MÔNICA CRISTINA PEREIRA MONTEIRO

FUNGOS ENDOFÍTICOS DE CAFEEIRO PRODUTORES DE COMPOSTOS ORGÂNICOS VOLÁTEIS E ENZIMAS EXTRACELULARES

LAVRAS - MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para a obtenção do título de Doutora.

Orientadora

Dra. Patrícia Gomes Cardoso

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Dra. Marisa Vieira de Queiroz	UFV
Dr. Eduardo Alves	UFLA
Dr. Márcio Pozzobon Pedroso	UFLA
Dr. Flávio Henrique Vasconcelos de Medeiros	UFLA

Dra. Patrícia Gomes Cardoso

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RESUMO

Micro-organismos endofíticos habitam o interior das plantas, tanto na parte aérea, como caules e folhas, quanto em raízes, sem ocasionar prejuízo aos seus hospedeiros. As interações dos endófitos com suas plantas hospedeiras são benéficas e podem estar relacionadas à sanidade das plantas, controlando nematoides, insetos e outros organismos. Este trabalho foi realizado com os objetivos de identificar fungos endofíticos isolados de *Coffea arabica* produtores de compostos orgânicos voláteis (COVs); avaliar o efeito dos COVs produzidos por fungos endofíticos sobre o crescimento de *Rhizoctonia solani*, *Fusarium oxysporum*, *Phoma* sp., *Botrytis cinerea*, *Fusarium solani*, *Fusarium verticillioides*, *Cercospora coffeicola*, *Aspergillus ochraceus* e *Pestalotia longisetula*; selecionar fungos endofíticos com potencial para o controle biológico de *A. ochraceus* inoculados em grãos de café e *F. verticillioides* inoculados em sementes de milho; identificar COVs produzidos pelo fungo endofítico *Acremonium* sp. (C19) utilizando cromatografia gasosa acoplada à espectrometria de massa (GC-MS) e avaliar a produção de enzimas extracelulares. Doze fungos endofíticos foram identificados como *Muscodor* spp. (9), *Simplicillium* sp. (2) e *Acremonium* sp. (1). Os compostos voláteis produzidos por estes fungos inibiram o crescimento de diferentes fungos fitopatogênicos com diferentes eficácias. *Muscodor coffeanum* (COAD 1842) apresentou efeito fungicida contra *A. ochraceus* em grãos de café. O crescimento de *F. verticillioides* foi inibido por seis fungos endofíticos, *Muscodor coffeanum* (1842,1899,1900), *M. vitigenus* (C20) e *Simplicillium* sp. (C18). Em relação aos COVs produzidos por *Acremonium* sp., verificou-se redução significativa no crescimento de fungos fitopatogênicos, entretanto, os fungos *A. ochraceus* e *F.oxysporum* foram menos susceptíveis aos COVs. O perfil de COVs de *Acremonium* foi investigado utilizando-se a técnica *headspace* SPME/GC-MS e os compostos encontrados pertencem a diferentes classes químicas. Em relação à produção de enzimas, todos os fungos foram produtores das enzimas celulases e pectinases, 11 produziram lipase, 9 produziram fitase e protease, e 4 produziram amilase. A atividade específica de endo β-1,4 glucanase e exo β-1,4 glucanase foi detectada para 12 e 10 fungos endofíticos, respectivamente. Este estudo demonstra que os fungos endofíticos isolados de *Coffea arabica* são fontes promissoras de metabólitos bioativos.

Palavras-chave: Fungos endofíticos. *Acremonium* sp. Biocontrole. *Coffea arabica*. *Muscodor* spp. Compostos orgânicos voláteis.

ABSTRACT

Endophytic microorganisms inhabit the interior of shoot, stems, leaves and roots, without causing harm to their hosts. The interactions of endophytes with their host plants are beneficial and can be related to plant health, controlling the growth of pathogens, nematodes, insects and other organisms. The objectives of this study were to identify endophytic fungi isolated from *Coffea arabica* producers of volatile organic compounds (VOCs), evaluate the effect of VOCs produced by endophytic fungi on the growth of *Rhizoctonia solani, Fusarium oxysporum, Phoma* sp., *Botrytis cinerea, Fusarium solani, Fusarium verticillioides, Cercospora coffeicola, Aspergillus ochraceus* and *Pestalotia longisetula*, select endophytic fungi with potential for biological control of *A. ochraceus* inoculated in coffee beans and *F. verticillioides* inoculated in corn seeds, identify VOCs produced by the endophytic fungus *Acremonium* sp. (C19) using gas chromatography mass spectrometry (GC- MS) and to assess production of extracellular enzymes. Twelve endophytic fungi were identified as *Muscodor* spp. (9) *Simplicillium* sp. (2) and *Acremonium* sp. (1). The volatiles produced by these fungi inhibited the growth of different phytopathogenic fungi with different efficiencies. *Muscodor coffeanum* (COAD 1842) showed fungicidal effect against *A. ochraceus* in coffee beans. The growth of *F. verticillioides* was inhibited by six endophytic fungi, *Muscodor coffeanum* (1842, 1899, 1900), *M. vitigenus* (C20) and *Simplicillium* sp. (C18). Regarding the VOCs produced by *Acremonium* sp. (C19), a significant reduction was found in the growth of pathogenic fungi, however, the fungus *A. ochraceus* and *F.oxysporum* were less susceptible to VOCs. The *Acremonium* VOCs profile was investigated using the SPME/GC-MS headspace technique and found compounds belonging to different chemical classes. For enzyme production, all fungi were producers of cellulase and pectinase enzymes, 11 produced lipase, 9 produced phytase and protease and 4 produced amylase. The specific activity of endo-β 1.4 glucanase and exo-β 1.4 glucanase were detected for 12 and 10 endophytic fungi respectively. This study demonstrates that the endophytic fungi of *Coffea arabica* are promising sources of bioactive metabolites.

Keywords: Endophytic fungi. *Acremonium* sp. Biocontrol. *Coffea arabica*. *Muscodor* spp. Volatiles organic compounds.

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1 INTRODUÇÃO

Fungos endofíticos formam um grupo polifilético, altamente diversificado e, funcionalmente, são definidos por sua ocorrência no interior de tecidos de plantas, sem causar prejuízos aos seus hospedeiros (HYDE; SOYTONG, 2008). Uma vez no interior da planta hospedeira, estes micro-organimos geralmente assumem um estado de repouso (latente) e podem permanecer nele durante toda a vida útil do tecido vegetal (RODRIGUEZ; REDMAN, 2008).

Diferentes espécies de fungos filamentosos já foram isoladas como endofíticos em diversas plantas. Esses fungos penetram na planta através de feridas causadas por insetos, principalmente em suas raízes, por aberturas naturais como estômatos e hidatódios, por estruturas de fungos patogênicos, como os apressórios, e por meio da transmissão via sementes. Além disso, podem utilizar-se da produção de enzimas hidrolíticas, como pectinases e celulases, para facilitar sua entrada no tecido vegetal (POLLI et al., 2012).

Na maioria dos casos estudados, as interações entre plantas e micro-organismos têm se mostrado benéficas. Produtos naturais obtidos a partir de endofíticos têm apresentado atividade antimicrobiana e, em muitos casos, atuam na proteção da planta hospedeira contra micro-organismos fitopatogênicos (GUNATILAKA, 2006; STROBEL; DAISY, 2003; STROBEL et al., 2004; TAN; ZOU, 2001; ZHANG; SONG; TAN, 2006). Estes micro-organismos também conferem aumento da resistência a estresses abióticos, como seca, pH e temperaturas adversas (BACKMAN; SIKORA, 2008; VERMA; KHARWAR; STROBEL, 2009).

Além disso, estes micro-organismos são considerados importantes fontes para triagem de agentes de biocontrole para suprimir pragas de plantas. Neste contexto, as espécies do gênero *Muscodor* são consideradas promissoras.

Estudos demonstraram que a micofumigação utilizando bioprodutos obtidos a partir de culturas de *Muscodor* spp. em grãos de cereais foi eficaz no controle de doenças de pós-colheita (MERCIER; JIMENEZ, 2004; STROBEL, 2006; SUWANNARACH et al., 2012; TALIBI et al., 2014). Em outro estudo verificou-se que misturas naturais e artificiais de COVs produzidos por *Muscodor albus* inibiram em 100% o crescimento de vários micro-organismos fitopatogênicos (EZRA; STROBEL, 2003).

Estudos têm sido realizados com endófitos isolados de plantas economicamente importantes, incluindo *Coffea arabica* (SANTAMARÍA; BAYMAN, 2005; VEGA et al., 2010). A cultura do café é de grande importância para o Brasil, especificamente para o estado de Minas Gerais. Sendo assim, este trabalho foi realizado com os objetivos de identificar fungos endofíticos isolados de *Coffea arabica* produtores de compostos orgânicos voláteis (COVs); avaliar o efeito dos COVs produzidos por estes fungos endofíticos sobre o crescimento de fungos fitopatogênicos; selecionar fungos endofíticos com potencial para o biocontrole de *Aspergillus ochraceus* inoculados em grãos de café e *Fusarium verticillioides* inoculados em sementes de milho; identificar COVs produzidos pelo fungo endofítico *Acremonium* sp. (C19), utilizando cromatografia gasosa acoplada à espectrometria de massa (GC-MS) e avaliar a produção de enzimas extracelulares, como amilase, celulase, lipase, pectinase, fitase, protease, endo β-1,4 glucanase e exo β-1,4 glucanase.

2 REVISÃO DE LITERATURA

2.1 Fungos endofíticos

Estudos sobre biodiversidade de fungos propõem que o número de espécies pode ser de 1,5 milhão. Entretanto, estima-se que de 70.000 a 100.000 espécies de fungos foram isoladas e identificadas até o momento, o que representa apenas 5% do total do número de fungos estimado (HAWKSWORTH, 2001). Os fungos são micro-organismos de grande importância, pois desempenham papéis ecológicos críticos no ciclo global do carbono e na reciclagem dos nutrientes, além de serem fundamentais para a sobrevivência de outros grupos de organismos. Além disso, apresentam grande potencial para a biotecnologia e para a agricultura (BAKER et al., 2008).

O termo endofítico foi primeiramente introduzido por De Bary (1866) e era aplicado a qualquer micro-organismo presente dentro do tecido das plantas. Atualmente, o conceito mais comumente empregado provém de Petrini (1991), segundo o qual fungos endofíticos são aqueles micro-organismos que habitam o tecido vegetal durante alguma fase do seu ciclo de vida, colonizando o tecido interno da planta, mas sem causar doença ao hospedeiro. O conceito de Schulz e Boyle (2005) acrescenta que são fungos cuja colonização nunca resulte em sintomas visíveis de doença no hospedeiro, em qualquer momento específico, ou seja, sob quaisquer condições, não ocorrerá evidência de sintomas da doença causada por esses fungos.

Esses simbiontes podem ter efeitos profundos na ecologia e na evolução vegetal, moldando as comunidades de plantas e manifestando fortes efeitos sobre a estrutura da comunidade e na diversidade de outros organismos associados (OMACINI et al., 2001).

Endófitos podem ser transmitidos de forma vertical ou horizontal. A transmissão vertical ocorre por meio das sementes e está intimamente associada a algumas espécies de plantas (gramíneas); já na transmissão horizontal, a entrada no tecido vegetal ocorre a partir do ambiente (BREM; LEUCHTMANN, 2002; RODRIGUEZ et al., 2009; SAIKKONEN et al., 2004).

De modo geral, dois grandes grupos de fungos endofíticos são reconhecidos com base na taxonomia, na planta hospedeira, na função ecológica e nas diferenças evolutivas. Os endófitos clavicipitaceous (C-endofítos) colonizam as gramíneas e os endófitos não clavicipitaceous (NC-endófitos) infectam plantas não vasculares, samambaias, coníferas e angiospermas (RODRIGUEZ et al., 2009).

A colonização bem sucedida por endófitos depende de muitas variáveis, incluindo tipo de tecido da planta, bem como o seu genótipo, a estirpe microbiana e fatores ambientais bióticos e abióticos. Além disso, a diversidade de fungos endofíticos pode variar em diferentes partes da planta hospedeira e em locais geograficamente distintos. Estudos indicam que as taxas de colonização podem ser diferentes entre cascas, ramos e folhas e alguns fungos têm especificidade por famílias de plantas hospedeiras (DURÁN et al., 2005; KUMAR; HYDE, 2004; RUBINI et al., 2005).

