LARISSA MAIA DE OLIVEIRA

BIOLOGICAL CONTROL OF *Botrytis cinerea* IN THE POST-HARVEST OF STRAWBERRY FRUITS BY *Muscodor* sp.

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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Fitopatologia, área de concentração Controle Biológico, para obtenção do título de Mestre.

Orientador
Dr. Jorge Teodoro de Souza

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CONTROLE BIOLÓGICO DE *Botrytis cinerea* EM PÓS- COLHEITA DE MORANGO POR *Muscodor* sp.

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APROVADA em 13 de abril de 2017.

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LAVRAS - MG

2017

Aos meus pais Luiz Carlos e Rita de Cássia, à minha irmã Laís e à minha filha Helena pelo amor incondicional

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“Grandes realizações não são feitas por impulsos, mas por uma soma de pequenas realizações”

Vincent Van Gogh

RESUMO

O mofo cinzento, causado por *Botrytis cinerea*, é uma das doenças mais importantes na pós colheita do morango. Este patógeno é responsável por grandes perdas em morangos embalados. Esporos latentes podem estar presentes na superfície dos frutos ainda no campo, mas os sintomas podem aparecer apenas depois da colheita. Para o controle do mofo cinzento no campo, são realizadas aplicações de fungicidas desde o início da floração. O morango é um dos frutos que mais possuem resíduos de agrotóxicos. Atualmente não existem fungicidas registrados para aplicação após a colheita dos morangos. Assim, a busca por agentes de controle biológico tem sido foco de grupos de pesquisas. Fungos que produzem compostos orgânicos voláteis (COVs) tem se mostrado eficiente no controle de fitopatógenos em diferentes sistemas. O objetivo deste trabalho foi isolar, selecionar e desenvolver um produto biológico a base de fungo produtor de COVs com capacidade de controlar *B. cinerea* em morangos embalados. Entre os fungos endofíticos isolados de diferentes plantas hospedeiras e testados contra *B. cinerea*, o isolado FTB01 obtido de folhas da planta parasita *Struthanthus* sp. mostrou ser capaz de inibir completamente o crescimento micelial de *B. cinerea* pela produção de COVs. Vários substratos foram testados para otimizar o crescimento e a produção de COVs e grãos de milheto foram escolhidos devido a facilidade de encontrar estes grãos e pelo seu baixo custo. Experimento usando grãos de milheto triturados e misturados com ágar-água mostraram a capacidade do isolado FTB01 para controlar *B. cinerea,* variando de acordo com a temperatura e o tempo de incubação do fungo antagonista em relação ao patógeno. No entanto, quando grãos de milheto colonizados pelo isolado FTB01 foram utilizados para confeccionar *sachets* e esses utilizados contra *B. cinerea*, constatou-se inibição completa de *B. cinerea*, independente da temperatura utilizada. Dez COVs produzidos pelo isolado FTB01 colonizando milheto foram identificados por meio de cromatografia gasosa acoplada a espectrometria de massas. O isolado FTB01 foi identificado como uma espécie do gênero *Muscodor* baseado na ausência de estruturas reprodutivas e análise da sequência da região ITS do DNA ribissômico. Com base nos resultados, *Muscodor* sp. FTB01 é um potencial agente biológico para controlar *B. cinerea* em morangos embalados.

Palavras-chave: Mofo cinzento, morangos embalados, compostos orgânicos voláteis.

ABSTRACT

Gray mold, caused by *Botrytis cinerea*, is one of the most important post-harvest diseases in strawberry fruits. This pathogen is responsible for great losses in packaged strawberries. Latent spores may be present on the surface of fruits harvested in the field, but symptoms appear only in post-harvest. To control the disease in the field, fungicide applications begin at flowering. Strawberry is one of the fruits with the highest amounts of pesticide residues. Currently, there are no fungicides registered for applications in post-harvest. Thus, the search for biological control agents has been the focus of several research groups. Fungi that produce volatile organic compounds (VOCs) were shown to control phytopathogens in different systems. The objective of this study were to isolate, select and develop a biological product based on VOCs production fungus with the capacity to control *B. cinerea* in packaged strawberries. Among the endophytic fungi isolated from different host plants and tested against *B. cinerea*, isolate FTB01 obtained from leaves of the parasitic plant *Struthanthus* sp. has been shown to completely inhibit mycelial growth of *B. cinerea* by the production of VOCs. Several substrates were tested to optimize the growth and production of VOCs and millet grains were ideal because of its ease to find and its relative low cost. Experiments employing grinded millet grains mixed with water-agar showed that the capacity of isolate FTB01 to control *B. cinerea* varied according to the temperature and the time of incubation of the antagonistic fungus in relation to the pathogen. However, when whole millet grains colonized by isolate FTB01 were used to fill sachets and those employed against *B. cinerea*, complete inhibition of the pathogen was observed regardless of the temperature of incubation. Ten VOCs were identified in the headspace of isolate FTB01 grown on millet grains by gas chromatography coupled to mass spectrometry. Isolate FTB01 was identified as a species in the genus *Muscodor* on the basis of the lack of reproductive structures and sequence analysis of ITS region of the ribosomal DNA. Based on the results, *Muscodor* sp. FTB01 is a potential biological agent to control *B. cinerea* in packaged strawberries.

Keywords: Gray mold, packaged strawberries, volatile organic compounds.

SUMÁRIO

CAPÍTULO 1

Controle de *Botrytis cinerea* em pós-colheita de morango por voláteis produzidos por *Muscodor* sp.

1 INTRODUÇÃO GERAL

O Brasil é um grande produtor e exportador de frutas e em 2010 foram exportadas 759 mil toneladas de frutas frescas gerando 609 milhões de dólares, segundo o Instituto Brasileiro de Frutas. O morango é um pseudofruto de grande importância econômica e social e, no Brasil, os principais estados produtores são Minas Gerais, Rio Grande do Sul, São Paulo, Espírito Santo, Paraná, Santa Catarina e Distrito Federal. Em Minas Gerais, a produção está concentrada no sul do estado. Devido a sua importância social, os produtores vêm incorporando tecnologias ao cultivo com o objetivo de obter frutos de melhor qualidade e atender as demandas do mercado internacional (ANTUNES; REISSER JÚNIOR, 2007).

A cultura do morangueiro é caracterizada pelo alto custo de produção, devido ao uso intensivo de mão de obra e embalagens, porém a rentabilidade por área é alta (SPECHT; BLUME, 2009).

