feijão e reduzem a qualidade fisiológica, sanitária, nutricional e comercial do produto (BOERSMA et al. 2015). Uma das principais doenças que ocorrem no feijoeiro é o mofo branco, causada pelo fungo fitopatogênico *Sclerotinia sclerotiorum* (Fig. 7) (LOBO, 2002; WENDLAND et al., 2014). Os prejuízos causados pelo patógeno em lavouras em diversas regiões estão relacionados com a redução da produtividade e queda da qualidade da semente. Ricardo et al. (2009) estimaram um dano econômico total de 36 milhões de reais na terceira safra de 2007 no estado de Goiás, causado pelo mofo-branco.

Figura 7 – Vagens de feijão infectadas por *S. sclerotiorum*. Fonte: campoenegocios.com.br

A principal forma de controle de fungos fitopatogênicos, mundialmente utilizada, ainda é o uso de fungicidas químicos. Esse processo além de ocasionar o surgimento de patógenos resistentes e pragas secundárias pode prejudicar à saúde dos produtores agrícolas e consumidores causando impactos ao meio ambiente. Outra desvantagem no uso de fungicidas químicos está relacionada à sua inespecificidade, podendo eliminar microrganismos já estabelecidos no solo que são benéficos às plantas aumentando assim, sua suscetibilidade aos patógenos de solo. Nos últimos anos, devido à pressão imposta pelas leis de impacto ambiental, a crescente preocupação da população com o meio ambiente e saúde, bem como pela necessidade de desenvolvimento de métodos de controle mais eficientes, surgiu a demanda por novas estratégias neste controle (PIRES et al, 2003; CARVALHO et al., 2015).

3.4 Biossíntese de antimicrobianos

Diferentes organismos no ambiente, principalmente fungos e bactérias chamados antagonistas, realizam complexa interação ecológica. Muitos desses microrganismos produzem biocompostos por meio de seu metabolismo secundário, os quais têm efeito contra demais microrganismos de um mesmo ecossistema, fenômeno esse denominado antibiose. Tais metabólitos são produtos de baixa massa molecular, geralmente produzidos durante a fase de crescimento tardio, componentes comumente extracelulares, não essenciais para o crescimento celular, mas servem como mecanismo de defesa/competição (SINGH et al. 2019, GOKULAN et al. 2014; MADIGAN *et al*., 2010)

Alguns grupos de bactérias cultiváveis são característicos pela capacidade de sintetizar metabólitos com atividade antifúngica. Dentre esses grupos, destacam-se os actinomicetos, representantes dos gêneros *Bacillus, Pseudomonas* spp. e *Burkholderia* spp., as quais são muito relevantes pelos seus produtos metabólicos, tendo uma grande plasticidade metabólica e fenotípica (MELO e AZEVEDO, 1998).

Esses antimicrobianos podem ser de interesse econômico, uma vez que podem ser utilizados como alternativa aos produtos comerciais, ou mesmo serem aplicados concomitantemente com esses produtos, visando efeitos sinérgicos. Uma parcela destas substâncias são de antifúngicos protetores - também denominados: convencionais e não-sistêmicos. Estes são caracterizados por apresentarem inespecificidade no mecanismo/sítio de ação bioquímica, possuindo forte efeito fungicida. Essas características são importantes, pois reduzem as chances seleção de microrganismos resistentes (GHINI e KIMATI, 2000).

3.4.1 *Pseudomonas* sp.

O gênero *Pseudomonas* é conhecido por colonizar com sucesso superfícies e tecidos internos de raízes e caules em altas densidades para sua sobrevivência e adaptação em diversos nichos ambientais, assim como por sua capacidade de produzir metabólitos secundários bioativos (WELBAUM et al. 2004). Várias espécies de *Pseudomonas* fuorescentes, incluindo *Pseudomonas fuorescens*, *Pseudomonas aeruginosa, Pseudomonas aureofaciens, Pseudomonas putida, Pseudomonas chlororaphis* e *Pseudomonas pyrrocinia* foram demonstrados *in-vitro* e *in-vivo* por seu antagonismo contra patógenos vegetais bacterianos e fúngicos (AL-HINAI et al. 2010).

Pseudomonas spp. produzem uma grande variedade de metabólitos secundários, incluindo antimicrobianos, antimitóticos, herbicidas, anti-helmínticos, nematicidas, fitotóxicos e de detecção de *quorum*. Além disso, muitas espécies também são conhecidas pela produção de sideróforos, enzimas hidrolíticas extracelulares, compostos orgânicos voláteis e hormônios promotores de crescimento de plantas. Muitos desses compostos são gerados por meio de vias metabólicas complexas e estão envolvidos na supressão competitiva e na inibição de patógenos de plantas (SHANMUGAIAH et al. 2010; SHAHID et al. 2017).

Dentre os compostos produzidos se destacam os pertencentes a família dos sideróforos, fenazinas, pirroles e indolinonas (NAVARRO et al., 2019). Os sideróforos mais estudados são as pioverdinas, com mais de 100 compostos descritos os quais possuem a capacidade de adquirir ferro em ambientes de baixa concentração (CEZARD et al., 2014). As fenazinas com atividade antimicrobiana são representadas principalmente pela fenazina-1-ácido carboxílica e a fenazina-1-carboxiamida, esta última apresenta também possível indução de resistência em plantas (SIMIONATO et al., 2017; SHANMUGAIAH et al, 2010; ZHANG et al., 2015). Dos compostos do grupo pirroles se destaca a pirrolnitrina, molécula com atividade antifúngica, nematicida e repelente (TAKEDA et al., 1990; NANDI et al., 2015). Já as moléculas pertencentes a família das indolinonas são estudadas na inibição do crescimento e da proliferação celular anormal, podendo ser utilizado no desenvolvimento de antitumorais (BENICIO et al., 2018; PRAKASH e RAJA, 2012).

3.4.1.1 *Pseudomonas aeruginosa* cepa LV

Microrganismos com atividade antagônica sobre *Xantomonas citri* pv. citri (Xcc 306) foram isolados de folhas de laranja infectadas com cancro cítrico, coletadas da cidade de Astorga, PR, Brasil. Dentre os microrganismos antagonistas isolados, uma cepa denominada LV, se destacou por apresentar ótima atividade antimicrobiana (RAMPAZO, 2004). Desde então esta cepa vem sendo estudada para a produção, extração e fracionamento de moléculas provenientes do sobrenadante livre de células.

No trabalho de Oliveira et al. (2011) foi isolada uma fração denominada F3 com alta atividade antimicrobiana frente aos fitopatógenos Xcc 306, *Xantomonas axonopodis* (LOPES et al., 2012) e *Xantomonas arborícola* pv. *pruni* (SILVA VASCONCELLOOS et al., 2014). Esta fração foi purificada aumentando sua atividade antimicrobiana frente às espécies de *Xantomonas* spp., a qual foi denominada F3d (DE OLIVEIRA et al., 2016), no entanto ainda houveram mudanças no processo de purificação, e a fração passou a ser chamada de F4A (PISTORI et al., 2018).

A fração F4A é composta por duas moléculas de fenazina (fenazina carboxílica e carboxiamida), uma molécula da família das indolinonas e um composto organometálico (PISTORI et al., 2018) que foi identificado como Fluopsina C (NAVARRO et al., 2019). A combinação destas quatro substâncias apresentou controle sobre o fungo fitopatogênicos *Phakopsora pachyrhizi* causador da ferrugem asiática em soja (BARAZETTI et al., 2019), e *Botrytis cinérea,* que teve seu crescimento inibido por fenazina carboxílica, molécula presente na fração F4A (SIMIONATO et al., 2017).

3.4.2 *Burkholderia* sp.

Bactérias do gênero *Burkholderia* vivem em diversos nichos ecológicos, incluindo solo, água, plantas, bem como animais e humanos (MAHENTHIRALINGAM et al. 2008). São classificadas em 4 grupos distintos, sendo: *Burkholderia. pseudomallei*, *Burkholderia glathei*, *Burkholderia xenovorans* e complexo *Burkholderia cepacia* (ou *Bcc*) (DEPOORTER et al., 2016). Esse último agrupa a maior parcela dos isolados produtores de bioativos antimicrobianos. Atualmente, o grupo *Bcc* compreende ao menos 19 espécies, dentre as quais estão *B. cepacia* (PALLERONI e HOLMES, 1981), *Burkholderia pyrrocinia* (VANDAMME *et al*., 2000), *Burkholderia cenocepacia* (VANDAMME *et al*., 1997), *Burkholderia pesudomultivorans* (PEETERS *et al*., 2013) *Burkholderia difusa e Burkholderia metallica* (VANLAERE *et al*., 2008).

Muitas características os tornam bons candidatos como agentes promotores de biocontrole e crescimento. Estes incluem a capacidade de (i) induzir resistência vegetal contra condições de estresse ambiental (MIOTTO-VILANOVA et al. 2016), (ii) utilizar sementes e exsudatos radiculares (ABE E NAKAZAWA 1994; LIM et al. 1994), (iii) fixação de nitrogênio (CABALLERO-MELLADO et al. 2007),(iv) colonizar a rizosfera da planta (CHEN et al. 2006; CHEN et al., 2007), e (v) produzir uma grande variedade de enzimas extracelulares e metabólitos secundários (VIAL et al. 2007).

São organismos versáteis, com capacidade para produção de metabólitos secundários de baixo peso molecular, (CIMERMANCIC et al. 2014) entre os quais muitos com propriedades antifúngicas, antibacterianas, herbicidas ou inseticidas. Cepas de *Burkholderia* são conhecidas por produzir pirrolnitrina (EL-BANNA e WINKELMANN 1998), fenazinas (CARTWRIGHT et al. 1995), sideróforos (DARLING *et al*., 1998), peptídeos antimicrobianos, como xilocandinas, cepacidinas (MEYERS et al. 1987), occidiofunginas (LU *et al*., 2009), burkholdinas (THOMSON e DENNIS, 2012), entre outros compostos.

Pirrolnitrina é um potente metabólito antifúngico e antibacteriano, desempenhando um papel na atividade de biocontrole contra fungos fitopatogênicos como *Rizoctonia solani* e F*usarium* spp. (BURKHEAD et al. 1998; HWANG et al. 2001). As fenazinas produzidas por espécies de *Burkholderia,* são a iodinina (BELL e TURNER, 1973), ácido 4,9 diidroxifenazina-1,6-dicarboxilico dimetil éster (CARTWRIGHT *et al*., 1995) e, mais recente identificada, fencomicina (HAN *et al*., 2014).

Burkholdinas agem como antifúngicos contra uma ampla gama de fitopatógenos interferindo na membrana celular (LU et al. 2009). A estrutura de occidiofungina possui peptídeos que interagem com a parede celular formando inclusões intracelulares e fazendo mudanças significativas na morfologia das hifas de fungos-alvo (LU et al. 2009). As xilocandinas carregam forte atividade antifúngica contra leveduras, incluindo *Candida albicans* (MEYERS et al. 1987). Cepacidinas compartilham estrutura com xilocandina, com forte atividade antifúngica contra fungos fitopatopatôgênicos incluindo *Pitium ultimum* no algodão e pepinos, *Plasmopora veticola* em uvas e *Septoria nodorum* e *Fusarium culmorum* no trigo (LEE et al. 2000).

Burkholderia metallica cepa RV7S3 foi isolada de amostras de solo coletadas em Rio Verde, Itararé, SP, Brasil. Em testes de disco difusão os metabólitos bioativos produzidos pela cepa RV7S3 apresentaram atividade antimicrobiana sobre *Rizoctonia. solani*, *Sclerotinia sclerotiorum* e *Alternaria alternata Mycosphaerella fijiensis*, *Botrytis cinerea* e *Candida albicans,* demostrando grande potencial antifúngico (MODOLON, 2019).

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APRESENTAÇÃO

O presente trabalho foi dividido em dois artigos já finalizados. O artigo I, intitulado "Effect of secondary metabolites produced by *Pseudomonas aeruginosa* on carpogenic germination of *Sclerotinia sclerotiorum*" está no modelo da revista Biological control. O artigo II, denominado: "Control of *Sclerotinia sclerotiorum* by extracellular metabolites from *Burkholderia metallica*" está no modelo da revista Plant Diseases.

