ANDRÉ ANGELO MEDEIROS GOMES

PROSPECTING OF ENDOPHYTIC FUNGI PRODUCING VOLATILE ORGANIC COMPOUNDS: TAXONOMY, IDENTIFICATION OF VOLATILES AND POTENTIAL USES FOR THE BIOLOGICAL CONTROL OF POSTHARVEST DISEASES

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 17 de fevereiro de 2017.

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Dedico à minha família!

"Há um tempo em que é preciso abandonar as roupas usadas, que já tem a forma do nosso corpo, e esquecer os nossos caminhos, que nos levam sempre aos mesmos lugares. É o tempo da travessia: e, se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos."

(Fernando Pessoa)

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BIOGRAFIA

André Angelo Medeiros Gomes, filho de Nascimento Gomes Pereira e Laura Clarinda de Medeiros Gomes, nasceu na cidade de Janaúba – MG, em 28 de março de 1987.

No ano de 2005 iniciou o curso de graduação em Agronomia pela Universidade Estadual de Montes Claros, obtendo o título de Engenheiro Agrônomo no ano de 2010.

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CAPÍTULO IV

Controle de antracnose e do mofo cinzento do morango, em pós colheita, através da micofumigação com *Muscodor coffeanum* CDA 739. Estratégia de micofumigação para controle de doenças pós colheita.

RESUMO

GOMES, André Angelo Medeiros, D.Sc., Universidade Federal de Viçosa, fevereiro de 2017. **Prospecção de fungos endofíticos produtores de compostos orgânicos voláteis: taxonomia, identificação dos voláteis, e potencial uso para o controle biológico de doenças pós-colheita.** Orientador: Olinto Liparini Pereira. Coorientadores: Marisa Vieira de Queiroz e Ueder Pedro Lopes.

Na busca por estratégias alternativas de controle de doenças pós colheita em frutas e hortaliças que visem a diminuição da utilização de fungicidas, foi realizado uma prospecção de fungos endofíticos capazes de emitirem compostos voláteis com propriedades antimicrobianas. Com a técnica de isolamento em cultivo paralelo, direcionada para isolamento de fungos produtores de voláteis antimicrobianos, foram obtidos isolados endofíticos de plantas de café e carqueja, que em ensaio preliminar inibiram o crescimento de *Aspergillus ochraceus* através da emissão de compostos voláteis. Ao esclarecer a identidade dos isolados endofíticos obtidos através de estudos comparativos de estruturas morfológicas, BLAST de sequências génicas depositadas no GenBank, e análises filogenéticas, constatou-se a presença de pelo menos três espécies distintas de *Muscodor,* e um novo táxon pertencente ao gênero *Simplicillium.* Isolados de *M. yucatanensis*, *Muscodor* sp. e *M. coffeanum* foram obtidos a partir de ramos de café, esse último foi também isolado de folhas de carqueja. A atividade antifúngica desses isolados foi demonstrada contra espécies de *Aspergillus* frequentemente associadas aos grãos de café, através da micofumigação *in vitro*. Adicionalmente, micofumigação com o isolado de *M. coffeanum* CDA 741 inibiu o crescimento de *A. ochraceus* inoculado em grãos de café. Os compostos presentes na mistura de voláteis emitida por cada isolado foi identificado por micro extração em fase sólida e cromatografia gasosa acoplada à espectrometria de massas. Uma nova espécie de *Simplicillium* endofítica de plantas de café (representada pela isolado CDA 734), será proposta com base em estudos comparativos de sua morfologia aliado às análises filogenéticas. *Simplicillium* sp. CDA

734 inibiu, *in vitro,* o crescimento de *A. ochraceus*, *A. tubingensis*, *A. sydowii* e *A. niger* através da micofumigação *in vitro*. Entre os compostos identificados na mistura de voláteis emitida por *Simplicillium* sp. CDA 734*,* 1-Propanone, 1-(5-methyl-2-furanyl)-, Cyclopropane, 1-ethoxy-2,2-dimethyl-3-(2-phenylethynyl)-, e 2-Propenoic acid, 3-(2-formyl-4-methoxyphenyl)-, ethyl ester, (E)- foram aqueles com maior porcentagem de pico por área. Em micofumigação de morangos orgânicos inoculados com *Colletotrichum acutatum* e *Botrytis cinerea*, *M. coffeanum* CDA 739 diminui 100 e 81% a incidência de antracnose e mofo cinzento nos frutos, respectivamente. Micofumigação com sachê contento grãos de centeio colonizados por *M. coffeanum* CDA 739 diminuiu significativamente a incidência de antracnose em frutos inoculados com *C. acutatum,* viabilizando a sua utilização em larga escala para controle de doenças pós colheita em morango através da micofumigação.

ABSTRACT

GOMES, André Angelo Medeiros, D.Sc., Universidade Federal de Viçosa, February, 2017. **Prospecting of endophytic fungi producing volatile organic compounds: taxonomy, identification of volatiles and potential uses for the biological control of postharvest diseases.** Adviser: Olinto Liparini Pereira. Co-advisers: Marisa Vieira de Queiroz and Ueder Pedro Lopes.

In the search for alternative strategies to control of postharvest diseases in fruits and vegetables aimed at reducing the use of fungicides, was conducted a prospection to endophytic fungi capable of emitting volatile compounds with antimicrobial properties. Through parallel growth technique, directed towards the isolation of fungi producing volatile antimicrobials, endophytic isolates of coffee and carqueja plants were obtained and in preliminary test inhibited the growth of *Aspergillus ochraceus* by the emission of volatile compounds. The presence of at least three distinct species of *Muscodor* was verified, and a new taxon of *Simplicillium* by clarifying the identity of the endophytic isolates obtained through comparative studies of morphological structures, BLAST of gene sequences and phylogenetic analyzes. *M. yucatanensis*, *Muscodor* sp. and *M. coffeanum* isolates were obtained from coffee branches, the latter also isolated in leaves of carqueja. The antimicrobial activity of these isolates was demonstrated against *Aspergillus* species frequently associated with coffee beans by *in vitro* mycofumigation. In addition, mycofumigation with *M. coffeanum* CDA 741 inhibited the growth of *A. ochraceus* inoculated in coffee beans. The compounds present in the volatile mixture emitted by each isolate were identified by solid-phase micro-extraction gas chromatography and mass spectroscopy. *Simplicillium* sp. CDA 734, a new species of endophytic Cordycipitaceae from coffee plants, will be proposed through comparative studies of its morphology combined with phylogenetic analyzes. *Simplicillium* sp. CDA 734 inhibited the growth of *A. ochraceus*, *A. tubingensis*, *A. sydowii* and *A. niger* by mycofumigation. Among the compounds identified in the volatile mixture emitted by

Simplicillium sp. CDA 734, 1-Propanone, 1-(5-methyl-2-furanyl)-, Cyclopropane, 1-ethoxy-2,2-dimethyl-3-(2-phenylethynyl)-, and 2-Propenoic acid, 3-(2-formyl-4-methoxyphenyl)-, ethyl ester, (E)- were those with the highest percentage of peak per area. In mycofumigation of organic strawberries inoculated with *Colletotrichum acutatum* e *Botrytis cinerea, M. coffeanum* CDA 739 decreases 100 and 81% the incidence of anthracnose and gray mold in the fruits, respectively. Mycofumigation through sachets containing rye grains colonized by *M. coffeanum* CDA 739 significantly reduced the incidence of anthracnose in fruits inoculated with *C. acutatum,* making possible its use in large-scale to control postharvest strawberry diseases through mycofumigation.

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INTRODUÇÃO GERAL

A demanda mundial por frutos e hortaliças vem crescendo expressivamente nos últimos anos, em virtude, principalmente, da conscientização da população acerca da importância de uma alimentação saudável e do reconhecimento de sua participação na prevenção de várias enfermidades, pois esses produtos são ricos em vitaminas essenciais, minerais, fibras e compostos que promovem a saúde humana, fornecendo proteção contra diversas doenças (Yahia, 2010; Key, 2011).

Apesar da diversidade e certa disponibilidade de produtos hortícolas no mercado, seu tempo de prateleira é limitado, principalmente por serem altamente perecíveis, e geralmente manuseados sob condições ambientais que aceleram sua deteriorização. Pelo fato de serem, em geral, tenros e suculentos, constituindo substratos ricos e adequados ao desenvolvimento microbiano, os produtos hortícolas estão sujeitos a danos físicos e fisiológicos e, consequentemente, às infecções pós-colheita (Benato e Cia, 2009).

As mais sérias causas de perdas pós-colheita em produtos hortícolas são, provavelmente, as infecções por micro-organismos, favorecidas por danos físicos e fisiológicos, que predispõem o produto à invasão de fitopatógenos. Os fungos estão mais frequentemente envolvidos com as podridões de frutos e hortaliças e destacam-se como importantes agentes de doenças pós-colheita, sendo o grupo de micro-organismos com maior frequência e atividade, responsável por 80 a 90% do total de perdas causadas por agentes microbianos (Prusky, 2011).

Atualmente, o controle de doenças em pós-colheita tem sido feito de maneira indiscriminada, basicamente pela aplicação de agrotóxicos, os quais, em muitos casos, sequer são produtos registrados para a cultura, representando, com isso, um sério risco à população. A utilização intensiva de agrotóxicos tem reconhecidamente promovido diversos problemas de ordem ambiental, como a contaminação dos alimentos, do solo, da água, dos animais, e promovendo desequilíbrios biológico (Schirra et al., 2011). Quando se trata de sua utilização em pós-colheita, a situação é ainda mais preocupante, devido ao índice elevado de resíduos que permanecem nos produtos hortícolas disponíveis ao consumidor (Anvisa, 2013).

Nos últimos 20 anos tem crescido significativamente as pesquisas envolvendo o controle alternativo de doenças de plantas, com especial ênfase no controle biológico. Entretanto pouca ênfase tem sido dada ao controle biológico de doenças pós-colheita, na qual a micofumigação destaca-se como promissora alternativa na redução de perdas pós-colheita causada por fitopatógenos. O conceito de micofumigação vem sendo estabelecido após a descrição de *Muscodor albus* Worapong, Strobel e W.M. Hes, *Ascomycota* que se destaca na produção de compostos voláteis antimicrobianos de amplo espectro contra patógenos humanos e de plantas (Worapong et al., 2001). Após sua descoberta, o processo de micofumigação, através do uso de *Muscodor albus*, foi patenteado para uso agrícola (US Patent N° 2004/0141,955 - Compositions related to a novel endophytic fungi and methods of use) e ambiental (US Patent N° 7,341,862 - Application of *Muscodor albus* to control harmful microbes in human and animal wastes) pelo seu descobridor, Prof. Gary Strobel da Montana State University. Posteriormente à descoberta de *M. albus*, outras espécies de *Muscodor* foram isoladas e descritas especialmente de plantas tropicais, apresentando também emissão de voláteis

potencialmente antimicrobianos (Daisy et al., 2002; Mitchel et al., 2002; González et al., 2009; Chu-Long et al., 2010; Kudalkar et al., 2012).

O objetivo desse estudo foi realizar uma prospecção pioneira de fungos endofíticos em quatro espécies de plantas no Parque Estadual da Serra do Brigadeiro – MG, que a exemplo do gênero *Muscodor*, possuam capacidade de produção de voláteis antimicrobianos, para uso no controle biológico de doenças pós-colheita. O Estudo enfatiza o esclarecimento da identidade de potenciais fungos isolados; a identificação dos compostos presentes na mistura de voláteis; a análise do potencial de micofumigação frente a outros fungos fitopatógenos; e teste da eficiência da micofumigação no controle de doenças pós colheita.

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CAPÍTULO I

Mycofumigation for controlling post-harvest diseases in fruits and vegetables

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Abstract

There are several causes of post-harvest losses in fruits and vegetables, and microbial infections are responsible for the greatest losses that occur during the transport, storage, and sale of these products. Chemical control is the most used method to combat post-harvest diseases in fruits and vegetables by directly applying synthetic fungicides to the product to be consumed. However, the indiscriminate use of fungicides may be associated with serious toxicity problems in humans and environmental imbalance. Mycofumigation, which is the use of volatile antimicrobial organic compounds produced by fungi to inhibit microbial growth, has become a promising alternative for controlling phytopathogenic fungi associated with post-harvest diseases in fruits and vegetables. The technique has some advantages relative to traditional disease control methods, for example, it does not require direct contact between the antagonist and the plant product, the antimicrobial volatiles diffuse easily in closed environments, they do not leave residues on the plant product to be consumed, and most of the antimicrobial volatile mixtures exhibit bioactivity against a wide range of microorganisms, including many phytopathogens associated with post-harvest diseases. This review highlights mycofumigation as a method for controlling post-harvest diseases in fruits and vegetables, emphasizing the effects of volatile compounds on phytopathogenic fungi and their potential to be applied during the transport and storage of fresh fruits and vegetables.

Keyword: biofumigation, *Muscodor*, antimicrobial volatiles

Abbreviations: VOCs - volatile organic compounds

Introduction

As fruits and vegetables are usually tender and juicy, they can become rich and adequate substrates for microbial growth and, consequently, post-harvest infections. These infections are usually responsible for the greatest post-harvest losses observed in horticultural products. For example, in citrus fruit, the *Penicillium digitatum* (Pers.) Sacc. fungus is responsible for more than 90% of post-harvest production losses [1].

Physical and physiological damage favors microbial infections, and fruits' and vegetables' natural resistance to disease decreases with maturation, favoring phytopathogen invasion. These phytopathogens require an entry site to start an infection and may become a serious problem in products stored for long periods of time [2].

Post-harvest decay during the supply chain has been identified as the greatest cause of post-harvest losses in fruits and vegetables, which results in significant economic losses [3]. It is estimated that approximately 20-25% of the fruits and vegetables harvested in developed countries are lost due to action/attack by phytopathogenic microorganisms during post-harvest handling. In developing countries, post-harvest losses are usually higher, especially due to inadequate storage methods and transport difficulties [4].

Fungi are often involved in the decay of fruits and vegetables. This microbial group stands out as important post-harvest disease-causing agents with the highest frequency and activity, and they are responsible for 80 to 90% of the total losses caused by microbial agents (Figure 1). Many fungal species within the most varied genera have been reported to be associated with post-harvest diseases in fruits and vegetables worldwide: *Penicillium* Link, *Aspergillus* P. Micheli, *Geotrichum* Link, *Botrytis* P. Micheli, *Fusarium* Link, *Alternaria* Nees, *Colletotrichum*, *Dothiorella* Sacc, *Lasiodiplodia* Ellis & Everh, *Phomopsis* Sacc. & Roum, *Cladosporium* Link, *Phytophthora* De Bary, *Pythium* Nees, *Rhizopus* Ehrenb, *Mucor* P. Micheli ex L., *Sclerotium* Tode, *Rhizoctonia* D.C. [5,6,7,8,9,10,11,12].

In addition to their potential to cause rot, some fungi that are associated with fruits and vegetables have high potential for mycotoxin production. These secondary metabolites exhibit bioactivity associated with toxic effects in humans, animals, and plants [13]. Several toxins produced by *Aspergillus*, *Penicillium*, and *Fusarium* species and their toxic effects on humans have been reported [14,15].

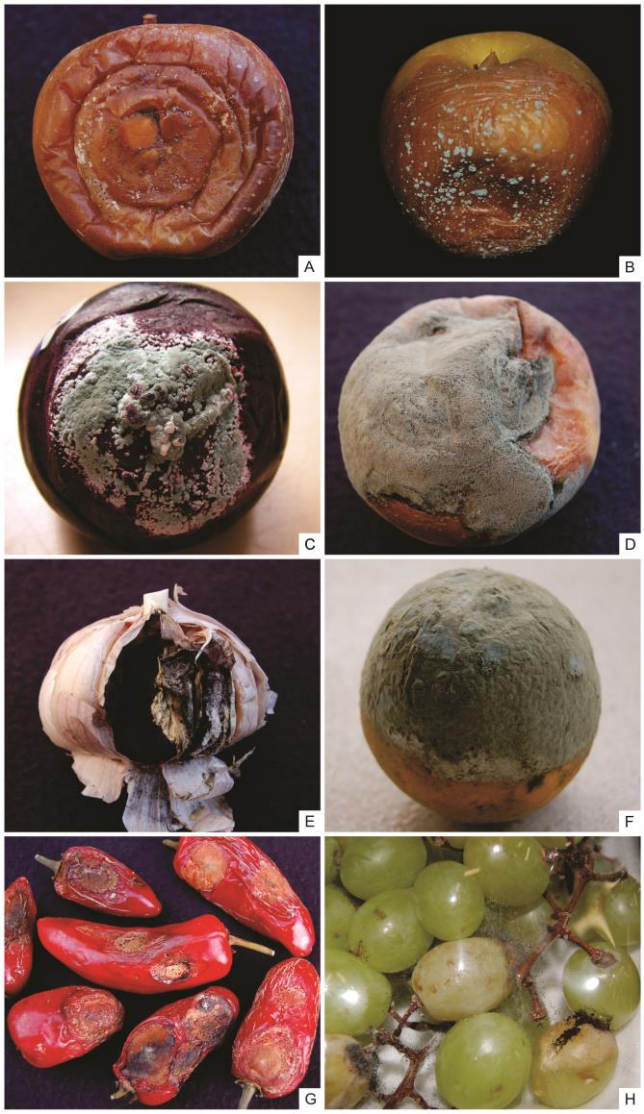

Figure 1. Post-harvest diseases of fruits and vegetables. Bitter Rot (A) and blue mold (B), postharvest decay of apple caused by the fungus *Colletotrichum* spp. and *Penicillium expansum* respectively; C - Decay of nectarine fruit caused by *P. expansum*; D - Brown Rot of peach caused by *Monilinia fructicola*; E - Black Mold caused by *Aspergillus niger* on garlic; F - Green mold caused by *P. digitatum* on citrus fruits; G - Anthracnose of pepper fruit caused by *Colletotrichum* sp.; H - Decay of table grapes caused by *Rhizopus* sp. and *Aspergillus* sp.

Practices have been adopted to reduce the incidence of fungi and consequent damage and losses caused by post-harvest diseases in fruits and vegetables, including manipulation of the storage environment and resistance induction. However, the main method used to combat post-harvest diseases in fruits and vegetables is by applying fungicides via spraying or even by immersing the horticultural products in fungicide solution [12,16].

Studies have indicated the efficiency of several fungicides with different active ingredients in combating post-harvest decay in fruits and vegetables. Solutions of borax,

sodium bicarbonate, and more recently synthetic fungicides such as sodium ortho-phenylphenate, imazalil, and thiabendazole are often used for controlling post-harvest decay in fruits and vegetables by immersing the fruit in fungicide solution [17,18]. One classic example is the use of 2,6-dichloro-4-nitroaniline to control post-harvest decay in peaches, plums, and nectarines [19]. Another very widespread technique involves using benzimidazoles to control post-harvest decay in cherries by application before and after fruit harvest [20].

Although the use of pesticides such as fungicides has positive aspects, the vast majority of products applied are extremely toxic, endangering human health and environmental balance. Several studies have demonstrated the presence and persistence of fungicide residues in fruits and vegetables [21, 22, 23]. The application of fungicides together with high temperatures for controlling post-harvest diseases led to increased 2,6-dichloro-4-nitroaniline residue levels in plum and nectarine and increased sodium o-phenylphenate residue levels in citrus fruit [24]. Imazil residue was also detected in citrus fruit after being applied post-harvest, and the residue level was associated with treatment method, where dip-treated fruit exhibited higher quantities of residue than fruit treated with the same fungicide and at the same concentration but by spraying [25].