Considerado típico de agricultura familiar, o morangueiro é cultivado principalmente por pequenos produtores e, ainda que sejam utilizados muitos defensivos para o controle de pragas e doenças, os prejuízos causados por patógenos são enormes, sobretudo na pós-colheita dos frutos. Dentre as principais doenças comumente associadas a perdas na pós-colheita de frutos de morango estão *Botrytis cinerea*, *Rhizopus stolonifer e Penicillium digitatum* (BAUTISTA-BAÑOS et al., 2003).

Devido a alta perecibilidade, os morangos tem uma vida de prateleira curta, e como alternativa para minimizar as perdas, é feito o armazenamento à baixas temperaturas, visto que, em relação à fungicidas, ainda não se encontram defensivos registrados para aplicação após a colheita (AGROFIT, 2017). O armazenamento e a correta conservação possibilita uma melhor comercialização dos frutos (CHITARRA; CHITARRA, 2005).

Buscando controlar as doenças, são realizadas várias aplicações de fungicidas (MERTELY; MACKENZIE; LEGARD, 2002) e o uso abusivo de defensivos químicos tem causado vários problemas ambientais e à saude humana (ANDRIGUETO; KOSOSKI, 2002). Neste cenário, um dos desafios da agricultura tem sido a busca por alternativas que resultem em alimentos saudáveis, de uma maneira sustentável, segura e viável economicamente (KHAN; ANWER, 2011; ANDRIGUETO; KOSOSKI, 2002). Assim sendo, estudos visando obtenção de tratamentos alternativos têm ocupado espaço na área da pesquisa, e como exemplo, para o controle de doenças, tem-se o uso de óleos e extratos vegetais, indução de

resistência em plantas e controle biológico, sendo que este último abrange vários tipos de interações antagonistas, na qual fungos entomopatogênicos e fungos endofíticos estão em destaque (ESPOSITO; AZEVEDO, 2010).

Dentre os fungos endofiticos, *Muscodor* é um gênero que tem chamado a atenção pela sua capacidade de produzir uma mistura de compostos orgânicos voláteis (COVs) que inibem e matam efetivamente uma ampla gama de bactérias e fungos patogênicos a humanos e plantas, além de controlar também nematóides, insetos e bactérias (STROBEL et al., 2001; RIGA; LACEY; GUERRA, 2008; HUANG et al., 2011; KUDALKAR et al., 2012). A grande maioria das espécies de *Muscodor* produzem compostos orgânicos voláteis, sendo encontrando comumente o ácido propanóico, 2-metil, 1-butanol, 3-metil, fenetil álcool (KUDALKAR et al., 2012). O tipo de meio de cultura onde *Muscodor* é cultivado interfere no seu comportamento, fazendo-o produzir diferentes COVs (EZRA; STROBEL, 2003). Devido as suas características, *Muscodor* vem sendo estudado e apresenta grande possibilidade de ser um biofumigante eficiente no controle biológico, especialmente em pós-colheita de frutos.

Em virtude da necessidade de controle alternativo de doenças e que aumente a competitividade de frutas no mercado, o presente trabalho teve o objetivo de isolar, selecionar e desenvolver um produto biológico a base de fungo produtor de COVs para controlar *B. cinerea* em morangos embalados.

2 REFERENCIAL TEÓRICO

2.1 O mofo cinzento no morango

Botrytis cinerea, o agente etiológico do mofo cinzento, é um fungo fitopatogênico que causa doença em mais de duzentas espécies de plantas, dentre elas, o morango (WILLIAMSON et al., 2007). *B. cinerea* é um patógeno necrotrófico, que ataca folhas, pecíolos, caule, botões florais e pétalas e causa podridões nos frutos em campo ou em pós-colheita (YU et al., 2006). É o maior causador de perdas por podridão dos frutos, sendo essa podridão ocasionada pela secreção de enzimas que degradam a parede celular, tais como poligalacturonases, metilesterases de pectina, celulases e hemicelulases, e metabólitos fitotóxicos (CHOQUER et al., 2007).

Para o controle do mofo cinzento, são realizadas aplicações de fungicidas no campo desde o início da floração (MERTELY; MACKENZIE; LEGARD, 2002). Contudo, os esporos podem estar presentes na superfície do fruto de forma latente, e somente após a colheita apresentar os sintomas e sinais de infecção (COSTA; VENTURA, 2006). Este fitopatógeno produz bastante micélio aéreo e conídios os quais são dispersados de um fruto infectado para frutos sadios logo, um único fruto doente é capaz de comprometer todos os frutos embalados (MLIKOTA GABLER et al., 2006).

De acordo com a Anvisa (2017), o morango é um dos alimentos que apresentou alto índice de resíduos, e revelou também resíduos de produtos fitossanitários que não são registrados para a cultura. Tal fato ocorre devido ao uso incorreto de agrotóxicos e por não respeitarem o período de carência recomendado. Além de ser prejudicial à saude humana e ao meio ambiente, o uso indiscriminado de defensivos químicos pode induzir *B. cinerea* a desenvolver resistência aos fungicidas (DIÁNEZ et al., 2002; CHEN et al., 2007).

Com a falta de produtos registrados para aplicação na pós-colheita associada ao rápido metabolismo do morango, principalmente os que são comercializados *in natura*, a vida de prateleira é muito curta, e, logo, o desenvolvimento de alternativas seguras para o controle de doenças de pós-colheita é fundamental para prolongar o tempo de viabilidade comercial do morango. O controle biológico usando microrganismos com atividade anti-fúngica tem apresentado resultados promissores, podendo ser alternativa para controlar o mofo cinzento em pós-colheita de morango (HUANG et al., 2011).

2.2 Controle biológico

A redução de inóculo ou dos sinais e sintomas provocados por um patógeno, ocasionada por um ou mais organismo vivo, exceto o homem, pode ser definido como controle biológico segundo Cook e Baker (1983). Apesar de ser um conceito amplo, controle biológico é muito utilizado para se referir ao controle de um patógeno por um antagonista (MICHEREFF; ANDRADE; MENEZES, 2005). O agente de controle biológico ou antagonista tem a capacidade de interferir nos processos vitais dos patógenos (MICHEREFF; ANDRADE; MENEZES, 2005) e vários são os tipos de interações envolvidas nos mecanismos de biocontrole, como a competição, onde o agente de biocontrole cresce mais rápido que o patógeno no local de infecção; o parasitismo, na qual os organismos disputam

nutrientes (MELO; AZEVEDO, 1998); a premunização, que é o uso de um virus de estirpe fraca para proteger a planta antes da colonização de uma estirpe forte; a antibiose, onde os organismos secretam metabólitos com atividade antimicrobiana, podendo inibir o crescimento ou matar outros organismos (AMORIM; REZENDE; ARMANDO, 2010).

