BÁRBARA COUTINHO MOURÃO CAVALCANTI

INTERACTION OF ENDOPHYTIC FUNGI OF THE GENUS *Muscodor* WITH ARABICA COFFEE PLANTS

LAVRAS – MG
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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Plantas Medicinais, Aromáticas e Condimentares, para obtenção do título de Doutora.

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Ficha catalográfica elaborada pelo Sistema de Geração de Ficha Catalográfica da Biblioteca Universitária da UFLA, com dados informados pelo(a) próprio(a) autor(a).

Mourão, Bárbara.
Interação de Fungos Endofíticos do Gênero *Muscodor* com Plantas de Café Arábica / Bárbara Mourão. - 2024.
72 p.

Orientador(a): Patrícia Cardoso.

Tese (doutorado) - Universidade Federal de Lavras, 2024.
Bibliografia.

1. Microbiologia. 2. Agricultura. 3. Bioquímica. I. Cardoso, Patrícia. II. Título.

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INTERACTION OF ENDOPHYTIC FUNGI OF THE GENUS *Muscodor* WITH ARABICA COFFEE PLANTS

INTERAÇÃO DE FUNGOS ENDOFÍTICOS DO GÊNERO *Muscodor* COM PLANTAS DE CAFÉ ARÁBICA

Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Plantas Medicinais, Aromáticas e Condimentares, para obtenção do título de Doutora.

APROVADA em 22 de março de 2024.

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AGRADECIMENTOS

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001. Agradeço imensamente à Universidade Federal de Lavras que proporcionou minha formação desde a graduação até o doutorado. Gratidão a todos os mestres e doutores que foram minha inspiração para trilhar este caminho.

RESUMO

Fungos endofíticos são encontrados associados a vegetais e sua interação com plantas de importância econômica e fitoterápica tem sido reportada podendo resultar no desenvolvimento de bioinoculantes e outros produtos biotecnológicos. O objetivo deste trabalho foi avaliar a interação de 12 fungos endofíticos do gênero *Muscodor* com as cultivares Catuaí Vermelho e Topázio de *Coffea arabica*, em relação ao crescimento vegetativo, às enzimas do sistema antioxidante, peroxidação lipídica e peróxido de hidrogênio das plantas, aos fito-hormônios produzidos pelos fungos e plantas, bem como à capacidade antioxidante e aos compostos fenólicos totais dos fungos e plantas.Também foi realizado o re-isolamento dos fungos das plantas inoculadas. O fungo *Muscodor coffeanum* (CML 4019) produziu mais ACC deaminase, auxinas com e sem L-Triptofano e giberelinas, além de uma maior concentração de compostos fenólicos totais e a maior atividade antioxidante. Já a produção de sideróforos foi maior para o fungo *M. coffeanum* (CML 4018). Após seis meses de cultivo, as plantas de café foram expostas ao ataque do bicho-mineiro, e foi observado menos danos nas plantas inoculadas com os fungos endofíticos. O crescimento inicial das plantas da cultivar Catuaí Vermelho inoculadas com *M. coffeanum* (CML 4014) e *Muscodor* sp. (CML 4015), e as da cultivar Topázio inoculadas com o fungo *M. coffeanum* (CML 4018) foi melhor para altura da planta, diâmetro do caule, número de folhas, comprimento e diâmetro da raiz principal, e biomassa seca total. Maior atividade da enzima superóxido dismutase foi obtida nas plantas inoculadas com *M. coffeanum* (CML 4011 e CML 4020) para as cultivares Catuaí Vermelho e Topázio, respectivamente. Foram re-isolados fungos com estruturas macro e microscópicas semelhantes à *Muscodor* das plantas inoculadas. Os resultados sugerem uma interação positiva entre fungos *Muscodor* e plantas de café, intensificando a produção de alguns metabólitos importantes para o crescimento e proteção das plantas.

Palavras-chave: antioxidantes; bicho-mineiro; compostos fenólicos; crescimento vegetativo; fito-hormônios.

ABSTRACT

Endophytic fungi are found associated with plants and their interaction with plants of economic and phytotherapeutic importance has been reported and may result in the development of bioinoculants and other biotechnological products. The objective of this work was to evaluate the interaction of 12 endophytic fungi of the genus *Muscodor* with the cultivars Catuaí Vermelho and Topázio of *Coffea arabica*, in relation to vegetative growth, enzymes of the antioxidant system, lipid peroxidation and hydrogen peroxide of plants, phytohormones produced by fungi and plants, as well as the antioxidant capacity and total phenolic compounds of fungi and plants. The fungi were also re-isolated from the inoculated plants. The fungus *Muscodor coffeanum* (CML 4019) produced more ACC deaminase, auxins with and without L-Tryptophan and gibberellins, in addition to a higher concentration of total phenolic compounds and the highest antioxidant activity. The production of siderophores was greater for the fungus *M. coffeanum* (CML 4018). After six months of cultivation, the coffee plants were exposed to leaf miner attack, and less damage was observed in plants inoculated with the endophytic fungi. The initial growth of Catuaí Vermelho cultivar plants inoculated with *M. coffeanum* (CML 4014) and *Muscodor* sp. (CML 4015), and those of the Topázio cultivar inoculated with the fungus *M. coffeanum* (CML 4018) were better for plant height, stem diameter, number of leaves, length and diameter of the main root, and total dry biomass. Greater activity of the enzyme superoxide dismutase was obtained in plants inoculated with *M. coffeanum* (CML 4011 and CML 4020) for the cultivars Catuaí Vermelho and Topázio, respectively. Fungi with macro and microscopic structures similar to *Muscodor* were re-isolated from the inoculated plants. The results suggest a positive interaction between *Muscodor* fungi and coffee plants, intensifying the production of some important metabolites for plant growth and protection.

Keywords: antioxidants; coffee leaf miner; phenolic compounds; vegetative growth; phytohormones.

INDICADORES DE IMPACTO

Fungos endofíticos são encontrados associados a vegetais e sua interação com plantas de importância econômica e fitoterápica tem sido reportada podendo resultar no desenvolvimento de bioinoculantes e outros produtos biotecnológicos. O objetivo deste trabalho foi avaliar a interação de 12 fungos endofíticos do gênero *Muscodor* com as cultivares Catuaí Vermelho e Topázio de *Coffea arabica*, em relação ao crescimento vegetativo, às enzimas do sistema antioxidante, peroxidação lipídica e peróxido de hidrogênio das plantas, aos fito-hormônios produzidos pelos fungos e plantas, bem como à capacidade antioxidante e aos compostos fenólicos totais dos fungos e plantas. Os resultados obtidos mostraram que os fungos endofíticos estudados conferiram maior resistencia ao ataque do bicho-mineiro às plantas, uma vez que as plantas inoculadas sofreram menor dano foliar, quando comparadas às não inoculadas. Os fungos contribuíram também para um melhor crescimento inicial das plantas de café, visto que as inoculadas apresentaram crescimento superior às não-inoculadas. Portanto, a interação entre *Muscodor* e *C. arabica* mostrou-se promissora para a promoção de crescimento, melhores respostas de defesa antioxidante e aumento da resistência do café contra o bicho-mineiro. Essa interação apresentou-se como uma associação específica, uma vez que demonstrou respostas variadas de acordo com as espécies e isolados diferentes dos fungos do gênero *Muscodor*, e as cultivares Catuaí Vermelho e Topázio. Dessa forma, os fungos endofíticos estudados na tese "Interaction of endophytic fungi of the genus *Muscodor* with arabica coffee plants" apresentam potencial aplicabilidade no biocontrole do bicho-mineiro, uma das principais pragas agrícolas do café, afetando diretamente na minimização da poluição ambiental e intoxicação de trabalhadores agrícolas e de consumidores, uma vez que atualmente o controle dessa praga é realizado por meio de defensivos químicos. Além desses fungos contribuírem possitivamente para com o crescimento inicial e defesa enzimática do café.

IMPACT INDICATORS

Endophytic fungi are found associated with vegetables and their interaction with plants of economic and phytotherapeutic importance has been reported and may result in the development of bioinoculants and other biotechnological products. The objective of this work was to evaluate the interaction of 12 endophytic fungi of the genus Muscodor with the cultivars Catuaí Vermelho and Topázio of Coffea arabica, in relation to vegetative growth, enzymes of the antioxidant system, lipid peroxidation and hydrogen peroxide of plants, phyto-hormones produced by fungi and plants, as well as the antioxidant capacity and total phenolic compounds of fungi and plants. The results obtained showed that the endophytic fungi studied provided greater resistance to leaf miner attack on plants, since inoculated plants suffered less leaf damage when compared to non-inoculated plants. The fungi also contributed to better initial growth of the coffee plants, as the inoculated plants showed higher growth than the non-inoculated ones. Therefore, the interaction between Muscodor and C. arabica showed promise for promoting growth, better antioxidant defense responses and increasing the resistance of coffee against the miner. This interaction presented itself as a specific association, as it demonstrated varied responses according to the different species and isolates of fungi of the genus Muscodor, and the cultivars Catuaí Vermelho and Topázio. Thus, the endophytic fungi studied in the thesis “Interaction of endophytic fungi of the genus Muscodor with arabica coffee plants” have potential applicability in the biocontrol of leafminer, one of the main agricultural pests of coffee, directly affecting the minimization of environmental pollution and intoxication agricultural workers and consumers, since this pest is currently controlled using chemical pesticides. In addition, these fungi positively contribute to the initial growth and enzymatic defense of coffee.

SUMÁRIO

1. INTRODUÇÃO

Fungos endofíticos são microrganismos que vivem de forma harmônica associados às plantas. Podem estar presentes em todos os órgãos vegetais, conferindo benefícios ao hospedeiro, como proteção contra patógenos, resistência a estresses, maior crescimento de raízes e partes aéreas, além de produzir compostos químicos como alcaloides, antibióticos, enzimas e hormônios.

Os fungos endofíticos do gênero *Muscodor* produzem compostos orgânicos voláteis (COVs) e não-voláteis com atividades bactericida, antifúngica, antiviral e antioxidante com aplicação biotecnológica em setores de interesse econômico, tais como agrícola, saúde, meio ambiente e alimentício. Os estudos relacionados à sua interação com plantas de importância econômica e fitoterápica têm sido realizados, possibilitando o conhecimento destas interações.

As espécies *Muscodor coffeanum* e *Muscodor* sp., isoladas de plantas de café silvestre, produzem compostos secundários eficientes nas atividades antifúngica, antibacteriana e antinematóide, sendo capazes de inibir vários fitopatógenos (*Aspergillus ochraceus*, *Botrytis cinerea*, *Cercospora coffeicola*, *Colletotrichum lindemuthianum*, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium verticillioides*, *Pestalotia longisetula*, *Pseudocercospora griseola*, *Rhizoctonia solani* e *Sclerotinia sclerotiorum*). Seus extratos de acetato de etila apresentam bioatividade, atuando como moduladores do processo de coagulação sanguínea junto a diferentes classes de proteases, desempenhando um papel na manutenção da hemostasia humana e como produtores de enzimas extracelulares.

Coffea arabica L. é uma das culturas de maior interesse econômico do país, sendo uma das bebidas mais consumidas no mundo. A bebida é comprovadamente uma fonte dietética importante de compostos fenólicos, com atividades antiinflamatória, antioxidante, neuroprotetora, antidepressiva e ansiolítica, conferindo ao consumidor regular da bebida à redução de doenças crônicas e síndromes metabólicas.

Esse trabalho teve como objetivo avaliar a interação de 12 fungos endofíticos do gênero *Muscodor* com as cultivares Catuaí Vermelho e Topázio de *C. arabica*, em relação ao crescimento vegetativo, às enzimas do sistema antioxidante, peroxidação lipídica e peróxido de hidrogênio das plantas, aos fito-hormônios produzidos pelos fungos e plantas, bem como à capacidade antioxidante e aos compostos fenólicos totais dos fungos e plantas.

2. REFERENCIAL TEÓRICO

2.1 Gênero *Muscodor*

O gênero de fungos endofíticos *Muscodor*, foi descrito em 2001 a partir da espécie tipo *Muscodor albus* (WORAPONG *et al.*,2001). Samarakoon *et al.* (2020), transferiram as espécies de *Muscodor* para o gênero *Induratia*. A proposta de mudança foi baseada na relação filogenética com as espécies dos gêneros *Induratia* e *Emarcea*, e na análise das sequências dos genes ITS, LSU, *rpb*2 e *tub*2. Recentemente, alguns dos mesmos pesquisadores que sugeriram a primeira transferência, propuseram o retorno das espécies para o gênero *Muscodor*, uma vez que as espécies que estavam em *Induratia* não apresentaram relação com a cultura do antigo holótipo de *Induratia apiospora*. (CEDEÑO-SANCHEZ, 2023).

Fungos endofíticos do gênero *Muscodor* são interessantes como fonte de novos metabólitos secundários bioativos que podem ter aplicação biotecnológica nos setores agrícola, da saúde, meio ambiente e alimentício. A biologia desses fungos apresenta características importantes, uma delas é a produção de compostos orgânicos voláteis (COVs) que apresentam atividades inseticida, antibacteriana, antifúngica e nematicida (SAXENA; STROBEL, 2020; STROBEL, 2006a, 2006b, 2011).

Com a intenção de diminuir os danos colaterais no meio ambiente e na saúde, existe um interesse crescente em métodos biológicos para o controle de fungos e bactérias patogênicas que ameaçam as lavouras e os produtos em armazenamento e transporte (GU *et al.*, 2007; SAXENA; STROBEL, 2020). Os COVs de *M. albus* mostram-se eficientes para tal controle, sendo estudado para a substituição do brometo de metila na fumigação do solo e nas biofumigações de hortifrutigranjeiros (GABLER *et al.*, 2006; SAXENA; STROBEL, 2020).

Outra utilidade promissora para os COVs de *M. albus* é no tratamento de dejetos humanos e animais, reduzindo a população de bactérias nocivas (MERCIER; MANKER, 2005; MERCIER; JIMNEZ, 2007). A Cleanwaste criou o produto WAGBAGs®, a partir dos COVs de *Muscodor*, que está sendo usado em hospitais e banheiros ambientais portáteis, além de ter uso amplo pelas forças de defesa dos Estados Unidos (STROBEL; EZRA, 2008).

Os COVs de *Muscodor* apresentam atividades inibitórias contra patógenos humanos *in vitro*, inibindo o crescimento de *Escherichia coli*, *Salmonella typhi*, *S. choleraesuis*, *Staphylococcus aureus*, *Mycobacterium marinum*, *Yersiniapestis* e *Pythiuminsidiosum*, possibilitando seu uso na fabricação de antibióticos e sanitizantes voláteis. Seus COVs também têm sido empregados no controle de fungos (*Aspergillus* spp., *Cladosporium* spp., *Paecilomyces variotii*, *Stachybotrys chartarum*, *Ulocladium botrytis*, *Wellemiasebi*) em moldes de construção (casas e edifícios sob condições úmidas, que emitem toxinas voláteis

que podem ser responsáveis por vários problemas respiratórios) (SAXENA; STROBEL, 2020).

Espécies de *M. coffeanum*, *M. yucatanensis* e *M. vitigenum*, isoladas de plantas saudáveis de café produziram COVs com atividade inibitória sobre *A. ochraceus, B. cinerea*, *C. coffeicola*, *F. oxysporum*, *F. solani*, *F. verticillioides*, *P. longisetula* e *R. solani* (MONTEIRO *et al.*, 2017). Guimarães *et al.* (2021) identificaram os COVs de isolados de *Muscodor* e observaram que esses podem não estar correlacionados com a atribuição filogenética dos fungos estudados. De acordo com os autores, os COVs de *M. coffeanum* apresentam eficientes atividades antifúngica, antibacteriana e antinematóide.

Os bioativos não voláteis desses fungos também estão sendo estudados e potenciais aplicações biológicas têm sido descritas (SAXENA; STROBEL, 2020; BASTOS et al., 2020). O extrato de acetato de etila de *M. indicus* apresenta-se como um eficiente bactericida, indicando um possível e eficiente antibiótico (BOPARAI *et al.*, 2015). Bem como, o do tipo selvagem de *M. yucatanensis* contém brefeldina A, que possui atividades antifúngica, antibacteriana e antiviral (BETINA, 1992) e o de suas variantes epigenéticas, ergosterol e xilaguaianol (QADRI *et al.*, 2017). Os extratos de clorofórmio de *M. darjeelingensis* apresentam metabólitos secundários com potenciais atividades anti-hiperuricêmica, e nos extratos de *M. indicus*, *M. strobelii*, *M. darjeelingensis* e *M. kashayum* atividade antioxidante via eliminação de radicais livres (KAPOOR; SAXENA, 2016; SAXENA; STROBEL, 2020).

Monteiro *et al.* (2017), descreveram a presença das enzimas celulase, pectinase, protease, fitase, lipase, amilase e atividade específica das enzimas endo β-1, 4 glucanase e exo β-1,4 glucanasenos extratos de acetato de etila dos fungos *M. coffeanum* e *M. yucatanensis*.