Intensive pesticide use for disease control has admittedly caused several environmentally related problems, such as contamination of food, soil, water, and animals; toxicity to farmers; resistance of pathogens to certain active ingredients in the pesticides; development of iatrogenic diseases (occurring due to pesticide use); biological imbalance, altering nutrient and organic matter cycling; elimination of beneficial organisms; and reduction of biodiversity, among others [24].

The identification of these problems has increased the demands for residue-free products, making it necessary to search for disease control/management techniques in fruits and vegetables that do not endanger consumers and to reduce the risk of toxicity to farmers and the environmental imbalance generated by using synthetic fungicides.

Mycofumigation for controlling post-harvest diseases

Studies involving alternative control of plant diseases have increased significantly over the last 20 years, particularly emphasizing biological control as a promising alternative for reducing synthetic fungicide use. The potential of several microorganisms

for controlling different disease-causing pathogens in fruits and vegetables has been reported [26, 27, 28, 29].

However, the development of commercial products intended for the biocontrol of post-harvest diseases has been limited, most likely due to the long time period necessary to identify, develop, and market the products, in addition to the process's high financial cost. Several features characterize a microorganism as an antagonist with potential for the development of commercial products, such as: genetic stability; effective at low concentrations; simple nutritional requirement; capacity to survive under adverse environmental conditions; effective against a wide range of phytopathogens in different products; resistant to the chemical products used in the post-harvest environment; compatible with commercial processing procedures; and lack of risk to human health [27].

The vast majority of the studies related to post-harvest biological control involve the use of fungi or bacteria as microbiological control agents. However, the positive effect on disease control/management is often only observed when the biological agent is directly applied to the fruits or vegetables. This effect may occur mainly due to the main antimicrobial action mechanisms triggered by antagonistic microorganisms, namely competition for space and nutrients, and antibiosis [4,29].

However, some questions have been raised regarding the introduction of antagonists to the human diet and concerns for human health and food security [29]. In addition, the fact that most registered biocontrol products, such as Biosave (*Pseudomonas syringae* Van Hall), Shemer (*Metschnikowia fructicola* Kurtzman & Droby), BioNext, Aspire™, Leasaffre International (*Candida oleophila* Kaisha & Iizuka), and Yield Plus (*Cryptococcus albidus* (Saito) C.E. Skinner), have similar application methods that involve directly applying a cell suspension to horticultural products can generate fear in the population regarding their consumption.

Mycofumigation is a different biological control strategy for post-harvest diseases in fruits and vegetables that can be an effective alternative to directly applying microorganisms to horticultural products. This strategy consists of the use of antimicrobial volatile organic compounds (VOCs) produced by fungi.

The concept of mycofumigation started developing with the description of *Muscodor albus* Worapong, Strobel & W.M. Hes, an endophytic fungus obtained from *Cinnamomum zeylanicum* Breyne, and its potential for emitting volatile compounds that inhibit the growth and/or promote the death of many plant pathogenic agents [30, 31].

A peculiarity of antimicrobial VOCs is that they can diffuse in the air, reaching difficult-to-access habitats in closed environments [32]. This property makes antimicrobial VOCs emitted by fungi an additional valuable strategy for post-harvest disease biocontrol. For example, without any direct contact between isolates, the *M. albus* volatiles inhibited growth of a wide range of fungal species, including *Aspergillus fumigatus* Fresen, *A. carbonarius* (Bainier) Thom, *A. flavus* Link, *A. niger* Tiegh, *A. ochraceus* Wilh, *Penicillium verrucosum* Dierckx, *P. digitatum* (Pers.) Sacc. *Fusarium culmorum* (Wm. G. Sm.) Sacc. *F. graminearum* Schwabe, *Botrytis cinerea* Pers, *Colletotrichum acutatum* J.H. Simmonds, *Geotrichum candidum* Link, *Monilinia fructicola* (G. Winter) Honey, and *Rhizopus* sp., important fungal species associated with post-harvest decay and mycotoxin production [31, 33, 33, 35].

Diversity of antimicrobial volatile organic compound-producing fungi

After the discovery of *M. albus*, many antimicrobial VOC-producing fungal species were identified (Table 1). The vast majority of these species were isolated from healthy plant tissue, especially from tropical plants commonly used in alternative medicine, such as *Ananas ananassoides* (Baker) L. B. Sm.), *Aegle marmelos* (L.) Corr., *Cinnamomum* spp. and *Myroxylon balsamum* (L.) Harms. [30, 36, 37, 38, 39].

Hitherto, most filamentous fungi related to antimicrobial volatile emission have belonged to phylum Ascomycota, order Xylariales, and other related ascomycetes are found in the classes Sordariomycetes, Dothideomycetes, and Leotiomycete, all of which are endophytic (Tab 1). In a more phylogenetically distant group, the basidiomycetes *Oxyporus latemarginatus* (Durieu & Mont.) Donk and *Schizophyllum commune* Fr. are also related to antimicrobial volatile production, and *S. commune* is noteworthy because, unlike the others, it was isolated from decomposing material, exhibiting a saprophytic lifestyle in nature.

In addition to filamentous fungi, some yeasts have the potential for emitting the VOCs described. *Aureobasidium pullulans* (de Bary & Löwenthal) G. Arnaud, *Saccharomyces cerevisiae* Meyen ex E.C. Hansen, *Candida intermedia* (Cif. & Ashford) Langeron & Guerra, *Wickerhamomyces anomalus* (E.C. Hansen) Kurtzman, Robnett & Bas.-Powers, and *Metschnikowia pulcherrima* Pitt & M.W. Mill. were reported emitting volatile compound mixtures that inhibit the growth of fungi associated with post-harvest decay in fruits and vegetables [40, 41, 42].

The identification of fungi associated with antimicrobial VOC production has been conducted through morphology studies and mainly by molecular analyses of the internal transcribed spacer (ITS) region sequences of their DNA. For species of the *Muscodor* genus, identification and even the proposal of new species have been performed via phylogeny based on ITS region sequencing, accompanied by the volatile compound production profile, as specialized structures in sexual and asexual reproduction have never yet been observed for this genus. This feature is useful for identifying and differentiating fungal species.

Antimicrobial volatile organic compounds (VOCs)

VOCs are solid/liquid carbon-based compounds that easily enter the gas phase via vaporization at 0.01 KPa and temperature close to 20ºC, i.e., exhibit high vapor pressure and low water solubility, which allows them to evaporate and diffuse easily through the air [16,43].

More than 250 VOCs have been identified from fungi, occurring in the form of mixtures of simple hydrocarbons, heterocyclic hydrocarbons, aldehydes, ketones, alcohols, phenols, thioalcohols, thioesters and their derivatives, including benzene and cyclohexanes [32].

VOCs may be derived from primary and secondary metabolic pathways of microorganisms. The microorganism releases VOCs as byproducts of primary metabolism when it decomposes substrates to extract nutrients necessary for its maintenance. In contrast, in secondary metabolism, VOC production is usually related to competition for resources in nutrient-poor environments [44].

The profiles of volatiles produced by a certain species or isolates may vary, depending on the substrate used for growth, incubation duration, nutrient type present, temperature, and other environmental parameters [32,45]. The same *M. albus* 620 isolate shows variation in volatile profile composition depending on the nutrient concentration in the growth medium, where the number of volatile compounds detected was higher in culture media that exhibited a greater quantity of the carbon source [46].

The VOCs produced by *Muscodor* species consist mainly of low-molecular-weight esters, alcohols, and acids, with differences between the compound mixtures produced by different species of the genus. However, the VOC mixture produced by most *Muscodor* species has antimicrobial bioactivity [47,48].

Muscodor species vary regarding the VOC mixture emitted. *M. crispans* Mitch, Strobel, Hess, Vargas & Ezra, for example, do not produce naphthalene or azulene derivatives, compounds observed in other species of the genus *Muscodor* [36]. In contrast, naphthalene predominates in the VOC mixture emitted by *M. vitigenus* Daisy, Strobel, Ezra & Hess, and the VOC mixture emitted by this fungus does not exhibit antifungal bioactivity, though it has previously demonstrated lethality in insects [49].

Gas chromatography/mass spectrometry analyses of the VOC mixture produced by *M. albus* reveal the presence of at least 28 different VOCs, representing at least five classes of organic substances, where the esters contributed the highest percentage in the mixture, followed by alcohols, acids, lipids, and ketones [31].

The antimicrobial action spectra of the compounds emitted by certain species or isolates seem to be affected by the compound mixture emitted by each isolate. Several studies have demonstrated that the volatile mixture among *Muscodor* species varies, and the action spectrum also varies, with some being more efficient in inhibiting the growth of certain fungi than others [31,37,38,3947,48,49].

Antimicrobial effects of the VOCs produced by fungi in post-harvest pathogens in fruits and vegetables

Most studies on the antimicrobial effects of volatiles produced by fungi involve *Muscodor* species (Figure 2), although the biological functions of the toxic compounds produced are still not well elucidated. Most *Muscodor* spp. isolates and other antimicrobial volatile-producing species are endophytic. VOC emission by these fungi may act as a defense mechanism for the host plant against pathogen attack, helping the antimicrobial VOC-producing endophyte survive by preventing colonization of the host plant by microorganisms that compete for the same ecological niche [31].

Toxicity from exposure to *M. albus* appears to be associated with combined action of the compounds present in the mixture. Each of the five classes of volatile compounds produced by the fungus (alcohols, esters, ketones, acids, and lipids) had some inhibitory effect against fungi and bacteria when tested alone but did not cause their death. However, they acted synergistically when collectively tested in the mixture, killing a wide range of fungi and bacteria pathogenic to plants and humans [31].

A recent attempt to elucidate the action mechanism of the volatile compounds emitted by *M. albus* shows DNA damage in *Escherichia coli* cells when exposed to VOCs

emitted by the fungus, which most likely resulted in the interruption of the replication and/or transcription processes; the compounds also caused morphological changes in the cells, generating increased fluidity of the cell membrane [50].

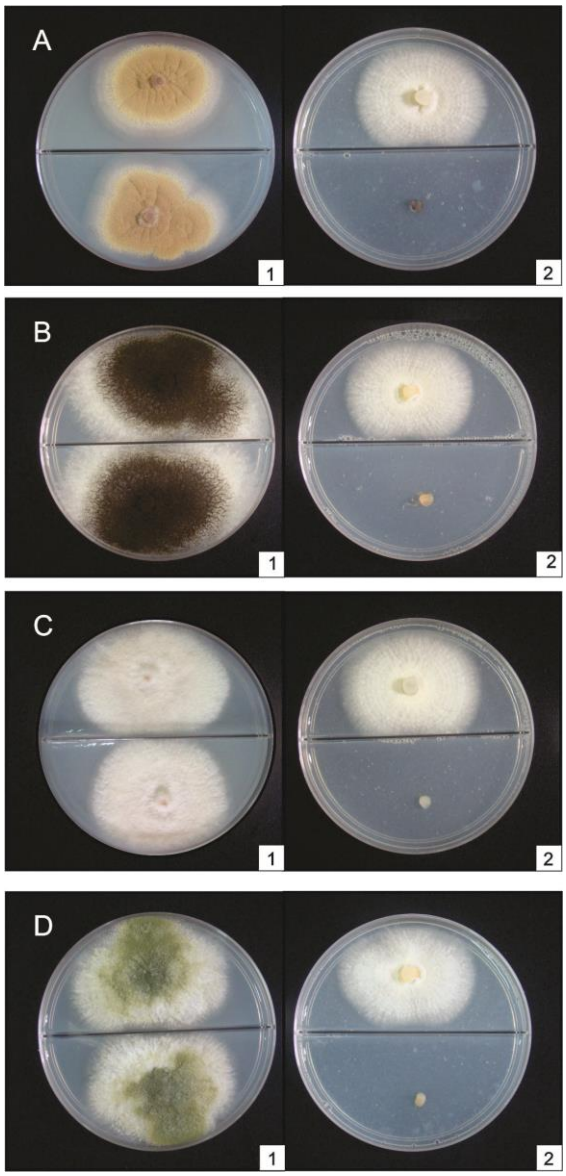

Figure 2. Effect *In vitro* of inhibition mycelial growth of *A. ochraceus* (A – 2); *A. niger* (B – 2); *F. semitectum* (C – 2); *A. flavus* (D – 2) after exposure to VOCs emitted by *Muscodor* sp. Control (1).

The antimicrobial potential of the compounds emitted by *M. albus* against diverse microbial groups among fungi, bacteria, and oomycetes has been described in the literature. Growth (*in vitro*) of *B. cinerea*, *A. fumigatus*, *Tapesia yallundae* Wallwork & Spooner, *Rhizoctonia solani* Kühn, *Sclerotinia sclerotiorum* (Lib.) de Bary, *Candida albicans* (C.P. Robin) Berkhout, *Pythium ultimum* Trow, *Verticillium dahliae* Kleb,

Phytophthora cinnamomi Rands, *E. coli*, *Bacillus subtilis, Staphylococcus aureus,* and *Micrococcus luteus,* representative of diverse groups of "fungi" and "bacteria", was inhibited, and their cells died after exposure to VOCs emitted by *M. albus* isolates. [30,31].

The effects of the VOCs emitted by *M. albus* 620 were reported (*in vitro*) against three important fungi frequently associated with post-harvest decay, *S. sclerotiorum*, *B. cinerea*, *Penicillium expansum* Link. The volatiles emitted by the *M. albus* 620 isolate exhibited significant effects in the germination of *B. cinerea* and *P. expansum* spores, preventing the conidia of these fungi to germinate and reducing *S. sclerotiorum* colony diameter growth. For both treatments, the source of *M. albus* 620 used was rye grain colonized by the fungus, and higher grain weight (0.25 g to 1.25 g/L) in each treatment corresponded to a stronger observed effect, where 1.25 g/L completely inhibited *B. cinerea* and *P. expansum* spore generation and *S. sclerotiorum* growth [51].

The volatiles emitted by *M. albus* were also tested against important toxin-producing fungi. Conidia of *Aspergillus carbonarius* (Bainier) Thom*, A. flavus, A. niger, A. ochraceus, P. verrucosum, F. culmorum*, and *F. graminearum* died or their germination was inhibited (*in vitro*) when exposed to volatiles produced by *M. albus* colonizing rye grain at 20º C. When conidia of the same fungi were separately exposed to the compounds most abundant in the compound mixture emitted by *M. albus*, isobutyric acid and 2-methyl-1-butanol, the same magnitude of effect was not observed [34].

In addition to *M. albus*, other *Muscodor* species have also been reported to inhibit the growth of fungi associated with post-harvest decay. VOCs emitted by *M. crispans* were effective against a wide range of phytopathogens, among which *B. cinerea*, *Colletotrichum lagenarium* Caruso & Kuc*, Fusarium avenaceum* (Fr.) Sacc.*, F. culmorum*, *Phytophthora palmivora* Butler (Butler)*, P. ultimum, S. sclerotiorum, G. candidum, A. fumigatus,* and *Curvularia luneta* (Wakker) Boedijn exhibited inhibited colony growth. Additionally, except for the last three, 24-hour exposure to the compound mixture emitted by *M. crispans* led to cell death [36].

The volatiles emitted by *M. strobelii* exhibited a broad spectrum of activity against yeasts, bacteria, and filamentous fungi and, among the fungi tested, the VOCs completely inhibited the growth of *Penicillium citreonigrum* Dierckx, *B. cinerea*, and *Aspergillus japonicus* Saito after three days of exposure. The mixture of compounds emitted by *M. strobelii* is different from the mixtures of other species of the genus *Muscodor*, exhibiting

4-octadecylmorpholine as the most abundant compound, along with tetraoxapropellan and aspidofractinine-3-methanol; the last two compounds are not encountered among the volatiles of the other *Muscodor* species [38].

Variation in compounds present in the VOC mixture among *Muscodor* species also occurred in *M. sultura,* where there is variation in the compound mixture profile compared with other *Muscodor* species, producing higher abundances of propanoic acid, 2-methyl, and thujopsene. The VOCs emitted by *M. sultura* exhibited antimicrobial bioactivity against a wide range of fungi, inhibiting the growth of *A. fumigatus*, *B. cinerea, C. lagenarium, Ceratocystis ulmi* (Buisman) C. Moreau, *Cercospora beticola* Sacc., *G. candidum, Mycosphaerella fijiensis* M. Morelet, *P. cinnamomi*, *P. palmivora*, *Pythium ultimum, R. solani*, *S. sclerotiorum,* and *V. dahliae* after two days of exposure, promoting death of their cells. Many of these species are important phytopathogenic fungi associated with post-harvest decay in fruits and vegetables [52].

Other *Muscodor* species, such as *M. musae*, *M. oryzae, M. suthepensis* and *M. equiseti* (N. Suwannarach & S. Lumyong), were described together with the antimicrobial potential of VOCs emitted. These VOCs showed antimicrobial activity against several microorganisms, including important post-harvest phytopathogens, such as *A. flavus, B. cinerea, Colletotrichum capsici* (Syd. & P. Syd.) Butler & Bisby*, Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., *Colletotrichum musae* (Berk. & Curtis) Arx*, Penicillium digitatum,* and *P. expansum*, and in most cases, the exposure to the compounds emitted by these *Muscodor* species inhibited 100% of phytopathogen growth and caused death of their cells [47].

Muscodor species are not the only fungi that have been reported to emit antimicrobial volatiles with the potential to inhibit growth and even kill post-harvest phytopathogenic fungi in fruits and vegetables. For *Myrothecium inundatum* Tode, *Phomopsis* sp., *Hypoxylon* sp., *Nodulisporium* sp., *Bionectria ochroleuca* (Schwein.) Schroers & Samuels*, Schizophyllum commune* RF., *Gloeosporium* sp., and *Gliocladium* sp., even though these fungi do not exhibit the same effects observed in *Muscodor* spp. compounds *in vitro*, the VOCs produced by isolates of these fungi reduced the growth of important fungi associated with post-harvest diseases, such as *Aspergillus ochraceus, A. flavus, A. fumigatus, B. cinerea, C. capsici, C. gloeosporioide, C. lagenarium, C. musae, G. candidum, Penicillium digitatum, Penicillium expansum, Phytophthora palmivora, Pythium ultimum,* and *Sclerotinia sclerotiorum* [53,54,55,56,57,58,59,60].

In addition to *in vitro* assays, some studies have been performed to elucidate the potential of VOCs produced by fungi to control post-harvest diseases in fruits and vegetables by mycofumigation of the horticultural product. The VOCs emitted by *Aureobasidium pullulans* yeast isolates inhibited (*in vitro*) conidial germination of post-harvest disease-causing phytopathogens in apple. Furthermore, when tested *in vivo*, the VOCs reduced the incidence of blue mold and bitter rot in apple caused by *Penicillium expansum* and *Colletotrichum acutatum,* respectively; however, the greatest effect was observed after directly applying the antagonists to the fruit [61]. In later tests (*in vivo*), VOCs of the same isolates significantly reduced *B. cinerea* and *P. expansum* infection in apple, as observed by the smaller size of damage in the fruit compared with the control treatment; in this assay, the antagonist was inoculated in culture medium deposited at the bottoms of glass boxes containing apples artificially inoculated with the phytopathogens, thus preventing direct contact between the antagonist and the fruit [62].

Other yeasts, such as *Candida intermedia, Wickerhamomyces anomalus*, and *Metschnikowia pulcherrima,* have been tested for post-harvest disease control in fruit. Isolates of these yeasts were used to control *B. cinera* colonization in strawberry and table grape. The VOCs emitted inhibited *B. cinera* growth *in vitro,* and the yeasts reduced disease severity when applied *in vivo*. However, the effect on the inhibition of disease development was more intense after directly applying yeast suspension to the strawberries inoculated with *B. cinera* [40,42].

