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New endophytic isolates of *Muscodor albus*, a volatile-antibiotic-producing fungus

David Ezra,[1] W. M. Hess[2] and Gary A. Strobel[1]

[1]Department of Plant Sciences, Montana State University, Bozeman, MT 59717, USA

[2]Department of Integrated Biology, Brigham Young University, Provo, UT 84602, USA

Correspondence
Gary Strobel
email-uplgs@montana.edu

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Muscodor albus, an endophytic fungus originally isolated from *Cinnamomum zeylanicum*, produces a mixture of volatile organic compounds (VOCs) in culture and its spectrum of antimicrobial activity is broad. Using the original isolate of *M. albus* as a selection tool, it has been possible to find other culturally and biochemically unique wild-type isolates of this organism existing as endophytes in a variety of other plant species, including *Grevillea pterifolia* (fern-leafed grevillea), *Kennedia nigriscans* (snake vine) and *Terminalia prostrata* (nanka bakarra) growing in the northern reaches of the Northern Territory of Australia. Interestingly, none of the new isolates had a culture morphology that was identical to the original isolate, nevertheless each possessed hyphal characteristics that resembled that isolate. Furthermore, their ITS-5·8S rDNA sequences were 96–99 % identical to that of *M. albus* and the isolates were considered *M. albus* on the basis of the DNA sequence data. However, the VOCs produced by these new isolates greatly differed in quality from the original strain by virtue of the production of naphthalene, naphthalene, 1,1′-oxybis-, and one or more other compounds. In bioassays with a range of test micro-organisms, including fungi and bacteria, each isolate possessed biological activity but the range of activity was great. Artificial mixtures of some of the VOCs mimicked the effects of the VOCs of the fungus. The value of these observations to the biology and practical uses of *M. albus* in agriculture and other applications is discussed.

INTRODUCTION

Several years ago, an unusual endophytic fungus was isolated from *Cinnamomum zeylanicum*, growing in a rainforest in Honduras (Strobel *et al.*, 2001; Worapong *et al.*, 2001). The fungus lacked spores and spore-producing structures. All attempts to induce spore formation failed and thus initially, this organism could not be readily identified. Nevertheless, the fungus had distinctive whitish hyphae that were rope-like, interwoven and widely varying in diameter. Partial ITS-5·8S and 18S rDNA sequence data obtained on this organism indicated that it was a xylariaceous fungus with no complete molecular identity to any other fungus in the family Xylariaceae. The most notable property of this organism was its ability to produce a mixture of volatile organic compounds (VOCs) that were lethal to a wide variety of human- and plant-pathogenic fungi and bacteria. This mixture of gases consisted primarily of various alcohols, acids, esters, ketones and lipids. Artificial mixtures of the VOCs mimicked the biological effects of the fungal VOCs when tested against a wide range of fungal and bacterial pathogens. Based on the available molecular biological, chemical and structural information, this organism was given a novel genus and species designation – *Muscodor albus*.

Abbreviations: ITS, internal transcribed spacer; VOC, volatile organic compound.

While VOC-producing fungi have been isolated and studied chemically in the past 30–40 years, none have been found that have such a comprehensive spectrum of antimicrobial activity as that of *M. albus* (Strobel *et al.*, 2001; McAfee & Taylor, 1999). Thus, it was of interest to learn whether other strains, variants or biotypes of this organism exist in nature and if they too possess biological activity via the production of VOCs. It was realized that in order to find other related *Muscodor* spp., the VOCs of *M. albus* could be used as a selection tool to effectively eliminate other competing endophytic micro-organisms and allow for only the growth of xylariaceous fungi including such organisms as xylaria, pestalotiopsis, daldinia and muscodor (Daisy *et al.*, 2002a). By this method, other species of muscodor have been isolated from tropical trees and vines and characterized by chemical and molecular biological techniques including *Muscodor roseus* and *Muscodor vitigenus* (Worapong *et al.*, 2002; Daisy *et al.*, 2002a, b). More recently, a strain of *M. albus* was obtained from *Myristica fragrans* growing in Thailand and it very closely resembled the original culture of *M. albus* in most chemical, structural and molecular biological characteristics (Sopalun *et al.*, 2003).

The search for new isolates of *M. albus* or other related organisms was centred in the monsoonal rainforest of the Northern Territory of Australia since this is where the second new species of muscodor was found, namely *M.*

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roseus (Worapong *et al.*, 2002). This fungus differed from *M. albus* in its reddish mycelial coloration and the quality of its volatiles (Worapong *et al.*, 2002). For a contrast in environmental conditions, plants in a temperate region of Australia were also sampled, in this case, the island of Tasmania. As a whole, it appears that the plants of Australia have an enormous diversity of endophytic micro-organisms that make bioactive metabolites (Strobel & Daisy, 2003). After collecting a number of specimens from woody plants in that area and using the VOCs of *M. albus* as a selection tool, at least seven widely differing isolates of an organism outwardly resembling muscodor were obtained. Each of these isolates was subjected to rigorous chemical analysis of its VOCs, subsequently analysed for its taxonomic position, and finally for its spectrum of biological activity. This report presents data describing these organisms, and how they compare to each other and to the original isolate of *M. albus*.