Também foi reportada a bioatividade dos extratos de acetato de etila a partir do filtrado da cultura dos fungos *M. coffeanum* e *M. yucatanensis* atuando como moduladores do processo de coagulação sanguínea junto a diferentes classes de proteases, desempenhando um papel na manutenção da homeostasia humana (BASTOS *et al.*, 2020). Cardoso *et al.* (2022) avaliando os efeitos de metabólitos não voláteis de *Muscodor* spp. sobre alterações tóxicas induzidas por espécies de Bothrops venenosas, observaram que espécies desse fungo diminuíram a hemólise e a trombólise induzidas pelo veneno dessas cobras. Bem como, não exerceram efeitos citotóxicos, demonstrando uso potencial dos metabólitos não voláteis de *Muscodor* spp. como moduladores enzimáticos.

Outra característica importante da biologia de *Muscodor* é a possibilidade de introduzir artificialmente esses fungos em uma planta hospedeira diferente (fato realizado pela primeira vez com *M. albus* em *Guazuma ulmifolia* Lam.), essa característica comprova que

esses fungos podem ser utilizados para potencializar a proteção natural de espécies vegetais através de inoculação (STROBEL *et al.*, 2007; SAXENA; STROBEL, 2020).

Quando inoculadas em feijão, Mota *et al.* (2021) e Hayashibara *et al.* (2022) observaram que *M. coffeanum* e *Muscodor* sp. reduziram os sintomas de doenças causadas por fitopatógenos (*Colletotrichum lindemuthianum, Sclerotinia sclerotiorum* e *Pseudocercospora griseola*) e promoveram o crescimento da cultura, melhorando a produtividade e a fitossanidade de *Phaseolus vulgaris* L.

2.2 *Coffea arabica*

O café é uma das principais *commodities* brasileiras, sendo uma das bebidas mais consumidas no mundo. Estima-se que são bebidas por ano cerca de 400 bilhões de xícaras de café. O Brasil é considerado o segundo maior mercado consumidor de café, atrás apenas dos Estados Unidos (DONG *et al.*, 2017; SACHS *et al.*, 2019; ABIC, 2021). A cultura cafeeira no Brasil teve início em 1727, e hoje o país é o maior produtor de café do mundo, seguido pelo Vietnã e Colômbia (YANAGIMOTO *et al.*, 2004). O estado de Minas Gerais é o maior produtor do país, sendo responsável por aproximadamente 70% de todo o café arábica produzido por ano (CONAB, 2023).

As plantações de café do país são compostas, principalmente, por cultivares de *C. arabica*. A cultivar Catuaí é resultante do cruzamento entre as cultivares Mundo Novo e Caturra. A cultivar Topázio é o resultado do cruzamento entre Mundo Novo e Catuaí Amarelo (BLANK; SEN; GROSCH, 1991; NOGUEIRA, 2005; EPAMIG, 2017).

Existem vários fatores que prejudicam a produtividade do café em todo o mundo. A ferrugem do café (*Hemileia vastatrix* Berkeley & Broome), o bicho-mineiro (*Leucoptera coffeella* Guérin-Méneville), a cercosporiose (*Cercospora coffeicola* Berkeley e Cooke) e a antracnose (*Colletotrichum* spp.) estão entre as principais causas da baixa produtividade. Geralmente, o controle dessas doenças é realizado por meio do uso inadequado de defensivos químicos, o que gera alguns problemas diretamente ligados à poluição ambiental e intoxicação de trabalhadores agrícolas e de consumidores (RUTHERFORD; PHIRI, 2006; SAVCI, 2012; RODRÍGUEZ *et al.*, 2016).Visando minimizar a utilização de agroquímicos e seus impactos, o emprego de agentes bióticos pode ser uma alternativa para o controle biológico em *C. arabica* (HADDAD *et al.*, 2014; ALDINA; INDARTI; WIBOWO, 2017; O'BRIEN, 2017; HYDE *et al.*, 2019; SAMARAKOON *et al.*, 2020; SAXENA; STROBEL, 2020).

Estudos apontam benefícios à saúde pela ingestão do café devido à presença de moléculas bioativas em sua composição química, tais como, alcalóides, compostos fenólicos, vitaminas, carboidratos e lipídios (NATELLA *et al.*, 2002; SOMOZA *et al.*, 2003; OKAMURA *et al.*, 2005). A molécula que mais se destaca na composição química do café é a cafeína, um alcalóide purínico que atua na inibição de alguns receptores de adenosina, estimulando a atividade neural, que por sua vez, promove o aumento da liberação de adrenalina, resultando no aumento da pressão arterial, metabolismo energético e capacidade respiratória, bem como o aumento dos níveis de dopamina, promovendo sensação de prazer e melhorando a capacidade cognitiva (CADONÁ *et al.*, 2019; GOEL, 2017).

As principais propriedades medicinais do gênero *Coffea* são: antioxidante (JUNG *et al.*, 2017; SOUZA *et al.*, 2020), antiinflamatória (KEMPF *et al.*, 2010; FREEDMAN *et al.*, 2012; AKASH *et al.*, 2014; WEBER *et al.*, 2020), antitumor (MICHAUD *et al.*, 2010; JIANG *et al.*, 2013; LIU *et al.*, 2015; MUT-SALUD *et al.*, 2016; SOUZA *et al.*, 2020; MONTENEGRO *et al.*, 2021), neuroprotetora (SMITH, 2002; DAVIS *et al.*, 2003; JOGHATAIE *et al.*, 2004; ARENDASH *et al.*, 2006), antidepressiva e ansiolítica (HIGDON; FREI, 2006; LUCAS *et al.*, 2014; SZOPA *et al.*, 2016; AMAGHNOUJE *et al.*, 2020) e de controle do diabetes (SARTORELLI *et al.*, 2010; DING *et al.*, 2014; ODEGAARD *et al.*, 2016; BONESI *et al.*, 2019; MINDARTI *et al.*, 2020).

Com relação aos estudos com fungos endofíticos de *C. arabica*, existem poucos trabalhos publicados. Santamaría e Bayman (2005) observaram que existe uma diferença entre a comunidade de fungos da superfície e do interior da folha de café e que tal diferença influencia na interação destes com outros microrganismos ali presentes. Em outro trabalho, 11 espécies de *Penicillium* foram encontradas por Vega *et al.* (2006) vivendo nos tecidos de *C. arabica* coletadas na Colômbia, no Havaí e nos Estados Unidos. Segundo os autores, quatro destas espécies produziram ocratoxina A. Sette *et al.* (2006) realizaram a caracterização molecular de 37 isolados de fungos endofíticos de cafeeiros e observaram sua atividade antimicrobiana contra diferentes bactérias patogênicas humanas.

Saucedo-García *et al.* (2014) descreveram a diversidade de comunidades de fungos endofíticos foliares de diferentes agroecossistemas de *C. arabica* de duas regiões de Veracruz no México. Em 2017, Monteiro e colaboradores selecionaram e identificaram os fungos endofíticos de café que produzem COVs e avaliaram seu efeito no crescimento de vários microrganismos fitopatogênicos. Durante uma bioprospecção de fungos endofíticos produtores de COVs antimicrobianos foi descoberta uma nova espécie, nomeada de *Simplicillium coffeanum* (GOMES *et al.*, 2018). A partir de testes de atividade

antimicrobiana, os autores constataram que a mistura de voláteis emitida por *S. coffeanum* inibiu o crescimento de quatro espécies de *Aspergillus*. Rodríguez *et al.* (2021) isolaram de *C. arabica* quatro novas espécies endofíticas: *Trichoderma botryosum*, *T. caeruloviride*, *T. lentissimum*e *T. pseudopyramidale*.

3. OBJETIVOS

3.1 Objetivo Geral

Avaliar a interação de 12 fungos endofíticos do gênero *Muscodor* com as cultivares Catuaí Vermelho e Topázio de *C. arabica*, em relação ao crescimento vegetativo, enzimas do sistema antioxidante, peroxidação lipídica e peróxido de hidrogênio das plantas, aos fito-hormônios produzidos pelos fungos e plantas, bem como à capacidade antioxidante e aos compostos fenólicos totais dos fungos e plantas.

3.2 Objetivos Específicos

- Inocular os 12 isolados fúngicos nas sementes das cultivares Catuaí Vermelho e Topázio de *C. arabica*;

- Semear as sementes inoculadas e as não inoculadas em vasos com substrato em casa de vegetação;

- Avaliar o crescimento inicial das plantas de *C. arabica* inoculadas e não inoculadas;

- Determinar a atividade da enzima SOD das plantas de *C. arabica* inoculadas e não inoculadas;

- Determinar a atividade de peroxidação lipídica e peróxido de hidrogênio das plantas de *C. arabica* inoculadas e não inoculadas;

- Determinar a produção dos fito-hormônios ACC deaminase, AIA e GA dos fungos e das plantas de *C. arabica* inoculadas e não inoculadas;

- Determinar a capacidade antioxidante e os compostos fenólicos totais dos fungos e plantas inoculadas e não inoculadas;

- Identificar os compostos fenólicos produzidos pelos fungos do gênero *Muscodor*;

- Re-isolar fungos endofíticos das plantas de *C. arabica* inoculadas e não inoculadas.

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4. ARTIGO 1

Muscodor - COFFEE PLANT INTERACTIONS: BENEFICIAL FUNGAL ENDOPHYTES

ABSTRACT

Endophytic fungi live harmoniously associated with plants, and may be present in all plant organs. This association usually confers benefits to the host. The study aimed to evaluate the vegetative growth, enzymes of the antioxidant system, and lipid peroxidation of *Coffea arabica* plants inoculated with 12 *Muscodor* species and the isolation of the initially inoculated fungi after 2 years. The endophytic fungi isolated from coffee from a secondary forest in Viçosa (MG), were inoculated in seeds of *C. arabica*, Catuaí Vermelho (IAC 144) and Topázio (MG 1190) cultivars. The inoculated seeds were sown in vases with substrate and kept in a greenhouse of 24 months. After the first six months of the experiment, the greenhouse was infested by coffee leaf miner (*Leucoptera coffeella*), and the coffee plants were exposed to the attack of this pest. It was observed that plants inoculated with *Muscodor* endophytes showed less damage caused by the insect when compared to non-inoculated plants. Catuaí Vermelho cultivar plants inoculated with *Muscodor coffeanum* (CML 4014) and *Muscodor* sp. (CML 4015) showed the highest values for plant height, stem diameter, number of leaves, main root length, main root diameter and total dry biomass; Topázio cultivar inoculated with the fungus *M. coffeanum* (CML 4018) showed highest stem diameter, number of plagiotropic pairs, number of leaves, leaf area, main root diameter and total dry biomass. The highest SOD enzyme activity was in Catuaí Vermelho cultivar inoculated with *M. coffeanum* (CML 4011) and Topázio cultivar inoculated with *M. coffeanum* (CML 4020). In control plants was observed the lowest activity of all enzymes when compared to the inoculated plants. Fungi with morphological structures similar to those of *Muscodor* were isolated from the inoculated coffee plants. The results showed that the interaction between *Muscodor* and *C. arabica*, when well established, shows promise for promoting growth and inducing the resistance coffee to the coffee leaf miner.

Keywords: Antioxidant metabolism. Coffee Leaf miner. SOD enzyme. Vegetative growth.

INTRODUCTION

Coffea arabica is a crop of economic and phytotherapeutic interest, since it has biological activities such as anti-inflammatory, antioxidant, neuroprotective, antidepressant and anxiolytic (SZOPA et al., 2016; JUNG et al., 2017; SACHS et al., 2019; AMAGHNOUJE et al., 2020; SOUZA et al., 2020; WEBER et al., 2020). Brazil is the largest coffee producer in the world, accounting for a third of world exports, and the second largest consumer, behind only the United States (SACHS *et al.*, 2019; VELOSO *et al.*, 2020; ABIC, 2021). Coffee plantations in the country are mainly composed of cultivars of *C. arabica.* Catuaí cultivar is the result of a cross between the Mundo Novo and Caturra cultivars.

Topázio cultivar is the result of the cross between Mundo Novo and Catuaí Amarelo. These cultivars are among the most planted in Brazil (BLANK; SEN; GROSCH, 1991; NOGUEIRA, 2005; EPAMIG, 2016).

Coffee rust (*Hemileia vastatrix* Berkeley & Broome), coffee leaf miner (*Leucoptera coffeella* Guérin-Méneville), brown eyespot (*Cercospora coffeicola* Berkeley and Cooke) and anthracnose (*Colletotrichum* spp.) are some of the factors that affect coffee productivity worldwide (RUTHERFORD; PHIRI, 2006; RODRÍGUEZ *et al.*, 2016). In order to minimize the use of agrochemicals and environmental impacts, the use of biotic agents as endophytic microorganisms can be an alternative for biological control of diseases in *C. arabica* (O'BRIEN, 2017; HYDE *et al.*, 2019; SAMARAKOON *et al.*, 2020; SAXENA; STROBEL, 2020).

Endophytic fungi are a source of volatile and non-volatile bioactive compounds that are important for the survival and maintenance of the health of the host plant, increasing its tolerance to stressful environments, reducing attacks by phytopathogens and improving its productivity, since they are actively involved in the metabolic control of their host (GOATES; MERCIER, 2009; CORRÊA *et al.*, 2014; LUGTENBERG; CARADUS; JOHNSON, 2016; EID *et al.*, 2019).

The endophyte-plant association can improve the survival environment for both organisms, as well as benefit the growth and increase crop yield, photosynthetic efficiency, nutrient and water use efficiency (TANNEY *et al.*, 2018; RABIEY et al., 2019; YAN *et al.*, 2019; da SILVA *et al.*, 2020; TORRES-MENDOZA; ORTEGA; CUBILLA-RIOS, 2020; TURBAT *et al.*, 2020). This interaction can also result in increased production of bioactive compounds originally produced by the plant, thus, by synthesizing these compounds, endophytes guarantee their host protection against herbivory and environmental adversities (GOUDA, 2016; RAJAMANIKYAM *et al.*, 2017; LATA *et al.*, 2018; MCMULLIN *et al.*, 2018; STROBEL, 2018). Some antioxidant enzymes, such as, superoxide dismutase (SOD)can increase and help to reduce oxidative damage caused by reactive oxygen species (ROS) (YANG *et al.*, 2013; LIU *et al.*, 2021).

The species *Muscodor coffeanum* and *Muscodor* sp., isolated from healthy wild coffee trees, when inoculated into *Phaseolus vulgaris* L. reduced the symptoms of diseases caused by phytopathogens and promoted plant growth, improving the productivity and plant health of the crop (MOTA *et al.*, 2021; HAYASHIBARA *et al.*, 2022). This is an important characteristic of the biology of fungi of the genus *Muscodor*, the possibility of artificially

introducing themselves into a different host plant, enhancing the natural protection of plant species through inoculation (STROBEL *et al.*, 2007; SAXENA; STROBEL, 2020).

However, no studies were found in the literature evaluating the inoculation of these endophytic fungi in their original plant host; so the objective of this study was to evaluate vegetative growth, antioxidant system enzymes and lipid peroxidation of *Coffea arabica* plants inoculated with 12 species of *Muscodor* and the isolation of the initially inoculated fungi after 2 years.

MATERIAL AND METHODS

Endophytic fungi

The endophytic fungi were isolated from healthy leaves and stems of *C. arabica* present in a secondary forest in Brazil and identified by Guimarães *et al.* (2021) as *Muscodor coffeanum* (CML 4009, CML 4010, CML 4011, CML 4012, CML 4014, CML 4016, CML 4017, CML 4018, CML 4019, CML 4020) and *Muscodor* sp. (CML 4013, CML 4015).

Seed inoculation

Discs of approximately 9 mm in diameter from the fungal colonies were cultivated in 2L Erlenmeyer flasks with PD medium for 30 days at room temperature. Coffee seeds, of the Catuaí Vermelho (IAC 144) and Topázio (MG 1190) cultivars, were disinfested in order to eliminate the epiphytic organisms, by immersion and constant agitation, in 70% ethanol for 2 minutes, in sodium hypochlorite (2% active chlorine) for 3 minutes, and again, in 70% ethanol, for 1 minute. Then, the material was triple-washed in autoclaved distilled water. The dry seeds were transferred to Petri dishes, containing PD culture medium and crushed mycelia of endophytic fungi, being immersed for 6 hours.

Coffee growing

After the inoculation procedure, the seeds were planted in 12 L pots, with substrate composed of 70% sieved soil and 30% tanned bovine manure, for each cubic meter 5 kg of simple superphosphate, 1 kg of potassium chloride and 1.5 kg of limestone were added (CFSMG, 1999). Soil analysis showed that the substrate mixture used had the following chemical attributes: pH = 4.8; P, K and Na (mg dm^{-3}) = 1272.81; 207.07 and 61.00; Ca, Mg, Al, H + Al, SB, t e T (cmolc dm^{-3}) = 4.89; 1.28; 0.10; 4.20; 9.43; 9.43 and 13.63; m and V (%) = 1.05 and 69.21; organic matter = 6.72 dag kg^{-1} ; P-Rem = 30.20 mg L^{-1} ; Zn, Fe, Mn,

Cu, B and S (mg dm^{-3}) = 5.40; 35.60; 15.30; 0.85; 0.18 and 455.00. Substrate texture: clayey, with 64.0 dag kg^{-1} of clay, 16.0 dag kg^{-1} of silt and 20.0 dag kg^{-1} of sand.

