Muscodor albus E-6, an endophyte of *Guazuma ulmifolia* making volatile antibiotics: isolation, characterization and experimental establishment in the host plant

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Muscodor albus is an endophytic fungus, represented by a number of isolates from tropical tree and vine species in several of the world's rainforests, that produces volatile organic compounds (VOCs) with antibiotic activity. A new isolate, E-6, of this organism, with unusual biochemical and biological properties, has been obtained from the branches of a mature *Guazuma ulmifolia* (Sterculiaceae) tree growing in a dry tropical forest in SW Ecuador. This unique organism produces many VOCs not previously observed in other *M. albus* isolates, including butanoic acid, 2-methyl-; butanoic acid, 3-methyl-; 2-butenal, 2-methyl-; butanoic acid, 3-methylbutyl ester; 3-buten-1-ol, 3-methyl-; guaiol; 1-octene, 3-ethyl-; formamide, *N*-(1-methylpropyl); and certain azulene and naphthalene derivatives. Some compounds usually seen in other *M. albus* isolates also appeared in the VOCs of isolate E-6, including caryophyllene; phenylethyl alcohol; acetic acid, 2-phenylethyl ester; bulnesene; and various propanoic acid, 2-methyl- derivatives. The biological activity of the VOCs of E-6 appears different from the original isolate of this fungus, CZ-620, since a Gram-positive bacterium was killed, and *Sclerotinia sclerotiorum* and *Rhizoctonia solani* were not. Scanning electron micrographs of the mycelium of isolate E-6 showed substantial intertwining of the hyphal strands. These strands seemed to be held together by an extracellular matrix accounting for the strong mat-like nature of the mycelium, which easily lifts off the agar surface upon transfer, unlike any other isolate of this fungus. The ITS-5.8S rDNA partial sequence data showed 99 % similarity to the original *M. albus* strain CZ-620. For the first time, successful establishment of *M. albus* into its natural host, followed by recovery of the fungus, was accomplished in seedlings of *G. ulmifolia*. Overall, isolates of *M. albus*, including E-6, have chemical, biological and structural characteristics that make them potentially useful in medicine, agricultural and industrial applications.

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INTRODUCTION

Muscodor is an endophytic fungal genus that is known from certain tropical tree and vine species in Central/South

America, and South Eastern Asia and Australia (Worapong *et al.*, 2001; Strobel *et al.*, 2001; Daisy *et al.*, 2002; Ezra *et al.*, 2004a; Atmosukarto *et al.*, 2005). New species and isolates in this fungal genus have been recognized on the basis of their partial 18S rDNA sequences as well as their unique physiological and biochemical properties (Daisy *et al.*, 2002; Sopalan *et al.*, 2003). In addition, several new isolates of *Muscodor albus* have recently been obtained that have nearly perfect ITS-5.8S rDNA partial sequence similarities

Abbreviations: (E)SEM, (environmental) scanning electron microscopy; TEM, transmission electron microscopy; VOC, volatile organic compound.

The GenBank/EMBL/DDBJ accession number for the partial ITS-5.8S rDNA-ITS sequence data of *M. albus* isolate E-6 is EF183509.

with the original isolate, CZ-620, of *M. albus* (Ezra *et al.*, 2004a; Atmosukarto *et al.*, 2005). A characteristic of *Muscodor* spp. is that each produces one or more VOCs that have biological activity (Strobel *et al.*, 2001; Ezra *et al.*, 2004a). In the case of *Muscodor vitigenus* the bioactive compound is naphthalene and the fungus has insect-repellent activity, whereas in the case of *M. albus* and *Muscodor roseus* a multitude of VOCs have antibiotic properties (Strobel *et al.*, 2001; Worapong *et al.*, 2002). Overall, it seems as if each new isolate of *Muscodor* spp. possesses a surprise in terms of its chemistry, biological activity or biological promise. Therefore, it seems warranted to find new isolates of this organism and to learn of their potential applications.

Ecuador is widely recognized as a world centre for biodiversity because it has a multitude of natural habitats ranging from the vast reaches of the upper Amazonian basin and the cloud forests of the high Andes to a number of coastal dry-monsoonal forests. These areas harbour rich plant and animal diversity and many of these species are endemic. Thus, it may follow that great microbial diversity is also associated with the diversity of the macro flora and fauna of this region (Strobel & Daisy, 2003). A search was launched in areas of Ecuador bearing biological resemblance to those parts of the world where other isolates of *M. albus* have been obtained. Plant parts were harvested and submitted to a selective isolation procedure to determine if they harbour novel *Muscodor* isolates (Daisy *et al.*, 2002; Ezra *et al.*, 2004a).