METHODS

Collecting, isolating, culturing and storing *M. albus*. The cultures of *M. albus* isolated and examined in this study were obtained as endophytes from small limbs of a number of tree and vine species in the Northern Territory of Australia. Three species, namely *Grevillea pterifolia* (fern-leafed grevillea), *Kennedia nigriscans* (snake vine) and *Terminalia prostrata* (nanka bakarra), are each native to the Northern Territory, and both the snakevine and nanka bakarra have medicinal uses by the Aborigines in this area, while the fern-leafed grevillea does not have any apparent medicinal applications. Many other plants around the Northern Territory, including *Erythrophelum chlorostachys* (ironwood), *Bambusa arnhemica* (native bamboo), *Eucalyptus tetrodonta* (stringy bark), *Brachychiton diversifolus* (kurrajong) were also sampled. In addition, many of the common forest trees of Tasmania were also sampled and some of the more notable ones included *Acacia dealbata* (silver wattle), *Nothofagus gunnii* (deciduous beech), *Eucalyptus coccifera* (Tasmanian snow gum) and *Dacrydium franklinii* (Huon pine).

A culture of *M. albus* was used as a selection tool to find and isolate other xylariaceous fungi, including the new isolates of *M. albus*. Potato dextrose agar (PDA) was poured into one quadrant of commercially available Petri plates that have the base half-plate separated into quadrants. Then, into that quadrant on each plate was placed a small plug of agar containing an actively growing culture of *M. albus*. The other three quadrants of each plate contained water agar. *M. albus* was incubated for 4 days at 23 °C before exposing the plant tissues being sampled to the VOCs of *M. albus* arising in the plates. The procedures used to isolate the original culture of *M. albus* were used to obtain the new isolates. The plant samples were treated with 70 % ethanol before excising the internal plant tissues and placing them onto the other three quadrants of each plate, containing water agar (Strobel *et al.*, 2001; Worapong *et al.*, 2001).

Endophytic fungi growing from the plant tissues, usually after 4–7 days, were then picked and recultured on PDA to determine culture purity. Pure fungi were tested at least three times by exposure to *M. albus* in order to exclude false positive growth in the initial screening.

The fungi obtained by these methods could best be stored by placing small pieces of agar supporting fungal growth on PDA in 15 % (v/v) glycerol and placing them at −70 °C. The fungi could also be stored after colonizing sterilized grain (including wheat, rye and barley), drying at room temperature and then placing them in sterile vials at −70 °C. The fungi, under these conditions, remained viable for over 2 years.

The cultures were each given a name designation, based on their plant source, as individual isolates of *M. albus* and deposited as living cultures at −70 °C in the Montana State University Mycological Collection (MONT) (Table 1).

Scanning electron microscopy. Once fungal isolates were obtained that outwardly resembled *M. albus* and possessed antimicrobial activity via VOCs, they were initially characterized by photographing and recording their cultural characteristics. Then, scanning electron microscopy was performed on each isolate suspected of being related to *Muscodor* spp. The fungal preparations were placed into 2 % glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·2–7·4) with Triton X, a wetting agent, aspirated for 5 min and left overnight. Then the fixed carnation leaf pieces supporting fungal growth were placed into no. 1 Whatman filter paper packets, made by folding the filter paper over a piece of cork (1·5 cm × 1·5 cm). The packets were tied with cotton string and two removable split shot sinkers (approx. 3·25 g each) were attached next to the packets to hold them under the surface of the dehydrating solutions and the liquid CO_2 during critical-point drying. The next day they were washed in six 15 min changes of water-buffer, followed by a 15 min change in 10 % ethanol, a 15 min change in 30 % ethanol, a 15 min change in 50 % ethanol and five 15 min changes in 70 % ethanol, and were left overnight or longer in 70 % ethanol. They were then rinsed for 15 min in 95 % ethanol and then for three 15 min changes in 100 % ethanol, followed by three 15 min changes in acetone. The dehydration process was done slowly, to minimize hyphal shrivelling. Ultimately, for scanning electron microscopy the fungal material was critical-point dried, gold sputter-coated and images were recorded with an XL30 ESEM FEG in the high-vacuum mode using the Everhart–Thornley detector.

Fungal DNA isolation. Each fungal test isolate was grown on PDA in a 9 cm Petri plate for 21 days at 25 °C. The mycelium was scraped directly from the surface of the agar culture and weighed. Nucleic acid (DNA) was extracted using the DNeasy Plant and Fungi Mini Kit (Qiagen) according to the manufacturer's directions. Agarose gel electrophoresis and a UV spectrophotographic system were used to record the data.

Amplification of internal transcribed spacer sequences (ITS) and 5·8S rDNA. The ITS regions of the tested fungus were amplified

Table 1. The *M. albus* isolates and their ITS-5·8S rDNA accession numbers in GenBank and their MSU culture collection deposit number

Isolate*	Accession no.	MSU deposit no.
TP-21	AY527045	2291
KN-26	AY527044	2289
KN-27	AY527046	2294
GP-100	AY555731	2293
GP-115	AY527047	2292
KN-205	AY597208	2288
GP-206	AY527048	2290
CZ-620	AF324336	2080

*The isolate name is formed from the first letter of the host genus and species: TP, *Terminalia prostrata*; GP, *Grevillea pteridifolia*; KN, *Kennedia nigriscans*; CZ, *Cinnamomum zeylanicum*.