ARTIGO I

Effect of secondary metabolites produced by *Pseudomonas aeruginosa* on carpogenic germination of *Sclerotinia sclerotiorum*

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ABSTRACT

Sclerotinia sclerotiorum (Lib.) de Bary is a filamentous phytopathogenic fungus known as white mold. The management of this disease is based on crop rotation and the use of chemical pesticides, which cause contamination in the environment and selected resistant isolates, therefore it is important to develop of new products for the control of this phytopathogen. In this context, the present study had as objective to evaluate the antifungal activity of F4A fraction, a group of bioactive produced by *Pseudomonas aeruginosa* LV strain, on the carpogenic germination of *S. sclerotiorum* sclerotia and its potential for infection control in bean plants. For this, sclerotia produced in a carrot-based substrate were inoculated in gerbox and treated with F4A fraction 25, 50, 100 e 200 µg.mL^{-1}. At the end of 55 days of incubation, it was possible to observe increasing inhibition of the carpogenic germination of sclerotia, with 100% inhibition in the concentration of 200 µg.mL^{-1}. The antifungal activity of F4A was also tested on the infection of white mold in detached leaves of common bean. The most efficient treatment for disease control was preventive + curative, in which 100% of the inhibition of fungal infection occurred at a concentration of 100 µg.mL^{-1}. The F4A fraction showed great potential for controlling *S. sclerotiorum*, however new strategies to decrease phytotoxicity must be considered.

Keywords: white mold, sclerotia, common beans, antimicrobial.

1. INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a filamentous cosmopolitan and necrotrophic fungus that causes the disease known as white mold or white rot, which is carried by the soil. It has low specificity with a wide range of hosts, over 400 species of plants, responsible for serious annual losses estimated in US$1.47 billion of many economically important crops, including soybeans (Lehner et al., 2017), beans, potato, and canola (Boland and Hall, 1994; Wang et al., 2019).

The characteristics of the disease are the development of white mycelium on stems and fruits, and the formation of sclerotia in infected tissues of the host plant or the soil. The fungus can pass 90% of its life cycle in the form of sclerotia, a form of resistance with internal aggregates of hyphae and an outer layer containing melanin, surviving for up to eight years, or until the moment it finds adequate conditions for germination. This can be myceliogenic, in which hyphae will be produced, directly infecting plant tissues, or carpogenic, with the production of apothecium, which releases infectious ascospores. The massive reproductive potential, together with long-term survival capacity, makes sclerotia central components in the epidemiology of the disease (Willbur et al., 2019; Xia et al., 2020). The management of white mold is based on crop rotation and chemical control with fungicides. Therefore, the use of fungicides cause contamination in the environment, as well as increasing the frequency and distribution of resistant isolates (Xia et al., 2020), making the development of new products important, mainly of natural origin.

Many bacteria use their secondary metabolism to produce bioactive compounds to reduce competition for nutrients in the natural environment. The complex nature of these biomolecules often makes the growth of competing species impossible, conditioning an adaptive advantage to the microorganisms that produce them (Dror et al., 2020). Members of the genus *Pseudomonas*, including pathogenic and non-pathogenic strains, can produce various extracellular secondary metabolites. These metabolites have different properties, they function as virulence factors, siderophores, biosurfactants, and antimicrobial agents, as well as in cell-cell signaling (Keswani et al., 2020).

These metabolites allow *Pseudomonas* spp. to adapt to different environments, colonize different hosts, and compete with other species of microorganisms. Secondary metabolites produced by *Pseudomonas* spp. show strong bioactivity, including phenazines, pyrrolnitrin antibiotics, pyo compounds, indole derivatives, peptides, glycosides, lipids and aliphatic compounds (Gross and Loper, 2009). These compounds have received interest from researchers due to their antibiotic, antitumor, antifungal properties (Bilal et al., 2017).

The F4A fraction is composed of secondary metabolites produced by *P. aeruginosa* LV strain, phenazine-1-carboxamide (PCN), phenazine-1-carboxylic acid (PCA), indolinone (IND), and fluopsin C (Navarro et al., 2019; Pistori et al., 2018). Previous studies carried out by the Microbial Ecology Laboratory of the State University of Londrina demonstrated the antibiotic effect of the secondary metabolites of the strain LV against bacterial phytopathogenic species, as *Xanthomonas* species and *Candidatus Liberibacter* (De Oliveira et al., 2011; De Oliveira et al., 2016; Lopes et al., 2012; Munhoz et al., 2017; Pistori et al., 2018; Silva Vasconcellos et al., 2014; Spago et al., 2014), and PCA antifungal activity on the phytopathogenic fungus *Botrytis cinerea* (Simionato et al., 2017).

In this context, the present study had as objective to evaluate the antifungal activity of F4A fraction, produced by *Pseudomonas aeruginosa* strain LV, on the carpogenic germination of *S. sclerotiorum* sclerotia and its potential infection control in bean plants.

2. MATERIAL AND METHODS

2.1 Microorganisms

The antagonist bacteria *P. aeruginosa* LV strain was isolated from an old citrus canker lesion on orange leaves (*Citrus sinensis* cv. Valencia) in Astorga - PR, Brazil (Rampazo, 2004). The genome sequence was deposited in GenBank under accession number CP058323 (BioProject PRJNA450135, BioSample SAMN08930812). The bacterial stocks were maintained in glycerol 40% at -20 °C and in liquid nitrogen.

The phytopathogen *S. sclerotiorum* was kindly provided by Prof. Dra. Maria Isabel B. Peña, Laboratory of Phytopathology and Agricultural Sciences, UEL, and stored in 0.9% saline solution containing agar plugs with fungal mycelium and maintained at 8 °C. The strains were deposited in the Microbial Culture Collection of the Microbial Ecology Laboratory, Londrina State University, Brazil.

2.2 Culture conditions to produce the F4A fraction

The culture conditions to produce metabolites of *P. aeruginosa* LV strain were patented (PI0803350-1 - INPI 09/12/2008; Andrade, 2008), and carried out with some modifications. Aliquots of 500 μL of cell suspension in the logarithmic phase ($D.O._{590nm} = 0.09 = 10^8$ $UFC.mL^{-1}$) were inoculated in gallons of fermentation (Thermo Scientific Nalgene, 10L) containing 5 L of Nutrient Broth added with copper chloride (NB - 2 $g.L^{-1}$ peptone; 1.2 $g.L^{-1}$ of meat extract; 0.005 $g.L^{-1}$ of $CuCl_2.2H_2O$; pH 6.8) and incubated at 28 °C in the dark for 10 days with a continuous supply of microfiltered air (Navarro et al., 2019).

2.3 Obtention of the F4A fraction

After 10 days of fermentation, the bacterial culture was centrifuged at 9000 rpm for 15 min at 4 °C and the cell-free supernatant was concentrated in a drying oven at 60 °C. The supernatant was then subjected to liquid-liquid extraction with dichloromethane in the proportion of 1:2 (v:v) three times in a separating funnel. The dichloromethane phase (DP) obtained was concentrated on a rotary evaporator (Rotavapor R 215, Büchi) under the vacuum system of -80 KPa at 45 °C.

The DP purification process was performed by Vacuum Liquid Chromatography (VLC), carried out on a glass column (350 mm ; 20 mm in diameter) filled with silica gel 60 (0.063-0.200 mm, Macherey-Nagel) coupled to a pump vacuum (~ 150 $mm.Hg^{-1}$). The fractionation of DP was carried out by the mobile phases (v/v),: hexane (100%; F1A and F1B); hexane:dichloromethane (1:1; F2A and F2B), dichloromethane (100%; F3A and F3B), dichloromethane:ethyl acetate (1:1; F4A and F4B) (Munhoz et al., 2017). The F4A fraction was collected and concentrated on a rotary evaporator at 45 °C.

2.4 Production of sclerotia

Sclerotia of *S. sclerotiorum* were produced in different substrates and incubation conditions, to verify which requirements were necessary for better production. In Erlenmeyers with 500 mL capacity, 100 g of rice, potato, and carrot were added separately, plus 50, 20, and 20 mL of distilled water respectively and sterilized for 20 min at 121 °C. PDA plugs (Potato Dextrose Agar) containing *S. sclerotiorum* mycelium were added to the Erlenmeyer, which were incubated for 20 days at 20 °C with two different lighting conditions, total darkness and 12 h day/night photoperiod. After the incubation period, the sclerotia were separated, cleaned and counting manually (Garcia et al., 2012).

2.5 Padronization of carpogenic germination of sclerotia

The sclerotia produced in the different substrates and incubation conditions were tested separately for their carpogenic germination. In transparent polystyrene gerboxes measuring 11x11 cm, 200g of soil (*ferralsol rhodic* - FAO, 1997) sterile by tindalization was added, moistened to 100% field capacity, in which 25 sclerotia (previously disinfected in a 70% alcohol solution and 2% sodium hypochlorite) was buried with a thin layer of soil. The boxes were incubated at 20 °C in different lighting conditions, in the dark and with a photoperiod of 12 h day/night, for 55 days. After the incubation period, the number of sclerotia that germinated cariogenic and how many apothecium were formed by sclerotia was verified (Taylor et al., 2018).

2.6 Inhibition of carpogenic germination by F4A

The gerboxes were prepared as mentioned previously (item 2.5), and 25 sclerotia produced in carrots in the dark, were buried and incubated at 20 °C with a 12 h day/night photoperiod. On the 25th day of incubation, 10 mL of F4A was sprayed on the soil at concentrations 25, 50, 100, and 200 $\mu g.mL^{-1}$, and as a control, water, and water + DMSO 0.5% were used. A second and third application of F4A was performed on the 35th and 45th days of incubation. Monitoring and quantification of carpogenic germination were performed every ten days after the first application of treatments until 55 days of incubation.

2.7 Test on detached leaf

Seeds of beans, *Phaseolus vulgaris* L. were planted in the substrate MECPLANT, and kept in a greenhouse with a controlled temperature of 25 °C. After seed germination, the seedlings were transplanted into 290 cm^3 tubes filled with soil (*ferralsol rhodic* - FAO, 1997) sterile by tindalization. The central leaves of the completely developed trifoliate were detached and transferred to Petri dishes 15 cm in diameter, with cotton moistened in the petiole. PDA plugs of 9 mm diameter containing mycelium of *S. sclerotiorum* were inoculated in the center of each leaf and incubated 20 ºC with a photoperiod of 12 h day/night (Miorini et al., 2019).

The leaves were treated with a preventive, curative, and preventive + curative treatment, which 500 µL per leaf of F4A (diluted in 0.5% DMSO, 0.25% mineral oil, and distilled water) was sprayed in concentrations 20, 40, 60, 80 and 100 $\mu g.mL^{-1}$. As a control, 0.5% DMSO, 0.25% mineral oil, and distilled water were used. In the preventive treatment the F4A fraction was sprayed on the leaves 24 h before the fungal inoculum, while in the curative F4A it was sprayed on the leaves 24, 48, and 72 h after the fungal inoculum. In the preventive + curative treatment, F4A was sprayed 24 h before and 24, 48, and 72 h after the fungal inoculum. The advance of the lesion on the leaves was monitored daily, measuring the diameter (mm) of the fungal mycelial growth.

2.8 Statistical analysis

The experiments were carried out in triplicate for each treatment and the results were evaluated by analysis of variance (ANOVA) using the Tukey test, with a 95% confidence level ($p < 0.05$), in the SigmaPlot 11.0 software.

3. RESULTS

3.1 Production of sclerotia and its carpogenic germination ratio

S. sclerotiorum incubated on a rice-based substrate, in the dark and with photoperiod, provided higher sclerotia yield, with an average of 1054 and 1504 sclerotia respectively. Potato and carrot substrates showed lower sclerotia yield with an average of 250 and 113 sclerotia, respectively. When incubated in the dark and with photoperiod, the carrot-based substrate showed no significant difference in the number of sclerotia produced (Fig. 1).

Concerning lighting conditions, the gerboxes incubated in the dark did not show carpogenic germination, therefore the results presented are only for gerboxes incubated with a photoperiod of 12 h day/night.

The sclerotia that showed the best carpogenic germination were those produced in the carrot-based substrate in the dark, with 100% of the germinated sclerotia and an average of 4.84 apotheciums formed by sclerotia. The sclerotia produced on the potato-based substrate in the dark also showed 100% carpogenic germination, but with an average of 4.13 apotheciums formed by sclerotia. Despite being the substrate that showed the best yield in the production of sclerotia, rice showed low carpogenic germination, with 18% of the germinated sclerotia and an average of 2.21 apotheciums formed by sclerotia (Fig. 2).

3.2 Inhibition of carpogenic germination by F4A

On the 25th day of incubation of sclerotia and the first application of F4A, small stipes had germinated demonstrating the break in the dormancy, being the effective day for the application of the product. On the 35th day of incubation and the second application of F4A, the inhibition of carpogenic germination was a crescent with increasing substance concentration (Fig. 3), and there was necrose in the stipes that germinated on the 25th day of incubation, that is, they were dark, without showing growth or viability characteristics.

On the 45th day of incubation and the third application of F4A, the inhibition of carpogenic germination of sclerotia was even more evident, with a crescent decrease of the germination as the concentration increased, 17%, 67%, 97%, and 100% at concentrations 25, 50, 100 and 200 µg.mL^{-1} respectively. At the end of the experiment with 55 days of incubation and 10 days after the last application of F4A, the inhibition of carpogenic germination was maintained, and the apotheciums that had germinated and were treated with F4A there was necrosed. With 44%, 96%, 100% and 100% inhibition at concentrations 25, 50, 100 and 200 µg.mL^{-1} respectively (Fig. 3).

