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Muscodor brasiliensis sp. nov. produces volatile organic compounds with activity against *Penicillium digitatum*

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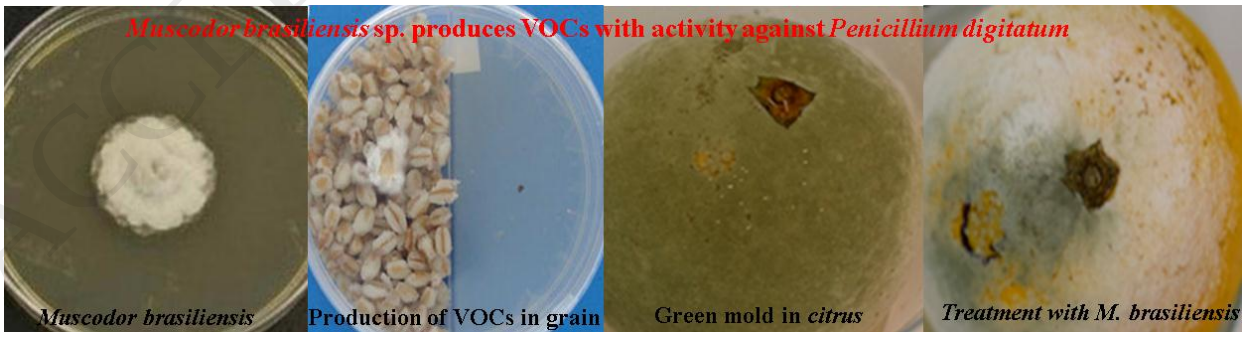

Abstract

Endophytic fungi belonging to *Muscodor* genus are considered as promising alternatives to be used in biological control due to the production of volatile organic compounds (VOCs). The strains LGMF1255 and LGMF1256 were isolated from the medicinal plant *Schinus terebinthifolius* and, by morphological data and phylogenetic analysis, identified as belonging to *Muscodor* genus. Phylogenetic analysis suggests that strain LGMF1256 is a new species, which is herein introduced as *Muscodor brasiliensis* sp. nov. The analysis of VOCs production revealed that compounds phenylethyl alcohol, α-curcumene, and E (β) farnesene until now has been reported only from *M. brasiliensis*, data that supports the classification of strain LGMF1256 as a new species. *M. brasiliensis* completely inhibited the phytopathogen *P. digitatum in vitro*. We also evaluated the ability of VOCs from LGMF1256 to inhibit the development of green mold symptoms by inoculation of *P. digitatum* in detached oranges. *M. brasiliensis* reduced the severity of diseases in 77%, and showed potential to be used for fruits storage and transportation to prevent the green mold symptoms development, eventually reducing the use of fungicides.

Keywords: Biocontrol; VOCs; Endophytic fungus; Plant diseases; Green mold

Introduction

The *Muscodor* genus was described as a sterile endophytic fungus isolated from *Cinnamomum zeylanicum* in Honduras (Worapong et al. 2001), thereafter the isolation of strains belonging to this genus has been reported in tropical rainforests in Australia, Central and South America, and Southeastern and Northeastern Himalayas (Zhang et al. 2010; Suwannarach et al. 2013; Saxena et al. 2015; Pena et al. 2017). The *Muscodor* genus comprises 18 legitimate species deposited at MycoBank database (http://www.mycobank.org) that have been described based on phylogenetic analysis of the *ITS1-5.8S-ITS2* sequences and volatile organic composition analysis (Zhang et al. 2010; Suwannarach et al. 2013; Saxena et al. 2015). Morphologically, these endophytic fungi are differentiated by colony and mycelial characteristics, that appear differ in hyphal arrangement (Strobel et al. 2001; Zhang et al. 2010).

Muscodor species are used in biological control due to the production of volatile organic compounds (VOCs) with antimicrobial properties (Strobel et al. 2001; Worapong et al. 2002; Pena et al. 2017). Interestingly, fungi of the genus *Muscodor* can inhibit a large variety of fungi, but do not inhibit fungi of the same genus (Ezra, Hess and Strobel, 2004). The use of VOCs is especially effective against postharvest diseases in view of the ability to act and diffuse through heterogeneous environments, whereas water-soluble antimicrobial agents are limited to proximal interactions via aqueous-phase diffusion (Worapong et al. 2001; Fialho et al. 2011; Hutchings et al. 2017). Thus, VOCs have already been used to prevent postharvest disease during storage and transport of grapes (Gabler et al. 2010) and peaches (Schanabel and Marcier 2006).

Among the postharvest diseases, the green mold caused by *Penicillium digitatum* (Macarisin et al. 2007; Marcet-Houben et al. 2012) has great impact in the citrus production worldwide, including in Brazil (FAOSTAT 2015), accounting for half of the total fruit wastage (Milind 2008). The green mold symptoms in oranges are

characterized by slight fruit discoloration in the affected region, and at an advanced stage, the mycelium assumes a greenish color due to spore production. The lesion generally shows rapid development and can easily compromise the entire fruit (Timmer et al. 2002). Control of green mold is mainly based on application of several fungicides and maturation regulators (Agrios 2005). However, the widespread use of fungicides for green mold control significantly increases the production costs and has also been associated with negative environmental effects and selection of resistant pathogens (Agrios 2005; Fischer et al. 2011; Talibi et al. 2014). In this scenario, promising results have been found for the biocontrol of this disease using yeasts from the Brazilian Cerrado (Sperandio et al. 2015). Therefore, the use of VOCs produced by *Muscodor* species for biocontrol (Worapong et al. 2001) could be an alternative treatment of postharvest diseases, such as green mold.