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using PCR and the universal ITS primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). PCR was performed in a 50 µl reaction vial containing 0·1 µg genomic DNA, 10 mM of each primer, 3 mM of the four dNTPs and 0·25 unit Taq polymerase (Novataq) in a 10× Taq buffer (Novataq) containing 500 mM potassium chloride, 15 mM magnesium chloride, 100 mM Tris/HCl (pH 8·8 at 25 °C) 1% Triton X-100. The following cycle parameters were maintained: 95 °C for 5 min followed by 39 cycles of 45 s at 95 °C, 45 s at 60 °C and 45 s at 72 °C followed by 5 min at 72 °C.

The PCR products were purified and desalted using the QIAquick PCR purification kit (Qiagen). The PCR product was cloned into a pGEM-T easy vector (Promega) according to the manufacturer's instructions.

Transformation and DNA extraction. Competent cells of *E.coli* strain DH5α were prepared by the CCNB80 method as described by Stinson *et al.* (2003a). Transformation of the cells with DNA was performed according to standard procedures. The transformed cells were plated on LB agar supplemented with 50 µg ampicillin ml^{-1} (Sigma), in the presence of IPTG and X-Gal for blue/white selection. White single colonies were grown in LB broth and DNA was extracted using a Perfectprep Plasmid Mini (Eppendorf) according to the manufacturer's instructions. Presence of the insert was confirmed by DNA digestion with *Eco*RI (Promega). Plasmid DNA extracted from the transformants, carrying the insert, was sequenced.

Cycle sequencing of the ITS regions and 5·8S rDNA. The plasmid inserts were sequenced by the Plant-Microbe Genomics Facility at Ohio State University using an Applied Biosystems 3700 DNA Analyser and BigDye cycle sequencing terminator chemistry and the universal primers T7 and Sp6.

Test fungi and bacteria. All plant-pathogenic fungi and other organisms used in the bioassay test system were obtained from Drs Don Mathre and Nina Zidack of the MSU Department of Plant Sciences or were a part of the Montana State University collection. All fungi and bacteria were grown on PDA and LB, respectively, at 23 °C prior to testing.

Bioassay test for volatile antimicrobials. A simple bioassay test system was devised that allows only for volatiles being the causative agents for any microbial inhibition being examined. Potato dextrose agar (PDA) was poured into all quadrants of commercially available Petri plates with the base half-plate separated into quadrants. Then, into one quadrant, a small plug of agar containing an actively growing culture of the tested isolate was placed. The plate was sealed and incubated for 4 days at 23 °C prior to exposure of the test fungi and bacteria to the VOCs of the *M. albus* isolate arising in the plates. Individual fungi were inoculated in the plate on a 3 mm× 3 mm × 3 mm plug of agar. Bacteria and *Saccharomyces cerevisiae* were streaked (1·5 cm streaks) on to the other quadrants of the plate, one organism in each quadrant. The dividing walls in the plate precluded the diffusion of any inhibitory soluble compounds emanating from the *M. albus* isolate to the organisms being tested. The plate was wrapped with two pieces of Parafilm and incubated at 23 °C. The growth of the yeast and bacteria was judged visually as described by Strobel *et al.* (2001). The linear growth of the filamentous fungi was measured from the edge of the agar inoculum plugs and compared with growth on a control plate. At the end of the assay, the viability of the each test fungus and bacterium was evaluated by transferring a portion of the original inoculum plug, or an area of the plate that had been streaked, on to a fresh plate of PDA and observing any growth developing within 2–3 days (Strobel *et al.*, 2001). Each bacterium and fungus that was subjected to testing was obtained from a freshly growing culture. In each case, appropriate growth and viability of each organism was noted in the experimental set-up.

Quantitiative and qualitative analyses of VOCs. The gases in the air space above the *M. albus* isolate mycelium growing in Petri plates were analysed as described previously (Strobel *et al.*, 2001; Ezra & Strobel, 2003). A solid-phase micro-extraction syringe was used for trapping the fungal volatiles. The fibre material (Supelco) was 50/30 divinylbenzene/carburen on polydimethylsiloxane on a stable flex fibre. The syringe 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 a gas chromatograph (Hewlett Packard 5890 Series II Plus) equipped with a mass-selective detector. A 30 m × 0·25 mm i.d. ZB Wax capillary column with a film thickness of 0·50 mm was used for the separation of the volatiles. The column was temperature programmed as follows: 25 °C for 2 min, then increasing to 220 °C at 5 °C min^{-1}. The carrier gas was helium (UltraHigh Purity; local distributor) and the initial column head pressure was 50 kPa. The helium pressure was ramped with the temperature ramp of the oven to maintain a constant carrier gas flow velocity during the course of the separation. 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 gas chromatograph. The chromatograph was interfaced to a VG 70E-HF double-focusing magnetic mass spectrometer operating at a mass resolution of 1500. The mass spectrum was scanned at a rate of 0·50 s per mass decade over a mass range of 35–360 Da. Data acquisition and data processing were performed on the VG SIOS/OPUS interface and software package. Initial identification of the unknowns produced by *M. albus* was made by library comparison using the NIST database.