3.3 Evaluation of the effect of the F4A fraction on the control of *S. sclerotiorum* in detached leaves of common bean

Bean leaves were infected with *S. sclerotiorum* and treated with F4A at concentrations 20, 40, 60, 80, and 100 µg.mL^{-1} in 3 different treatment. In the preventive treatment, the concentration of 100 µg.mL^{-1} was shown to be more efficient, retarding fungal development, however with 96 h of incubation F4A can't prevent the development of the fungus, which covers the leaf completely at the end of the fifth day of incubation (Fig. 4).

In the curative treatment, the fungal development occurs faster, presenting greater difficulty in controlling the infection. The concentration of 100 µg.mL^{-1} was able to delay fungal growth. However, with 72 h of incubation, the fungus started to grow again, covers the leaf completely (Fig. 5).

In the preventive + curative treatment, at 60 µg.mL^{-1} concentration, it is possible to observe a delay in fungal development, followed by 80 µg.mL^{-1}. However, it is not enough to prevent the fungus from continuing its development. The concentration of 100 µg.mL^{-1} controlled the growth of *S. sclerotiorum* from the beginning of the incubation, maintaining the fungal inhibition until the end of the experiment (Fig 6). Nevertheless , the higher and necessary concentrations to control the fungal infection cause phytotoxicity in the treated leaves, which have necrotic spots due to their extension.

4. DISCUSSION

S. sclerotiorum (Lib.) de Bary is one of the most devastating and cosmopolitan plant pathogens. Sclerotias make an important role in disease cycles, as they are the source of inoculum and the main structures for long term survival. The massive reproductive potential, with the long-term survival capacity, makes sclerotia a central component of the epidemiology of the disease (Wang et al., 2019).

When inoculated in rice, *S. sclerotiorum* showed higher sclerotia production, with an average yield of 1300 structures; when inoculated in potato and carrot the yield fell drastically, with an average of 250 and 113 sclerotia respectively, data similar to those found in the work of Ethur et al., (2016). The difference in the sclerotia yields in the different culture media can be explained by the fact that rice has a higher carbohydrate content (NEPA, 2011), and possibly because it has a greater contact surface compared to the other substrates. These data corroborate with the results of Garcia et al., (2012), who verified that substrates with a high carbohydrate content resulted in higher fresh weight and number of sclerotia Budge and Whipps, (1991) tested different concentrations of sucrose and found that the higher the concentration of sugar, the more sclerotia were formed.

The sclerotia are resting structures that present a reduced level of hydration. In the early stages of sclerotia development, droplets are visible on the hyphae of *S. sclerotiorum*, these droplets are exudates composed mainly of water and soluble carbohydrates. While carbohydrates are being excreted, part of it is being converted into insoluble carbohydrates, which will be used for storage and structural components (Cooke, 1969; Wang et al., 2018). Therefore, possibly substrates with higher amounts of carbohydrates have a greater supply of conditions for the formation of sclerotia.

However, when carpogenic germination was tested, the sclerotia produced in carrot and potato showed better germination, with apothecium formation in 100% of the sclerotia tested; the sclerotia produced in rice showed only 18% germination of the incubated sclerotia. As in the work of Budge and Whipps (1991), in which sclerotia produced in the lowest concentrations of carbohydrates germinated more quickly with a greater apothecium production. However, it is not known for certain why this happens.

The F4A fraction showed excellent activity on the carpogenic germination of *S. sclerotiorum* sclerotia, completely inhibiting the formation of apothecium in sclerotia treated with 200 µg.mL^{-1} of the bioactive metabolite. Studies using natural compounds on the inhibition of carpogenic germination of *S. sclerotiorum* sclerotia, mostly use plant extracts. In the study by Monteiro et al., (2012), the extracts used were ineffective, with no antifungal activity on the germination of sclerotia; Ojaghian et al., (2014) treated sclerotia with 2g.L^{-1} of ginger extract, reaching up to 75% of the inhibition of carpogenic germination, lower data and with a concentration 1000 times higher than that found in our study. Sumida et al., (2018) used methanolic extract produced by *Trichoderma asperelloides*, which showed antifungal activity, but in a reduced form, with only 40% inhibition of carpogenic germination of sclerotia at a concentration of 4 mg.mL^{-1}. The antifungal action of F4A possibly occurs due to the action of phenazine and fluopsin C present in the fraction. Phenazins are oxidized in the cell generating reactive oxygen species (ROS), which have as their main target mitochondria (Briard et al., 2019).

The treatment of bean leaves, infected with *S. sclerotiorum*, with F4A proved to be effective in controlling the development of white mold. However, the higher concentrations necessary to control fungal infection cause phytotoxicity in the treated leaves. This phytotoxicity is possibly due to fluopsin C present in the F4A fraction, an organocupric compound, produced by the strain LV during the remediation of copper present in the culture medium (Gionco et al., 2017). Fluopsin C is a potent antimicrobial compound but has shown moderate toxicity in animal cell culture (Navarro et al., 2019), there is still no report on the level of selectivity that fluopsin C can present in plants. Copper, present in the molecule, is a plant micronutrient that becomes phytotoxic in high concentrations. Stunted growth, leaf epinastia, and chlorosis are visible symptoms of strong metal toxicity. In lower concentrations, these macroscopic symptoms are less pronounced or even absent, but cellular processes can be affected (Chatterjee and Chatterjee, 2000; Cuypers et al., 2002).

5. CONCLUSIONS

The F4A fraction, a secondary metabolite produced by *P. aeruginosa* strain LV, has great antifungal potential in the control of *S. sclerotiorum*, in addition to inhibiting the carpogenic germination of sclerotia, as well as causing the death of treated apotheciums. When applied to the leaves of infected bean plants, it controls fungal development. However, a phytotoxicity effect was observed in some concentrations used, possibly by the presence of fluopsin C. New formulations should be studied to improve the selectivity and reduce the phytotoxicity of the F4A fraction.

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FIGURES LEGEND

Figure 1 - Number of sclerotia produced in potatoes, carrots and rice, under different light conditions. Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2 - Percentage of carpogenic germination of sclerotia produced in potatoes, carrots and rice, in the dark and with photoperiod 12 h day/nigth (data from gerboxes incubated only with photoperiod). Bars with different letters are significantly different ($p \leq 0.05$).

Figure 3 - Percentage of carpogenic germination of sclerotia from *S. sclerotiorum*, treated with F4A after 35, 45 and 55 days of incubation. Bars with different letters are significantly different ($p \leq 0.05$).

Figure 4 - Fungal growth diameter (mm) in detached bean leaves infected with *S. sclerotiorum* and treated with sprinkling of F4A at concentrations 20, 40, 60, 80 and 100 µg.mL^{-1} in a preventive treatment.

Figure 5 - Fungal growth diameter (mm) in detached bean leaves infected with *S. sclerotiorum* and treated with sprinkling of F4A at concentrations 20, 40, 60, 80 and 100 µg.mL^{-1} in a curative treatment.

Figure 6 - Fungal growth diameter (mm) in detached bean leaves infected with *S. sclerotiorum* and treated with sprinkling of F4A at concentrations 20, 40, 60, 80 and 100 µg.mL^{-1} in a preventive and curative treatment.

Figure 1

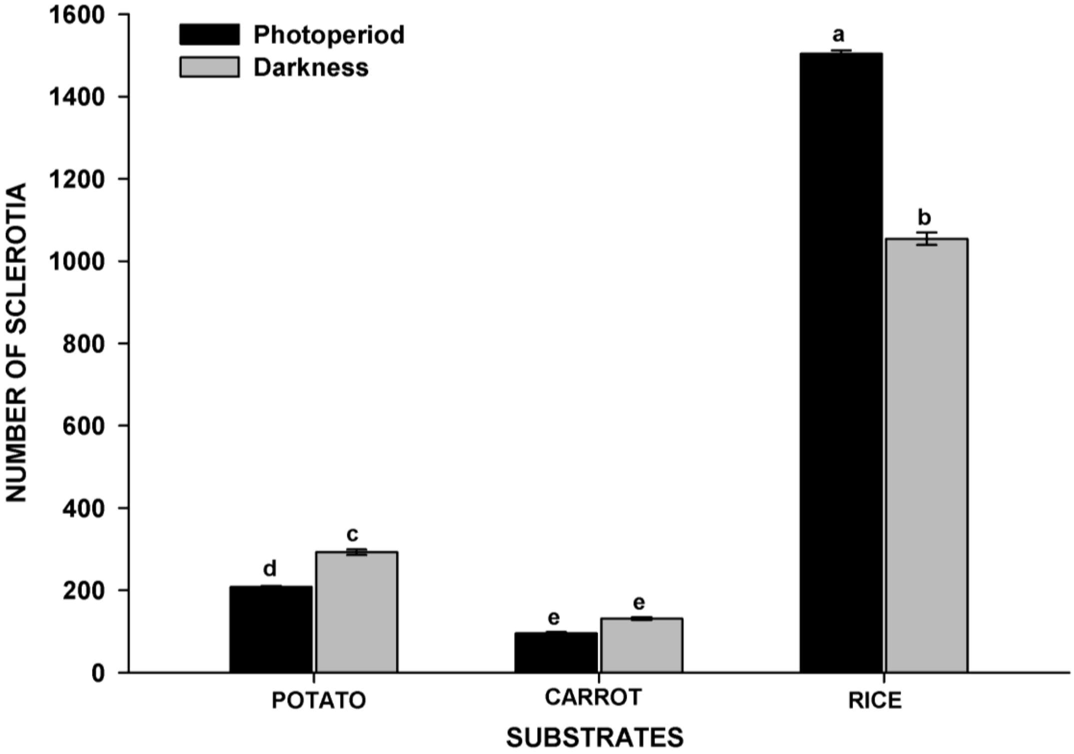

Photoperiod
Darkness
NUMBER OF SCLEROTIA
1600
1400
1200
1000
800
600
400
200
0
a
b
c
d
e
e
POTATO
CARROT
RICE
SUBSTRATES

Figure 2

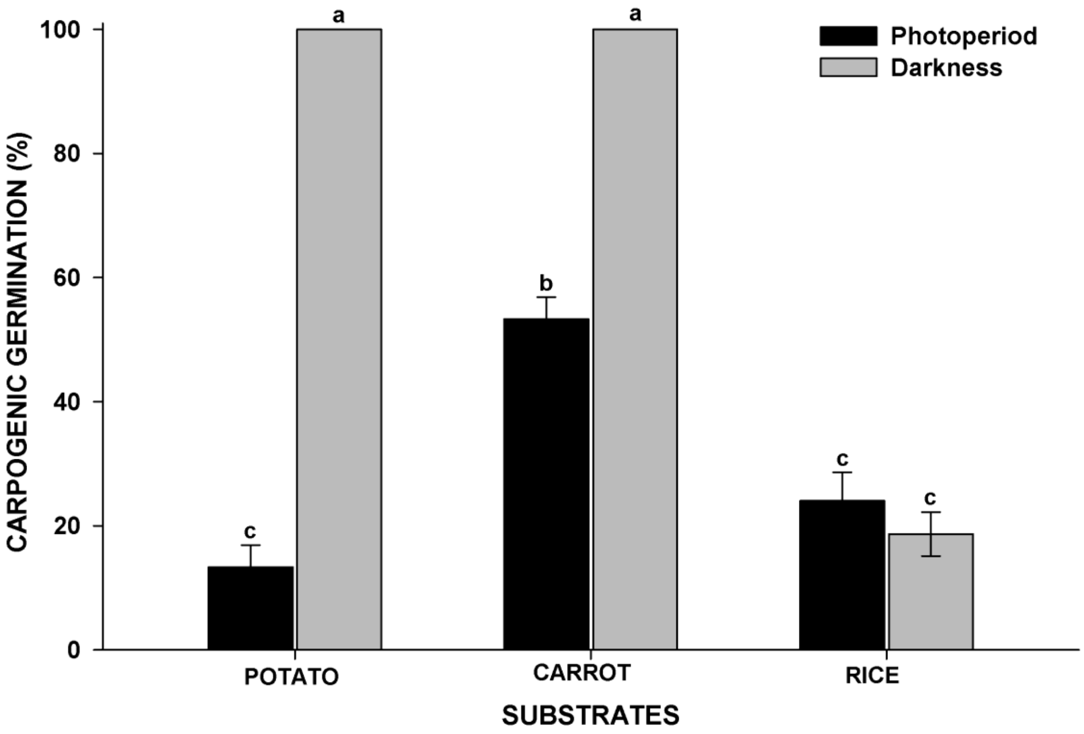

100
80
60
40
20
0
CARPOGENIC GERMINATION (%)
a
a
b
c
c
c
Photoperiod
Darkness
POTATO
CARROT
RICE
SUBSTRATES