Há algumas décadas atrás, o controle biológico de pragas e doenças passou a ser foco de pesquisas na busca por tratamentos alternativos aos defensivos químicos, atedendo a demanda por alimentos mais saudáveis, sem resíduos de agrotóxicos, produzido de maneira sustentável e sem poluir o meio ambiente (BETTIOL et al., 2009; PEÑA, 2014). Em 2008 foi registrado o primeiro fungicida biológico comercial, à base de *Trichoderma harzianum*, sendo este um antagonista para o controle de doenças de plantas (BETTIOL, 2009). A comercialização de produtos biológicos, principalmente a base de fungos e bactérias, para o controle de pragas e doenças de plantas já é uma realidade no mercado de defensivos desde a década de 80 e a cada dia que passa vem ganhando mais espaço na agricultura (BETTIOL, 2009). Atualmente, existem mais de 70 produtos comerciais de biocontrole à base de fungos, bactérias e vírus registrados no Brasil, segundo a ABCBio (2017).

Com o aumento da demanda por produtos para o controle biológico, houve também o aumento na busca desses agentes de biocontrole com diferentes modos de ação (ZHANG et al., 2010). Alguns agentes de biocontrole possuem como mecanismo de ação a produção de COVs, que são metabólitos secundários, sendo estes compostos eficientes para inibir e matar patógenos de humanos e de plantas, e que já apresentam resultados promissores para o controle de fitopatógenos, especialmente durante o transporte e comercialização, onde tem-se um ambiente fechado (HUANG et al., 2011; EZRA; STROBEL, 2003).

Fungos do gênero *Muscodor* vem sendo descritos desde 2001 e tem sido foco de pesquisa devido a sua capacidade de produzir uma mistura de compostos orgânicos voláteis com ação antagonista à fitopatógenos (STROBEL, 2001). Vários estudos mostraram a sua eficiência como agente de biocontrole em pós-colheita de frutos (BANGUELA-CASTILLO et al., 2015; MLIKOTA GABLER et al., 2006), sendo uma alternativa de aplicação nos frutos após serem colhidos, visto que para algumas culturas há registro de produtos fitossanitários apenas para a pré-colheita, como é o caso do morango (AGROFIT, 2017).

2.3 Compostos orgânicos voláteis produzidos por fungos

Compostos orgânicos voláteis são substâncias de baixa molaridade e baixo peso molecular (VESPERMANN; KAI; PIECHULLA, 2007). Plantas, bactérias, fungos filamentosos e leveduras (podem produzir COVs através de processos metabólicos com capacidade de inibir e matar microrganismos (KULAKIOTU; THANASSOULOPOULOS; SFAKIOTAKIS, 2004; GU et al., 2007; KOITABASHI, 2005; HUANG et al., 2011; FIALHO et al., 2010; INSAM; SEEWALD, 2012). Os COVs são produzidos por diversos gêneros de fungos, como *Trichoderma* (BOMFIM, 2010), *Candida* (HUANG et al., 2011), *Fusarium* (JELEN et al., 1995), *Muscodor* (STROBEL et al., 2001) entre outros.

Espécies de fungos do gênero *Muscodor*, descritos recentemente, tem chamado a atenção pelo potencial em inibir e matar uma ampla gama de bactérias e fungos patogênicos a humanos e plantas, além de controlar também nematóides e insetos através desses compostos orgânicos voláteis produzidos (STROBEL et al.,2001; RIGA; LACEY; GUERRA, 2008; HUANG et al., 2011; KUDALKAR et al., 2012). Além de ser um mecanismo de defesa desenvolvido pelos fungos, os compostos orgânicos voláteis produzidos podem contribuir para diferenciar as espécies dentro do gênero *Muscodor*, visto que suas espécies apresentam variações quanto a produção de COVs (KUDALKAR et al., 2012).

Strobel e colaboradores (2001) relataram alguns microrganismos controlados por *M. albus,* como *Pythium ultimum, Phytophthora cinnamomi, Rhizoctonia solani, Ustilago hordei, Stagnospora nodorum, Sclerotinia sclerotiorum, Aspergillus fumigatus, Verticillum dahliae, Tapesia yallundae, Candida albicans, Escherichia coli, Staphylococcus aureus, Micrococcus luteus.* Trabalhando com *M. sutura*, Kudalkar e colaboradores (2012) obtiveram alguns microrganismos inibidos completamente, como *Aspergillus fumigatus, Botrytis cinerea, Colletrotrichum lagenarium, Ceratocystis ulmi, Cercospora beticola, Geotrichum candidum, Mycospharella fijiensis, Phythophthora cinnamomi, Phytophthora palmivora, Pythium ultimum, Rhizoctonia solani, Sclerotinia sclerotiorum, Verticillium dahliae.*

A grande maioria das espécies de *Muscodor* produzem COVs, sendo encontrando comumente o 1-butanol, 3-metil, fenetil álcool, ácido propanoico, 2-metil, dentre outros (KUDALKAR et al. 2012). Kudalkar (2012) analisou por GC-MS (*Gas Chromatography-Mass Spectrometry*) os COVs produzidos por diferentes espécies de *Muscodor* e comparou os resultados com os dados listados no NIST (*The National Institute of Standards and*

Technology). Para *M. sutura*, a maioria dos compostos voláteis encontrados foram ácido propanóico, 2-metil e thujopseno com ação anti-fungos; para *M. albus* ácido propanoico, 2-metil, naftaleno e derivados de azuleno, com ação anti-fungos e bactérias; para *M. vitigenus* somente naftaleno, com ação anti-insetos; para *M. crispans* ácido propanoico, 2-metil e vários ésteres, com ação anti-fungos e bactérias; e para *M. fengyangensis* derivados de azuleno e naftaleno e vários outros, com ação anti-fungos e bactérias (KUDALKAR et al., 2012). Para *M. yucatanensis* foram identificados COVs como octano, 2-metil butil acetato, 2-pentil furan, cariofileno e aromadendrene (MACIAS-RUBALCAVA et al., 2010).

Com a produção de COVs é possível a biofumigação utilizando produtos provenientes do cultivo de espécies de *Muscodor* e tem se mostrado eficiente no controle de patógenos de solo, sementes e principalmente em doenças associadas a pós colheita de frutos (MERCIER; JIMENEZ, 2007; SUWANNARACH et al., 2012). Fungos endofíticos do gênero *Muscodor* tem sido foco de pesquisas e os resultados mostram que estas espécies são promissoras na área de biocontrole, e consequentemente, a descoberta e caracterização de novos isolados tem ganhado espaço nos estudos (ZHANG et al., 2010). Além do mais, por produzirem COVs, a busca por novas moléculas com ação antimicrobiana e até mesmo herbicida tem sido considerada relevante.

