

DEIZIANE DA CONSOLAÇÃO DUTRA

**FUMIGATION WITH *Muscodor albus* VOLATILES FOR THE CONTROL
OF POSTHARVEST DECAY**

Dissertação apresentada à
Universidade Federal de Viçosa,
como parte das exigências do
Programa de Pós-Graduação em
Fitopatologia, para obtenção do
título de *Magister Scientiae*.

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“Tudo o que um sonho precisa para ser realizado é alguém que acredite que ele possa ser realizado.”
(**Roberto Shinyashiki**)

A Deus,
À minha mãe Maria José,
Aos meus irmãos Adriano, Flávio,
Mariana e Eliane,
À minha avó Maria Estelina e
À Minha tia Maria das Graças

Dedico!

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“Se eu vi mais longe, foi por estar de pé sobre ombros de gigantes.” (Isaac Newton).

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BIOGRAFIA

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RESUMO

DUTRA, Deiziane da Consolação, M. Sc., Universidade Federal de Viçosa, fevereiro de 2013. **Fumigação com voláteis de *Muscodor albus* para controle de doenças pós-colheita.** Orientador: Olinto Liparini Pereira.

Doenças pós-colheita causam perdas expressivas de frutas e hortaliças desde o momento da colheita até o consumo, especialmente em países em desenvolvimento. O controle biológico, particularmente por meio de biofumigação, tem sido apontado como alternativa para reduzir esse problema. Entretanto, para o uso no controle de doenças de plantas, o conhecimento prévio sobre a etiologia é essencial. Um levantamento sistemático foi, portanto, conduzido no mercado local de Viçosa e na CEASAMINAS, a fim de gerar informações sobre doenças pós-colheita no Estado de Minas Gerais. Os fungos associados às doenças pós-colheita foram isolados, identificados e posteriormente submetidos a testes *in vitro* e *in vivo* visando verificar o potencial de *Muscodor albus*, fungo endofítico produtor de voláteis antimicrobianos, em controlar doenças pós-colheita de frutas e hortaliças por fumigação biológica. O teste *in vitro* foi conduzido utilizando-se placas bipartidas contendo meio BDA, nas quais *M. albus* foi previamente cultivado por seis dias em um dos compartimentos. Os discos contendo micélio de cada patógeno foram dispostos no outro compartimento de cada placa e cultivados por sessenta horas. Os voláteis antimicrobianos de *M. albus* apresentaram ação fungistática ou fungicida a depender do fungo fitopatogênico testado. Para o teste *in vivo* de micofumigação em pós-colheita foi selecionado o patossistema *Botrytis cinerea* - morango em virtude dos resultados *in vitro* e da importância econômica do mofo cinzento para a cultura. Morangos adquiridos sem a aplicação de agrotóxicos foram inoculados por ferimentos e dispostos em caixas plásticas de 11L contendo culturas de *M. albus*. As culturas de *M. albus* foram crescidas em placas de Petri, contendo BDA e os morangos inoculados foram dispostos em bandejas, de forma a não haver contato direto entre o fungo e os frutos. A micofumigação proporcionou um controle efetivo do mofo cinzento e de podridões totais em morango em pós-colheita.

ABSTRACT

DUTRA, Deiziane da Consolação, M. Sc., Universidade Federal de Viçosa, February, 2013. **Fumigation with *Muscodor albus* volatiles for the control of postharvest decay.** Adviser: Olinto Liparini Pereira.

Postharvest diseases cause considerable losses of fruits and vegetables, from the harvest to their consumption, especially in developing countries. The biological control, particularly by biofumigation, has been pointed as an alternative to reduce postharvest losses. However, for using in the plant disease control, the previous knowledge regarding etiology is essential. A systematic etiological survey was, therefore, conducted in the local market of Viçosa and CEASAMINAS to generate information about the postharvest diseases in Minas Gerais state. The fungi associated with postharvest diseases were isolated, identified and subsequently submitted to *in vitro* e *in vivo* tests to verify the potential of *Muscodor albus*, an endophytic fungus that produces antimicrobials volatiles, in controlling postharvest diseases of fruits and vegetables by biological fumigation. *In vitro* test was conducted using bipartite Petri dishes with PDA medium, in which *M. albus* was previously grown for six days in one of the compartments. The mycelial plugs of each pathogen were placed in the other compartment of each plate and cultivated for sixty hours. The *M. albus* antimicrobial volatiles presented fungistatic or fungicide action depending on the phytopathogenic fungi tested. For the *in vivo* postharvest micofumigation test, the pathosystem *Botrytis cinerea* – strawberry was selected because of the *in vitro* results and the economic importance of gray mold for the crop. Strawberries produced with no pesticides use were wound-inoculated and placed in 11L plastic boxes containing *M. albus* cultures. The *M. albus* cultures were grown in Petri dishes containing PDA and the inoculated strawberries were placed in packaging in a way that had no direct contact between fungi and fruits. The micofumigation effectively controlled gray mold and the total decay in postharvest strawberries.

GENERAL INTRODUCTION

In the last thirty years the world has gone through a significant increase in the demand for food, especially fruits and vegetables. This has been accompanied by a considerable increase in fruit and vegetable production in developing countries, leading Brazil to reach an important position between the top three producers, followed by China and India (FAO, 2003). The world population growth as well as the awareness of the population concerning the health benefits of eating fruits and vegetables plays an important role in such increase (FAO, 2003; Huang, 2004).

Despite the effort to increase the availability of food in the world, much of what is produced never reaches the consumer (Bourne, 1977). Postharvest damage represents one of the most severe causes of fruit and vegetable losses. The economic cost is proportionally greater than those due to losses in the field, because of the harvest, transportation and storage costs, that are added to those of the production. Especially in developing countries, the deterioration of fruit and vegetables still results in expressive economic losses, reaching 50% depending on the commodity and country. Inadequate infrastructure and technology are pointed as a drawback to the developing countries competition with the industrialized ones (Prusky, 2011).

Causes of postharvest losses involve physiological damage, besides several organism combinations. Spoilages caused by fungi are generally a consequence of the acidity of raw fruits (FAO, 2003). They consist of spots that affect the appearance, of rot that change the consistency and flavor, and the production of mycotoxins often acutely toxic to humans, animals, and plants (Sanhueza & Janisiewicz, 2006).

Some pathogens can penetrate fruits or vegetables while still in the field, remain in a latent form and develop in tissues only after harvest (Prusky et al., 1983; Prusky, 1996; Beno-Moualem & Prusky, 2000). Most storage pathogens require a wound to facilitate their penetration, since they are incapable of penetrating directly the cuticle or epidermis of their hosts (Barkai-Goland, 2001). Most of the postharvest losses occur during transport, handling, storage, and processing, due to

mechanical damage caused by inappropriate methods of handling the products, which lead to the occurrence of wounds that favor the infection by microorganisms. Additionally, fruits and vegetables are very attractive to several bacteria and fungi due to their water and nutrient contents, and the attack is eased due to the loss of the most intrinsic resistance that protects them during their development (Droby et al. 1992).

Knowledge on the etiology and symptoms of each disease is important to prevent problems on other fruits and vegetables or to prevent disorders in the future. It allows the evaluation of losses and contributes to recommend the appropriate measure of control. Proper identification of the disease and its causal agent make it possible to avoid waste of time, money and further losses caused by the incorrect application of chemicals. Despite its great importance, the etiological study of postharvest diseases has been neglected in Brazil.

Some non-systematic surveys have demonstrated the etiology of fungal diseases in important food in Brazil (Harrington et al., 2005; Henz et al., 2007; Silva & Pereira, 2008; Duarte et al., 2010; Lopes et al., 2010; Nascimento et al., 2010). A systematic gathering approach, especially in distribution centers, certainly could reveal new fungal-host associations that cause losses in fruits and vegetables.

To reduce postharvest losses some techniques have been developed and applied during storage conditions and transportation. The control, modification of the atmosphere, appropriate packaging, low temperatures, humidity, hygiene practices and ethylene production have been considered to maintain the products quality (Bachmann & Earles, 2000; Chitarra & Chitarra, 2005). Despite the use of these beneficial practices, such as modern storage facilities and techniques, potent chemicals and pesticides have been widely applied for disease control (Eckert & Ogawa, 1988). Frequently, these products are not even registered for application in certain products (ANVISA, 2011).