As associações que esses micro-organismos apresentam com as plantas hospedeiras podem ser complexas, uma vez que colonizam os tecidos vivos do hospedeiro. A interação entre endófitos e seus hospedeiros varia de mutualismo a parasitismo e depende do estado fisiológico, da espécie e dos constituintes químicos da planta. Além disso, as condições abióticas também afetam essas interações (GILBERT; STRONG, 2007).

Sendo assim, estes micro-organismos podem ter desenvolvido estreitas associações biológicas e o resultado destas associações reflete na produção de substâncias bioativas pelo fungo endofítico (BACON; WHITE, 2000). Os

metabólitos secundários produzidos por fungos endofíticos em seus hospedeiros podem ser utilizados como defesas químicas contra herbívoros, patógenos e outros organismos competidores (SCHULZ; BOYLE, 2005; STROBEL, 2006).

O potencial do uso de fungos endofíticos como agentes no controle biológico de doenças e pragas em plantas, na indução de resistência na planta hospedeira, na promoção de crescimento vegetal, no aumento da tolerância ao estresse pela seca ou calor em gramíneas e na produção de metabólitos secundários de interesse para a saúde humana tem sido demonstrado em diferentes estudos (ARNOLD; HERRE, 2003; CAO et al., 2009; DINGLE; MCGEE, 2003; HAMAYUN et al., 2009; LI et al., 2007; MARQUEZ et al., 2007; MOUSA; RAIZADA, 2013; REDMAN et al., 2002; STROBEL et al., 2001; WANG et al., 2013; ZHANG et al., 2008).

Um aspecto interessante é que o número de metabólitos secundários produzidos por fungos endofíticos é maior do que para qualquer outro grupo de micro-organismos, o que está associado à alta diversidade encontrada em plantas, o que favorece ainda mais o seu estudo (ZHANG; SONG; TAN, 2006).

2.2 Metabólitos secundários produzidos por fungos endofíticos

A descoberta de novos compostos químicos naturais é muito importante para a formulação de novas drogas e os fungos endofíticos têm recebido cada vez mais atenção. Isso porque a produção de compostos bioativos por endofíticos, especialmente aqueles exclusivos de suas plantas hospedeiras, é importante não só a partir de uma perspectiva ecológica, mas também do ponto de vista bioquímico e molecular (KUSARI et al., 2012).

Desde a descoberta da penicilina e sua aplicação como antibiótico, o século XX acompanhou a descoberta, o isolamento e a caracterização química de ampla variedade de produtos naturais a partir de fungos, incluindo alcaloides,

terpenoides, flavonoides, ácidos fenólicos, quinonas, esteroides e outros (HOFFMEISTER; KELLER, 2007; TAN; ZOU, 2001). Alguns compostos são conhecidos por apresentarem funções antibióticas, imunossupressoras, anticancerígenas e de controle biológico (GUNATILAKA, 2006; JOSEPH; PRIYA, 2011; STROBEL et al., 2004; TAN; ZOU, 2001; YU et al., 2010; ZHANG et al., 2005). Em relação ao termo controle biológico, este pode ser definido como um produto contendo organismos vivos, seus metabólitos ativos e/ou sub-produtos com atividade direta na fisiologia da planta e/ou controle de doenças de plantas (WILSON, 1997).

Em experimentos utilizando fungos endofiticos *in vitro* foi demonstrado que algumas espécies apresentam a capacidade de inibir o crescimento de fungos fitopatogênicos. Os fungos endofíticos *Dothideomycetes* sp., *Alternaria tenuissima*, *Thielavia subthermophila*, *Alternaria* sp., *Nigrospora oryzae*, *Colletotrichum truncatum* e *Chaetomium* sp. inibiram o crescimento de *Sclerotinia sclerotiorum* e *Fusarium oxysporum* (KUMAR et al., 2011; KUMAR; KAUSHIK, 2013).

O endofíto *Acremonium zeae* produz o antibiótico pirrocidina (A e B), que tem atividade antifúngica significativa contra os fungos produtores de micotoxinas *Aspergillus flavus* e *Fusarium verticillioides* (WICKLOW et al., 2005). Em adição, fungos pertencentes ao gênero *Phomopsis* são conhecidos como produtores de metabólitos secundários com diversas atividades biológicas, incluindo compostos antimicrobianos, antifúngicos, antimaláricos e antitumorais.

Os fungos endofíticos também têm relevância para a agricultura. O fungo *Scolecobasidium humicola*, encontrado em raízes de tomate, demonstrou capacidade de aumentar a biomassa vegetal utilizando uma fonte orgânica de nitrogênio. Esta descoberta é o primeiro relato de *S. humicola* como endofítico

com habilidade de melhorar o crescimento vegetal (MAHMOUD; NARISAWA, 2013).

Os fungos endofíticos também são considerados agentes eficazes no controle biológico de insetos. As espécies de *Neotyphodium* são reportadas por sua produção de metabólitos bioprotetores. O alcaloide lolina, produzido por *Neotyphodium uncinatum*, confere aumento da resistência de sua planta hospedeira contra insetos herbívoros (BLANKENSHIP et al., 2001; LEHTONEN et al., 2005; SCHARDL et al., 2007). Além disso, os endófitos podem produzir os mesmos ou metabólitos secundários similares aos metabólitos produzidos por seu hospedeiro. A droga anticâncer taxol (paclitaxel) foi inicialmente isolada a partir de *Taxus brevifolia* (uma planta medicinal). Entretanto, o fungo *Taxomyces andreanae*, isolado como endofítico desta planta, também produziu o mesmo composto (STIERLE et al., 1995). Diferentes grupos de fungos endofíticos, tais como *Seimatoantlerium tepuiense*, *S. nepalense*, *Pestalotiopsis microspora*, *Periconia* sp., *Metarhizium anisopliae*, *Colletotrichum gloeosporioides*, *Gliocladium* sp. e *Fusarim solani*, têm sido relatados por sintetizarem o taxol (ALVIN; MILLER; NEILAN, 2014; BASHYAL et al., 1999; DENG et al., 2009; GANGADEVI; MUTHUMARY, 2008; HEINIG; SCHOLZ; JENNEWEIN, 2013; LI et al., 1998; SREEKANTH et al., 2009; STROBEL et al., 1996). A transferência horizontal de genes tem sido proposta para a biossíntese de taxol por fungos endofíticos. Há relatos de que os fungos endofíticos contêm genes previamente identificados em *Taxus* spp. (KUMARAN; KIM; HUR, 2010; ZHANG et al., 2008).

Outros compostos bioativos que são coproduzidos pelas plantas, bem como por endófitos, incluem a camptotecina, um alcaloide produzido pelos endofíticos *Entrophospora infrequens* e *Rhizopus oryzae*, ambos isolados da planta *Nothapodytes foetida*. Esse composto apresenta ação contra tumores em útero, ovário e cólon. A camptotecina é conhecida comercialmente como

Camptosar (AMNA et al., 2006; LORENCE; NESSLER, 2004; PURI et al., 2005).

A podofilotoxina também é sintetizada pelos fungos endofíticos *Trametes hirsuta, Phialocephala fortinii* e *Fusarium oxysporum.* Esse composto é utilizado como uma droga anticancerígena e seu mecanismo de ação se limita à inibição da topoisomerase II, bloqueando, assim, o ciclo celular e, consequentemente, afetando a integridade do genoma, levando à apoptose e à morte celular (EYBERGER; DONDAPATI; PORTER, 2006; KOUR et al., 2008; PURI et al., 2006).

Além da produção de compostos bioativos, os fungos endofíticos são excelentes fontes de enzimas industriais (CORREA et al., 2014; LI et al., 2012). A produção de enzimas pelos fungos endofíticos está relacionada à especificidade entre a planta hospedeira e o fungo (PEIXOTO JUNIOR; AZEVEDO; ARAÚJO, 2002).

A produção de enzimas também é uma forma de conferir resistência ao hospedeiro. Quando o fungo endofítico coloniza a superfície da planta, ele produz enzimas, tais como celulase, proteases, lipases, beta-1,3 glucanases e quitinases. Além disso, estas enzimas também têm uma função para suprimir a atividade de agentes patogênicos de plantas, degradando a parede celular de fungos e oomicetos (GAO; DAI; LIU, 2010).

Fungos endofíticos, como *Acremonium terricola*, *Aspergillus japonicus*, *Cladosporium cladosporioides*, *C. sphaerospermum*, *Fusarium lateritium*, *Monodictys castaneae*, *Nigrospora sphaerica*, *Penicillium aurantiogriseum*, *P. glandicola*, *Pestalotiopsis guepinii*, *Phoma tropica*, *Phomopsis archeri* e *Xylaria* sp., são promissores em processos biotecnológicos que envolvem a produção de pectinases, celulases, xilanases e proteases (BEZERRA et al., 2012).

2.2.1 Compostos orgânicos voláteis produzidos por fungos

Muitos micro-organismos produzem uma variedade de metabólitos secundários, muitos dos quais são voláteis. Compostos orgânicos voláteis (COVs) referem-se, de modo geral, a substâncias de baixa polaridade que facilmente entram na fase gasosa através da vaporização a 0,01 kPa e temperatura próxima de 20 °C, apresentando, portanto, alta pressão de vapor e baixa solubilidade em água (PAGANS; FONT; SÁNCHEZ, 2006). De modo geral, COVs são ativos em concentrações muito baixas e, devido à sua volatilidade, esses compostos podem ser transportados para grandes distâncias em ambientes estruturalmente heterogênios, como, por exemplo, o solo, que é composto por sólidos, líquidos e gases, sendo ideais "infoquímicos" (KORPI; JÄRNBERG; PASANEN, 2009; MINERDI et al., 2009; MORATH; HUNG; BENNETT, 2012; PAGANS; FONT; SÁNCHEZ, 2006; WHEATLEY, 2002).

Aproximadamente 250 COVs foram identificados a partir de fungos e foram encontrados compostos pertencentes a diferentes classes químicas, como aldeídos, álcoois, ésteres, lactonas, terpenos e compostos aromáticos (MINERDI et al., 2009; MORATH; HUNG; BENNETT, 2012). A maioria dos fungos emite diferentes misturas de compostos voláteis, sendo o perfil específico produzido por cada espécie ou consórcio microbiano fortemente dependente de temperatura, pH, umidade, nutrientes e idade da cultura (BENNETT; INAMDAR, 2013).

Isolados de *Muscodor albus* apresentaram perfis de produção de COVS diferentes e o número de compostos detectados foi maior em meios de cultura que exibiram maior quantidade da fonte de carbono (KORPI; JÄRNBERG; PASANEN, 2009). Além disso, foi constatado que meios enriquecidos foram mais eficazes na inibição de um conjunto de patógenos de plantas (EZRA; STROBEL, 2003).

Outros fungos endofíticos, tais como *Phomopsis* sp., *Nodulisporium* sp., *Hypoxylon* sp., *Gliocladium* sp. e a espécie *Latemarginatus oxyporus*, são relatados como produtores de compostos voláteis que controlam a decadência de frutas (LEE et al., 2009; SINGH et al., 2011; STINSON et al., 2003; TOMSHECK et al., 2010).

Testes *in vitro* com o fungo *Nodulisporium* sp. revelaram que os COVs produzidos inibiram e/ou mataram fungos fitopatogênicos de 12 plantas diferentes. Os resultados de ensaios *in vivo* também foram satisfatórios, uma vez que foi realizada a micofumigação para o controle de doenças de pós-colheita em citrus causadas pelos fungos *Penicillium digitatum* e *Penicillium expansum* (SUWANNARACH et al., 2013).

O fungo *Hypoxylon* sp., isolado de *Persea indica*, produziu uma mistura de COVs com atividade antimicrobiana contra *Botrytis cinerea*, *Phytophthora cinnamomi*, *Cercospora beticola* e *Sclerotinia sclerotiorum.* Os autores sugerem que os COVs podem desempenhar algum papel na biologia do fungo e na sobrevivência da planta hospedeira (TOMSHECK et al., 2010).

O endófito *Phomopsis* sp. produziu uma mistura de COVs que também apresentou propriedades antifúngicas. A mistura artificial dos COVs imitou os efeitos antibióticos deste organismo com a maior bioatividade contra patógenos de plantas, incluindo *Pythium*, *Phytophthora*, *Sclerotinia*, *Rhizoctonia*, *Fusarium*, *Botrytis*, *Verticillium* e *Colletotrichum*. Entretanto, este isolado não conseguiu sobreviver na presença dos gases inibitórios de *Muscodor albus* (SINGH et al., 2011).