At the time of planting, PD medium with crushed mycelia were also added to the holes next to the seeds with parchment. The pots were kept in a greenhouse for 2 years in a completely randomized design (CRD), with automatic irrigation.

Vegetative growth

After 24 months of cultivation, both cultivars were evaluated regarding the following morphological attributes: plant height – PH (cm), measured from the collar to the apical bud; stem diameter – SD (mm), measured in the collar region; number of pairs of plagiotropic branches – NPP; number of leaves – NS; leaf area – LA (mm^2), calculated using the ImageJ® Software; main root length – RL (cm), measured from the branching region to the root cap; main root diameter – RD (mm), measured in the branching region; total dry biomass – TDB (g), oven drying at 60 ºC. Assessments were performed in triplicate.

Superoxide Dismutase activity (SOD)

After 24 months of cultivation, young and undamaged leaves of both cultivars were collected and immediately frozen in liquid nitrogen and stored in an ultra-freezer (-80º C).

The extraction of plant material for the analysis of superoxide dismutase (SOD) was performed following the protocol of Biemelt *et al.* (1998). Frozen leaves (0.2 g) were ground in liquid nitrogen with 50% polyvinylpolypyrrolidone (PVPP). Then, the samples were homogenized in 1,500 µL of extraction buffer (potassium phosphate, EDTA, ascorbic acid and water) and centrifuged at 13,000 g for 10 minutes at 4 ºC. Subsequently, the supernatant was collected and subjected to analysis. All analyzes were performed in triplicate for three plants of each treatment.

Superoxide dismutase activity was determined by its ability to inhibit nitrotetrazolium blue (NBT) photoreduction according to the protocol of Giannopolitis and Ries (1977) with modifications. Aliquots of the supernatant were added to the incubation buffer (50 mM potassium phosphate – pH 7.8, 14 mM methionine, 0.1 µM EDTA, water, 75 µM NBT and 2 µM riboflavin). Subsequently, the incubation medium and the blank sample were distributed in ELISA plates that were illuminated with a 20W fluorescent lamp for 7 min. The absorbance of the incubation medium was read at 560 nm in a spectrophotometer. Activity was expressed as U SOD min^{-1} g^{-1} FW.

Lipid Peroxidation (MDA) and Hydrogen Peroxide (H_2O_2)

After 24 months of cultivation, young and undamaged leaves of both cultivars were collected and immediately frozen in liquid nitrogen and stored in an ultra-freezer (-80º C).

The extraction of plant material for lipid peroxidation (MDA) and hydrogen peroxide (H_2O_2) concentration analyses were carried out following the protocol by Buege and Aust (1978). Frozen leaves (0.2 g) were ground in liquid nitrogen with 50% polyvinylpolypyrrolidone (PVPP). Then, the samples were homogenized in 1,500 µL of 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 g for 15 minutes at 4 ºC. Subsequently, the supernatant was collected and submitted for analysis. All analyses were performed in duplicate for three plants of each treatment.

The quantification of lipid peroxidation by the TBARS method (thiobarbituric acid) was determined using the protocol by Buege and Aust (1978), where aliquots of the supernatant were added to the reaction medium (0.5% thiobarbituric acid – TBA and 10% trichloroacetic acid – TCA) and taken to a water bath at 95 ºC for 30 min. Subsequently, the reaction was stopped by rapid cooling on ice, and the absorbance of the supernatant was read in a spectrophotometer at 532nm and the value of nonspecific absorption at 600 nm. The malondialdehyde (MDA) concentration was calculated using the absorption coefficient of 155 mM cm^{-1} and the results were expressed as ηmol MDA g^{-1} FM.

The quantification of hydrogen peroxide was determined by the Velikova protocol *et al.* (2000), in which aliquots of the supernatant were added to the incubation buffer (10 mM potassium phosphate – pH 7.0, and 1M potassium iodide). Subsequently, the incubation medium and the blank samples were distributed in ELISA plates, which were taken to read the absorbance at 390 nm, in a spectrophotometer. The H_2O_2 concentration was calculated according to a standard curve and the results were expressed as mmol H_2O_2 g^{-1} FM.

Re-isolation of fungi

Healthy roots, stems and leaves of *C. arabica* were superficially disinfested, following the same protocol above. Subsequently, the plant material was transferred to Petri dishes, containing PDA culture medium, and left for 15 days, aiming at the growth of colonies. Then, the colonies were isolated, photographed and taken for morphological identification.

Statistical analysis

The experiment was conducted in a completely randomized design (CRD) in a factorial scheme. The factors consisted of 2 coffee plants cultivars, 12 fungal isolates of the

genus *Muscodor* and 2 additional controls (2 x 12 + 2). Each treatment had 3 replications, totaling 78 experimental units.

The interrelationships between variables and treatments were assessed using exploratory Principal Component Analysis (PCA). To choose the number of components, we observed those that had eigen values greater than 1 ($\lambda > 1$) (FRAGA *et al.*, 2015) and an accumulated percentage of at least 80% explanation of the total variation (JOHNSON; WICHERN, 1998). In the graph of the two-dimensional projection of variables and treatments from the PCA, the variables were discriminated according to their percentage contribution (%) in relation to the components. The contribution was determined by the ratio of the square of the variable's coordinates in the scatterplot to the total of the component. The contribution was represented in a color scale. All analyzes were performed using the statistical package R version 4.1.2 (R Core Team, 2021). The "factoextra" library was used for PCA (KASSAMBARA; MUNDT, 2020).

Evaluation of injured leaf areas of coffee plants attacked by coffee leaf miner

After six months of coffee plant cultivation, the greenhouse where it was home to the experiment was infested by coffee leaf miner (*L. coffeella*) and all plants were attacked. Given this, it was observed that the plants inoculated with the endophytic fungi had less damage caused by the attack when compared to the control plants. Eighteen months after the attack, the leaf areas injured by the attack were evaluated using the ImageJ® program, where the total leaf areas and the injured leaf areas were recorded and their proportions calculated. Assessment was performed in triplicate. The data obtained were subjected to analysis of variance (ANOVA) with the significance of the effects verified by the F test at 5% probability.

RESULTS

All parameters of vegetative growth evaluated after inoculation with endophytic fungi showed an increase in the plants compared to non-inoculated plants. Catuaí Vermelho cultivar plants inoculated with the endophytic fungi *M. coffeanum* (CML 4014) and *Muscodor* sp. (CML 4015) showed higher values for the variables: plant height, stem diameter, number of leaves, main root length, main root diameter and total dry biomass, respectively, when compared to the non-inoculated plants. Topázio cultivar plants inoculated with the fungus *M. coffeanum* (CML 4018) showed higher values for the variables: stem diameter, number of

plagiotropic pairs, number of leaves, leaf area, main root diameter and total dry biomass, compared to the non-inoculated plants (Figures 1 and 2).

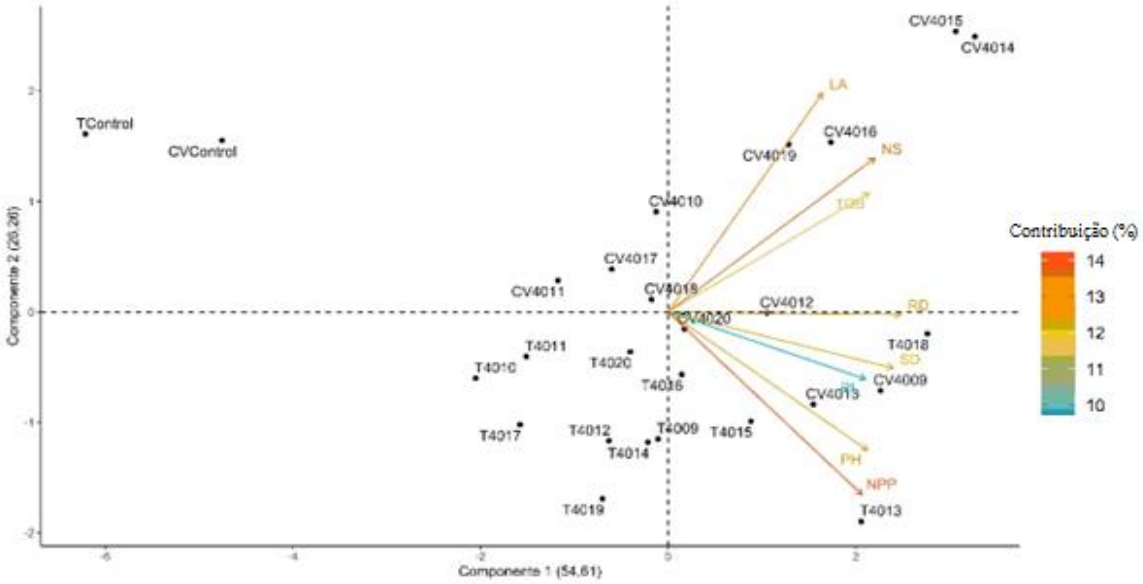

Figure 1 – Two-dimensional projection of endophytic fungi and agronomic variables into the two main components (CP1 x CP2) for the Catuaí Vermelho and Topázio cultivars.Caption –Plant height (PH); Stem diameter (SD); Number of plagiotropic pairs (NPP); Number of leaves (NS); Leaf area (LA); Main Root Length (RL), Main Root Diameter (RD); Total dry biomass (TDB).

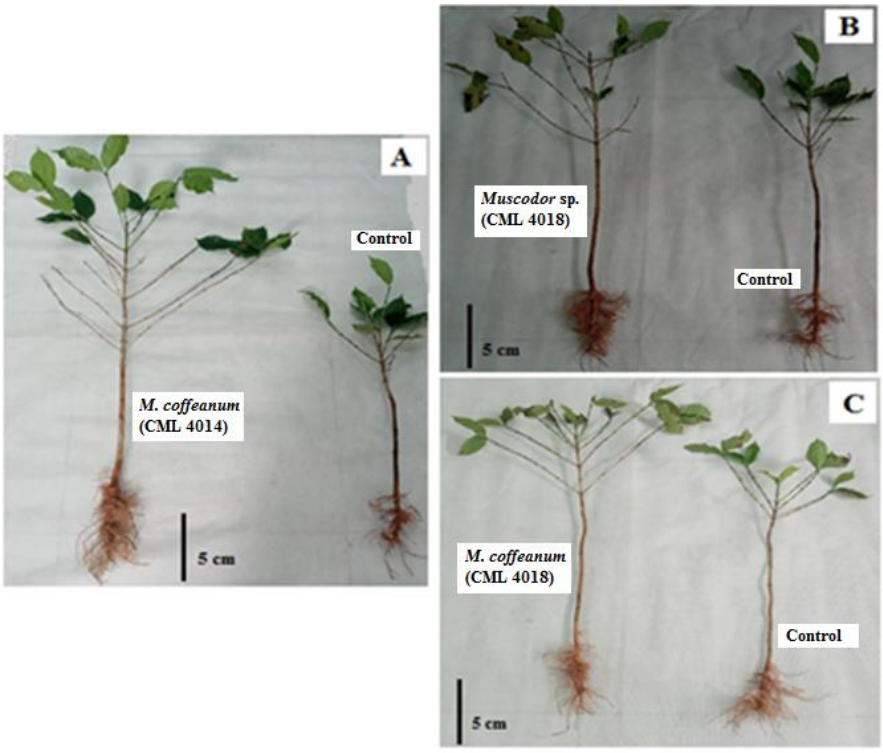

Figure 2 – *Coffee arabica* Catuaí Vermelho cultivar inoculated with *M. coffeanum* (CML 4014) – A; *Muscodor* sp. (CML 4015) – B and *C. arabica* Topázio cultivar inoculated with *M. coffeanum* (CML 4018) – C, and its respective controls at 24 months after inoculation.

Principal component analysis (PCA) explained 80.87% of the variability observed in the data for the Catuaí Vermelho and Topázio cultivars (Figure 1). The obtained results showed the formation of four groups of variables: first group composed by leaf area; second group by number of leaves and number of plagiotropic pairs; third group formed by total dry biomass, main root diameter, stem diameter and plant height; and fourth group composed by length of the main root. Considering the positioning of our treatments, plants of the Catuaí Vermelho cultivar inoculated with the endophytic fungi *M. coffeanum* (CML 4014, 4016 and 4019) and *Muscodor* sp. (CML 4015) showed a positive correlation with number of leaves, main root diameter, total dry biomass and leaf area, while plants inoculated with *M. coffeanum* (CML 4009, 4012 and 4020) and *Muscodor* sp. (CML 4013) showed a positive relationship with number of plagiotropic pairs, length and diameter of root, and height plant. Plants of the Topázio cultivar inoculated with *M. coffeanum* (CML 4018 and 4016) and *Muscodor* sp. (CML 4013 and 4015) showed a positive correlation with diameter of root and stem, and number of plagiotropic pairs. A clustering tendency can also be observed among the fungi *M. coffeanum* (CML 4010, 4011, 4017 and 4019) inoculated in the cultivar Catuaí Vermelho, and *M. coffeanum* (CML 4009, 4010, 4011, 4012, 4014, 4017, 4019 and 4020) inoculated in the Topázio cultivar, which presented more intermediate values in all variables. Non-inoculate plants were not influenced by the evaluated morphological parameters.

Catuaí Vermelho cultivar plants had highest SOD enzyme activity when inoculated with the fungus *M. coffeanum* (CML 4011), when compared to the non-inoculate plants. Lipid peroxidation (MDA) was higher in plants inoculated with *M. coffeanum* (CML 4014) and lower in plants inoculated with the fungus *Muscodor* sp. (CML 4013), while the concentration of H_2O_2 was higher in control plants and lower in plants inoculated with *M. coffeanum* (CML 4019). For the Topázio cultivar, the highest activity of the SOD enzyme was in coffee plants inoculated with the fungus *M. coffeanum* (CML 4020), when compared to the control. Lipid peroxidation (MDA) was higher in plants inoculated with *M. coffeanum* (CML 4017) and the lowest in plants inoculated with *M. coffeanum* (CML 4010), and the concentration of H_2O_2 was also higher in control plants and lower in plants inoculated with the fungus *M. coffeanum* (CML 4011) (Figure 3).

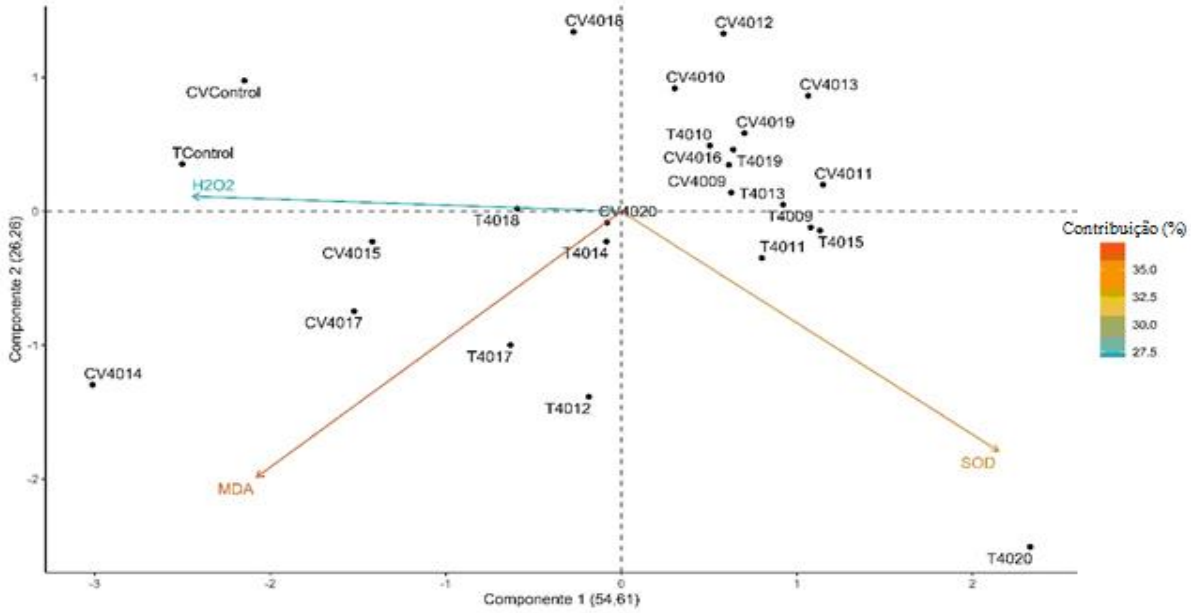

Figure 3 – Two-dimensional projection of endophytic fungi and the superoxide dismutase (SOD), and lipid peroxidation (MDA) and hydrogen peroxide (H_2O_2) in the two main components (CP1 x CP2) for the Catuaí Vermelho and Topázio cultivars.