In the last years, public and scientific concerns about the presence of synthetic chemicals in food supplies and in the environment, the development of fungicide-resistant strains of postharvest pathogens, and the lack of replacement fungicides, have alarmed legal enforcers and consumers about the use of synthetic

fungicides, motivating the search for alternative approaches (Wisniewski & Wilson, 1992; Janisiewicz & Korsten, 2002; Abano & Sam-Amoah, 2012).

Biological control, or biocontrol, which is already successfully used in farming practices around the world, has attracted the interest of researchers and consumers for use in postharvest handling process (Hajek, 2004). It is believed that the use of postharvest biocontrol presents several advantages over its use in manage soil borne and foliar pathogens, including the possibility of maintaining the stability of the physical environment (temperature, humidity etc.), the ability to readily access the desired location to be treated, the economic feasibility of the technique in storage conditions, and the reduced competition between the microorganism used in biocontrol with native microorganisms (Chitarra & Chitarra, 2005; Droby et al., 2009). A number of studies have been conducted involving the search for biological control agent in which the yeasts and bacteria have been reported to inhibit postharvest decay of fruit effectively (Qin et al., 2004; Droby et al., 2009).

Currently in the market there are, at least, two registered products for postharvest use: Biosave, a product based on a *Pseudomonas syringae* variety, which is registered in the USA for controlling pome fruit, citrus, cherry, and potato diseases; and “Shemer”, a *Metschnikowia fructicola* yeast formulation, registered in Israel, used against postharvest diseases of sweet potatoes, table grapes, strawberries, peppers, and carrots (Droby et al., 2009). The application of both products consists of a spray or dip of harvested vegetables. The product application over the fruit surface is necessary because of the mode of action of each biocontrol agent which is competition for space and nutrients.

The necessity of direct application of microorganism in the fruit surface might lead the consumers to reject the food, which may hamper the product marketing. An alternative to deal with this problem is the use of organisms which are capable to produce volatile antibiotic substances that make possible the biofumigation of the harvested product (Strobel et al., 2001; Lee et al., 2009).

Muscodor albus, an endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* (cinnamon tree) from Honduras, described by Woropong et al. (2001) has attracted the attention of several researchers in the last years. The

isolate 620 of *M. albus* is characterized as a deuteromycetous (*Mycelia Sterilia*) endophytic species and is related to the ascomycetous group *Xylaria* due to 96 – 98 % homology of its 18S rDNA (2089 bp) (Strobel et al., 2001). The fungus is capable of producing gases with a musty odor under many cultural conditions. The volatile products of *M. albus* were analyzed quantitatively and qualitatively using gas chromatography and mass spectroscopy GC/MS and belonged to the chemical classes of alcohols, esters, ketones, acids, and lipids (Strobel et al., 2001). However, according to Ezra & Strobel (2003) the composition of the medium used to support the growth of this fungus greatly influences the quality and effectiveness of the volatiles emitted.

The potential lethality of *M. albus* to several plant pathogenic fungi and bacteria has been successfully evaluated (Strobel et al., 2001) as well as to nematodes and certain insects (Strobel, 2006; Riga et al., 2008; Lacey et al., 2009). However, its effect on many fungi causing postharvest disease in the tropics was never tested.

There are a number of pathogens that have been tested both *in vitro* and *in vivo* assays against the volatile antimicrobials produced by *M. albus*. Species that were already tested *in vitro* are the following: *Pythium aphanidermatum*, *Fusarium culmorum*, *Stachybotrys chartarum*, *Glomerella cingulata* (Atmosukarto et al., 2005); *Phytophthora erythroseptica*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Penicillium expansum* (Schotsmans et al., 2008); *Pythium ultimum*, *Phytophthora cinnamomi*, *Rhizoctonia solani*, *Verticillium dahliae*, *Ustilago hordei*, *Stagnospora nodorum*, *Aspergillus fumigatus*, *Fusarium solani*, *Cercospora beticola*, *Candida albicans*, *Xylaria* sp., *Tapesia yallundae* (Strobel et al., 2001); *Helminthosporium solani*, *Mucor pyriformis* (Mercier et al., 2007); *Fusarium sambucinum* (Corcuff et al., 2006); *Colletotrichum acutatum*, *Colletotrichum coccodes*, *Geotrichum candidum*, *Monilinia fructicola*, *Rhizopus* sp., *Penicillium digitatum* (Mercier & Jiménez, 2004) and *Geotrichum citri-aurantii* (Mercier & Smilanick, 2005). Species which have been tested *in vivo* were: *S. sclerotiorum*, *B. cinerea* and *P. expansum* on apple fruit (Mercier & Jiménez, 2004; Schotsmans et al., 2008); *F. sambucinum* on stored potatoes (Corcuff et al., 2006); *P. digitatum* and *G. citri-aurantii* on lemon (Mercier & Smilanick, 2005); *M. fructicola* on peaches (Mercier & Jiménez, 2004)

and *Tilletia caries* on seeds and soil treatment (Blair & Mercier, 2011). *Rhizoctonia solani* was already tested in soil and substrate against the volatiles from *M. albus* to verify the potential to control damping-off of broccoli seedlings (Mercier & Jiménez, 2009).

Generally, in the initial evaluation process of the microorganism capability to control another for biological control, an *in vitro* assay is required. It is an important step in the screening process of biocontrol agents of plant diseases, since it is the base to further *in vivo* study involving the wounded fruits (Nunes, 2012). This is, therefore, the reason for conducting *in vitro* study using *Muscodor albus* as antagonist against several fungi causing plant diseases. For further *in vivo* tests, the pathosystem is usually chosen according to the crop regional economic importance and qualities.

Strawberries are fruits with great socioeconomic importance (Tanaka et al., 2005), rich in natural antioxidants (Heinonen et al., 1998) and extremely perishable (Kader, 1991). They are, therefore, attractive for researches around the world.

It consists of an herbaceous plant belonging to the family Rosaceae. The domesticated strawberry *Fragaria x ananassa* Duch., is a hybrid that was first found in France in the 18th century, resulting from a cross between *Fragaria chiloensis* and *Fragaria virginiana* (Hummer & Janick, 2009).

The crop can be grown for fresh fruits, for frozen pulp or to make preserves. Currently, the United States of America is the largest strawberry producer in the world, with a production of about 1.3 million tons in 2011, while the numbers for this in Brazil was 3 thousand tons (FAOSTAT, 2011). Strawberry is among the most widely consumed fruits throughout the world, and there are some Brazilian regions where strawberry cultivation is one of the most important agricultural activities (Tanaka et al., 2005). The great economic importance throughout the world led it to take a place among the species included in The International Treaty on Plant Genetic Resources for Food and Agriculture, an international agreement whose purpose is fight against hunger and poverty that is essential to guarantee food security (FAO, 2009).

Strawberry has a very short shelf-life, generally, less than a week even under ideal conditions. One of the most important reasons for that is the *B. cinerea* attack, which causes gray mold, one of the most serious and economically important plant diseases worldwide (Dean et al., 2012). During storage, transit and market, secondary rot can be caused, becoming the infected fruit soft, watery, and light brown. The infectious process may start in the flowers, remain quiescent until fruits mature, when the pathogen develop abundantly causing fruit rots and producing many conidia for further autoinfection (Lattanzio et al.,1996; Kovach et al., 2000; Wszelaki & Mitcham, 2003; Holz et al., 2004).

One of the most common strategies used in strawberry diseases management is the field application of fungicides (Maas, 1998; Wedge et al., 2007). However, the use of some chemicals has become restricted. Reasons for that include the development of resistant strains of pathogens, concern about health risk of the chemical residues on fruits in addition to other damages, such as negative effects on pollination and fruit malformation (Wedge et al., 2007; Mercier et al., 2010). In Brazil, strawberry is the second in detections of irregularities on active ingredient (a.i.), losing only to pepper (ANVISA, 2011). These problems have stimulated researchers to search for alternatives to handle postharvest diseases of strawberry, including gray mold rot. Biological control is one of these important strategies. It has been investigated under field conditions (Lima et al., 1997; Boff et al., 2002; Cota et al., 2008; Kowalska, 2011). Studies involving postharvest diseases biological control in strawberry, however, are few and generally they involve the use of yeast application (Zhang et al., 2007; Vagelas et al., 2009).