Muscodor albus, um fungo endofítico isolado de *Cinnamomum zeylanicum*, produziu COVs que inibiram e/ou mataram uma ampla variedade de espécies de fungos e bactérias (STROBEL et al., 2001). Além disso, os COVs produzidos por *M. albus* sugerem a possibilidade de controlar fungos causadores de doenças de pós-colheita em maçãs e pêssegos (MERCIER; JIMENEZ, 2004).

Outras espécies de *Muscodor* caracteristicamente produzem uma matriz de COVs com atividade antimicrobiana, nematicida e inseticida (GUERRA, 2008; LACEY; NEVEN, 2006; RIGA; LACEY; STROBEL, 2006). Desde a primeira descrição da espécie *Muscodor albus* (CZ- 620), outras espécies novas têm sido isoladas a partir de plantas nativas, em regiões tropicais e subtropicais (DAISY et al., 2002; GONZALEZ et al., 2009; KUDALKAR et al., 2012; MESHRAM; KAPOOR; SAXENA, 2013; MITCHELL et al., 2008; SAXENA; MESHRAM; KAPOOR, 2015; SUWANNARACH et al., 2013; WORAPONG et al., 2002; ZHANG et al., 2010).

Estes compostos naturais com propriedades antimicrobianas podem representar alternativas aos fungicidas sintéticos (TALIBI et al., 2014). Consequentemente, o conceito de micofumigação se desenvolveu a partir da descrição de *M. albus*, uma vez que COVs com ação antimicrobiana podem se difundir no ar, atingindo habitats de difícil acesso em ambientes fechados (MORATH; HUNG; BENNETT, 2012). A utilização de isolados de *M. albus* na agricultura tem sido patenteada como um biofumigante antimicrobiano para o controle de doenças de pós-colheita, fungos presentes no solo e doenças transmitidas via semente (MERCIER et al., 2009; STROBEL; MANKER; MERCIER, 2005).

A primeira demonstração prática de seus efeitos contra um patógeno foi em sementes de cevada infectadas, resultando em 100% de controle da doença (STROBEL et al., 2001). Além disso, os solos podem ser pré-tratados com uma formulação *M. albus*, a fim de evitar o desenvolvimento de mudas infectadas. Outra vantagem é que estes compostos podem ser utilizados para inibir ou matar patógenos em áreas fechadas, sem a necessidade de contato direto com o produto, minimizando a manipulação de produtos tratados e, ainda, permitindo o tratamento de frutas frágeis ao tratamento regular com fungicidas (MERCIER; JIMENEZ, 2004).

Sendo assim, o conceito de micofumigação apresenta potencial para substituir o uso de substâncias perigosas que são atualmente aplicadas em alimentos e em solos. Em adição, os COVs produzidos por *M. albus* demonstraram ser seguros, eficazes e ecologicamente corretos (STROBEL, 2006).

Tabela 1 - Espécie, hospedeiro e posição taxonômica de endófitos relatados como produtores COVs

Espécie	**Hospedeiro**	**Posição taxonômica**
Muscodor albus	*Cinnamomum zeylanicum*	Ascomycota, Sordariomycetes, Xylariales
M. kashayum	*Aegle marmelos*	Ascomycota, Sordariomycetes, Xylariales
M. crispans	*Ananas ananassoides*	Ascomycota, Sordariomycetes, Xylariales
M. roseus	*Grevillea pteridifolia*	Ascomycota, Sordariomycetes, Xylariales
M. oryzae	*Oryza rufipogon*	Ascomycota, Sordariomycetes, Xylariales
M. musae	*Musa acuminata*	Ascomycota, Sordariomycetes, Xylariales
M. cinnanomi	*C. bejolghota*	Ascomycota, Sordariomycetes, Xylariales
M. strobelii	*C. zeylanicum*	Ascomycota, Sordariomycetes, Xylariales
M. darjeelingensis	*C. camphora*	Ascomycota, Sordariomycetes, Xylariales
M. tigerii	*C. camphora*	Ascomycota, Sordariomycetes, Xylariales
M. suthepensis	*C. bejolghota*	Ascomycota, Sordariomycetes, Xylariales
M. yucatanensis	*Bursera simaruba*	Ascomycota, Sordariomycetes, Xylariales
M. vitigenus	*Paullinia paullinioides*	Ascomycota, Sordariomycetes, Xylariales
M. equiseti	*Equisetum debile*	Ascomycota, Sordariomycetes, Xylariales
M. sutura	*Prestonia trifidi*	Ascomycota, Sordariomycetes, Xylariales

M. fengyangensis	*Actinidia chinensis*	Ascomycota, Sordariomycetes, Xylariales
Gliocladium sp.	*Eucryphia cordifolia*	Ascomycota, Sordariomycetes, Hypocreales
Phomopsis sp.	*Odontoglossum* sp.	Ascomycota, Dothideomycetes, Pleosporales

Fonte: Gomes, Queiroz e Pereira (2015).

Recentemente, a espécie *M. coffeanum* isolada de *Coffea arabica* no Brasil foi descrita. Este isolado também apresenta micélio estéril e difere de outras espécies de *Muscodor* por apresentar hifas e estruturas derivadas de diferentes tamanhos. Além disso, *M. coffeanum* é distinto, filogeneticamente, de outras *Muscodor* spp. (HONGSANAN et al., 2015). Na Tabela 1 estão destacados alguns fungos endofíticos produtores de COVs com ação antimicrobiana.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1

Normas do periódico *Bioscience Journal*

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Antimicrobial activity of endophytic fungi from coffee plants

RESUMO

Fungos endofíticos são uma fonte promissora para a descoberta de compostos com potencial biotecnológico. Os objetivos, neste estudo, foram selecionar e identificar fungos endofíticos de *Coffea arabica* que produzem compostos orgânicos voláteis (COVs), avaliar o efeito dos compostos orgânicos voláteis produzido por fungos endofíticos sobre o crescimento de *Rhizoctonia solani*, *Fusarium oxysporum*, *Phoma* sp., *Botrytis cinerea*, *Fusarium solani*, *Fusarium verticillioides*, *Cercospora coffeicola* e *Pestalotia longisetula*, e selecionar fungos endofíticos com potencial para controle biológico de *Aspergillus ochraceus* inoculado em grãos de café e *F. verticillioides* inoculado em sementes de milho. Um isolado de *Muscodor albus* foi utilizado como ferramenta de seleção para fungos endofíticos produtores de COVs. Dentre os 400 fungos endofíticos isolados, 11 foram capazes de crescer na presença de COVs produzidos por *M. albus*. Estes fungos foram identificados como *Muscodor* spp. (9) e *Simplicillium* sp., de acordo com pesquisas na base de dados UNITE, usando sequências de DNA do espaçador transcrito interno (ITS). Os COVs produzidos por fungos endofíticos inibiram o crescimento dos fungos fitopatogênicos, em comparação com o controle com diferentes eficácias. Os COVs produzidos por *Muscodor coffeanum* (COAD 1842) apresentaram efeito fungicida contra *A. ochraceus* em grãos de café. Seis fungos endofíticos inibiram completamente o crescimento de *F. verticillioides* inoculado em sementes de milho. Neste estudo demonstra-se que os fungos endofíticos produtores de compostos voláteis isolados de *Coffea arabica* são fontes promissoras de compostos bioativos.

Palavras-chave: *Aspergillus ochraceus*. *Fusarium verticillioides*. Inibição. *Muscodor* spp. Compostos voláteis.

ABSTRACT

Endophytic fungi are a promising source for discovery of compounds with biotechnological potential. The aim of this study was to select and identify endophytic fungi from *Coffea arabica* that produce volatile organic compounds (VOCs), evaluate the effect of the VOCs produced by endophytic fungi on the growth of *Rhizoctonia solani*, *Fusarium oxysporum*, *Phoma* sp., *Botrytis cinerea*, *Fusarium solani*, *Fusarium verticillioides*, *Cercospora coffeicola* and *Pestalotia longisetula*, and select endophytic fungi with potential for biological control of *Aspergillus ochraceus* inoculated in coffee beans and *F. verticillioides* inoculated in corn seeds. An isolate of *Muscodor albus* was used as selection tool for VOC producing fungi. Among the 400 endophytic fungi isolates, 11 were able to grow in the presence of VOCs produced by *M. albus*. These fungi were identified as *Muscodor* spp. (9) and *Simplicillium* sp. according to searches in UNITE database using DNA sequences of internal transcribed spacer (ITS). The VOC's produced by endophytic fungi inhibited the phytopathogenic fungi growth with different efficacies, compared to the control. The VOCs produced by *Muscodor coffeanum* (COAD 1842) showed fungicidal effect against *A. ochraceus* on coffee beans. Six endophytic fungi completely inhibited growth of *F. verticillioides* inoculated in corn seeds. This study demonstrates that the volatile-compound producing endophytic fungi, isolated from *Coffea arabica*, are promising sources of bioactive compounds.

Keywords: *Aspergillus ochraceus*. *Fusarium verticillioides*. Inhibition. *Muscodor* spp. Volatiles compounds.

1 INTRODUCTION

Endophytic fungi colonize living tissues of various plants, establishing mutualistic relationship without causing any symptom of disease (PETRINI, 1991; AZEVEDO et al., 2000; HYDE; SOYTONG, 2008). Their distribution within plants is ubiquitous but varies according to plant tissue (root, leaf, stems and fruits) and from strain to strain (TAN; ZOU, 2001). Endophytes have received considerable attention because of their ability to produce several novel compounds including terpenoids, alkaloids, phenylpropanoids, polyketides, aminoacids, and phytohormones (STROBEL et al., 2001; TEJESVI et al., 2007).

Some metabolites produced by endophytic fungi can help the host plant tolerate biotic and abiotic stress, protect plants against diseases and from insect and nematode attack, as well as favor the growth of crop plants (KOGEL et al., 2006). In addition, endophytic fungi have been reported to reduce the growth of the different phytopathogenic fungi (EZRA; STROBEL, 2003; ZHANG et al., 2010; SUWANNARACH et al., 2012; SAXENA et al., 2015). *Muscodor* species produce a mixture of volatile organic compounds that open new possibilities for the biological control of microbial decay in food and agriculture by biofumigation (STROBEL et al., 2001; DAISY et al., 2002; MERCIER;SMILANICK 2005; GRIMME et al., 2007).

Studies have been conducted with endophytes using species of plants that have economic significance, especially coffee crops (SANTAMARIA; BAYMAN, 2005; VEGA et al., 2005a, 2006b, SETTE et al., 2006a; SAUCEDO-GARCIA et al., 2014). Due to the economic importance of this crop and biotechnological potential of endophytic fungi, the aims of this study were to isolate and identify endophytic fungi from *Coffea arabica* in Brazil that produce volatile organic compounds (VOCs), evaluate the effect of the VOCs produced by endophytic fungi on the growth of phytopathogenic fungi and select

endophytic fungi with potential for biological control of *A. ochraceus* in coffee beans and *F. verticillioides* in corn seeds.

2 MATERIAL AND METHODS

2.1 Sample collection and isolation

Field surveys were carried out during 2011 in the Zona da Mata region, Viçosa municipality, Minas Gerais, Brazil to obtain endophytic fungi on native coffee plantations. Coffee tissue parts were rinsed in sterile distilled water for 1 min and dried. Small pieces (4–5 mm) of apparently healthy tissue were then disinfected in 70% ethanol for 1 min followed by 2.5% sodium hypochlorite for 3 min and washed in sterile distilled water. Fragments were placed in Petri dishes with Potato Dextrose Agar (PDA - Acumedia®) amended with chloramphenicol 100 ppm and incubated at 25°C. Hyphal tips of fungal colonies emerging from plant tissue pieces were transferred to PDA dishes and incubated at 25°C. The cultures were stored in tubes on PDA at 10°C.

2.2 Screening of VOC producing isolates

Screening of endophytic fungi that produce VOCs was conducted as described by Strobel et al., (2001) with modifications. A culture of the original isolate of *Muscodor albus* (strain CZ620) was used as selection tool for VOC producing fungi. *Muscodor albus* was placed and grown on one side of the plate for 7 days at 25ºC. A mycelial disk of each endophytic isolate (5 mm diameter) was deposited on the opposite side. Each isolate was tested in three replicates. The plate was wrapped with Parafilm® and incubated at 25ºC for one week. The experiment was performed twice and only isolates able to grow in the presence of VOCs produced by *M. albus* were selected for identification.