Comparable analyses were conducted on Petri plates containing only PDA, and the compounds obtained therefrom, mostly styrene, were subtracted from the analyses done on plates containing the fungus. Final identification of the majority of the compounds was done by acquiring or making authentic samples of the tentatively identified compounds and then subjecting them to the same conditions of trapping, separation and mass spectroscopy as described above. However, a number of compounds were not available and thus their identification remains tentative. As a first approximation, the quantitative analysis of each compound found in fungal cultures is based on its relative peak area obtained after GC-MS analysis.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

After exposing the stem tissues of various plant species to the VOCs of *M. albus*, a number of endophytic fungi appeared on the Petri plates from most plant samples after 4–7 days. These isolates were purified by picking hyphal tips and transferring them to PDA plates. Many of them were shown to be isolates of pestalotiopsis, xylaria and other related species (Daisy *et al.*, 2002a). However, seven fungi appeared that produced odours and they survived repeated exposure to *M. albus*; these were kept for further examination. In addition, each of these fungi fitted the basic criterion of bearing no spores, like the original muscodor that was isolated (Worapang *et al.*, 2001). Furthermore, each fungus produced odours that resembled those of the original culture of *M. albus*, which was used in a direct comparison. These sterile odoriferous fungi only appeared in plants

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obtained from the Northern Territory of Australia and not those from Tasmania. Furthermore, only *Grevillea pterifolia* (collected at 13° 07′ 947″ S, 132° 27′ 435″ E), *Kennedia nigriscans* (collected at 12° 57′ 991″ S, 132° 48′ 818″ E) and *Terminalia prostrata* (12° 12′ 137″ S, 132° 47′ 066″ E) yielded such sterile odoriferous fungi.

Each of the seven fungi was cultured on PDA and the growth patterns recorded (Fig. 1). Each pattern was distinctive from that of the original *M. albus* (CZ-620) on the basis of its cultural characteristics (Fig. 1). Each isolate developed a whitish mycelium with the exception of KN-27, which was tan, and GP-100, which appeared pinkish on PDA. The cultures also differed in growth rate, with CZ-620 and TP-21 being the most rapid after 10 days (Fig. 1), and in general culture morphology; e.g. GP-100 showed fuzziness of the mycelium, while GP-115, TP-21 and GP-100 produced a series of concentric rings that may be related to the response of the cultures to being in alternating cycles of light and darkness (Fig. 1).

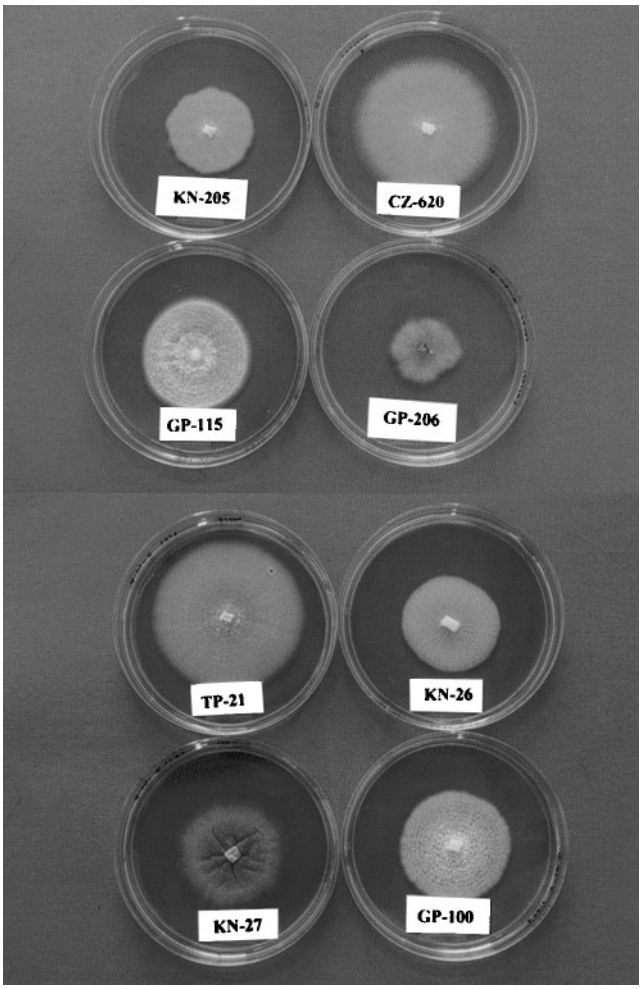

Fig. 1. The mycelial colonies of the new isolates of *M. albus* grown on PDA for 10 days. The original isolate of *M. albus* is also shown as CZ-620. The new isolates are each designated with a two-letter abbreviation (from the scientific name of their respective host plant) and a lab culture number.