From this study, we identified one endophyte that resembled *Muscodor* spp. and produced VOCs with antibiotic activity. This organism has unique properties, relative to all other *M. albus* isolates, in terms of the VOCs that it produces as well as its biological properties. It also possesses some unusual mycelial structural features involving intricate hyphal intertwining and cementing that allows the entire mycelial mat, on an agar surface, to be easily lifted from it. In turn, with the successful cultivation of *Guazuma ulmifolia*, the host plant, we have succeeded in re-establishing the fungus in its host. It has not previously been possible to obtain a successful introduction and establishment of *Muscodor* spp. in any plant. This finding has enormous implications for the eventual use of these organisms as inoculants on plants or plant parts to provide protection against certain pests and pathogens. The isolation of the fungus, its biological features and activities, along with studies showing its re-establishment in its host plant, are described in this report.

METHODS

Isolating, culturing and storing *M. albus* E-6. The culture of *M. albus* used in this study was obtained as an endophyte from small limbs in the crown of *Guazuma ulmifolia* (Sterculiaceae; plant E-6) found at the Bosque Protector Cerro Blanco preserve near Guayaquil, Ecuador, at 2° 10' 43" S; 80° 01' 41" W. During this study, samples of stem tissues from at least 29 different plants (mostly tree and vine

species) were collected from several Ecuadorian cloud forests, two high alpine forests, and regions of the Amazonian rain forest. Plant samples (given a numerical designation) were exposed to the VOCs of *M. albus* strain CZ-620 (Strobel *et al.*, 2001) as a means to facilitate, by selective pressure, the isolation of any new isolate of *M. albus* or related organisms (Ezra *et al.*, 2004a; Atmosukarto *et al.*, 2005). Plants growing in the same region as plant E-6, such as *Clavija* sp., *Pseudobombax millei*, *Dyospiros ebemifolia*, *Brosimum alicastrum* and *Aspidospermaulei* sp., did not yield any antibiotically active VOC-producing fungi. One fungus, designated E-6, was recovered from *G. ulmifolia* under these selective culture conditions and was initially shown to produce antibiotically active VOCs by virtue of the bioassay test (Strobel *et al.*, 2001). To store the fungus, PDA plugs containing the E-6 mycelium were placed in 15 % glycerol and stored at -70 °C. However, the best storage conditions for the fungus were obtained by growing it on sterilized barley and placing the infested grains at -70 °C. The fungus was deposited as no. 2331 in the living mycological culture collection at Montana State University.

Test fungi and bacteria. All plant-pathogenic fungi used in the bioassay test system were obtained from Drs Don Mathre and Nina Zidak of the MSU Department of Plant Sciences. *Candida albicans* and bacterial cultures were supplied by the MSU Department of Microbiology. All fungi were grown on potato dextrose agar (PDA), and bacteria on Luria Broth agar (LBA) at 23 °C; only freshly transferred cultures (4–7 days old), unless otherwise stated, were used in the fungal bioassay tests.

Scanning (SEM) and other electron microscopies. Isolate E-6, grown on PDA, was processed for SEM. Many agar pieces containing the fungus were placed into filter paper packets and suspended in 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2–7.4) with Triton X (a wetting agent). Tissues were aspirated for 5 min and left overnight as previously described (Ezra *et al.*, 2004a). For SEM the fungal material was critical-point dried, gold sputter-coated and images were recorded with a Philips XL30 ESEM FEG in high-vacuum mode using the Everhart–Thornley detector.

Because it appeared that 'exploratory' hyphae of the fungus were developing within 24 h of a mycelial mat being placed near to its host, observations of this relationship could not be seen by regular SEM techniques. Instead, carefully prepared samples were examined by environmental SEM (ESEM) as described by Castillo *et al.* (2005). Since this technique is non-disruptive and relies on the presence of free water to generate images, it was successfully used to examine the interactions occurring between the tightly interwoven fungal mat and various plant surfaces. Thus, freshly prepared specimens with intact hyphal connections from the mycelial mat to the plant were examined by ESEM and images were recorded with an XL30 ESEM FEG. A gaseous secondary electron detector was used with a spot size of 3, at 15 kV. The temperature was 4 °C, with a chamber pressure which ranged from 5 to 6 torr (666–800 Pa), providing humidity up to 100 % at the sample.