The potential of volatiles produced by *M. albus* to control post-harvest diseases in fresh fruit by mycofumigation was also studied. Mycofumigation of apple with *M. albus* culture controlled blue mold (*Penicillium expansum*) and gray mold (*Botrytis cinerea*) in apples inoculated with the phytopathogens, without requiring direct contact between the fruit and the *M. albus* culture. The same was observed in peaches inoculated with *Monilinia fructicola*, where fumigation with *M. albus* culture promoted complete control of brown rot in an assay performed using closed plastic boxes. In organic table grape ('Thompson Seedless' and 'Red Seedless' varieties), mycofumigation with *M. albus* culture in plastic boxes reduced the incidence of post-harvest decay [35,63,64].

Mycofumigation with *Oxyporus latemarginatus* isolate culture also reduced development of gray mold caused by *B. cinera* in apples [65]. In citrus, mycofumigation with *Nodulisporium* sp. isolate culture controlled green mold decay in *Citrus limon* caused by *Penicillium digitatum* and blue mold decay in *Citrus aurantifolia* and *C. reticulata* caused by *P. expansum* [57].

Conclusion

Mycofumigation is a promising alternative for reducing post-harvest losses in fruits and vegetables caused by fungi. The method has potential to be applied during the transport and storage of fresh fruits and vegetables, where the presence of antimicrobial VOCs, such as compound mixtures produced by *M. albus* cultures, may increase the shelf lives of these horticultural products by reducing the incidence of post-harvest diseases. The potential of some fungi to emit VOCs able to inhibit or cause death of important phytopathogenic fungi associated with post-harvest decay, without requiring direct contact with the product to be consumed, together with the wide range of microorganisms sensitive to VOCs from fungal species, makes mycofumigation an interesting method for controlling post-harvest diseases, which, unlike traditional methods, reduces risks to human health and environmental contamination.

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Taxon	Host	Lifestyle	Site isolation	Taxonomic position	Reference Number
Filamentous fungi					
Muscodor albus	*Cinnamomum zeylanicum*	Endophytic	Honduras	Ascomycota, Sordariomycetes, Xylariales	30
M. kashayum	*Aegle marmelos*	Endophytic	India	Ascomycota, Sordariomycetes, Xylariales	37
M. crispans	*Ananas ananassoides*	Endophytic	Bolivian	Ascomycota, Sordariomycetes, Xylariales	36
M. roseus	*Grevillea pteridifolia*	Endophytic	Honduras	Ascomycota, Sordariomycetes, Xylariales	66
M. oryzae	*Oryza rufipogon*	Endophytic	Thailand	Ascomycota, Sordariomycetes, Xylariales	47
M. musae	*Musa acuminata*	Endophytic	Thailand	Ascomycota, Sordariomycetes, Xylariales	47
M. cinnanomi	*C. bejolghota*	Endophytic	Thailand	Ascomycota, Sordariomycetes, Xylariales	39
M. strobelii	*C. zeylanicum*	Endophytic	India	Ascomycota, Sordariomycetes, Xylariales	38
M. darjeelingensis	*C. camphora*	Endophytic	India	Ascomycota, Sordariomycetes, Xylariales	67
M. tigerii	*C. camphora*	Endophytic	India	Ascomycota, Sordariomycetes, Xylariales	68
M. suthepensis	*C. bejolghota*	Endophytic	Thailand	Ascomycota, Sordariomycetes, Xylariales	47
M. yucatanensis	*Bursera simaruba*	Endophytic	Mexico	Ascomycota, Sordariomycetes, Xylariales	69
M. vitigenus	*Paullinia paullinioides*	Endophytic	Peru	Ascomycota, Sordariomycetes, Xylariales	49
M. equiseti	*Equisetum debile*	Endophytic	Thailand	Ascomycota, Sordariomycetes, Xylariales	47
M. sutura	*Prestonia trifidi*	Endophytic	Colombia	Ascomycota, Sordariomycetes, Xylariales	52
M. fengyangensis	*Actinidia chinensis*	Endophytic	China	Ascomycota, Sordariomycetes, Xylariales	48
Hypoxylon sp.	*Persea indica*	Endophytic	Canary Islands	Ascomycota, Sordariomycetes, Xylariales	55

Nodulisporium sp.	*Myroxylon balsamum*	Endophytic	Ecuador	Ascomycota, Sordariomycetes, Xylariales	56
Nodulisporium sp.	*Lagerstroemia loudoni*	Endophytic	Thailand	Ascomycota, Sordariomycetes, Xylariales	57
Myrothecium inunduatum	*Acalypha indica*	Endophytic	India	Ascomycota, Sordariomycetes, Hypocreales	53
Gliocladium sp.	*Eucryphia cordifolia*	Endophytic	USA	Ascomycota, Sordariomycetes, Hypocreales	60
Trichoderma atroviride				Ascomycota, Sordariomycetes, Hypocreales	70
Bionectria ochroleuca	*Nothapodytes foetida*	Endophytic	India	Ascomycota, Sordariomycetes, Hypocreales	58
Phomopsis sp.	*Odontoglossum* sp.	Endophytic	Ecuador	Ascomycota, Sordariomycetes, Diaporthales	54
Phoma sp.	*Larrea tridentata*	Endophytic	USA	Ascomycota, Dothideomycetes, Pleosporales	71
Gloeosporium sp.	*Tsuga heterophylla*	Endophytic	USA	Ascomycota, Leotiomycetes, Helotiales	59
Oxyporus latemarginatus	*Capsicum annum*	Endophytic		Basidiomycota, Agaricomycetes	65
Schizophyllum commune		Saprofit	Chile	Basidiomycota, Agaricomycetes	72
Yeast fungi					
Aureobasidium pullulans		Saprophytic		Ascomycota, Dothideomycetes, Dothideales	61 / 62
Saccharomyces cerevisiae				Ascomycota, Saccharomycetes, Saccharomycetales	40 / 41
Candida intermedia				Ascomycota, Saccharomycetes, Saccharomycetales	42
Wickerhamomyces anomalus				Ascomycota, Saccharomycetes, Saccharomycetales	40
Metschnikowia pulcherrima				Ascomycota, Saccharomycetes, Saccharomycetales	40

Table 1. Fungal species related to the antimicrobial volatile organic compounds production..

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CAPÍTULO II

Brazilian *Muscodor* species endophytic from coffee and carqueja plants producing antimicrobial volatiles.

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Abstract

Several endophytic fungi have been reported to have produced bioactive metabolites. Some like *Muscodor* species have the capacity to emit volatile compounds with antimicrobial properties with broad spectrum against human and plant pathogens. The aim of this study was to prospect the *Muscodor* species producing antimicrobial VOCs, in tropical plants used in alternative medicine and coffee plants in Brazil. A total of 11 fungal isolates producing volatile metabolites was obtained by a parallel growth technique using *M. albus* 620 as a reference strain (eight from coffee plants and three from carqueja plants). Phylogenetic relationships revealed the presence of at least three distinct species, *M. coffeanum*, *M. yucatanensis* and *Muscodor* sp. SPME/GC/MS analyses of the VOCs in the headspace above the mycelium from *Muscodor* species 10 days old cultures on PDA revealed the volatile profile emitted by *M. coffeanum* CDA 741, *M. coffeanum* ACJ01, *M. yucatanensis* CDA 736 and *Muscodor* sp. CDA 724.

Volatile organic compounds of all *Muscodor* isolates tested had some effect on the growth of at least one of the *Aspergillus* species tested in the test of antimicrobial activity *in vitro*. *M. coffeanum* isolates showed antimicrobial activity against all *Aspergillus* species tested (*Aspergillus ochraceus, A. sclerotiorum*, *A. elegans*, *A. foetidus*, *A. flavus*, *A. tamari*, *A. tubingensis*, *A. sydowii*, *A. niger*, *A. caespitosus*, *A. versicolor* and *A. expansum*), sometimes by decreasing the growth rate or for the most part, by fully inhibiting colony growth. Fifty-eight percent of the target species died after six days exposure to VOCs emitted by *M. coffeanum* CDA 741, in addition to the inhibition of growth in *A. ochraceus* inoculated into coffee beans. A discovery of new *Muscodor* isolates, especially in different ecological niches with high activity of competition and antagonism, is a promising source of biological control agent adapted to a particular environment that can be used on a specific site. The total inhibition of growth in *A. ochraceus* in coffee beans by VOCs emitted by *M. coffeanum* CDA 741 opens up a prospect that plants which have *M. coffeanum* as an endophyte may be protected from attacks by this plant pathogen.

Keywords: biological control, fungal volatiles, *Muscodor coffeanum,* postharvest diseases

Introduction

Endophytic fungi are characterized by their ability to colonize plant tissues without causing any visible symptoms of disease for at least a part of their life cycle (Schulz and Boyle 2005; Piepenbring 2015). Their presence can promote the development of their host by producing a range of metabolites that may protect the host plant against different stress conditions, such as pathogenic invasions and drought (Yu et al. 2010; Suwannarach et al. 2013). Several endophytic fungi have been reported to have produced bioactive metabolites. However, little emphasis has been given to the production of volatile compounds with antimicrobial properties. Research involving the capacity of a fungus to emit antimicrobial volatile organic compounds (VOCs) has intensified since the discovery of *Muscodor albus,* an endophytic fungus isolated from *Cinnamomum Zeylanicum,* Breyne that stands out in the production of antimicrobial volatiles with broad spectrum against human and plant pathogens (Worapong et al. 2001; Strobel et al. 2001).

After the discovery of *M. albus*, other species of fungi have been linked with the production of antimicrobial VOCs. Most of these species have been isolated from healthy plant tissue, especially in tropical plants used in alternative medicine, such as *Ananas ananassoides* (Baker) L.B. Smith, *Aegle marmelos* (L.) Corr., *Cinnamomum* spp., and *Myroxylon balsamum* (L.) Harms (Mitchell et al. 2008; Suwannarach et al. 2010; Meshram et al. 2013; Meshram et al. 2014) as well as the *Muscodor* species which belong to the phylum Ascomycota, order Xylariales, whilst other related Ascomycota belong to the Sordariomycetes, Dothideomycetes and Leotiomycete class, which are predominant in the endophytic life style (Gomes et al. 2015).

Gas Chromatography / Mass Spectrometry analyses of the mixture of VOCs produced by *M. albus* showed the presence of at least 28 different compounds

representing at least five classes of organic substances, where the esters contribute a higher percentage to the mixture, followed by alcohols, acids, lipids and ketones (Strobel et al. 2001).

VOCs present in the mixture emitted by *Muscodor* can vary from species to species. *M. albus,* for example, produces 1-Butanol, 3-methyl-, acetate and 1-Butanol, 3-methyl- in greater abundance. *M. vitigenus* already stands out in the production of naphthalene and its derivatives (Strobel et al. 2001; Daisy et al. 2002). Differences in the volatile profile have also been observed between individual isolates of the same *Muscodor* species (Ezra et al. 2004). Such variation in the volatile profile can exert influence on the antimicrobial action spectrum of volatiles emitted.

The versatility of endophytic fungi like the *Muscodor* species for the production of bioactive metabolites makes it a promising microbial control agent for use in the biological control of plant pathogens. Currently, few studies have been undertaken to investigate the potential of endophytic fungi to emit VOCs with antimicrobial properties in Brazil. The aim of this study was to prospect the *Muscodor* species producing antimicrobial VOCs, in tropical plants used in alternative medicine and coffee plants in Brazil, to identify these VOCs, and evaluate their antimicrobial activity against the *Aspergillus* species often associated with coffee beans.

Materials and methods

Microorganisms

Aspergillus species used in this study were provided by Coleção de Cultura de Microrganismos do Departamento de Ciências dos Alimentos – UFLA. *M. albus* CZ 620 was provided by Montana State University Mycological Collection.

Isolation and screening of endophytic fungi

Leaves and branches intact and asymptomatic from *Baccharis trimera* Less, *Hyptis brevipes* Poit, *Ottonia anisum* Sprengel and *Coffea arabica* L plants were collected in the Atlantic Forest biome at Parque Estadual da Serra do Brigadeiro forest reserve (20° 42′ 55″ S, 42° 26′ 51″ W), located in the Zona da Mata region, Minas Gerais state, Brazil. The plant tissue was gently washed with tap water, cut into fragments of 0.5 x 0.5 cm of size, disinfested according Zhang et al. (2010) by immersion in 75% ethanol for 30 s, followed by immersion in 1% sodium hypochlorite for 10 min and finally washed three times in sterile distilled water. Isolation of endophytic fungi proceeded according to the parallel growth isolation technique, adapted of Worapong et al. (2001) and Ezra et al. (2004). Both sides of a two compartment plastic Petri dish were loaded with Potato Dextrose Agar (PDA). A micelial plug of 5 mm diameter from a 10 days old culture of *M. albus* CZ 620 producing antimicrobial volatile metabolites was inoculated on one side of the dish and grown for 10 days at 20 ± 2 ºC in absence of light. Plant fragments was then placed on the another side of the dish. The dish was sealed with pvc plastic film and incubated at 20 ± 2 ºC in absence of light. Fungal hyphae emerging from the fragments

were transferred to another Petri dish without the presence of *M. albus* CZ 620 and incubated in the same previous conditions.

Screening for production of VOCs with antimicrobial properties was performed by the ability of endophytic fungus inhibit the growth of *Rhizopus stolonifer* (Ehrenb.) Vuill., *Botrytis cinerea* Pers and *Aspergillus ochraceus* G. Wilh through of parallel growth technique previously mentioned. Both sides of a two compartment plastic Petri dish were loaded with PDA. A micelial plug of 5 mm diameter from a 10 days old culture of endophytic fungi supposedly producing antimicrobial volatile metabolites was inoculated on one side of the dish and grown for 10 days at 20 ± 2 ºC in absence of light. A micelial plug of 5 mm diameter from a 7 days old culture of plant pathogenic fungus was then placed on the another side of the dish. The dish was sealed with pvc plastic film and incubated at 20 ± 2 ºC in absence of light for 48 h. The influence of endophytic isolate on the growth of plant pathogen fungi was assessed by the presence or absence of mycelial growth in the inoculated dishes.

Morphological analysis

The isolates were grown in potato dextrose agar (PDA, Sigma-Aldrich), 2% malt extract agar (MEA, Kasvi) and synthetic nutrient deficient agar (SNA) with and without presence of dry and autoclaved plant tissue (corn stover, branches and leaves pine) at 25ºC to induce the formation of reproductive structures. Micro-morphological characteristics were observed with a light microscope (Olympus BX53, Japan).

DNA extraction, PCR amplification, sequencing and phylogenetic analysis

Genomic DNA was extracted from colonies originated from fragment of hyphae tip, grown in PDA at 25º C with twelve daily hours of light for 7 days. Fungal mycelium was scraped in the colony margins and DNA extraction proceeds according to protocol established by Pinho et al. (2012) whose the genomic DNA is extracted by Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.) with adaptations.

Sequences of the Internal Transcribed Spacer (ITS) and partial 28S (rRNA) regions of rDNA were amplified using primers ITS1 and ITS4, LR0R and LR5, respectively (White et al. 1990; Vilgalys and Hester 1990). Polymerase Chain Reaction (PCR) products were purified and sequenced in Macrogen (South Korea). Primers used for sequencing were the same used for amplification of the fragments. Nucleotide sequences were edited on software BioEdit 7.2.5 (Hall 2013). All sequences were verified manually and ambiguous nucleotide positions were clarified using sequences of both DNA strands. The sequences were subjected to analysis on Basic Local Alignment Search Tool (BLAST) on GenBank - National Center for Biotechnology Information (NCBI) and Unite databases. Sequences with high similarity to each locus were taken along with representatives of each fungal species obtained in this study. Sequences obtained in this study and sequences take on GenBank – NCBI were aligned using MUSCLE (Edgar 2004) and manually corrected on software MEGA 7.0 (Kumar et al. 2016). Alignment regions with ambiguous sequences were excluded from the analysis and Gaps (insertions and deletions) were treated as missing data.

Phylogenetic analysis of Bayesian Inference was performed on CIPRES portal (Miller et al. 2010) using MrBayes v 3.2 (Ronquist et al 2012), based on Markov Monte Carlo Chain (MCMC) with 10,000,000 generations using the nucleotide substitution

model informed by the Akaike Information Criterion (AIC) on software MrModeltest v.2.3 (Posada and Crandall 1998). Trees were sampled every 1000 generations, burning 25% of all trees obtained. Posterior probabilities (Rannala and Yang 1996) were determined in the most consensus tree among the 7500 remaining trees.

Antifungal activity of volatile metabolites produced by *Muscodor* spp. against *Aspergillus* species

The antifungal activity of VOCs emitted by representative isolates of *Muscodor* species obtained in this study was tested against *Aspergillus* species often associated with coffee beans (*Aspergillus sclerotiorum, A. caespitosus, A. elegans, A. expansum, A. flavus, A. foetidus, A. niger, A. sydowii, A. tamari, A. tubingensis, A. versicolor,* and *A. ochraceus*) by adaptation of the parallel growth technique described by Worapong et al. (2001) and Ezra et al. (2004). Both sides of a two compartment plastic Petri dish were loaded with PDA. A micelial plug of 5 mm diameter from a 10 days old culture of the *Muscodor* isolate was inoculated on one side of the dish and grown for 10 days at 20 ± 2 ºC in absence of light. A micelial plug from the margin of 7 days old culture of each *Aspergillus* sp. was then placed on the other side of the dish. The dish was sealed with pvc plastic film and incubated at 20 ± 2 ºC in absence of light for 6 days. *Aspergillus* sp. growing without the presence of *Muscodor* isolate was the control. The inhibition percentage of *Aspergillus* sp. was measured through growth rate of colony and its viability was investigated by subculture of the pathogen on tested dish into a non-treated PDA dish. The experiment was repeated twice with five replicates.

Antifungal activity of volatile metabolites produced by *M. coffeanum* CDA 741 against *A. ochraceus* inoculated on coffee beans

The antifungal activity of VOCs emitted by *M. coffeanum* CDA 741 was tested against *A. ochraceus* associated with coffee beans. The assay was performed on plastic Petri dishes with two compartments. Both sides were loaded with PDA, and a micelial plug of 5 mm diameter from a 10 days old culture of *M. coffeanum* CDA 741 was inoculated on one side of the dish and grown for 10 days at 20 ± 2 ºC in absence of light. Five autoclaved coffee beans inoculated with *A. ochraceus* by immersion in a suspension of conidia (10^5 conidia.mL^{-1}) for 30s were placed on the another side of the dish. The dish was sealed with pvc plastic film and incubated at 20 ± 2 ºC in absence of light. Autoclaved coffee beans inoculated with *A. ochraceus* and placed on Petri dish without the presence of *M. coffeanum* CDA 741 was the control 1, and autoclaved coffee beans placed on Petri dish was the control 2. The effect of treatment was evaluated by observation of the emergence of *A. ochraceus* conidiophores on coffee beans using a stereoscopic microscope. The experiment was repeated twice with five replicates (dishes).

Identification of VOCs

The VOCs produced by representative isolates of *Muscodor* species were identified by solid-phase micro-extraction gas chromatography and mass spectroscopy (SPME-GC/MS), adapted from Strobel et al. (2001). The VOCs were extracted with a SPME syringe (SULPECO, USA), 50/30 µm divinylbenzene/carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) on StableFlex fiber (SULPECO, USA) from the headspace above a 10 days old culture of each fungal

endophytic grown on PDA in a headspace vial of 20 mL. The SPME fiber was placed through a small hole drilled in the vial septum, and the adsorption was allowed to continue for 60 min with the fiber cooled by liquid nitrogen (CF-SPME), as described by Moreira et al. (2014).