Scanning electron microscopy of the mycelium of each of the cultures revealed all of the features that have been associated with this group of organisms, including right-angle branching, the intertwining of hyphae into rope-like strands, hyphal coiling, and sometimes submerged growth under the agar surface (Worapong *et al.*, 2001) (Fig. 2a–d). One culture (KN-27), however, consistently preferred growing in a submerged state, while still developing hyphal coils, hyphal stranding and right-angle branching (Fig. 2d).

The partial ITS-5·8S rDNA sequences of the seven isolates were obtained (see Table 1 for GenBank accession numbers) and compared to sequences of all fungi in GenBank. There was 96–99 % sequence identity of these isolates to the original isolate of *M. albus*, CZ-620, (Table 2; sequence alignment available as supplementary data with the online version of the paper at http://mic.sgmjournals.org), making a very tight and clustered relationship among the ITS-5·8S rDNAs among all of the organisms whose sequences are now on deposit at the GenBank listed as *M. albus* (Strobel *et al.*, 2001; Worapong *et al.*, 2001; Sopalun *et al.*, 2003) (Table 2). Extreme difficulty was encountered with isolate KN-205, in which five unsuccessful attempts were made to get total comparable partial ITS-5·8S rDNA sequence information. This unexpected result may be related to the presence of shorter sequences in isolate KN-205 that bear sequence similarity to the primers that were used, but only allow for partial reading of the ribosomal gene. Nevertheless, the very small partial sequences of this organism that were obtained showed similarity to the ITS-5·8S rDNA of *M. albus* (see the sequence alignment available as supplementary data).

Although there are differences in colony morphology among the seven new isolates, their hyphal morphologies, with few exceptions, are quite comparable. In addition, they each produce VOCs and their partial ITS-5·8S rDNA sequences are similar to the original *M. albus* (designated CZ-620) and to each other (Table 2). We therefore designate them as new individual isolates of *M. albus*.

VOC production by the new isolates of *M. albus*

Each of the seven new isolates of *M. albus*, as well as the original isolate of this fungus (CZ-620), was subjected to analysis of the VOCs in a 10-day-old culture using the techniques as described. Final identification of the VOCs was done using authentic compounds obtained commercially and synthesized (Strobel *et al.*, 2001; Daisy *et al.*, 2002b). At least one compound that was not available has been given a tentative identification and this is indicated in Table 3. Quite surprisingly, none of the new isolates of *M. albus* produced any of the esters that are commonly known for the original *M. albus* isolate (CZ-620) or the relatively new isolate of *M. albus* from *Myristica fragrans* obtained in Thailand (Sopalun *et al.*, 2003). However, with

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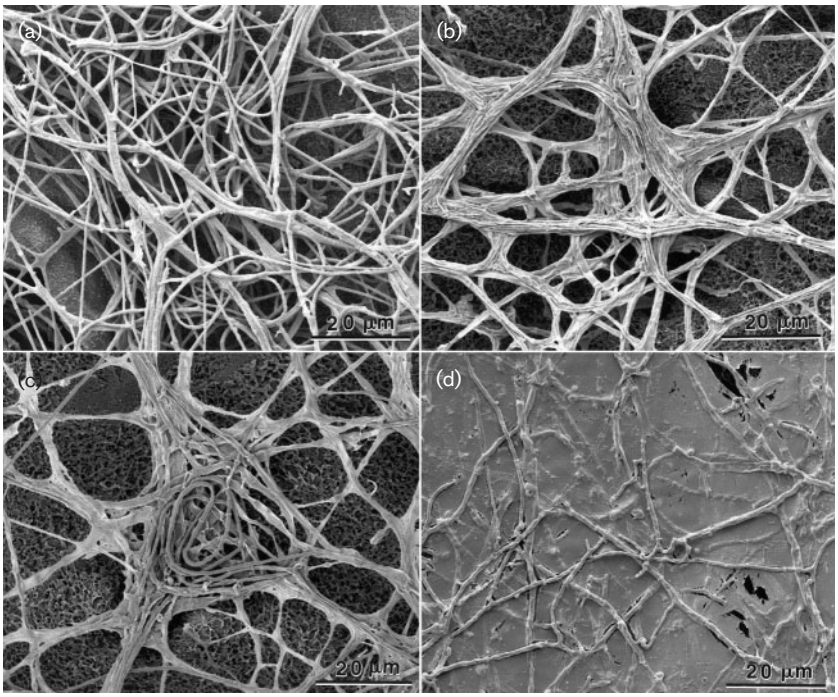

Fig. 2. Scanning electron micrographs of the common hyphal characteristics of *M. albus* including (a) right-angle branching; (b) winding of hyphae into rope-like strands; (c) coiling of hyphae; and (d) hyphae growing submerged in PDA.

one exception, isolate TP-21, each new isolate of *M. albus* produced propanoic acid, 2-methyl- and the azulene derivative bulnecene. Also, all the new isolates produced naphthalene and an unknown compound having a retention at ~37–38 min (Table 3). Likewise, with the exception of KN-26 they each produced naphthlene, 1,1′-oxybis. Furthermore, with the exception of TP-21 and GP-100 each produced 1-butanol, 3-methyl-.