Camino Real is a short-day strawberry variety developed at University of California in 2001. Its fruits are firm and dark red, presenting a good shelf life and very good flavor. It is late maturing and relatively resistant to rain damage, in addition, it is reported as being quite resistant to *Verticillium* wilt, *Phytophthora* crown rot and relatively resistant to anthracnose crown rot (Phillips & Reid, 2008). However, according to producers, this variety is highly susceptible to gray mold. They observed that the Camino real big calyx is a kind of shelter for *B. cinerea* and it creates a favorable condition for pathogen survival (U. P. Lopes, personal communication).

CHAPTER I

FUNGI ASSOCIATED WITH FRUITS AND VEGETABLES POSTHARVEST DECAY

INTRODUCTION

The world population growth as well as the awareness of health benefits of eating fruit and vegetable has led to an expressive worldwide increase in the demand of fruits and vegetables in the last years.

Infections caused by microorganisms including fungi, however, cause serious postharvest losses in food, especially in developing countries, where the infrastructure and technology for food storage are not enough adequate. Most of the infections caused by microorganisms are eased by damage caused during transport and storage processes.

Knowledge regarding etiology and symptoms is essential to plan control strategies, since it allow the recommendation of appropriate measures of control and prevention of disease occurrence in fruit and vegetable.

The purpose of this study was to generate information about the etiology of fungal diseases that occur on harvested fruits and vegetables in Minas Gerais.

MATERIALS AND METHODS

Sample collection and isolation

From July 2010 to November 2012, fruits and vegetables, exhibiting rot symptoms were monthly collected, packaged according to Shurtleff & Averre (1997) and conducted to the Laboratório de Patologia de Sementes e de Pós-colheita (Departamento de Fitopatologia, Universidade Federal de Viçosa) where they were first examined for the possible presence of fungal fruiting structures. The samples were periodically taken in the CEASAMINAS, in Contagem store (Metropolitan Region of Belo Horizonte) and local market in Viçosa, both in the State of Minas Gerais.

Standard procedures were done for the direct or indirect isolation (Alfenas et al., 2007) of pure cultures of necrotrophic fungi that causes decay in the harvested crops. Cultures were preserved according to Dhingra & Sinclair (1995): successive subcultures in potato and carrot medium, preservation in silica gel and preservation in saline solution.

Morphological studies

The material naturally infected was examined under a stereomicroscope (20X). When the reproductive structures was absent, fragments were removed from the fruit, disinfected in 70% ethanol for 1 min, followed by 1% sodium hypochlorite for 3 min and washed in sterile distilled water. Later, these were placed in Petri dishes with Potato-Dextrose-Agar (PDA - Acumedia[®]) with rifamicyn at 4µg/L and incubated at 25°C.

The elucidation of the identity of the collected fungi was based on morphological studies of the vegetative and reproductive structures using a light microscope OLYMPUS CX31 (400X). The structures were collected and prepared between slides and cover slips using lactophenol for the provisional identification of fungi.

The genus or species was elucidated using a dichotomous key and the collected material was compared to that described in the published literature to determine the species. When the pathogenic fungi did not produce reproductive structures, the sporulation was induced using media poor in nutrients, according to Dhingra & Sinclair (1995).

RESULTS

Postharvest pathogens identified

Ninety isolates identified to genus or to species level were found associated with rot in forty-two different fruits and vegetables (**Table 1, Figures 1 and 2**). Eight of those pathogens were found associated to postharvest disease in each culture for the first time: *Chalara* sp. on banana; *Colletotrichum* sp., *Ceratocystis* sp. and *Lasiodiplodia* sp. on passion fruit; *Acrostalagmus* sp. on pumpkin; *Ceratocystis* sp. on chili; *Ceratocystis paradoxa* on immature coconut and *Fusarium boothii* on tomato (**Figure 3**).

Table 1. Postharvest pathogens found in the systematic survey in the CEASAMINAS, on Contagem store (Metropolitan Region of Belo Horizonte) and local market in Viçosa, both in the State of Minas Gerais.

Hosts	Fungi
Apple	<i>Alternaria</i> sp.; <i>Colletotrichum</i> sp.
Apricot	<i>Botrytis</i> sp.; <i>Penicillium</i> sp.
Arracacha	<i>Penicillium</i> sp.
Avocado	<i>Colletotrichum</i> sp.; <i>Lasiodiplodia</i> sp.; <i>Rhizopus stolonifer</i>
Banana	<i>Colletotrichum musae</i> ; <i>Challara</i> sp.
Beet	<i>Colletotrichum</i> sp.
Blueberry	<i>Botrytis</i> sp.
Carrot	<i>Challara elegans</i>
Cashew	<i>Mucor hiemalis</i> ; <i>Cladosporium</i> sp.
Chayote	<i>Colletotrichum truncatum</i>
Chili	<i>Ceratocystis</i> sp.
Coconut	<i>Lasiodiplodia</i> spp.
Eggplant	<i>Phytophthora capsici</i> ; <i>Alternaria</i> sp.
Genipap	<i>Penicillium</i> sp.
Grape	<i>Cladosporium</i> sp.
Guava	<i>Colletotrichum</i> sp.; <i>Guignardia psidii</i> ; <i>Penicillium</i> sp.; <i>Rhizopus stolonifer</i>
“Jambo”	<i>Rhizopus stolonifer</i>
“Jilo”	<i>Phytophthora capsici</i>
Kaki	<i>Mucor hiemalis</i>
Kiwi	<i>Cladosporium</i> sp.
Mango	<i>Alternaria</i> sp.; <i>Colletotrichum</i> sp.; <i>Lasiodiplodia</i> sp.
Melon	<i>Alternaria</i> sp.; <i>Fusarium</i> spp.; <i>Cladosporium</i> sp.
Nectarine	<i>Monilinia fructicola</i> , <i>Penicillium expansum</i> , <i>Rhizopus stolonifer</i>
Okra	<i>Phytophthora capsici</i> ; <i>Rhizopus stolonifer</i>
Onion	<i>Aspergillus niger</i> ; <i>Stemphyllium solani</i>
Orange	<i>Penicillium italicum</i> ; <i>Phyllosticta citricarpa</i> <i>Alternaria</i> sp.; <i>Colletotrichum</i> sp.; <i>Cladosporium</i> sp.; <i>Fusarium</i> spp.; <i>Didymella bryoniae</i> ;
Papaya	<i>Phytophthora palmivora</i>
Passion Fruit	<i>Colletotrichum</i> spp.; <i>Ceratocystis</i> sp.; <i>Lasiodiplodia</i> sp.; <i>Fusarium</i> sp.
Peach	<i>Monilinia fructicola</i> ; <i>Penicillium expansum</i> ; <i>Mucor</i> sp.
Pear	<i>Alternaria</i> sp.
Pepper	<i>Alternaria</i> sp.
Pineapple	<i>Embellisia</i> sp.
Plum	<i>Cladosporium</i> sp.; <i>Monilinia fructicola</i> ; <i>Penicillium expansum</i>
Pumpkin	<i>Acrostalagmus</i> sp.; <i>Fusarium</i> sp.; <i>Rhizopus stolonifer</i>
Squash	<i>Didymella bryoniae</i> ; <i>Phytophthora capsici</i>
Strawberry	<i>Botrytis cinerea</i> ; <i>Pestalotia longisetula</i> ; <i>Rhizopus stolonifer</i> ; <i>Colletotrichum</i> spp.
Starfruit	<i>Colletotrichum</i> sp.
String Bean	<i>Phytophthora capsici</i>
Sweet potato	<i>Rhizopus stolonifer</i>
Tomato	<i>Alternaria</i> sp.; <i>Fusarium boothii</i> ; <i>Fusarium</i> sp.; <i>Cladosporium</i> sp.
Yam	<i>Cladosporium</i> sp.; <i>Penicillium</i> sp. <i>Fusarium</i> sp.
Zucchini	<i>Colletotrichum</i> sp.; <i>Phytophthora capsici</i>