Figure 3

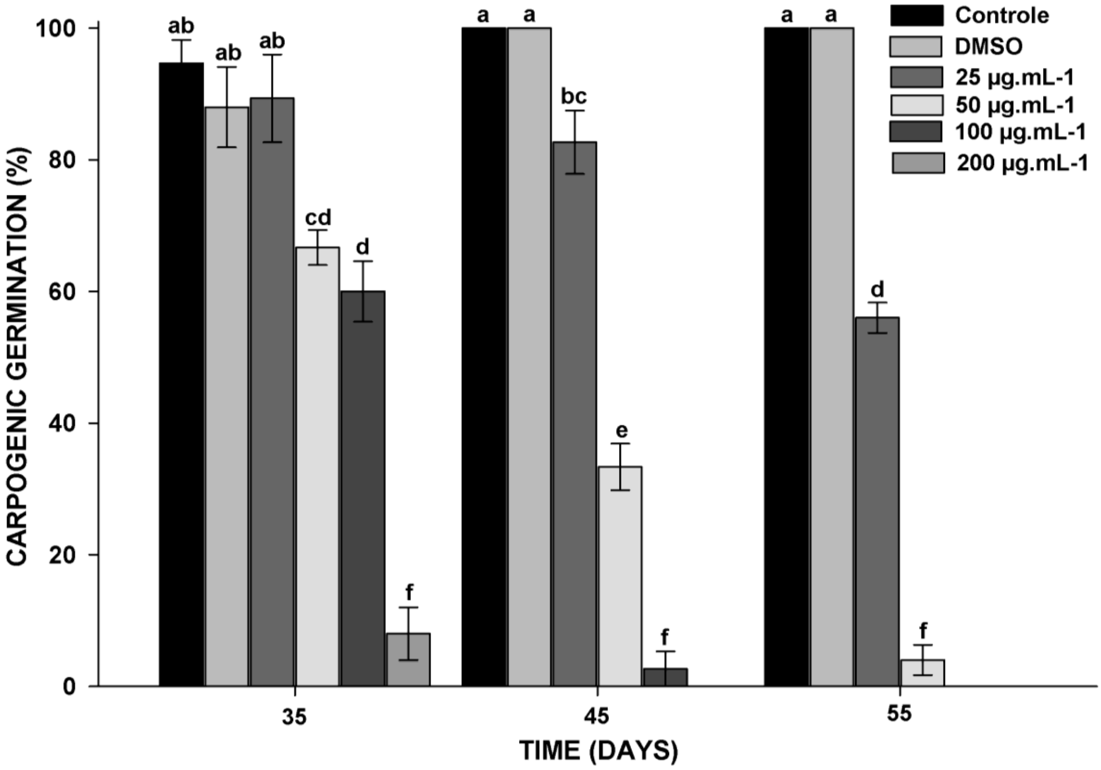

CARPOGENIC GERMINATION (%)
0
20
40
60
80
100
35
45
55
TIME (DAYS)
Controle
DMSO
25 µg.mL-1
50 µg.mL-1
100 µg.mL-1
200 µg.mL-1
ab
ab
ab
cd
d
f
a
a
bc
e
f
a
a
d
f

Figure 4

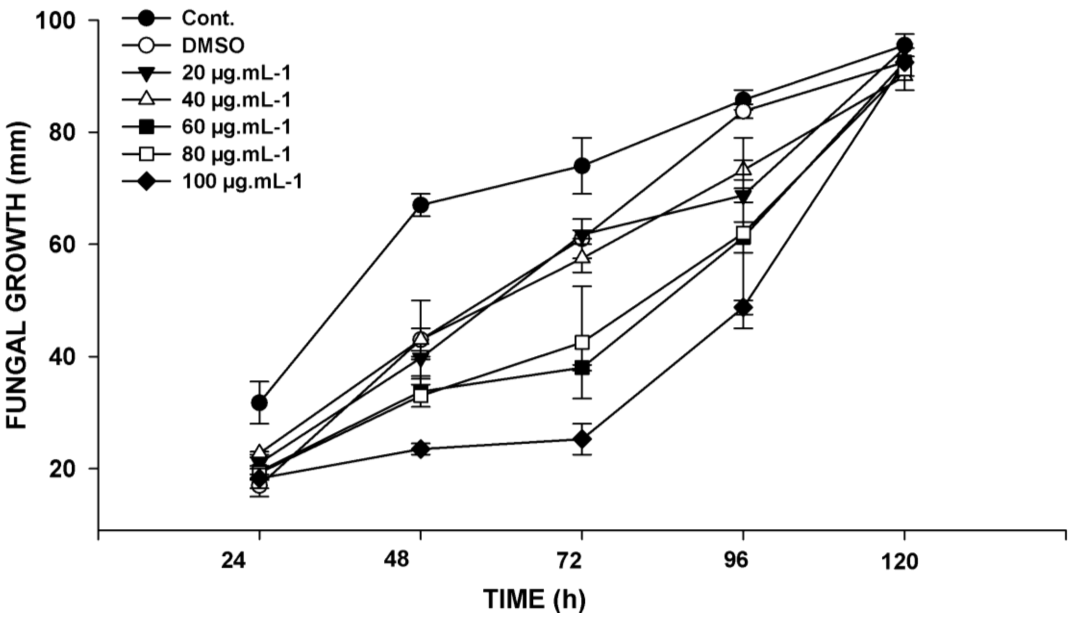

Cont.
DMSO
20 µg.mL-1
40 µg.mL-1
60 µg.mL-1
80 µg.mL-1
100 µg.mL-1
FUNGAL GROWTH (mm)
100
80
60
40
20
24
48
72
96
120
TIME (h)

Figure 5

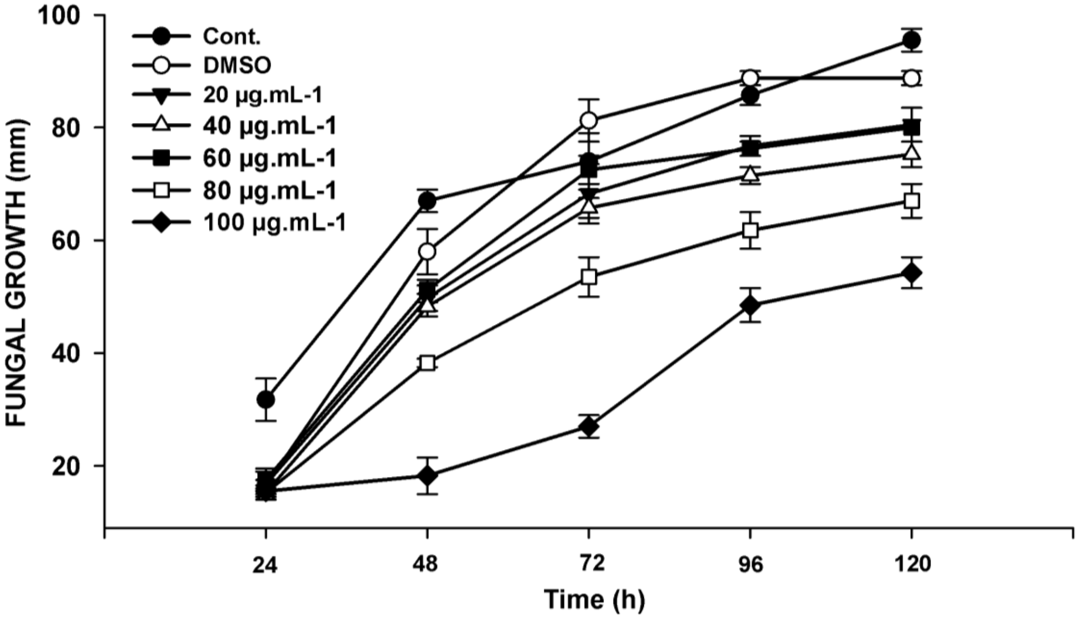
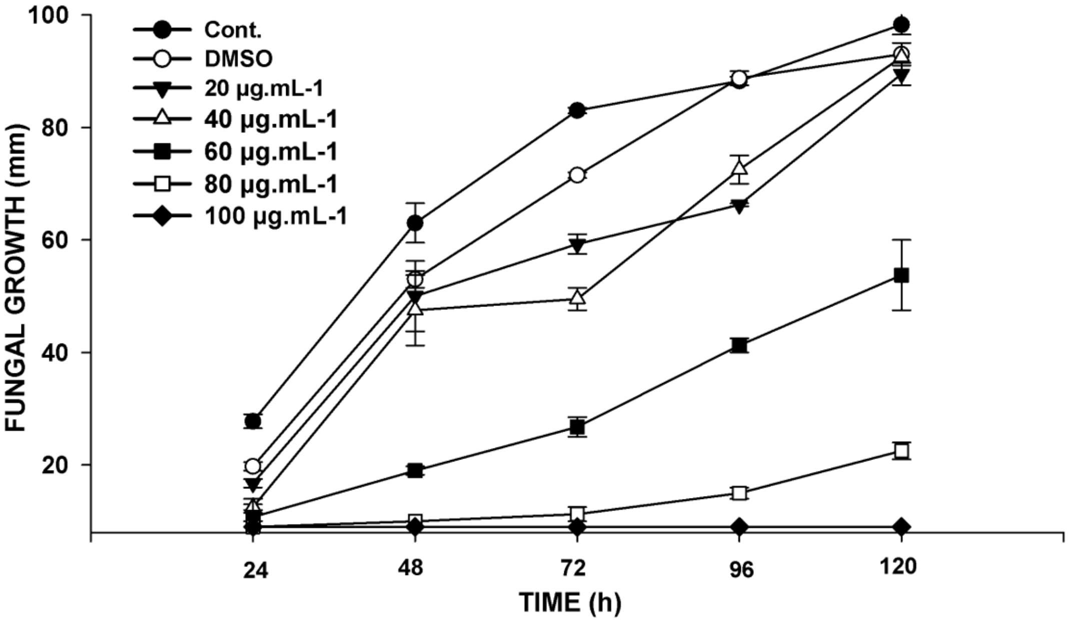

100
80
60
40
20
FUNGAL GROWTH (mm)
Cont.
DMSO
20 µg.mL-1
40 µg.mL-1
60 µg.mL-1
80 µg.mL-1
100 µg.mL-1
24
48
72
96
120
Time (h)

Figure 6


100
80
60
40
20
FUNGAL GROWTH (mm)
24
48
72
96
120
TIME (h)
Cont.
DMSO
20 µg.mL-1
40 µg.mL-1
60 µg.mL-1
80 µg.mL-1
100 µg.mL-1

ARTIGO II

Control of *Sclerotinia sclerotiorum* by extracellular metabolites from *Burkholderia metallica*

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ABSTRACT

Sclerotinia sclerotiorum (Lib.) de Bary is a phytopathogenic filamentous fungus that produces resistance structures called sclerotia, which can remain viable in the soil for years. White mold has a range of more than 400 species of host plants and is one of the main pathogens of common beans, which can affect seed yield. The use of chemical pesticides in the treatment of the disease causes contamination on environment and selects resistant fungal, so the development of new products of natural origin is important. In this context, the present study had as objective evaluate the antifungal action of extracellular secondary metabolites produced by *Burkholderia metallica* strain RV7S3 in controlling *in vitro* and *in vivo* the phytopathogen *Sclerotinia sclerotiorum*. For this, sclerotia were inoculated in gerbox, treated with the dichloromethane phase (DP) of the bacterial secondary metabolites, and after 55 days of incubation the concentration that showed 100% inhibition of carpogenic germination was 1,000 µg.mL^{-1}. The DP phase showed good results in the treatment of bean plants infected with *S. sclerotiorum*, maintaining 40% of viable plants treated with 1,000 µg.mL^{-1} without causing phytotoxicity. Analysis by scanning electron microscopy showed that in treated plants, fungal development is inhibited, with intense branching and hyphae retraction, which have rounded extremities. The treatment with secondary metabolites produced by the RV7S3 strain showed excellent results without causing damage to the plant, however future studies need to be carried out to optimize production and increase the yield of the FRV7 fraction, as it would present a better percentage of plant survival in lower concentrations. of product.

Keywords: sclerotia, scanning electron microscopy, white mold, gerbox.

INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a filamentous cosmopolitan fungus also known as white mold, has no specificity of hosts (Boland and Hall, 1994). Symptoms associated with the disease include rot or wilt that can cause host plants to collapse and die. The wide range of hosts makes it difficult to control the disease, especially because the fungus produces resistance structures called sclerotia, which can remain viable in the soil for years and under appropriate environmental conditions to germinate in the form of mycelium or producing apotheciums that release millions of ascospores. These processes allow the fungus to act as a pathogen transported by soil and air, respectively (Bardin and Huang, 2001; Rothmann and McLaren, 2018).

S. sclerotiorum is one of the main pathogens of common beans (*Phaseolus vulgaris* L.), which can affect seed yield in number, weight, or total number of pods per plant. Recommended management strategies are crop rotation, wider spacing between sowing lines, and treatment with chemicals (Vieira et al., 2010), but the latter can cause contamination in the environment, as well as increasing the frequency and distribution of fungal isolates resistant (Li et al., 2006).