Tissues were prepared for thin-section transmission electron microscopy (TEM) studies by fixation in 2 % glutaraldehyde in sodium cacodylate buffer (pH 7.2–7.4) for 2 h at room temperature. After fixation, tissues were washed six times with 1 : 1 water-buffer solution, and post-fixed and stained with buffered 1 % osmium tetroxide for 2 h at 0–4 °C. After washing six times with the water-buffer, the tissues were dehydrated in a graded series of ethanol and embedded in Spurr's resin (Spurr, 1969). Tissues were sectioned, stained with Reynolds' alkaline lead citrate (Reynolds, 1963) and images were recorded with an FEI Technai 12 transmission electron microscope.

Fungal DNA isolation and acquiring ITS-5.8S rDNA sequence information. A pure E-6 culture, growing on PDA, was used as a source of DNA after incubation for 7–10 days at 25 °C using the

Rapid Homogenization: Plant Leaf DNA Amplification kit (Cartagen). Some of the techniques used were similar to those used to genetically characterize other *M. albus* isolates from Australia (Ezra *et al.*, 2004a). Squares of the cultured mycelia (0.5 cm²) were cut from 1 week old cultures. The agar was scraped from the bottom of the pieces, in order to exclude as much agar as possible. The pieces were placed into 1.5 ml Eppendorf vials and incubated for about 10 min at -80 °C. The DNA was then extracted according to the instructions of the kit manufacturer. Extracted DNA was diluted (1:9) in double-distilled, sterile water and 1 µl samples were used for PCR amplification. The ITS1-5.8S-ITS2 rDNA sequence was amplified by PCR using the primers ITS1 (TCCGTAGGTGAACCTGCGGG) and ITS4 (TCCTCCGCTTATTGATATGC). The PCR procedure was carried out in a 14 µl reaction mix containing 1 µl DNA extracted from the fungal culture (1:9 dilution), 0.5 µl primer ITS1 and 0.5 µl primer ITS4, 7 µl RedMix plus PCR mix with 1.5 mM MgCl₂ (GeneChoice) and 5 µl ddH₂O PCR grade (Fisher Scientific). The PCR amplification was performed in a Biometra personal cycler: 96 °C for 5 min followed by 35 cycles of 95 °C for 45 s, 50 °C for 45 s and 72 °C for 45 s, followed by a 72 °C cycle for 5 min. The PCR products were examined using gel electrophoresis, on a 1.3 % agarose gel for 30 min at 100 V with TAE buffer (GelXL Ultra V-2 from Labnet International) or the Wealtec GES cell system. Gels were soaked in a 0.5 µg ml⁻¹ ethidium bromide solution for 5 min and then washed in distilled water for 5 min. Gel imaging was performed under UV light in a Bio-Imaging System (model 202D; DNR-Imaging Systems). A ~500 bp PCR product was purified using the UltraClean PCR Clean Up DNA Purification kit (MO BIO Laboratories). Purified products were sent to Danyel Biotech, Rehovot, Israel, for direct PCR sequencing. Sequencing was performed on both strands of the PCR product using ITS1 and ITS4 primers and DYEnamic ET terminators on a MegaBACETM1000 analysis system. Sequences were submitted to GenBank on the NCBI website (<http://www.ncbi.nlm.nih.gov>). Sequences obtained in this study were compared to the GenBank database using the BLAST software on the NCBI website: <http://www.ncbi.nlm.nih.gov/BLAST/>.

Bioassay test for volatile antimicrobials. A relatively simple bioassay test system was devised that allowed only for VOCs from the fungus being the active agents for any microbial inhibition being examined, as previously described (Strobel *et al.*, 2001). A 2.5 cm wide strip of agar was removed from the mid-portion of a standard PDA Petri dish, then *M. albus* was inoculated and grown on one side of the plate for varying time periods prior to testing. The test fungus or bacterium was placed on to the agar half moon strip on the opposite side of the plate. Individual fungi were then inoculated on the test side of the plate on a 3 mm³ plug of agar. Bacteria and the yeast culture were simply streaked (1.5 cm long) on to the PDA on the test side of the plate. The plate was wrapped with two individual pieces of Parafilm and incubated at 23 °C. The growth of test organisms was visually judged on the basis of any new microbial density appearing on the area of the agar that had been inoculated. Eventually, the linear growth of the filamentous fungi (as measured from the edge of the agar inoculum plugs) as well as the viability of each test fungus and bacterium were evaluated. The test with each designated assay organism was repeated at least three times.