It is noted that the original *M. albus* isolate (CZ-620) does not make naphthalene or naphthalene, 1,1′-oxybis- but does produce two different nonanones, ethanol and acetic acid, 2-phenylethyl ester and many esters which were not detected in the new *M. albus* isolates (Table 3). Also, in the most recent analysis of the original *M. albus* (CZ-620) VOCs, no bulnecene was detected, for unknown reasons, but the unknown compound of mass 204 Da appearing at ~37–38 min, was detectable in the VOCs of this isolate (Strobel *et al.*, 2001) (Table 3). A number of other volatiles found in the original analysis of the gases of this fungus (Strobel *et al.*, 2001) were not detected in this analysis (Table 3). The reasons for this may relate to the fungus having been continually transferred: bio-typical selection may have occurred; however this fungal isolate has retained its biological activity.

Table 2. Percentage DNA identity of the ITS-5·8S rDNA sequence of the different new *M. albus* isolates to *M. albus* CZ-620, *M. roseus* and *M. vitigenus*

M. albus isolate	*M. albus* CZ-620	*M. roseus*	*M. vitigenus*
TP-21	99	99	95
KN-26	99	98	95
KN-27	99	99	96
GP-100	96	96	96
GP-115	98	98	95
KN-205	99	99	95
GP-206	99	99	96

The isolates having similar major compounds appearing as VOCs are GP-206, KN-205, GP-115 and KN-27 (Table 2). Although the VOCs appear in differing amounts and ratios, the compound in greatest abundance in all of the new isolates of *M. albus* is naphthalene (Table 2). This is comparable to *M. vitigenus*, whose only detectable VOC is naphthalene (Daisy *et al.*, 2002b).

Biological activities of the VOCs of the *M. albus* isolates

The noticeable variation in VOC production among the new isolates of *M. albus*, as contrasted to the original isolate, may suggest that the biological activities of these isolates would be different, given the broad spectrum of test microorganisms used to assay them (Table 4). Overall, they have comparable biological activities against fungi and bacteria (Table 4). Those most alike in their spectrum of inhibition and being able to cause cell death were isolates KN-26 and KN-27 (Table 4). However, when evaluated on the same basis, isolate KN-205 was the most active followed by CZ-620 and TP-21. Most of the test organisms were, at the least, inhibited by the VOCs of the new isolates of *M. albus* (Table 4). However, isolate GP-115 had the lowest biological activity of all of the *M. albus* isolates tested followed

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Table 3. GC/MS analysis of the volatile compounds produced by various isolates of *M. albus*

Samples of gases were trapped from 10-day-old cultures and subjected to GC/MS as described in Methods.

Retention time (min : s)	Total area* (%)	Possible compound†	Molecular formula	Mass (Da)
CZ-620				
4 : 59	2·43	Ethyl acetate	$C_4H_8O_2$	88
5 : 50	8·74	Propanoic acid, 2-methyl-,methyl ester	$C_5H_{10}O_2$	102
6 : 11	10·2	Ethanol	C_2H_6O	46
8 : 17	2·2	Acetic acid, 2-methylpropyl ester	$C_6H_{12}O_2$	116
10 : 24	1·7	Propanoic acid, 2-methyl-,butyl ester	$C_8H_{16}O_2$	144
11 : 24	17	1-Butanol, 2-methyl, acetate	$C_7H_{14}O_2$	130
13 : 27	3·2	Propanoic acid, 2-methyl-,3-methylbutyl ester	$C_9H_{18}O_2$	158
13 : 30	4·12	†Propanoic acid, 2-methyl-,pentyl ester	$C_9H_{18}O_2$	158
14 : 03	10	1-Butanol, 3-methyl	$C_5H_{12}O$	88
17 : 22	11·6	4-Nonanone	$C_9H_{18}O$	142
19 : 06	6·64	2-Nonanone	$C_9H_{18}O$	142
29 : 33	11·65	Acetic acid, 2-phenylethyl ester	$C_{10}H_{12}O_2$	164
37 : 12	10·5	Unknown	$C_{15}H_{24}$	204
TP-21				
29 : 06	91·8	Naphthalene	$C_{10}H_8$	128
37 : 45	4·47	†Naphthalene, 1,1′-oxybis-	$C_{20}H_{14}O$	270
38 : 18	3·74	Unknown	$C_{15}H_{24}$	204
KN-26				
14 : 44	1·28	1-Butanol, 3-methyl-	$C_5H_{12}O$	88
24 : 38	3·09	Propanoic acid, 2-methyl-	$C_4H_8O_2$	88
28 : 17	1·66	Azulene, 1,2,3,5,6,7,8,8a-octanydro-1,4 dimethyl-7-(1-methyethyenyl)-,[1*S*-(1.alpha.,7.alpha.,8a.beta)] (bulnecene)	$C_{15}H_{24}$	204
29 : 12	89·89	Naphthalene	$C_{10}H_8$	128
38 : 19	4·06	Unknown	$C_{15}H_{24}$	204
KN-27				
14 : 54	0·7	1-Butanol, 3-methyl-	$C_5H_{12}O$	88
24 : 41	4·1	Propanoic acid, 2-methyl-	$C_4H_8O_2$	88
28 : 18	3·07	Azulene, 1,2,3,5,6,7,8,8a-octanydro-1,4 dimethyl-7-(1-methyethyenyl)-,[1*S*-(1.alpha.,7.alpha.,8a.beta)] (bulnecene)	$C_{15}H_{24}$	204
29 : 13	77·25	Naphthalene	$C_{10}H_8$	128
37 : 47	3·44	†Naphthalene, 1,1′-oxybis-	$C_{15}H_{24}$	270
38 : 21	11·35	Unknown	$C_{15}H_{24}$	204
GP-100				
24 : 44	2·61	Propanoic acid, 2-methyl-	$C_4H_8O_2$	88
28 : 12	1·93	Azulene, 1,2,3,5,6,7,8,8a-octanydro-1,4 dimethyl-7-(1-methyethyenyl)-,[1*S*-(1.alpha.,7.alpha.,8a.beta)] (bulnecene)	$C_{15}H_{24}$	204
29 : 07	87·26	Naphthalene	$C_{10}H_8$	128
37 : 46	5·19	†Naphthalene, 1,1′-oxybis-	$C_{15}H_{24}$	270
38 : 19	2·99	Unknown	$C_{15}H_{24}$	204
GP-115				
14 : 46	4	1-Butanol, 3-methyl-	$C_5H_{12}O$	88
24 : 40	3·15	Propanoic acid, 2-methyl-	$C_4H_8O_2$	88
28 : 14	4·2	Azulene, 1,2,3,5,6,7,8,8a-octanydro-1,4 dimethyl-7-(1-methyethyenyl)-,[1*S*-(1.alpha.,7.alpha.,8a.beta)] (bulnecene)	$C_{15}H_{24}$	204
29 : 09	74·9	Naphthalene	$C_{10}H_8$	128
37 : 44	1·67	†Naphthalene, 1,1′-oxybis-	$C_{15}H_{24}$	270
38 : 19	11·1	Unknown	$C_{15}H_{24}$	204