2.3 Molecular identification

The genomic DNA was extracted from pure cultures grown on PDA using a Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A). The internal transcribed spacer (ITS) was amplified using primers ITS1 and ITS4 (WHITE et al., 1990). PCR products were purified and sequenced by Macrogen, South Korea. The sequences were edited using BioEdit software (HALL, 1999). A BLAST search was performed to check for similarity with other sequences and identification was performed according to searches in UNITE database (NILSSON et al., 2014).

2.4 Bioassay for volatile antimicrobials

The inhibitory antimicrobial activity of VOCs produced by endophytic fungi from coffee was tested against the following phytopathogenic fungi: *Rhizoctonia solani* (LAPS 369), *Fusarium oxysporum* (LAPS 152), *Phoma* sp. (DFP 01), *Botrytis cinerea* (LAPS 300), *Fusarium solani* (LAPS 298), *Fusarium verticillioides* (CML 1896), *Cercospora coffeicola* (CML 2984) and *Pestalotia longisetula* (DFP 02). Endophytes were cultivated in PDA medium in Petri dishes and incubated at 25°C for 7 days. After this period, the phytopathogenic fungi were transferred to the other side of the plate. The plates were incubated at 25°C for 7 days. Phytopathogenic fungal growth was measured and compared with control plates without endophytic fungi. The colony diameters (cm), were measured and classified the according to following scale: T-Total inhibition (0); P-Partial inhibition (1-2.0); N- No inhibition (≥2.1). The experiment was repeated twice with three replicates.

2.5 Biofumigation with endophytic fungi from coffee

The VOCs produced by endophytic fungi were tested against *A. ochraceus* (SCM 1.15), producer of sclerotia and ochratoxin A in coffee grains (coffee in the dried bean and hulled coffee), isolated belonging to the Culture Collection of the Departament of Food Sciences (CDCA; Federal University of Lavras, Minas Gerais, Brazil), and *F. verticillioides* (CML1896), in corn grains. Grains of coffee and corn were surface disinfested by immersion in 70% ethanol for 3 min, sodium hypochlorite at 2.5% for 5 min and three times with sterile distilled water. After air-dying coffee and corn grains, they were inoculated by immersion in a suspension of *A. ochraceus* (1.5×10^5 conidia mL^{-1}) and *F. verticillioides* (2.0×10^5conidia mL^{-1}) spores, respectively. In bipartite Petri dishes containing PDA medium, the endophytes were cultivated for 7 days at 25°C. After this period, coffee grains inoculated with *A. ochraceus* and corn grains inoculated with *F. verticillioides* were placed on the other side of the plate. The effect of volatile compounds produced was evaluated by the presence or absence of growth in grains inoculated with *A. ochraceus* and *F. verticillioides.* The control treatment consisted of grains inoculated with plant pathogens without the presence of the endophytic fungi. To assess the fungistatic and fungicidal action of the volatile compounds the coffee grains and corn seeds, they were transferred to PDA medium after 7 days of exposure to volatile compounds. The experiment was repeated twice with three replicates.

3 RESULTS AND DISCUSSION

3.1 Endophytic fungi from *Coffea arabica*

A total of 620 fragments were obtained from stems (391), leaves (113) and fruits (116) of the *Coffea arabica*. Among the 400 endophytic fungi isolated from stems (261), fruits (97), and leaves (42), eleven (stems 7 and leaves 4) were able to grow in the presence of VOCs produced by *M. albus*. Colonies of 11 endophytic fungi on PDA were white, cottony with slow growth and absence of sporulation. The fungi were identified as *Muscodor coffeanum* (3), *Muscodor vitigenus* (4), *Muscodor yucatanensis* (2) and *Simplicilium* sp. (2), according to searches in UNITE database using DNA sequences of internal transcribed spacer (ITS) (Table 1).

Table 1 - Identification of the isolated endophytic fungi producing volatile compounds.

Isolate	Origin	Accession no.
M. coffeanum (COAD1842)	Leaf Stem	KM514680
M. coffeanum (COAD1899)	Leaf	KM514681
M. coffeanum (COAD1900)	Leaf	KP862879
M. vitigenus (C20)	Stem	KU094049
M. vitigenus (HZM10)	Stem	KU094053
M. vitigenus (HZM39)	Stem	KU094054 KU094055
M. vitigenus (HZM41)	Stem	KU094055
M. yucatanensis (HZM60)	Leaf	KU094056
M. yucatanensis (HZM64)	Leaf	KU094052
Simplicillium sp. (C18)	Stem	KU094050
Simplicillium sp. (C12)	Stem	KU094051

Muscodor is a genus of sterile endophytic fungi, all species of this genus were characterized by the production of volatile organic compounds (VOCs) that inhibit the growth of other microorganisms (STROBEL et al., 2001a; STROBEL, 2006b; STROBEL, 2011c; STINSON et al., 2003; MERCIER; JIMENEZ, 2004; MERCIER; MANKER 2005; MERCIER et al., 2007; WORAPONG; STROBEL, 2009; ZHANG et al., 2010; SUWANNARACH et al., 2012; KUDALKAR et al., 2012; SAXENA et al., 2015).

The species *M. vitigenus*, identified in our study was first isolated from *Paullinia paullinioides* by Daisy et al. (2002). These authors report that this specie produces compounds such as styrene, benzaldehyde, butylated hydroxytoluene, toluene, naphthalene and a number of minor benzene derivatives and that the compound naphthalene, causes modifications in insect behaviour.

Muscodor yucatanensis, one of the species identified, is a recognized producer of an intense musty odor. Colonies, when grown on PDA, usually form a whitish, flocculose colony with an uncolored reverse and a mycelium that grows slowly (GONZÁLEZ et al., 2009).

Two isolates of the genus *Simplicillium* also were identified in our study. The species *S. lonosoniveum* and *S. lamellicola* were isolated from coffee plants but not as endophytes and they have been exploited as biological control agent (ZARE et al., 2001; WARD et al., 2010).

Muscodor coffeanum reported in this study (COAD 1842, COAD 1899 and COAD 1900) is a new species isolated from leaves and stems from coffee plants in Brazil (HONGSANAN et al., 2015).

3.2 Biological activity of the VOC's produced by endophytic fungi

The action of VOCs produced by endophytic fungi was tested against a spectrum of phytopathogenic fungi and fungi associated postharvest diseases (Table 2). The VOCs produced by endophytic fungi exhibited antifungal activity with different efficiency. The phytopatogenic fungi *R. solani, C. coffeicola* and *Phoma* sp., were completely suppressed by VOCs produced by most endophytic fungi, whereas fungi like *B. cinerea, A. ochraceus* and *F. verticillioides* showed sensitivity to VOCs.

Previous works with VOCs produced by *Muscodor albus* presented antimicrobial potential against fungi and bacteria. The growth of *Botrytis cinerea, Aspergillus fumigatus, Rhizoctonia solani, Sclerotinia sclerotiorum, Pythium ultimum, Verticillium dahliae, Phytophthora cinnamomi, Candida albicans, Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus* was inhibited or the fungi died after exposure to VOCs of *M. albus* (WORAPONG et al., 2001; STROBEL et al., 2001). Moreover, other *Muscodor* species have been described to inhibit the growth of fungi associated with post-harvest decay (MITCHELL et al., 2008).

None of the endophytic fungi showed total inhibition against *F. oxysporum*. *Fusarium* species may be less susceptible to VOCs (FIALHO et al., 2010). The VOCs produced by endophytic fungus *M. yucatanensis* were lethal to *Colletotrichum* sp., *Phomopsis* sp., *Guignardia mangiferae*, *Phythophthora capsici, P. parasitica, Rhizoctonia* sp., and *Alternaria solani* but there was no complete growth inhibition of *F. oxysporum* when compared with the control (MACÍAS-RUBALCAVA et al., 2010).

Strobel et al., (2001) also found similar results, among several tested fungi the phytopathogenic fungi *Fusarium solani* was more resistant to the VOCs produced by *M. albus*. In addition, the artificial mixtures of VOCs

produced by *Gliocladium* sp. partially inhibited *F. oxysporum* (STINSON et al., 2003).

In our study, the VOCs produced by *Simplicillium* sp. (C12, C18) also exhibited antifungal activity, the isolate C12 completely inhibit the growth of *R. solani*, *C. coffeicola* and *Phoma* sp., whereas isolate C18 inhibited total growth of *C. coffeicola*. To our knowledge, our study is one of the few that has reported the antifungal activity of *Simplicillium* sp. through production of VOCs. Thus, these isolates may be candidates for more detailed studies involving biological control. Moreover, further studies are needed to understand how these compounds act and to know their effect on these organisms.

Table 2 - Action of volatile organic compounds produced by endophytic fungi in inhibition growth of phytopathogenic fungi.

Endophytic fungi	Phytopathogenic fungi								
	B. cinerea	***A. ochraceus***	***F. solani***	***R. solani***	***F. verticillioides***	***C. coffeicola***	***F. oxysporum***	***Phoma* sp.**	***P. longisetula***
M. coffeanum (COAD1842)	T	P	N	T	T	T	N	T	P
M. coffeanum (COAD1899)	T	N	P	T	T	P	N	T	T
M. coffeanum (COAD1900)	T	N	N	T	T	P	P	P	P
M. vitigenus (C20)	P	N	N	T	T	T	N	T	T
M. vitigenus (HZM10)	P	N	N	T	N	T	P	T	T
M. vitigenus (HZM39)	P	N	N	T	N	T	N	T	T
M. vitigenus (HZM41)	P	T	N	P	N	T	P	T	P
M. yucatanensis (HZM60)	T	N	P	T	P	T	P	P	P
M. yucatanensis (HZM64)	T	P	N	T	P	T	N	T	P
Simplicillium sp. (C12)	P	N	P	T	P	T	N	T	P
Simplicillium sp. (C18)	P	N	N	P	N	T	N	P	P

T-Total inhibition (0); P-Partial inhibition (1-2.0 mm); N- No inhibition (≥2 mm).

3.3 Activity of VOCs against seed pathogens

The endophytic fungus *M.* c*offeanum* (COAD 1842) presented fungicidal activity since it completely inhibited the mycelial growth of *A. ochraceus*. The endophytic fungi *Simplicillium* sp. (C12), *M.* c*offeanum* (COAD 1900) and *M.* c*offeanum* (COAD 1899) showed fungistatic activity. Endophytic fungi also showed growth inhibition of *F. verticillioides* in corn seeds. Among the eleven evaluated endophyte fungi the isolates *M.* c*offeanum* (COAD 1842), *M. coffeanum* (COAD 1899), *M. coffeanum* (COAD 1900), *M. vitigenus* (C20), *Simplicillium* sp. (C12) and *Simplicillium* sp. (C18) showed total growth inhibition of *F. verticillioides* (Table 3).

The fungus *A. ochraceus* is reported as producer of ochratoxin A (OTA) in coffee beans, and its presence, as well as the production of OTA in coffee, is undesirable because it may be used as a trade barrier, affecting the economies of producing countries (SUAREZ-QUIROZ et al., 2004). The fungi, producers of volatile compounds with broad antimicrobial activity, isolated from *C. arabica* have potential for biotechnological applications.

These findings open new possibilities for developing mycofumigation as a post-harvest treatment, since, *Muscodor* spp. and *Simplicilium* stand out as potential candidates for biocontrol agents in post-harvest technology, constituting an alternative to replace chemical fungicides.Characterization studies on the bioactive metabolites of the potent fungal strains from *C. arabica* and their use as biocontrol agents are in progress.

Fusarium verticillioides is one of the most commonly reported soil-borne fungal pathogens infecting maize (*Zea mays* L.), one of the most important cereal grains grown worldwide. This fungus produces secondary metabolites such as fumonisins (FB), especially fumonisin B1 (FB1), which affects human and animal health (BACON et al., 1996). Since *F. verticillioides*

is endophytic in maize and is almost universally associated with maize and maize products, it is very important to control this species in this agriculturally important commodity. Furthermore, root colonization by *F. verticillioides* has been considered the initiator of systemic infection that eventually results in the fungus producing fumonisins in kernels. Seed treatment with biocontrol agents is an appropriate method for biocontrol of soil-borne plant pathogens in the spermosphere and rhizosphere (KERRY, 2000).

Table 3 - *Aspergillus ochraceus* and *F. verticillioides* inhibited by volatile organic compounds produced by endophytic fungi.