Pesquisas que buscam encontrar alternativas para o uso de fungicidas químicos, especialmente para a pós-colheita de frutos, e que levam em consideração o fato de obter um bioproduto que não promova a contaminação ambiental, não ofereça riscos à saude humana e seja prático e viável, relatam a eficiência de bioprodutos à base de *Muscodor* e tem ganhado espaço em universidades públicas e empresas privadas.

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CAPÍTULO 2

Volatiles produced by *Muscodor* sp. in millet grains control *Botrytis cinerea* in packaged strawberries

To be submitted to Biological Control

ABSTRACT

Botrytis cinerea is a pathogen commonly associated with post-harvest losses of strawberry fruits. In this study the objective was to isolate, select and develop a biological product to control *B. cinerea* in packaged strawberries. Among the various endophytic fungi tested, one isolate (FTB01) from *Struthanthus* sp. was shown to completely inhibit mycelial growth of *B. cinerea* through the production of volatile organic compounds (VOCs). Millet grains were chosen as the ideal substrate for VOCs production because of its ease to find and its relative low cost. Grinded millet grains were mixed with water-agar and used as a growth medium to assess the capacity of isolate FTB01 to control *B. cinerea* in different inoculation times of the biological control agent in relation to the pathogen and on different temperatures. Inhibition of mycelial growth in this medium was influenced both by the time of incubation and temperature. However, when whole millet grains colonized by isolate FTB01 were used to fill sachets and those employed against *B. cinerea*, complete inhibition was observed, indepent of the temperature. A total of 10 VOCs were produced by the fungus and identified by gas chromatography coupled to mass spectrometry. The endophytic fungus has never produced spores in the culture media used and was identified as a species of the genus *Muscodor* on the basis of ITS (Internal Transcribed Spacer) sequencing. The use of sachets containing millet colonized by *Muscodor* is promising and can extend the shelf life of packaged strawberries.

Keywords: Gray mold, post-harvest, volatile organic compounds, biological control.

1 INTRODUCTION

The genus *Muscodor* was introduced in 2001 (Worapong et al., 2001) and currently contains 19 described species. These non-sporulating fungi are endophytic and may be isolated from various plant species (Worapong et al., 2001; Macías-Rubalcava et al., 2010; Daisy et al., 2002; Mitchell et al., 2010; Sopalun et al., 2003; Worapong et al., 2001, 2002; YUAN et al., 2011). The colony of the fungus has a whitish color and during its growth on Potato Dextrose Agar (PDA) exudes a strong odor (Macías-Rubalcava et al., 2010), which is a striking feature of the genus. *Muscodor* is closely related to *Xylaria*, another common endophytic genus (Worapong et al., 2001). *Muscodor albus*, the best-known representative species, is able to grow in a wide range of temperatures ranging from 3 to 25ºC in PDA (Schotsmans et al., 2008). During its growth it produces a variety of volatile organic compounds (VOCs), even at low temperatures (Mercier et al., 2010), and therefore has potential to be used in biofumigation.

The VOCs produced by *Muscodor* have activity against bacteria, fungi, oomycetes, insects and nematodes (Daisy et al., 2002; Grimme et al., 2007; Strobel et al. 2001; Strobel & Daisy, 2003; Worapong et al., 2001; Lacey & Neven, 2006). The bioactive molecules include alcohols, acids, esters, ketones and lipids (Ezra et al., 2004; Strobel, 2006). Compounds produced by *M. yucatanensis* on PDA were active against *Phytophthora capsici*, *P. parasitica*, *Rhizoctonia* sp. and *Alternaria solani* (Macías-Rubalcava et al., 2010). Differences in the production of VOCs may be observed among species and isolates (Sopalun et al., 2003). For *M. sutura* the compounds with antifungal activity normally found are propanoic acid, 2-methyl and thujopsene; in *M. albus* the most frequent were propanoic acid, 2-methyl naphthalene and azulene derivatives with antifungal and antibacterial activity; for *M. vitigenus* the most frequent is naphthalene with activity against insects; for *M. cinnamomi* the most frequent were propanoic acid, 2-methyl, methyl ester and β-humuleno; *M. crispans* produces propanoic acid, 2-methyl and various ester with antifungal and antibacterial effects; and in *M. fengyangensis* azulene derivatives and naphthalene were detected and these have antifungal and antibacterial activity (Kudalkar et al., 2012). Although the activity of the mixture of VOCs produced by individual isolates of *Muscodor* is easily demonstrated, determining the individual compound responsible for the effects may be a challenge.

The concentration and diversity of the VOCs produced by *Muscodor* spp. depends on the substrate used for growth (Ezra & Strobel, 2003). *Muscodor* spp. grown in rye and barley has been used in bioassays to select agents active in post-harvest and soil fumigation (Gabler et al., 2006; Mercier & Jiménez, 2004; Mercier et al., 2007; Stinson et al., 2003; Strobel et al., 2005). Other commonly used substrates are paddy rice (Banguela-Castillo et al., 2015), and PDA (Strobel et al., 2001).

Strawberries and many succulent organs suffer heavy losses in post-harvest caused by pathogens such as *Botrytis cinerea* (Bautista-Baños et al., 2003). According to Anvisa (2017), strawberry fruits contain high levels of toxic residues that result from an inadequate use of pesticides in the field. Even these high concentrations of pesticides are not enough to inhibit *B. cinerea* in post-harvest. Strawberries are marketed in packages wrapped with a plastic film that favors the growth and development of post-harvest fungi and for this reason its shelf-life is estimated in approximately five days (Henrique & Cereda, 1999). This sealed environment also favors the application of *Muscodor* species as biofumigants. Other authors have successfully demonstrated the activity of *Muscodor* species against *B. cinerea* in grapes (Mlikota Gabler et al., 2006; Mercier et al., 2010). There are some commercial products on the market based on *Muscodor*, such as ARABESQUE™, ANDANTE™, GLISSADE™ all with *M. albus* isolate QST 20799. However, in Brazil, there are no products registered with *Muscodor* sp.