Based on the reasoning presented so far, a decision was made to study the Brazilian medicinal plant *Schinus terebinthifolius* as a source of *Muscodor* isolates able to act against the citrus green mold disease. Endophytes from *S. terebinthifolius* have shown high antimicrobial activity (Dos Santos et al. 2016; Tonial et al. 2016); however, *Muscodor* species have not been previously reported as part of the endophytic community of this medicinal plant. Hence, the following objectives were established for this work i) isolate *Muscodor* strains from *S. terebinthifolius*, ii) conduct morphological, VOCs, and phylogenetic analyses to check if the isolated strains belong to the *Muscodor* genus, and iii) investigate the ability of the isolated strains to inhibit *P. digitatum* development.

Materials and Methods

Biological material

Muscodor sp. LGMF1254, previously obtained by our research group (Pena et al. 2017) was used as a selective factor to obtain new isolates of this genus, since it is reported in the literature that *Muscodor* isolates were resistant to *Muscodor* VOCs. *P. digitatum* LGMF1507 was obtained from contaminated market oranges and its identity was confirmed through *ITS* DNA sequence analysis (GenBank accession No.: KY978834). All the strains were deposited in the LabGeM Culture Collection, Federal University of Parana (UFPR), Curitiba, Brazil.

***Muscodor* isolation**

Ten leaves of *Schinus terebinthifolius,* without scratches, or wounds, from healthy plants were collected in Curitiba, Brazil (25°26′52.5″S 49°14′00.3″W). The leaves surfaces were disinfected by immersion in 70% (v/v) ethanol for 1 min, followed by incubation in 2.5% NaClO (v/v available chlorine) for 4 min and washed in 70% ethanol for 30 s. The leaves were rinsed twice with sterilized distilled water for 6 min and cut into fragments of 7 × 7 mm and transferred to Petri dishes containing potato dextrose agar (PDA) medium (pH 5.8) and a previously cultivated inoculum (5 day old) of *Muscodor* sp. LGMF1254. The Petri dishes were incubated at 24°C. Emerging hyphae for the next 2 weeks were transferred to a new PDA plate for further analyses. Two isolates, named as LGMF1255 and LGMF1256, were then isolated and deposited in the LabGeM Culture Collection, UFPR, Brazil.

Morphological characterization

Macroscopic analysis. To assess the differences in growth rates and colony morphology, strains LGMF1255 and LGMF1256 were grown in Petri dishes containing PDA, malt extract agar medium (MEA; malt extract 20.0 g/L, dextrose 20.0 g/L, peptone

1.0 g/L, agar 15.0 g/L), Sabouraud agar (SDA; peptone 15 g/L, dextrose 45g/L, agar 15g/L), or a minimal medium (MM; $NaNO_3$ 6 g/L, KH_2PO_4 1.5 g/L, $MgSO_4.7H_2O$ 0.5 g/L, KCl 0.5 g/L, $FeSO_4$ 0.01 g/L, $ZnSO_4$ 0.01 g/L, dextrose 10 g/L, agar 15.0 g/L), at 24 °C for 8 days. The characteristics of colonies were analyzed by visual inspection, and the growth rate was evaluated by measuring the colony area with the software Image J (Rasband 2016).

Light microscopy analysis. Microcultures of isolates LGMF1255 and LGMF1256 were grown separately based on Kern and Blevins (1997). The isolates were grown in PDA medium at 24 °C for 5 and 10 days, and the slides were stained with lactophenol cotton blue or analyzed directly with distilled water. The slides were examined on Axio Imager Z2 (Carl Zeiss, Jena, DE), with software Metafer 4/VSlide (Metasystems, Altlussheim, DE), by differential interference contrast (DIC) illumination. Micrometry was carried out with the Image J software (Rasband 2016) (National Institutes of Health, Bethesda, MD) with 50 measurements per structure.

Scanning electron microscopy (SEM). The isolates were grown on PDA at 24 °C for 10 days, and fixed in Karnovsky solution (2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.05 M sodium cacodylate buffer, $CaCl_2$ 0.001 M, pH 7.2) for 24 h. The samples were dehydrated in ascending series of ethanol solutions (10%, 30%, 50%, and 70%) for 10 min each step, then ethanol 95% for 15 min, and finally maintained 72 h in 100% ethanol. The samples were brought to a critical point, dried, and coated with 80-nm gold. The observed images of structures and arrangements of mycelium were captured under low-vacuum conditions in a VEGA 3 TESCAN microscope.

DNA extraction, sequencing, and phylogenetic analysis

Genomic DNA extraction was performed using the UltraClean™ Microbial DNA Kit (MO Bio, Carlsbad, CA). For amplification of the *ITS* region of ribosomal DNA, primers ITS1 and ITS4 (White and Marrow 1990; Gerrits Van Den Ende and De Hoog 1999) were used. All the amplicons were purified using the enzymes Exo1 and FastAP™ (GE Healthcare, USA) and were sequenced using the BigDye Sequencing Kit (Applied Biosystems). The sequences were analyzed on an ABI3500 DNA Sequencer (Applied Biosystems). The BioEdit software was applied to edit the sequences, which were compared with sequences from the type species available in the GenBank database of NCBI (http://www.ncbi.nlm.nih.gov/) and Mycobank (http://www.mycobank.org/), and aligned in the CLUSTAL_X v.1.81 software. The best-fit model was identified in web-based MODELTEST 3.7: Kimura 2 parameters plus Gamma were selected. The Bayesian inference phylogenetic analysis was carried out with 10.000 generations by means of the MrBayes software, v.3.1.1, using *Xylaria berteri* (GenBank accession No. GU324749) as an outgroup. Sequences obtained in this study were deposited in GenBank under the following accession numbers *ITS*: KY924493 (LGMF1255) and KY924494 (LGMF1256). The final alignment of ITS was deposited in TreeBASE under accession number 21938.