The analysis of VOCs was conducted with a Finnigan Trace DSQ GC/MS equipped with an ion trap mass spectrometer from Thermo Scientific (West Palm Beach, FL, USA); equipped with a HP-5MS column (30 m × 0.25 mm × 0.25 μm) with a helium flow of 1.5 mL min^{-1}. The injector was operated in splitless mode for 10 min with an injector temperature of 250 °C. The oven temperature ramp started at 50 °C, increased to 70 °C at a rate of 5 °C min^{-1}, maintained for 1 min and then increased to 280 °C at a rate of 10 °C min^{-1}, maintained for 2 min. The mass spectrometer was operated in electron ionization mode (EI) with energy of 70 eV. Data acquisition and data processing were performed by software systems. The VOCs produced by endophytic fungi were identified by comparing the obtained mass spectra with the National Institute of Standards and Technology (NITS) library and by comparing the calculated Kováts retention index with compounds under higth quality match indicated by NITS library. Comparative analysis were conducted on control vials containing only PDA. The compounds present in the control were removed from data set obtained from the GC/MS of endophytic fungi.

Statistical Analysis

The data obtained in this study were evaluated by one-way ANOVA, and the significance of the treatments was determined by Tukey's HSD for multiple comparisons ($P \leq 0.05$).

Results

Isolation and identification

A total of 11 fungal isolates producing volatile metabolites was obtained by a parallel growth technique using *M. albus* 620 as a reference strain. Eight isolates were obtained from the branches of coffee plants and three from *B. trimera* (common name: Carqueja) leaves.

ITS-5.8S sequences of rDNA from endophytic isolates showed at least 99% similarity with *Muscodor* sequences by BLAST in GenBank and UNITE databases. Comparative studies of morphological characteristics indicate that although belonging to the genus *Muscodor*, no conidia nor sporulation structures were observed under laboratory conditions.

Bayesian inference analysis of ITS-5.8S sequences derived from all *Muscodor*-like isolates revealed phylogenetic relationships with the *Muscodor* genus. Among the isolates obtained from coffee plants, isolates CDA 724 and CDA 740 are phylogenetically close as well as isolates CDA 747 and CDA 738, and are grouped in the same cluster with *M. equiseti*, *M. sutura* and *M. vitigenus* (Fig. I). Isolates CDA 736 and CDA 744 are phylogenetically close to *M. yucatanensis* forming a clade. Isolates CDA 743 and CDA 741 comprise a group forming a clade with *M. coffeanum* together with isolates ACJ01, ACJO6 and ACJ08 obtained from carqueja leaves (Fig. I).

Antifungal activity of *Muscodor* spp. volatiles against the *Aspergillus* species

The antifungal activity of VOCs emitted by different *Muscodor* isolates obtained in this study were tested against the *Aspergillus* species which are often associated with

coffee beans. The volatile metabolites produced by CDA741 and ACJ01 isolates (both phylogenetically identified as *M. coffeanum*) showed antifungal activity against all *Aspergillus* species tested, sometimes by decreasing the growth rate or for the most part, by fully inhibiting colony growth (Table I). Fifty-eight percent of the target species died after six days exposure to VOCs emitted by *M. coffeanum* CDA 741 isolated from coffee plants. VOCs emitted by isolate CDA 736 (phylogenetically identified as *M. yucatanensis*) fully inhibited the growth of *A. sclerotiorum*, *A. versicolor,* and *A. tamari,* causing the demise of the latter. VOCs emitted by *Muscodor* sp. CDA 724 fully inhibited the growth of *A. tamari*, *A. ochraceus,* and *A. niger* killing the last two.

VOCs emitted by *M. coffeanum* CDA 741 in addition to inhibiting growth in all *Aspergillus* species tested, nullified growth in *A. ochraceus* when inoculated into coffee beans. Where $P \leq 0.01$ *M. coffeanum* CDA 741 the inhibition of growth by VOCs in *A. ochraceus* inoculated into coffee beans was significant, and no growth in *Aspergillus* was observed in coffee beans treated with *M. coffeanum* CDA 741 VOCs (Fig. II).

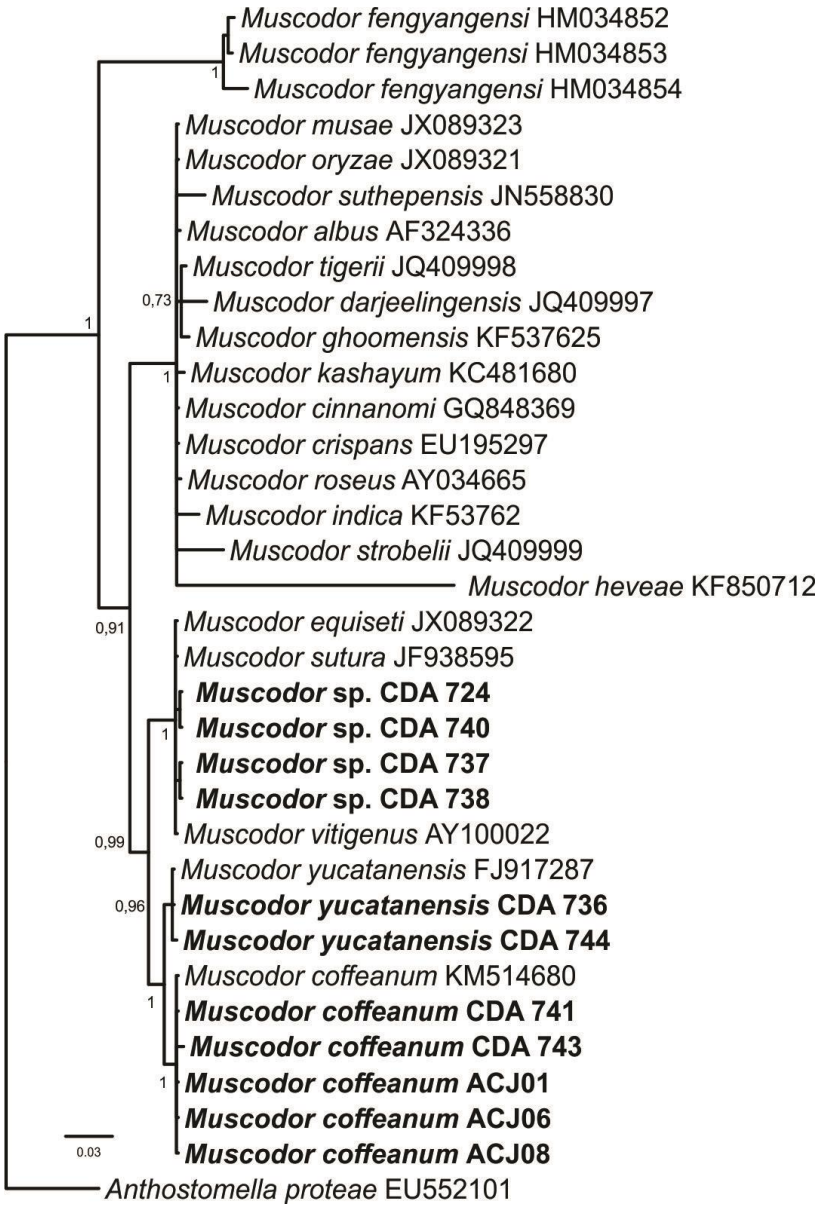

Fig. I Bayesian Inference tree of *Muscodor* using ITS-5.8S sequences of rDNA. The posterior probability values are indicated at the nodes. The *Muscodor* isolates from this study is highlighted in bold. The analyses included 33 *Muscodor* specimens and was rooted with *Anthostomella proteae* (Xylariaceae) for out-group**.** Bar = 0.03 substitutions per nucleotide position.

Table I. Effect of the VOCs emitted by *Muscodor* isolates on the growth of *Aspergillus* species.

Aspergillus species	Growth ratio after 6 days of exposure (% vs. control)			
	M. coffeanum **CDA 741**	***M. coffeanum*** **ACJ01**	***M. yucatanensis*** **CDA 736**	***Muscodor*** **sp. CDA 724**
A. ochraceus	0 Dead	0 Dead	98.6 ± 1.3	0
A. sclerotiorum	0 Dead	0 Dead	0 Dead	91.1 ± 2.7
A. elegans	0	0	100 ± 4.2	100 ± 5.6
A. foetidus	0 Dead	0 Dead	97.7 ± 1.2	100 ± 1.6
A. flavus	0	0 Dead	99.3 ± 3.8	99.5 ± 2.9
A. tamari	0 Dead	0 Dead	0 Dead	0 Dead
A. tubingensis	0 Dead	0	99.0 ± 1.3	51.9 ± 4.2
A. sydowii	0 Dead	0	70.6 ± 0.9	41.7 ± 1.0
A. niger	0 Dead	17.6 ± 1.3	91.9 ± 2.9	0
A. caespitosus	0	0	79.8 ± 5.9	72.2 ± 3.1
A. versicolor	0	43.8 ± 3.7	0	100 ± 8.9
A. expansum	39.7 ± 1.1	64.5 ± 1.7	92.3 ± 2.0	97.3 ± 11.6

Note: Growth ratio was calculated as the fraction of the value of colony diameter grown in the presence and in the absence of *Muscodor* isolate and expressed as percentage.
Tests were repeated three times and means ± standard deviation were calculated.
Viabiliity of each *Aspergillus* species was evaluated after 6 days in fresh PDA dishes (free of *Muscodor* VOCs)

VOCs produced by *Muscodor* species obtained in this study

SPME/GC/MS analyses of the VOCs in the headspace above the mycelium from *Muscodor* species 10 days old cultures on PDA revealed the volatile profile emitted by *M. coffeanum* CDA 741, *M. coffeanum* ACJ01, *M. yucatanensis* CDA 736 and *Muscodor* sp. CDA 724 (Table II - V).

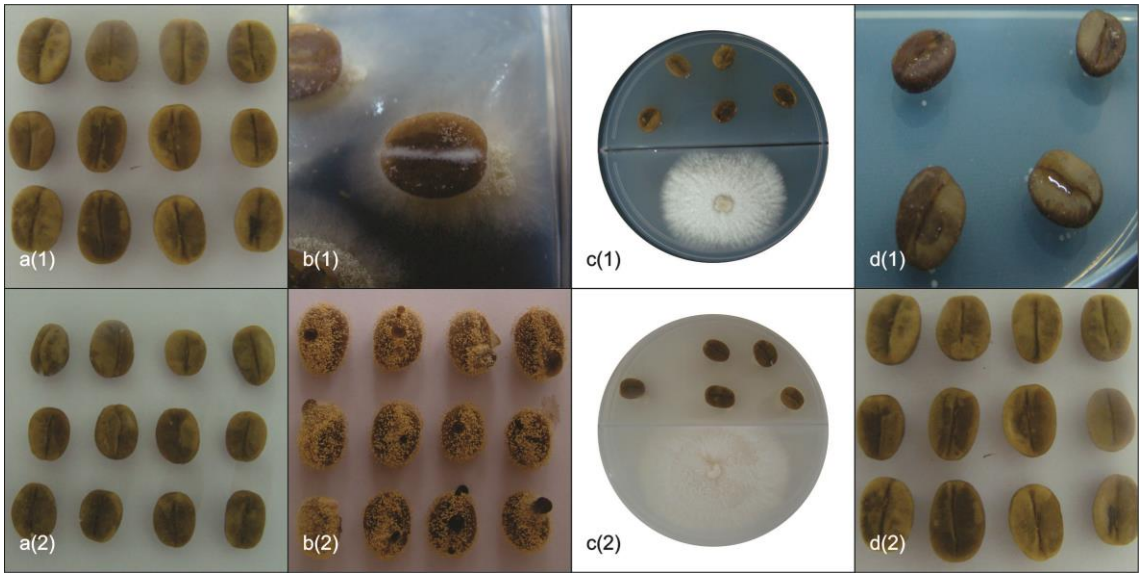

Fig. II Mycofumigation of *A. ochraceus* in coffee beans with *M. coffeanum* CDA 741 VOCs. a(1 – 2), Control I, coffee beans without inoculation of *A. ochraceus* and non-fumigated. b (1 – 2), Control II, coffee beans inoculated with *A. ochraceus.* c(1 – 2), d(1 – 2) coffee beans inoculated with *A. ochraceus* and mycofumigated with *M. coffeanum* CDA 741.

Table II. GC/MS analysis of the volatile compounds emitted by *Muscodor coffeanum* CDA 741.

RT (min)	Compound	Molecular formula	peak area %	Kovats RI	Calculated Kovats
14.25	Pyrimidine, 2-chloro-4-ethyl-6-methyl-	$C_7H_9ClN_2$	0.91	1174	1099
15.58	Cyclosativene	$C_{15}H_{24}$	9.18	1125	1150
16.05	Tricyclo[4.3.1.1(3,8)]undecane, 3-methoxy-	$C_{12}H_{20}O$	4.8	1273	1166
17.8	4-Amino-3,5-diethylpyridine	$C_9H_{14}N_2$	7.8	1411	1422
18.73	Imidazo[5,1-f][1,2,4]triazine-2,7-diamine	$C_5H_6N_6$	4.6	1570	1698
19.43	Dimethyl-[4-[2-(3-methylisoxazol-5-yl)vinyl]phenyl]amine	$C_{14}H_{16}N_2O$	0.82	1812	1805
20.13	4(1H)-Pyrimidinone, 2,3-dihydro-1-methyl-6-(4-pyridinyl)-2-thioxo-	$C_{10}H_9N_3OS$	1.6	1943	1829
20.82	2-Phenyl-6-chloro-benzofuran	$C_{14}H_9ClO$	1.51	1861	1852
22.77	1-Methyl-2-nitro-4-(1,2,2-trimethyl-cyclopentyl)-benzene	$C_{15}H_{21}NO_2$	8.93	1951	1946

Table III. GC/MS analysis of the volatile compounds emitted by *Muscodor coffeanum* ACJ01.

RT (min)	Compound	Molecular formula	peak area %	Estimated Kovats RI	Calculated Kovats
7.97	Phenylethyl Alcohol	$C_8H_{10}O$	11.1	1136	989
14.02	1,2-Bis[methyl(trimethylene)silyloxy]propane	$C_{11}H_{24}O_2Si_2$	12.37	1081	1096
15.58	Cyclosativene	$C_{15}H_{24}$	20.04	1125	1149
15.81	But-3-en-2-one, 4-(2-chloro-6-fluorophenyl)-	$C_{10}H_8ClFO$	4.4	1391	1158
15.94	1-Ethyl-12-oxatetracyclo[5.2.1.1(2,6).1(9,11)]dodecane	$C_{13}H_{20}O$	7.55	1391	1162
18.48	6,6,9a-Trimethyl-5,5a,6,7,8,9,9a,9b-octahydronaphtho[1,2-c]furan-1(3H)-one	$C_{15}H_{22}O_2$	3.24	1807	1661
21.55	Cyclopropa[3,4]cyclohepta[1,2-a]naphthalene, 1,1a,1b,2,3,7b,8,9,10,10a-decahydro-5-methoxy-10-methylene-	$C_{18}H_{22}O$	4.32	1823	1875
22.77	2-(4a,8-Dimethyl-7-oxo-1,2,3,4,4a,7-hexahydronaphthalen-2-yl)-propionic acid	$C_{15}H_{20}O_3$	14.46	1980	1946

Table IV. GC/MS analysis of the volatile compounds emitted by *Muscodor yucatanensis* CDA 736

RT (min)	Compound	Molecular formula	peak area %	Estimated Kovats RI	Calculated Kovats
12.63	Pyrazine, isopropenyl-	$C_7H_8N_2$	1.22	988	1078
12.86	1,3,5,6,7-Pentamethylbicyclo[3.2.0]hepta-2,6-diene	$C_{12}H_{18}$	2.95	1121	1081
13.6	Cyclosativene	$C_{15}H_{24}$	13.24	1125	1091
13.82	Bicyclo[2.2.2]octa-2,5-diene, 1,2,3,6-tetramethyl-	$C_{12}H_{18}$	9.16	1168	1094
14.01	Cyclosativene	$C_{15}H_{24}$	2.93	1125	1097
15.59	Cyclosativene	$C_{15}H_{24}$	26.9	1125	1150
16.18	2,3,5-Trimethyl-6-ethylpyrazine	$C_9H_{14}N_2$	0.42	1220	1171
16.39	1,2-Bis(diethylphosphino)ethane	$C_{10}H_{24}P_2$	1.46	1269	1179
17.26	Tricyclo[4.4.0.0(2,7)]dec-8-ene-3-methanol, à,à,6,8-tetramethyl-, stereoisomer	$C_{15}H_{24}O$	6.07	1325	1257
17.8	2H-Pyrazol-3-ol, 5-furan-2-yl-	$C_7H_6N_2O_2$	4	1473	1422
18.32	1-Penten-3-one, 1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	$C_{14}H_{22}O$	0.48	1557	1637
21.14	(Z,Z)-3-Methyl-3H-cyclonona(def)biphenylene	$C_{18}H_{14}$	0.51	1882	1863
21.21	Benzo[c]2,7-naphthiridine-4,5(3H,6H)-dione	$C_{12}H_8N_2O_2$	0.38	1896	1865
22.21	9H-Xanthen-9-one, 4-methoxy-	$C_{14}H_{10}O_3$	0.67	1963	1896

22.76	2-Cyanomethyl-2-methyl-8-methoxy-1,2,3,4-tetrahydroquinoline	$C_{13}H_{16}N_2O$	5.94	1981	1945
22.87	(Z,Z)-3-Methyl-3H-cyclonona(def)biphenylene	$C_{18}H_{14}$	1.43	1882	1926

Table V. GC/MS analysis of the volatile compounds emitted by *Muscodor* sp. CDA 724.

RT (min)	Compound	Molecular formula	peak area %	Kovats RI	Calculated Kovats
12.03	Tricyclo[4.4.0.0(2,7)]dec-3-ene, 8-isopropyl-1,3-dimethyl-, (1S,2R,6R,7R,8S)-(+)-	$C_{15}H_{24}$	3.78	1300	1070
12.22	Cycloisolongifolene	$C_{15}H_{24}$	6.96	1197	1072
12.71	Cyclosativene	$C_{15}H_{24}$	0.65	1125	1079
12.88	Copaene	$C_{15}H_{24}$	2.53	1221	1082
13.51	Cyclosativene	$C_{15}H_{24}$	9.01	1125	1090
13.81	Cyclosativene	$C_{15}H_{24}$	9.67	1125	1094
14.02	12-Oxatetracyclo[4.3.1.1(2,5).1(4,10)]dodecane, 11-isopropylidene-	$C_{14}H_{20}O$	3.24	1272	1097
15.58	1H-Cyclopropa[a]naphthalene, decahydro-1,1,3a-trimethyl-7-methylene-, [1aS-(1aà,3aà,7aá,7bà)]-	$C_{15}H_{24}$	38.49	1398	1150
16.2	1-Dimethyl(pentafluorophenyl)silyloxycyclopentane	$C_{13}H_{15}F_5OSi$	0.54	1293	1172
17.31	3,5-Dimethyl-1-dimethylisopropylsilyloxybenzene	$C_{13}H_{22}OSi$	0.69	1339	1268
21.54	4-Trimethylsilyl-9,9-dimethyl-9-silafluorene	$C_{17}H_{22}S$	1.46	1693	1875
22.76	2-Methyl-5-methoxy-3-(á-aminopropyl)-indole	$C_{13}H_{18}N_2O$	3.62	1920	1945

Discussion

The genus *Muscodor* has been isolated from healthy plant tissue in tropical regions throughout the world. Currently 19 species of *Muscodor* have already been described as isolated from different host plants. In this study 11 *Muscodor* isolates were obtained from coffee and carqueja plants in the Atlantic Forest biome in Brazil by an isolation technique specific to the *Muscodor* genus. The initial identification of these isolates was made by

comparative studies of morphological characteristics and the BLAST of ITS-5.8S sequences in the Genbank and UNITE databases. All sequences showed similarity of at least 99% with *Muscodor* sequences. Through Bayesian inference analysis with ITS-5.8S sequences of all *Muscodor* species described to date, phylogenetic relationships revealed the presence of at least three distinct species, *M. coffeanum*, *M. yucatanensis* and *Muscodor* sp. (Fig. I).