Endophytic fungi	***A. ochraceus* in Coffee in the dried pod**	***A. ochraceus* in Hulled coffee**	***F. verticillioides* in corn seed**
M. coffeanum (COAD1842)	+	+	+
M. coffeanum (COAD1899)	±	±	+
M. coffeanum (COAD1900)	±	±	+
M. vitigenus (C20)	-	-	+
M. vitigenus (HZM10)	-	-	±
M. vitigenus (HZM39)	-	-	±
M. vitigenus (HZM41)	-	-	±
M. yucatanensis (HZM60)	-	-	±
M. yucatanensis (HZM64)	-	-	±
Simplicillium sp. (C12)	-	-	+
Simplicillium sp. (C18)	±	±	+

Total inhibition (+); Partial inhibition (±); No inhibition (-).

4 CONCLUSION

Volatile compound producing endophytic fungi were isolated from *C. Arabica*. Among the 400 fungi, 11 isolates were able to grow in the presence of VOCs produced by *M. albus*. The VOC producing fungi belong the genus *Muscodor* (9) and *Simplicillium* (2). The volatile compounds produced by *M. coffeanum* (COAD 1842) showed fungicidal activity against *A. ochraceus* and six isolates inhibited the growth of *F. verticillioides*.

The present study demonstrates the potential of fungal endophytes from *C. arabica* with antimicrobial action, since, plant pathogens were inhibited or killed by endophytic fungi, producers of volatile organic compounds.

CONFLICT OF INTEREST

No conflict of interest declared.

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ARTIGO 2

Normas do periódico *Ciência e Agroteconologia*
Artigo em fase de submissão no periódico *Ciência e Agrotecnologia*

Volatile organic compounds produced by *Acremonium* sp. with antimicrobial action

RESUMO

Acremonium sp. (C19), isolado como fungo endofítico de *Coffea arabica*, foi avaliado quanto à produção de compostos orgânicos voláteis (COVs), como parte de seu modo de ação contra fungos fitopatogênicos (*Botrytis cinerea*, *Aspergillus ochraceus*, *Fusarium solani*, *Fusarium oxysporum*, *Fusarium verticillioides*, *Cercospora coffeicola*, *Pestalotia longisetula* e *Phoma* sp.). COVs produzidos por *Acremonium* sp. (C19) causaram redução significativa no crescimento de *B. cinerea* e *P. longisetula* e a inibição completa de *C. coffeicola*, *R. solani*, *Phoma* sp. e *F. solani*. Em contraste, os fungos *A. ochraceus* e *F.oxysporum* foram menos susceptíveis aos COVs do que outras espécies testadas. O perfil de COvs produzidos por *Acremonium* sp. (C19) foram investigados utilizando-se a técnica de SPME-GC-MS e revelaram compostos, tais como etanol, 3-metil-1-butanol e 2-metil-1-butanol, dentre outros. A bioatividade dos COVs produzidos sugere que *Acremonium* sp. (C19) desempenha papel importante na defesa da planta hospedeira contra fungos fitopatogênicos. Além disso, para o nosso conhecimento, este estudo é o primeiro a identificar compostos orgânicos voláteis com ação antimicrobiana de *Acremonium* sp. (C19) isolado como endofítico de plantas de café no Brasil.

Palavras-chave: Potencial antimicrobiano. *Acremonium* sp. Prospecção. Fungos endofíticos. GC-MS.

ABSTRACT

Acremonium sp. (C19) isolated as an endophytic fungi from *Coffea arabica* was evaluated for volatile organic compound (VOC) production as a part of its mode of action against phytopathogenic fungi (*Botrytis cinerea*, *Aspergillus ochraceus*, *Fusarium solani*, *Fusarium oxysporum*, *Fusarium verticillioides*, *Cercospora coffeicola*, *Pestalotia longisetula* and *Phoma* sp.). VOCs produced by *Acremonium* sp. caused significant reduction in the growth of *B. cinerea* and *P. longisetula* and complete inhibition of *C. coffeicola*, *R. solani*, *Phoma* sp., and *F. solani*. In contrast, the fungi *A. ochraceus* and *F.oxysporum* were less susceptible to VOCs than other species tested. The profile of *Acremonium* sp. VOCs were investigated using SPME combined wih Gas chromatography-mass spectrometry technique and revealed compounds such as ethanol, 3-methyl-1-butanol, 2-methyl-1-butanol among others. The VOCs bioactivity suggest that *Acremonium* sp. (C19) plays an important mutualistic role by host defensive response against pathogens of plants. Besides that, to our knowledge, this study is the first to identify VOCs with antimicrobial action of *Acremonium* sp. (C19) isolated as endophytic fungus coffee plants in Brazil.

Keywords: Antimicrobial potential. *Acremonium* sp. Bioprospection. Endophytic fungi. GC-MS

1 INTRODUCTION

Endophytic microorganisms inhabit the interstitial spaces of plant tissues and cause no apparent symptoms of their presence in the plant (BACON and WHITE, 2000). The relationship existing between the fungi and the plant can vary from symbiotic to pathogenic (STROBEL and DAISY, 2003). These interactions are dependent on species, physiological state, chemistry of the host and environmental conditions (ESPINOSA-GARCIA et al., 1993, GILBERT and STRONG, 2007).

The endophytic fungi can be beneficial to diverse host plants. The secondary metabolites produced by endophytes have action against insects, pathogens or competitors, increase the fitness of plants by enchancing their tolerance to abiotic stress and promote plant growth (REDMAN et al., 2002; ARNOLD and LEWIS, 2005; RUBINI et al., 2005; SCHULZ and BOYLE 2005, RAHMAN and SAIGA 2005; BAILEY, et al., 2008; JOHNSTON-MONJE and RAIZADA, 2011). Different biological classes of natural products with antibacterial, antifungal, antitumor and antiviral properties have been reported from endophytic fungi (VERMA; KHARWAR; STROBEL, 2009; ALY et al., 2010).

Among the metabolites produced by endophytic fungi, the volatile organic compounds (VOCs) have been highlighted due to their biological activity against fungi, bacteria, and insects, presenting potential use in agriculture (MORATH; HUNG; BENNETT, 2012). The species of the genus *Acremonium* are reported as a rich source of biologically active secondary metabolites including β-lactam antibiotics, cyclosporins, lolitrem, acremines and prenylated phenol inhibitors of N-SMase (MOUSSAIF et al., 1997; ADINARYANA et al., 2003; ASSANTE et al., 2005; FRASER et al., 2013). However, to our knowledge few studies have reported the production of VOCs

from *Acremonium* species with antimicrobial action. Thus, this study aimed were to investigate of the action VOCs from *Acremonium* sp. (C19) against the phytopathogenic fungi, *Botrytis cinerea*, *Aspergillus ochraceus*, *Fusarium solani*, *Fusarium oxysporum*, *Fusarium verticillioides*, *Cercospora coffeicola*, *Pestalotia longisetula* and *Phoma* sp. and to identify the compounds emitted by *Acremonium* sp. (C19) with the solid phase microextration (SPME)-gas chromatographic technique.

2 MATERIALS AND METHODS

2.1 Isolate *Acremonium* sp. (C19)

The strain of *Acremonium* sp. (C19) used in this study was obtained from stems of *Coffea arabica* located in the Zona da Mata Mineira, Viçosa – MG and currently belongs to the collection at the Fungi Bioprospecting and Genetics Laboratory (Biogen) of the Federal University of Lavras, Brazil. The fungus was identified based on its molecular biology. The culture was maintained in MilliQ water at 4°C. The isolate was reactivated on potato dextrose agar (PDA) and incubated at 25°C for 7 days.

2.2 Microbial inhibitory activity

The inhibitory antimicrobial activity of VOCs produced by *Acremonoium* sp. (C19) was tested against the fungi: *Rhizoctonia. solani*, *Fusarium oxysporum*, *Phoma* sp., *Botrytis cinerea*, *Fusarium. solani*, *Fusarium verticillioides*, *Cercospora coffeicola*, *Aspergillus ochraceus*, and *Pestalotia longisetula*. The endophytic fungus was cultivated in PDA medium in bipartide Petri dishes and incubated at 25°C for 7 days. After this period, phytopathogenic

fungi were transferred to the other side of the plate. The plates were incubated at 25°C for 7 days. Phytopathogenic fungi growth was measured and compared with control plates without endophytic fungi. The diameter of the colonies (mm) were measured. The experiment was repeated twice with three replicates.

2.3 Characterization of VOCs

The analyzes of VOCs produced by *Acremonium* sp. (C19) were conducted at the at the Center for Chemical Analysis and Prospecting – CAPQ, Department of chemistry of the Federal University of Lavras - UFLA. *Acremonium* sp. (C19) was inoculated in 20 mL SPME vials containing PDA culture medium. After 7 days of incubation, the VOCs were extracted via headspace - SPME (ARTHUR and PAWLISZYN, 1990). The SPME extraction was performed as follows: a DVB/CAR/PDMS (Divinylbenzene, Carboxen, and Polydimethylsiloxane) fiber, extraction temperature of 55°C with 250 rpm sample agitation, 35 min extraction time and 2 min desorption time in the GC injector. A GC-MS QP 2010 Ultra (Shimadzu, Japan) gas chromatograph coupled with a mass spectrometer equipped with an AOC-5000 (Shimadzu, Japan) automatic injector for liquids and gases and an HP-5 (5% phenyl-95% dimethyl siloxane) 30 m × 0.25 mm × 0.25 m column was used to separate the VOCs. The injector, interface and ion detector temperatures were 250°C, 240°C, and 200°C, respectively. The injector was operated in the splitless mode. The carrier gas was grade 5.0 He with a flow of 1.0 mL min^{-1}. The GC oven temperature was increased at a rate of 3°C min^{-1} from 40°C to 160°C and then at 10°C min^{-1} to 240°C.

The VOCs were identified by comparing the mass spectrum obtained via the Automated Mass Spectral Deconvolution and Identification System (AMDIS) v. 2.6 software to those in the NIST library using the Mass Spectral Search Program v. 1.7 (NIST, Washington DC, USA) software. For the mass spectra comparison, only spectra with a similarity better than 80% were considered. In order to improve identification, experimental retention indices (RI Exp) were obtained by injecting a homologous series of alkanes and comparing to those reported in the literature (RI Lit) (ADAMS, 2007; NIST, 2013).

3 RESULTS

3.1 Biological activities of the VOCs the *Acremonium* sp. (C19)

The endophytic fungi showed antagonistic property during the preliminary test. The exposure to volatile compounds for seven days was enough to severely reduce *B. cinerea*, *P. longisetula* and *F. verticillioides* and totally inhibit the growth of *F. solani*, *R. solani*, *C. coffeicola* and *Phoma* sp. However, the fungi *A. ochraceus* and *F. oxysporum* responded differently to VOCs produced by *Acremonium* sp. (C19), these fungi presented as more tolerant during the exposure to VOCs, as their growth rates were similar to the control (Table 1).

Table 1 - Influence of the VOCs of *Acremonium* sp. (C19) on other fungi.

Phytopathogenic fungi	**Growth (cm) after 7 days of expossure**	**Growth (cm) the pathogen in the absence of VOCs after 7 days**
B. cinerea	2.8	6.2
A. ochraceus	5.8	6.0
C. coffeicola	0.0	5.5
F. solani	0.0	6.0
F. verticillioides	3.0	5.7
F. oxysporum	5.2	5.8
R. solani	0.0	6.3
P. longisetula	2.5	4.6
Phoma sp.	0.0	5.4

3.2 Chemical composition of the fungal volatiles

The VOCs present in the headspace of *Acremonium* sp. (C19), were investigated using SPME combined wih GC-MS technique. The DVB/CAR/PDMS fiber was chosen as a non-specific fiber and revealeded the presence of at least 25 VOCs consisting mainly of alcohols, esters and terpenes (Table 2). The most intense peaks in the chromatogram were ethanol, 3-methyl-1-butanol, 2-methyl-1-butanol and two non-identified compounds. The mass spectra of these unidentified peaks reveals a pattern fragmentation of non-oxigenated sesquiterpenes, although the correct structure could not be assigned.

Table 2 - GC/MS analysis of the VOCs of *Acremonium* sp. (C19).