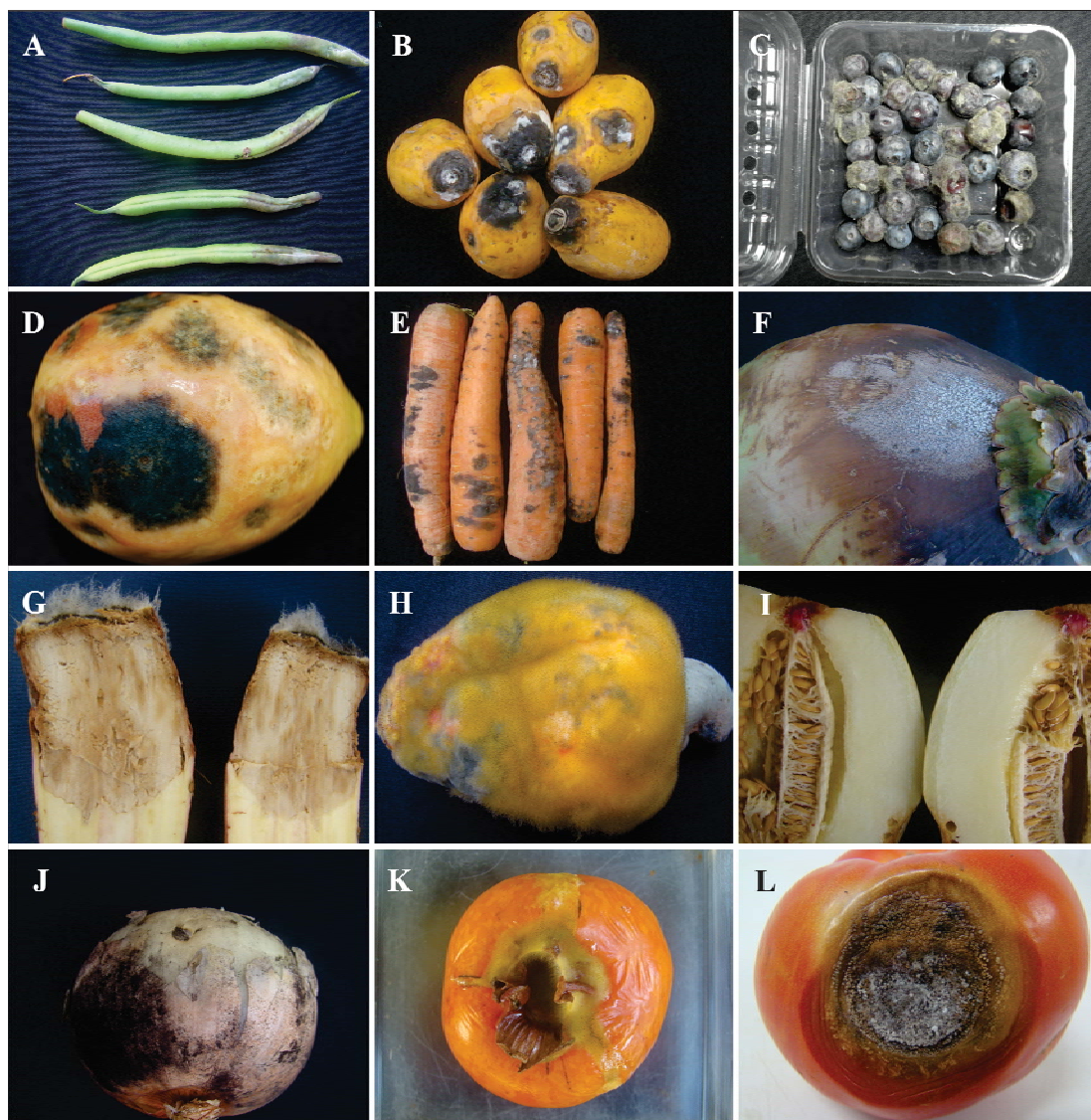


Figure 1. Examples of cultures where postharvest fungi were collected: A - String Bean (*Phytophthora capsici*); B - Papaya (*Didymella bryoniae*); C - Blueberry (*Botrytis* sp.); D - Guava (*Guignardia psidii*); E - Carrot (*Challara elegans*); F - Coconut (*Lasiodiplodia* sp.); G - Sweet potato (*Rhizopus stolonifer*.); H - Cashew (*Mucor hiemalis*); I - Melon (*Fusarium* sp.); J - Onion (*Aspergillus niger*); K - Kaki (*Mucor hiemalis*); L - Tomato (*Colletotrichum* sp.).

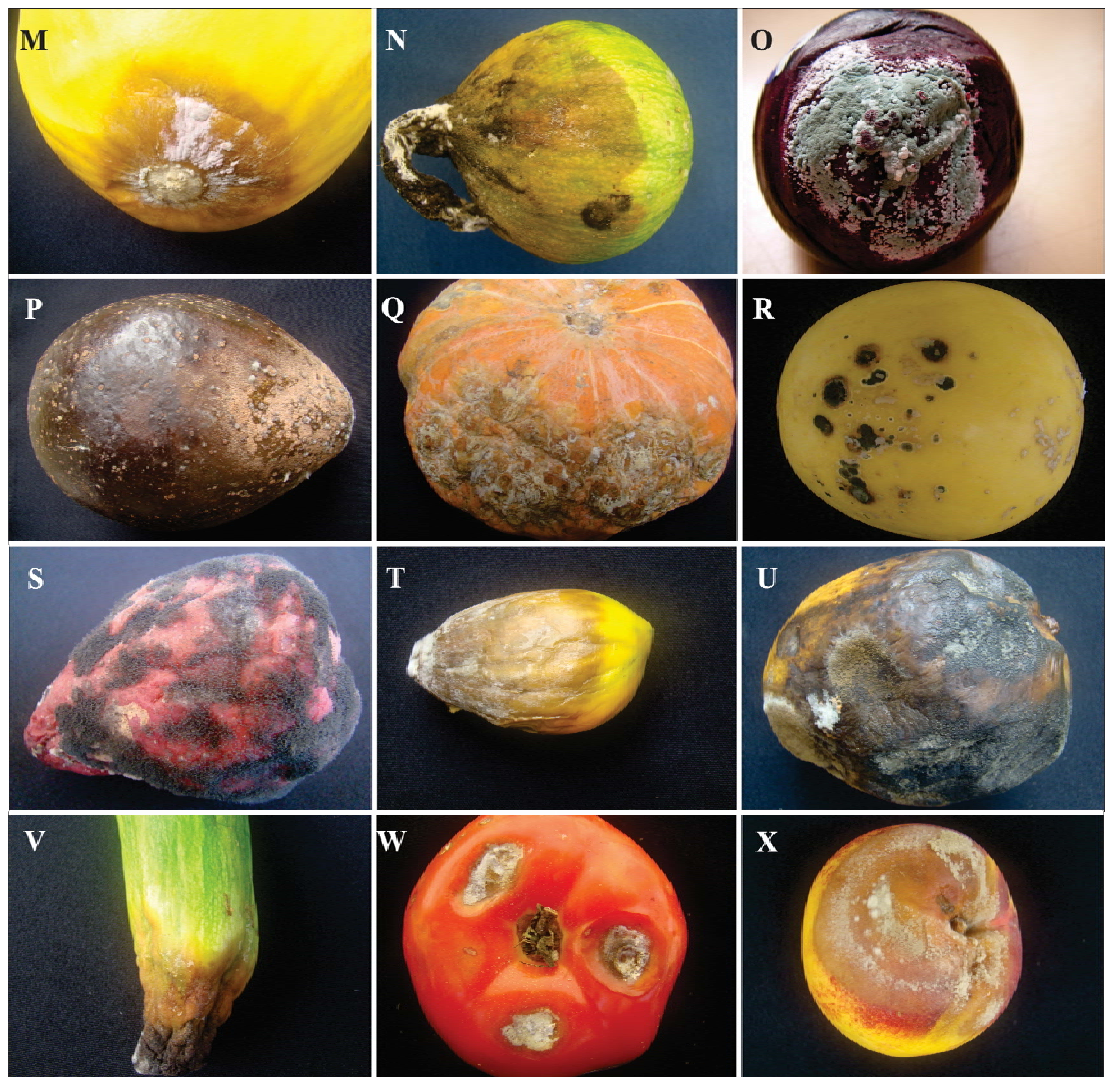


Figure 2. Examples of cultures where postharvest fungi were collected: M - Melon (*Fusarium* sp.); N - Squash (*Didymella bryoniae*); O - Plum (*Penicillium expansum*); P - Avocado (*Colletotrichum* sp.); Q - Pumpkin (*Fusarium* sp.); R - Melon (*Alternaria* sp.); S - “Jambo” (*Rhizopus stolonifer*); T - “Jilo” (*Phytophthora capsici*); U - Mango (*Lasiodiplodia theobromae*); V - Zucchini (*Phytophthora capsici*); W - Tomato (*Alternaria* sp.); X - Nectarine (*Monilinia fructicola*).