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Table 3. cont.

Retention time (min : s)	Total area* (%)	Possible compound†	Molecular formula	Mass (Da)
KN-205				
14 : 48	0·78	1-Butanol, 3-methyl-	$C_5H_{12}O$	88
24 : 41	2·41	Propanoic acid, 2-methyl-	$C_4H_8O_2$	88
28 : 18	3·6	Azulene, 1,2,3,5,6,7,8,8a-octanydro-1,4 dimethyl-7-(1-methyethyenyl)-,[1*S*-(1.alpha.,7.alpha.,8a.beta)] (bulnecene)	$C_{15}H_{24}$	204
29 : 13	69·67	Naphthalene	$C_{10}H_8$	128
37 : 46	7·34	†Naphthalene, 1,1′-oxybis-	$C_{15}H_{24}$	270
38 : 21	14·6	Unknown	$C_{15}H_{24}$	204
GP-206				
14 : 45	2·13	1-Butanol, 3-methyl-	$C_5H_{12}O$	88
24 : 42	0·88	Propanoic acid, 2-methyl-	$C_4H_8O_2$	88
28 : 13	5·85	Azulene, 1,2,3,5,6,7,8,8a-octanydro-1,4 dimethyl-7-(1-methyethyenyl)-,[1S-(1.alpha.,7.alpha.,8a.beta)] (bulnecene)	$C_{15}H_{24}$	204
29 : 08	71·15	Naphthalene	$C_{10}H_8$	128
37 : 45	4·4	†Naphthalene, 1,1′-oxybis-	$C_{15}H_{24}$	270
38 : 20	14·5	Unknown	$C_{15}H_{24}$	204

*Compounds making up less than 0·7 % of the total detectable VOCs are not included; therefore each column does not necessarily equal the expected 100 % total.
†Compound identity not confirmed by retention time and mass spectral data using an authentic compound as a standard.

closely by isolates GP-100 and GP-206 (Table 4). Since their qualitative VOC production is comparable, the differences in activity between isolates may relate to the quantitative production of the VOCs by these isolates. This cannot be readily measured by our GC/MS methods. This explanation seems reasonable, since one of the most active new isolates is TP-21 and it produces only three detectable VOCs, which were also detected in isolates GP-100 and GP-206.

Table 4. The VOC bioassay of the various isolates of *M. albus* as compared to the original isolate, CZ-620