Qualitative analysis of *M. albus* E-6 volatiles. The method used to analyse the gases in the air space above the 7-day old culture of the *M. albus* mycelium growing in Petri plates was similar to that used for the original isolate of *M. albus*, strain CZ-620 (Strobel *et al.*, 2001). First, a baked solid-phase micro-extraction syringe (Supelco) consisting of 50/30 divinylbenzene/carburene on polydimethylsiloxane on a stable flex fibre was placed through a small hole drilled in the side of the Petri plate and exposed to the vapour phase for 45 min. The syringe was then inserted into the splitless injection port of a Hewlett Packard 6890 gas chromatograph (GC) containing a

30 m × 0.25 mm i.d. ZB Wax capillary column with a film thickness of 0.50 µm. The column was temperature programmed as follows: 30 °C for 2 min, then increasing to 220 °C at 5 °C min⁻¹. The carrier gas was ultra-high-purity helium (local distributor) and the initial column head pressure was 50 kPa. Prior to trapping the volatiles, the fibre was conditioned at 240 °C for 20 min under a flow of helium gas. A 30 s injection time was used to introduce the sample fibre into the GC. The GC was interfaced to a Hewlett Packard 5973 mass selective detector (mass spectrometer, MS) operating at unit resolution. The MS scanned at a rate of 2.5 scans per second over a mass range of 35–360 Da. Data acquisition and data processing were performed on the Hewlett Packard ChemStation software system. Initial identification of the unknowns produced by *M. albus* was made through library comparison using the NIST database (National Institute of Standards and Technology).

Comparable analyses were conducted on Petri plates containing only PDA, and the compounds obtained therefrom, mostly styrene and its derivatives, were subtracted from the analyses done on plates containing the fungus. Tentative identification, based on comparative mass spectral information between observed data and those in the NIST database, was made for 22 fungal VOCs. Final confirmatory identification was made on 9 of the 22 compounds by comparing GC/MS data of authentic standards with the data on the fungal VOCs.

Sourcing of authentic standards for fungal VOCs. Many of the organic alcohols, esters and acids were purchased from Aldrich/Sigma; bulnesene was a gift of Dr C. H. Heathcote, University of California, Berkeley. Acetic acid, 2-phenylethyl ester was prepared by standard organic synthesis by Dr Chris Markworth of Montana State University using previously described methods (Hoeffle *et al.*, 1978).

Inoculation of *G. ulmifolia* and recovery of *M. albus* E-6. Seeds of *G. ulmifolia* were recovered from several fruit pods of this plant. The seeds were scarified by exposing them to boiling water for 30 s and then placing them on wetted paper mats in a disinfected plastic box for 3–4 weeks, after which germination began. Ultimately, the young seedlings were planted in sterilized soil, watered with sterile distilled water and fertilized every 2 weeks with an inorganic fertilizer containing N, P, K at a ratio of 20/10/20. The plants were placed in a high-humidity greenhouse having both natural and incandescent light sources for several months until used. When the plants had grown 5–7 cm (2 months old) they were used for inoculation studies. All plant inoculations were done by placing a 2-week-old mycelial mat 0.70 × 1.3–1.5 cm, carefully teased from the PDA agar surface, over the young growing leaf, followed by making small pin-point punctures through the mat into the plant surface below with a sterilized sharp knife point. The mats, with no added sticking agent, readily adhered to the leaf surface and a few droplets of water were added on alternate days. All inoculated plants (seedlings or larger plants) were always intact in the presence of 100 % relative humidity, indirect light, and an exposure of at least 6 days to the fungal mats. At the end of the incubation time, the fungal mats were lifted away from the plant surfaces and the plant or plant part swabbed with a Kimwipe having been dampened with a 70 % ethanolic/water solution. Effectively, this treatment decontaminated the plant surface from unwanted microbes along with any surface-borne hyphae of *M. albus*. The surface-treated leaves were then placed on PDA containing 10 p.p.m. chloroamphenicol to discourage bacterial growth and encourage the emergence of any *M. albus* hyphae that had become established within the leaf tissues of *G. ulmifolia*. Plants not having been inoculated with *M. albus*, but wounded with the knife blade, served as controls. In addition, at least 12 seeds of *G. ulmifolia* having been scarified and also treated with 95 % ethanol and then flamed and plated on PDA, served as an additional control to determine if the plants were already likely carriers of the fungus E-6.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

After subjecting the stems of various plant species to the VOCs of *M. albus* CZ-620, endophytic fungi appeared on the Petri plates from many of the 29 Ecuadorian plant samples after 4–10 days. To purify these isolates, hyphal tips were picked and transferred to PDA plates. Ultimately, many of these were shown to be isolates of *Pestalotiopsis*, *Xylaria* and other related ascomycetes (Atmosukarto *et al.*, 2005; Ezra *et al.*, 2004a). However, one fungus was isolated that produced odours, and had a tough mycelial mat that survived repeated exposure to *M. albus* isolate CZ-620 (Ezra *et al.*, 2004a). Like the original isolate of *M. albus*, this fungus did not produce spores, and also had a ropy mycelium and produced strong characteristic odours. This fungus, designated E-6, was isolated from *Guazuma ulmifolia* (Fig. 1A), but from none of the other 29 plants collected in Ecuador. It is now stored as isolate 2331 in the living mycological culture collection of Montana State University.