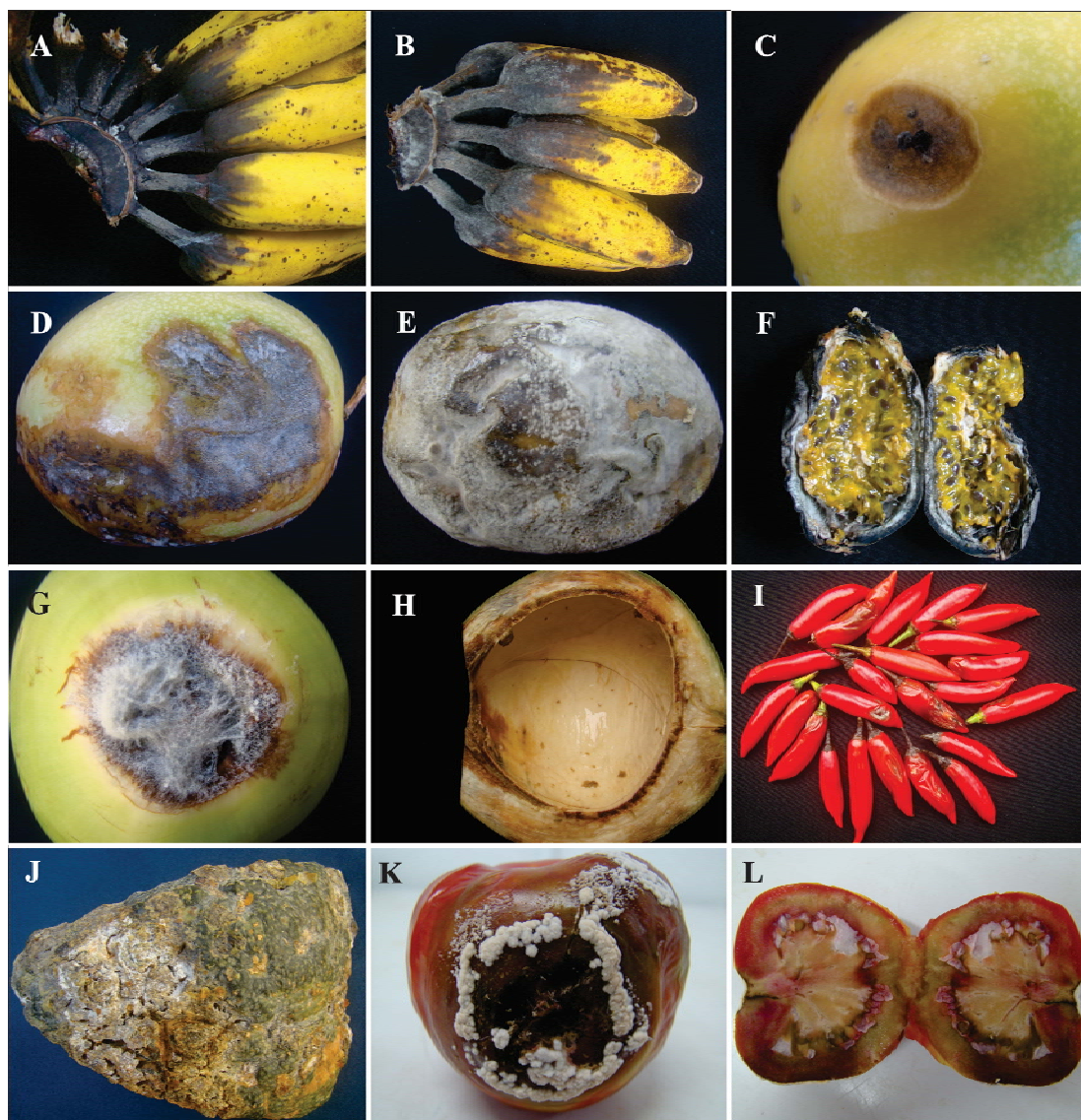


Figure 3. New postharvest association found during the systematic survey. A-B: *Chalara* sp. on banana; C: *Ceratocystis* sp. on passion fruit; D: *Colletotrichum* sp. on passion fruit; E-F: *Lasiodiplodia* sp. on passion fruit; G-H: *Ceratocystis* sp. on immature coconut; I: *Ceratocystis* sp. on chili; J: *Acrostalagmus* sp. on pumpkin; K-L: *Fusarium boothii* on tomato.

DISCUSSION

A systematic survey of fungi associated with fruits and vegetables was performed and revealed species that cause highly important diseases on fruits or vegetables in other countries, but that, according to Mendes & Urban (2013), had never been reported in Brazil or are new worldwide diseases. One of those important pathogens is *C. paradoxa*, which was found causing internal postharvest rot disease on immature coconut. This species was also reported causing post-harvest diseases in banana, starfruit, guava and pineapple (Reyes et al., 1998; Junqueira et al., 2001; Ploetz, 2003). This pathogen causes stem bleeding (Warwick & Passos, 2009; Freire & Martins, 2010) and fruit basal rot of coconut at field conditions (Rossetti, 1955; Camargo & Gimenes-Fernandes, 1997; Tzeng & Sun, 2009; Tzeng et al., 2010) and external post-harvest disease on coconut (Tzeng & Sun, 2009; Tzeng et al., 2010). Thus, this is the first report of post-harvest internal rot disease on immature coconut caused by *C. paradoxa*. Recently, Lopes et al (2012) reported the detection of *C. paradoxa* in the sap of infected coconut palms. Maybe this is one way used by the fungus to enter inside the fruits with posterior manifestation of post-harvest internal rot, without apparent external symptoms.

In the same way, other associations, such as: *F. boothii* (on tomato), *Ceratocystis* sp. (on chili), *Acrostalagmus* sp. (on pumpkin), *Chalara* sp. (on banana) *Colletotrichum* sp., *Lasiodiplodia* sp. and *Ceratocystis* sp. (on passion fruit) are worldwide novelties for each fruit or vegetable.

On the other hand, the etiology of postharvest diseases revealed the frequent occurrence of certain fungi in several different crops, such as *Colletotrichum* spp., *R. stolonifer* and *Botrytis* spp., causing disease on fruits and vegetables. Those fungi are highly aggressive and have a wide host range leading to significant postharvest losses. In Brazil, *Colletotrichum* spp. are generally associated with anthracnose on tropical fruits, *R. stolonifer* is responsible for soft rot of postharvest fleshy organs and *B. cinerea* to gray mold in several crops (Kimati et al., 2005; Henz, 2006).

Botrytis species, besides causing diseases in postharvest fruits and vegetables, are important pathogens of nursery, ornamental, and field and orchard plants (Dean,

2012). It may be quiescent in the host tissue for varying periods, remaining on its 'non-aggressive' form until the expansion of lesions (Elad et al., 2004). *Colletotrichum* species, in the same way, also may infect fruits in the field, leaves, stems, petioles and flowers of plants, besides the postharvest stage and may equally remain in a quiescent form for some time (Kimati et al., 2005). *Rhizopus stolonifer*, in turn, constitutes a problem mainly as causal agent in postharvest diseases, rarely in the field. It invades the wounded tissues of fleshy organs, being considered as a less aggressive pathogen, once it is unable to penetrate directly the tissues (Barkai-Golan, 2001).

Cladosporium, *Alternaria*, *Mucor*, *Stemphylium*, *Penicillium*, *Aspergillus*, *Monilinia*, *Geotrichum*, and *Fusarium* are other important decay agents which affect harvested fruits and vegetables and, given the appropriate conditions, develop and cause decay (Barkai-Golan, 2001). Those fungal genus were also found in several collected samples.

This study had therefore, an important role in showing the diversity of fungi involved in postharvest decay of fruits originated from CEASAMINAS, at Metropolitan Region of Belo Horizonte, and in local market in Viçosa, Minas Gerais state and will serve as a basis for a postharvest disease catalog to assist pathological postharvest losses surveys.

CHAPTER II

BIOFUMIGAÇÃO *IN VITRO* DE FUNGOS CAUSADORES DE DOENÇAS PÓS-COLHEITA POR *Muscodor albus*

INTRODUCTION

One of the most important strategies to control plant diseases is biological control. This strategy is advantageous for postharvest diseases mainly because of the possibility to maintain the temperature and humidity under control on storage environment.