Identification of volatile organic compounds

The identification of VOCs produced by isolates LGMF1255 and LGMF1256 was performed using solid-phase microextraction coupled gas chromatography with mass spectrometry (SPME-GC-MS). For trapping the VOCs produced, the cultures were set up with 5 mL of PDA in a 20 mL glass test tube closed with a rubber stopper and incubated at 24 °C; a syringe with a stable, flexible fiber made of 50/30 divinylbenzene (DVB)/carboxen on polydimethylsiloxane (PMDS) (Supelco, Sigma-Aldrich) was employed. The syringe was manually inserted into a small hole in the rubber stopper of

the test tube, and the fiber was exposed to the air space above the 10 days older mycelium for 60 min and immediately desorbed into the hot injector of the gas chromatograph coupled to a mass spectrometer.

Volatile constituents were analyzed on a Shimadzu GC-2010 gas chromatographer coupled with a GCMS-QP2010 Plus mass spectrometer, fitted with a Shimadzu AOC-20i auto sampler and equipped with a Rtx-5MS capillary column (30 m × 0.25 mm × 0.25 µm). The device was operated at programmed temperatures from 60 °C to 250 °C changed at 3 °C min, and at an injector temperature of 250 °C, with an injection of 15 min. The interface ion source was at 300 °C, analyzing molecular fragments between 40 and 350 m/z, with helium as a carrier gas at a flow rate of 1.02 mL/min, in the ionization mode: electron impact 70 eV. The constituents of the volatiles were identified by calculating retention indexes (RI) and compared with mass spectra from the NIST08 library and with data from the literature (Adams 2007). Compounds identified in control test tubes, and the silylated compounds from the column were subtracted from the analysis. Experimental RIs were determined by means of the *n*-alkane series(C_9–C_{24}) standard, using the equations of Van der Dool and Kratz (Van Den Dool and Kratz 1963). The components were quantified according to the peak area, without correction for response factors. Each condition (PDA and PDA plus *Muscodor* isolate) was analyzed in triplicate.

Activity against *Penicillium digitatum*

Inhibition of P. digitatum mycelial development. The production of VOCs with antifungal activity, against *Penicillium digitatum* (LGMF1507), by strains LGMF1255 and LGMF1256 were evaluated primarily in PDA media using plates with a divisor (Pena et al. 2017). The strain that showed the highest antifungal activity was selected for

evaluation of VOCs production in autoclaved wheat or oatmeal grains as a nutrient source. Petri dishes with two compartments were used, one compartment containing PDA (10 mL) for the phytopathogen development and in the other wheat or oatmeal. Mycelial discs (5-mm diameter) of 10-day-old colonies of selected strain were added to the compartments containing wheat, or oatmeal. After 7 days, an inoculum of *P. digitatum* LGMF1507 (1-mm diameter) was placed in the PDA compartment. As a positive control only the phytopathogen was inoculated. The Petri dishes were sealed with Parafilm® and Scotch® tape and incubated at 24 °C for 8 days. The inhibition analysis was performed comparing the growth of the pathogen (colony area: cm^2) in comparison to control.

Biocontrol assay in fruits

The experiment was conducted using 40 organic sweet oranges (*Citrus sinensis* (L) Obsbeck). The fruits were disinfected, first immersed in 70% (v/v) ethanol for 1 min, followed by immersion in 2.5% NaClO (v/v available chlorine) for 3 min and washed with 70% ethanol for 30 seconds and finally the fruits were rinsed twice with sterilized distilled water. The *P. digitatum* LGMF1507development in citrus fruits was performed as described by López-Pérez (2014). To evaluate the ability of endophytic strain to inhibit the *P. digitatum* development, each orange was placed in disinfected transparent plastic cylinders (volume of 500 mL, r = 5.5 cm, h = 8 cm) and the fruit was inoculated with 10 µL of phytopathogen spore solution (10^6 conidia/mL) deposited into 12 small injuries distributed in an area of 1 cm^2. In the treatment, a culture plate without the lid containing the *Muscodor* strain, growth on PDA for 20 days was deposited inside of the plastic cylinder. In the positive control only the phytopathogen was inoculated, and the negative control consists in the inoculation of a saline solution (NaCl 0.85%), instead of the phytopathogen. As fungicide control, Magnate 500 EC® (2 mL/L), a systemic imidazole, was sprayed directly over the injuries. The experiment was conducted using 10 oranges

for each condition. The cylinders were closed and sealed with the Scotch® tape and incubated at 24º C. The development of green mold in oranges were evaluated daily, until the oranges in the positive control group were fully covered by the green mold, but no later than 10 days after inoculation. The ability of selected strain to inhibit *P. digitatum* development in fruit was evaluated comparing the diameter of lesion in the treatment with the control.

