ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC FUNGI FROM COFFEE PLANTS

ATIVIDADE ANTIMICROBIANA DE FUNGOS ENDOFÍTICOS DE PLANTAS DE CAFÉ

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

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

INTRODUCTION

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

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

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

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control of *A. ochraceus* in coffee beans and *F. verticillioides* in corn seeds.

MATERIAL AND METHODS

Sample collection and isolation

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

Screening of VOC producing isolates

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

Molecular identification

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

Bioassay for volatile antimicrobials

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

Biofumigation with endophytic fungi from coffee

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

RESULTS AND DISCUSSION

Endophytic fungi from *Coffea arabica*

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

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

Isolate	Origin	Accession no.
M. c*offeanum* (COAD 1842)	Leaf	KM514680
M.coffeanum (COAD 1899)	Leaf	KM514681
M.coffeanum (COAD 1900)	Leaf	KP862879
M. vitigenus (C20)	Stem	KU094049
M. vitigenus (HZM10)	Stem	KU094053
M. vitigenus (HZM39)	Stem	KU094054 KU094055
M. vitigenus (HZM41)	Stem	KU094055
M. yucatanensis (HZM60)	Leaf	KU094056
M. yucatanensis (HZM64)	Leaf	KU094052
Simplicillium sp. (C18)	Stem	KU094050
Simplicillium sp. (C12)	Stem	KU094051

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

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

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

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

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

Biological activity of the VOC's produced by endophytic fungi

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

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

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

Strobel et al., (2001) also found similar results, among several tested fungi the phytopathogenic fungi *Fusarium solani* was more resistant to the VOCs produced by *M. albus*. In addition, the artificial mixtures of VOCs produced by *Gliocladium* sp. partially inhibited *F. oxysporum* (STINSON et al., 2003).

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

Activity of VOCs against seed pathogens

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

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

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

Fusarium verticillioides is one of the most commonly reported soil-borne fungal pathogens infecting maize (*Zea mays* L.), one of the most important cereal grains grown worldwide. This fungus produces secondary metabolites such as fumonisins (FB), especially fumonisin B1 (FB1), which affects human and animal health (BACON et al., 1996). Since *F. verticillioides* is endophytic in maize and is almost universally associated with maize and maize products, it is very important to control this species in this agriculturally important commodity. Furthermore, root colonization by *F. verticillioides* has been considered the initiator of systemic infection that eventually results in the fungus producing fumonisins in kernels. Seed treatment with biocontrol agents is an appropriate method for biocontrol of soil-borne plant pathogens in the spermosphere and rhizosphere (KERRY, 2000).

Table 2. Action of volatile organic compounds produced by endophytic fungi on growth inhibition of phytopathogenic fungi T-Total inhibition (0); P-Partial inhibition (1-2.0 cm); N- No inhibition (≥2 cm).

Endophytic fungi	**Phytopathogenic fungi**								
	B. cinerea	***A. ochraceus***	***F. solani***	***R. solani***	***F. verticillioides***	***C. coffeicola***	***F. oxysporum***	***Phoma* sp.**	***P. longisetula***
M. coffeanum (COAD 1842)	T	P	N	T	T	T	N	T	P
M. coffeanum (COAD 1899)	T	N	P	T	T	P	N	T	T
M. coffeanum (COAD 1900)	T	N	N	T	T	P	P	P	P
M. vitigenus (C20)	P	N	N	T	T	T	N	T	T
M.vitigenus (HZM10)	P	N	N	T	N	T	P	T	T
M. vitigenus (HZM39)	P	N	N	T	N	T	N	T	T
M.vitigenus (HZM41)	P	T	N	P	N	T	P	T	P
M.yucatanensis (HZM60)	T	N	P	T	P	T	P	P	P
M. yucatanensis (HZM64)	T	P	N	T	P	T	N	T	P
Simplicillium sp. (C12)	P	N	P	T	P	T	N	T	P
Simplicillium sp. (C18)	P	N -n	N	P	N	T	N	P	P

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

Endophytic fungi	*A. ochraceus* in Coffee in the dried bean	*A. ochraceus* in Hulled coffee	*F. verticillioides* in corn seed
M. coffeanum (COAD 1842)	+	+	+
M. coffeanum (COAD 1899)	±	±	+
M. coffeanum (COAD 1900)	±	±	+
M. vitigenus (C20)	-	-	+
M.vitigenus (HZM10)	-	-	±
M. vitigenus (HZM39)	-	-	±
M. vitigenus (HZM41)	-	-	±
M. yucatanensis (HZM60)	-	-	±
M. yucatanensis (HZM64)	-	-	±
Simplicillium sp. (C12)	-	-	±
Simplicillium sp. (C18)	±	±	+

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

CONCLUSIONS

Volatile compound producing endophytic fungi were isolated from *C. arabica*, among the 400 fungi, 12 isolates were able to grow in the presence of VOCs produced by *M. albus*.

The VOC producing fungi belong the genus *Muscodor* (9) *Simplicillium* (2) and *Acremonium* (1). The volatile compounds produced by *M. coffeanum* (COAD 1842) showed fungicidal activity against *A. ochraceus* and six isolates inhibited the growth of *F. verticillioides*.

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

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

PALAVRAS-CHAVE: *Aspergillus ochraceus*. *Fusarium verticillioides*. Inibição. *Muscodor* spp. Compostos voláteis.

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