Products are registered for biocontrol in which bacteria and yeasts are the controlling agent. Their mode of action is competition for space and nutrients. They are, therefore, sprayed or dipped on fruit surface, leading the consumers to reject the product, hampering the product market.

Muscodor albus, an endophytic volatile producing fungus, is an important alternative to deal with this problem since its volatile compounds are capable to kill several pathogens by micofumigation. It has been successfully tested as its potential to control plant pathogenic fungi by micofumigation worldwide. However, it was never tested against pathogens that cause diseases in tropical environment.

The purpose of this study was evaluating the capability of *in vitro* biofumigation of some pathogens that causes postharvest diseases by *M. albus*.

MATERIALS AND METHODS

Fungal pathogens for *in vitro* bioassay test

Some of the pathogenic isolates used in the *in vitro* test were obtained from the culture collection of the “Laboratório de Patologia de Sementes e de Pós-colheita, of the Departamento de Fitopatologia at Universidade Federal de Viçosa” which were: *Chalara elegans*, *Pilidium concavum*, *Geotrichum* sp., *Fusarium solani*, *Gliomastix murorum*, *Acrostalagmus luteo-albus* (two isolates), *Mucor hiemalis*, *Cercospora apii*, *Dothiorella dominicana*, *Fusarium semitectum*, *Absidia cylindrospora*, *Verticillium theobromae*, *Ceratocystis fimbriata*, *Colletotrichum magna*, *Glomerella cingulata*, *Neofusicoccum kwambonambiense* and *Aspergillus flavus*.

Some others were selected among those found in the etiological survey and were: *Cladosporium* spp. (three isolates), *B. cinerea*, *Aspergillus niger*, *Fusarium boothii*, *Colletotrichum* spp. (two isolates), *Guignardia psidii*, *Pestalotia longisetula*, *Fusarium oxysporum* (two isolates), *Colletotrichum truncatum*, *Embellisia allii* (two isolates), *Cylindrocladium* sp., *Penicillium expansum*, *Penicillium italicum*, *Lasiodiplodia theobromae*, *Mucor hiemalis*, *Trichotecium roseum*, *Monilinia fructicola*, *Colletotrichum gloeosporioides*, *Botrytis* sp., *Rhizopus stolonifer*, *Alternaria solani*, *Geotrichum candidum*, *Ceratocystis* sp., *Stemphyllium solani* and *Phytophthora palmivora*.

Muscodor albus isolate

Muscodor albus isolate 620 was obtained from Dr. G. Strobel, Montana State University, and deposited in the culture collection of the “Laboratório de Patologia de Sementes e de Pós-colheita of the Departamento de Fitopatologia at Universidade Federal de Viçosa”. It was previously described by Woropong et al. (2001).

***In vitro* bioassay test for micofumigation**

Muscodor albus was grown on PDA that was prepared using extract of 200 g of boiled potatoes, 500 mL; dextrose, 20 g; agar, 17 g and distilled water, 500 mL. It was very important that PDA was prepared according to prescription because previous assays showed that when the fungus was grown in the synthetic PDA it was less efficient in controlling the same microorganism.

Twenty mL of PDA were poured into partitioned Petri dishes that allow only volatile compounds effects over the microorganisms (Strobel et al., 2001). *Muscodor albus* was placed and grown on one side of the plate for six days. Then, five plugs (8 cm diameter) were placed on the opposite side. Each isolate was tested in four replications, totaling twenty plugs of each one. Individual fungi were also placed on the test side of the plate, without *M. albus* presence, that was used as a control. The plate was wrapped with two layers of Parafilm[®] and incubated at 25°C for further sixty hours, when the first evaluation was done. The growth of the test microorganisms was visually assessed. The microorganisms evaluated were, then, removed from the presence of *M. albus* and placed in another PDA plate in order to evaluate the effect (Strobel et al., 2001). The effect was judged after three days. The experiment was performed twice. For those isolates which results remained uncertain the experiment was conducted once again.

The evaluation consisted of classifying the effect caused by *M. albus* volatile compounds on the pathogenic isolates, when there was, as fungicide or fungistatic. The base for evaluation was the results obtained at the first (*M. albus* exposure for 60 hours) and second (after *M. albus* exposure for 60 hours) steps, when the percentage of plugs that had their growth inhibited was measured.

RESULTS

***In vitro* effect of *M. albus* on pathogenic fungi**

Most tested pathogens were killed (**Table 2; Figure 4: A- F**). However, the volatiles of *M. albus* also had an initial inhibitory effect over some pathogens, but further viability test showed that those pathogens were not killed: *A. luteo-albus*

(from ginger) (**Figure 4: G – I**), and *A. luteo-albus* (from banana), *F. solani* (from ginger). A few of them remained viable: *P. longisetula* (from strawberry) (**Figure 4: J – L**), *F. oxysporum* (from ginger) and *F.oxysporum* (from taro). The effect of *M. albus* volatiles was classified regarding fumigation activity as fungicide or fungistatic (**Table 2**). The proportion of survivor or dead plugs for the same pathogens was sometimes changed depending on the experiment, but in most cases it does not harm to experiment evaluation and effect classification. For some pathogens, however, such as *Verticillium theobromae*, the numbers of survivor or dead plugs were variable and doubtful and were among half of plugs tested. For that pathogen the effect was classified as “uncertain” (**Table 2**).

Table 2. Effect of *Muscodor albus* volatiles on several postharvest pathogens.

Fungi	Experiment 1			Experiment 2		
	Growth inhibition (%)	Effect	Growth inhibition (%)	Effect		
	After 60 h exposure to volatiles	After viability test	After 60 h exposure to volatiles	After viability test		
<i>Chalara elegans</i>	100	95	+	100	100	+
<i>Pilidium concavum</i>	100	95	+	100	100	+
<i>Geotrichum</i> sp.	100	100	+	100	75	+
<i>Fusarium solani</i>	70	0	•	100	0	•
<i>Gliomastix murorum</i>	90	0	•	50	0	x
<i>Acrostalagmus luteoalbus</i> (isolate 1)	100	0	•	100	0	•
<i>Acrostalagmus luteoalbus</i> (isolate 2)	100	0	•	100	0	•
<i>Mucor hiemalis</i>	100	45	x	85	75	+
<i>Cercospora apii</i>	100	75	+	90	75	+
<i>Cladosporium</i> sp. (isolate 1)	100	100	+	100	100	+
<i>Cladosporium</i> sp. (isolate 2)	100	100	+	100	75	+
<i>Cladosporium</i> sp. (isolate 3)	100	100	+	100	100	+
<i>Dothiorella dominicana</i>	100	100	+	100	100	+
<i>Fusarium boothii</i>	100	100	+	100	100	+
<i>Fusarium semitectum</i>	100	100	+	100	100	+
<i>Botrytis cinerea</i>	100	100	+	75	70	+
<i>Aspergillus niger</i>	100	100	+	100	100	+
<i>Colletotrichum</i> sp. (isolate 1)	100	90	+	100	100	+
<i>Colletotrichum</i> sp. (isolate 2)	100	100	+	100	100	+
<i>Absidia cylindrospora</i>	100	95	+	100	100	+
<i>Verticillium theobromae</i>	100	40	x	100	60	x
<i>Guignardia psidii</i>	100	25	x	100	70	+
<i>Pestalotia longisetula</i>	0	0	-	0	0	-
<i>Fusarium oxysporum</i>	10	0	-	75	25	-
<i>Colletotrichum truncatum</i>	100	100	+	100	100	+
<i>Embellisia</i> sp. (isolate 1)	100	100	+	100	100	+
<i>Embellisia</i> sp (isolate 2)	100	65	x	100	100	+
<i>Cylindrocladium</i> sp.	0	0	-	55	0	x
<i>Penicillium expansum</i>	100	95	+	50	0	-
<i>Penicillium italicum</i>	100	100	+	100	100	+
<i>Fusarium</i> sp.	0	0	-	70	0	-
<i>Lasiodiplodia</i> sp.	0	0	-	75	0	•
<i>Mucor</i> sp.	100	75	+	100	100	+
<i>Trichothecium</i> sp.	100	100	+	100	70	+
<i>Ceratocystis fimbriata</i>	100	100	+	100	75	+
<i>Monilinia fructicola</i>	100	100	+	100	100	+
<i>Colletotrichum magna</i>	100	100	+	100	100	+
<i>Colletotrichum gloeosporioides</i>	100	100	+	100	70	+
<i>Glomerella cingulata</i>	100	100	+	80	75	+
<i>Botrytis</i> sp.	100	100	+	100	100	+
<i>Aspergillus flavus</i>	95	95	+	100	100	+
<i>Rhizopus stolonifer</i>	75	75	+	100	100	+
<i>Alternaria solani</i>	100	100	+	100	100	+
<i>Geotrichum candidum</i>	100	100	+	100	75	+
<i>Neofusicoccum kwambonambiense</i>	100	35	x	100	15	•
<i>Ceratocystis</i> sp.	100	80	+	100	100	+
<i>Stemphyllium solani</i>	100	100	+	100	100	+
<i>Phytophthora palmivora</i>	100	100	+	100	95	+

+ = Fungicide effect; - = No effect; x = Uncertain; • = Possible fungicide effect.