In this study, we report the isolation and selection of a *Muscodor* strain that is able to effectively control post-harvest losses caused by *B. cinerea* in packaged strawberries. The volatiles produced by strain FTB01 of *Muscodor* were shown to be effective to inhibit mycelial growth and spore germination of *B. cinerea.*

2 MATERIAL AND METHODS

2.1 Isolation and selection of VOCs-producing fungi

Isolation of VOCs-producing fungi was done according to the method described by Strobel (2001). Samples of leaves, petioles and stems of *Mangifera indica, Siparuna guianensis, Morus* sp. and *Struthanthus* sp. were collected, cut in fragments of approximately 1 cm^2 and surface sterilized by immersing in distilled sterilized water for 30 sec, 70% ethanol for 30 sec, 2% sodium hipoclorite for 3 min, 70% ethanol for 30 sec, and rinsed in distilled

sterilized water for 30 sec. All samples were collected in the municipalities of Lavras and Ijaci, Minas Gerais State. These fragments were transferred to 9 cm diameter Petri plates containing PDA, where isolate HZM 39 of *M. vitigenus* grown for 3 days. This isolate was kindly provided by Dr. Olinto L. Pereira, Federal University of Viçosa, Viçosa, MG, Brazil. Plates were incubated at 25º C for approximately 12 days and all colonies that grew from the fragments were transferred to new plates, purified and kept in the refrigerator for further studies. Long-term storage was done by placing mycelial discs in sterile distilled water at 4ºC or in mineral oil at room temperature.

The isolates obtained from different host plants were tested for their ability to inhibit *B. cinerea in vitro*. For this, a 5 mm mycelial disc of each isolate was transferred to one of the sections of Petri plates divided into two sections containing PDA and two days later a mycelial disc of *B. cinerea* was transferred to the other section of the Petri plate. *B. cinerea* was obtained from the Mycological Collection of Lavras (CML) mycological collection (Federal University of Lavras, accession number 2317). The plates were wrapped with plastic film and incubated at 25ºC with 12 h photoperiod for 3 days. To verify the viability of *B. cinerea*, the mycelial discs exposed to the isolates were placed individually in new Petri plates containing PDA. Mycelial discs showing fungal growth after 3 days were considered viable.

2.2 Substrate selection

All experiments described below were done with isolate FTB01 selected as described above. To select the best substrate for growth and VOCs production, the fungus was grown on the following media: 1) malt extract agar (MEA) composed of 20 g of malt extract (Himedia) and 20 g of agar per litre; 2) potato dextrose agar (PDA; Himedia) at 39 g per litre; 3) rice; 4) maize; 5) millet medium; the last three composed of 166.7 g of grinded grain and 20 g of agar per litre. A 5 mm micelial disc of the fungus was transferred to one section of plates divided into two sections containing each of the media described above and three days later a mycelial disc of *B. cinerea* was placed on the other section of the Petri plate filled with PDA. The growth of *B. cinerea* was evaluated five days after transference and incubation at 25ºC with 12 h photoperiod.

2.3 Inhibition of mycelial growth and germination of conidia *in vitro*

Millet was chosen as the growth substrate for isolate FTB01 and its chemical composition is shown on table 1. Millet was used in two ways: 1) millet medium prepared with 166.7 g of grinded millet, 20 g of agar, and distilled water to one litre; 2) 100 g of whole millet grains and 100 mL of distilled water were placed in transparent plastic bags measuring 30 x 40 cm. Both millet medium and whole millet grains were autoclaved for 20 min at 120ºC. The bags containing whole millet grains were seeded with 15 mycelial discs of isolate FTB01 and incubated for 21 days at 25ºC. Whole grains colonized by isolate FTB01 were used to perform assays to study mycelial growth and germination of *B. cinerea* conidia. Mycelial growth and conidia germination of *B. cinerea* was determined on Petri plated divided into two sections in the following manner: one of the sections was filled with 10 ml of millet medium or 8 g of whole millet grains colonized by isolate FTB01 for 21 days and the other section of the Petri plates was filled with 10 ml of PDA. Mycelial discs of isolate FTB01 were only transferred to the millet medium and mycelial discs of *B. cinerea* were only transferred to the PDA medium. Plates with millet medium received micelial discs of isolate FTB01 and were incubated for 0, 2, 4 and 6 days at 5, 10, 15, 20, 25 and 30ºC. Following the incubation, mycelial discs of *B. cinerea* were placed on the PDA section of the Petri plates and incubated for three days in the different temperatures mentioned above and 12 h photoperiod. The assay was done in a factorial scheme with four incubation times, six temperatures and three replicates. In another assay, Petri plates were filled with whole grains colonized with isolate FTB01 for 21 days in one section and the PDA section received mycelial discs of *B. cinerea* on the same day and were incubated at 5, 10, 15, 20, 25 and 30ºC. A similar assay was conducted to evaluate conidia germination, but instead of PDA,

Table 1. Chemical composition of the millet grains used in this study.

Macronutrients ($g.Kg^{-1}$)					
N	**P**	**K**	**Ca**	**Mg**	**S**
30.33±0.81	6.20±0.95	4.93±0.81	0.77±0.15	1.93±0.15	0.93±0.06
Micronutrients ($mg.Kg^{-1}$)					
Zn	**Cu**	**Fe**	**Mn**	**B**	
53.30±1.11	6.47±0.70	110.40±5.43	15.10±0.61	6.83±1.59	
Total proteins ($g.kg^{-1}$)					
191.58±6.78					

water-agar 2% was used in the other section that received 100 µL of a suspension containing $2x10^5$ *B. cinerea* conidia/mL. Petri plates were incubated at 5, 10, 15, 20, 25 and 30ºC as described above and conidia germination was evaluated 48h after spreading the suspension. For all experiments described above the controls consisted of plates containing *B. cinerea* only. These assays were installed in a completely random design with three replicates.