Principal component analysis (PCA) explained 80.87% of the variability observed in the data for the Catuaí Vermelho and Topázio cultivars (Figure 3). The obtained results showed the formation of three groups of variables: first group composed by SOD enzyme; second group by lipid peroxidation (MDA); and third group formed by H_2O_2. Considering the positioning of our treatments, plants of the Catuaí Vermelho cultivar inoculated with the endophytic fungi *M. coffeanum* (CML 4014, 4017 and 4020) and *Muscodor* sp. (CML 4015) showed a positive correlation with lipid peroxidation (MDA).Plants of the Topázio cultivar inoculated with *M. coffeanum* (CML 4020, 4011 and 4009) and *Muscodor* sp. (CML 4015) showed a positive relationship with SOD enzyme, while the plants inoculated with *M. coffeanum* (CML 4017, 4012 and 4014) showed a positive correlation with lipid peroxidation (MDA), and plants inoculated with *M. coffeanum* (CML 4018) with H_2O_2. A clustering tendency can also be observed among the fungi *M. coffeanum* (CML 4009, 4010, 4011, 4012, 4013, 4016, 4018 and 4019) inoculated in the Catuaí Vermelho cultivar, and *M. coffeanum* (CML 4010, 4013 and 4019) inoculated in the Topázio cultivar, which presented more intermediate values in all variables. Non-inoculate plants were directly influenced by H_2O_2 (Figure 3).

Fungi morphological structures, mycelial and colonial, similar to those of the *Muscodor* were isolated at inoculated coffee plants (Figure 4).

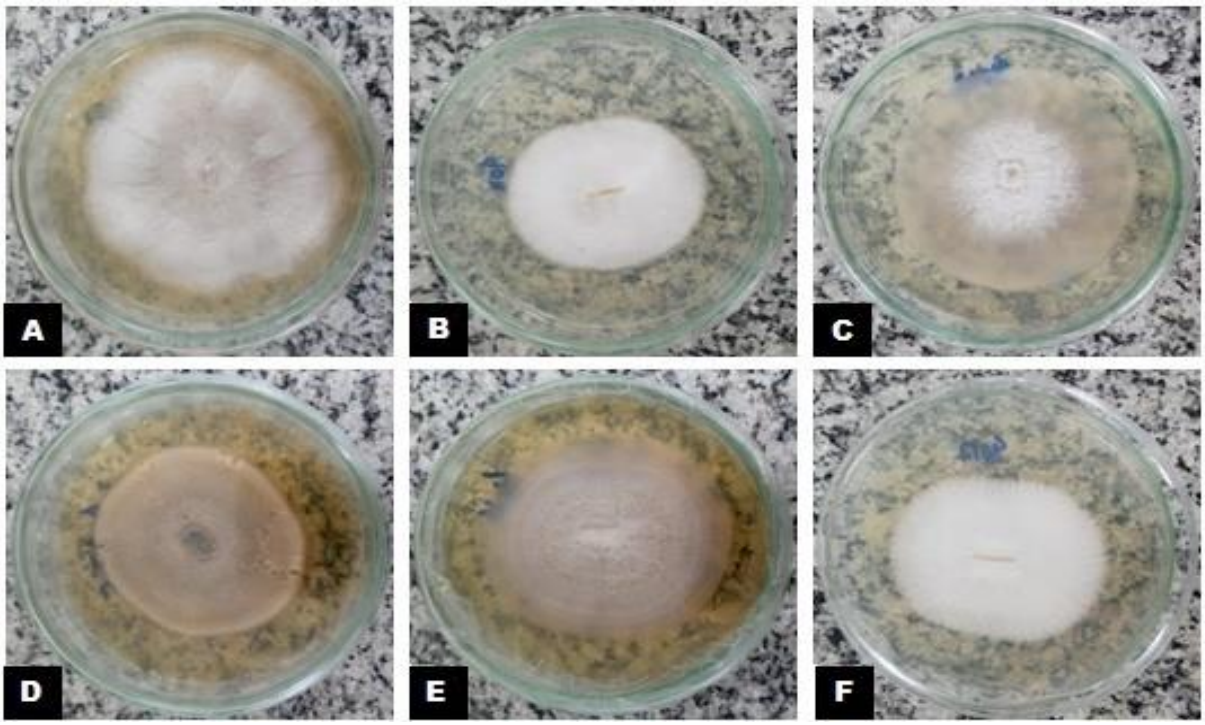

Figure 4 – Colonies of fungi isolated from stems of *C. arabica* Catuaí Vermelho cultivar inoculated with endophytic fungi of the genus *Muscodor*. A – *M. coffeanum* (CML 4010); B – *M. coffeanum* (CML 4011); C – *Muscodor* sp. (CML 4013); D – *Muscodor* sp. (CML 4015); E – *M. coffeanum* (CML 4016); F – *M. coffeanum* (CML 4017).

The calculation of the injured leaf areas showed that, for both cultivars, non-inoculate plants had most damaged by the coffee leaf miner attack when compared to the inoculated plants (11.88% – Catuaí Vermelho, and 6.59% – Topázio). The plants of the Catuaí Vermelho cultivar least damaged were those inoculated with the *M. coffeanum* (CML 4011, 4009, 4014 and 4012) (Table 5 and Figure 5). Topázio cultivar plants inoculated with *M. coffeanum* (CML 4020 e 4017) and *Muscodor* sp. (CML 4015) were least damaged by the leaf miner (Table 5).

Table 5 – Calculations of injured leaf areas of *C. arabica* Catuaí Vermelho and Topázio cultivars.

	Catuaí Vermelho cultivar	**Topázio cultivar**
Treatment	**ILA (%)**	**ILA (%)**
Control	11.88b	6.59b
M. coffeanum (CML 4009)	1.25a	0.88a
M. coffeanum (CML 4010)	3.04a	2.03a
M. coffeanum (CML 4011)	1.14a	1.19a
M. coffeanum (CML 4012)	1.31a	0.77a
Muscodor sp. (CML 4013)	1.71a	1.70a
M. coffeanum (CML 4014)	1.29a	0.66a
Muscodor sp. (CML 4015)	2.02a	0.45a
M. coffeanum (CML 4016)	1.44a	0.67a
M. coffeanum (CML 4017)	1.54a	0.48a
M. coffeanum (CML 4018)	1.91a	1.63a
M. coffeanum (CML 4019)	1.41a	0.68a
M. coffeanum (CML 4020)	1.48a	0.42a

Means followed by the same letters in the column do not differ from each other by the Skott-Knott test ($p<0.05$).

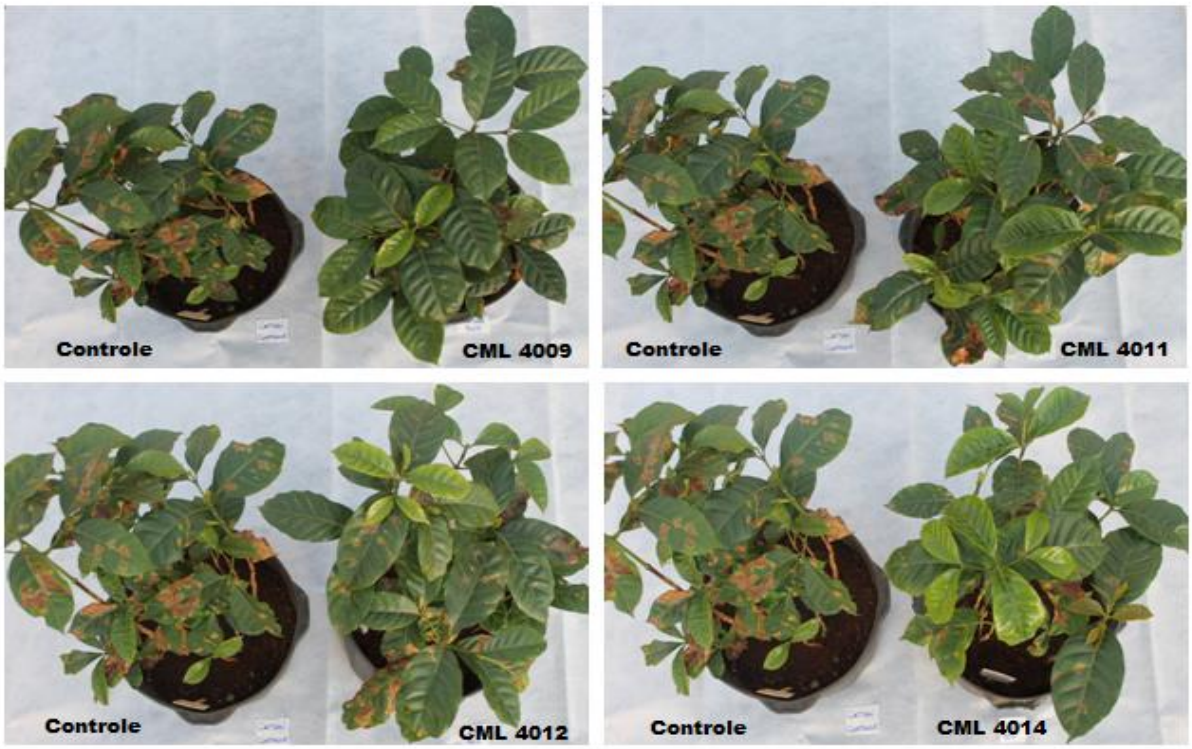

Figure 5 – *Coffea arabica* Catuaí Vermelho cultivar: control plant compared with plants inoculated with *M. coffeanum* (CML 4009, CML 4011, CML 4012 and CML 4014), under attack by leaf miner.

DISCUSSION

Our study demonstrated a positive relationship between the endophytic fungi *M. coffeanum* and *Muscodor* sp. and *C. arabica* plants with benefits in the vegetative growth of inoculated plants (plant height, stem diameter, number of plagiotropic pairs, number of leaves, leaf area, main root length, main root diameter and total dry biomass), when compared to non-inoculated plants. Similar results were observed previously by Hayashibara *et al.* (2022); endophytic fungi of the genus *Muscodor* in the *Muscodor*-bean interaction, mainly *M. coffeanum* (CML 4018 and 4020) and *Muscodor* sp. (CML 4015), proved to be efficient in promoting growth of *Phaseolus vulgaris* L. plants, and can be used in strategic management for the crop, as they can improve the productivity and plant health of beans. Therefore, we can infer that the fungi *M. coffeanum* (CML 4014 and 4018) and *Muscodor* sp. (CML 4015), which stood out in this study, indicate that they are promising in promoting the growth of *C. arabica*.

In *C. arabica* Catuaí Vermelho cultivar, leaf area, number of leaves and total dry biomass are directly related to the photosynthetic nutritional supply, which reflect on larger plants, observed in plants inoculated with *M. coffeanum* (CML 4014, 4016 and 4019) and *Muscodor* sp. (CML 4015), which showed higher values for plant height, and which were also positively influenced by the parameters of this group. In an other group, the parameters stem diameter, root diameter and length, plant height and number of plagiotropic pair are also directly related with supply of nutrients, since the greater the root diameter and length, the vegetative growth, this which can be observed in plants of Catuaí Vermelho cultivar inoculated with the fungi *M. coffeanum* (CML 4009, 4012 and 4020) and *Muscodor* sp. (CML 4013), and plants of Topázio cultivar inoculated with *M. coffeanum* (CML 4016 and 4018) and *Muscodor* sp. (CML 4013 and 4015), positively influenced by these parameters (Figure 1).

Our results showed that coffee plants inoculated with endophytic fungi when exposed to coffee leaf miner attack showed greater activity of the enzyme superoxide dismutase (SOD). Although White Jr. and Torres (2010) defend the hypothesis that the increase in the synthesis of antioxidant system enzymes in plants inoculated with endophytes may be due to the plant's response to ROS produced by fungi, our results showed that among the inoculated plants there were difference in the values referring to the production of antioxidant enzymes, signaling the possibility that some isolates of fungi of the genus *Muscodor* have enhanced the coffee's defense response against the formation of free radicals resulting from exposure to coffee leaf miner attack. The synthesis of bioactive metabolites by endophytic fungi can be

regulated by environmental changes, and endophytic fungi can have an important influence on the plant's metabolic process, thus being directly related to the plant's defense system, inducing systemic resistance to pathogens (ALY *et al.*, 2010; GAO *et al.*, 2010).

Our results showed that some of the isolates of endophytic fungi of the genus *Muscodor* also reduced the MDA content of coffee plants inoculated with them, when compared to the non-inoculate plants. Malondialdehyde (MDA) is produced by lipid peroxidation of the cell membrane when the plant is undergoing some type of stress (HENDGES *et al.*, 2015). The MDA content is used as an indicator of the degree of oxidative damage (OLIVEIRA; GOMES-FILHO; ENÉAS-FILHO, 2010). This suggests that the presence of endophytic fungi in the inoculated plants may have contributed to the reduction of damage caused by the stress of the coffee leaf miner attack. The reduction of MDA in plants treated with *Bacillus subtilis* shows its ability to maintain the integrity of the cell membranes of these plants (LANGARO *et al.*, 2014).

The leaves of coffee plants inoculated with endophytic fungi of the genus *Muscodor* were less damaged by the coffee leaf miner attack, indicating the possibility of interaction *Muscodor-C.arabica* increased the resistance of these plants, since fungi are actively involved in the metabolic control of their host, inducing systemic resistance and increasing tolerance to pathogens. This can be corroborated by the correlation between the lower foliar damage of plants inoculated with endophytic fungi and the results of the analysis of enzymes of the antioxidant system and lipid peroxidation of their respective plant extracts (Figure 3 and Table 5). The responses to both foliar damage and antioxidant system enzymes varied between species, and between different isolates of the same species of endophytic fungi of the genus *Muscodor*, and between cultivars of *C. arabica* studies, this can be explained by the fact that the endophytic fungus-host association is specific (SAIKKONEN *et al.*, 1998; SCHULZ; BOYLE, 2005). *Leucoptera coffeella* (coffee leaf miner) is currently the main pest of coffee cultivation in Brazil and other countries, with the application of chemical products being the most efficient control method, but it increases production costs and harms the environment and health human. The development of cultivars resistant to the coffee leaf miner is an alternative to minimize attacks by the insect. However, little is known about the nature of resistance to *L. coffeella*, researchers believe that the coffee plant's resistance to the coffee leaf miner may be linked to plant’s metabolism (MEDINA-FILHO; CARVALHO; MONACO, 1977; GUERREIRO-FILHO; SILVAROLLA; ESKES, 1999; GUERREIRO-FILHO; MAZZAFERA, 2000; RAMIRO *et al.*, 2004).

The isolation of endophytic fungi of the genus *Muscodor* carried out in plants showed an efficient inoculation methodology. All fungi isolated in plants presented the same micro and macro morphologies described by Guimarães *et al.* (2021), as branched hyaline hyphae, with cauliflower-like structures observed in some of the isolates, colonies with flaky or cottony surfaces, and colored ranging from beige to white.

CONCLUSION

Catuaí Vermelho cultivar inoculated with *M. coffeanum* (CML 4014) and *Muscodor* sp. (CML 4015) and Topázio cultivar inoculated with *M. coffeanum* (CML 4018) presented the highest values for growth. The vegetative growth of *C. arabica* inoculated with different species and different isolates of endophytic fungi of the genus *Muscodor* was varied. The antioxidant defense response of *C. arabica* inoculated with different species and different isolates of endophytic fungi of the genus *Muscodor* was also varied. Catuaí Vermelho cultivar inoculated with *M. coffeanum* (CML 4011) and Topázio cultivar inoculated with *M. coffeanum* (CML 4020) presented the smallest leaf area injured by the coffee leaf miner. The interaction between *Muscodor* and *C. arabica*, when well established, shows promise for promoting growth and increasing the resistance of coffee to the leaf miner.

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5. ARTIGO 2

Muscodor spp. AND *Coffea arabica*: PRODUCTION OF PHYTOHORMONES AND SIDEROPHORES

ABSTRACT

The endophyte-plant association can improve the survival environment for both organisms, as well as benefit crop growth and yield. *Coffea arabica* is a crop of economic interest, and is quite vulnerable to climate change and sensitive to other environmental conditions, which have a direct and indirect effect on its productivity. The objective of this study was to evaluate the activity of ACC deaminase, production of IAA, GA and siderophores of endophytic fungi, and the production of IAA and GA of coffee plants inoculated with endophytic fungi. Endophytic fungi *Muscodor* spp. were isolated from healthy wild coffee plants and inoculated in seeds of *C. arabica*. After 2 years, leaves were evaluted for phytohormones. The fungi *Muscodor coffeanum* (CML 4019) produced greater amounts of ACC deaminase, auxins and gibberellins, and *M. coffeanum* (CML 4018) produced more siderophores. Coffee plants inoculated with *M. coffeanum* (CML 4019) produced more IAA and GA compared to non-inoculated plants, indicating that the presence of the fungus may have contributed to the greater production of phytohormones by the plants evaluated.

Keywords: Arabica coffee. Bioactive metabolites. Plant defense system. Promotion of growth. Secondary metabolism.

INTRODUCTION

The understanding that plants and microorganisms work together as a dynamic unit to face environmental challenges has led to the holobiont concept (LYU *et al.*, 2021). Among plant-associated microorganisms, endophytes are those that can inhabit plant tissues without causing external signs of infection or other harmful effects to host plants (BURRAGONI; JEON, 2021). The symbiotic connection between plants and microbiota, as well as how this positive interaction acts synergistically, has been essential for exploring alternative methods that can be used to reduce or replace the use of agrochemicals in order to promote a more sustainable agriculture (AHMAD *et al.*, 2018).