Statistics

The results on growth in different media, the antifungal effect against *P. digitatum* on PDA, grains, and on the detached oranges were analyzed according to the last day of evaluation, and normality of the residual distribution was verified (Shapiro and Wilk 1965).The obtained means were grouped by the Tukey test ($p \leq 0.05$) and the Student *t* test, or the mean pair comparison was conducted when there was a pair of responses due to variable function. For quantitative analysis, regression analyses were conducted, with adjustment of curves and functions by linear, quadratic, and cubic models (Supporting material S4). The R program (Core Team 2013) was used to implement the functions and to generate the routines of the statistical tests, with the aid of the ExpDes package and Agricolae package (Mendiburu 2010) for statistical functions implementations. All the experiments above were conducted at least in triplicate.

Results and Discussion

Isolation and identification of *Muscodor* isolates

In this study, we present the prospection of two *Muscodor* isolates, LGMF1255 and LGMF1256, obtained from the medicinal plant *S. terebinthifolius* against the causal agent of citrus green-mold, *P. digitatum*.

Two endophytic isolates from leaves of *S. terebinthifolius*, LGMF1255 and LGMF1256, showed morphological characteristics consistent with the genus *Muscodor*, such as ivory mycelium, without reproductive structures (Figure 1) and with a musty odor. Isolate LGMF1255 showed faster growth on PDA, MEA, and SDA, than MM medium (Figure 1.1) after 8 days of incubation (Supporting material Figures S1, S2). Using light microscopy, hyaline thin-walled septate hyphae, diameter of 0.45 to 2.04 µm ($\bar{x} = 1.05$ µm, SD = 0.355 µm), with right angles between branches and coiling structures in the center (Figure 1) were observed. SEM showed structures previously described for the *Muscodor* genus, called cauliflower-like or bloom structures (Figure 1.1G) and vesicles (Figure 1.1H, Table S2). It was also possible to notice the hyphal growth interlacing on PDA (Figure 1.1I) and no specific organization of the mycelium (Figure 1.1J).

Isolate LGMF1256 had a dense mycelium, with appearance of multiple layers of growth in colonies and irregular edges on PDA. On MEA, its appearance was also dense but more uniform than on PDA (Figure 1.2A and 1.2C). On SDA (Figure 1.2D), the colonies showed a regular colony border, with prominent radial lines along the mycelium extension and a dense mycelium. The area of colonies on the eighth day was 4.79, 4.29, or 4.24 cm² on PDA, MEA, and SDA, respectively. The growth was slower on MM than on the other media, with colony area of 2.35 cm² on average, after 8 days (Figure 1.2B and figure S2). Light microscopy analysis of isolate LGMF1256 revealed hyaline septate hyphae, thin walls with diameter of 0.79 to 2.19 µm ($\bar{x}$= 1.45µm, SD = 0.34 µm), and straight angles between branches (Figure 1.2E and 1.2F, Table S2). The hyphae showed interlacing and formed ropelike strands. SEM revealed cauliflower-like structures (Figure 1.2G), and no specific organization of the mycelium was observed (Figures 1.2H, 1.2I and 1.2J). Strain LGMF1255 showed the presence of coiling hyphae; unlike in isolate LGMF1256, where these structures were not observed (Table S2). Coiling hyphae are

common among *Muscodor* species; however, this characteristic is not associated with the phylogenetic analysis, since it is reported for different species belonging to different phylogenetic groups into *Muscodor* genus (Daisy et al. 2002; Kudalkar et al. 2012; Meshram et al. 2013; Meshram et al. 2014).

Phylogenetic analysis

ITS sequence analysis included 22 *Muscodor* sequences, with 18 from type strains recognized as legitimate species by the Mycobank database (Mycobank.org) and the outgroup *Xylaria berterii*. The alignment contained 371 characters, with 289 conserved sites, 82 variables and 42 parsimony-informative. Based on *ITS* sequence analysis, *Muscodor* species were grouped in two clades (Figure 2), and isolates LGMF1255 and LGMF1256 were in Clade 2. Strain LGMF1255 was found to be close related to species *M. vitigenus*, *M. equiseti*, and *M. sutura* showing 100% sequence similarity with these strains, a cryptic group into *Muscodor* genus, in which it is not possible to perform species identification based on ITS phylogenetic analysis (Pena et al. 2017).

The position of isolate LGMF1256, as a sister species of *M. yucatanensis* and *M. coffeanum*, but located in a separate branch, suggests this strain as a new species in the *Muscodor* genus, named *Muscodor brasiliensis*. All the branches and clades in the phylogenetic analysis are supported by high posterior probability values (> 0.94). The designation of *M. brasiliensis* as a new species is also supported by micromorphology analysis - hyphae diameter of 2.19 μm, in comparison to *M. equiseti*, *M. yucatanensis*, and *M. coffeanum* (Table S2) that have greater diameters: 2.9, 4.0 and 5.5 μm, respectively (González et al.2009; Suwannarach et al.2013; Hongsanan et al.2015). Names of *Muscodor* species are commonly related with its host (González et al.2009; Swannarach et al.2013; Hongsanan et al.2015), however we obtained two different

species from the same host, consequently, we proposed the name of *M. brasiliensis*, based on the country where it was isolated.