On PDA, isolate E-6 developed an almost whitish-hyaline mycelium that toward the colony centre turned white with ageing, making it distinct from all other isolates of this organism in our collection (Ezra *et al.*, 2004a). Notably, the E-6 fungus was capable of easily being lifted in mass from the agar surface with a scalpel. This appears to be something unique among all endophytic fungi studied to date. It seems that the organization of the hyphal strands is such to have allowed for the apparent strength and maintenance of the integrity of the mycelial mat. SEM of the top and bottom sides of the mycelial mat of E-6 revealed intertwining of hyphae into rope-like strands, with subsequent multiple side branches developing into a mesh-like interconnected mass of hyphae (Fig. 1B). There also appears to be an extracellular matrix of materials (probably polysaccharide) associated with the hyphae (Fig. 1B). This seems to be similar to the matrix of material associated with *M. albus* isolate I-41.3s (Atmosukarto *et al.*, 2005). Undoubtedly, these structural features contribute to the strong mat-like nature of the mycelium.

Partial ITS-5.8S rDNA-ITS sequence data of isolate E-6 were obtained and deposited as entry EF183509 in GenBank. A BLAST search of the database indicated at least 99 % sequence identity (564/567 bases) between the sequence of isolate E-6 and the original isolate of *M. albus*, CZ-620 (GenBank accession no. AF324336). This sequence information on E-6 further adds to an exceedingly tight and clustered relationship among the ITS-5.8S rDNAs among all of the organisms whose sequences are now on deposit at GenBank listed as *Muscodor* spp. (Atmosukarto *et al.*, 2005; Ezra *et al.*, 2004a). Therefore, this fungus will be referred to as *Muscodor albus* E-6 in this report.

VOC production from *M. albus* E-6

M. albus E-6 produced at least 22 VOCs and 9 of these could be positively identified on the basis of a GC/MS

comparison with authentic standards obtained from commercial sources as well as organic synthesis (Table 1) (Strobel *et al.*, 2001). The remaining compounds were identified primarily on the basis of their mass spectral properties as compared to the NIST database. Of the compounds produced by this organism the most abundant was propanoic acid, 2-methyl-, followed by propanoic acid, 2-methyl-methyl ester and 1 butanol, 2-methyl (Table 1). Although these compounds have been detected in *M. albus* CZ-620, they are present in relatively low abundance (Strobel *et al.*, 2001). Interestingly, none of the other isolates of *M. albus* produce the butanoic or butenal derivatives as does E-6, making it unique in this respect (Table 1) (Strobel *et al.*, 2001; Atmosukarto *et al.*, 2005; Ezra *et al.*, 2004a). Likewise, a number of other volatiles appear that are unique to this isolate, including guaiol; formamide, *N*-(1-methylpropyl); 2-furanmethanol; and 1-octene, 3-ethyl-; along with some azulene and naphthalene compounds (Table 1). Furthermore, other compounds usually seen in *M. albus* isolates also appeared in the VOCs of isolate E-6, including caryophyllene; phenylethyl alcohol; acetic acid, 2-phenylethyl ester; bulnesene; and various propanoic acid, 2-methyl- derivatives.

Biological activities of the VOCs of *M. albus* E-6

When grown for 4–7 days at 23 °C on PDA, *M. albus* E-6 had maximum lethality to other fungi and bacteria when placed in the bioassay Petri plate test system (Strobel *et al.*, 2001). The test organisms were selected to include a broad taxonomic representation of major plant and some human bacterial and fungal pathogens. Microbes including yeasts and others representing each of the major classes of fungi were tested along with both Gram-positive and Gram-negative bacteria. The following test microbes were 100 % inhibited and died after a 2 day exposure to the gases of E-6: *Botrytis cinerea*, *Candida albicans*, *Phytophthora cinamomi*, *Escherichia coli* and *Bacillus subtilis* (Table 2). After a 4 day exposure to *M. albus* E-6, the following test organisms were also dead: *Pythium ultimum*, *Verticillium dahliae*, *Mycosphaerella fijiensis* and *Aspergillus fumigatus* (Table 2). But, even after a 4 day exposure to E-6, *Fusarium solani*, *Rhizoctonia solani*, *Trichoderma viride* and *Sclerotinia sclerotiorum*, which showed some initial inhibition by E-6, never succumbed to its VOCs (Table 2). These results are in sharp contrast to results of similar tests conducted in parallel with *M. albus* CZ-620, wherein *R. solani* seemed to be one of the most susceptible of the test organisms (G. A. Strobel, unpublished). Similarly, isolate CZ-620 kills *E. coli*, but does not kill *Bacillus* spp. (Strobel *et al.*, 2001). Furthermore, *S. sclerotiorum* is another test organism that is extremely sensitive to *M. albus* isolate CZ-620 (Strobel *et al.*, 2001).