2.4 Chemical analyses of VOCs produced by isolate FTB01

Volatiles produced by isolate FTB01 grown for 21 days at 25ºC on whole millet grains placed inside "Supelco" tubes were extracted through solid phase microextraction (SPME) and analyzed by gas chromatography coupled to a mass spectrometer (GC-MS Shimadzu QP 2010 Plus, Japan). Volatiles of whole millet grains without isolate FTB01 served as controls. SPME extraction was operated in the headspace mode for VOCs extraction with the following parameters: fiber DVB/CAR/PDMS (Divinylbenzene, Carboxen, and Polydimethylsiloxane), extraction temperature of 55 °C, extraction time of 35 min and desorption time in the GC injector of 1 min. A GC-MS QP 2010 Ultra (Shimadzu, Japan) gas chromatograph coupled to a mass spectrometer equipped with a HP-5 column (5% phenyl-95% dimethylsiloxane) with dimensions 30 m × 0.25 mm × 0.25 µm was used for separation and identification of the volatiles produced. The injector temperature was 250 °C, the interface temperature 240 °C and the ion source temperature 200 °C. The injector was operated in splitless mode. The carrier gas was He 5.0 at 1.0 mL min^{-1}. The temperature program of the GC oven was as follows: an increase from 40 °C to 160 °C at 3 °C min^{-1} and then an increase to 240 °C at 10 °C min^{-1}. Initial identification of the unknown volatiles produced by FTB01 was done as follow: the mass spectra of each peak in the chromatogram was extracted using the Automated Mass Spectral Deconvolution and Identification System (AMDIS) v.2.63 software. The identification of VOCs was performed by comparing the peak mass spectra in samples with spectra in the NIST (National Institute of Standards and Technology) Mass Spectral Library Search Program v. 1.7 (NIST, Washington, DC, USA) and by comparing the retention indices obtained experimentally (RI Exp.) with the retention rates in the literature (RI Lit.) (NIST, 2013; Adams, 2007). For comparing mass spectra, we considered only peaks with a spectra similarity greater than 80 %. The experimental retention indices were obtained by the injection of a homologous series of alkanes. Peaks were identified in the chro-

matograms only when mass spectra data agreed with the retention indices.

2.5 Control of *B. cinerea* in packaged strawberries

To assess the control of *B. cinerea* in packaged strawberries were made sachets with one face made of plastic and the other of paper were prepared. They were filled with 40 g of millet grains colonized for 21 days at 25ºC by isolate FTB01 (Figure 1). The sachets were placed with the plastic face upwards on the bottom of standard commercial plastic trays containing eight strawberries. The trays were incubated at 10, 15, 20ºC and 7 days later the incidence of *B. cinerea* was assessed. These assays were installed in a completely random design with three replicates containing eigth fruits per replication.

2.6 DNA isolation and identification

Isolate FTB01 was grown in liquid malt extract (ME) medium at 25ºC for 11 days. The mycelium was collected on filter paper, weighted and grinded in liquid nitrogen. DNA was extracted using the Wizard Genomic DNA Purification kit (Promega) according to the manufacturer's directions. A fragment of the internal transcribed spacer (ITS) of the ribosomal DNA was amplified and sequenced with primers ITS5 F (5' GGA AGT AAA AGT CGT AAC AAG G 3') and ITS4 R (5' GGT CCG TGT TTC AAG ACG G 3') as described by White (1990). The amplified fragment was visualized in 1% agarose gel electrophoresis and sequenced by the sanger method at Macrogen, Korea. Sequences were assembled with Sequencher v.5 and compared with other sequences deposited in public databases using BLASTn.

Figure 1. Sachet filled with 40 g of millet grains colonized by isolate FTB 01.

3 RESULTS

3.1 The VOCs-producing isolate is a species of *Muscodor*

A total of 5 fungal isolates were obtained from different plant species. From these, isolate FTB01 was the only one able to completely inhibit the growth of *B. cinerea* in *in vitro* bioassays and was selected for further studies. Isolate FTB01 presented a withish color in different culture media, including MEA, PDA and millet medium; absence of spores or any other reproductive structures and a strong odor. The sequence of the ITS region of the ribosomal DNA of isolate FTB01 was 98.7% identical when compared to *Muscodor yucatanensis* B110 (accession number FJ917287) and 99.8% identical when compared to *Muscodor coffeanum* CDA739 (accession number KM514680), confirming that this isolate belongs in the genus *Muscodor*.

3.2 *Muscodor* sp. FTB01 inhibits *B. cinerea in vitro*

The inhibition of *B. cinerea* mycelial growth varied according to the interval it was transferred to the Petri plates in relation to *Muscodor* and also according to the temperature of incubation in grinded millet medium (Table 2). When *B. cinerea* and *Muscodor* were

Table 2. *In vitro* mycelial growth of *Botrytis cinerea* exposed to *Muscodor* sp. FTB01 grown on grinded millet. These experiments were done in Petri plates divided into two sections. Millet medium were placed in one of the sections and PDA was placed in the other section. Mycelial discs of *Muscodor* were transferred to millet medium and PDA received mycelial discs of *B. cinerea* at different times. Plates were incubated at different temperatures for three days before the evaluation of mycelial growth.

Temperature	Grinded millet			
	Days of incubation			
	0	2	4	6
	Mycelium growth (cm)			
5ºC	2.04	0.76	1.08	0
10ºC	2.79	2.53	2.2	1.75
15ºC	3.23	0	0.68	0
20ºC	1.83	0.48	0	0
25ºC	1.50	0.49	0	0
30ºC	0.78	0	0	0

transferred to the plates at the same time (0 days) there was a lower inhibition of mycelial growth than when *B. cinerea* was trasnferred 2 to 6 days after *Muscodor* (Table 2). Lower temperatures (5-10ºC) were not favorable to the activity of *Muscodor* sp. when *B. cinerea* was transferred to the plates 0-4 days after *Muscodor*. When *B. cinerea* was transferred 6 days after *Muscodor*, there was no growth of *B. cinerea* mycelium, but not at 10ºC (Table 2). On the other hand, whole millet grains colonized for 21 days by *Muscodor* sp. FTB01 inhibited both mycelial growth and conidia germination of *B. cinerea*, independent of the temperature of incubation (Figure 2 and 3).

3.3 *B. cinerea* produces sclerotia upon exposure to *Muscodor* VOCs

Analyses of scanning electron microscopy images showed that the phytopathogen *B. cinerea* begins to produce primordia of sclerotia when exposed to *Muscodor* sp. FTB01 at 21 days at 20ºC. These structures were not observed in control plates (Figure 2 and Figure 4). Sclerotia completely formed were not observed because *B. cinerea* dies 1 day after exposure to *Muscodor* VOCs and does not recover after its transference to fresh plates.

Figure 2. *In vitro* assay to evaluate *B. cinerea* mycelial growth inhibition on plates by *Muscodor* sp. FTB01 grown for 21 days on whole millet grains and incubated at different temperatures. The upper panel shows the complete lack of *B. cinerea* mycelial growth on plates incubated at different temperatures with *Muscodor* sp. FTB01 on whole millet grains. The lower panel shows the mycelial growth of *B. cinerea* on control plates containing only autoclaved whole millet grains.