In this study *M. coffeanum* was isolated from two different host plants (*Coffea arabica* and *Bacchans trimera*) and as in other *Muscodor* species showed no host specificity (Ezra et al. 2004). *Muscodor coffeanum* was described by A. A. M. Gomes, D. B. Pinho and O. L. Pereira (Hongsanan et al. 2015) as endophytic in coffee plants and was the first study relating to *Muscodor* in Brazil featuring the Rubiaceae plant family. To date reports on its presence are restricted to Brazil. *Baccharis trimera* (Asteraceae) is a South American plant used in traditional medicine for the treatment of rheumatism, hepatobiliary disorders and diabetes (Simões et al. 2016). Extracts of *B. trimera* have shown anti-inflammatory and analgesic properties (Gené et al. 1995). To our knowledge, this is the first time that *M. coffeanum* has been isolated from *B. trimera* .

Muscodor yucatanensis was first described by González et al. 2009 as an endophyte from *Bursera simaruba* in Mexico, a plant native to tropical regions with medicinal properties. However, in this study, *M. yucatanensis* CDA 736 was isolated from coffee plants and, to our knowledge, this is the first time that *M. yucatanensis* has been reported in Rubiaceae.

It was not possible to identify four isolates obtained in this study (*Muscodor* CDA 724, *Muscodor* CDA 740, *Muscodor* CDA 737 and *Muscodor* CDA 738) at the species level, and together with *M. equiseti*, *M. sutura* and *M. vitigenus* they were grouped in the same clade. Currently the description of new species of *Muscodor* has been arrived at by

phylogenetic species recognition coupled with the phenotype of the volatile profile emitted (Meshram et al. 2013; Suwannarach et al. 2013). However, a number of *Muscodor* isolates that group in the same monophyletic clade with another species of *Muscodor* have been proposed as a new species simply on account of their different volatile profile (Suwannarach et al. 2013). Care and caution are necessary for a new species proposition based on differences in the volatile profile because certain studies indicate that profiles of volatile metabolites may be the result of a variety of factors, including growing media and environment, and enzyme activity for biosynthesis of such metabolites (Morath et al. 2012).

The lack of more robust morphological characteristics for *Muscodor* taxonomy coupled with the inconsistent pattern of volatile profile among isolates make it difficult to identify the *Muscodor* species. An alternative for better discriminating the *Muscodor* species could be the use of other molecular markers, looking for other DNA regions more informative to the phylogeny of this group as has happened with other fungi (Giraldo et al. 2015; Lombard et al. 2015).

Volatile profile analysis was performed for *M. coffeanum* CDA 741, *M. coffeanum* ACJ01, *M. yucatanensis* CDA 736 and *Muscodor* sp. CDA 724 (Table II-V). This is the first time the volatile profile emitted by *M. coffeanum* has been revealed. Among the compounds emitted by *M. coffeanum* CDA 741 there are at least 9 volatiles that could possibly be identified by making comparisons with the NIST library and Kovats retention index (Table II). The isolate ACJ01, which also belongs to *M. coffeanum,* emitted a volatile profile different from *M. coffeanum* CDA 741 with only one compound (Cyclosativene) shared by these isolates (Table III). *Muscodor coffeanum* CDA 741 and *M. coffeanum* ACJ01 were isolated from different host plants (from coffee and carqueja plants respectively) and although they belong to the same species, the composition of the

volatile profile seems to vary between them. The difference in the mixture of VOCs emitted by isolates from the same species has also been observed in *M. albus* isolates (Ezra et al. 2004). The characteristic mixture of volatile compounds produced by each *Muscodor* species suggests adaptation to a unique ecological role in its respective ecosystem (González et al. 2009).

The profile of volatiles emitted by *M. yucatanensis* CDA 736 was different from that of other *M. yucatanensis* isolates reported (González et al. 2009). Among 16 volatiles identified pyrazine derivatives, sesquiterpenes, alcohols and benzene derivatives were present. Cyclosativene was the compound with the highest percentage of peak per area present in the mixture emitted by *M. yucatanensis* CDA 736.

Muscodor sp. CDA 724 also produced a mixture of VOCs different from other *Muscodor* reported on, with at least 12 volatiles identified by comparison with the NIST library and Kovats retention index. 1H-Cyclopropa[a]naphthalene, decahydro-1,1,3a-trimethyl-7-methylene-, [1aS-(1aα,3aα,7aβ,7bα)]-, was the compound with the highest relative proportion (38.49%). The emission of a naphthalene derivative has also been observed in other *Muscodor* species, especially in *M. vitigenus* (Daisy et al. 2002), species phylogenetically close to *Muscodor* sp. CDA 724.

The test of antifungal activity of VOCs emitted by selected *Muscodor* isolates representative of each species obtained in this study was against the *Aspergillus* species often associated with coffee beans and mycotoxin production (Batista et al. 2003). Volatile organic compounds of all *Muscodor* isolates tested had some effect on the growth of at least one of the *Aspergillus* species tested. However, *M. coffeanum* isolates were the most effective in the inhibition and / or demise of the *Aspergillus* species tested, in addition to the inhibition of growth in *A. ochraceus* inoculated into coffee beans. The mixture of VOCs emitted by *Muscodor* isolates has shown antimicrobial activity against

a wide range of microorganisms including plant pathogen fungi such as the *Aspergillus* species (Strobel et al. 2006). Strobel et al. (2001) reported that VOCs of *M. albus* had low inhibitory effects against fungi and bacteria when tested separately, but when tested in a mixture they acted synergistically and killed a broad range of fungi and bacteria.

The production of antimicrobial VOCs by endophytic fungi can act as a defense mechanism for host plants against the attack of plant pathogens, which favors the survival of the fungi that produce them, by preventing the colonization of their host plant tissue by other microorganisms competing for the same ecological niche (Morath et al. 2012). Since the discovery of antimicrobial activity of VOCs emitted by *Muscodor*, they have been considered for use in agricultural, medical and industrial applications (Strobel 2006). A discovery of new *Muscodor* isolates, especially in different ecological niches with high activity of competition and antagonism, is a promising source of biological control agent adapted to a particular environment that can be used on a specific site. *Muscodor* isolates naturally present in coffee plants with antimicrobial activity against the *Aspergillus* species producing mycotoxins can act to protect their host plant from attack by these plant pathogens. The total inhibition of growth in *A. ochraceus* in coffee beans by VOCs emitted by *M. coffeanum* CDA 741 opens up a prospect that plants which have *M. coffeanum* as an endophyte may be protected from attacks by this plant pathogen. Further studies have to be undertaken to elucidate the mechanism of the action of VOCs, and establish methods of application of *Muscodor* sp. as a biological control agent.

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Table VI. List of *Muscodor* isolates used for phylogenetic analysis.

Species	Strain		Origin	Host	Reference
Muscodor musae	CMU-MU3	ex-type	Thailand	*Musa acuminata*	Suwannarach et al. 2013
Muscodor equiseti	CMU-M2	ex-type	Thailand	*Equisetum debile*	Suwannarach et al. 2013
Muscodor oryzae	CMU-WR2	ex-type	Thailand	*Oryza rufipogon*	Suwannarach et al. 2013
Muscodor suthepensis	CMU462	ex-type	Thailand	*Cinnamomum bejolghota*	Suwannarach et al. 2013
Muscodor sutura	SR-2011	ex-type	Colombia	*Prestonia trifida*	Kudalkar et al. 2012
Muscodor albus	620	ex-type	Honduras	*Cinnamomum zeylanicum*	Worapong et al. 2001
Muscodor tigerii	2CCSTITD	ex-type	India	*Cinnamomum camphora*	Saxena et al. 2014
Muscodor fengyangensis	ZJLQ151	ex-type	China	*Pseudotaxus chienii*	Zhang et al. 2010
Muscodor fengyangensis	ZJLQ070		China	-	Zhang et al. 2010
Muscodor fengyangensis	ZJLQ374		China	*Pseudotaxus chienii*	Zhang et al. 2010
Muscodor kashayum	16AMLWLS	ex-type	India	*Aegle marmelos*	Meshrama et al. 2013
Muscodor cinnamomi	CMU-Cib 461	ex-type	Tailand	*Cinnamomum bejolghota*	Suwannarach et al. 2010
Muscodor yucatanensis	B110	ex-type	Mexican	*Bursera sirnaruha*	Gonzalez et al. 2009
Muscodor vitigenus	P15	ex-type	Peru	*Paullinia paullinioides*	Daisy et al. 2002
Muscodor crispans	B-23	ex-type	Bolivia	*Ananas ananassoides*	Mitchell et al. 2008
Muscodor roseus	A3-5	ex-type	Australia	*Grevillea pteridifolia*	Worapong et al. 2002
Muscodor darjeelingensis	1CCSTITD	ex-type	India	*Cinnamomum camphora*	Saxena et al. 2014
Muscodor ghoomensis	6 CCSTITD	ex-type	India	*Cinnamomum camphora*	Meshram et al. 2015
Muscodor indica	6(b)CCSTITD	ex-type	India	*Cinnamomum camphora*	Meshram et al. 2015
Muscodor heveae	RTM5-IV3	ex-type	Thailand	*Hevea brasiliensis*	Siri-udom et al. 2015
Muscodor coffeanum	COAD1842	ex-type	Brazil	*Coffea arabica*	Hongsanan et al. 2015
Muscodor strobelii	#6610	ex-type	India	*Cinnamomum zeylanicum*	Meshram et al. 2014
Muscodor coffeanum	CDA 741		Brazil	*Coffea arabica*	In this study
Muscodor coffeanum	CDA 743		Brazil	*Coffea arabica*	In this study

Muscodor coffeanum	ACJ01	Brazil	*Baccharis trimera*	In this study
Muscodor coffeanum	ACJ06	Brazil	*Baccharis trimera*	In this study
Muscodor coffeanum	ACJ08	Brazil	*Baccharis trimera*	In this study
Muscodor yucatanensis	CDA 736	Brazil	*Coffea arabica*	In this study
Muscodor yucatanensis	CDA 744	Brazil	*Coffea arabica*	In this study
Muscodor sp.	CDA 724	Brazil	*Coffea arabica*	In this study
Muscodor sp.	CDA 738	Brazil	*Coffea arabica*	In this study
Muscodor sp.	CDA 740	Brazil	*Coffea arabica*	In this study
Muscodor sp.	CDA 737	Brazil	*Coffea arabica*	In this study

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CAPÍTULO III

A new species of *Simplicillium*, endophytic from coffee plants, emitting antimicrobial volatiles

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Abstract

The genus *Simplicillium* was described by Gams W. & Zare to accommodate morphologically similar groups of Cordycipitaceae with exclusively, or predominantly, solitary phialides that had formed a monophyletic basal lineage to the other taxa of *Lecanicillium***.** Like some members of Cordycipitaceae, certain *Simplicillium* species exhibit antimicrobial activity against important plant pathogens. The aim of this study is to describe a new species of *Simplicillium* obtained in healthy tissue from coffee plants, to evaluate its antimicrobial activity against *Aspergillus* species through the emission of antimicrobial volatiles. Two *Verticillium*-like isolates from coffee branches were obtained by a parallel growth technique using *M. albus* CZ 620 as a reference strain. micro-morphological characteristics and phylogenetic relationships inferred by Bayesian inference analyses showed that *Simplicillium* sp. CDA 734 is a new taxon of *Simplicillium*. In the test for antimicrobial activity, the mixture of volatiles emitted by *Simplicillium* sp. CDA 734 inhibited growth in *Aspergillus ochraceus*, *A. tubingensis*, *A. sydowii* and *A. niger* on PDA. Among the compounds identified in the volatile mixture

emitted by *Simplicillium* sp. CDA 734, 1-Propanone, 1-(5-methyl-2-furanyl)-, Cyclopropane, 1-ethoxy-2,2-dimethyl-3-(2-phenylethynyl)-, and 2-Propenoic acid, 3-(2-formyl-4-methoxyphenyl)-, ethyl ester, (E)- were those with the highest percentage of peak per area. In conclusion, in this study, we have described a new species of *Simplicillium,* which is endophytic in coffee plants, with potential for use as a biocontrol agent through the emission of antimicrobial volatiles.

Keywords: new species, Cordycipitaceae, antifungal volatiles

Introduction

The genus *Simplicillium* was described by Gams W. & Zare (Zare and Gams 2001) to accommodate morphologically similar groups consisting of four species with exclusively, or predominantly, solitary phialides that had formed a monophyletic basal lineage to the other taxa of *Lecanicillium* according to ITS sequences (Zare et al. 2000). Both the genera, *Simpliciilium* and *Lecanicillium*, were segregated from the former *Verticillium* sect. *Prostrata* W. Gams that was introduced by Gams (1971) for species producing prostrate conidiophores and are often poorly differentiated by a fine, white or yellowish mycelium.

Simplicillium morphology resembles *Lecanicillium*, but with mostly solitary phialides arising from aerial hyphae, usually prostrate and little differentiated from the subtending hyphae. These phialides are discrete, aculeate and narrow, with a very narrow tip. Conidia adhering in globose slimy heads or imbricate chains, are short-ellipsoidal to subglobose or obclavate, not cyanophilic (Zare and Gams 2001). *Simplicillium* was allocated to the Cordycipitaceae family when Sung et al. (2007) sought to refine the classification of *Cordyceps* and Clavicipitaceae validated the family Cordycipitaceae according to the type of *Cordyceps militaris* and phylogenetic relationships based on analyses consisting of five to seven loci, including the nuclear ribosomal small and large subunits (nr*SSU* and nr*LSU*), the elongation factor 1α (*tef1*), and the largest and second largest subunits of RNA polymerase II (*rpb1* and *rpb2*), β-tubulin (*tub*), and mitochondrial ATP6 (*atp6*).

Currently, the genus *Simplicillium* consists of nine species, *S. lanosoniveum* (F.H. Beyma) Zare & W. Gams, *S. obclavatum* (W. Gams) Zare & W. Gams, *S. lamellicola* (F.E.V. Sm.) Zare & W. Gams, *S. chinense* F. Liu & L. Cai, *S. subtropicum* Nonaka,

Kaifuchi & Masuma, *S. minatense* Nonaka, Kaifuchi & Masuma, *S. cylindrosporum* Nonaka, Kaifuchi & Masuma, *S. aogashimaense* Nonaka, Kaifuchi & Masuma, and *S. sympodiophorum* Nonaka, Kaifuchi & Masuma. Although the taxonomy of *Simplicillium* was studied by using materials collected from around the world, the majority of isolates examined were from the continent of Asia (Zare and Gams 2001; Liu and Cai 2012; Nonaka et al. 2013).

Simplicillium species have been isolated from different environments such as soil, freshwater, plants, and other parasitizing fungi (Liu and Cai 2012; Nonaka et al. 2013) and like other members of Cordycipitaceae that include entomopathogenic and mycoparasitic Ascomycetes, certain *Simplicillium* species exhibit antimicrobial activity against important plant pathogens. For example, the type of *S. lamellicola* that was isolated from *Agaricus bisporus* (edible mushrooms) (Zare and Gams 2001) and other isolates of the species have shown antimicrobial activity against bacteria and fungi plant pathogenics (Le Dang et al. 2014). *Simplicillium. lanosoniveum* is another example of *Simplicillium* with antimicrobial activity which has been isolated as a hyperparasitizing fungus that causes rust plant disease, and has been used as a biological control agent for rusts, such as *Hemileia vastatrix*, *Phakopsora pachyrhizi* and *Uromyces pencanus* (Ward et al. 2011; Ward et al. 2012).

The antimicrobial activity of *Simplicillium* isolates against other fungi has been characterized mainly by parasitism. Blum (2006) defined parasitism as the activity that a number of microorganisms frequently express through infection (penetration and colonization), enzymatic degradation and consumption of the nutrients belonging to the cells of other organisms. However, in addition to parasitism, other biological control mechanisms may be associated with the antimicrobial activity of *Simplicillium*, such as the emission of volatile organic compounds (VOCs) with antimicrobial action, as in the

case of *Muscodor* species that emit a specific mixture of VOCs capable of inhibiting growth or even killing a wide range of microorganisms (Strobel et al. 2001; Suwannarach et al. 2013). The aim of this study is to describe a new species of *Simplicillium* obtained in healthy tissue from coffee plants, to evaluate its antimicrobial activity against *Aspergillus* species through the emission of antimicrobial VOCs and to identify the compounds present in the mixture of volatiles.

Materials and methods

Isolation of endophytic fungi and screening to emission of antimicrobial volatiles

Leaves and branches intact and healthy of *Coffea arabica* L plants were collected in the Atlantic Forest biome at Parque Estadual da Serra do Brigadeiro (PESB) forest reserve (20° 42′ 55″ S, 42° 26′ 51″ W), located in the Zona da Mata region, Minas Gerais state, Brazil. The plant tissue was gently washed with tap water, cut into fragments of 0.5 x 0.5 cm of size, disinfested according Zhang et al. (2010) by immersion in 75% ethanol for 30s, followed by immersion in 1% sodium hypochlorite for 10 min and finally washed three times in sterile distilled water. The isolation of endophytic fungi proceeded according to the parallel growth isolation technique, adapted of Worapong et al. (2001) and Ezra et al. (2004). Both sides of a two compartment plastic Petri dish were loaded with Potato Dextrose Agar (PDA). A micelial plug of 5 mm diameter from a 10 days old culture of *Muscodor albus* CZ 620 producing antimicrobial volatile metabolites was inoculated on one side of the dish and grown for 10 days at 20 ± 2 ºC in absence of light. Plant fragments was then placed on the other side of the dish. The dish was sealed with pvc plastic film and incubated at 20 ± 2 ºC in absence of light. Fungal hyphae emerging

from the fragments were transferred to another Petri dish without the presence of *M. albus* CZ 620 and incubated in the same previous conditions.

Screening for production of VOCs with antimicrobial properties was performed by the ability of endophytic fungi inhibit the growth of *Rhizopus stolonifer* (Ehrenb.) Vuill., *Botrytis cinerea* Pers and *Aspergillus ochraceus* G. Wilh through of parallel growth technique previously mentioned. Both sides of a two compartment plastic Petri dish were loaded with PDA. A micelial plug of 5 mm diameter from a 10 days old culture of endophytic fungi supposedly producing antimicrobial volatile metabolites was inoculated on one side of the dish and grown for 10 days at 20 ± 2 ºC in absence of light. A micelial plug of 5 mm diameter from a 7 days old culture of plant pathogenic fungus was then placed on the other side of the dish. The dish was sealed with pvc plastic film and incubated at 20 ± 2 ºC in absence of light for 48 h. The influence of endophytic isolate on the growth of plant pathogenic fungi was assessed by the presence or absence of mycelial growth in the inoculated dishes.