Secondary metabolites are relatively low molecular weight molecules, biosynthesized in specialized pathways from primary metabolites, they are not directly involved in the growth, development or reproduction of producing microorganisms, but they can play important ecological roles in interactions with other organisms (Tyc et al., 2017; Vinale et al., 2014). The enormous structural diversity of these compounds also explains their wide range of activities and functions, such as mediating communication within a species or between different species, in defense against competitors, acquisition of nutrients, and even symbiotic interactions (Macheleidt et al., 2016; Sid Ahmed et al., 2003). Many bacterial species produce secondary bioactive metabolites, of which members of the genera, *Pseudomonas*, *Bacillus, Serratia, Streptomyces*, and *Burkholderia* are prominent sources (Keswani et al., 2020; Thapa and Grove, 2019).

Burkholderia is an incredibly diverse, versatile, and cosmopolitan genus of Gram-negative bacteria, a diversity of isolates of this genus are known for their ability to produce secondary metabolites with properties, antimicrobial, antifungal, herbicides and/or insecticides (Depoorter et al., 2016). Many of the metabolites of *Burkholderia* that have antimicrobial activity have low molecular weight, such as pyrrolinitrin(El-Banna and Winkelmann, 1998), phenazines (Pierson and Pierson, 2010), siderophores (Darling et al., 1998); as well as antimicrobial peptides, such as xylocandins (Meyers et al., 1987), occidiofungins (Lu et al., 2009) and burkholdins (Thomson and Dennis, 2012). Therefore, microbial metabolites can be another tool in the control and assist in problems of resistance and contamination of pesticides due to the versatility in the structure and low toxicity to non-target organisms. In this context, the present study had as objective evaluate the antifungal action of extracellular secondary metabolites produced by *B. metallica* strain RV7S3 in controlling *in vitro* and *in vivo* the phytopathogen *S. sclerotiorum*.

MATERIAL AND METHODS

Microorganisms

The antagonist bacteria *B. metallica* RV7S3 strain was isolated from soil samples from Rio Verde, Itararé - SP, Brazil. The bacterial stocks were maintained in glycerol 40% at -20 °C and in liquid nitrogen. The phytopathogen *Sclerotinia sclerotiorum* was kindly provided by Professor Dra Maria Isabel B. Peña, Laboratory of Phytopathology and Agricultural Sciences, UEL, and stored in 0.9% saline solution (9 g.L^{-1} of NaCl) containing agar plugs with mycelium fungal and maintained at 8 °C. The strains were deposited in the Microbial Culture Collection of the Microbial Ecology Laboratory, Londrina State University, Brazil.

Culture conditions to produce of the fraction with antifungal action

The bacterium *B. metallica* RV7S3 strain was incubated on Nutrient Agar at 28 °C for 24 h. Aliquots of 1 ml of cell suspension at a concentration of 1.5x10^{8} CFU.mL^{-1} (McFarland scale) were inoculated in 1 L of Nutrient Broth in Erlenmeyer 2 L and kept at 28 °C under orbital shaking at 150 rpm for 6 days (Modolon, 2019).

Extraction and purification of RV7S3 metabolites

The bacterial culture was centrifuged at 9000 rpm for 15 min at 4 °C and the cell-free supernatant was concentrated in a drying oven at 60 °C. The supernatant was subjected a liquid-liquid extraction with dichloromethane in the proportion of 1:2 (v/v), ten times, in a separating funnel. The dichloromethane phase (DP) obtained was concentrated on a rotary evaporator (Rotavapor R 215, Büchi) under the vacuum system of -80 KPa at 45 °C.

The process of purifying the FRV7 bioactive fraction from the DP was carried out according to Modolon , (2019). A Flash Chromatography (FC), performed on a glass column (100 mm; 10 mm in diameter) filled with silica gel 60 (0.04-0.063 mm, Macherey-Nagel) and with the mobile phase n-hexane:ethyl acetate (7:3). The collected fractions were subjected to Thin Layer Chromatography (TLC) (ALUGRAM® Xtra SIL G/UV$_{254}$), with an eluent system composed of n-hexane:ethyl acetate (7:3). The fraction with antifungal activity has a retention factor (Rf) of 0.45.

Disc diffusion test

Sterile paper discs (9 mm diameter) were impregnated with 10 µL of the DP and the purified fraction FRV7 diluted in chloroform in the concentrations 125, 250, 500 µg.disc^{-1} and 6.25, 12.5, 25 µg.disc^{-1},respectively, as control discs were used to which only the diluent (chloroform) was applied. The discs were inoculated at opposite ends, 10 mm from the edge of the Petri dish (85 mm) containing Potato Dextrose Agar medium. Agar plugs containing active mycelium of *S. sclerotiorum* grown in PDA, were inoculated in the center of each plate and incubated at 20 °C with a photoperiod of 12 h day/night for 7 days. The radius of fungal mycelial growth was measured daily (mm) (Simionato et al., 2017).

Minimum Inhibitory Concentration

The MIC values for S. sclerotiorum were determined using a 10-fold serial dilution method in Petri dishes with PDA, where 10 mL of PDA were mixed in melted agar in the respective concentrations of compound DP (3.9; 7.8; 15.6; 31.25; 62.5; 125; 250; 500; 1000 µg.mL-1) and FRV7 (0.098; 0.2; 0.39; 0.78; 1.56; 3.13; 6.25; 12.5; 25; 50 µg.mL-1). Agar plugs of 6 mm diameter containing the *S. sclerotiorum* mycelium were inoculated in the center of each plate and incubated at 20 °C for 7 days. The diameter of the fungal mycelial growth was measured daily (mm) (Simionato et al., 2017).

Inhibition of carpogenic germination of *S. sclerotiorum*

In a transparent polystyrene gerboxes measuring 11x11cm, 200g of soil (*ferralsol rhodic* - FAO, 1994) sterile by tindalization was added, moistened to 100% field capacity, in which 25 sclerotia (previously disinfected in a 70% alcohol solution and 2% sodium hypochlorite) were buried with a thin layer of soil. The boxes were incubated at 20 °C with a 12 h day/night photoperiod for 55 days. The sclerotia used were produced in chopped carrots, which were inoculated with agar plugs containing *S. sclerotiorum* mycelium and incubated at 20 °C in the dark for 20 days (Costa and Costa, 2006; Garcia et al., 2012).

On the 25th day of incubation, DP was diluted in 0.5% DMSO + distilled water in concentrations 125, 250, 500, and 1000 µg.mL^{-1}, and 10 mL were sprayed on the soil. As a control, water and water + 0.5% DMSO was used. A second and third application of DP was performed on the 35th and 45th days of incubation. Monitoring and quantification of carpogenic germination were performed every ten days after the first application of treatments until 55 days.

Test on detached leaf

The central leaves of the completely developed trifoliate of bean plants were detached and transferred to Petri dishes of 15 cm in diameter, with cotton moistened in the petiole. PDA plugs of 9 mm diameter containing mycelium of *S. sclerotiorum* were inoculated in the center of each leaf and incubated 20 ºC with a photoperiod of 12 h day/night (Farzand et al., 2019b; Miorini et al., 2019).

The leaves were treated with a preventive, curative and preventive + curative treatment, in which 500 µL per leaf DP diluted in 0.5% DMSO, 0.25% mineral oil and distilled water was sprayed in concentrations 125, 250, 500 and 1000 µg.mL^{-1}. As a control, 0.5% DMSO, 0.25% mineral oil and distilled water were used. In the preventive treatment the fraction was sprayed on the leaves 24 h before the fungal inoculum, while in the curative the fraction was sprayed on the leaves 24, 48 and 72h after the fungal inoculum. In the preventive + curative treatment, DP was sprayed 24h before and 24, 48 and 72h after the fungal inoculum. The advance of the lesion on the leaves was monitored daily, measuring the diameter (mm) of the fungal mycelial growth.

Greenhouse test

Seeds of bean, *Phaseolus vulgaris* L. pv were planted in the substrate MECPLANT (composition: pine bark, vermiculite, acidity concealer and mineral fertilizers), and kept in a greenhouse at a controlled temperature of 25 °C. After seed germination, the seedlings were transplanted into 290 cm^3 tubes filled with soil (*ferralsol rhodic* - FAO, 1994) sterile by tindalization.

The experimental design was a randomized block design (RBD) with a preventive + curative regime using 4 concentrations of DP and 5 replicates. For this, 5 mL of syrup containing DP were sprayed per plant, 24h before and 24, 48 and 72 h after the fungal inoculum. The DP fraction was diluted in 0.5% DMSO, 0.25% mineral oil and distilled water in concentrations 125, 250, 500 and 1000 $\mu g.mL^{-1}$. As a positive and negative control, plants inoculated and not inoculated with the fungus were used, respectively, and treated with the diluent.

The first application of treatments was carried out when the plants were in stage V3, where the 1st trifoliate is fully developed, then the plants were kept in a humidity chamber wrapped by transparent plastic bags and maintained at 20 °C with photoperiod 12 h day/night until the end of the experiment. Twenty-four hours after preventive application, agar plugs containing active mycelium of *S. sclerotiorum* grown in PDA were inoculated into the axial buds of the primary leaves. Then, the 1st, 2nd and 3rd application of DP (curative system) were performed with an interval of 24h between each application. The next day after the 3rd application, the plants were removed from the humidity chamber and the percentage of death/survival was estimated.

Scanning Electron Microscopy

To analyze the behavior of *S. sclerotiorum* front the antifungal effect of the metabolites produced by the RV7S3 strain, plugs of 5 mm in diameter were collected from the test performed on a detached leaf with a preventive + curative system in concentrations 500 and 1000 $\mu g.mL^{-1}$, leaf control and DMSO + mineral oil control, 24 h after the 3rd curative application of DP. The collected samples were kept in a fixative solution composed of 2.5% glutaraldehyde, 2% paraformaldehyde and 0.1M sodium cacodylate buffer (pH 7.2) at 4 °C for 24 h. After fixing the samples were washed 3 times in 0.1M sodium cacodylate buffer (pH 7.2) for 10 min. Then, dehydrated in a series of ethanol (30, 50, 70, 90 and 100%) for 10 min for 3 times, then dried in a critical point with CO^2 (BALTEC CPD 030 Critical Point Dryer), coated with gold (BALTEC SDC 050 Sputter Coater) and observed in Scanning Electron Microscopy (SEM) (FEI Quanta 200 operating at 30 kV).

Statistical analysis

The experiments were carried out in triplicate for each treatment and the results were evaluated by analysis of variance (ANOVA) using the Tukey test, with a 95% confidence level ($p < 0.05$), in the SigmaPlot 11.0 software.

RESULTS

Disc diffusion test

To evaluate the antifungal activity of the secondary metabolites produced by *B. metallica* strain RV7S3, first tested using the disk diffusion method. The DP fraction retarded the growth of *S. sclerotiorum* since the first concentration tested, 125 µg.mL^{-1}. However, not enough to prevent the fungus from taking the plate in 120 h of incubation. The 250 and 500 µg.mL^{-1} concentrations showed antifungal activity with 73% and 84% inhibition, respectively, at the end of the seventh day of incubation (Fig. 1).

In tests using the FRV7 fraction, concentrations 6.25 and 12.5 µg.mL^{-1} delayed the development of *S. sclerotiorum* from the first day of incubation, maintaining the fungal growth at baseline levels. However, with 144 h of incubation the fungus got to overcome the fraction disc, growing across the plate at the end on the seventh day. The concentration of 25 µg.mL^{-1} maintained the fungal inhibition until the sixth day of incubation, showing small growth at the end of the evaluation (Fig. 2).

Minimum Inhibitory Concentration

With 72 h of incubation, *S. sclerotiorum* completely has grown up in the control plates. In the plates with the concentration of 15.63 µg.mL^{-1} of DP, and the following ones, did not show fungal growth. However, over the days *S. sclerotiorum* overcame some concentrations. At the end of 168 h of incubation, the fungus took the plates with concentrations of 3.9; 7.81; 15.63; 31.25 µg.mL^{-1}. The concentration of 62.5 µg.mL^{-1} showed 98.5% inhibition and the minimum inhibitory concentration (MIC) for DP was 125 µg.mL^{-1} with 100% fungal inhibition in agar (Fig. 3).

The FRV7 fraction showed antifungal activity with lower concentrations in comparison to FD. With 72 h of incubation, *S. sclerotiorum* has grown up completely in the control plates, concentrations from 0.098 to 0.78 µg.mL^{-1} maintained the fungal growth at baseline levels. However, over the days the fungus overcame these concentrations. At the end of 168 h of incubation, the fungus grown up completely at concentrations 0.098; 0.2; 0.39; 0.78; 1.56; 3.13; 6.25; 12.5 µg.mL^{-1}. In the concentration of 25 µg.mL^{-1}, the inhibition was 30.8% and the MIC was 50 µg.mL^{-1} showing 100% of the fungal inhibition (Fig. 4).

