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The same host but a different *Muscodor*: A new *Muscodor albus* isolate from wild pineapple (*Ananas ananassoides*) with potential application in agriculture

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ABSTRACT

Muscodor is an endophytic fungal genus whose members produce volatile organic compounds (VOCs) with broad antimicrobial, nematocidal and insecticidal activities. This study describes the isolation and characterization of a new strain of *Muscodor albus*, designated as *M. albus* aa3, from wild pineapple (*Ananas ananassoides*) plants collected in Havana, Cuba. *In vitro* cultures of *M. albus* aa3 on both potato dextrose agar media (PDA) and paddy produced VOCs with antimicrobial activity against *Escherichia coli* and plant pathogens, including several species of the *Phytophthora* genus and the wood rot fungus *Fomitiporia maxonii*; but innocuous to the beneficial mycopathogen *Trichoderma koningii*. GC/MS analysis indicated the unique composition of the mixture of VOCs emitted by aa3, in which sesquiterpenes represent the most abundant compounds. VOCs emitted during the growth of *M. albus* aa3 on paddy grains protected Persian lime (*Citrus* × *latifolia* Tanaka) fruits from infection by *Phytophthora nicotianae* Breda de Haan, suggesting the potential use of this fungus for postharvest biofumigation. Isolation of *M. albus* from wild pineapple points out this plant as a susceptible host to be colonized by distinct species of the *Muscodor* genus.

1. Introduction

Endophytes are a group of organisms that colonize plant tissues and typically produce inconspicuous infections. *Muscodor* is an endophytic fungal genus belonging to the *Ascomycetes* and is closely related to the endophytic group *Xylaria* (Worapong et al., 2001). *Muscodor* species display a sterile mycelium and produce an array of low-molecular weight volatile organic compounds (VOCs) with broad antimicrobial, nematocidal and insecticidal activities (Lacey and Neven, 2006; Riga et al., 2008; Strobel, 2006). Up to date, sixteen *Muscodor* species have been isolated from tropical and subtropical native wild plants collected in rainforests over the world (Daisy et al., 2002; González et al., 2009; Kudalkar et al., 2012; Meshram et al., 2014, 2013; Mitchell et al., 2008; Saxena et al., 2015, 2014; Suwannarach et al., 2013, 2010; Worapong et al., 2002, 2001; Zhang et al., 2010).

Muscodor albus Worapong, Strobel and Hess (Worapong et al., 2001) and *Muscodor crispans* (Mitchell et al., 2008) are two *Muscodor* species that exhibit distinctive characteristics. *M. albus* displays whitish colonies with rope-like and coiling hyphae whilst *M. crispans* has white to red color-changing colonies at sun light and hyphae with cauliflower-like structures (Mitchell et al., 2010).

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Several strains of *M. albus* have been isolated from tropical trees and vine species around the world (Atmosukarto et al., 2005; Banerjee et al., 2014, 2010; Ezra et al., 2004; Sopalun et al., 2003; Strobel et al., 2007, 2001), indicating a wide distribution range of this species. In contrast, *M. crispans* has been merely isolated from an *Ananas ananassoides* (Baker) L.B.Sm (wild pineapple) plant found in the Bolivian Amazon (Mitchell et al., 2008). *A. ananassoides* is a bromeliad native from Central and South American regions extending from Costa Rica to Paraguay. The plant is valuable as an ornamental and is traditionally propagated vegetatively. This practice leads to a passive transmission of pathogens and non-pathogenic organisms like fungal endophytes.

Fumigation using bioproducts obtained from cultures of *Muscodor* spp. on cereal grains has shown to be effective in controlling soil-borne, seed-borne and postharvest diseases, as well as building molds (Mercier and Jimenez, 2007; Suwannarach et al., 2012). Consequently, *Muscodor* species are considered a promise for biocontrol (Strobel, 2006; Talibi et al., 2014), and the discovery and characterization of new isolates and taxa from this genus have gained the interest of mycologists (Zhang et al., 2010).

Here, with the aim to find alternatives to chemical fungicides, we sought out for the putative presence of *Muscodor crispans* in wild pineapple plants gathered in a botanic collection in Havana, Cuba. Unexpectedly, instead of *M. crispans*, we isolated a new strain of *M. albus* (designated *M. albus* aa3), a fact that points out *A. ananassoides* as a host for different species of the genus *Muscodor*. Besides, it was demonstrated that VOCs produced by the new isolate have growth-inhibitory and/or biocide action against *Escherichia coli* and several species of crop pathogens. Finally, we present elements that suggest the potential application of *M. albus* aa3 grown on paddy grains as a postharvest biofumigant.