Comparative morphological studies

The isolates were grown in potato dextrose agar (PDA, Sigma-Aldrich), 2% malt extract agar (MEA, Kasvi) and synthetic nutrient deficient agar (SNA) with and without presence of dry and autoclaved plant tissue (corn stover, branches and leaves pine) at 25ºC to induce the formation of reproductive structures. Micro-morphological characteristics were observed with a light microscope (Olympus BX53, Japan).

DNA extraction, PCR amplification, sequencing and phylogenetic analysis

Genomic DNA was extracted from colonies originated from a single spore, grown in PDA at 25º C with twelve daily hours of light for 7 days. Fungal mycelium was scraped in the colony margins and DNA extraction proceeds according to protocol established by Pinho et al. (2012) whose the genomic DNA is extracted by Wizard® Genomic DNA Purification Kit (Promega Corporation, WI, U.S.A.) with adaptations.

Sequences of the Internal Transcribed Spacer (ITS) and partial 28S (rRNA) regions of rDNA were amplified using primers ITS1 and ITS4, LR0R and LR5, respectively (White et al. 1990; Vilgalys and Hester 1990). Polymerase Chain Reaction (PCR) products were purified and sequenced in Macrogen (South Korea). Primers used for sequencing were the same used for amplification of the fragments. Nucleotide sequences were edited on software BioEdit 7.2.5 (Hall 2013). All sequences were verified manually and ambiguous nucleotide positions were clarified using sequences of both DNA strands. The sequences were subjected to analysis on Basic Local Alignment Search Tool (BLAST) on GenBank - National Center for Biotechnology Information (NCBI) and UNITE database. Sequences with high similarity to each locus were taken along with representatives of each fungal species obtained in this study. Sequences obtained in this study and sequences take on GenBank – NCBI were aligned using MUSCLE (Edgar 2004) and manually corrected on software MEGA 7.0 (Kumar et al. 2016). Alignment regions with ambiguous sequences were excluded from the analysis and Gaps (insertions and deletions) were treated as missing data.

Phylogenetic analysis of Bayesian Inference were performed on CIPRES portal (Miller et al. 2010) using MrBayes v 3.2 (Ronquist et al 2012), based on Markov Monte Carlo Chain (MCMC) with 10,000,000 generations using the nucleotide substitution

model informed by the Akaike Information Criterion (AIC) on software MrModeltest v.2.3 (Posada and Crandall 1998). Trees were sampled every 1000 generations, burning 25% of all trees obtained. Posterior probabilities (Rannala and Yang 1996) were determined in the most consensus tree among the 7500 remaining trees.

Antifungal activity of VOCs emitted by *Simplicillium* sp. nov. against *Aspergillus* species

The antifungal activity of VOCs emitted by *Simplicillium* sp. was tested against *Aspergillus* species often associated with coffee beans by adaptation of the parallel growth technique described by Worapong et al. (2001) and Ezra et al. (2004). Both sides of a two compartment plastic Petri dish were loaded with PDA. A micelial plug of 5 mm diameter from a 10 days old culture of *Simplicillium* sp. was inoculated on one side of the dish and grown for 10 days at 20 ± 2 ºC in absence of light. A micelial plug from the margin of 7 days old culture of *Aspergillus* sp. was then placed on the other side of the dish. The dish was sealed with pvc plastic film and incubated at 20 ± 2 ºC in absence of light for 6 days. *Aspergillus* sp. growing without the presence of *Simplicillium* sp. was the control. The inhibition percentage of *Aspergillus* sp. was measured through growth rate of colony and its viability was investigated by subculture of the pathogen on tested dish into a non-treated PDA dish. The experiment was repeated twice with five replicates.

VOCs emitted by *Simplicillium* sp. nov.

The VOCs emitted by *Simplicillium* sp. were identified by solid-phase micro-extraction gas chromatography and mass spectroscopy (SPME-GC/MS), adapted of Strobel et al (2001). The VOCs were extracted with a SPME syringe (SULPECO, USA), 50/30 µm divinylbenzene/carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) on StableFlex fiber (SULPECO, USA) of headspace above a 10 days old culture of *S.* coffeanum CDA 734 grown on PDA in a headspace vial of 20 mL. The SPME fiber was placed through a small hole drilled in the vial septum, and the adsorption was allowed to continue for 60 min with the fiber cooled by liquid nitrogen (CF-SPME), as described by Moreira et al. (2014).

The analysis of VOCs was conducted with a Finnigan Trace DSQ GC/MS equipped with an ion trap mass spectrometer from Thermo Scientific (West Palm Beach, FL, USA); equipped with a HP-5MS column (30 m × 0.25 mm × 0.25 µm) with a helium flow of 1.5 mL min^{-1}. The injector was operated in splitless mode for 10 min with an injector temperature of 250 °C. The oven temperature ramp started at 50 °C, increased to 70 °C at a rate of 5 °C min^{-1}, maintained for 1 min and then increased to 280 °C at a rate of 10 °C min^{-1}, maintained for 2 min. The mass spectrometer was operated in electron ionization mode (EI) with energy of 70 eV. Data acquisition and data processing were performed by software systems. The VOCs produced by *S. coffeanum* CDA 734 were identified by comparing the obtained mass spectra with the National Institute of Standards and Technology (NITS) library and by comparing the calculated Kováts retention index with compounds under higth quality match indicated by NITS library. Comparative analysis were conducted on vials control containing only PDA. The compounds present in the control were removed from data set obtained from the GC/MS of endophytic fungi.

Results

Two *Verticillium*-like isolates from coffee branches were obtained by a parallel growth technique using *M. albus* CZ 620 as a reference strain. They were classified as members of *Simplicillium* by using a combination of micro-morphological characteristics and ITS-5.8S BLAST sequences.

Bayesian inference analysis of partial 28S rDNA sequences derived from isolates obtained in this study and other closely related taxa showed the phylogenetic relationships between them (Fig. I). The isolates obtained in this study (*Simplicillium* sp. CDA 734 and *Simplicillium* sp. CDA 735) were grouped in the same cluster together with other species of *Simplicillium*. The dataset consisted of 38 sequences representing 12 genera of Cordycipitaceae, *Pochonia* (Clavicipitaceae), *Bionectria* (Bionectriaceae), *Calonectria* (Nectriaceae), *Hypocrea* (Hypocreaceae) and *Ceratocystis moniliformis* as out- group.

Phylogenetic relationships inferred by Bayesian inference analysis of ITS-5.8S sequences with all *Simplicillium* species described to date, *Simplicillium* sp., and other genera of Cordycipitaceae show that the isolates obtained in this study (*Simplicillium* sp. CDA 734 and *Simplicillium* sp. CDA 735) grouped in the same clade with *Simplicillium* species, are phylogenetically close but clearly distinct from *S. chinense* (Fig. II). The dataset consisted of 46 sequences representing 7 genera of Cordycipitaceae, including all *Simplicillium* species described to date, and *Pochonia chlamydosporia* (Clavicipitaceae) as out-group.

In the test for antimicrobial activity, the mixture of VOCs emitted by *Simplicillium* sp. CDA 734 inhibited growth in *Aspergillus ochraceus*, *A. tubingensis*, *A. sydowii* and *A. niger* on PDA (Table I). SPME/GC/MS analysis of the VOCs in the headspace above the mycelium from ten day old cultures on PDA revealed the presence of at least 11

volatiles that could possibly be identified by comparison with the NIST library and Kovats retention index (Table II). Among the compounds identified were 1-Propanone, 1-(5-methyl-2-furanyl)-, Cyclopropane, 1-ethoxy-2, 2-dimethyl-3-(2-phenylethynyl)-, and 2-Propenoic acid, 3-(2-formyl-4-methoxyphenyl)-, ethyl ester, (E)- were those with the highest percentage of peak per area.

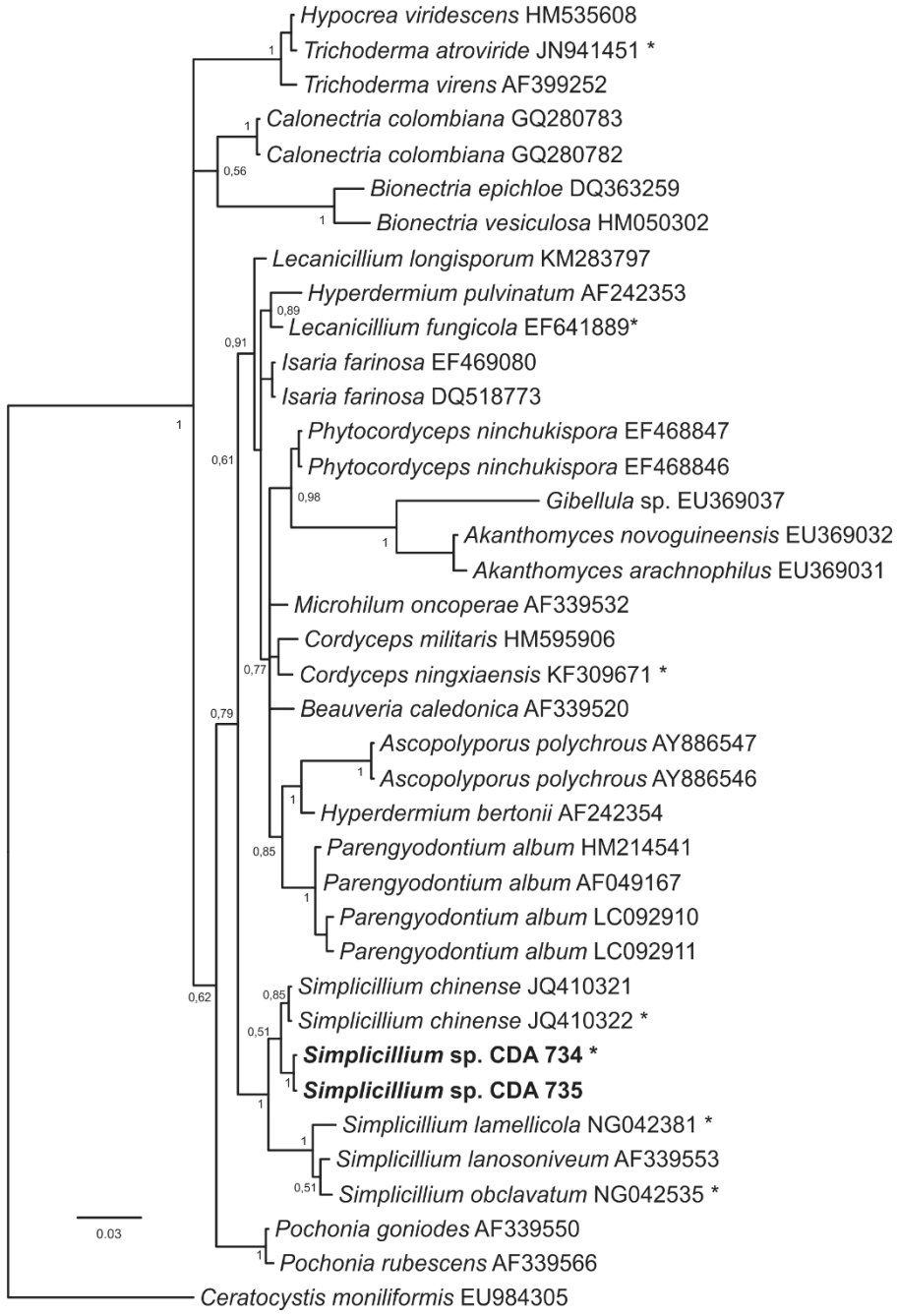

Fig. I Bayesian Inference tree showing the phylogenetic relationship between *Simplicillium* sp. and closely taxa based on partial 28S rDNA sequences. The posterior probability values are indicated at the nodes. The isolates from this study is highlighted in bold. The tree is rooted with *Ceratocystis moniliformis*. Asterisks indicate the type strains.

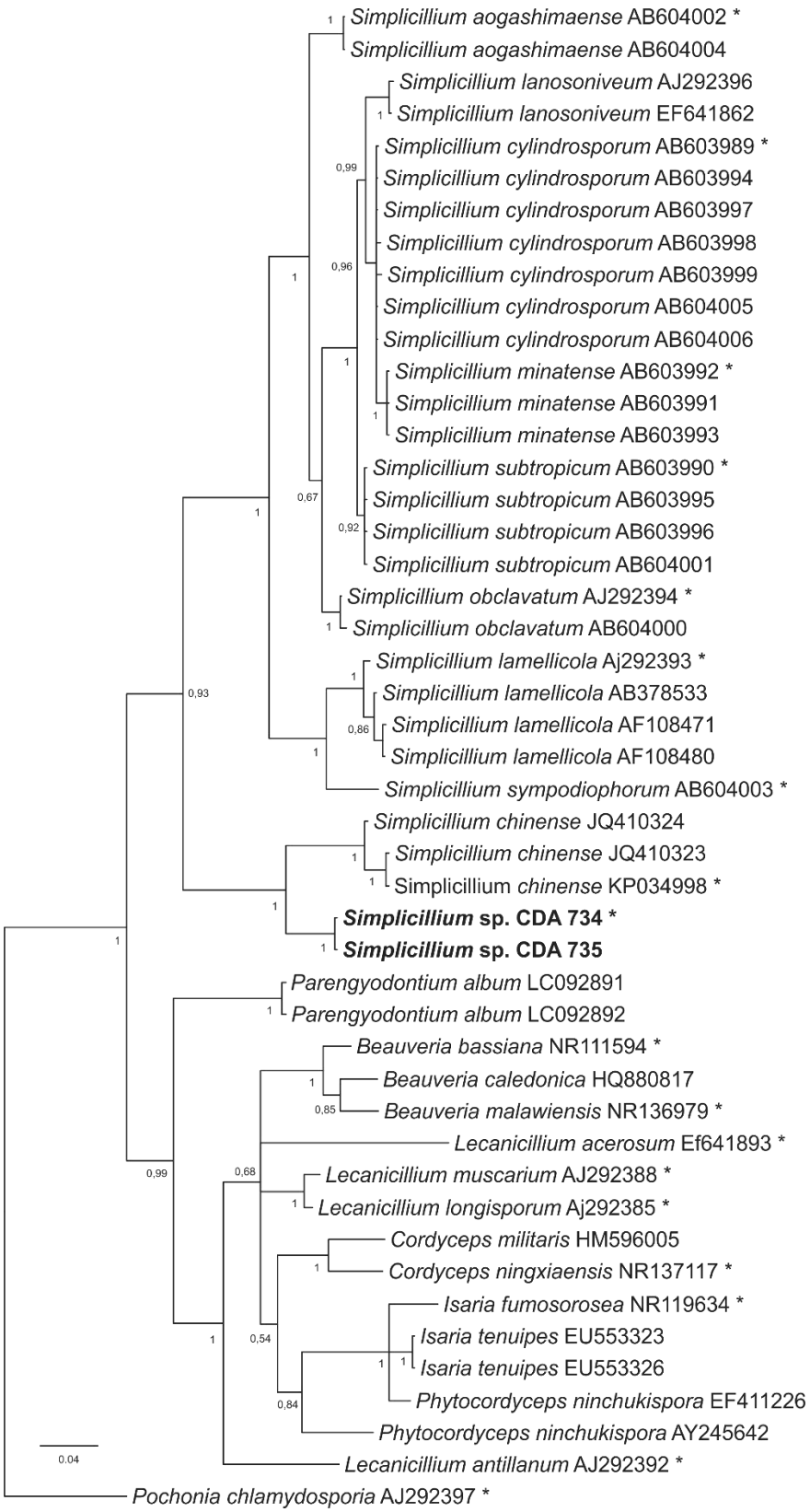

Fig. II Bayesian Inference tree of *Simplicillium* and closely Cordycipitaceae using ITS-5.8S sequences of rDNA. The posterior probability values are indicated at the nodes. The Simplicillium isolates from this study is highlighted in bold. The tree is rooted with *Pochonia chlamydosporia*. Asterisks indicate the type strains.

Table I. Effect of the VOCs emitted by *Simplicillium* isolates on the growth of *Aspergillus* species.

	Growth ratio after 6 days of exposure (% vs. control)	
Aspergillus species	*Simplicillium* sp. CDA 734	*Simplicillium* sp. CDA 735
A. ochraceus	0	78.1 ± 1.3
A. foetidus	100 ± 0.6	97.3 ± 1.2
A. flavus	100 ± 3.3	99.5 ± 2.0
A. tamari	100 ± 2.4	100 ± 1.1
A. tubingensis	67.1 ± 3.9	96.6 ± 1.3
A. sydowii	67.4 ± 1.4	27.0 ± 1.7
A. niger	49.0 ± 2.1	57.1 ± 1.7
A. caespitosus	96.6 ± 3.0	97.34 ± 4.6
A. expansum	99.0 ± 2.6	98.2 ± 1.8

Note: Growth ratio was calculated as the fraction of the value of colony diameter grown in the presence and in the absence of *Muscodor* isolate and expressed as percentage. Tests were repeated three times and means ± standard deviation were calculated.

Table II. GC/MS analysis of the volatile compounds emitted by *Simplicillium* sp. CDA 734.

RT (min)	Compound	Molecular formula	peak area %	Kovats RI	Calculated Kovats
8.93	1-Propanone, 1-(5-methyl-2-furanyl)-	$C_8H_{10}O_2$	9.94	1067	1017
14.02	12-Oxatetracyclo[4.3.1.1(2,5).1(4,10)]dodecane, 11-isopropylidene-	$C_{14}H_{20}O$	3.79	1272	1097
16.2	1-Dimethyl(pentafluorophenyl)silyloxycyclopentane	$C_{13}H_{15}F_5OSi$	1.28	1293	1172
18.3	3-Buten-2-one, 4-(2,5,6,6-tetramethyl-1-cyclohexen-1-yl)-	$C_{14}H_{22}O$	0.66	1519	1634
18.65	Cyclopropane, 1-ethoxy-2,2-dimethyl-3-(2-phenylethynyl)-	$C_{15}H_{18}O$	11.17	1582	1686
19.82	6-Methyl-5-oxo-5,8-dihydro-(1,2,4)-triazolo[3,4-c](1,2,4)-triazine	$C_5H_5N_5O$	0.76	1624	1819
20.45	(Z,Z)-3-Methyl-3H-cyclonona(def)biphenylene	$C_{18}H_{14}$	1.85	1882	1840
20.82	6-Methyl-5-oxo-5,8-dihydro-(1,2,4)-triazolo[3,4-c](1,2,4)-triazine	$C_{14}H_{16}N_2O$	1.65	1812	1852
22.77	2-Propenoic acid, 3-(2-formyl-4-methoxyphenyl)-, ethyl ester, (E)-	$C_{13}H_{14}O_4$	16.67	1857	1946
23.07	1H-Indole, 3-methyl-2-(2'-pyridyl)-	$C_{14}H_{12}N_2$	2.64	1921	1978
25.76	Unknown		21.49		2472

The compounds were identified by the similarity of mass and Kováts retention index whit NITS library. Level of confidence of 5% was adopted for Kováts RI comparison.

Taxonomy

Simplicillium sp. CDA 734 (to be proposed as new)

MycoBank: 00000

GenBank: 00000 (ITS), 00000 (LSU)

Systematic position: Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Cordycipitaceae.