Inhibition of carpogenic germination

After 25 days of incubation, small stipes germinated with the breaking of the sclerotia dormancy, so that day the DP was sprayed on the soil of each gerbox. On the 35th day of incubation, the inhibition of carpogenic germination was crescent with an increasing concentration of the applied fraction (Fig. 5).

On the 45th day of incubation, the inhibition of carpogenic germination was 17.3%; 50.7%; 82.7% and 100% at concentrations 125, 250, 500 and 1000 µg.mL^{-1}, respectively. Some stipes and apotheciums that germinated and were treated with DP presented necrosed without showing growth or viability characteristics. Therefore, at the end of the experiment, with 55 days of incubation and 10 days after the last application of DP, the percentage of inhibition of the sclerotia germination was even higher, with 68%; 90.7 and 100% at concentrations of 250, 500 and 1000 µg.mL^{-1} respectively (Fig. 5).

Treatment of detached bean leaves

In the preventive system, the concentrations 125, 250 and 500 µg.mL^{-1} showed no inhibition on the fungus, following in parallel with the development of the controls. The 1000 µg.mL^{-1} concentration showed up to be more efficient, delaying fungal development. However, with 72 h of incubation DP was unable to prevent the progression of fungal development, which took the leaf completely at the end of the seventh day of incubation (Fig. 6).

The curative system was not efficient in controlling the infection because the development of the fungus was close to that of the control. The 1000 µg.mL^{-1} concentration slightly retarded the growth, but not enough to prevent the fungus from taking the leaf completely (Fig. 7).

In the preventive + curative treatment, at a concentration of 500 µg.mL^{-1}, it is possible to see a slight delay in the development of *S. sclerotiorum*. However, the fungus continues to grow, taking the leaf on the sixth day of incubation. The concentration of 1000 µg.mL^{-1} inhibited fungal development, maintaining it until the end of the experiment (Fig. 8).

Control of *S. sclerotiorum* infection in common bean

From the results obtained in the detached leaves test, it was decided to use the preventive + curative system in the treatment of *S. sclerotiorum* infection in bean plants. Twenty-four hours after inoculation, the fungus showed white and cottony hyphae on the plug, as well as small lesions in the around region in the inoculum. On the second evaluation day, the fungus caused some petioles to fall.

On the fourth day, 24 h after the last curative application of DP, the positive control was completely infected, with white hyphae throughout the plant; at concentrations 125 and 250 µg.mL^{-1}, the stem was tipped over. However, there were some leaves with a healthy aspect. Three days after removal from the humidity chamber, 20% and 40% of the plants treated with 500 and 1000 µg / mL, respectively, survived the infection by white mold (Fig. 9).

Ultrastructural evaluation

In the positive control sheets, it is possible to observe a cotton carpet, composed of infection hyphae (ih) and branched hyphae (bh) throughout the leaf surface (Fig. 10D). Also, these hyphae showed amorphous extracellular polysaccharides (AEP) associated with their surfaces (Fig. 10E and F). In the leaves treated with 500 µg / mL (Fig. 10G, H and I) it is possible to observe that the carpet of cotton hyphae on the leaf surface is absent (Fig. 10G).

After infecting the inside of the leaf, the hyphae begin to emerge in the regions close to trichomes (tr) and leaf ribs (lr) (Fig. 10G) and come into direct contact with DP present in the adaxial region of the leaves. As a defense strategy, the hyphae avoid touching the leaf surface, growing upwards and using the trichomes as support. However, the leaves continued to be treated with DP (3 curative applications), which caused the retraction of mycelial growth, forming hyphae each time more branched and with their short and rounded ends (Fig. 10H and I).

The absence of AEP associated with hyphae was also observed. The negative control leaves (Fig. 10A, B and C) present healthy cells with the presence of two types of trichome (Fig. 10B) and stomata (Fig. 10C). Figure 10 J, K and L are of the leaves treated with 1000 µg.mL^{-1}, a concentration that completely inhibited the development of *S. sclerotiorum* without causing phytotoxicity in its leaves or lesions caused by white mold infection.

DISCUSSION

The DP and FRV7 fractions produced by *B. metallica* strain RV7S3, showed antifungal activity on *S. sclerotiorum*. However, as expected, the FRV7 fraction showed better inhibition at lower concentrations, due to purification by flash column, selecting only molecules of interest, which exhibit antifungal activity. The MIC of DP and FRV7 on the white mold were 125 µg.mL^{-1} and 50 µg.mL^{-1}, respectively.

There are few reports regarding *B. metallica*, there are some papers of isolates from pulmonary infections (Carraro et al., 2018; Vanlaere et al., 2008) and recent papers of strains isolates from soil samples (Hall et al., 2015; Wang et al., 2018). This species has already been used as an inoculant from a microbial consortium with plant growth promoting activity, which consists of seven bacteria from different groups, among them, *B. metallica* 026_G06, which shows antagonism against fungi: *Monographella nivalis*, *Gibberella zeae, Stagonospora nodorum, Colletotrichum graminicola* and *Penicillium sp*., but without clarifying the mechanism of action and characterization of the active metabolite (Bullis et al., 2018).

Among the metabolites produced by *Burkholderia*, the compound 2-hydroxymethyl-chroman-4-one (Kang et al., 2004) has characteristics similar to the FRV7 fraction, which has a colorless band and has Rf = 0.42 with the eluent system in similar proportions (n-hexane: ethyl acetate (3: 2)) as well as showing antifungal activity on *S. sclerotiorum*.

A previous study by Modolon (2019) demonstrated that *B. metallica* strain RV7S3 showed high antifungal activity for some fungi, and some bacteria like *Ralstonia* sp., *Staphylococcus. aureus., Candida. albicans, Rhizoctonia solani, S. sclerotiorum,* and *Alternaria alternata*. The bioactive molecule of the RV7S3 strain is in the process of identification. The FRV7 fraction showed antifungal activity on *S. sclerotiorum*. However, its yield is very low, 1.35 mg.L^{-1}, therefore only DP was tested on the carpogenic germination of *S. sclerotiorum* and the infection in bean plants.

The sclerotia of *S. sclerotiorum* plays an important role in disease cycles, as they produce inoculum and are the main structures for long-term survival (Wang et al., 2019). Sclerotia of *S. sclerotiorum* treated with DP, it was possible to observe from the first application, the inhibition of carpogenic germination, which was maintained until the end of the evaluation. In addition to inhibiting the formation of apotheciums, this structure necrosed when treated with the bioactive molecule, without showing viability characteristics.

One of the main virulence factors of *S. sclerotiorum* is the production of oxalic acid, which plays an important role in the pathogenesis of white mold infections, as it decreases the pH of infected tissues by increasing the activity of enzymes secreted by the pathogen (Bateman and Beer , 1965), is toxic to host tissues, impairs plant defenses and facilitates colonization (Noyes and Hancock, 1981), in addition to inhibiting the antioxidant system of plants, which causes an increase in reactive oxygen species, causing cell damage (Cessana et al., 2000).

The DP fraction showed good results in the treatment of bean plants infected by *S. sclerotiorum*, keeping 40% of the plants viable without causing phytotoxicity. In SEM analysis it is possible to observe that the fungus hyphae penetrate the plant structures, infecting the intra and intercellular spaces, initiating the infection, and the external development in the plant, forming cottony mycelium, as described by Lumsden and Wergin (1980). In plants treated with PD, fungal development is inhibited, with intense branching and retraction with shortening of the hyphae, which have swollen and rounded ends. Data also found in the work by Chen et al., (2014), in which the observations in SEM revealed that hyphae trated with *Bacillus subitilis* EDR4 strain supernatant, were shortened with irregular and swollen tips.

Other studies have also evaluated the antifungal activity of cumeric acid (Sun et al., 2017) and fengicin produced by *Bacillus amyloliquefaciens* FZB42 (Farzand et al., 2019a), and a volatile organic compound produced by *Bacillus velezensis* VM11 (Massawe et al., 2018), showing shortening of hyphae, however with different morphological changes to our work. The antifungal action of bioactive molecules is still unknown, but it is believed that there is a correlation with the accumulation of reactive oxygen species (ROS), causing harmful effects to cellular components, however new experiments need to be carried out to confirm this hypothesis.

DP showed excellent results, but it is expected that the FRV7 fraction will present even better data with lower work concentrations. For this, future studies need to be performed to optimize the production of the metabolites of *B. metallica* strain RV7S3, improving fraction yield.

CONCLUSION

The DP produced by *B. metallica* strain RV7S3 showed antifungal activity on *S. sclerotiorum*, inhibiting the carpogenic germination of sclerotia and the infection of white mold in bean plants without causing any other side effect, such as phytotoxicity. Likewise, FRV7 purified fraction has a potent antifungal activity with promising data with lower working concentrations and more pronounced effects. However, the low productivity of the fraction did not allow testing in vivo, to alleviate this problem, new studies are being done to optimize the production of FRV7.

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FIGURES LEGEND

Figure 1 - Radius of growth of *S. sclerotiorum* (mm) in disk diffusion test with DP. Bars with different letters are significantly different ($p \leq 0.05$).

Figure 2 - Radius of growth of *S. sclerotiorum* (mm) in disk diffusion test with FRV7. Bars with different letters are significantly different ($p \leq 0.05$).

Figure 3 - *S. sclerotiorum* inhibition curve in the plate dilution test with the DP fraction.

Figure 4 - *S. sclerotiorum* inhibition curve in the plate dilution test with the FRV7 fraction.

Figure 5 - Percentage of carpogenic germination of *S. sclerotiorum* sclerotia, treated with DP after 35, 45 and 55 days of incubation. Bars with different letters are significantly different ($p \leq 0.05$).

Figure 6 - Fungal growth diameter (mm) in bean leaves infected with *S. sclerotiorum* and treated with DP in a preventive system.

Figure 7 - Fungal growth diameter (mm) in bean leaves infected with *S. sclerotiorum* and treated with DP in a curative system.

Figure 8 - Fungal growth diameter (mm) in bean leaves infected with *S. sclerotiorum* and treated with DP in a preventive + curative system.

Figure 9 - Common bean infected with *S. sclerotiorum* and treated in a preventive + curative system with DP. Cont. = Negative control; DMSO = Positive Control; Magnitude, µg / mL.

Figure 10 - Scanning electron microscopy of the antifungal effect of DP against *S. sclerotiorum*. (**A - C**) Negative control; (**D - F**) Positive control; (**G - I**) Treatment with 500 µg / mL of FD; (**J - L**) Treatment with 1000 µg / mL FD. In the magnitudes of 300x (**A, D, G, J**: bar 500 µm), 1,200x (**B, E, H, K**: bar 100 µm) and 3,000x (**C, F, I, L**: bar 20 µm).

Figure 1

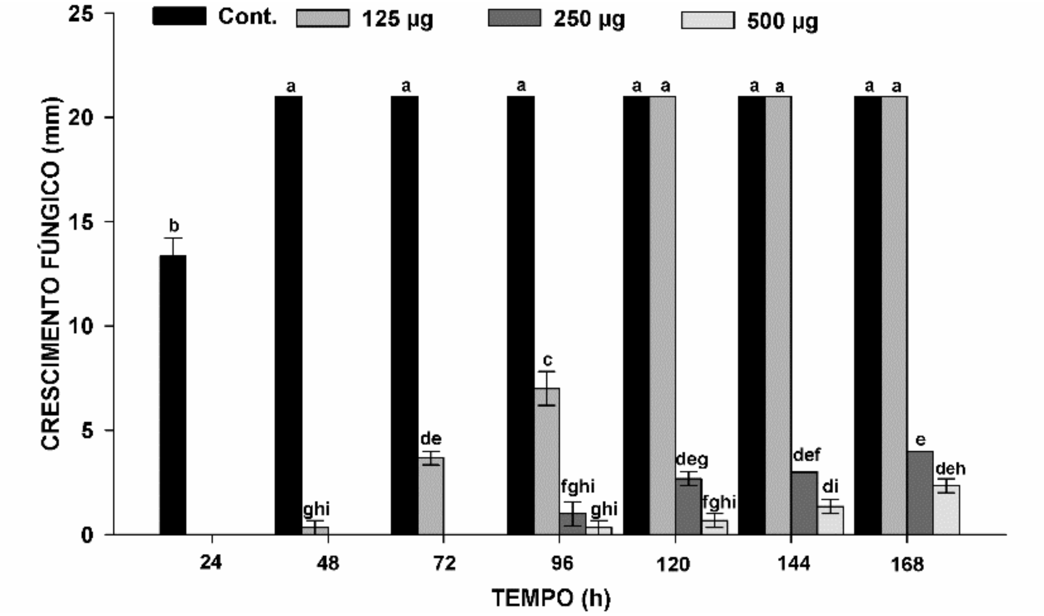

Cont.
125 µg
250 µg
500 µg
CRESCIMENTO FÚNGICO (mm)
25
20
15
10
5
0
b
a
ghi
a
de
a
c
fghi
ghi
a a
deg
fghi
a a
def
di
a a
e
deh
24
48
72
96
120
144
168
TEMPO (h)