2. Materials and methods

2.1. Isolation of endophyte fungi from the wild pineapple (*A. ananassoides*)

The source for endophyte isolations consisted of *A. ananassoides* plants kept as part of the *Ananas* collection of the Research Institute on Tropical Fruits (IIFT) in Havana, Cuba, in April 2010. The origin of the plants is unknown, and they are systematically vegetatively propagated. Plant leaves were cut in fragments (3 cm) and sterilized under a laminar flow hood according to the following procedure: incubation in 96% ethanol for 3 min, 0.5% NaOCl for 1 min, 96% ethanol for 0.5 min. Subsequently, the leaves were cut in small pieces (0.5 × 1.0 cm), the alcohol excess was air dried and the fragments were placed onto potato dextrose agar medium (PDA) plates to allow the growth of endophyte fungi (Fig. 1A). Petri plates were examined periodically during 15 days. Once fungus hyphae were observed, their tips were aseptically cut out from the medium and placed on fresh PDA.

2.2. Microorganisms

All plant fungi and *E. coli* used as targets in the bioassay test were obtained from the collection of the IIFT. All microorganisms were grown on PDA at 25 ± 2 °C and the fungal bioassay tests were conducted using fresh cultures.

2.3. Detection of the antimicrobial activity of volatile organic compounds

Emission of volatiles with antibiotic activity was estimated following the bioassay plate test previously described by Strobel et al. (2001). In brief, a plug of media containing the putative endophyte was placed on one half of a PDA agar Petri plate (9.0 cm diameter) where a diametrical stripe of 1 cm was previously removed (Fig. 1B). After 7 days, the target organism was placed on the opposite half. To test the production and the antibiotic activity of volatiles from the endophyte grown on paddy grains, one half of the PDA media was completely removed from Petri plates (9.0 cm diameter). Target microorganisms were inoculated on the remaining PDA half and three endophyte-colonized paddy grains were placed in the side without medium (Fig. 3B). All plates were wrapped with two layers of Parafilm™ and incubated at 25 ± 2 °C in the dark. The growth of the target organisms was visually analyzed. Eventually, the linear growth of the filamentous fungi (using as reference the edge of the agar inoculum plugs) and the viability of each target fungus and bacterium were evaluated. For viability test, an agar plug containing the target fungus was placed in a fresh PDA Petri plate, or the target bacterium was re-streaked in a fresh medium. In both cases, the growth was evaluated 2–3 days after the inoculation (Strobel et al., 2001). In parallel, target microorganisms were allowed to grow in the absence of endophytes to assess their growth parameters under normal conditions. Target microorganisms grew normally in the presence of endophyte-free paddy grains.

2.4. Scanning electron microscopy

Scanning electron microscopy was performed as previously described (Castillo et al., 2005). Mycelia from *M. albus* aa3 were incubated in a solution containing 2% gluteraldehyde, 0.1 mol L^{-1} sodium cacodylate buffer (pH 7.2–7.4) and 0.02% Triton X100. The solution was aspirated for 5 min and incubated overnight. After 24 h, samples were rinsed as follows: six times in distilled water, 15 min in sodium cacodylate buffer (v:v) 15 min/change in 10% ethanol, 15 min/change in 30% ethanol, 15 min/change in 50% ethanol, five times of 15 min/changes in 70% ethanol. Samples were incubated overnight in 70% ethanol and rinsed six times for 15 min in 95% ethanol, three times in 15 min/changes in absolute ethanol and three times in 15 min/changes in acetone. Finally, the samples were dried up to the critical point and gold sputter coated. Images were recorded with an XL30 ESEM FEG in the high vacuum mode using the Everhart–Thornley detector. Hyphae were measured using Image J software (available online: http://rsb.info.nih.gov/ij/).

2.5. Manipulation of *M. albus* isolate aa3

To stimulate the sporulation or the induction of fruiting bodies of *M. albus* aa3, leaf pieces of carnation (*Dianthus caryophyllus*) and wild pineapple were placed on top of actively growing hyphae cultivated on PDA. Alternatively, the fungus was also cultivated on different media including Oat Agar, Water Agar, V8 Agar, Czapeck Agar, Modified Czapeck Agar, and Potato Sucrose Agar. In all the conditions and media tested, *M. albus* aa3 was grown in natural and artificial lights, and in the dark.

To preserve the fungus, small pieces of PDA media containing hyphae were placed into vials containing 15% glycerol and stored at −70 °C (Mitchell et al., 2008). Fungus cultures on paddy grains were stored at 4 °C in distilled water.

2.6. Fungal DNA isolation and ITS1-5.8S-ITS2 rDNA sequence analysis

A seven day old culture of *M. albus* aa3, grown on PDA, was used as DNA source. Approximately 0.1 g of mycelium was ground using liquid nitrogen and homogenized with 1 mL of the extraction buffer (100 mmol L^{-1} Tris-HCL pH 8.0; 50 mmol L^{-1} EDTA pH 8.0; 500 mmol L^{-1} NaCl; 0.07% 2-mercaptoethanol). After addition of