Identification of volatile organic compounds

Once VOCs were used for *Muscodor* species identification, we analyzed this characteristic of both isolates LGMF1255 and LGMF1256. In *M. brasiliensis* LGMF1256 14 VOCs were detected, and *Muscodor* sp. LGMF1255 analysis yielded 12 chemical compounds overall. All the compounds present in the control were disregarded, as were silylated compounds belonging to the equipment column. Both strains produced 10 compounds in common (Table 1). The compounds produced by both strains were octylformate, 2-phenyl ethyl acetate,2-undecanone, β-elemene, *n*-tetradecane, α-guaiene, β-selinene, aciphyllene, α-bulnesene, and pogostol. Among them, many already have been found to be produced by other microorganisms or plants, and their bioactive properties have not been described (Strobel et al. 2007; Swamy and Sinniah 2015). The active compound, phenylethyl alcohol, detected in *M. brasiliensis* LGMF1256 was reported inhibiting the growth of gram-negative microorganisms by interfering with DNA synthesis, cell membranes stability (Corre et al. 1990), and may be related to the inhibition of *P. digitatum*, as observed in our experiments. This compound also is produced by *M. albus* (Strobel et al. 2007) and *M. fengyangensis* (Zhang et al. 2010), strains with antimicrobial activity. The most representative VOC produced by both isolates was pogostol, isolated first from *Pogostemon cablin*, plant from the family Lamiaceae, compound that has being already used as an antimicrobial agent (Swamy and Sinniah 2015). Compound α-curcumene, produced by *Curcuma zedoaria*, causes apoptotic effect on ovarian tumor cells (Shin 2013), and has been found in some fungi and bacteria (Korpi et al. 2009). However, it is the first report of α-curcumene production by a *Muscodor*

species (Table 1), which can also be used to differentiate *M. brasiliensis* from the other species of the *Muscodor* genus.

Taxonomic description

Muscodor brasiliensis Pena, Servienski& Kava, **sp. nov.**

MycoBank No.: MB#820079

GenBank accession No.: *ITS*: KY924494; *RPB2*: MF510171

Etymology. Named after the country in which it was isolated, Brazil.

The fungus in nature is associated with the plant *Schinus terebinthifolius* and is an Ascomycete with a sterile mycelium. The colonies of *M. brasiliensis* grown on PDA, MEA, and SDA media, with diameters of 4.79, 4.79, and 4.24 cm respectively (Table S2), at 24 °C, 8 days after inoculation. Growth covered the Petri dishes of 9 cm PDA in 3 to 4 weeks and produced a characteristic odor of the genus that was also detected in the other culture media used. The light microscopy analysis revealed hyaline hyphae, with diameter of 0.79 to 2.19 µm ($\bar{x}$ = 1.45 µm, SD = 0.34 µm) septate, branched at a right angle, thin-walled. The hyphae were intertwining and forming rope-like strands. No conidia or other sporulation structures were observed. *M. brasiliensis* produces, α-curcumene and *n*-pentadecane as exclusively VOCs, compared to others *Muscodor* species.

Specimen examined: Brazil, Paraná, Curitiba 25°26′52.5′′S 49°14′00.3′′W. *Muscodor brasiliensis* is an endophytic fungus from internal tissue of leaves of *Schinus terebinthifolius* (*Anacardiaceae*) isolated in September 2013 by Servienski, A. & Kava, V. (Holotype: 91050, ex-type culture: LGMF1256).

Antifungal activity of *Muscodor brasiliensis* against *Penicillium digitatum*

As the VOCs produced by *Muscodor* species are related to high biotechnological potential we decided to analyze the ability of the new species, *M. brasiliensis* LGMF1256 to control an important post-harvest pathogen, *P. digitatum.* Strains LGMF1255 and LGMF1256, on PDA medium, inhibited *P. digitatum* growth by 70% and 100%, respectively. In view of the great inhibition caused by the VOCs produced by *M. brasiliensis* LGMF1256, this strain was selected for the analysis of VOCs production using wheat or oatmeal as a nutritional source. Similarly, on these substrates, the VOCs produced completely inhibited the phytopathogen growth (Figure 3, Table 2), data that suggest a great potential to be used in industrial scale (Table 2).

The potential use of *M. brasiliensis* LGMF1256 to control the green mold during fruit transportation was demonstrated in the experiment with detached organic orange fruits artificially infected with *P. digitatum* LGMF1507. *M. brasiliensis* LGMF1256 was able to suppress the green-mold lesion size by approximately 77% in the detached organic oranges (Figure 4), lesions reached 25.2 cm^2 in the positive control (Figure 4C), and 5.9 cm^2 in the treatment with *M. brasiliensis* LGMF1256 (Figure 4F; Table S1 - Tukey at the significance level of 0.05%,). In the negative control, green-mold symptoms were not observed, and the fungicide treatment completely inhibited the pathogen development.

The reduction (77%) in the severity of green mold symptoms, suggests that LGMF1256 VOCs have great potential to disease control. Similar results were obtained by Suwannarach et al. (2013) with *M. suthepensis* for biofumigation-based to control tangerine rot, also caused by *P. digitatum*. The use of VOCs to inhibit microbial growth has been studied for different purposes and there are already indications for the use of

these compounds to combat citrus diseases. Fialho et al. (2011) reported a high inhibitory activity (87% inhibition) of the VOC ethyl acetate produced by *Saccharomyces cerevisiae* against *Phyllosticta citricarpa*, the causal agent of citrus black spot (CBS). In addition, previous results from our research group demonstrated the potential of one *Muscodor* strain, LGMF1254, suppressing *P. citricarpa* growth (Pena et al. 2017).

The presence and mixture of several types of VOCs (3-methyl-1-butanol, naphthalene and propanoic acid), is necessary for *M. albus* to inhibit the phytopathogens *S. sclerotiorum*, *Pythium ultimum*, and *Rhizoctoniasolani*, indicating the synergism among the different components of the mixture (Ezra et al. 2004). In this view, it is more probably that a mix of compounds, instead of a single compound, can be responsible for the biological activity of *M. brasiliensis*. The use of a mix of compounds is particularly interesting as a treatment of phytopathogens because the compounds may represent different mechanisms operating at the same time, preventing the selection of resistant strains (Tonial et al. 2017).