The promotion of plant growth through the activity of endophytic microorganisms has been recognized for crops, including coffee (ASAD *et al.*, 2023). It has been demonstrated that endophytic fungi have the function of improving the ability of plants to absorb nutrients from the soil, improving mineral availability and nitrogen fixation (DUONG *et al.*, 2020). Furthermore, they assist in essential functions in plants, including the production and/or modulation of plant growth regulators (HASSAN, 2017).

Endophytic fungi can regulate several phytohormones (auxins, cytokinin, ethylene), and also increase the bioavailability of nutrients in the soil (production of siderophores, solubilization of phosphorus and iron), and prevent damage to plants by releasing enzymes, volatile compounds, which inhibit the activities of pathogens and induce systemic resistance (SEGARAN; SATHIAVELU, 2019; ADELEKE; AYANGBENRO; BABALOLA, 2022).

In coffee cultivation, there are reports of different endophytic populations, including bacteria and fungi (HOANG *et al.*, 2020; DUONG *et al.*, 2021; CHAUDHARY *et al.*, 2022; ASAD *et al.*, 2023). LU *et al.* (2022) evaluated the diversity of endophytic fungi present in healthy coffee leaves and there in vitro antagonistic capacity against phytopathogens that affect coffee crops. The endophytic fungi *Acremonium*, *Muscodor* and *Simplicillium*, isolated from coffee, have antagonistic activity against *Aspergillus ochraceus* inoculated in coffee beans, in addition to synthesizing bioactive compounds that affect the development of the pathogen *Fusarium verticillioides* (MONTEIRO *et al.*, 2017).

The study of the interaction of endophytic fungi with their host plants expands the possibility of using fungi as biocontrol agents, as well as biostimulants and biofertilizers, producing secondary metabolites important for these functions. The objective of this study was to evaluate the activity of ACC deaminase, production of IAA, GA and siderophores of endophytic fungi, and the production of IAA and GA of coffee seedlings inoculated with endophytic fungi.

MATERIAL AND METHODS

Endophytic Fungi Used and Growth Conditions

The endophytic fungi were isolated from healthy wild coffee plants from secondary forest in Viçosa (MG), and were identified as *Muscodor coffeanum* (CML 4009, CML 4010, CML 4011, CML 4012, CML 4014, CML 4016, CML 4017, CML 4018, CML 4019, CML 4020) and *Muscodor* sp. (CML 4013, CML 4015) (GUIMARÃES *et al.*, 2021).

Fungal inocula were prepared from discs of approximately 9 mm in diameter of fungal colonies and were cultivated in 2L erlenmeyer flasks with BD medium for 30 days at 25 ºC temperature.

Seed inoculation

Discs of approximately 9 mm in diameter from the fungal colonies were cultivated in 2L Erlenmeyer flasks with PD medium for 30 days at room temperature. Coffee seeds, of the

Catuaí Vermelho (IAC 144) and Topázio (MG 1190) cultivars, were disinfested in order to eliminate the epiphytic organisms, by immersion and constant agitation, in 70% ethanol for 2 minutes, in sodium hypochlorite (2% active chlorine) for 3 minutes, and again, in 70% ethanol, for 1 minute. Then, the material was triple-washed in autoclaved distilled water. The dry seeds were transferred to Petri dishes, containing PD culture medium and crushed mycelia of endophytic fungi, being immersed for 6 hours.

Coffee growing

After the inoculation procedure, the seeds were planted in 12 L pots, with substrate composed of 70% sieved soil and 30% tanned bovine manure, for each cubic meter 5 kg of simple superphosphate, 1 kg of potassium chloride and 1.5 kg of limestone were added (CFSMG, 1999). Soil analysis showed that the substrate mixture used had the following chemical attributes: pH = 4.8; P, K and Na (mg dm^{-3}) = 1272.81; 207.07 and 61.00; Ca, Mg, Al, H + Al, SB, t e T (cmolc dm^{-3}) = 4.89; 1.28; 0.10; 4.20; 9.43; 9.43 and 13.63; m and V (%) = 1.05 and 69.21; organic matter = 6.72 dag kg^{-1} ; P-Rem = 30.20 mg L^{-1} ; Zn, Fe, Mn, Cu, B and S (mg dm^{-3}) = 5.40; 35.60; 15.30; 0.85; 0.18 and 455.00. Substrate texture: clayey, with 64.0 dag kg^{-1} of clay, 16.0 dag kg^{-1} of silt and 20.0 dag kg^{-1} of sand.

At the time of planting, PD medium with crushed mycelia were also added to the holes next to the seeds with parchment. The pots were kept in a greenhouse for 2 years in a completely randomized design (CRD), with automatic irrigation.

Determination of ACC deaminase Activity of Endophytic Fungi

ACC deaminase activity was assessed by quantifying α-ketobutyrate produt of deamination of ACC. Discs of approximately 5 mm in diameter from *Muscodor* fungi colonies were used as inocula and transferred to tubes containing 10 mL of ammonium-free synthetic medium (MS) (15 g glucose, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.6 g of K_2HPO_4, 0.15 g of KCl, 3 mM of ACC as carbon source per liter of H_2O and 1 mL of the following trace elements per liter of stock solution: 0.005 g of $FeSO_4 \cdot 7H_2O$, 0.006 g of $MnSO_4 \cdot H_2O$, 0.004 g of $ZnSO_4 \cdot H_2O$ and 0.002 g of $CoCl_2$), following the methodology described by Yedidia *et al.* (1999).The fungi were cultivated for 25 days, during which time 3 samples were taken (10, 17 and 25 days). Subsequently, cultures were resuspended in 1 mL of 0.1 M Tris buffer (pH 8.5) and manually homogenized. Then, 200 µL of the extracts were added to 25µL of toluene and shaken vigorously for 30 seconds, and mixed with 20 µL of ACC (0.5 M solution), after an incubation period of 15 min at 30 °C, 1 mL of 0.56 N HCl. The mixtures were centrifuged at

10,000 g for 10 min. Subsequently, 1 mL of the supernatants were mixed with 800 µL of 0.56N HCl and 300 µL of 2,4-dinitrophenylhydrazine (0.2 g in 100 mL of 2N HCl), and incubated for 30 min at 30 °C. Then, 2mL of NaOH 2N were added. The absorbance of the mixture was measured at 540 nm. The analysis was performed in triplicate, and ACC deaminase activity was expressed as µmol of α-ketobutyrate mg $^{-1}$ protein h $^{-1}$.

Production of Indole-3-acetic Acid (IAA) by Endophytic Fungi

The production of indole-3-acetic acid was evaluated using the Salkowski technique according to Blanco *et al.* (2021). To prepare the inoculum, a disc of approximately 5 mm in diameter of *Muscodor* fungi colonies was cultivated in 10 mL of CZAPEK-DOX broth supplemented with tryptophan (1 and 2 mg/mL), and without tryptophan (L-trp; Sigma – Aldrich), all samples were made in triplicates and kept at 25 °C, with constant stirring at 120 rpm for 31 days. Controls were composed of CZAPEK-DOX medium, with and without tryptophan. Evaluations were carried out at different times (15, 20, 23, 26, 28 and 31 days), where 100 µL of supernatant were removed from each sample and transferred to a microplate to be mixed with 200 µL of Salkowski reagent (H_2SO_4 (7.9 M), $FeCl_3$ (12 g/L)) and incubated for 30 min at 25 ºC in the dark. The reddish-pink color was considered positive for IAA production. Subsequently, optical densities were measured using a spectrophotometer at 530 nm. The quantification of IAA was determined based on a calibration curve prepared with standard IAA, in a range from 0 to 300 µg/mL, where the r^2 value was calculated and, using the straight-line equation, the concentration of IAA was calculated produced by each fungus. The experiment was carried out in triplicate and the results obtained were expressed in µg/mL.

Production of Gibberellins (GA) by Endophytic Fungi

The biosynthesis of gibberellin-like compounds was estimated by colorimetry as described by Holbrook, Edge e Bailey (1961). To prepare the inoculum, discs of approximately 5 mm in diameter from *Muscodor* fungi colonies were cultivated in 10 mL of CZAPEK-DOX broth and incubated for 26 days, during this time, 4 evaluations were carried out (20, 22, 24 and 26 days). To obtain pure extracts, 2 mL of supernatants were collected and then mixed with 2 mL of ethyl acetate, the solvent was evaporated at room temperature. Subsequently, the crude extracts were dissolved in methanol and mixed with 2 mL of 21.9% zinc acetate, and incubated for 2 min. Then, 2 mL of 10.6% potassium ferrocyanide were added and the mixtures were centrifuged at 2000 rpm for 15 min. A volume of 5 mL of 30%

HCl was added to 5 mL of the supernatants and incubated at 20 °C for 75 min. The absorbances of the samples were measured at 430 nm using a spectrophotometer. Gibberellic acid (GA3) was used to prepare the standard curve and quantified from the range 10 to 100 mg dissolved in absolute alcohol and expressed in µg/mL. The experiment was carried out in triplicate.

Production of Siderophores by Endophytic Fungi

The production of siderophores was detected by the Chromium Azurol-S test (CAS): solution A - 72.9 mg of HDTMA (hexadecyltrimethylammonium bromide) dissolved in 40 mL of distilled water, and solution B - 60.5 mg of chromium azurol sulfonate , dissolved in 50 mL of distilled water, added to a solution of 0.0027 g of $FeCl_3$ in 10 mL of HCl (10 mM) (SCHWYN; NEILANDS, 1987), in a substrate based on King's medium B - 1.5 g /L of K_2HPO_4, 1.5 g/L of $MgSO_4$ $7H_2O$, 15 mL/L of glycerol, 15 g/L of agar (GLICKMANN; DESA-SAUX, 1995).Both solutions (A and B) were mixed and sterilized separately. Subsequently, the culture medium and solution were mixed in a 9:1 ratio, respectively, and transferred to Petri dishes. Discs of approximately 5 mm in diameter of *Muscodor* fungi colonies, with 5 days of growth, were inoculated on plates with King's solid medium B and Chromium Azurol-S (CAS) and incubated in the dark at 30 °C for 10 days. Then, the plates were qualitatively evaluated for the appearance of a yellow or orange halo zone around the fungal discs in blue agar medium. The experiment was carried out in triplicate.

Production of Indole-3-acetic Acid (IAA) by Coffee Seedlings

Auxin extraction and quantification (IAA) were performed according to the protocol described by Anthony and Street (1970). The plant material (60 mg of fresh leaf tissue) was ground in a mortar with liquid nitrogen, then resuspended in 2 mL of buffer solution (0.01 M Na-phosphate pH 7) and centrifuged at 17,000 g for 15 min. Then, the supernatant was recovered and added to 2 mL of 100% (w/v) trichloroacetic acid (TCA) and 2 mL of Ehrlich reagent (Dimethylaminobenzaldehyde – DMAB). Simultaneously, the blank was prepared with the buffer solution (0.01 M Na phosphate) and the standard curve with commercial IAA. The mixtures were incubated for 20 minutes in the dark and their absorbances were measured at 530 nm on a microplate spectrometer (Thermo Scientific™ Multiskan™). The analysis was carried out in triplicate, and the results obtained were expressed in µg IAA.mg^{-1}fresh biomass.

Production of Gibberellins (GA) by Coffee Seedings

GA extraction and quantification were performed according to the protocol of Graham and Henderson (1961). It was prepared 200 mL of Folling-Wu reagent - phosphomolybdic acid (85% molybdic acid, 10% sodium tungstanate, 10% sodium hydroxide and phosphoric acid). The reagent was prepared in two phases: in the first, 7 g/L of molybdic acid, 1 g/L of sodium tungstanate were added, and subsequently 40 mL of 10% sodium hydroxide and 40 mL of distilled water were added. The solution was boiled for 40 minutes to remove traces of ammonium present in molybdic acid. In the second phase, the solution was cooled and 70 mL of distilled water and 25 mL of 85% phosphoric acid were added to it. Then, the solution was made up to 200 mL with distilled water and stored in a refrigerator.

Subsequently, a mixture was made in a proportion of 1 mL of samples to 3 mL of phosphomolybdic acid reagent. The reaction time was 60 min in a boiling water bath. Time zero was estimated after 2 min of submerging the test tubes in a boiling water bath; after 60 min, the tubes were removed from the water bath and the temperature was lowered using an ice bath. When at room temperature, the color intensity was observed and the optical density was measured at 780 nm on a microplate spectrophotometer (Thermo Scientific™ Multiskan™). The experiment was carried out in triplicate, and the results obtained were expressed in µg GA.mg^{-1}fresh biomass.

Statistical analysis

The data obtained were subjected to analysis of variance and the means of the treatments were compared using the Skott-Knott test ($p<0.05$). The interrelationships between variables and treatments were assessed using exploratory Principal Component Analysis (PCA). To choose the number of components, we observed those that had eigen values greater than 1 ($\lambda > 1$) (FRAGA *et al.*, 2015) and an accumulated percentage of at least 80% explanation of the total variation (JOHNSON; WICHERN, 1998). In the graph of the two-dimensional projection of variables and treatments from the PCA, the variables were discriminated according to their percentage contribution (%) in relation to the components. The contribution was determined by the ratio of the square of the variable's coordinates in the scatterplot to the total of the component. The contribution was represented in a color scale. All analyzes were performed using the statistical package R version 4.1.2 (R Core Team, 2021). The “factoextra” library was used for PCA (KASSAMBARA; MUNDT, 2020).

RESULTS

Before evaluating the production of IAA and GA of coffee plants inoculated with endophytic fungi, the characterization of endophytic fungi was carried out by evaluating the production of activity of ACC deaminase, production of IAA, GA and siderophores.

The ACC deaminase activity was grester for *M. coffeanum* (CML 4019). *Muscodor coffeanum* (CML 4020) also stood out in the production of this enzyme when compared to other fungi of the genus. However, from the 17th day onwards there was a drop in production. The fungi *Muscodor* sp. (CML 4013) and *M. coffeanum* (CML 4016) had the lowest productions of ACC deaminase activity (Figure 1).

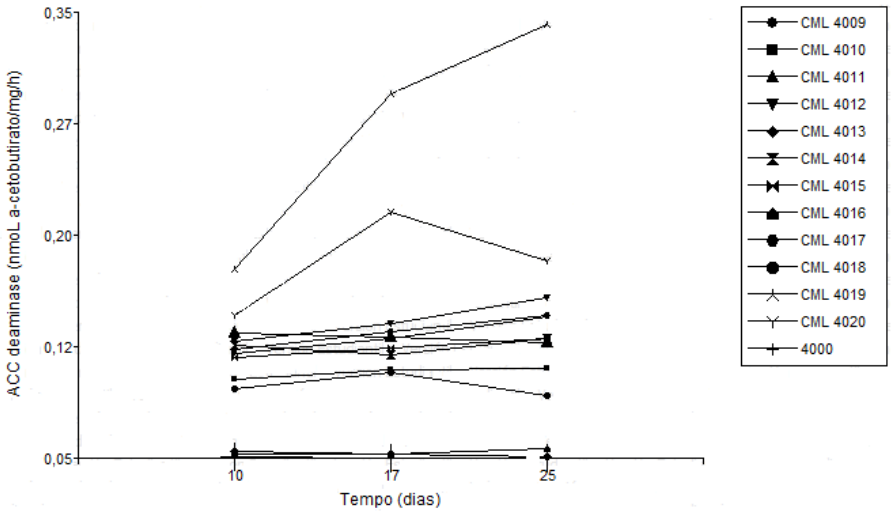

Figure 1 – ACC deaminase activity of endophytic fungi over 25 days.

The IAA production by endophytic fungi showed that for the medium supplemented with tryptophan (1 mg/mL) the fungus *M. coffeanum* (CML 4019) presented, between days 15 and 28, a higher production, standing out from the others fungi. For the medium with 2 mg/mL of tryptophan, the production of IAA by the fungi was more homogeneous, with *M. coffeanum* (CML 4019) showing the highest production peak (day 26). As for the medium without tryptophan, *M. coffeanum* (CML 4019) showed a peak in IAA production on day 20, followed by a sudden drop and growth in production over the days (23 to 31).However, it was *M. coffeanum* (CML 4011) which presented the highest production peak (day 31) (Figure 2).