Figure 2

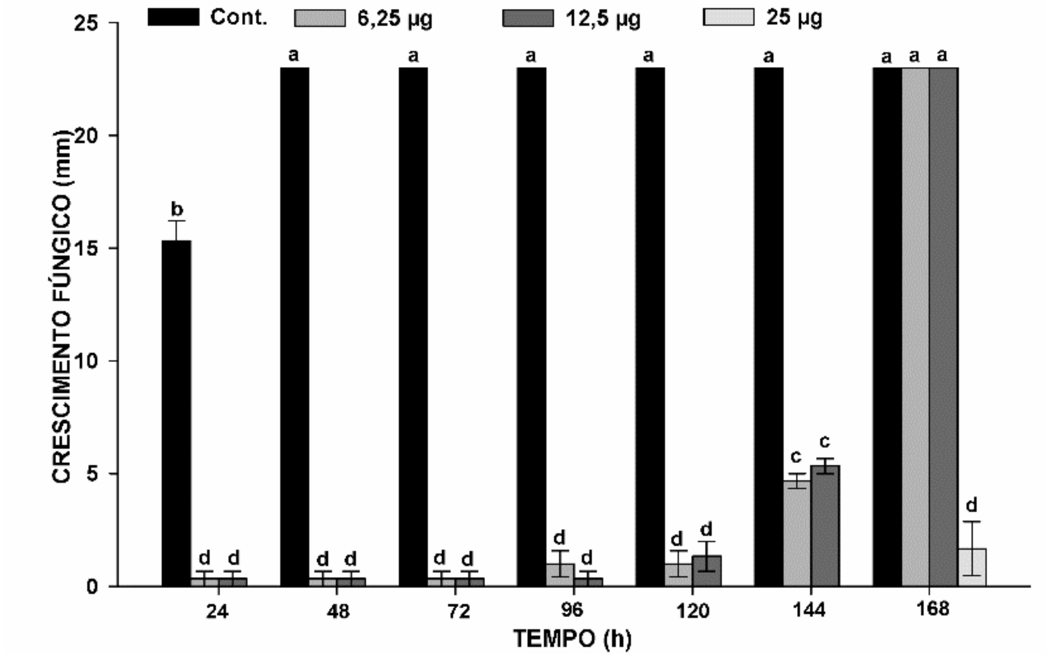
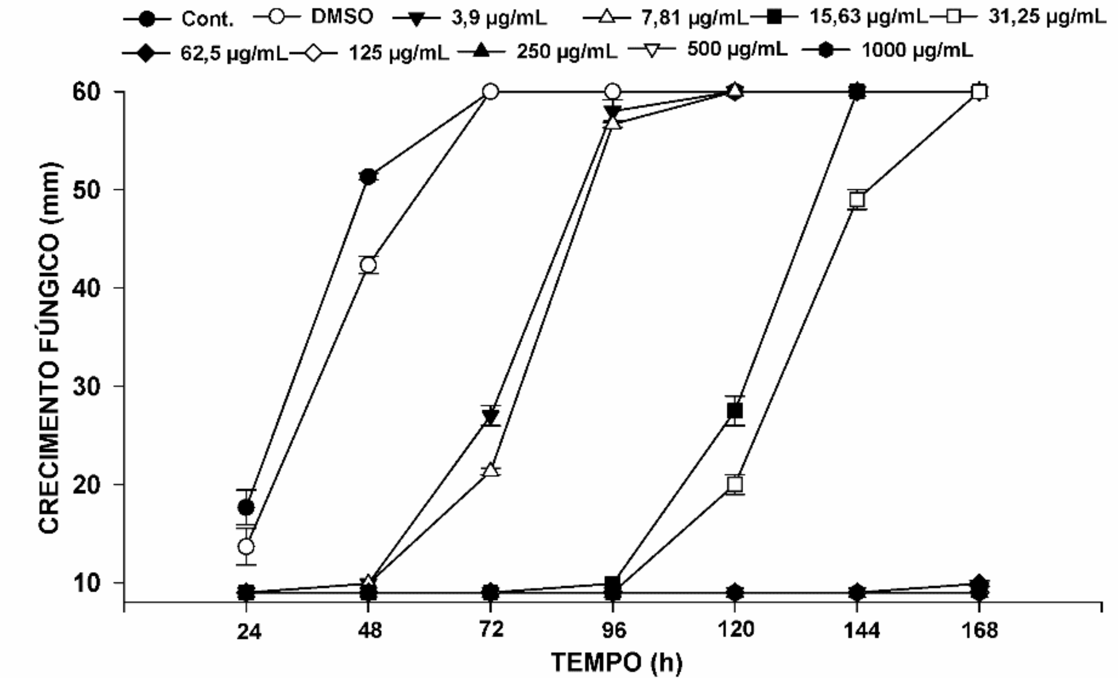

Cont.
6,25 µg
12,5 µg
25 µg
CRESCIMENTO FÚNGICO (mm)
25
20
15
10
5
0
b
d d
a
d d
a
d d
a
d d
a
d d
a
c c
a a a
d
24
48
72
96
120
144
168
TEMPO (h)

Figure 3



Cont.
DMSO
3,9 µg/mL
7,81 µg/mL
15,63 µg/mL
31,25 µg/mL
62,5 µg/mL
125 µg/mL
250 µg/mL
500 µg/mL
1000 µg/mL
CRECIMENTO FÚNGICO (mm)
60
50
40
30
20
10
24
48
72
96
120
144
168
TEMPO (h)

Figure 4

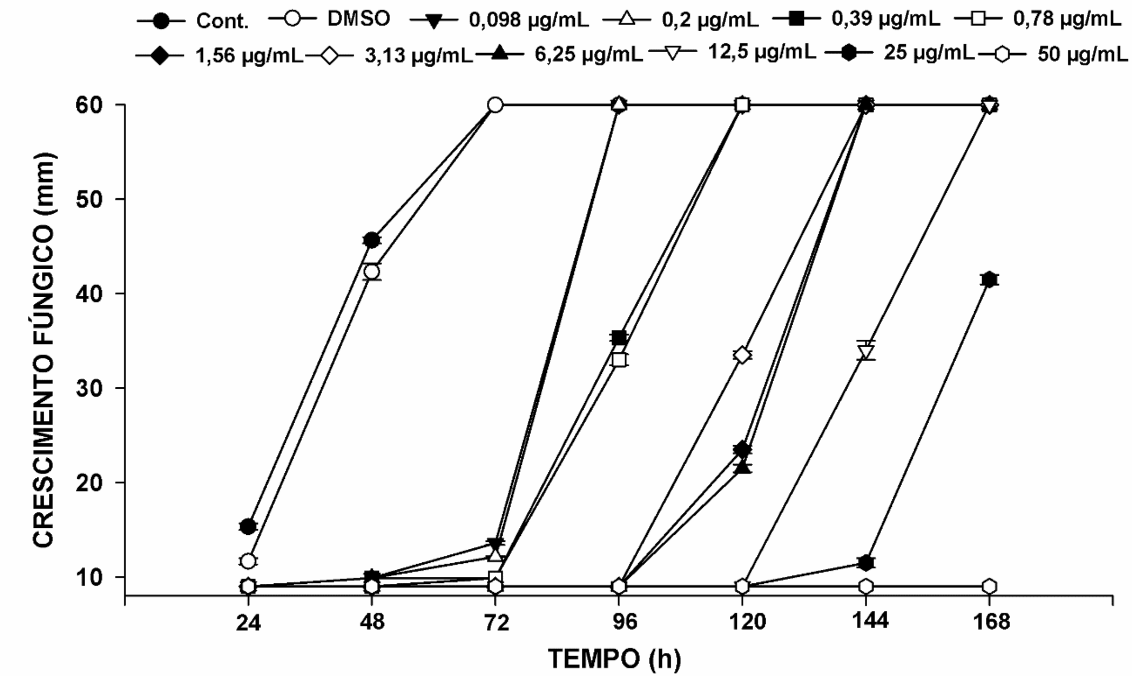

Cont.
DMSO
0,098 µg/mL
0,2 µg/mL
0,39 µg/mL
0,78 µg/mL
1,56 µg/mL
3,13 µg/mL
6,25 µg/mL
12,5 µg/mL
25 µg/mL
50 µg/mL
CRESCIMENTO FÚNGICO (mm)
60
50
40
30
20
10
24
48
72
96
120
144
168
TEMPO (h)

Figure 5

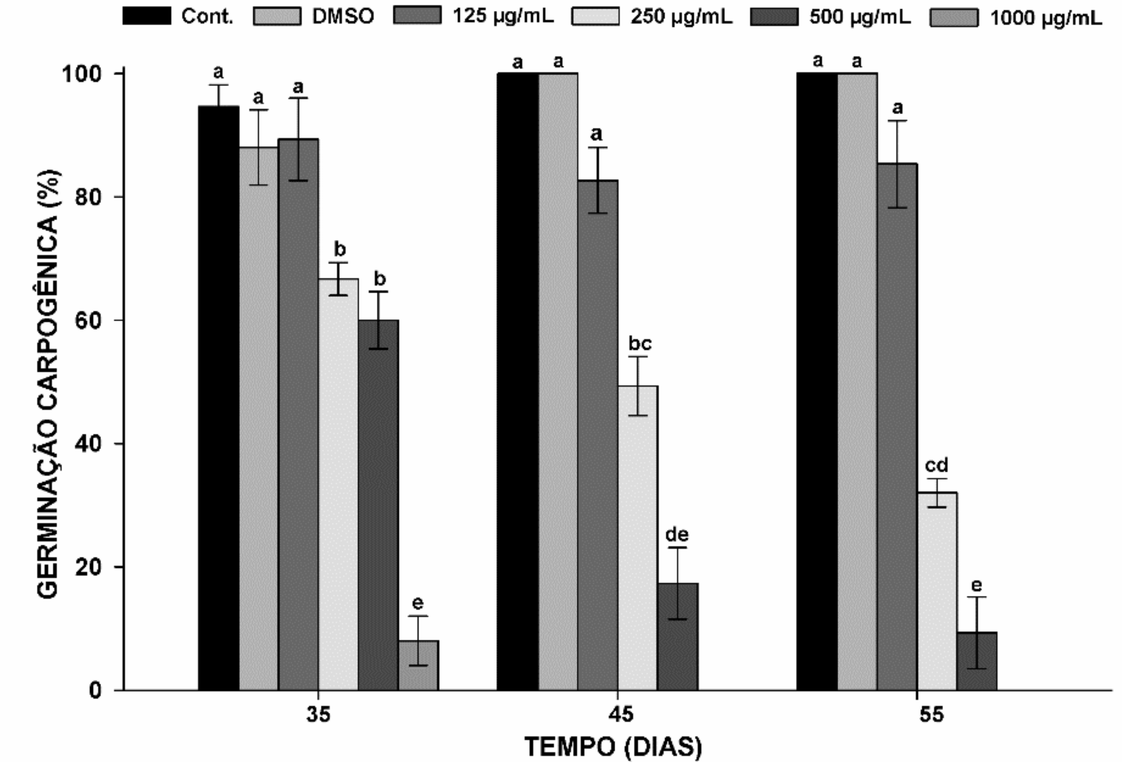

Cont.
DMSO
125 µg/mL
250 µg/mL
500 µg/mL
1000 µg/mL
GERMINAÇÃO CARPOGÊNICA (%)
100
80
60
40
20
0
a
a
a
b
b
e
a
a
a
bc
de
a
a
a
cd
e
35
45
55
TEMPO (DIAS)

Figure 6

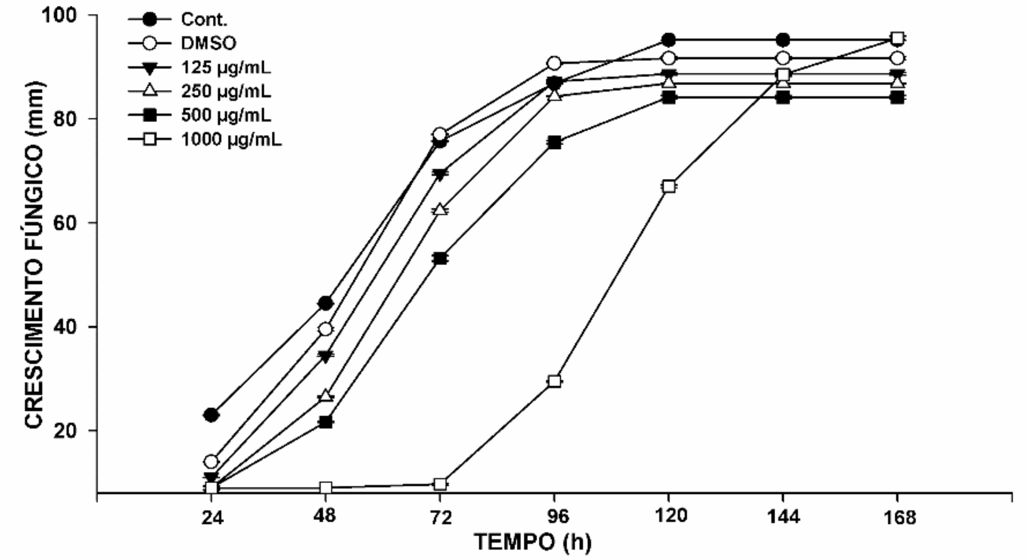

Cont.
DMSO
125 µg/mL
250 µg/mL
500 µg/mL
1000 µg/mL
CRESCIMENTO FÚNGICO (mm)
100
80
60
40
20
24
48
72
96
120
144
168
TEMPO (h)