M. brasiliensis LGMF1256 was able to suppress the green-mold lesion size by approximately 77% in the detached organic oranges (Figure 4), lesions reached 25.2 cm^2 in the positive control (Figure 4C), and 5.9 cm^2 in the treatment with *M. brasiliensis* LGMF1256 (Figure 4F; Table S1 - Tukey at the significance level of 0.05%,). In the negative control, green-mold symptoms were not observed, and the fungicide treatment completely inhibited the pathogen development.

In conclusion, *Schinus terebinthifolius* is a host of two strains belonging to the *Muscodor* genus: *Muscodor* sp. LGMF1255 and the new species *Muscodor brasiliensis* LGMF1256. The VOCs produced by *M. brasiliensis* were able to significantly decrease the severity of green mold symptoms on oranges, suggesting the potential use of these

compounds during the fruits storage and transportation to inhibit green mold development, mainly in organic fruits.

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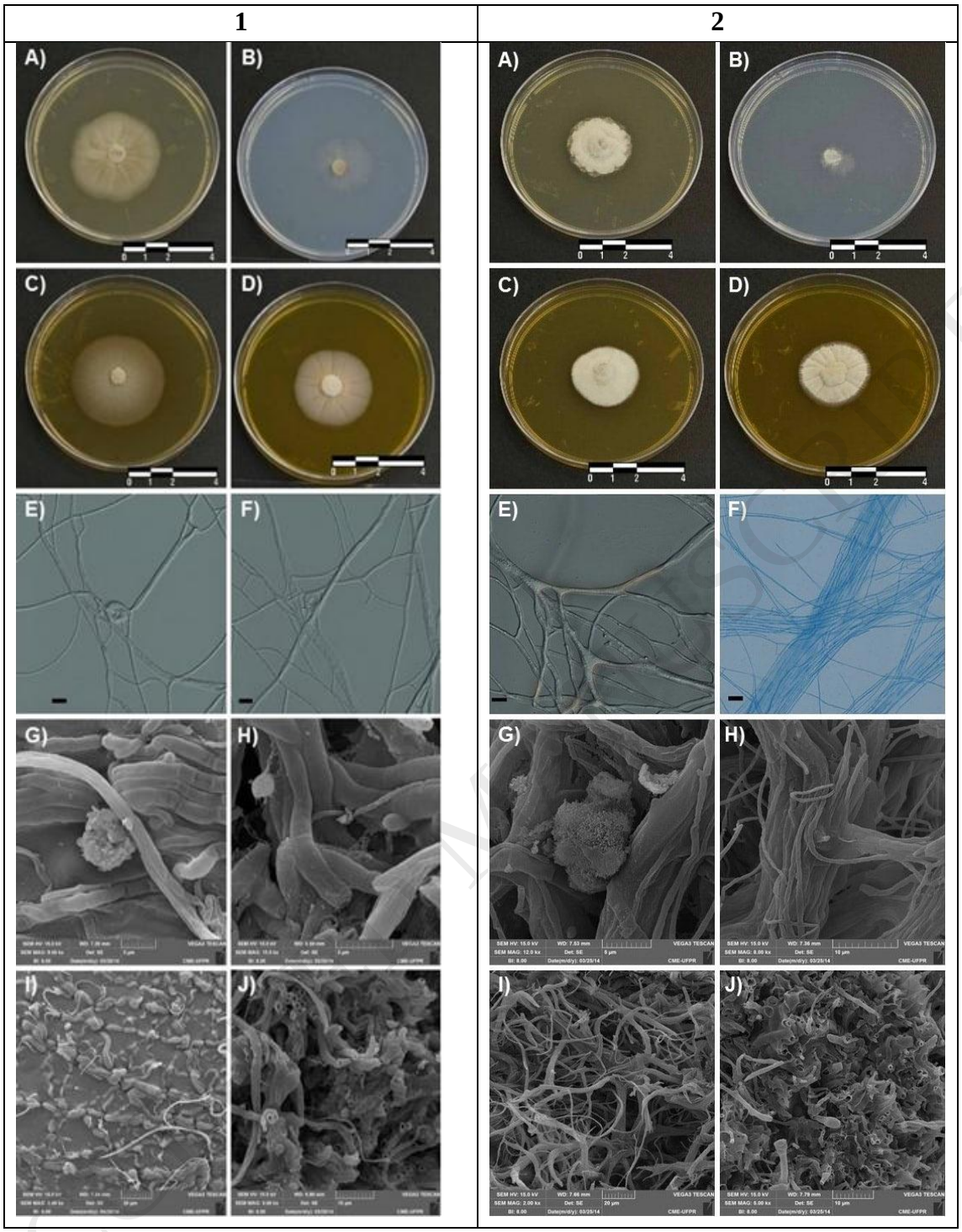
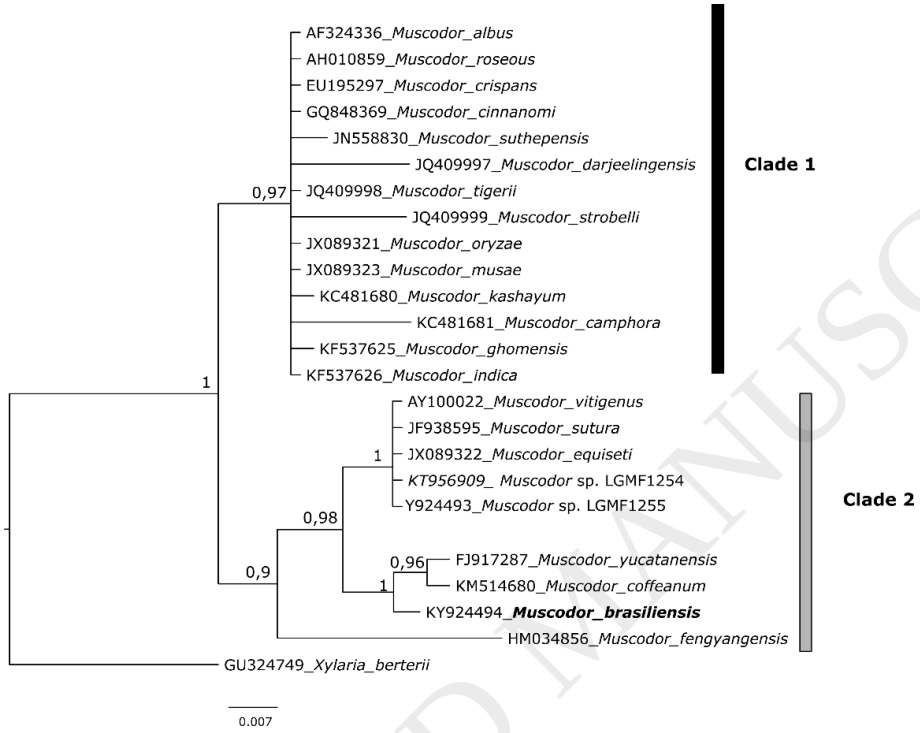
Figures captions