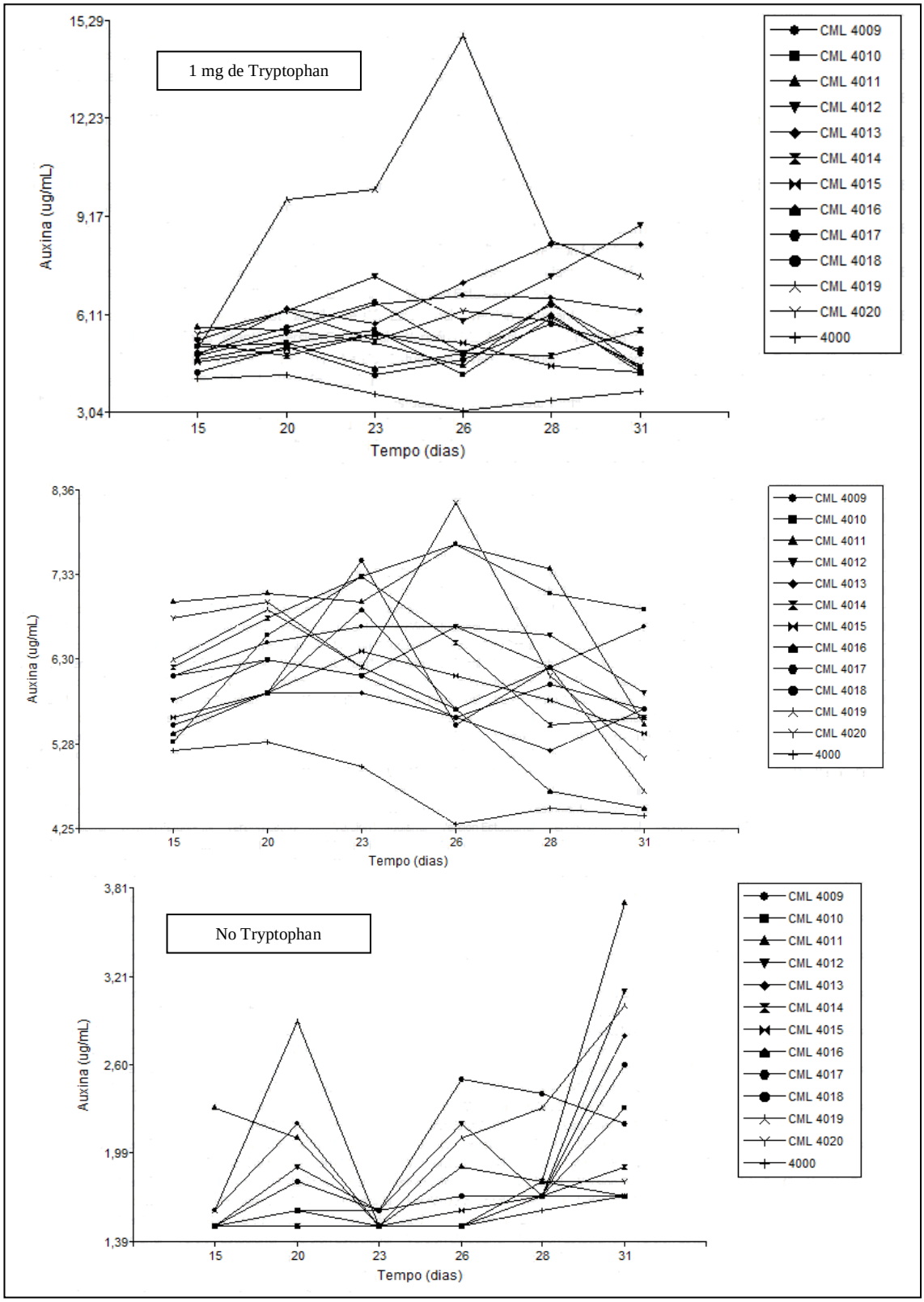

Figure 2 – Indole-3-acetic acid production by *Muscodor* spp. over 31 days in CZAPEK-DOX medium with two concentrations of L-tryptophan and without tryptophan.

GA production was higher for *M. coffeanum* (CML 4019), with a maximum peak on the 26th day, followed by *M. coffeanum* (CML 4016), where both fungi had an increase in

production over the days of the experiment. The fungus *M. coffeanum* (CML 4009) showed the lowest GA production (Figure 3).

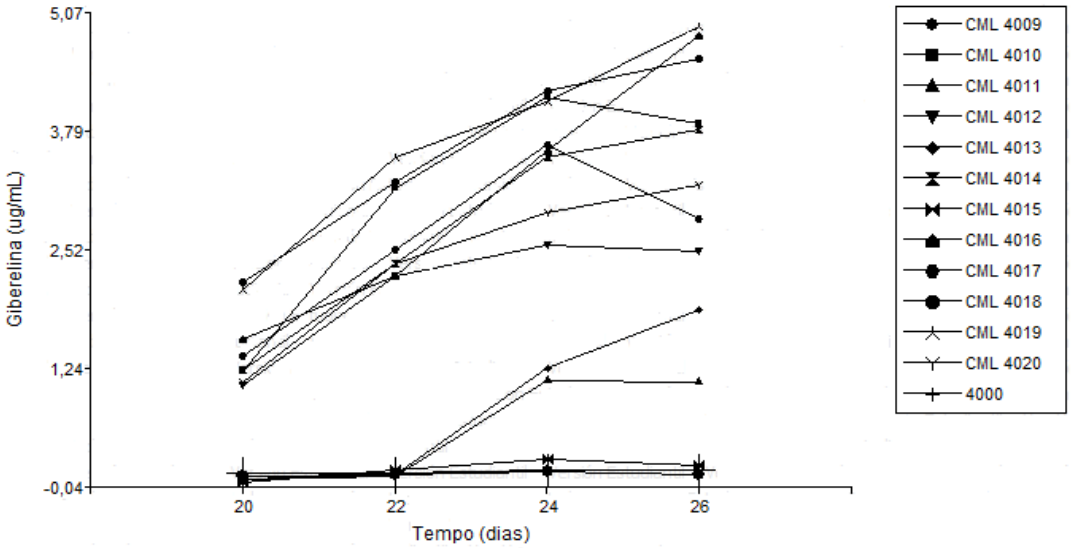

Figure 3 – Gibberellins production by endophytic fungi over 26 days.

The siderophore production was greater for *M. coffeanum* (CML 4018), and *Muscodor* sp. (CML 4013) and *M. coffeanum* (CML 4014) had the lowest production (Table 1).

Table 1 – Siderophores production by *Muscodor* spp..

Fungi	Siderophores
M. coffeanum (CML 4009)	48.69b
M. coffeanum (CML 4010)	55.92a
M. coffeanum (CML 4011)	36.56b
M. coffeanum (CML 4012)	25.00c
Muscodor sp. (CML 4013)	12.74c
M. coffeanum (CML 4014)	12.90c
Muscodor sp. (CML 4015)	16.02c
M. coffeanum (CML 4016)	47.44b
M. coffeanum (CML 4017)	46.86b
M. coffeanum (CML 4018)	64.40a
M. coffeanum (CML 4019)	50.99b
M. coffeanum (CML 4020)	54.84a

Means followed by the same letters in the column do not differ from each other by the Skott-Knott test ($p<0.05$).

In the Catuaí and Topazio cultivar plants inoculated with *M. coffeanum* (CML 4019 and 4011) were observed higher productions of AIA. For Catuaí cultivar plants (0.075 µg AIA.mg^{-1} fresh biomass and 0.070 µg AIA.mg^{-1}fresh biomass), and non-inoculated plants had the lowest production (0.0055 µg AIA.mg^{-1} fresh biomass). For Topázio cultivar plants (0.080

µg AIA.mg^{-1} fresh biomass and 0.079 µg AIA.mg^{-1} fresh biomass), and non-inoculated plants showed the lowest production (0.0041 µg AIA.mg^{-1} fresh biomass) (Table 2).

Table 2 – Indole-3-acetic acid production by *C. arabica* Catuaí and Topázio cultivars inoculated with endophytic fungi and non-inoculated.

	Catuaí cultivar	**Topázio cultivar**
Treatment	**AIA (µg AIA.mg^{-1}freshbiomass)**	**AIA (µg AIA.mg^{-1}freshbiomass)**
Control	0.0055c	0.0041d
M. coffeanum (CML 4009)	0.057a	0.072a
M. coffeanum (CML 4010)	0.054a	0.070a
M. coffeanum (CML 4011)	0.070a	0.079a
M. coffeanum (CML 4012)	0.062a	0.077a
Muscodor sp. (CML 4013)	0.060a	0.058b
M. coffeanum (CML 4014)	0.013b	0.025c
Muscodor sp. (CML 4015)	0.027b	0.053b
M. coffeanum (CML 4016)	0.014b	0.038b
M. coffeanum (CML 4017)	0.044a	0.045b
M. coffeanum (CML 4018)	0.018b	0.017c
M. coffeanum (CML 4019)	0.075a	0.080a
M. coffeanum (CML 4020)	0.033b	0.023c

Means followed by the same letters in the column do not differ from each other by the Skott-Knott test ($p<0.05$).

In the Catuaí and Topazio cultivar plants inoculated with *M. coffeanum* (CML 4019 and 4016) was observed highest productions of GA. For Catuaí cultivar plants (0.044 µg GA.mg^{-1} fresh biomass and 0.041 µg GA.mg^{-1} fresh biomass, respectively) and the lower was for non-inoculated plants (0.005 µg GA.mg^{-1} of fresh biomass). For Topázio cultivar plants (0.046 µg GA.mg^{-1} fresh biomass and 0.042 µg GA.mg^{-1} fresh biomass, respectively) were the highest, and the lowest production was from non-inoculated plants (0.005 µg GA.mg^{-1} fresh biomass) (Table 3).

Table 3 – Gibberellins production by *C. arabica* Catuaí and Topázio cultivars inoculated with endophytic fungi and non-inoculated plants.

	Catuaí cultivar	**Topázio cultivar**
Treatment	**GA (µg GA.mg^{-1}fresh biomass)**	**GA (µg GA.mg^{-1}fresh biomass)**
Control	0.005d	0.005d
M. coffeanum (CML 4009)	0.033b	0.009d
M. coffeanum (CML 4010)	0.012c	0.014c
M. coffeanum (CML 4011)	0.015c	0.019c
M. coffeanum (CML 4012)	0.020b	0.024b
Muscodor sp. (CML 4013)	0.018c	0.021b
M. coffeanum (CML 4014)	0.011c	0.011c
Muscodor sp. (CML 4015)	0.019c	0.019c
M. coffeanum (CML 4016)	0.041a	0.042a
M. coffeanum (CML 4017)	0.028b	0.037b
M. coffeanum (CML 4018)	0.011c	0.011c
M. coffeanum (CML 4019)	0.044a	0.046a
M. coffeanum (CML 4020)	0.029b	0.040a

Means followed by the same letters in the column do not differ from each other by the Skott-Knott test ($p<0.05$).

The principal components analysis (PCA) explained 80.09% of the variability of the factors for the two axes. The variables related to the first axis formed four groups: the first group composed of gibberellins, the second composed of ACC deaminase, the third of IAA, and the fourth group, referring to the production of siderophores. *Muscodor* spp. also were divided into four groups: in the first *M. coffeanum* (CML 4019) was shown to be correlated with the production of IAA and ACC deaminase, in the second *M. coffeanum* (CML 4012) showed direct relationships with the production of IAA, in the third *M. coffeanum* (CML 4020) was shown to be correlated with the production of gibberellins and in the fourth group *M. coffeanum* (CML 4010, 4017 and 4018) were shown to be correlated with the production of siderophores (Figure 4).

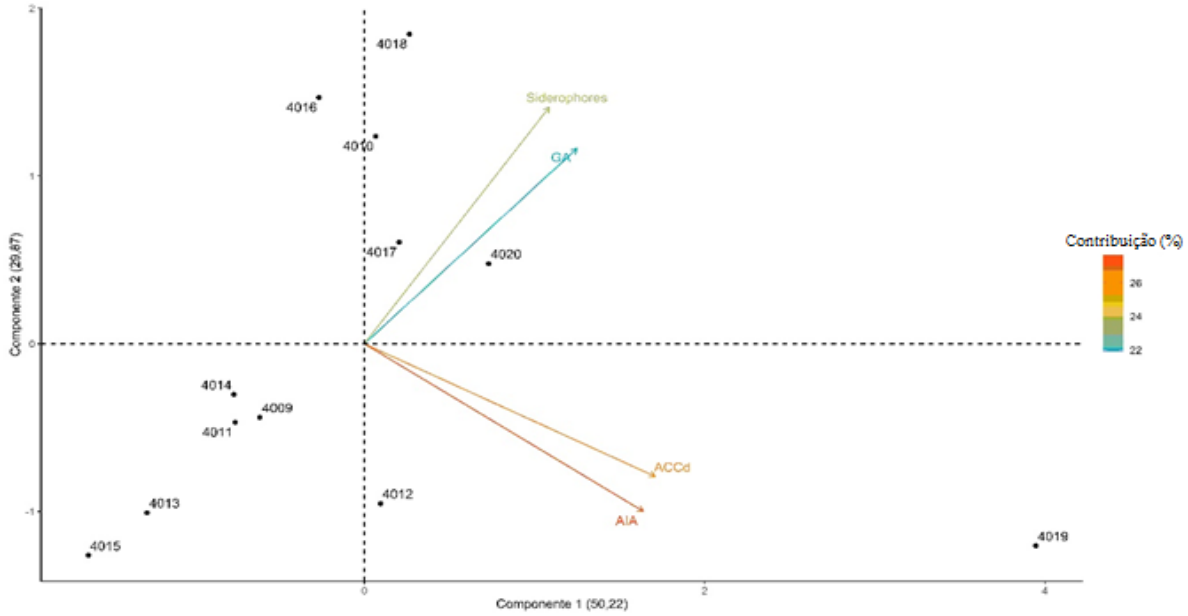

Figure 4–Two-dimensional projection of endophytic fungi and the phytohormones ACC deaminase (ACCd), indole-3-acetic acid (AIA) and gibberellins (GA) and siderophores in the two main components (CP1 x CP2).

The analysis of main components showed the separation of endophytic fungi of the genus *Muscodor* into four groups organized according to the biochemical characteristics of these fungi, as demonstrated by the direction and amplitude of the corresponding vectors. The fungi *M. coffeanum* (CML 4009, 4011, 4014 and 4016) and *Muscodor* sp. (CML 4013 and 4015) showed a negative correlation with the production of ACC deaminase, IAA, GA and siderophores, therefore it is not possible to characterize them biochemically in relation to these variables (Figure 4).

DISCUSSION

The use of endophytic fungi adapted to extreme habitats with ACC deaminase activity is a known strategy to minimize the negative effects of abiotic stress on agricultural crops (REHMAN *et al.*, 2022). *Muscodor coffeanum* (CML 4019) stood out for its ACC deaminase activity, and could be an ally in promoting the growth of plants under stress conditions, since it may be able to hydrolyze ACC into ammonium and α-ketobutyrate for use as a source of nitrogen.

Studies have shown that plant growth promotion can be attributed to the secretion of plant growth-promoting secondary metabolites (phytohormones and siderophores) and to the ability of endophytic fungi to mobilize insoluble phosphate and supply nitrogen to host plants (DAI; YU; LI, 2008 ; KHAN *et al.*, 2012; WAQAS *et al.*, 2012; WAQAS; KHAN; LEE,

2014; KHAN *et al.*, 2015; DENG; CAO, 2017). The ability to produce indole-3-acetic acid (IAA) is one of the most valued characteristics of microorganisms used to promote plant growth, since IAA participates in several physiological processes, including elongation, cell division, germination, vascular development and root growth (WAQAS *et al.*, 2012; SHI *et al.*, 2017).

Our study showed that endophytic fungi of the genus *Muscodor* showed IAA production in the concentration range of 5.0 – 14.8 ug mL^{-1} via the L-tryptophan (Trp)-dependent pathway. The same was observed by Waqas *et al.* (2012), when studying cucumber endophytic fungi grown in the field, they observed that *Phoma glomerata* produced IAA in the range of 3.89 μg/mL^{-1} with Trp. Khan *et al.* (2016), when studying 17 endophyte fungi isolated from *Boswellia sacra* (an economically important incense tree), they concluded that only one strain of *Aureo basidium* sp. demonstrated potential to produce IAA. Studies show that the majority of endophytic fungi evaluated produce IAA through L-tryptophan-dependent pathways, however, there are few that produce auxin through Trp-independent pathways (WAQAS *et al.*, 2012; KHAN *et al.*, 2016; NUMPONSAK *et al.*, 2018). In our work, *M. coffeanum* (CML 4011 and 4019) showed IAA production also in the absence of L-tryptophan, being capable of synthesizing auxins using Trp-dependent and -independent pathways, in addition to being the fungi with the highest production of the phytohormone (Figure 2).Extracts from the leaves of coffee plants inoculated with these same fungi also showed the highest IAA production, following the same pattern as in vitro evaluation, suggesting that the presence of endophytic fungi inoculated in coffee plants may have contributed to the greatest production of auxin by the plants evaluated.

The GA production is an important characteristic that allows endophytic fungi to promote plant growth and decrease abiotic stress (HAMAYUN *et al.*, 2015). The literature shows that *Aspergillus fumigatus*, *Chrysosporium pseudomerdarium*, *Gibberella fujikuroi*, *Neurospora crassa*, *Penicillium citrinum*, *Phaeosphaeria* sp., and *Sesamum indicum* can improve plant growth and crop productivity through the production of GA (CHOI *et al.*, 2005; KAWAIDE, 2006; HAMAYUN *et al.*, 2009). *Muscodor coffeanum* (CML 4016 and 4019) produced significant amounts of GA, suggesting that their interactions with coffee can improve the quality and productivity of this crop, since when evaluating extracts from the leaves of plants inoculated with endophytic fungi, we observed that the plants that produced the most GA were those inoculated with these same fungi.

Siderophore-producing microorganisms can inhibit plant pathogens by eliminating available iron from the environment (AHMED; HOLMSTRÖM, 2014). Our study showed

that the endophytic fungus *M. coffeanum* (CML 4018) was the largest producer of siderophores after 27 days of growth, suggesting that the interaction of this fungus with the coffee plant can result in better responses in the agronomic performance of the crop, such as observed by Hayashibara *et al.* (2022), when he studied the bean crop and demonstrated that *M. coffeanum* (CML 4018) contributed to improving the productivity and plant health of *Phaseolus vulgaris* L.