Colonies white, compact, with moderate aerial mycelium, grows slowly on PDA, at a daily average of 3.2 mm, reaching 30-33 mm diam. in 10 days at 25 °C with a photoperiod of 12 hours, reverse cream-colored. Phialides produced on prostrate aerial hyphae, rarely in whorls of 2-3, gradually tapering towards the apex, 11-44 (-70) x 1.0 - 2.4 µm. Conidia in subglobose to ellipsoidal heads at the apex of the phialides. Usually the first-formed conidium is a macroconidium and the subsequent ones are microconidia. Macroconidia spindle-shaped, slightly curved, 1-celled, measuring 5.3–8.8 x 1.0-1.6 µm; microconidia cylindrical to fusiform, sometimes slightly curved, 1-celled, measuring 2.2-3.8 x 0.8-1.5 µm.

Notes: Morphologically, *Simplicillium* sp. CDA 734 resembles *S. lamellicola.* But the cylindrical to fusiform microconidia of *Simplicillium* sp. CDA 734 (2.2-3.8 µm) differ from oval to ellipsoidal and are longer than those of *S. lamellicola* (2.0-3.0 µm). *Simplicillium* sp. CDA 734 is phylogenetically close to *S. chinense,* but clearly distinct (Fig. II) and the conidia of *S. chinense* are formed in chains while macroconidia and microconidia of *Simplicillium* sp. CDA 734 are formed in heads.

Material examined: BRAZIL, Viçosa, in the region of Zona da Mata, state of Minas Gerais, on branches of *Coffea arabica*, D. B. Pinho, November, 2013, (VIC44077 to be proposed as **holotype**, to be proposed as **culture ex-type** COAD2057).

Additional specimens examined. BRAZIL, Viçosa, in the region of Zona da Mata, state of Minas Gerais, on branches of *Coffea arabica*, D. B. Pinho, November 2013: *Simplicillium* sp. CDA 735 (culture COAD 2061; ITS sequence GenBank 0000; 28S rRNA sequence GenBank 0000.

Etymology: Name reflects the host genus *Coffea*, from which the species was isolated.

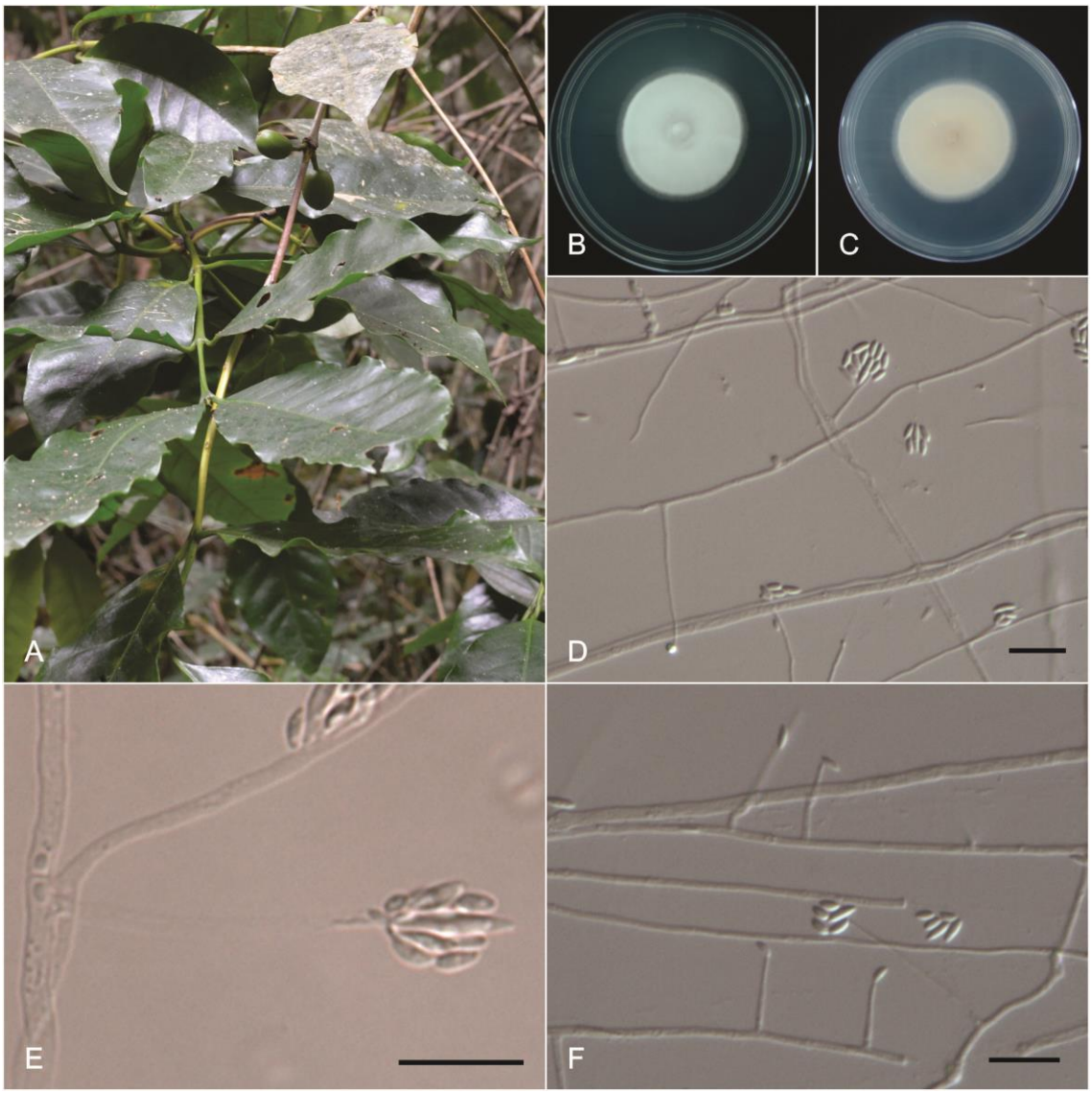

Fig. III. *Simplicillium* sp. CDA 734. **A**, Host plant; **B**, colony; **C -** Colony reverse; **D-F,** Hypha, phialides and conidia. Scale bars = 10 µm

Discussion

In selective isolation of endophytic fungi from coffee plants by the parallel growth technique with *M. albus* CZ 620 as the reference strain two isolates were obtained which in the screening for production of VOCs with antimicrobial properties, inhibited growth in *A. ochraceus*.

The isolates were identified as belonging to the genus *Simplicillium* by morphological comparisons with other similar taxa coupled with ITS-5.8S sequences BLAST. According to Nokada et al. (2013) species of *Simplicillium* can clearly be distinguished by using a combination of morphological characteristics and ITS sequences. However, phylogenetic analyzes were inferred to better position the isolates obtained in this study within *Simplicillium* and Cordycipitaceae. Bayesian inference analysis with partial 28S rDNA sequences from isolates obtained in this study and other closely related taxa showed that *Simplicillium* sp. CDA 734 have close affinities with other species of *Simplicillium.* Additionally, Bayesian inference analysis of ITS-5.8S sequences with all *Simplicillium* species described to date, showed that *Simplicillium* is a monophyletic group and *Simplicillium* sp. CDA 734 was placed within the *Simplicillium* clade as a new lineage (bootstrap = 100%), (Fig. II). The phylogenetic analyses coupled with morphological studies supported the introduction of the new species of *Simplicillium*.

Simplicillium sp. CDA 734 differs from the majority of the *Simplicillium* species described as it was isolated from coffee plants, and lives as endophytic in healthy coffee branches. Species of *Simplicillium* have been isolated principally from soil and other parasitizing fungi (Zare and Gams 2001; Nonaka et al. 2013). The mixture of VOCs emitted by *S. coffeanum* CDA 734 showed antimicrobial activity, reducing the growth

rate in *A. ochraceus*, *A. tubingensis*, *A. sydowii* and *A. niger* (fungi frequently associated with coffee beans). Members of Cordycipitaceae and other close Hypocreales such as Clavicipitaceae have been related to parasitizing fungi and insects (Ward et al. 2011; Ortiz-Urquiza et al. 2015). However, to our knowledge, this is the first time that a *Simplicillium* species has inhibited the growth of another organism by emission of antimicrobial VOCs.

The volatile profile emitted by *Simplicillium* sp. CDA 734 appears to be unique and different from the volatile profile of other fungi reported in its emission of antimicrobial VOCs. Fungi capable of emitting antimicrobial volatiles have been reported emitting different profiles of volatiles and this variation can occur even among isolates of the same species (Ezra et al. 2004). The characteristic mixture of volatile compounds produced by each species suggests adaptation to a unique ecological role in their respective ecosystems (González et al. 2009).

In conclusion, Cordycipitaceae species have demonstrated positive effects as biocontrol agents and *Simplicillium* species have been related to parasitizing fungi and plant parasitic nematodes (Ward et al. 2012; Le Dang et al. 2014). In this study, we have described a new species of *Simplicillium,* which is endophytic in coffee plants, with potential for use as a biocontrol agent through the emission of antimicrobial VOCs.

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CAPÍTULO IV

Controle de antracnose e do mofo cinzento do morango, em pós colheita, através da micofumigação com *Muscodor coffeanum* CDA 739: estratégia de micofumigação para controle de doenças pós colheita.

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Abstract

Postharvest infections caused by *Botrytis cinerea* and *Colletotrichum* spp. can generate significant production losses in fresh market strawberries. An alternative to the application of fungicides in postharvest disease control is the use of antimicrobial volatile organic compounds produced by fungi through Mycofumigation. The aim of this study was to evaluate the potential of *Muscodor coffeanum* CDA 739 in the control of gray mold and anthracnose rot in strawberry fruits by mycofumigation. Volatiles emitted by M. *coffeanum* CDA 739 inhibited the growth of *B. cinerea, C. acutatum,* and other phytopathogenic fungi *in vitro* tests, and reduced 75 and 100% respectively, the incidence of gray mold and anthracnose in strawberries inoculcated with these plant pathogens. The mixture of volatiles emitted by *M. coffeanum* CDA 739 revealed the presence of alcohols, sesquiterpenes, halogenated hydrocarbons and pyrazole derivatives via GC/MS analyses. Mycofumigation of strawberries inoculated with *C. acutatum* through the use of sachets containing rye colonized by *M. coffeanum* CDA 739 significantly reduced the incidence

of anthracnose. The mycofumigation with *M. coffeanum* CDA 739 is an alternative to control of gray mold and anthracnose in postharvest strawberry, and the elaboration of mycofumigation method through sachets containing substrate colonized by *M. coffeanum* CDA 739 makes possible its use in large scale.

Palavras chave: controle biológico, voláteis antifúngicos, método de micofumigação

1. Introdução

O morangueiro (*Fragaria* x *ananassa* Duch.), é uma planta herbácea pertencente à família Rosaceae, subfamília Rosoideae. Nativa das regiões de clima temperado da Europa e das Américas, atualmente é cultivado em mais de 60 países em diferentes regiões no mundo, sendo que as principais cultivares plantadas atualmente são híbridos oriundos de cruzamentos naturais das espécies *Fragaria virginiana* e *Fragaria vesca* (Stauct, 1988; Antunes et al., 2011)

A produção mundial de morango em 2014 foi de 8,11 milhões de toneladas, com os EUA se destacando como maior produtor (FAO, 2014). No Brasil, a produção de morango ainda é baixa, porém, vem crescendo gradativamente nos últimos anos, se concentrando principalmente nos estados de Minas Gerais, São Paulo e Rio Grande do Sul (Dias et al., 2007; Antunes e Peres, 2013)

O morango, é uma das "frutas' mais apreciadas em todo mundo, destacando-se pela sua coloração, aroma e sabor, que o faz ser altamente demandado tanto para consumo "in natura" quanto para o processamento industrial. No entanto, diversos fatores estão associados à sua produção, dentre eles a incidência de doenças, que afeta negativamente a produtividade e a qualidade dos frutos. Entre as doenças que afetam a cadeia produtiva do morango, o mofo cinzento e a antracnose dos frutos são de especial importância devido aos seus agentes causais poderem estar associados aos frutos em condições de campo e em pós colheita (Costa e Ventura, 2006).

O mofo cinzento do morangueiro é causado pelo fungo *Botrytis cinerea*, e ocorre de forma generalizada em regiões onde se cultiva morango, a doença é caracterizada pela formação sobre os frutos de uma massa de micélio de cor cinza, de onde vem o nome da doença (Simon et al., 2005; Costa e Ventura, 2006).

A antracnose em frutos de morango causada por *Colletotrichum* spp. tem sido constatada em diversas regiões produtoras de morango no Brasil, incidindo em frutos em qualquer fase de seu desenvolvimento. Os sintomas nos frutos são caracterizados pela formação de lesões deprimidas de consistência firme, de coloração escura. Em condições de alta umidade e temperatura adequada, são observadas massas rosadas de esporos do patógeno sobre as lesões (Tanaka et al. 1994; Costa e Ventura, 2006)

Na agricultura convencional, essas doenças são geralmente controladas por tratamentos com fungicidas que são aplicados próximo à floração e repetidos até a colheita, dependendo da incidência da doença e do intervalo de carência de aplicação do fungicida (Romanazzi et al., 2013). No entanto, a utilização intensiva de agrotóxicos para o controle de doenças, tem, reconhecidamente promovido diversos problemas de ordem ambiental, como a contaminação da água, do solo e dos alimentos (Schirra et al., 2011). A ANVISA (2013) destaca a preocupação com alto índice de resíduos agroquímicos que permanecem em produtos hortícolas que chega à mesa do consumidor.

Uma alternativa para controle de doenças de plantas, especialmente doenças pós colheita em frutos, é a utilização de compostos orgânicos voláteis antimicrobianos produzidos por fungos através da micofumigação. O conceito de micofumigação vem sendo estabelecido após a descrição de *Muscodor albus* Worapong, Strobel e W.M. Hes, *Ascomycota* que se destaca na produção de compostos voláteis antimicrobianos de amplo espectro contra patógenos humanos e de plantas (Worapong et al., 2001).

O objetivo deste trabalho foi avaliar o potencial de compostos voláteis antimicrobianos emitidos por *Muscodor coffeanum* CDA 739 no controle do mofo cinzento e podridão de antracnose em frutos de morango orgânico por meio da micofumigação com *M. coffeanum* CDA 739.

2. Material e Métodos

2.1. Isolado utilizados

Muscodor coffeanum CDA 739, foi previamente isolado de plantas de café no Brasil (Hongsanan et al., 2015), e mantido em Batata Dextrose Ágar a 4 ºC. *Botrytis cinerea*, *C. acutatum* e demais isolados fitopatogênicos utilizados neste trabalho foram cedidos pela Coleção de Cultura de Microrganismos do Departamento de Ciências dos Alimentos – UFLA e Coleção Octávio Almeida Drummond - UFV.

2.2. Frutos de morango

Morangos orgânicos da cultivar San Andreas foram utilizados para realização dos ensaios (*in vivo*) de controle do mofo cinzento e da podridão de antracnose em pós colheita. A colheita dos morangos foi realizada no mesmo dia de montagem dos experimentos.

2.3. Bioensaio (*in vitro*) de compostos voláteis produzidos por *M. coffeanum* CDA 739 contra fungos fitopatogênicos

A atividade antimicrobiana de compostos voláteis produzidos por *M. coffeanum* CDA 739 foi testada contra *Aspergillus niger, A. ochraceus, A. flavus, B. cinerea, C. acutatum, Fusarium semitectum, Penicillium expansum* e *Rhizopus stolonifer,* através da técnica de crescimento paralelo (Worapong et al., 2001; Ezra et al., 2004), com modificações. Ambos compartimentos de uma placa de Petri de plástico com uma subdivisão foram preenchidos com batata dextrose ágar (BDA). Disco de 5 mm

(diâmetro) contendo micélio de *M. coffeanum* CDA 739 foi inoculado em um dos compartimentos da placa, e incubado por 10 dias à 20 ± 2 ºC, na ausência de luz. Disco de micélio da margem de uma cultura de 7 dias do fungo fitopatogênico foi posteriormente inoculado no outro compartimento da placa de Petri. A placa foi selada com filme plástico pvc e incubada à 20 ± 2 ºC, na ausência de luz, durante 6 dias. Discos contendo micélio do fungo fitopatogênico em placa de Petri sem a presença de *M. coffeanum* CDA 739 foi o controle. A porcentagem de inibição de cada fitopatógeno foi mensurada através da taxa de crescimento da colônia e sua viabilidade foi avaliada pela remoção do disco de micélio do fitopatógeno exposto aos voláteis de *M. coffeanum* CDA 739 e sua deposição em outra placa de Petri com BDA sem a presença de *M. coffeanum* CDA 739. O experimento foi repedido duas vezes com cinco repetições.

2.4. Identificação dos compostos voláteis emitidos por *M. coffeanum* CDA 739

Os compostos produzidos por *M. coffeanum* CDA 739 foram identificados por cromatografia gasosa acoplada à espectrometria de massas com micro extração em fase sólida (SPME/GC/MS) de acordo com Strobel et al. (2001), com adaptações. Os voláteis foram extraídos com uma seringa SPME (SULPECO, USA), 50/30 µm divinylbenzene/carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) em fibra StableFlex (SULPECO, USA) do espaço acima de uma cultura de *M. coffeanum* CDA 739 cultivada em BDA em frasco de headspace de 20 mL. A fibra SPME foi colocada através de um pequeno orifício perfurado no septo do frasco, e a adsorção foi contínua por 60 min com a fibra resfriada com nitrogênio líquido, como descrito por Moreira et al. (2014).

Os voláteis foram analisados em um Finnigan Trace DSQ GC/MS equipado com espectrômetro de massas ion trap da Thermo Scientific (West Palm Beach, FL, USA),

equipado com uma coluna HP-5MS (30 m × 0.25 mm × 0.25 μm) com fluxo de hélio de 1,5 mL min^{-1}. O injetor foi operado no modo splitless por 10 min, com temperatura de 250 ºC. A rampa de temperatura do forno iniciou em 50 ºC, aumentando para 70 ºC a uma taxa de 5 ºC min^{-1}. Mantida por 1 min e posteriormente aumentada para 280 ºC a uma taxa de 10 ºC min^{-1}, mantida por 2 min. O espectrômetro de massas foi operado no modo de ionização eletrônica (EI) com energia de 70 eV. Os dados de aquisição e processamento foram processados no software do sistema. Os voláteis produzidos por *M. coffeanum* CDA 739 foram identificados através da comparação de seu espectro de massas com os da biblioteca do National Institute of Standards and Technology (NITS) e pela comparação do índice de retenção de Kováts calculado para cada composto presente na amostra com o índice de Kováts de compostos com alta similaridade de massas indicados pela biblioteca NITS. Análises comparativas foram realizadas em frascos contendo apenas BDA. Os compostos presentes no controle foram removidos do conjunto de dados obtidos da GC/MS de *M. coffeanum* CDA 739.

2.5. Eficiência de compostos voláteis produzidos por *M. coffeanum* CDA 739 no controle do mofo cinzento e da podridão de antracnose em frutos de morango.

O experimento foi conduzido com morangos (cv. San Andreas), produzido em sistema de cultivo orgânico, cidade de Ervália-MG, Brasil. Frutos livres de injúrias e doenças foram selecionados e colocados em caixas plásticas (36,5 x 26 x 13 cm). Os frutos foram inoculados de acordo com Capobiango et al., (2016), com modificações. Cada fruto foi equidistantemente ferido 5 vezes com uma alça de inoculação esterilizada. Posteriormente, 10 μL de suspensão de conídios de *B. cinerea* (1×10^5 ml^{-1}) ou *C. acutatum* (1×10^5 ml^{-1}) foram pipetados nos ferimentos. Frutos inoculados com 10 μL

de água destilada autoclavada formam usados como o controle da inoculação (Controle 1). O efeito à exposição de voláteis emitidos por *M. coffeanum* CDA 739 foi testado ao colocar 10 placas de Petri (60 x 15 mm), contendo cultura de 10 dias de *M. coffeanum* CDA 739 em BDA, nas caixas plásticas. O controle foi realizado com morangos inoculados com fitopatógenos e sem a presença de *M. coffeanum* CDA 739 (Controle 2). As caixas foram fechadas e mantidas a 19 ± 2 ºC com 12 horas diárias de luz por 5 dias. Foram avaliados o número de frutos com lesão e tamanho da lesão. O delineamento experimental utilizado foi inteiramente casualizado com 5 repetições (caixa contendo 24 frutos). O experimento foi repetido uma vez.