Figure 1. Morphological characteristics of isolates LGMF1255 (box 1) and LGMF1256 (*Muscodor brasiliensis*) (box 2) after 8 days of growth. Macromorphology of isolate LGMF1255 (*Muscodor* sp.) (box 1) cultivated on PDA (1A), MM (1B), MEA (1C), and SDA (1D). Micromorphological aspects of isolate LGMB1255 showing coiling formation (1E, F), cauliflower-like structures (1G), vesicles (1H), hyphae interlacing (1I), and mycelium arrangement (1J). Macromorphology of isolate LGMF1256 on PDA (2A), MM (2B), MEA (2C), and SDA (2D). Micromorphology aspects showing mycelium development (2E-F), cauliflower-like structures (2G) and mycelium arrangements (2H, 2I,2J).



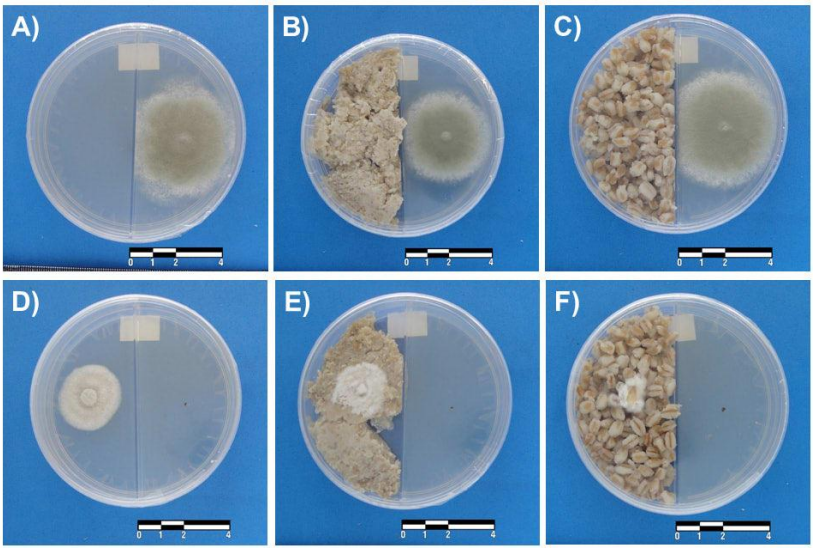
Notes: The scale bars on panels A–D are 4 cm. The scale bars on light microscopy images (E and F) are 10 µm. In the SEM images, the scale is 5 µm (G), 10 µm (H and J), and 20 µm (I).

Figure 2: Bayesian phylogenetic tree of *Muscodor* species based on the partial sequences of the ITS region (ITS1-5.8S-ITS2).