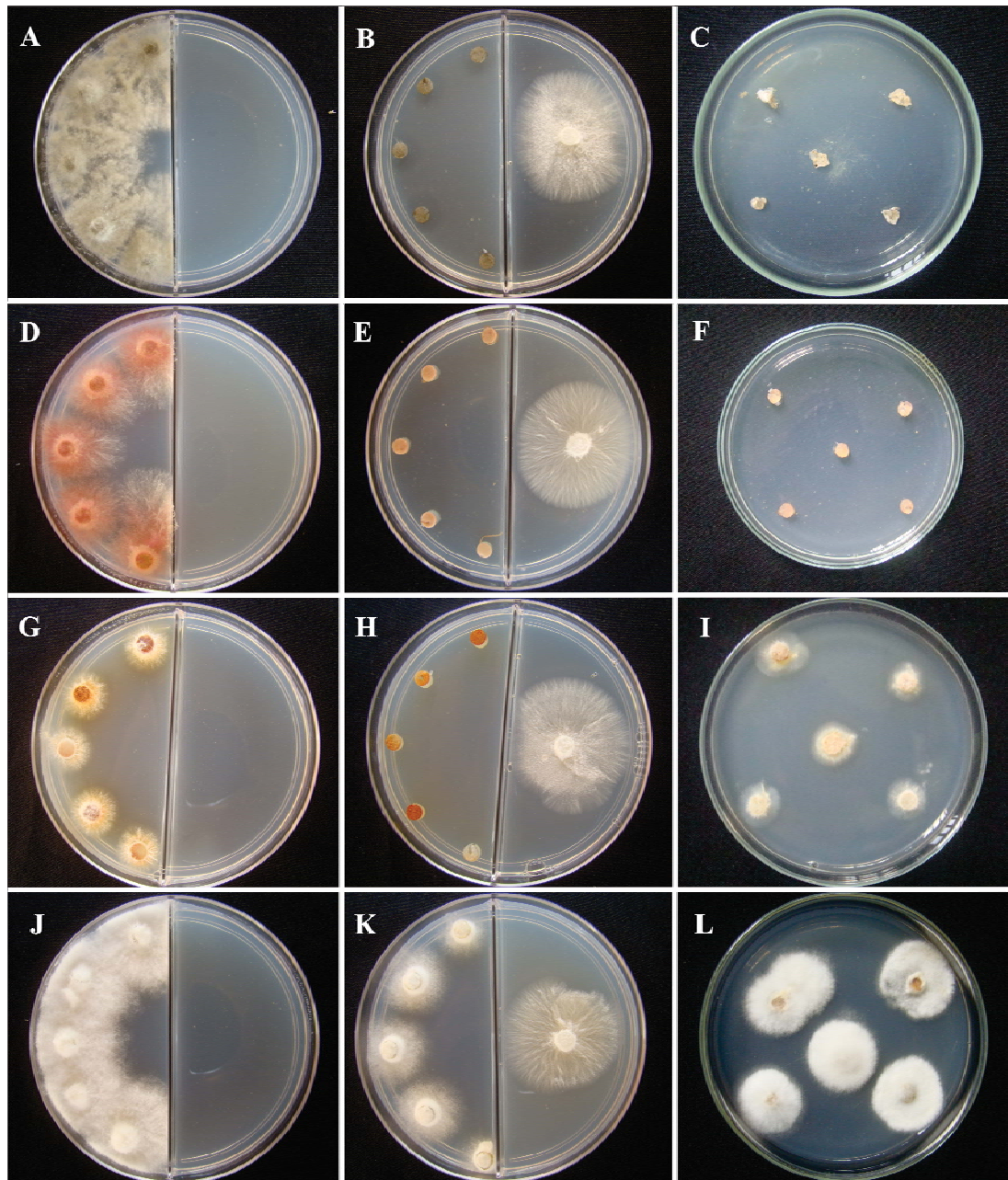


Figure 4. Effect of volatiles produced by *Muscodor albus* on different postharvest pathogens. From left to right: Control; effect after 60 hours of exposure to volatiles; viability after 60 hours of exposure to *Muscodor albus*. Fungicide effect on: A – C: *Botrytis cinerea*; D – F: *Fusarium bootii*. Fungistatic effect on: G – I: *Acrostalagmus luteo-albus*. No effect on: J – L: *Pestalotia longisetula*.

DISCUSSION

As it has been discussed by several researchers, postharvest diseases often cause considerable losses of fruits and vegetables during storage and transportation. Biological control, however, has been pointed as an alternative approach to deal with postharvest spoilage, especially because of the possibility to maintain the stability of the physical environment and the economic feasibility of the technique in storage conditions. In this context, the volatile antibiotic producing microbes have been considered as attractive organisms to be applied in postharvest spoilage control, due to their fumigation capability.

The *in vitro* assay, that tested *M. albus* volatiles against several pathogens showed efficiency in killing many pathogens, confirming that *M. albus* is an important promise for the control of fruit and vegetable postharvest diseases because of its broad-spectrum fungicidal activity. Some of the important pathogens that were killed by volatiles of *M. albus in vitro* were: *C. elegans*, which causes black root rot on carrots, *Colletotrichum* spp. and *Cladosporium* spp. that are important causative agent of several diseases in different cultures, among others.

The *in vitro* test results for some pathogens, such as: *B. cinerea*, *M. fructicola*, *G. candidum*, fungicide effect; and *F. solani*, fungistatic effect; were the same when compared to those previously described in the literature (Mercier & Jiménez, 2004; Atmosukarto et al., 2005). On the other hand, for *G. cingulata* result determined was different from that described in the literature as fungistatic (Atmosukarto et al., 2005). This indicates that may be a variation in sensitivity of volatiles produced by *M. albus* in different isolates belonging to the same species. Such work is important for reveal pathogens that will possibly be successfully controlled by *M. albus in vivo* or in soil later, as have been already happening with some pathogens (Mercier & Smilanick, 2005; Mercier & Jiménez, 2004; Mercier et al., 2009; Schotsmans et al., 2008).

Among pathogens that attack strawberry, tested for viability against *M. albus* volatiles, *P. concavum*, *B. cinerea*, *A. niger*, *Colletotrichum* sp., *P. longisetula*, *Cylindrocladium* sp., *G. candidum* and *N. kwambonambiense*, only *P. longisetula*, *N.*

kwambonambiense and *Cylindrocladium* sp., were not killed by *M. albus*. According to this result, an integration of methods, including micofumigation by *M. albus*, would be an interesting alternative to strawberry diseases control where those pathogens occur at the same time. Another possibility would be the endophytes discoveries that are effective against them.

CHAPTER III

FUMIGATION WITH *Muscodor albus* VOLATILES FOR THE CONTROL OF STRAWBERRY GRAY MOLD

INTRODUCTION

Strawberries have a great socioeconomic importance due to its nutritional characteristics, but are extremely perishable. The gray mold, caused by *B.cinerea*, is one of the most important reasons for strawberry losses worldwide. It is, nowadays the most important strawberry disease.

Currently, fungicides application constitutes the most common strategy to control strawberry gray mold. Consequently, in Brazil, strawberry is historically included among food with the highest irregularity register regarding the pesticides use.

On the other hand, biological control has been detached as an important way to control plant disease in the last years. Despite the use of biocontrol practices in organic strawberry, postharvest losses still being expressive. It is, therefore, important to search for alternative strategies to control gray mold in strawberry postharvest.