The effect of the age of E-6 and the test organisms on the results of the bioassay tests was examined. Commonly, only fresh or rapidly growing colonies of test organisms are used to carry out biological assays of *M. albus* (Strobel *et al.*,

Fig. 1. *G. ulmifolia* plant tissues, and photographs and electron microscopy images of *M. albus*. (A) Mature leaf and fruit of *G. ulmifolia*. The inset shows a *G. ulmifolia* flower. (B) Scanning electron micrograph of the upper surface of a culture of *M. albus*. The inset shows a scanning electron micrograph of the lower surface. (C) *M. albus* on a young leaf. (D) Environmental scanning electron micrograph of *M. albus* showing exploratory hyphal cells (EH). (E) *M. albus* showing fungal hyphae (FH) growing from an infested leaf surface to the agar surface. (F) Transmission electron micrograph of *G. ulmifolia* plant cells (PC) infested with *M. albus* fungal hyphae (FH).

Table 1. GC/MS analysis of the volatile compounds produced by *M. albus* E-6

Several minor peaks and the breakthrough peak were omitted from the total analysis since they represent only 1 % of the total area. Compounds found in the control PDA plate are not included in this table. RT, retention time.

RT (min : s)	Total area (%)	m/z	Possible compound	Mol. mass (Da)
4:58	32.4	102	*Propanoic acid, 2-methyl-methyl ester	102
7:07	1.2	116	Butanoic acid, 2-methyl-	116
7:24	1.0	116	Butanoic acid, 3-methyl-	116
9:35	1.3	84	2-Butenal, 2-methyl-	84
10:19	28.0	130	*1 Butanol, 2-methyl-	130
12:20	5.9	158	Butanoic acid, 3-methylbutyl ester	158
12:24	4:2	158	*Propanoic acid, 2-methyl, 2-methylbutyl ester	158
13:51	1.1	118	Propanoic acid, 2-hydroxy-2-methyl-methyl ester	118
14:07	1.0	86	3-Buten-1-ol, 3-methyl-	86
15:36	1.6	140	1-Octene, 3-ethyl-	140
16:12	1.1	142	*4-Nonanone	142
18:21	1.4	204	Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4aR- <i>trans</i>)	204
19:54	1.0	204	1H-cycloprop[e]azulene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,4,7-tetramethyl-, [1aR-(1a.alpha.)]	204
20:02	3.8	222	Guaiol	222
20:25	5.7	204	*Caryophyllene	204
22:33	100	88	*Propanoic acid, 2-methyl-	88
24:36	1.8	101	Formamide, N-(1-methylpropyl)	101
24:55	1.0	98	2-Furanmethanol	98
25:57	1.0	204	*Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethylenyl)-, [1S-(1.alpha., 7.alpha.,8a.beta.)] (common name: bulnesene)	204
28:25	7.0	164	*Acetic acid, 2-phenylethyl ester	164
30:26	17.1	122	*Phenylethyl alcohol	122
36:06	14.02	222	(-)-Globulol	222

*Denotes that the spectrum and retention time of this component were identical to those of an authentic standard compound. All other compounds are reported as the compounds best matching the spectrum in the NIST database.

2001). Experiments were set up to examine the impact of the age variation in both E-6 and test fungal cultures in order to establish its effect in the outcome of the bioassays. *Pythium ultimum* is a rapidly growing organism and it was found that the susceptibility of this organism to the volatiles of E-6 is related to its age as well as the age of the E-6 culture. For instance, when a 5-day-old culture of E-6 was used in the bioassay test against a 2-day-old culture of *P. ultimum* there was 87.3(±4.8) % inhibition of growth, compared with 100 % inhibition of growth of an 11 day old *P. ultimum* culture. Likewise, when a 12-day-old E-6 culture was tested against a 2-day-old *P. ultimum* culture there was only 57.1(±6.2) % inhibition of growth but 99.6(±1.3) % inhibition with an 11-day-old test culture of *P. ultimum*. Thus, as the E-6 culture ages there is a tendency for its bioactivity to decrease, with the best activity being observed at 4–8 days, whereas an increase in the age of the *P. ultimum* test culture results in an increase in its biological sensitivity to the volatiles of *M. albus* E-6. Interestingly, it had been previously demonstrated, using proton transfer reaction mass spectroscopy to constantly monitor the volatiles of a *M. albus* CZ-620 culture, that the production of volatiles declines as a function of age of the culture and a decrease in the nutrient source, but no data