	Compound	**IR Exp.**	**IR Lit.**
Alcohols			
1	Etanol	-	-
2	1-propanol	-	558
3	2-methyl-1-propanol	626	622
4	3-methyl-1-butanol	734	734
5	2-methyl-1-butanol	735	738
6	phenylethyl alcohol	1113	1114
Acids			
1	2-methyl-propanoic acid	810	793
Esters			
1	ethyl acetate	612	613
2	methyl 2-methyl-propanoate	681	685
3	ethyl 2-methyl-propanoate	752	762
4	2-methylpropyl acetate	768	767
5	3-methyl-1-butanol acetate	874	876
6	2-methyl-1-butanol acetate	876	880
Ketones			
1	2-nonanone	1090	1091
2	4-heptanone	869	871
Terpenes			
1	longifolene (iso)	1387	1390
2	β-elemene	1392	1390
3	β-cedrene	1421	1420
4	α bergamotene	1438	1436
5	α guaiene	1441	1439

6	non-oxygenated sesquiterpene*	1488	-
7	β-guaiene (cis)	1493	1492
8	β-guaiene (trans)	1506	1502
9	α bulnesene	1506	1505
10	non-oxygenated sesquiterpene*	1657	-

4. DISCUSSION

Fungi belonging to the genus *Acremonium* have been reported for their ability to produce secondary metabolites with antimicrobial action (WICKLOW et al., 2005; ARNONE et al., 2009; FRASER, et al., 2013; XIAO et al., 2014).The action of VOCs produced by *Acremonium* sp. (C19) was evalueted against *B. cinerea, A. ochraceus, F. solani, F. oxysporum, F. verticillioides, C. coffeicola, R. solani, Phoma sp*., and *P. longisetula*. Tests on bipartite Petri dishes showed a significant reduction of the growth *B. cinerea* and *P. longisetula* and complete inhibition of *C. coffeicola*, *R. solani*, *Phoma* sp., and *F. solani*.

Similar results involving VOCs with antimicrobial action were found in microrganisms such as *Candida intermedia* (C410), that inhibited the conidial germination and mycelial growth of *B. cinerea* (HUANG et al., 2011), *Penicillium expansum* (R82) which revealed antifungal activity against mycelial growth of important postharvest pathogens (ROUISSI et al., 2013), *Streptomyces globisporus* (JK-1) that inhibited *Botrytis cinerea* growth (LI et al., 2012 a) and showed effective control of *Penicillium italicum* (LI et al., 2010 b), *Gliocladium* sp., with production of lethal VOCs to *Pythium ultimum* and *Verticillum dahliae* (STINSON, et al., 2003). In addition, VOCs produced by *Muscodor* species showed strong biological activity against bacteria, fungi and

insects (STROBEL et al., 2001; DAISY et al., 2002; MERCIER and SMILANICK, 2005; LACEY et al., 2006).

In our study, not all fungi tested responded equally to the natural volatiles produced by *Acremonium* sp. (C19), the fungi *A. ochraceus* and *F.oxysporum* were less susceptible to VOCs. The susceptibility of the fungi to antimicrobial VOCs may vary according to the chemical nature and mode of action of the antimicrobial compound produced (WALKER et al., 1995). In addition, each species presents different sensitivities to the toxic effect of the volatile compounds. Furthermore, previous works reported that *A. ochraceus* was poorly controlled by compounds like acid isobutiric and 2-metil-1-butanol, produced by *M. albus* (BRAUN et al., 2012), and *Fusarium* species also are reported to be more resistant to antimicrobial VOCs (FIALHO et al., 2010).

In order to understand the nature of volatile substances produced by *Acremonium* sp. (C19), it was necessary to identify the components in the atmosphere. The VOCs, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-methyl-propanoic acid and phenethyl alcohol identified in this study were also reported in previous works (MERCIER and JIMENEZ, 2004; MIYAZAWA et al., 2008; STOPPACHER et al., 2010; FIALHO et al., 2010; ROUISSI et al., 2013;). The VOCs, 3-methyl-1-butanol and 2-methyl-1-butanol are reported as lipophilic compounds with high affinity for the plasma membrane and therefore present higher toxicity (HEIPIEPER et al., 1994).

A comparative analises the VOCs of *Acremonium* sp. (C19) revealed many compounds in common with those produced by *Muscodor* spp. (EZRA and STROBEL, 2003; EZRA; HESS;STROBEL, 2004; STROBEL, 2006; ZHANG et al., 2010; SUWANNARACK et al., 2010; KUDALKAR et al., 2012). Some of these compounds were: 3-methyl-1-butanol, 2-methyl-1-butanol,

2-methyl-propanoic acid, 2-methylpropyl acetate, phenylethyl alcohol and 2-nonanone.

According to Strobel et al., (2001), one of the most biologically active compounds among the VOCs of *M. albus* is 1-butanol, 3-methyl-, acetate. It alone produced complete inhibition of some of test assay fungi, showing potential antifungal. The presence of VOCs with antimicrobial action in *Acremonium* sp. is important in undestandings the biology of the fungus as well as their evolutionary relationship with theirs hosts.

This finding may be due to the fact that *Muscodor albus* (CZ620) was used as a selection tool in this study, enabling growth of endophytic fungi resistant to the volatiles of *M. albus* (CZ620). The VOCs of *M. albus* have been used effectively to eliminate other competing endophytic microorganisms, allowing the growth of Xylariaceous fungi and particularly other species of *Muscodor* (EZRA; HESS; STROBEL, 2004).

The presence to VOCs with antimicrobial action in *Acremonium* sp. is important to understand the biology of the fungus as well as its evolutionary relationship with its hosts. Herein, *Acremonium* sp. (C19) produced a mixture of volatile organic compounds (VOCs) with antimicrobial activities when cultured under *in vitro* conditions. Therefore, this isolate may make up new source of volatile compounds with important biotechnological applications.

CONCLUSION

This study report *Acremonium* sp. (C19) as a endophyte that producer of bioactive VOCs, since, showed antimicrobial activity against phytopathogenic fungi. Besides that, to our knowledge, this study is the first to identify VOCs

with antimicrobial action of *Acremonium* sp. (C19) isolated as endophytic fungus coffee plants in Brazil. The presente study leads to the need of further in depth studies on compounds bioactive produced by *Acremonium* sp. (C19) from *Coffea arabica*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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ARTIGO 3

Normas do periódico *Journal of Agricultural Science and Technology*
Artigo submetido no períodico *Journal of Agricultural Science and Technology*

Enzyme production by *Muscodor* and *Simplicillium* species from *Coffea arabica*

RESUMO

Micro-organismos endofíticos são aqueles que passam todo ou parte de seu ciclo de vida colonizando o interior dos tecidos de plantas hospedeiras, sem lhes causar qualquer dano aparente. Em vários estudos tem sido demonstrada a capacidade dos fungos endofíticos de produzirem metabólitos secundários. Dentre os metabólitos produzidos, destaca-se a produção de enzimas. O conhecimento da produção de enzimas por fungos endofíticos pode fornecer informações sobre suas possíveis aplicações biotecnológicas. Neste contexto, o objetivo deste estudo foi avaliar a atividade das enzimas amilase, celulase, lipase, pectinase, fitase, protease, endo β-1,4 glucanase e exo β-1,4 glucanase produzidas por doze fungos endofíticos isolados de plantas de café. Todos os fungos endofíticos apresentaram atividade celulolítica e pectinolítica; 10 foram produtores de lipase e protease; 9 produziram fitase e 4 foram produtores de amilase. A atividade específica das enzimas endo β-1,4 glucanase e exo β-1,4 glucanase foi detectada em 12 e 10 fungos endófitos, respectivamente. Nete estudo demonstra-se que os fungos endofíticos dos gêneros *Muscodor* e *Simplicilium*, produtores de compostos orgânicos voláteis isolados de *Coffea arabica*, são promissoras fontes de enzimas extracelulares.

Palavras-chave: Celulases, Enzimas extracelulares, *Muscodor* spp., *Simplicillium*

ABSTRACT

Endophytic microorganisms are those that spend all or part of their life cycle colonizing the interior of tissues in host plants without causing them any apparent harm. Several studies have demonstrated the ability of endophytic fungi to produce various secondary metabolites. Among the metabolites produced, enzyme production stands out. The knowledge of enzyme production by endophytic fungi may provide insights into their possible biotechnological applications. In this context, the aim of this study was to evaluate the activity of amylase, cellulase, lipase, pectinase, phytase, protease, endo β-1.4 glucanase and exo β-1.4 glucanase enzymes produced by twelve endophytic fungi isolated from coffee plants. All endophytic fungi presented cellulolytic, pectinolytic and lipolytic activity, 11 for protease, 9 for phytase and 4 for amylase. The specific activity of endo β-1.4 glucanase and exo β-1.4 glucanase enzymes were detected for 12 and 10 endophytic fungi, respectively. This study demonstrates that endophytic fungi of the genus *Muscodor* and *Simplicilium*, volatile compound producers isolated from *Coffea arábica*, are promising sources of extracellular enzymes.

Keywords: Extracellular Enzymes. Cellulases. *Muscodor* spp. *Simplicillium* sp.

INTRODUCTION

The term endophyte was first introduced by De Bary in the 19th century and was applied to any microorganism that resides inside the plant tissue. According to Petrini (1991), “endophytic fungi are those microorganisms that inhabit the plant tissue during some stage of their life cycle, and colonize the internal tissue of the plant, but without causing disease to the host”. These microorganisms protect their hosts against insect-pests and pathogenic microorganisms and provide several benefits to the host plant (Azevedo et al., 2000). In recent years, screening of new bioactive secondary metabolites from endophytes has received considerable attention, and a wide variety of metabolites have been isolated and characterized (Tan and Zou, 2001; Strobel et al., 2004).

In addition, these microorganisms are important producers of enzymes. Some studies have shown endophytic fungi have the capability to produce extracellular enzymes, such as amylases, lipases, proteases, pectinases and cellulases (Maria et al., 2005; Bischoff et al., 2009; Bezerra et al., 2012; Onofre et al., 2013; Sunitha et al., 2013; Desire et al., 2014). The production of enzymes is essential for endophytic fungi to colonize the plant. They produce enzymes that hydrolyze the cell walls of their hosts and some of these enzymes have the function of suppressing the invasion of pathogenic microorganisms (Gao et al., 2010; Sunitha et al., 2013).

The endophytic fungi have been found associated with economically important plants including *Coffea arabica* (Santamaría and Bayman, 2005; Veja et al., 2006; Vega et al., 2010). The coffee crop in Brazil, being the largest producer and exporter of coffee in the world, has great socioeconomic importance (Silva et al., 2015). The study of fungi associated with this plant becomes relevant, since the endophytic fungi are producers of metabolites useful

to agriculture and industry. Therefore, this study aimed to evaluate the potential of twelve endophytic fungi isolated from coffee plants, volatile organic compound producers, for the production of amylase, cellulase, lipase, pectinase, phytase, protease, endo β-1.4 glucanase and exo β-1.4 glucanase enzymes.

MATERIALS AND METHODS

Microorganisms

The fungi used in this study were obtained as endophytes of *Coffea arabica* plants located in the region of Mata Mineira, Viçosa - MG. Twelve fungi, producers of volatile organic compounds, were identified and selected to verify the production of extracellular enzymes (Table 1). The endophytic fungi cultures currently are a part of the collection at the Fungi Bioprospecting and Genetics Laboratory (Biogen) of the Federal University of Lavras - UFLA, Brazil. The isolates were reactivated on PDA medium and were incubated at 25°C for 7 days.

Table 1 - Origin of the isolates utilized in this study and their GenBank accession numbers.

Isolate	Origin	Accession no.
Muscodor coffeanum (COAD1842)	Stem	KM514680
Muscodor coffeanum (COAD1899)	Leaf	KM514681
Muscodor coffeanum (COAD1900)	Leaf	KP862879
Muscodor vitigenus (C20)	Stem	KU094049
Muscodor vitigenus (HZM10)	Stem	KU094053
Muscodor vitigenus (HZM39)	Stem	KU094054
Muscodor vitigenus (HZM41)	Stem	KU094055
Muscodor yucatanensis (HZM60)	Leaf	KU094056
Muscodor yucatanensis (HZM64)	Leaf	KU094052
Simplicillium sp. (C18)	Stem	KU094050
Simplicillium sp. (C12)	Stem	KU094051
Acremonium sp. (C19)	Stem	KU094048

Enzyme activity

The ability of endophytic fungi to produce Amylases, Cellulases, Lipases, Pectinases, Phytase, and Proteases were qualitatively assessed on solid medium. Fragments of the endophytic fungi grown in PDA for 7 days were transferred to the center of Petri dishes containing the solid medium with substrates specific for each enzyme.