2.6. Estabelecimento de estratégia de micofumigação com *M. coffeanum* para controle de doenças pós colheita em frutos. Antracnose de morango como modelo de controle de doença

Para aprimorar a micofumigação com *M. coffeanum* e estabelecer uma tecnologia compatível com a produção em larga escala foi testado método de cultivo e aplicação de *M. coffeanum* via sachês. *M. coffeanum* CDA 739 foi cultivado em grãos de centeio (triturados e autoclavados) durante 21 dias a 20 ± 2 ºC, na ausência de luz. Grãos colonizados por *M. coffeanum* CDA 739 foram depositados em sachês de tecido não tecido (TNT9 6 x 7 cm, 5 g por sachê. O efeito da micofumigação no controle de podridão de antracnose do morango foi testado através da exposição de frutos inoculados com *C. acutatum* (como anteriormente mencionado) aos voláteis emitidos por *M. coffeanum* CDA 739 colonizando grãos de centeio em caixas plástica gerbox (11 x 11 x 3 cm) hermeticamente fechadas (Fig. I). Foram testadas 4 diferentes concentrações do agente antifúngico (0, 5, 10, 15, g de grãos de centeio colonizado por caixa). Frutos não

inoculados com *C. acutatum* foram utilizados no controle. As caixas foram fechadas e lacradas com filme plástico pvc e mantidas a 19 ± 2 ºC com 12 horas diárias de luz por 5 dias. Foram avaliados o número de frutos com lesão de antracnose e o tamanho da lesão. Delineamento experimental inteiramente casualizado foi utilizado com 3 repetições (cada repetição consistiu em 3 caixas gerbox contendo 8 frutos cada).

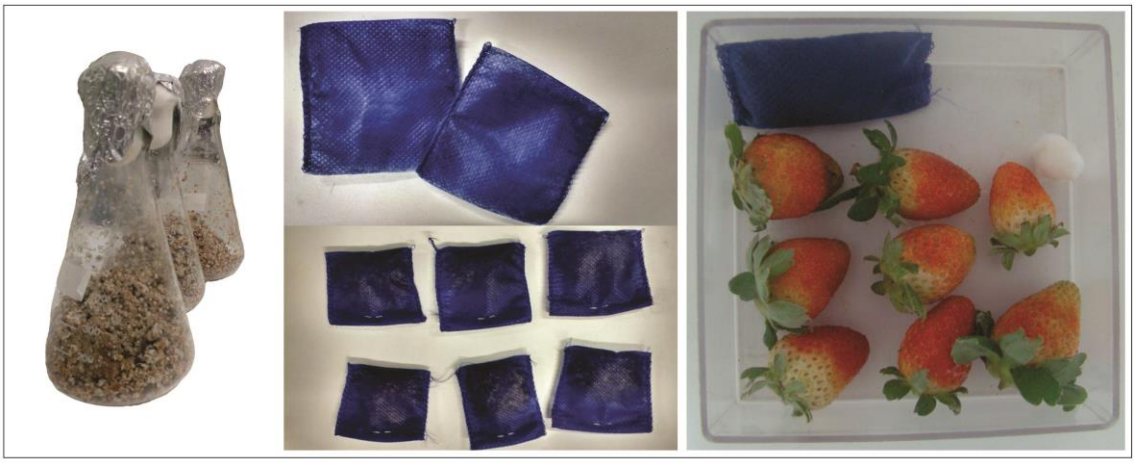

Fig. I. Estratégia de micofumigação com sachês contendo grãos de centeio colonizados por *M. coffeanum* CDA 739.

2.7 Análises Estatísticas

Os dados obtidos foram submetidos a análise de variância (ANOVA) e a significância dos tratamentos foi determinada por Tukey's HSD para múltiplas comparações ($P \leq 0.05$).

3. Resultados

3.1. Efeito dos voláteis emitidos por *M. coffeanum* CDA 739 contra fungos fitopatogênicos

Com exceção de *R. stolonifer,* os voláteis produzidos por *M. coffeanum* CDA 739 mostraram atividade antifúngica contra todos os outros fungos fitopatogênicos testados no bioensaio (*in vitro*) através da técnica de crescimento paralelo. O crescimento micelial de *B. cinerea*, *C. acutatum*, *A. flavus*, *A. niger*, *A. ochraceus, P. expansum* e *F. semitectum* foi 100% inibido pela exposição aos voláteis emitidos por *M. coffeanum* CDA 739. Apenas para *A. niger* e *P. expansum,* não foi observado crescimento quando esses foram transferidos para outra placa de Petri sem a presença de *M. coffeanum* CDA 739, após 6 dias de exposição.

3.2. Análise dos voláteis emitidos por *M. coffeanum* CDA 739

Analises de SPME/GC/MS revelaram o perfil de voláteis emitidos por *M. coffeanum* CDA 739. Através da comparação de espectros de massas obtidos com os da biblioteca NITS e pela comparação do índice de retenção de Kováts calculado para cada composto presente na amostra com o índice de Kováts de compostos com alta similaridade de massas indicados pela biblioteca NITS foi possível identificar pelo menos 11 das unidades voláteis presente na mistura emitida por *M. coffeanum* CDA 739 (Tabela I).

Tabela I. Análise de SPME/GC/MS dos compostos emitidos por *M. coffeanum* CDA 739

RT (min)	Composto	Formula molecular	Área do pico %	Kovats RI estimado	Kovats calculado
7,96	Phenylethyl Alcohol	$C_8H_{10}O$	5,89	1116	989
13,53	Cyclosativene	$C_{15}H_{24}$	1,68	1125	1090
15,59	Cyclosativene	$C_{15}H_{24}$	7,66	1125	1150
16,19	1-Dimethyl (pentafluorophenyl)silyloxycyclopentane	$C_{13}H_{15}F_5OSi$	0,74	1200	1171
17,17	Bis(2-chloroethyl)ethylamine	$C_6H_{13}Cl_2N$	1,76	1117	1236
18,73	Phenyl-1,2-diamine, N,4,5-trimethyl-	$C_9H_{14}N_2$	9,01	1530	1698
19,27	5H-2,3-Benzodiazepine, 1-phenyl-	$C_{15}H_{12}N_2$	0,83	1990	1800
20,75	Benzoic acid, 4-phenoxy-	$C_{13}H_{10}O_3$	1,72	1913	1850
21,54	4-Amino-6-methoxy-2-trifluoromethylquinoline-3-carbonitrile	$C_{12}H_8F_3N_3O$	1,35	1951	1875
22,24	1H-Pyrazole-4-carboxaldehyde, 5-chloro-3-methyl-1-phenyl-	$C_{11}H_9ClN_2O$	1,76	1822	1896
22,76	2-Methyl-5-methoxy-3-(β-aminopropyl)-indole	$C_{13}H_{18}N_2O$	4,74	1920	1954

3.3. Controle do mofo cinzento e antracnose em frutos de morango através da micofumigação com *M. coffeanum* CDA 739

A atividade antifúngica de *M. coffeanum* CDA 739 foi testada contra morangos artificialmente inoculados com *B. cinerea* e *C. acutatum* (10^5 conídios/mL). Em tratamento controle, onde não ocorreu a exposição dos morangos inoculados aos voláteis emitidos por *M. coffeanum* CDA 739 a incidência de mofo cinzento e antracnose nos frutos foi de 75 e 100% respectivamente. Micofumigação com *M. coffeanum* CDA 739 cultivado em BDA foi significativamente efetiva contra os dois fitopatógenos testados (*B. cinerea* e *C. acutatum*). A exposição aos voláteis emitidos por *M. coffeanum* CDA 739 inibiu completamente *C. acutatum,* evitando o surgimento de antracnose nos frutos

micofumigados, e reduziu em 81% o desenvolvimento de mofo cinzento causado por *B. cinerea* (Fig II).

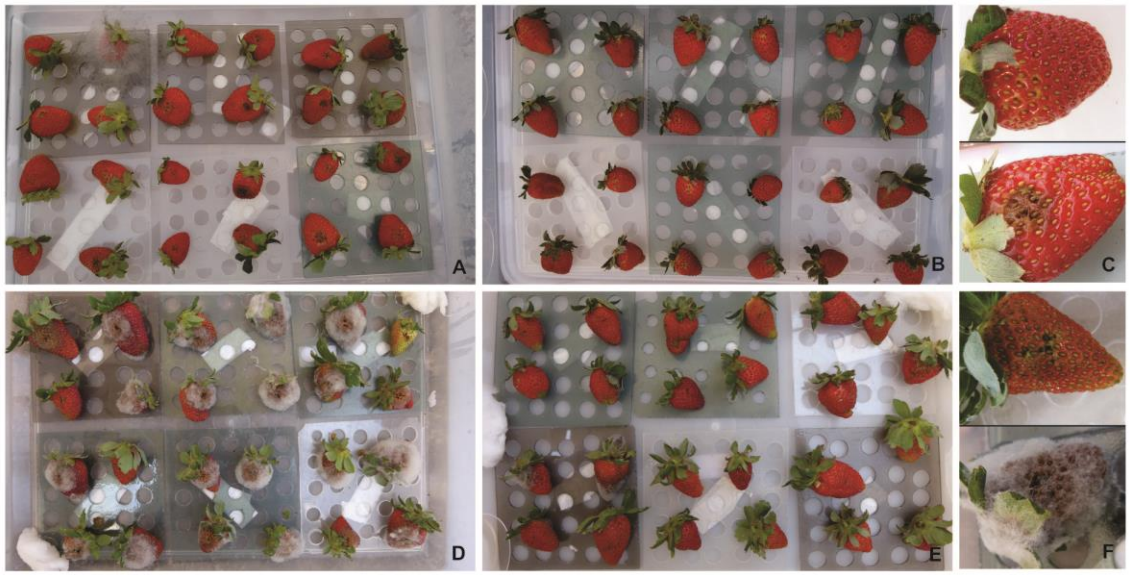

Fig. II. Micofumigação de frutos inoculados com *C. acutatum* e *B. cinerea* com *M. coffeanum* CDA 739. A- frutos de morango inoculados com *C. acutatum* e sem tratamento com *Muscodor*; B- frutos de morango inoculados com *C. acutatum* e micofumigado com *M. coffeanum* CDA 739; C- contraste do efeito da fumigação em frutos inoculados com *C. acutatum*; D- frutos de morango inoculados com *B. cinerea* e sem tratamento com *Muscodor*; E- frutos de morango inoculados com *B. cinerea* e micofumigado com *M. coffeanum* CDA 739; F- contraste do efeito da fumigação em frutos inoculados com *B. cinerea.*

3.4. Micofumigação com sachê contendo *M. coffeanum* CDA 739

Intenso crescimento micelial foi observado sob os grãos de centeio inoculados com *M. coffeanum* CDA 739, demonstrando a capacidade do fungo em crescer nesse substrato. A capacidade de micofumigação de *M. coffeanum* CDA 739 cultivado em grãos de centeio foi avaliada contra frutos de morango inoculados com *C. acutatum*. A deposição de sachês de TNT, contendo grãos de centeio colonizados por *M. coffeanum* CDA 739, em caixas gerbox contendo morangos, diminuiu significativamente a incidência de antracnose, entre 41,6% a 57,3 %. Entre as concentrações testadas do

micofumigante (5g, 10g e 15g de centeio colonizado por caixa gerbox), não ocorreu diferença significativa da incidência de antracnose e tamanho da lesão (Fig. III).

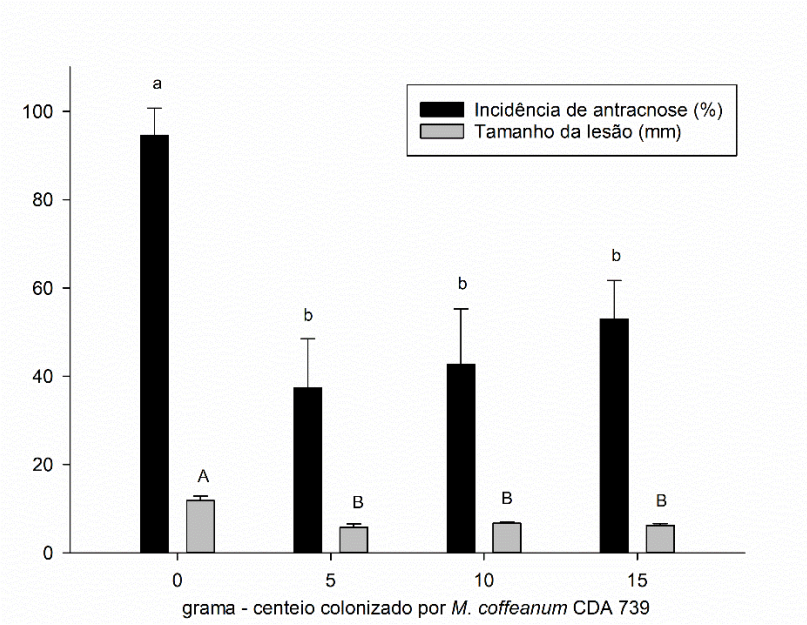

Fig. III. Efeito da micofumigação, com sachê contendo grãos de centeio colonizados por *M. coffeanum* CDA 739, em frutos de morango inoculados com *C. acutatum*. Barra preta representa a incidência da doença em porcentagem. Barra cinza representa o tamanho da lesão em milímetros. Barra de erro representa o desvio padrão das repetições. Letras diferente em minúsculo ou maiúsculo representa diferença significativa ($P \leq 0.05$) entre os tratamentos para cada variável.

4. Discussão

Muscodor coffeanum CDA 739, fungo endofítico isolado de plantas de café no Brasil (Hongsanan et al., 2015), foi testado quanto à sua atividade antifúngica, através da micofumigação contra duas importantes doenças pós colheita em morango, causadas pelos fungos *B. cinerea* e *C. acutatum.* Em ensaios *in vitro*, voláteis emitidos por *M. coffeanum* CDA 739 inibiram o crescimento de *B. cinerea* e *C. acutatum* e de outros fungos frequentemente associados a doenças em plantas. Outras espécies de *Muscodor* foram relatadas emitindo voláteis antimicrobianos capazes de matar ou inibir o crescimento de micro-organismos fitopatogênicos *in vitro* (Strobel, 2006; Suwannarach et al., 2013). São diversos os compostos encontrados e relatados no perfil de voláteis

emitidos por espécies de *Muscodor*, sendo que a composição da mistura de voláteis parece variar entre as espécies e até mesmo entre isolados de uma mesma espécie de *Muscodor* (Ezra et al., 2004).

A mistura de voláteis emitidos por *M. coffeanum* CDA 739 revelou a presença de álcoois, sesquiterpenos, hidrocarbonetos halogenados e derivados de pirazole. Derivados de pirazole são conhecidos por apresentarem ampla diversidade de propriedades biológicas, tais como anti-inflamatórias, antimicrobianas, antioxidante, antifúngica, etc. (Bekhit e Abdel-Aziem, 2004; Damljanović et al. 2009)

A micofumigação com *M. coffeanum* CDA 739 em frutos de morango inoculados com *B. cinerea* e *C. acutatum* diminuiu significativamente a incidência de mofo cinzento e antracnose, respectivamente. Efeito significativo de micofumigação com *M. albus* já foi observado no controle de podridões pós colheita em maçã, pêssego, e uva (Mercier e Jiménez, 2004; Mercier et al., 2010). Li et al., (2015) observaram controle de podridões pós colheita em citros, causadas por *Monilia fruticola* e *Penicillium digitatum*, após micofumigação dos frutos com *Ceratocystis fimbriata*.

Na tentativa de estabelecer um método eficaz que possa ser utilizado em larga escala para tratamento de doenças pós colheita em frutos, foi desenvolvida uma estratégia de micofumigação com *M. coffeanum* CDA 739, através de sachês de TNT contendo grãos de centeio colonizados por *M. coffeanum* CDA 739. Para validação desse método utilizou-se a micofumigação de morangos inoculados com *C. acutatum* como modelo de controle de doença. A micofumigação de morangos inoculados com *C. acutatum,* através da utilização de sachês contendo centeio colonizado por *M. coffeanum* CDA 739, reduziu significativamente a incidência de antracnose (41,6% a 57,3 %). Embora a micofumigação com *M. coffeanum* CDA 739 em placas de BDA tenha sido mais eficiente,

inibindo 100% a incidência de antracnose nos frutoss, o estabelecimento do método de micofumigação com sachês possibilita a sua aplicação em larga escala.

Esta é a primeira vez que é demonstrado o potencial de *M. coffeanum* para controlar podridões pós colheita. A eficiência da micofumigação com *M. coffeanum* CDA 739 no controle do mofo cinzento e da podridão de antracnose em frutos de morango demostra o potencial de *M. coffeanum* para ser utilizado contra outras doenças pós colheita. A elaboração de método de micofumigação através de sachês contendo substrato colonizado por *M. coffeanum* CDA 739 possibilita a sua utilização em larga escala. Entretanto, novos estudos estão sendo conduzidos com intuito de avaliar a eficiência da micofumigação com sachê de *M. coffeanum* CDA 739 para outras doenças pós colheita, assim como, aperfeiçoar o método com a utilização de novos substratos e tempo de exposição.

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CONCLUSÕES GERAIS

Três espécies de *Muscodor* (*M. coffeanum*, *M. yucatanensis* and *Muscodor* sp.) ocorrem endofíticamente em cafeeiro no Brasil. Além de *M. coffeanum* também ocorrer endofíticamente em plantas de carqueja.

E efeito da micofumigação "*in vitro"* utilizando *M. coffeanum* é dependente da espécie de *Aspergillus* testada. Adicionalmente a micofumigação com *M. coffeanum* CDA 741 inibiu o crescimento de *A. ochraceus* inoculado em grãos de café.

Simplicillium sp. CDA 734*,* endofítico em plantas de café, será proposta como nova, regendoo Código Internacional de Nomenclatura para algas, fungos e plantas.

Simplicillium sp. CDA 734 inibiu, o crescimento de *A. ochraceus*, *A. tubingensis*, *A. sydowii* e *A. niger* através da micofumigação *in vitro*.

Em micofumigação de morangos orgânicos inoculados com *Colletotrichum acutatum* e *Botrytis cinerea*, *M. coffeanum* CDA 739 diminui 100 e 81% a incidência de antracnose e mofo cinzento nos frutos, respectivamente.

A micofumigação com sachê contento grãos de centeio colonizados por *M. coffeanum* CDA 739 diminuiu significativamente a incidência de antracnose em frutos inoculados com *C. acutatum,* viabilizando a sua utilização em larga escala para controle de doenças pós colheita em morango através da micofumigação.

A micofumigação é uma alternativa promissora para reduzir as perdas pós colheita em frutas e hortaliças causadas por fungos. O método tem potencial para ser aplicado durante o transporte e armazenamento de frutas e hortaliças frescas, podendo aumentar o tempo de prateleira desses produtos pela redução na incidência de doenças pós colheita.