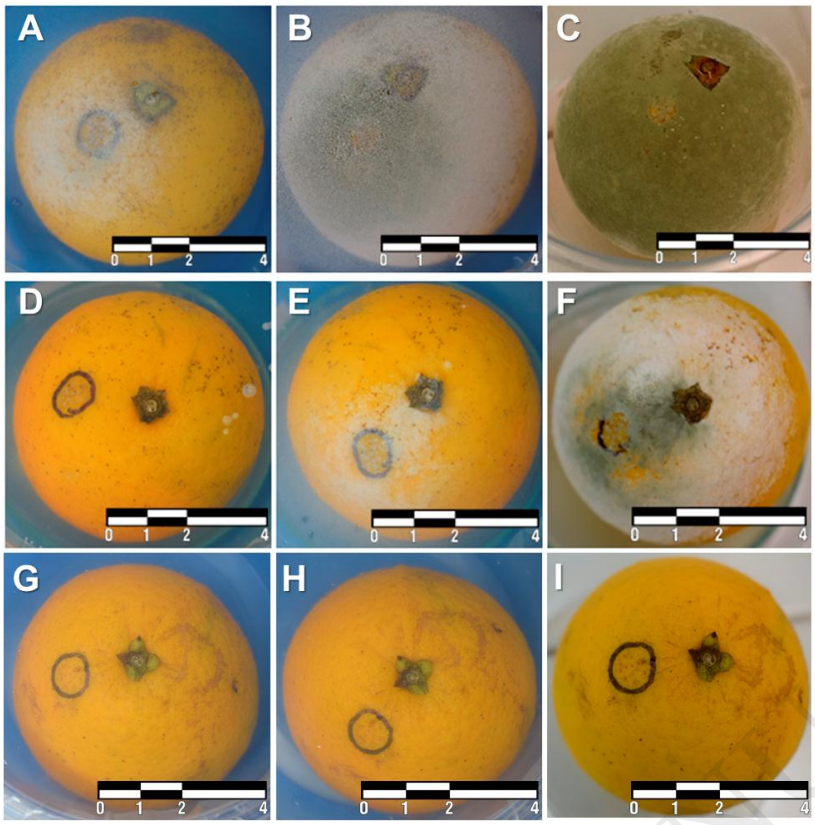
Notes: *Xylaria berteri* (GU324749) as outgroup. Scale bar: 0.006 substitutions per nucleotide position. Values at the nodes indicate Bayesian posterior probabilities.

Figure 3: Antifungal activity of VOCs produced by *Muscodor brasiliensis* LGMF1256, cultivated in different nutritional sources, against *Penicillium digitatum* LGMF1507.



Notes: **A–C)** positive control: only *P. digitatum* LGMF1705; Antifungal activity of VOCs produced by *M. brasiliensis* in PDA **D),** oatmeal **E)** and wheat **F).** The scale bars on panels represent 1, 2 and 4 cm.

Figure 4: Inhibition of green-mold symptoms in detached organic oranges caused by *Muscodor brasiliensis* LGMF1256 VOCs produced in PDA.



Notes: **A**, **B**, and **C**: positive control group with *Penicillium digitatum* on the 4th day**,** 5th day, and 6th day, respectively. **D, E**, and **F**: treatment with *M. brasiliensis* LGMF1256 on the 4th day**,** 5th day, and 6th day, respectively. **G, H** and **I**: treatment with the fungicide on the 4th day**,** 5th day, and 6th day, respectively

Table 1: Main volatile compounds produced by *Muscodor* sp. LGMF1255 and *Muscodor brasiliensis* LGMF1256 on potato dextrose agar (PDA) at 24°C after 10 days by SPME-GC-MS analysis

***Muscodor* sp. LGMF1255**				
RT (min)	AI	Average area (%)	AI[§] -	Possible compound
13.01	1135	7.21	1131	**Octylformate**
16.807	1224	3.84**	1254	**2-phenyl ethyl acetate**
19.423	1284	1.48	1293	**2-undecanone**
22.81	1362	1.13	1359	*neoio*-dihydrocarveolacetate
23.68	1382	6.12	1390	**β-elemene**
24.457	1400	1,01	1400	***n*-tetradecane**
24.81	1408	1.28	1409	*Z*- caryophyllene*
25.68	1429	4.59	1439	**α- guaiene***
27.603	1475	2.67	1489	**β-selinene**
28.07	1486	1.54**	1501	**Aciphyllene**
28.33	1492	6.53	1509	**α-bulnesene**
33.73	1628	62.60	1651	**Pogostol**
***Muscodor brasiliensis* LGMF1256**				
RT (min)	AI	Average area (%)	AI[§] -	Possible compound
10.903	1083	16.98	1106	phenylethylalcohol*
13.020	1135	8.15	1131	**octyl formate**
16.803	1224	12.17	1254	**2- phenylethylacetate***
18.950	1273	0.97**	1293	**2-undecanone***
23.680	1382	2.73**	1390	**β-elemene**
24.453	1400	1.24	1400	***n*-tetradecane**
25.677	1429	3.50	1439	**α-guaiene ***
27.213	1465	3.83	1479	α-curcumene
27.513	1473	3.57	1454	*E*-(β)-farnesene*
27.610	1475	2.46**	1489	**β-selinene**
28.073	1486	0.66	1501	**Aciphyllene**
28.330	1492	5.96	1509	**α-bulnesene**
28.643	1500	1.28**	1500	*n*-pentadecane
33.727	1628	36.51	1651	**Pogostol**

Notes: RTx retention time, AI arithmetic index; AI[§] arithmetic index by Adams, 2007; *Compounds identify by NIST and Adams, 2007; **Average obtained by two measures; Bold compounds names are produced by *Muscodor sp.* LGMF1255 and *Muscodor brasiliensis* LGMF1256.

Table 2: Percentage inhibition of radial growth (PIRG) of *Penicillium digitatum* LGMF1507 after 5 days of exposure to volatile organic compounds (VOCs) produced by *Muscodor brasiliensis* LGMF1256 on different nutrient sources.

Medium	PIRG of *P. digitatum*
Negative control	0 a *
PDA	99.5 b
Wheat	98.4 b
OatMeal	97.9 b
S-W=	**0.60*** **
CV (%)=	2.5

Notes: * Original Medium Averages; ** Means followed by the same letter in the column do not differ by the Tukey test at 5% probability; *** Values S-W in bold mean normal distribution by Shapiro-Wilk at .05 significance level. Negative control represents the evaluation without inoculation of *M. brasiliensis*.