Figure 7

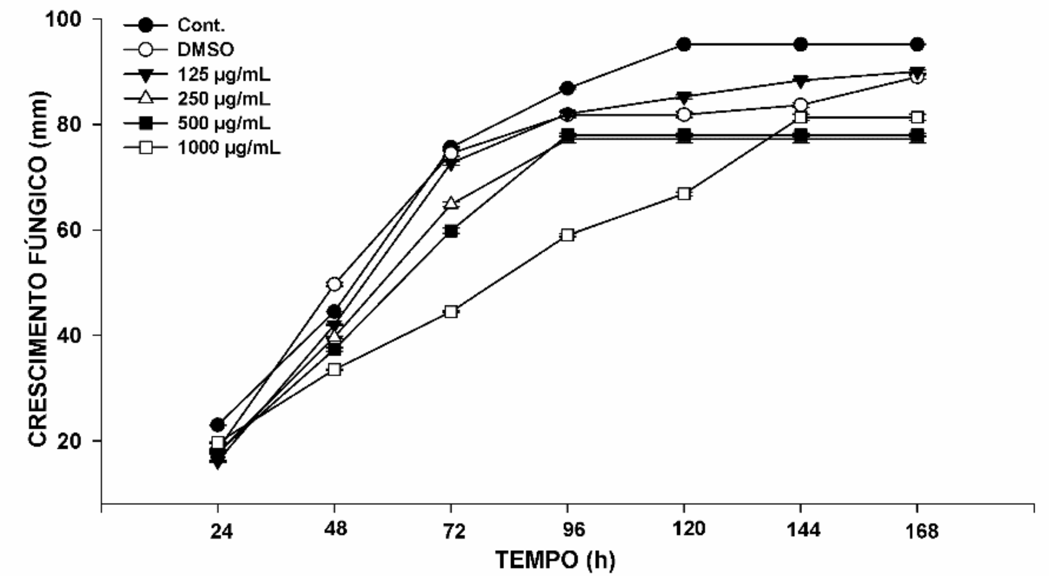

Cont.
DMSO
125 µg/mL
250 µg/mL
500 µg/mL
1000 µg/mL
CRESCIMENTO FÚNGICO (mm)
100
80
60
40
20
24
48
72
96
120
144
168
TEMPO (h)

Figure 8

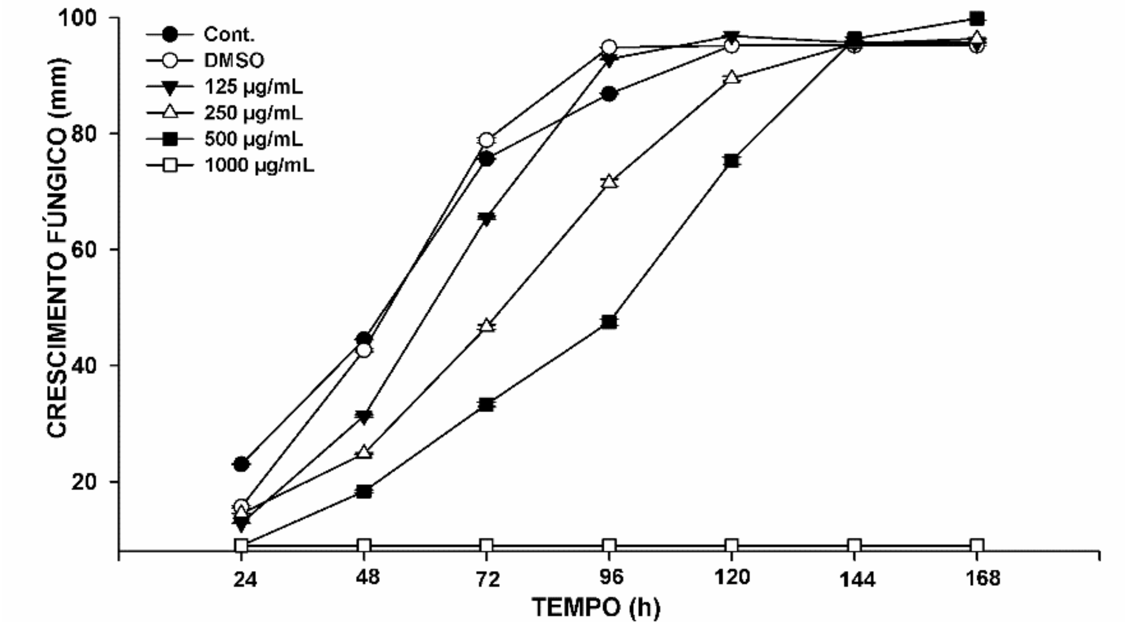

Cont.
DMSO
125 µg/mL
250 µg/mL
500 µg/mL
1000 µg/mL
CRESCIMENTO FÚNGICO (mm)
100
80
60
40
20
24
48
72
96
120
144
168
TEMPO (h)

Figure 9

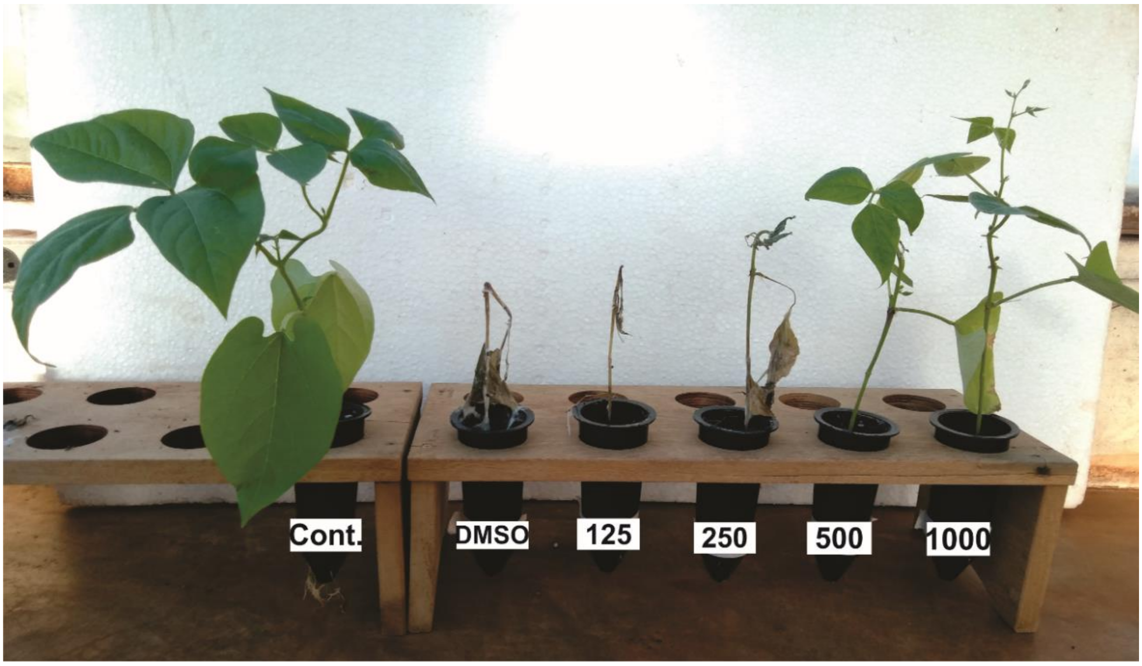
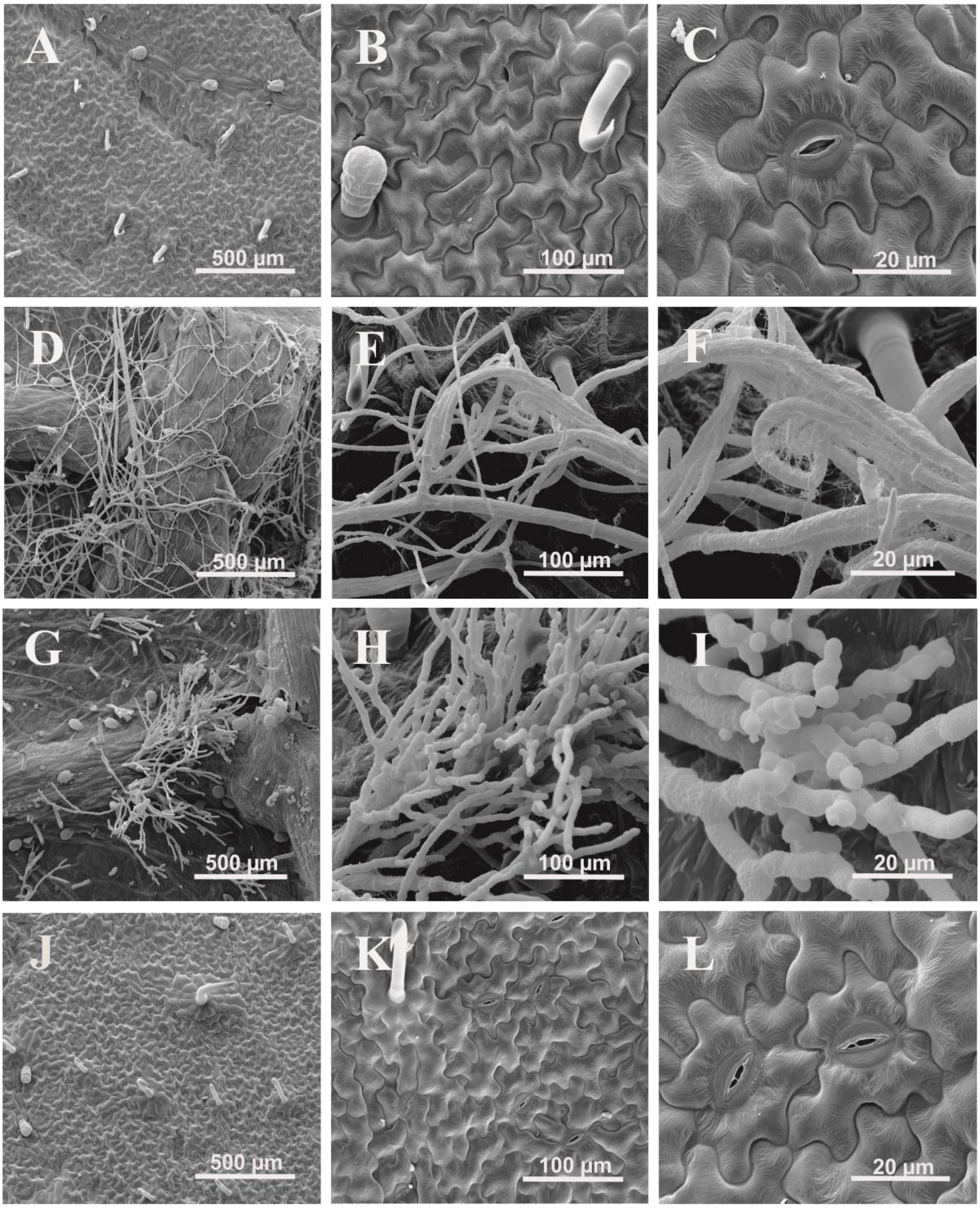


Figure 10


A
500 µm
B
100 µm
C
20 µm
D
500 µm
E
100 µm
F
20 µm
G
500 µm
H
100 µm
I
20 µm
J
500 µm
K
100 µm
L
20 µm

CONCLUSÕES

A fração F4A apresenta potencial antifúngico no controle de *S. sclerotiorum*, no entanto novas estratégias para diminuir a fitotoxicidade desta molécula devem ser consideradas. Os metabólitos secundários produzidos pela cepa RV7S3, também demostraram ação inibitória sobre o fungo, tanto sobre a germinação carpogênica de escleródios, como no tratamento da infecção em feijoeiro. No entanto estudos futuros precisam ser realizados para otimizar a produção e aumentar o rendimento da fração FRV7, pois esta apresentaria melhores porcentagens de sobrevivência de plantas em concentrações menores de produto.