Amylase: Amylase activity was assessed by growing the fungi in soluble starch (0.2%); glucose (0.1 %); yeast extract, (0.01%); peptone (0.05 %); Agar (1. 6%). The plates were incubated at 28°C for 7 days.

Cellulase: The medium for cellulose production consisted of the following: 0.2% $NaNO_3$, 0.1% K_2HPO_4, and 0.05% KCl, 0.02% peptone, 0.2%

Carboxymethylcellulose (CMC) and 1.7% agar. The plates were incubated at 28°C for 7days.

Lipase: The fungi were grown in medium containing Tween 20 (1.0%) as substrate, peptone (1.0%); NaCl (0.5%); $CaCl_2.2H_2O$, (0.01%); Agar (1.8%). The fungi were cultured at 30°C for 7 days.

Pectinase: The fungi were cultured in solid buffered mineral medium (KH2PO4, 0.2%; K2HPO4, 0.7% ; (NH4)2SO4, 0.1% ; MgSO4.7H2O, 0.1% yeast extract; 0.06%; citrus pectin, 0.3%; agar, 1.3%). The fungi were inoculated and maintained at 25°C for 7 days. After this time, mycelial disks were removed and transferred to the Macllvaine buffered medium (Agar, 1.3%; citrus pectin, 0.25%; solution of C6H8O7, 0.1M, 369 mL; solution of Na2HPO4, 0. 2 M, 631 mL) and, then incubated at 40°C for 48 hours.

Phytase: The fungi were cultured in medium containing phytic acid (C6H18O24P6), 0.5%; NaNO3, 0.3%; MgSO4.7H2O, 0.05%; KCl, 0.05%; FeSO4, 0.012%; CaCl, 0.06%; ZnSO4, 0.01%; Agar, 1.5%. The fungi were cultured for 7 days at 25°C.

Protease: For estimating the protease activity the medium contained, gelatin, 1.0%; skim milk, 1.0%; sodium citrate buffer 0.1 M (400 mL) and Agar, 1.8%. The fungi were incubated at 25°C for 7 days.

After the incubation period, the plate was flooded with iodine (2.0 g KI and 1.0 g iodine in 300 mL distilled water) for 3 to 5 min. The formation of a clear halo around the colonies was considered a positive result, indicating the presence of the given enzyme. The calculation of enzymatic index (EI) was performed via the ratio of the average degradation halo diameter and the average colony diameter, as proposed by Hankin and Anagnostakis (1975).

Enzyme activity assays for endo β-1, 4 glucanase and exo β-1, 4

The medium for cellulase production consisted of the following reagents: 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.02 g of peptone, and 0.2% of different inducers, Carboxymethylcellulose (CMC) and Avicel were used as inducers to produce endoglucanase and exoglucanase, respectively. Cultivation was conducted in 250 mL Erlenmeyer flasks containing 50 mL of production medium with the respective inducers. The Erlenmeyer flasks were incubated in a rotary shaker at 28ºC and 150 rpm, for 12 days. After the incubation period, the content of each flask was centrifuged and the enzymatic activities were determined. The enzymes in this study were analyzed according to Miller (1959) with modifications. For endoglucanase assays were performed in reaction tubes containing 125 µL of 2% CMC solution in 50 mM sodium citrate buffer (pH 4.8) with 125 µL of the enzymatic supernatant. The tubes were incubated at 50ºC for 10 min, and then 250 µL of DNS (3.5-dinitrosalicilic acid) was added to stop the reaction. The exoglucanase assays were performed according to Lever (1972) with modifications. The assays were conducted in reaction tubes containing 450 µL of 1% (w/v) Avicel, respectively, in 0.05 mM sodium acetate buffer, pH 5.0, with 50 µL of crude enzyme solution. The tubes were incubated at 50ºC for 30 min. To measure the glucose released, 1% ρ-hydroxybenzoic acid hydrazide was added (PAHBAH). The readings were performed in a spectrophotometer at 540 nm. One unit (U/mL) of enzyme activity was defined as the amount of enzyme that produces 1 µmol of glucose per minute under the assay conditions.

Protein determination

Protein concentrations were determined by the Bradford method, (1976), using bovine serum albumin (BSA) as standard.

Statistical analysis

The statistical analysis was performed with SISVAR 5.1 software (Statistical Analysis System) (Ferreira, 2008), using the Scott Knott test at 5% significance to compare the best isolates that produced endoglucanase and exoglucanase enzymes.

RESULTS AND DISCUSSION

Enzymatic profile of endophytes fungi

Twelve endophytic fungi were able to produce most of the extracellular enzymes evaluated (Table 2). These enzymes may help the endophytes to enter and colonize their hosts. Furthermore, the production of these enzymes by endophytic fungus acts as a resistance mechanism against pathogen invasion (Choi et al., 2005; Sunitha et al., 2013). According to the methodology employed, some isolates revealed significant substrate degradation zones, whereas other isolates showed fewer degradation areas, indicating different extracellular enzyme production levels. The hydrolysis halo diameter is useful in the selection of strains with high polysaccharide degradation levels and it is fast and simple to perform (Ten et al., 2004).

Cellulolytic and pectinolytic activities were observed in all the endophytic fungi, whereas the lipase and protease activities were only not present in *M. coffeanum* (COAD 1900) and *Simplicillium* sp. (C12). Amylolytic activity was observed in *M. coffeanum* (COAD 1900) and *M. vitigenus* (HZM10, HZM39, HZM41). Phytase activity was present in all species of *Muscodor* with EI ranges between 2.8 and 4.5. The specific phytase activity by *M. coffeanum* (COAD 1899) was evaluated after culture condition optimization. There was an 11 fold increased in the specific activity (Alves et al., 2016). Commercially, phytases are produced only by a few numbers of microorganisms, which justifies the importance of production by an endophytic fungus.

Table 2 - Enzymatic Index (EI) values.

Endophytic fungi	Cel[1]	Pec[2]	Phy[3]	Lip[4]	Amy[5]	Pro[6]
M. coffeanum (COAD1842)	2.1	2.1	3.3	3.0	-	1.5
M. coffeanum (COAD1899)	2.3	2.0	4.5	1.0	-	1.2
M. coffeanum (COAD1900)	2.7	1.8	4.4	-	2.1	1.3
M. vitigenus (C20)	4.0	2.8	2.8	1.0	-	1.3
M. vitigenus (HZM10)	1.4	1.8	3.4	2.7	2.0	1.4
M. vitigenus (HZM39)	1.7	2.3	3.2	2.0	1.9	1.6
M. vitigenus (HZM41)	1.5	2.1	2.6	1.0	2.2	1.3
M. yucatanensis (HZM60)	1.6	1.9	3.2	1.0	-	1.2
M. yucatanensis (HZM64)	1.8	1.7	3.7	1.0	-	1.2
Simplicillium sp.(C12)	1.4	2.0	-	1.0	-	-
Simplicillium sp. (C18)	2.3	2.0	-	1.0	-	1.6
Acremonium sp. (C19)	3.0	2.0	-	1.0	-	1.9

[1]Cellulase, [2]Pectinase, [3]Phytase, [4]Lipase, [5]Amylase, [6]Protease. Not detected (-).

Quantitative assessment of Endo-β 1. 4glucanase and Exo β-1.4 glucanase

Tests in solid media permit rapid screening for the presence or absence of extracellular enzymes and therefore we performed screening for six important enzymes. In this study we focused on Endo-β 1. 4glucanase and Exo β-1.4 glucanase activity. Cellulolytic enzymes present biotechnological application in various industries including the food, pharmaceutical, environmental and agricultural industries (Kuhad et al., 2011).

Analyzing the values obtained in the production of endo β-1.4 glucanase, all fungi were able to produce this enzyme. *M. coffeanum* (COAD 1842) and *Simplicillium* sp. (C12) were the best producers compared to the other fungi, with specific activity of 11. 89 and 10.04 Umg^{-1} respectively (Table 3). Regarding the production of exo β-1.4 glucanase, *M. vitigenus* (HZM41) was

the best producer with values of 4.48 Umg^{-1} and specific activity for the fungi *Simplicillium* sp. (C18) and *M. coffeanum* (COAD 1900) was not detected, despite showing positive production in the total cellulase evaluation. This fact can be explained by the variety of enzymes that comprise the cellulolytic complex that act jointly to degrade cellulose. Moreover, the selection method for enzyme producing fungi, using the revelation of the degradation halo and subsequent calculation of enzymatic index, merely allows rapid observation of positive and negative, but does not provide the production intensity (Kasana et al., 2008).

To our knowledge, our study is one of the few reporting enzyme activity of endophytic species of *Muscodor* and *Simplicillium* genera isolated from coffee plants. These genera are described as promising agents for biological control. Species of *Muscodor* emit a mixture of volatile organic compounds that inhibit or kill a broad range of pathogenic microorganisms and also insects. Also, many studies report the use of these compounds in post-harvest disease control and soil micofumigation (Strobel et al., 2001; Stinson et al., 2003; Mercier and Jimenez, 2004; Mercier and Manker, 2005; Strobel, 2006; Mercier et al., 2007; Worapong and Strobel, 2009; Zhang et al., 2010; Strobel, 2011; Suwannarach et al., 2013). Species of *Simplicillium* have been isolated and reported as promising biological control agents (Zare and Gams, 2001; Ward et al., 2010; Ward et al., 2012). In addition, *Muscodor coffeanum* (COAD 1842, COAD 1899 and COAD 1900), also used in our study, were described as new species by Hongsanan et al., (2015).

Table 3 - Total activity (U/mL) and specific enzymatic activity of endoglucanase and exoglucanase (U/mg) of endophytic fungi.

Endophytic fungi	Activity of β-1,4 glucanase			
	Endo (U/mL)		Exo (U/mg)	
	Total	Specific	Total	Specific
M. coffeanum (COAD1842)	1.42	11.89[a]	0.10	0.59[a]
M. coffeanum (COAD1899)	1.19	5.58[b]	0.11	1.11[a]
M. coffeanum (COAD1900)	0.62	1.98[c]	-	-
M. vitigenus (C20)	0.78	6.43[b]	0.18	0.98[a]
M. vitigenus (HZM10)	1.18	9.99[a]	0.27	1.54[a]
*M. vitigenus (*HZM39)	0.32	2.71[c]	0.08	0.46[a]
*M. vitigenus (*HZM41)	0.53	4.33[c]	0.78	4.48[a]
M. yucatanensis (HZM60)	0.90	7.06[b]	0.81	4.37[a]
M. yucatanensis (HZM64)	0.76	5.98[c]	0.22	1.60[a]
Simplicillium sp. (C18)	0.91	7.78[b]	-	-
Simplicillium sp. (C12)	1.27	10.04[a]	0.29	1.86[a]
Acremonium sp. (C19)	0.78	6.43 [b]	0.22	1.39 [a]

Means followed by the same letter do not differ by the Scott-Knott test (0.05).
Not detected (-).

CONCLUSION

Considering the results of this study, it was concluded that the evaluated endophytic species of *Muscodor* and *Simplicillium* isolated from coffee plant fungi have potential in the production of extracellular enzymes. Studies of endophytic fungi, especially of new species, are interesting, since the endophytic fungi present potential for exploration. Due to the limited number of studies demonstrating the enzymatic activity of endophytes, mainly fungi of the *Muscodor* genus, this work opens new perspective for the study of these species for the production and industrial application of these enzymes.

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CONFLICT OF INTEREST

No conflict of interest declared.