had been previously presented indicating an apparent age relatedness to the age of the test organism to the VOCs of *M. albus* (Ezra *et al.*, 2004b).

Plant inoculation studies

Attempts have been made by various investigators to artificially establish *M. albus* CZ-620 into a plethora of agriculturally important crop plants but with no success (G. Strobel & D. Ezra, unpublished; D. Manker, unpublished). Ultimately, the idea of these experiments was to determine if the host/endophyte relationship may confer disease or pest resistance to the host crop plant. In no case, however, in any of these studies was the original host of *M. albus* plant tested since all plants from which *M. albus* has been isolated have been tropical trees or vines not usually found in temperate areas or research facilities located therein. However, in the case of *M. albus* E-6 its tropical host is *Guazuma ulmifolia*, and it was possible to grow a multitude of these plants in the MSU plant growth facility. Leaves on the developing seedlings were inoculated directly with mycelial mats, followed by puncture wounds through the mats into the leaf surface (Fig. 1C). Initially, as examined by ESEM, the fungus seemed to develop

Table 2. Response of several test fungi and bacteria to the volatiles of *M. albus* E-6

The test organism was exposed to the VOCs of *M. albus* for the times given. Percentage inhibition was measured relative to the growth of the control culture. The viability was tested after the original inoculum was replaced on a regular PDA plate and checked for growth.

Test organism	Inhibition (%) after 2 days exposure	Alive after 2 days exposure	Alive after 4 days exposure
<i>Pythium ultimum</i>	96.1 ± 2.9	Yes	No
<i>Phytophthora cinnamomi</i>	100	No	No
<i>Trichoderma viride</i>	26.8 ± 4.7	Yes	Yes
<i>Aspergillus fumigatus</i>	90.0 ± 10.9	Yes	No
<i>Botrytis cinerea</i>	100	No	No
<i>Fusarium solani</i>	47.5 ± 6.1	Yes	Yes
<i>Rhizoctonia solani</i>	68.3 ± 6.8	Yes	Yes
<i>Verticillium dahliae</i>	100	Yes	No
<i>Sclerotinia sclerotiorum</i>	83.9 ± 8.6	Yes	Yes
<i>Mycosphaerella fijiensis</i>	100	Yes	No
<i>Escherichia coli</i>	100	No	No
<i>Bacillus subtilis</i>	100	No	No
<i>Candida albicans</i>	100	No	No

exploratory hyphae which were produced by the mycelial mat after 24 h (Fig. 1D). These hyphae were numerous and were directed towards the plant tissue itself. Initially, it appeared that each directional hypha became intertwined with other developing hyphae after it became attached to the host plant tissues (Fig. 1D). The fungus then apparently entered the host plant tissues.

A week after inoculation, the mycelial mats were removed from the leaves. No apparent damage to the leaves was noted except for the puncture wounds themselves. A control set of leaves was also tested but without the fungal inoculum having been placed on the leaves. The leaves were excised and treated with 70 % ethanol on a Kimwipe tissue to kill surface contaminating microflora. Small leaf pieces cut through the original inoculation sites were placed on PDA with chloroamphenicol and incubated for several days. A fungus with mycelia resembling *M. albus* (Fig. 1E) developed after 48 h in 4/8 of the inoculation spots on leaf designated A and in 8/15 of the spots on the leaf designated leaf B. In at least four of these cases the fungus emerged at least 2–4 mm from the original wound site, suggesting that tissue colonization had occurred well away from the inoculation site. The recovered fungi (representative isolates A and B) were both subjected to partial ITS-5.8S rDNA analysis and the results in both cases were identical to the original *M. albus* E-6 (GenBank EF183509). Isolates A and B were subsequently reinoculated into young leaves of *G. ulmifolia* and the results successfully repeated.