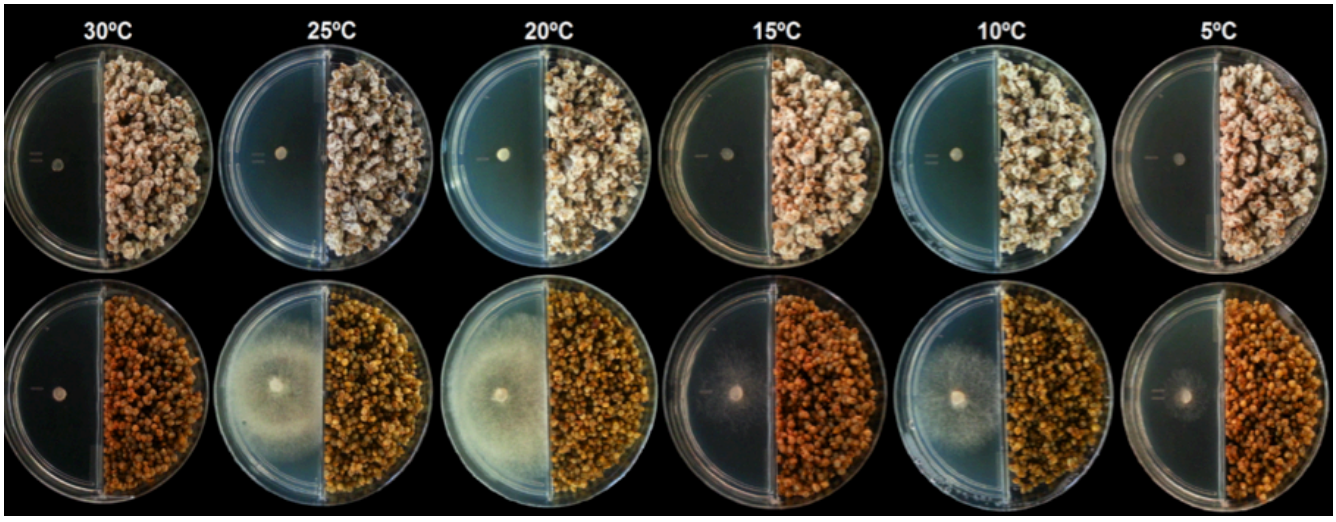
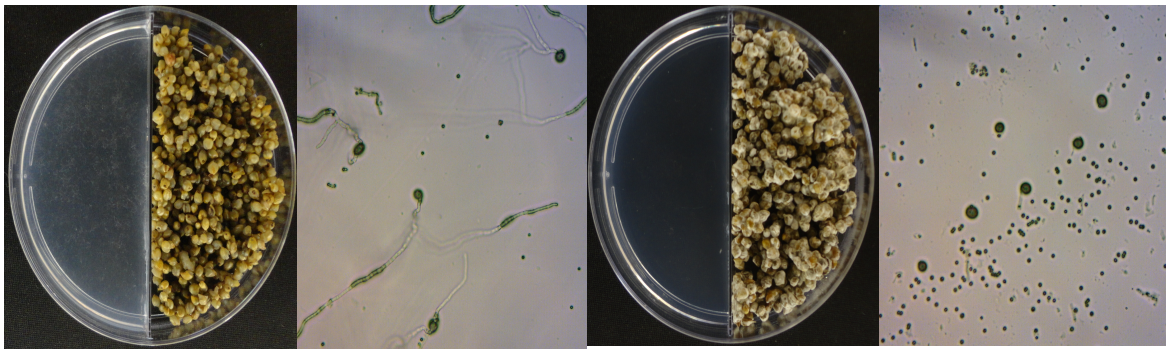


Figure 3. *In vitro* assay to evaluate *B. cinerea* conidial germination inhibition on plates by *Muscodor* sp. FTB01 grown for 21 days on whole millet grains and incubated at different temperatures. Image of germinated conidia of *B. cinerea* on control plates with autoclaved whole millet grains and non-germinated conidia on plates containing *Muscodor* on the other section of the Petri plate at 20ºC.



3.4 Chemical identity of the VOCs produced by *Muscodor* sp. FTB01

The compounds emitted by the *Muscodor* sp. FTB01 grown on the whole millet grains were identified by GC/MS. A total of 10 compounds were identified (Table 3) by comparison with the GC/MS library. These VOCs were not detected in the non-inoculated controls. It was not possible to determine the relative concentration of each compound in these analyses.

Figure 4. Scanning Electron Microscopy image showing the growth of *B. cinerea* in the untreated control (without *Muscodor*) and the formation of sclerotia primordia (black arrow) by *B. cinerea* exposed to the VOCs produced by *Muscodor* sp. FTB01 (with *Muscodor*).

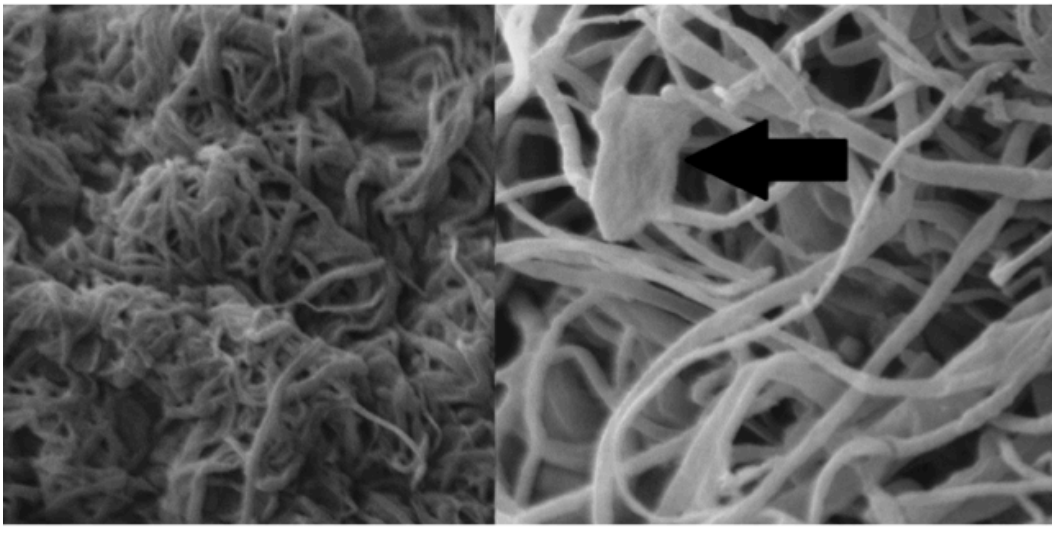


Table 3. VOCs produced by *Muscodor* sp. FTB01 on whole millet grains.