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ANEXO

Figura 1 - Morfologia dos fungos endofíticos isolados de *Coffea arabica* 93

Figura 2 - Ação dos compostos orgânicos voláteis (COVs) produzidos por fungos endofíticos na inibição do crescimento de fungos fitopatogênicos 94

Figura 3 - Efeito dos compostos orgânicos voláteis (COVs) produzidos por *Muscodor coffeanum* (COAD 1842) em grãos de café incoculados com *Aspergillus ochraceus* 96

Figura 4 - Efeito de compostos orgânicos voláteis (COVs) produzidos por fungos endofíticos em grãos de milho incoculados com *Fusarium verticillioides* 96

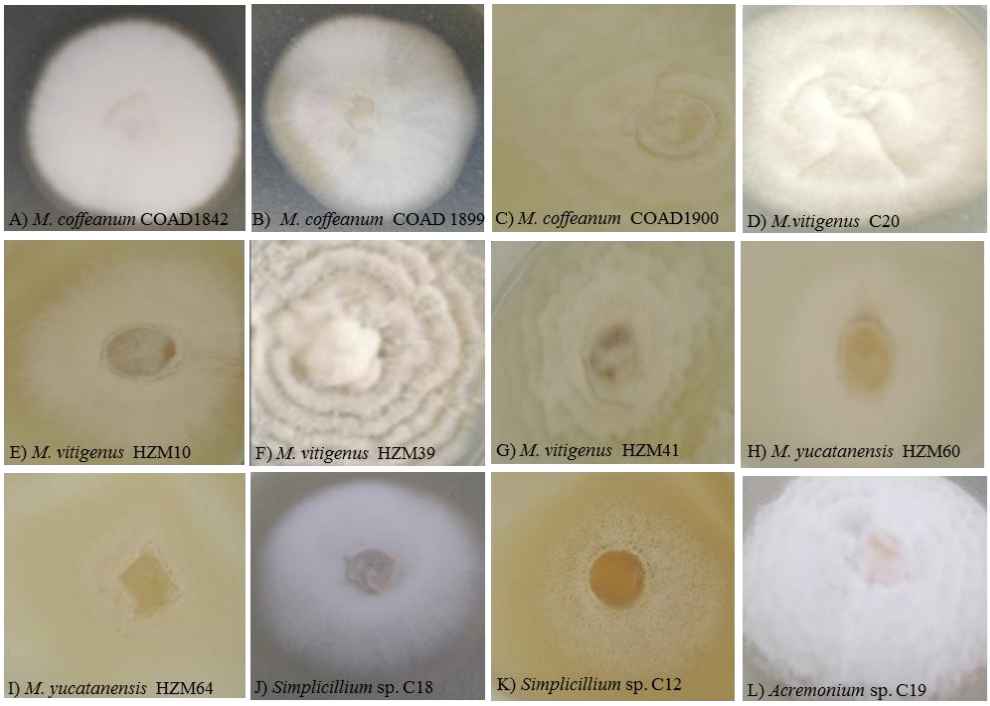
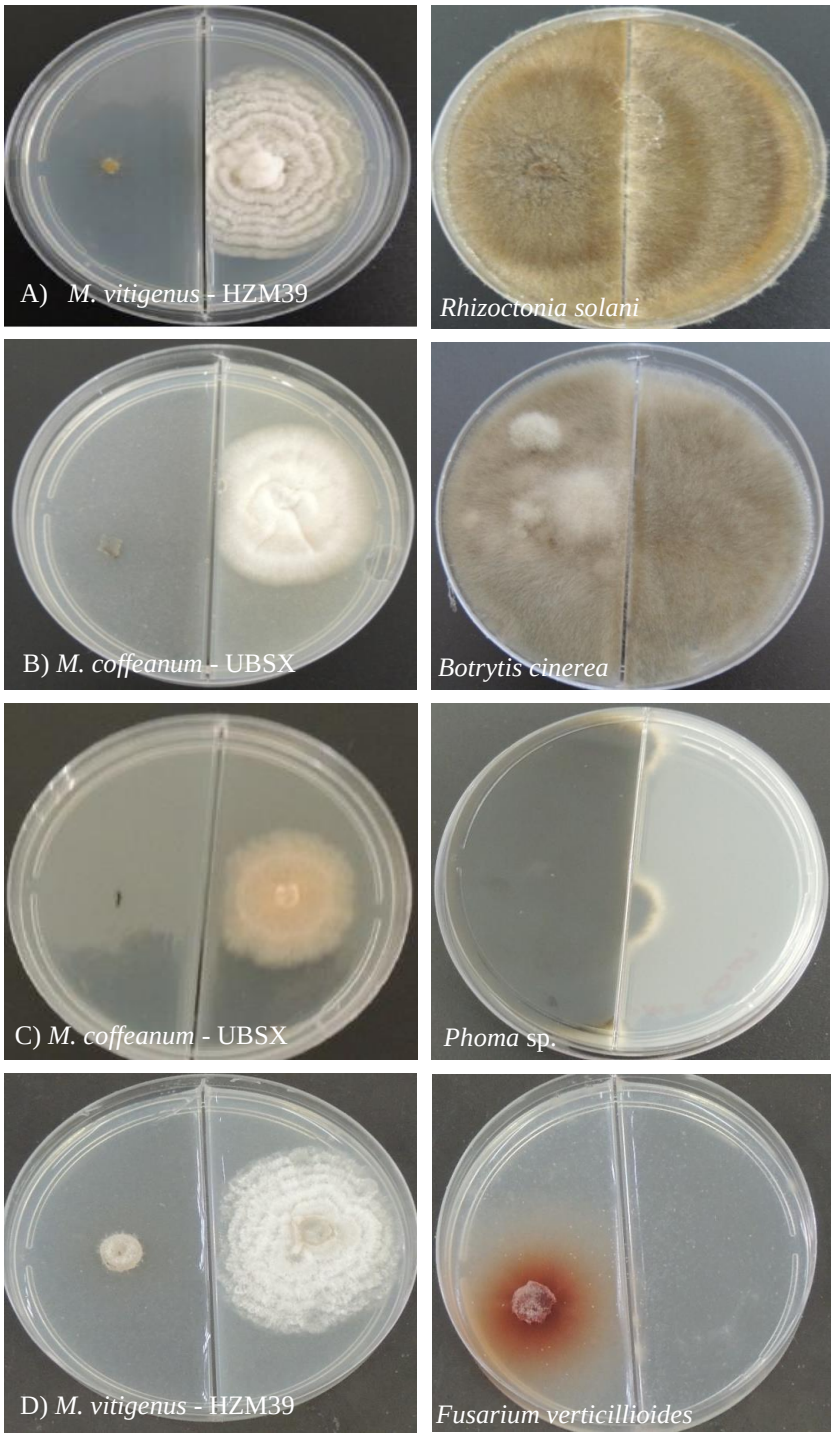


Figura 1. Morfologia dos fungos endofíticos isolados de *Coffea arabica*.


A) M. vitigenus - HZM39
Rhizoctonia solani
B) M. coffeanum - UBSX
Botrytis cinerea
C) M. coffeanum - UBSX
Phoma sp.
D) M. vitigenus - HZM39
Fusarium verticillioides

Continua...

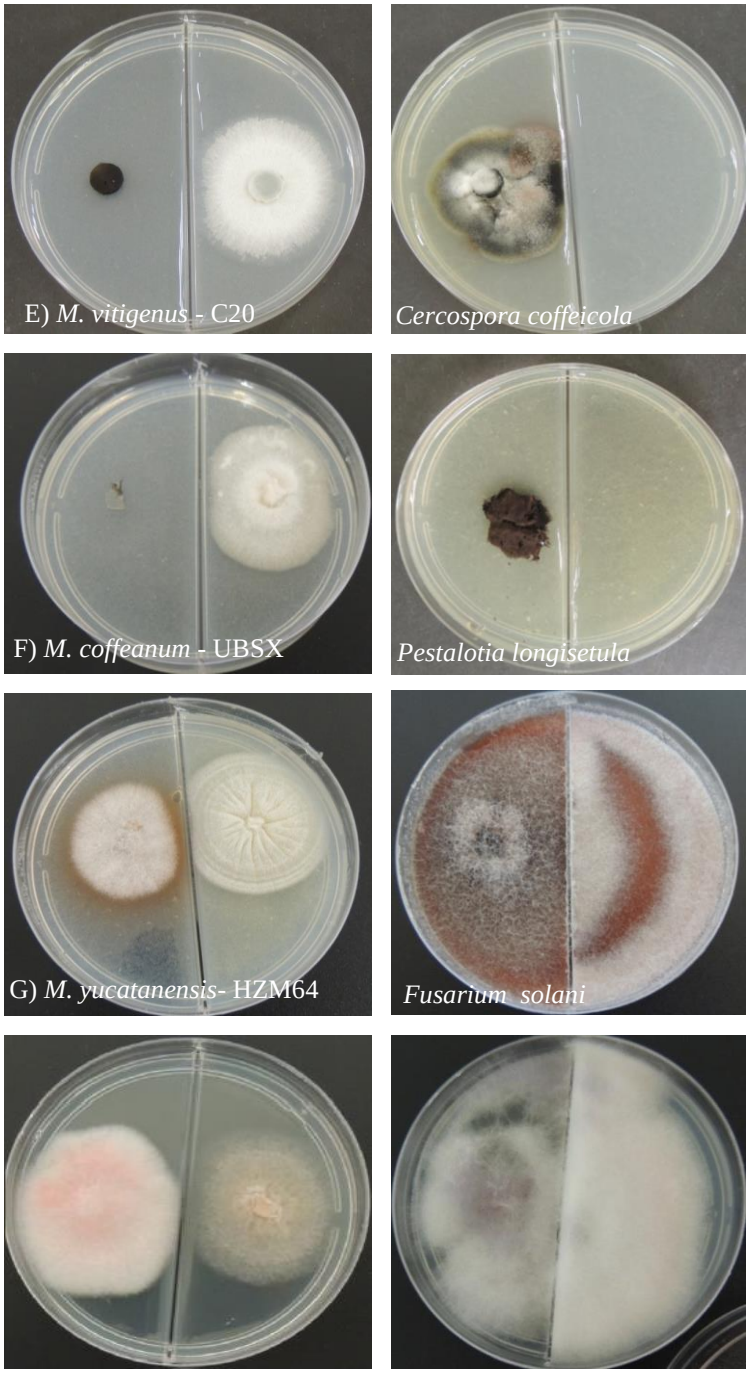


Figura 2. Ação dos compostos orgânicos voláteis (COVs) produzidos por fungos endofíticos na inibição do crescimento de fungos fitopatogênicos.

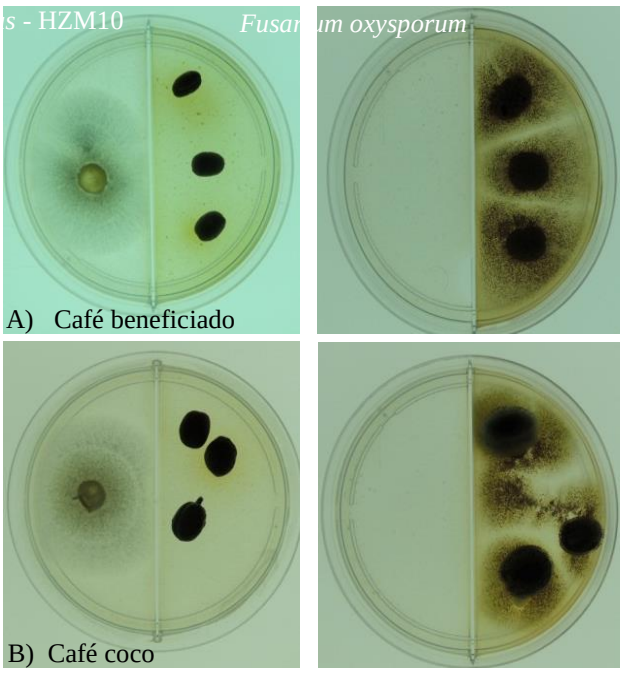


Figura 3. Efeito dos compostos orgânicos voláteis produzidos por *Muscodor coffeanum (*COAD 1842) em grãos de café inoculados com *Aspergillus ochraceus*. A) Café beneficiado (B) Grãos de café coco (B).

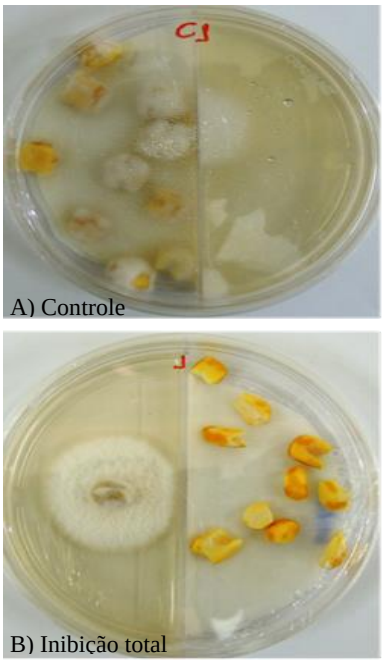


Figura 4. Efeito dos compostos orgânicos voláteis (COVs) produzidos por fungos endófitos em *Fusarium verticillioides* inoculados em grãos de milho. A) Controle. B) inibição total de crescimento de *Fusarium verticillioides*.