Furthermore, both isolates A and B had biological activities against the bioassay fungal test organisms identical to those

of *M. albus* E-6, and each had the same ropy mycelium as seen in the original isolate of *M. albus* E-6 (Fig. 1B). In the control experiments, it was not possible to isolate a fungus resembling *M. albus* E-6 from the leaves (successful recoveries per lesion per control test leaf were 0/13 and 0/12 in leaf A, and 0/14 and 0/14 in leaf B). Furthermore, it was not possible to isolate the fungus from 14 *G. ulmifolia* seeds that had been pre-treated with 70 % ethanol, flamed to remove the excess alcohol and then placed on to water agar plates for 3 weeks. Thus, the evidence supports the conclusion that it was possible to get *M. albus* E-6 re-established in its native host plant under the conditions described and that the fungus had not been pre-established in the plants that were under investigation.

The host plant/fungus relationship was further examined by TEM. The tissues examined were those in which only *M. albus* E-6, as the sole endophyte, was present as determined by culturing of test locations on the leaf. It appeared that the fungal hyphae were mostly developing intercellularly (Fig. 1F). This condition is probably the most common since if the hyphae were mainly intracellular one would expect a greater manifestation of a pathological condition in the host tissues (Fig. 1F). Some intracellular hyphae were observed, but this was probably in cells that had been injured by wounding in the original inoculation technique. In nature, the hyphae of *M. albus* are probably primarily located intercellularly since intracellular locations may result in tissue pathology and this condition is not observed either in nature or in the inoculated *G. ulmifolia* seedlings in the greenhouse.

Conclusions

The relationship of *M. albus* E-6 to its host seems intriguing since there were no external symptoms or signs on its original host plant, *G. ulmifolia*, that suggested the presence of a micro-organism. *M. albus* E-6 can be considered a true endophytic micro-organism, as is *M. albus* CZ-620 (Strobel *et al.*, 2001). *G. ulmifolia* is in the plant family Sterculiaceae (now Malvaceae); this now makes at least eight tropical plant families with which the fungal genus *Muscador* is associated. It is now becoming clear that *Muscador* is more widely distributed than originally supposed (Strobel *et al.*, 2001).

M. albus E-6 makes a series of compounds that collectively act to kill other microbes (Table 2). It is tempting to consider that this fungus lives in its host in a symbiotic condition, providing protection from pathogens while surviving and growing on plant nutrients. Now, as a result of the work on this isolate of *Muscador*, this hypothesis can be tested using *G. ulmifolia* trees that have been inoculated with E-6, which has become established therein. Appropriate generalized pathogens could be used to challenge seedlings that have the endophytic E-6 organism.

One of the biggest mysteries surrounding *Muscador* spp. is an understanding of any aspect of their life cycle. For

instance, spore production under laboratory conditions has never been observed, making it difficult to imagine how the organism may be dispersed in nature. It was not possible to acquire a successful establishment of E-6 in wounded hypocotyls of young germinating seedlings. This experiment was repeated at least 10 times with no success. It would seem that the hypocotyl would be vulnerable tissue if the fungus were associated with moist plant litter, wherein it could easily become associated with the newly developing plant. However, one conclusion from this study is that *M. albus* E-6 can enter its host via a wound in an appropriate host tissue such as a leaf (Fig. 1). Because they seem to be vulnerable, it may be that insect-produced wounds on developing leaves serve as a portal of entry for the fungus, which may move into leaf tissues from plant debris previously infested with the fungus. The newly inoculated plant could then serve as a host for the fungus as it moves throughout its tissues in the course of time.

It seems as if *M. albus* E-6 is behaving in a comparable manner to its relatives on other continents (Ezra *et al.*, 2004a; Strobel *et al.*, 2001). That is, it makes antibiotic volatiles but the composition of these gases is unique to this organism (Table 1). Only a few of these compounds were acquired to confirm compound identity, but not enough were available to carry out inhibition studies with the compounds themselves (Strobel *et al.*, 2001). Nevertheless, the spectrum of biological activity is generally unique to this isolate since both representative Gram-negative and Gram-positive bacteria, *E. coli* and *B. subtilis*, were killed on exposure to the VOCs and reduced activity was seen against organisms such as *R. solani* and *S. sclerotiorum* (Strobel *et al.*, 2001)(Table 2).

The potential uses of *Muscodor* for agriculture, industrial and medicinal applications have not escaped our attention (Strobel & Daisy, 2003; Mercier & Jimenez, 2004; Stinson *et al.*, 2003). It seems as if, for each new *Muscodor* that is found, additional potential applications become apparent. For instance, the discovery of *M. vitigenus*, making only naphthalene as its primary VOC product, has implications for causing aversion to insects as its main use. In the case of *M. albus* E-6, it seems that for the first time a *Muscodor* isolate has been found that can kill a Gram-positive bacterium (Table 2).

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