The pronounced effect of endophytic fungi in increasing the growth and yield attributes of various crops is well established in the literature (ALI *et al.*, 2018; ANITH *et al.*, 2018; ISMAIL *et al.*, 2018; QINGMIAO *et al.*, 2012). Therefore, the inoculation of endophytic fungi in plants of economic importance plays a fundamental role in improving crop growth and nutrient production, sustaining soil productivity and reducing the levels of chemical fertilizers and fungicides used (CHEN *et al.*, 2021).

CONCLUSION

Muscodor coffeanum (CML 4019) produced more ACC deaminase and the phytohormones IAA and GA, and *M. coffeanum* (CML 4018) was the largest producer of siderophores. The coffee plants inoculated with the fungus *M. coffeanum* (CML 4019) were those that produced the most AIA and GA, indicating that the presence of the endophytic fungus inoculated in the coffee plants has a positive correlation with greater production of phytohormones by the plants evaluated. Endophytic fungi of the genus *Muscodor* are efficient in promoting coffee growth and increasing crop productivity, with *M. coffeanum* (CML 4018 and 4019) being good candidates for initial growth-promoting inoculants for coffee.

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6. ARTIGO 3

PHENOLIC COMPOUNDS AND ANTIOXIDANT ACTIVITY OF *Muscodor* spp. AND COFFEE SEEDLINGS INOCULATED WITH ENDOPHYTIC FUNGI

ABSTRACT

The genus *Muscodor* produces volatile and non-volatile organic compounds with biological activity. The production of phenolic compounds and antioxidant activity of fungi and of plants inoculated have been little reported. Therefore, the objective of this work was to identify the total phenolic compounds and determine the antioxidant activity of *Muscodor* species isolated from leaves and stems of *Coffea arabica*, and two coffee cultivars inoculated with these endophytic species. Total phenolic compounds (TPC) were determined using the Folin-Ciocalteau method and their identification by HPLC-DAD. To determine the antioxidant activity, analyzes of free radical scavenging (DPPH), oxygen radical absorption capacity (ORAC) and total antioxidant capacity (TAC) were performed. *Muscodor coffeanum* (CML 4019) showed the highest concentration of total phenolic compounds (53.6 mgEAG/g), highest elimination of DPPH free radicals (276.7 μg/g) and highest absorption capacity of the oxygen radical (154.3 μmolET/g). Based on standards, HPLC-DAD analysis indicated the presence of 11 phenolic compounds produced by *Muscodor* spp.: catechin, theobromine, trigonelline, vanillin, and the caffeic, chlorogenic, ferulic, gallic, o-coumaric, p-coumaric and syringic acids. The Catuaí cultivar inoculated with *M. coffeanum* (CML 4019) also showed a higher concentration of TPC (1.71 mgEAG/g), a higher scavenging of DPPH free radicals (136.51 μg/g) and a higher ORAC (27. 31 μmolET/g). For the Topázio cultivar, the plants inoculated with the fungi *M. coffeanum* (CML 4018 and 4014) stood out in terms of TPC concentration (0.62 mgEAG/g) and ORAC (13.43 μmolET/g), and concentration of TPC (0.61 mgEAG/g) and total antioxidant capacity (TAC) (0.66 mgEAA/g), respectively. The antioxidant activity showed a direct correlation between antioxidant capacity and the presence of phenolic compounds. The production and concentration of phenols varied among isolates of the same species of the genus *Muscodor*.

Keywords: Caffeic acid. Folin-Ciocalteau. HPLC-DAD. Phenols. Trigonelline.

INTRODUCTION

Phenolic compounds are widely found in plants, being present in various plant foods such as fruits, vegetables and cereals, as well as in beverages of plant origin such as teas, wine and coffee (MANACH *et al.,* 2004; CHEYNIER, 2005; FARAH; DONANGELO, 2006). These compounds are considered antioxidants because they have the ability to prevent, delay and remove oxidative damage by inhibitingor capturing free radicals and reactive oxygen species (XU *et al.*, 2017; da SILVA *et al.,* 2020). In addition, the association of some phenols determines the coffe equality, playing a fundamental role in the formation of the specific aroma and flavor of the drink (VARIYAR *et al.,* 2003; FARAH *et al*., 2006; FARAH; DONANGELO, 2006).

Coffee is one of the main Brazilian commodities, with *C. arabica* and *C. canephora* Pierre ex A. Froehner being the most commercially important and the most consumed beverages in the world (DONG *et al.*, 2017). The coffee drink is proven to be an important dietary source of phenolic compounds, and because they have antioxidant properties, these compounds give the regular consumer of the drink the reduction of chronic diseases and metabolic syndromes (HARUMI KONDO; IKEWAKI, 2012; SOARES *et al.*, 2020; SOARES *et al.*, 2022).

The endophytic fungal genus *Muscodor*, described by Worapong *et al.* (2001), has currently been transferred to the genus *Induratia* (SAMARAKOON *et al.*, 2020). The proposed change was based on the phylogenetic relationship with species of the genera *Induratia* and *Emarcea*, and on the analysis of the sequences of the ITS, LSU, rpb2 and tub2 genes. Recently, it was proposed to return the species to the genus *Muscodor*, since the species that were in *Induratia* were not related to the culture of the ancient holotype of *Induratia apiospora* (CEDEÑO-SANCHEZ, 2023). Based on the analysis of the phylogeny of three loci (ITS, rpb2,and tub2), the morphological characterization and production of volatile organic compounds (VOCs) from *Muscodor* isolates, Guimarães *et al*. (2021) identified nine isolates of *Muscodor coffeanum* and two of *Muscodor* sp. isolated from stems and leaves of *Coffea arabica* L. found in a secondary forest in Brazil.

Muscodor coffeanum and *Muscodor* sp. produce volatile compounds efficient in antifungal, antibacterial and antinematode activities, being able to inhibit phytopathogens (MONTEIRO *et al.*, 2017; GUIMARÃES *et al.*, 2021; MOTA *et al.*, 2021). Its ethyl acetate extracts show bioactivity, acting as modulators of the blood clotting process along with different classes of proteases, playing a role in maintaining human hemostasis and as producers of extracellular enzymes (BASTOS *et al.*, 2020; MONTEIRO *et al.*, 2020; CARDOSO *et al.*, 2022.

There are studies on the production of phenolic compounds by endophytic fungi isolated from medicinal plants, as well as their antioxidant capacity (LAVANYA *et al.*, 2021; POLLI *et al.*, 2020; da SILVA *et al.*, 2020; DAS *et al*., 2018). However, there are still few studies that determine the production of these compounds by endophytes isolated from coffee.

Therefore, the objective of this work was to evaluate the production and identification of total phenolic compounds of *Muscodor* spp., and to evaluate the antioxidant activity of fungi and coffee plants associated with these endophytic fungi.

MATERIAL AND METHODS

Endophytic fungi

The endophytic fungi used in this work were isolated from coffee from a secondary forest in Viçosa (MG) identified by Guimarães *et al.* (2021): *Muscodor coffeanum* (CML 4009, CML 4010, CML 4011, CML 4012, CML 4014, CML 4016, CML 4017, CML 4018, CML 4019, CML 4020) and *Muscodor* sp. (CML 4013, CML 4015).

Seed inoculation

Discs of approximately 9 mm in diameter from the fungal colonies were cultivated in 2L Erlenmeyer flasks with PD medium for 30 days at room temperature. Coffee seeds, of the Catuaí Vermelho (IAC 144) and Topázio (MG 1190) cultivars, were disinfested in order to eliminate the epiphytic organisms, by immersion and constant agitation, in 70% ethanol for 2 minutes, in sodium hypochlorite (2% active chlorine) for 3 minutes, and again, in 70% ethanol, for 1 minute. Then, the material was triple-washed in autoclaved distilled water. The dry seeds were transferred to Petri dishes, containing PD culture medium and crushed mycelia of endophytic fungi, being immersed for 6 hours.

Coffee growing

After the inoculation procedure, the seeds were planted in 12 L pots, with substrate composed of 70% sieved soil and 30% tanned bovine manure, for each cubic meter 5 kg of simple superphosphate, 1 kg of potassium chloride and 1.5 kg of limestone were added (CFSMG, 1999). Soil analysis showed that the substrate mixture used had the following chemical attributes: pH = 4.8; P, K and Na (mg dm^{-3}) = 1272.81; 207.07 and 61.00; Ca, Mg, Al, H + Al, SB, t e T (cmolc dm^{-3}) = 4.89; 1.28; 0.10; 4.20; 9.43; 9.43 and 13.63; m and V (%) = 1.05 and 69.21; organic matter = 6.72 dag kg^{-1} ; P-Rem = 30.20 mg L^{-1} ; Zn, Fe, Mn, Cu, B and S (mg dm^{-3}) = 5.40; 35.60; 15.30; 0.85; 0.18 and 455.00. Substrate texture: clayey, with 64.0 dag kg^{-1} of clay, 16.0 dag kg^{-1} of silt and 20.0 dag kg^{-1} of sand.

At the time of planting, PD medium with crushed mycelia were also added to the holes next to the seeds with parchment. The pots were kept in a greenhouse for 2 years in a completely randomized design (CRD), with automatic irrigation.

Preparation of Fungal and Plant Extracts

Fungal extracts were prepared from five discs of approximately 5 mm in diameter from the fungal colonies and were transferred to 2 L Erlenmeyers with 1 L of potato-dextrose

medium, which were left static for 30 days at room temperature. Cultures in liquid medium were vacuum filtered and extracted twice by liquid-liquid partition with ethyl acetate (1:2). The extracts were concentrated and dried in a rotary evaporator at 40°C.

Plant extracts were obtained from 50 mg of frozen in liquid nitrogen and pulverized aliquots of Catuaí Vermelho and Topázio coffee leaves. Plant materials were sonicated during 30 min in 2 mL of 70% hydroethanolic solution. The extracts were purified by centrifuged at 10,000 rpm for 10 min. The supernatants were collected and diluted (from 1 to 32 times).

Total Phenolic Compounds and Antioxidant Capacities

The analysis of total phenolic compounds and antioxidants capacities were performed using an Infinite 200 spectrophotometer (A-5082; Tecan, Grödig, Salzburg). All treatments were evaluated in triplicate.

Sample preparation

The fungal (10 mg/mL) and plant (25 mg/mL) hydroethanolic extracts were sequential diluted up to a proportion of 1/128 in 96-well microplates.

Determination of Total Phenolic Compounds (TPC)

The quantification of TPC was performed based on the Folin-Ciocalteau method, with modifications (SINGLETON; ROSSI, 1965). An aliquot of fungal extracts (50 μL) and plant extracts (20 μL) was transferred to 96-well microplates. Both were mixed with 120 μL of Folin-Ciocalteau ethanolic solution (10%) and 120 μL of sodium carbonate (7%). Then, at room temperature, the samples were incubated for 2 hours away from light and the absorbances were read at 760 nm. The calibration curve of gallic acid (Sigma-Aldrich®, Brazil, 98% purity) comprised concentration range from 0.0078 to 0.25 mg/mL ($y = 6.6918 + 0.2452$, $R^2 = 0.9947$) were employed to determine TPC content. The results were expressed in milligrams of gallic acid equivalent per gram of extract (mgEAG/g).

Determination of Antioxidant Activity

- Analysis of the Oxygen Radical Absorption Capacity (ORAC)

The oxygen radical absorption capacity was determined by the method of Ou, Hampsch-Woodill and Prior (2001). In a 96-well black microplate, 25 μL of fungal or plant extracts and 150 μL of fluorescein (70 mM) prepared in phosphate buffer (75 mM; pH 7.4) were added. The microplate was pre-incubated for 10 minutes at 37°C in the TECAN

equipment. Subsequently, 30 μL of the radical AAPH [2,2'-Azobis (2-amidinopropane) dihydrochloride] (12 mM) were added. The microplate fluorescence was recorded every minute during 150 minutes. The Trolox standard was used as a positive control. As a negative control, 30 μL of potassium phosphate buffer (75 mM; pH 7.4) were used instead of AAPH. ORAC values were calculated using a regression equation for the Trolox concentration and the liquid are aunder the curve (AUC) ($AUC_{sample} - AUC_{control}$) using PRISM® software and expressed in micromole of Trolox equivalent per gram of extract (μmolET/g).

- Free Radical Elimination Activity (DPPH)

The antioxidant capacity of fungal and plant extracts, the DPPH (2,2-diphenyl-2-picryl-hydrazyl) radical scavenging were performed used, based on the methodology of Brand-Williams, Cuvelier and Berset (1995). For each sample, sequential dilutions in ethanol were performed, from the extract in the original concentration to the proportion 1/128. In 96-well microplates, 250 μL aliquots of DPPH ethanolic solution (60 μg/mL) were added to 50 μL of extracts. Subsequently, the samples were incubated for 60 minutes in the dark and at room temperature. Ethanol was used as a negative control. The absorbance of the samples was measured in a spectrophotometer at 517 nm. The percentage of inhibition of DPPH free radicals (I%) was calculated according to the equation $I(\%) = [(A_{standard} - A_{sample}) / A_{standard}] \times 100$, where $A_{standard}$ corresponds to the absorbance of the DPPH ethanolic solution and A_{sample} corresponds to the absorbance of the DPPH solution + extract. Free radical scavenging capacity was expressed by IC_{50} (μg/mL), corresponding to the extract concentration capable of inhibiting 50% of DPPH radicals.

- Analysis of Total Antioxidant Capacity (TAC)

Total antioxidant capacity was determined by the molybdate reduction protocol according to Prieto, Pineda and Aguilar (1999). In 2 mL microtubes, 50 μL of fungal or plant extracts and 1 mL of TAC reagent solution (monobasic sodium phosphate (28 mM); ammoniummolybdate (4 mM); sulfuric acid (0.6 M), in equal proportions). The microtubes were incubated for 90 minutes in a water bath at 95°C. After reaching room temperature, a 200 μL aliquot of the samples was pipette into 96-well microplates and its absorbance was measured in a spectrophotometer at 695 nm. The calibration curve was created using ascorbic acid standard (Sigma-Aldrich®, Brazil, 99.7% purity) at concentrations from 0.002 to 0.5 mg/mL, generating the equation: $y = 6.80x + 0.0716$, $R^2 = 0.9946$. The results obtained were expressed in milligrams of ascorbic acid equivalents per gram of extract (mgEAA/g).

HPLC-DAD Analysis of Phenolic Compounds in Fungal Extracts

HPLC-DAD analysis of phenolic composition of fungal extracts was performed on a Shimadzu liquid chromatography (Shimadzu Corp., Japan) with DAD using the Shim-Pack VPODS column (4.6 mm × 250 mm × 5 μm) and 20 μL of each sample. The mobile phase used was (A) water/acetic acid (98:2; v/v) and (B) methanol/water/acetic acid (70:28:2; v/v/v). The gradient was linear at a flow rate of 1.0 mL/min from 0% to 20% solvent B for 5 minutes, from 20% to 40% solvent B for 20 min, 40% to 45% solvent B for 18 min, 45% to 80% solvent B for 7 minutes and 80% to 0% solvent B for 5 min, followed by washing with solvent A and column-equilibration for 10 min. Diode array detection was performed at 280 nm. The analyzed phenolic compounds were identified by comparing their retention times with their respective standards (AMORIM *et al.*, 2018). Fifteen standards were evaluated: catechin, resveratrol, theobromine, trigonelline, vanillin and also the caffeic, chlorogenic, ferulic, gallic, m-coumaric, o-coumaric, p-coumaric, rosmarinic, syringic and trans-cinnamic acids.

Statistical Analysis

Data from the analyzes of oxygen radical absorption capacity (ORAC), free radical scavenging activity (DPPH), total antioxidant capacity (TAC) and total phenolic compounds (TPC) were submitted to analysis of variance (ANOVA) and the means of treatments were compared using the Scott-Knott test ($p<0.05$).

RESULTS

The quantification of total phenolic compounds, the analysis of the oxygen radical absorption capacity (ORAC), the free radical elimination activity (DPPH) and the analysis of total antioxidant capacity (TAC) at a concentration of 10 mg/mL of *Muscodor* spp. are shown in Table 1.

Muscodor coffeanum (CML 4019, CML 4018) and *Muscodor* sp. (CML 4013) produced the largest amount of phenolic compounds, 53.6 mgEAG/g, 52.3 mgEAG/g and 52.1 mgEAG/g, respectively. The other fungal isolates showed levels below 50 mgEAG/g.

From the comparison of the retention times of 15 standards, the results of the preliminary screening through HPLC-DAD analysis indicated the presence of 11 phenolic compounds in the analyzed fungal extracts being catechin, theobromine, trigonelline, vanillin and also the caffeic, chlorogenic, ferulic, gallic, o-coumaric, p-coumaric, and syringic acids (Table 2). Ferulic acid and trigonelline were produced by 10 of the 12 fungi analyzed and

caffeic acid by seven, while the remaining phenolic compounds were produced by five or fewer fungi (Figure 1).