The purposes of this study were to conduct *in vivo* assays of micofumigation by *M. albus* aiming the control of total decay caused by natural infections in strawberry. To conduct *in vivo* assays of micofumigation by *M. albus* involving the pathosystem *B. cinerea* – strawberry with the purpose of controlling gray mold.

MATERIALS AND METHODS

***In vivo* bioassay test for micofumigation**

***Botrytis cinerea* inoculum**

This fungus was previously isolated from infected strawberries and preserved in silica gel according to Dhingra & Sinclair (1995). Conidia suspensions were prepared by removing conidia from a fungus colony, grown for 15 days at 22°C on PDA medium, with a loop and suspended in sterile distilled water. Conidia concentration of *B. cinerea* was adjusted to 1×10^5 conidia/mL.

Strawberry

Visually healthy fruits were obtained in the morning, at Ervália municipality Minas Gerais, and transported to “Laboratório de Patologia de Sementes e de Pós-Colheita, of the Departamento de Fitopatologia at Universidade Federal de Viçosa”. They were maintained at room temperature till the installation of the experiment. The strawberries were produced without pesticide application, and when necessary, a formulation of *Clonostachys rosea* was used in the field, to control strawberry diseases. The strawberry variety ‘Camino Real’ was chosen because of its high susceptibility to *B. cinerea*, as reported by the producers.

Micofumigation activity against gray mold disease

The volatile compounds released by *M. albus* were evaluated for their effects on the development of *B. cinerea* on artificially inoculated strawberries. For this purpose, *M. albus* was cultured PDA as described for the *in vitro* test. It was grown on the center of the plate containing 20 mL of PDA for six days. Each healthy fruit was placed in a strawberry packaging, which maintains the fruits separated from each other. The packaging can accommodate up to 12 fruits. Each strawberry fruit was wounded in the equatorial region, at the inoculation moment, using the tip of the

micropipette used to inoculate 50 μL of *B. cinerea* conidia suspension (1×10^5 conidia/mL).

Each package was arranged in an 11- L plastic box containing four plates with six-day-old *M. albus* cultures and the containers were closed. The boxes were stored at 25°C in the dark. After 5 days of fumigation the gray mold incidence was evaluated. The experimental unit consisted of one box containing one packaging with twelve strawberries. Each replication consisted of one box. The experiment was performed with two replications and conducted twice; packaging containing strawberry fruits inoculated and non-inoculated without the presence of *M. albus* was used as control. The experimental design was completely randomized. Data were subjected to analysis of variance using SAS (SAS Institute, 2003). Treatment means were compared by the Tukey test at 5% probability. Graphics was constructed using SigmaPlot 11.0 (Systat Software, Inc. San Jose, CA 95110).

RESULTS

Control of gray mold of strawberry

On the first experiment, the volatile compounds produced by *M. albus* were able to significantly reduce the incidence of gray mold on strawberries (**Figure 5**). The means of inoculated and uninoculated controls were not statistically different for both gray mold and total decay. However, the mean of inoculated and micofumigated strawberries were different from control as well as for total decay (**Figure 6A**).

In the same way, on the second experiment, the volatile compounds produced by *M. albus* were also able to significantly reduce the disease incidence on strawberry. The means of inoculated and uninoculated controls were not statistically different for total decay. For inoculated and micofumigated fruits the means significantly differed from inoculated control (**Figure 6B**).



Figure 5. Symptoms of gray mold on strawberries micofumigated with *Muscodor albus*. A-C: non-micofumigated with *M. albus* and non-inoculated with *Botrytis cinerea*, D-F: non-micofumigated with *M. albus* and inoculated with *B. cinerea*, and G-I: micofumigated with *M. albus* and inoculated with *B. cinerea*.

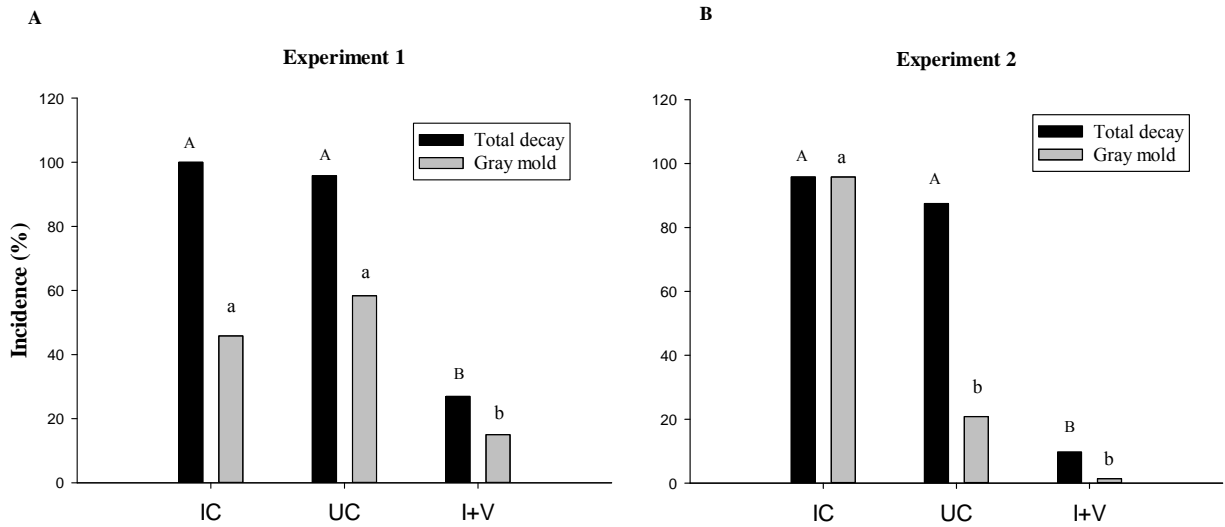


Figure 6. Efficiency of micofumigation with *Muscodor albus* volatiles on gray mold on strawberries. Means followed by the same letter in the column did not differ significantly from each other by Turkey's test ($P = 5\%$). IC: Inoculated control; UC: Uninoculated control; I+V: Inoculated and micofumigated strawberries.

It was not observed disorders on the strawberries exposed to the volatiles, however, the strawberries kept at temperature of 25°C for 5 days slightly changed the color of the pulp, a natural physiological process, evidenced by control that presented this change in color, in the same way.

DISCUSSION

Biofumigation of strawberries with *M. albus*, to control the inoculated pathogen *B. cinerea*, the causal agent of gray mold was shown to be a promising approach, once a continuous fumigation time of 5 days at temperature of 25°C controlled wound and natural infections. These results are comparable to gray mold control by *M. albus* in apples and brown rot of peaches (Mercier & Jiménez, 2004) and green mold of citrus in stored lemon (Mercier & Smilanick, 2005), but in

different conditions. Because of inoculum of *B. cinerea* were previously killed in the *in vitro* fumigation experiment, it is also likely that the pathogen was killed in wounds by the biofumigation treatment.

Although strawberries are highly perishable and have a short shelf life due to its characteristics of being non-climateric, with a thin and delicate epidermis, requiring great care during harvest, is not available yet an efficient and economically viable method to preserve the quality of fresh fruits. The results are, at least in Viçosa - MG, the common exposure at room temperature on store shelves, leading to losses of texture, color, taste and aroma. *Muscodor albus* has also been tested as its fungicidal activity at cold storage temperature against some postharvest pathogens (Mercier et al., 2010; Schotsmans et al., 2008), however, those conditions are rarely employed commercially for strawberries destined for fresh consumption in Brazil.

Biofumigation with *M. albus* may be an important alternative for organic cultivation, but in conventional cultivation it can also be interesting to protect the fruit and reduce the need for further disease control treatments, especially fungicide application, that has been a recurring problem in strawberries, due to irregularities concerning chemical products for disease control. However, further research is needed in order to determine more reliably the concentrations required for efficacy.

This was the first study in Brazil involving the volatile organic compounds produced by *M. albus*. This study revealed that *M. albus* was efficient to control gray mold and total decay in strawberry, indicating that it can be an important alternative to control diseases in strawberry.

CONCLUSIONS

1. Several fungi were associated with postharvest diseases in fruits and vegetables collected in the local market of Viçosa and CEASAMINAS, including new reports for both Brazil and worldwide;
2. The volatiles produced by *M. albus* had fungicide or a possible fungistatic activity, depending on the fungal species or even isolate;
3. The volatiles produced by *M. albus* were able to reduce the gray mold incidence and the total decay of strawberries.

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