Peak	KI Calculated	KI Literature	Time	Compounds	Match
1			1.97	Ethanol	96
2			2.61	Acetic acid ethyl ester	97
3	733	731	4.21	1-Butanol, 3-methyl	96
4	737	739	4.30	1-Butanol, 2-methyl	96
5	753	756	4.61	Isobutyrate<ethyl>	94
6	846	848	7.12	Butanoic acid, 2-methyl, Ethyl ester	95
7	1000	1000	13.30	3-octanol	91
8	1123	1122	19.04	Phenethyl alcohol	88
9	1456	1456	34.18	Dehydroaromandendrene	94
10	1523	1532	36.92	Epiglobulol	88

3.5 Control of *B. cinerea* in packaged strawberries by *Muscodor* sp. FTB01

Seven days after incubation at 10, 15 and 20ºC the number of fruits with *B. cinerea* incidence was determined. Most fruits in the control showed no symptoms of *B. cinerea* whereas most fruits treated with *Muscodor* sp. FTB01 were healthy (Table 4 and Figure 5). At 20ºC, the control, treatment showed higher incidence of diseased fruits than the other temperatures (Figure 5).

Figure 5. Aspect of the strawberries treated with sachets containing whole millet grains colonized by *Muscodor* sp. FTB01 (treatment) and whole millet grains without *Muscodor* (control) at different temperatures. The upper panels at each temperature show the fruits at the beginning of the experiment (Time 0) and the lower panels show the fruits at the end of experiment (7 days later).

Table 4. Mean number of strawberry fruits with *B. cinerea* (incidence) at the beginning of the experiment (Time 0) and at the end of the experiment (7 days later) in different temperatures. Fruits were either treated with a sachet containing whole millet grains colonized by *Muscodor* FTB01 (treatment) or with a sachet containing millet grains without *Muscodor* (control). The values are means of 3 replicates containing 8 fruits per replicate.

	7 days after	
Temperature	***% of B. cinerea* incidence**	
(ºC)	**control**	**treatment**
10	75	0
15	96	0
20	100	0

4 DISCUSSION

Strawberries have a short shelf-life as fresh fruits because of the occurrence of *Botrytis cinerea* and other post-harvest pathogens (Labuza & Breene, 1989; Bautista-Baños et al., 2003). Attempts to control these pathogens with fungicides make strawberry one of the fruits with the highest amounts of pesticide residues (Anvisa, 2016). In this study, an endophytic fungus able to produce volatile organic compounds was obtained, identified as a species in the genus *Muscodor* and tested for its activity *in vitro* and *in vivo* against *B. cinerea. Muscodor* sp. FTB01 was able to extend the shelf-life of packaged strawberries depending on the temperature.

Our studies show that *Muscodor* is an endophyte easily found in diverse tree species. Five isolates were obtained and one was chosen as an excellent producer of volatiles able to completely inhibit *B. cinerea in vitro*. Other authors also report that this genus is found as an endophyte in tropical plant species (Mitchel at al., 2008; Peña, 2014; Banguela-Castillo et al., 2015; Gomes et al., 2015). The identification of isolate FTB01 as a member of the genus *Muscodor* was done on the basis of ITS sequences, because this is the only fragment of DNA available for most isolates reported to date. Isolate FTB01 was most closely related to *M. yucatanensis* and *M. coffeanum* with ITS sequences, but we preferred not to define a species because this region is widely known for its poor resolution to identify fungi at the species-level (Druzhinina & Kubicek, 2005; Samson et al., 2014). This appears also to be true for *Muscodor* because species delineation is not clear in several cases, such as in the study reported by Peña (2014) that observed the same ITS sequences for two isolates of *Muscodor*

with different sequences for a fragment of the *rpb*2 (RNA *Polymerase* II) gene. Therefore, other regions with more resolution need to be employed to improve species delineation in the genus *Muscodor*. Malkus (2006) has proven that a fragment of the *rpb*2 gene is useful in phylogenetic analysis of fungi at the species and genus level. We initiated studies to amplify a fragment of the *rpb*2 gene, which is available for a few species of the genus.

During the course of our studies, millet grains were chosen as an ideal substrate to grow *Muscodor* sp. FTB01 because the fungus is able to grow well on it and produce VOCs that inhibit *B. cinerea*, besides that, millet grains are ease to find and have a relatively low cost. In millet medium lower temperatures and shorter intervals of *B. cinerea* transference were not effective to suppress mycelial growth, especially at 10ºC. Other authors found similar effects for lower temperatures (Mlikota Gabler et al., 2006), however, no explanations were found for this behavior.

The fact that *B. cinerea* produces sclerotia primordia upon exposure to the VOCs produced by *Muscodor* is a possible response to the stressful conditions and an attempt to scape the noxious atmosphere created by the VOCs. In general, formation of sclerotia by fungi occurs under stress conditions (Butler, 1966).

Muscodor produces a mixture of VOCs and its composition depends on the species and the growth substrate. The VOCs produced by these fungi have been suggested to have taxonomical applications in species definition (Kudalkar, 2012). As examples, *M. albus* typically produces 1-butanol, 3-methyl- followed by 1-butanol, 3 methyl-, acetate (Strobel et al., 2001), *M. vitigenus* produces mainly naphthalene (Daisy et al., 2002), *M. yucatanensis* produces octane, 2-methyl butyl acetate, 2-pentyl furan, caryophyllene, aromadendrene (Macias-Rubalcava et al., 2010), *M. sutura* produces propanoic acid, 2-methyl, thujopsene (Kudalkar et al., 2012).

The activity of *Muscodor* against *B. cinerea* was already demonstrated by other authors (Mercier et al., 2007; Mercier & Jimenez, 2009; Strobel, 2006; Banguela-Castillo et al., 2015), but products registered to control this pathogen in post-harvest are lacking in Brazil. Because *Muscodor* spp. does not produce spores, formulations must rely of mycelium. The formulations available with *Muscodor* comprise ARABESQUE™, ANDANTE™, GLISSADE™ with *M. albus* QST 20799 isolate (Biopesticide Registration Action Document, 2005). Among the advantages of *Muscodor* as a biocontrol agent is its lack of pathogenicity to humans and animals. This characteristic is extremely desirable in treatments of fresh

produce such as strawberries. In our study, *Muscodor* grown on whole millet grains was able to control *B. cinerea* on strawberries. We are still conducting experiments to optimize the treatment of these fruits in plast packages commonly used to commercialize strawberries.

In conclusion, this study reports the isolation, selection and identification of *Muscodor* FTB01 able to completely inhibit *B. cinerea in vitro* and increased the shelf-life of strawberries. This isolate has the potential to be developed into a commercial product to control post-harvest decays in strawberries and contribute to decrease the amount of pesticide residues in these fruits.

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