Results of DPPH free radical scavenging activity were expressed in IC_{50} (referring to the mg/mL concentration capable of inhibiting 50% of the DPPH radical) and showed that *M. coffeanum* (CML 4019) presented IC_{50} of 276.7 µg/mL, being significantly lower than the other extracts, showing a greater capacity for scavenging the DPPH radical.

Regarding the analysis of the oxygen radical absorption capacity, *M. coffeanum* (CML 4019 and CML 4018) and *Muscodor* sp. (CML 4013) showed the highest ORAC (154.3 µmolET/g, 150.3 µmolET/g and 149.9 µmolET/g, respectively).

The largest total antioxidant capacity was obtained by *M. coffeanum* (CML 4012) and *Muscodor* sp. (CML 4013), 46.6 mgEAA/G and 44.5 mgEAA/G, respectively.

Table 1 – Oxygen radical absorption capacity (ORAC), free radical scavenging activity (DPPH), total antioxidant capacity (TAC) and total phenolic compounds (TPC) of *Muscodor* spp.

Treatment	**DPPH IC_{50} (µg/mL)**	**ORAC (µmolET/g)**	**TAC (mgEAA/g)**	**TPC (mgEAG/g)**
M.coffeanum (CML 4009)	685.0f	132.5c	11.2e	46.1c
M.coffeanum (CML 4010)	1177.0i	134.5c	7.9f	46.7c
M.coffeanum (CML 4011)	433.3c	131.4c	11.4e	45.7c
M.coffeanum (CML 4012)	416.0c	131.9c	46.6a	45.8c
Muscodor sp. (CML 4013)	334.3b	149.9a	44.5a	52.1a
M.coffeanum (CML 4014)	1088.7h	140.5b	26.4b	48.8b
Muscodor sp. (CML 4015)	657.0f	125.0e	22.1c	43.5e
M.coffeanum (CML 4016)	572.3e	122.1f	7.1f	42.4f
M.coffeanum (CML 4017)	599.7e	127.8d	7.9f	44.4d
M.coffeanum (CML 4018)	472.3d	150.3a	23.3c	52.3a
M.coffeanum (CML 4019)	276.7a	154.3a	26.6b	53.6a
M.coffeanum (CML 4020)	1020.0g	119.1h	18.2d	41.0h
CV (%)	2,63	1,41	2,71	1,42

Means followed by the same letters in the column do not differ according to the Scott-Knott test ($p<0.05$).

Table 2 – Phenolic compounds produced by *Muscodor* spp.

Treatment	Caffeic Acid	Chlorogenic Acid	Ferulic Acid	Gallic Acid	O-coumaric Acid	P-coumaric Acid	Syringic Acid	Catechin	Theobromine	Trigonelline	Vanillin	Total
M. coffeanum (CML 4009)	X		X			X				X		4
M. coffeanum (CML 4010)		X	X				X	X	X	X	X	7
M. coffeanum (CML 4011)	X		X		X	X				X		5
M. coffeanum (CML 4012)	X		X							X		3
*Muscodor*sp. (CML 4013)	X		X			X			X	X		5
M. coffeanum (CML 4014)	X	X	X	X				X	X	X		7
*Muscodor*sp. (CML 4015)	X		X	X		X				X		5
M. coffeanum (CML 4016)			X							X		2
M. coffeanum (CML 4017)	X											1
M. coffeanum (CML 4018)			X	X								2
M. coffeanum (CML 4019)				X					X	X		3
M. coffeanum (CML 4020)		X	X	X						X		4

Quantification of total phenolic compounds, oxygen radical absorption capacity (ORAC), free radical elimination activity (DPPH) and total antioxidant capacity (CAT) of coffee seedling from the Catuaí and Topázio cultivars inoculated with *Muscodor* spp. after 2 years at a concentration of 25 mg/mL are shown in Table 3.

Coffee plants from the Catuaí cultivar inoculated with *M. coffeanum* (CML 4019 and CML 4014) showed the highest levels of phenolic compounds, 1.71 mgEAG/g and 1.31 mgEAG/g, respectively. The plants inoculated with the other isolated presented levels below 0.85 mgEAG/g. For the Topázio cultivar, plant extracts from plants inoculated with *M. coffeanum* (CML 4019, 4018 and 4014) showed the highest levels of phenolic compounds, 0.91 mgEAG/g, 0.62 mgEAG/g and 0.61 mgEAG/g, respectively.

The results of the DPPH free radical scavenging activity showed that coffee plants from the Catuaí cultivar inoculated with *M. coffeanum* (CML 4019) presented an IC50 of 136.51 µg/mL, being significantly lower than plants inoculated with the other isolates of *Muscodor*, exhibiting greater scavenging capacity of the radical DPPH. The DPPH free radical scavenging activity for the coffee plants from the Topázio cultivar were not significant.

The oxygen radical absorption capacity showed that coffee plants from the Catuaí cultivar inoculated with *M. coffeanum* (CML 4020 and CML 4019) presented the highest oxygen radical absorption capacities (29.87 µmolET/g and 27.31 µmolET/g, respectively). Coffee plants from the Topázio cultivar inoculated with *M. coffeanum* (CML 411, 4018 and 4016) presented the highest oxygen radical absorption capacities (30.63 µmolET/g, 13.43 µmolET/g and 13.36 µmolET/g, respectively).

The total antioxidant capacity showed that coffee plants from the Catuaí cultivar was the one that presented the highest CAT value when compared to the other extracts analyzed (2.97 mgEAA/g). Coffee plants from the Topázio cultivar inoculated with *M. coffeanum* (CML 4014) and *Muscodor* sp. (CML 4013) presented 0.66 mgEAA/g and 0.64 mgEAA/g, respectively, representing the highest CAT values compared to plants inoculated with the other isolates of *Muscodor*.

Table 3 – Total phenolic compounds (TPC), oxygen radical absorption capacity (ORAC), free radical scavenging activity (DPPH) and total antioxidant capacity (TAC) of *C. arabica* Catuaí and Topázio cultivars inoculated with *Muscodor* spp. and non-inoculated plants.

Treatment	Catuaí TPC (mgEAG/g)	Topázio TPC (mgEAG/g)	Catuaí ORAC (µmolET/g)	Topázio ORAC (µmolET/g)	Catuaí TAC (mgEAA/g)	Topázio TAC (mgEAA/g)	Catuaí DPPH IC_{50} (µg/g)
Control	0.76d	0.45e	21.64c	7.88e	2.97a	0.32f	702.96d
M. coffeanum (CML 4009)	0.69e	0.55c	19.11d	10.01d	0.91h	0.45d	234.71a
M. coffeanum (CML 4010)	0.61f	0.44e	21.64c	8.84e	1.33f	0.56b	269.41b
M. coffeanum (CML 4011)	0.47h	0.48d	15.41e	30.63a	0.59i	0.57b	319.46b
M. coffeanum (CML 4012)	0.53g	0.55c	18.46d	11.21c	1.31f	0.43e	262.13b
Muscodor sp. (CML 4013)	0.36j	0.56c	18.43d	10.26d	0.41j	0.64a	401.16c
M. coffeanum (CML 4014)	1.31b	0.61b	21.91c	11.19c	1.69e	0.66a	186.06a
Muscodor sp. (CML 4015)	0.82c	0.33f	21.14c	8.75e	1.58e	0.46d	145.63a
M. coffeanum (CML 4016)	0.42i	0.57c	18.06d	13.36b	0.67i	0.45d	272.81b
M. coffeanum (CML 4017)	0.58f	0.46e	19.06d	9.61d	2.76b	0.59b	206.26a
M. coffeanum (CML 4018)	0.71e	0.62b	21.36c	13.43b	1.11g	0.58b	275.51b
M. coffeanum (CML 4019)	1.71a	0.91a	27.31b	10.17d	2.16c	0.53c	136.51a
M. coffeanum (CML 4020)	0.84c	0.33f	29.87a	11.37c	1.84d	0.42e	297.06b
CV (%)	2.08	2.99	7.07	4.99	5.47	3.31	17.55

Means followed by the same letters in the column do not differ according to the Scott-Knott test ($p<0.05$).

DISCUSSION

Muscodor coffeanum (CML 4018, CML 4019) and *Muscodor* sp. (CML 4013) produced highest concentrations of total phenolic compounds and presented antioxidant potential, with the highest oxygen radical absorption capacities (Table 1). Moreover, the extract of *M. coffeanum* (CML 4019) showed the greatest capacity for scavenging the DPPH radical. Therefore, we can infer that the antioxidant activity of these extracts may be linked to the higher concentration of total phenolic compounds present in them, demonstrating a direct relationship between the presence of such phenols and the antioxidant capacity of fungal extracts. Phenolic compounds play an important role in stabilizing lipid oxidation, thus being associated with antioxidant activity as demonstrated in the work by Huang *et al.* (2007) with

endophytic fungi from traditional Chinese medicinal plants, where the antioxidant activity of the fungal cultures analyzed is directly correlated with the presence of phenolic compounds.

When analyzing plants of the Catuaí cultivar inoculated with the endophytic fungus *M. coffeanum* (CML 4019), the same behavioral pattern as the fungal extract was observed, where the plant extract presents the highest concentration of total phenolic compounds, the greatest capacity for elimination of the DPPH radical and the second highest oxygen radical absorption capacity (Table 3).Therefore, we can also infer that the antioxidant activity of this extract may be linked to the higher concentration of total phenolic compounds present in it.

Wheat bran is described in the literature as an important antioxidant agent due to the presence of considerable amounts of phenolic compounds, such as caffeic, chlorogenic, ferulic, vanillic acids, among others (SOARES, 2002). Fernandes *et al.* (2009) determined the content of total phenolic compounds in wheat bran and crude extract of *Alternaria alternata*, isolated from *C. arabica* (13.47 μgEAG/mg and 16.90 μgEAG/mg, respectively). These values are considerably lower when compared to our results, considering that the phenolic compounds present in wheat bran are also produced by the endophytic fungi studied here (Table 2).

Endophytic fungi of the genus *Muscodor* produce phenolic compounds such as trigonelline and p-coumaric, ferulic, chlorogenic, and caffeic acids (Table 2).The presence of these compounds in the fungal extracts evaluated can be explained by the fact that these fungi were isolated from coffee plants, since studies confirm the correlation between potentially medicinal secondary compounds produced by endophytic fungi and their host plants (STIERLE *et al.*, 1993; KRINGS *et al.*, 2007; KAPOOR; SAXENA, 2016; TIWARI; BAE, 2020). These phenols are linked to the quality of the coffee, providing aroma and flavor to the drink. However, phenolic compounds in high concentration are related to the defense of coffee plants, inhibiting insects and pests, such as coffee rust (*Hemileia vastatrix*) and associated with the low tasting quality of the drink (CLIFFORD, 1985; CLIFFORD, 1987; ALVES *et al.*, 2006; FARAH; DONANGELO, 2006; KITZBERGER *et al.*, 2014; RIBEIRO *et al.*, 2016).

Muscodor coffeanum and *Muscodor* sp. synthesize trigonelline, being present in 10 of the fungal extracts analyzed (Table 2). Trigonelline has stood out for explaining the quality of the coffee drink (RIBEIRO *et al.*, 2018). It also has antibacterial biological activity and neuroprotective and hypoglycemic medicinal properties (LAUKALEJA; KRUMA, 2018; CHOWDHURY *et al.*, 2018; QIU *et al.*, 2020). Guimarães *et al.* (2021) showed that *M.*

coffeanum (CML 4019), one of the trigonelline producers, has efficient antibacterial activity against *Staphylococcus aureus, Enterococcus faecalis* and *E. faecium*.

Muscodor coffeanum (CML 4014) produces chlorogenic, caffeic and ferulic acids, and it also showed promising results regarding oxygen radical absorption and total antioxidant capacities (Table 1), suggesting that its antioxidant potential may be linked to the production of these phenols. Chlorogenic acid plays an important role in the adaptation of plants to stress, indicating that this secondary compound may have specific physiological functions in the coffee plant, linked to the control of germination and cell growth, through the regulation of indole-3-acetic acid levels (CLIFFORD, 1985; CLIFFORD, 1987; FARAH; DONANGELO, 2006). The chlorogenic acid of coffee was able to reduce the oxidative stress of diseased cells and it eliminated damage to the DNA of breast cancer cells due to its antioxidant activity (ALVES; CASAL; OLIVEIRA, 2009; NUHU, 2014; DURÁN et al., 2017; LAUKALEJA; KRUMA, 2018). Caffeic and ferulic acids have shown a preventive effect in dermatological treatments due to their photoprotective and antioxidant activities, and acts against the growth of colonandrectal cancer (MAGNANI *et al.*, 2014).

It was also observed that *M. coffeanum* (CML 4019) stood out among the others based on its antioxidant potential (Table 1). However, Kapoor and Saxena (2016) showed that this result is still high when compared to fungi extract in India, with 133.3 μg/mLbeingthehighest IC_{50} found by the authors. The plant extract from the Catuaí cultivar inoculated with the endophytic fungus *M. coffeanum* (CML 4019) also stood out in relation to its antioxidant capacity to eliminate DPPH free radicals (Table 3). Thus, we can infer that the presence of the endophytic fungus inoculated in the coffee plant may have contributed to the plant's greater antioxidant capacity to eliminate DPPH free radicals.

The oxygen radical inhibitory capacity of endophytic fungi is probably associated with their life history strategy to overcome the metabolically aggressive environment of the host plant (KAPOOR; SAXENA, 2016). Mena, Ramírez and Arango (2021) studying the fungus *Phanero chaete* sp. found a value of 19 μmolET/g for the evaluated fungal extract in ethyl acetate. In addition, the extract of the endophytic fungus *Cladosporium* sp. isolated from leaves of *Annona cacans* Warm. presented a ORAC result of 856 μmolET/g (HERNÁNDEZ-TASCO *et al.*, 2022). The fungus *Phanero chaete* sp. did not maintainan endophytic-plant relationship and presented a very low oxygen radical inhibitory capacity, when compared to the results obtained from several endophytic fungi evaluated (HERNÁNDEZ-TASCO *et al.*, 2022). Comparing our results with those found by Hernández-Tasco *et al.* (2022) for the endophytic fungus *Cladosporium* sp., we observed that the oxygen radical inhibitory capacity

value for this fungus was higher, thus suggesting that the metabolic conditions of *A. cacans* more aggressive for the survival of its plant hosts than those found in *C. arabica*.

According to Kapoor and Saxena (2016), oxygen radical inhibitory capacity related to the antioxidant activity of fungi, which is linked to overcoming oxidative stress, based on the defense mechanism of plants. Our results showed such a relationship, since *M. coffeanum* (CML 4019) stood out for its greater ability to scavenge the DPPH radical and *Muscodor* sp. (CML 4013) showed considerably higher total antioxidant capacity when compared to the other fungi analyzed, which may explain why they also had the highest oxygen radical inhibitory capacities (Table 1).

Muscodor sp. (CML 4013) and *M. coffeanum* (CML 4019) presented one of the highest values of total antioxidant capacity, and they also presented the highest capacities for absorbing the oxygen radical and the highest levels of total phenolic compounds (Table 1), indicating that its antioxidant potential is directly related to the presence of phenolic compounds. This relationship was also found by several authors, when they observed that the antioxidant activity of the studied endophytic fungi was directly correlated with the presence of phenolic compounds (CAKIR *et al.*, 2003; PROESTOS *et al.*, 2006; HUANG *et al.*, 2007; LIU *et al.*, 2007; KANDASAMY; KANDASAMY, 2014; TAVARES *et al.*, 2018). This behavior was also observed in the plant extract of the Catuaí cultivar inoculated with *M. coffeanum* (CML 4019), showing a direct relationship between the presence of the endophytic fungus inoculated in the coffee seedlings and the best antioxidant responses of this plant.

Plant extracts from the Topázio cultivar inoculated with *Muscodor* sp.(CML 4013) also presented highest values of total antioxidant capacity. Coffee plant extracts inoculated with *M. coffeanum* (CML 4014, 4019 and 4018) and the extracts from the same fungi presented the highest levels of total phenolic compounds and the second highest oxygen radical absorption capacity, respectively (Table 4 and1).Therefore, the presence of endophytic fungi inoculated in coffee plants may have contributed to the better antioxidant responses of the plants evaluated.

CONCLUSION

Total phenolic compounds and production of some phenols varied between species and between different isolates of the same species. *Muscodor coffeanum* (CML 4019) showed the highest production of total phenolic compounds. The most common phenolic compounds in the genus were trigonelline and ferulic acid, synthesized by most of *Muscodor* spp. Some of the phenols produced by these fungi are also present in the host plant, being important for

the quality of the coffee, providing aroma and flavor to the drink. *Coffea arabica* inoculated with *M. coffeanum* (CML 4019) showed the greater production of phenolic compounds, indicating that the interaction fungus/seedling has a positive effect contributing to better antioxidant responses.

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7. CONSIDERAÇÕES FINAIS

A interação entre *Muscodor* e *C. arabica* mostrou-se promissora para a promoção de crescimento, melhores respostas de defesa antioxidante e aumento da resistência do café contra o bicho-mineiro. Essa interação apresentou-se como uma associação específica, uma vez que demonstrou respostas variadas de acordo com as espécies e isolados diferentes dos fungos do gênero *Muscodor*, e as cultivares Catuaí Vermelho e Topázio.