Test organism	Percentage inhibition (and viability)†							
	CZ-620	TP-21	KN-26	KN-27	GP-100	GP-115	KN-205	GP-206
Pythium ultimum	100 (D)	100 (D)	100 (D)	100 (D)	64 ± 54 (A)	33 ± 34 (A)	100 (D)	55 ± 48 (A)
Phytophthora cinnamomi	100 (D)	100 (D)	100 (D)	100 (D)	50 ± 3 (A)	80 ± 17 (D)	100 (D)	76 ± 33 (A)
Aphanomyces cochliodiodes	100 (D)	100 (D)	100 (D)	100 (D)	100 (D)	98 ± 3 (D)	100 (D)	100 (D)
Aspergillus fumigatus	100 (A)	100 (A)	100 (A)	100 (A)	100 (A)	100 ± 11 (A)	100 (D)	100 (A)
Aspergillus ochraceus	100 (D)	100 (D)	100 (D)	100 (D)	100 (A)	100 ± 19 (A)	100 (D)	100 (A)
Fusarium culmorum	69 ± 19 (A)	69 ± 9 (A)	56 ± 10 (A)	63 ± 2 (A)	43 ± 4 (A)	30 ± 15 (A)	63 ± 8 (A)	31 ± 18 (A)
Rhizoctonia solani	100 (D)	100 (D)	100 (D)	100 (D)	100 (D)	66 ± 13 (A)	100 (D)	80 ± 13 (A)
Glomerella cingulata	100 (D)	100 (D)	100 (D)	100 (D)	70 ± 25 (A)	97 ± 7 (D)	100 (D)	100 (D)
Sclerotinia sclerotiorum	100 (D)	100 (D)	100 (D)	100 (D)	100 (D)	53 ± 47 (A)	100 (D)	100 (D)
*Saccharomyces cerevisiae**	100 (A)	0 (A)	0 (A)	0 (A)	0 (A)	0 (A)	0 (A)	0 (A)
*Escherichia coli**	100 (D)	100 (A)	100 (D)	100 (D)	100 (D)	100 (A)	100 (D)	0 (A)
*Bacillus subtilis**	100 (A)	0 (A)	0 (A)	0 (A)	0 (A)	0 (A)	100 (A)	0 (A)

*These bacteria and the yeast were streaked on to the test area of the plate and an indication of growth was made if colony development eventually occurred. After appropriate exposure to the VOCs of *M. albus* the streaked area was sampled and restreaked on to a regular plate to test for viability. The inhibition values were calculated as percentage growth inhibition 72 h after exposure to the gas producers as compared to an untreated control test organism as described in Methods. Tests were repeated five times and means ± SD calculated.
†(D) and (A) in each column refer to dead and alive, respectively. Survival of each test organism was evaluated 6 days after exposure.

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Saccharomyces cerevisiae was the most resistant test organism used in our assays. It survived all *M. albus* VOCs even though it was 100 % inhibited by CZ-620 (Table 4). The other resistant micro-organism was the spore-forming bacterium *Bacillus subtilis* which was not killed by any isolate of *M. albus*; however, it was inhibited by CZ-620 and KN-205 (Table 4). Another notable survivor of *M. albus* VOCs was *Fusarium culmorum*, which was more or less inhibited by each isolate but not killed by any isolate under the conditions of this test. Likewise, those isolates that totally inhibited *Pythium ultimum* also killed it. *P. ultimum* survived under conditions in which it was only partially inhibited in its growth e.g. in response to GP-100, GP-115 and GP-206 (Table 4). *Phytophthora cinnamomi* behaved in an almost identical manner (Table 4).

Artificial mixtures of some of the main compounds, or derivatives thereof, that were present in the VOCs of the *M. albus* isolates were tested against several plant-pathogenic fungi. The artificial VOCs were optimized in quantity and quality for their biological activity. The plant pathogens were used in a Petri plate test according to established procedures in which a small plastic vial containing the test solution was placed in the plate along with the test fungus (Strobel *et al.*, 2001). Many individual compounds, as well as mixtures of various compounds, were examined in the plate test. The most biologically active mixture contained naphthalene, propanoic acid and butanol, 3-methyl, at a ratio of 9 : 45·5 : 45·5 (w/v/v), respectively. *Pythium ultimum* responded with 100 % inhibition, after a 6 day exposure, at a minimum inhibitory concentration (MIC_{100}) of 15 μl in the test vial. The test organisms also died after exposure to this mixture of VOCs. Butanol, 3-methyl-, naphthalene and propanonic acid alone each had MIC_{100} values of 100 μl or greater. Other test fungi including *Rhizoctonia solani* and *Sclerotia sclerotiorum* responded to the artificial mixture in nearly the same manner as *P. ultimum*.

Conclusions

Clearly, all the isolates of the sterile, VOC-producing xylariaceous fungus *M. albus* make compounds that are inhibitory, and in many cases lethal, to various test organisms including plant-pathogenic fungi, including yeasts and bacteria. Previously, it had been established that the main VOCs responsible for the inhibitory activity of *M. albus* isolate CZ-620 were esters, alcohols and acids (Strobel *et al.*, 2001). This work shows that the new isolates of *M. albus* make other VOCs such as naphthalene and an alcohol, an acid, and/or other naphthalene/azulene derivatives that possess biological activity both *in vivo* and in artificial mixtures. Interestingly, no individual compound by itself possessed major antifungal activity, but a combination of compounds was required, as was previously determined for *M. albus* isolate CZ-620 (Strobel *et al.*, 2001). It was also previously noted that naphthalene, the sole distinguishing VOC of *M. vitigenus*, was produced in great enough quantities to cause modifications in insect behaviour (Daisy *et al.*, 2002b). It may be the case that the new isolates of *M. albus* have comparable anti-insect activities; if they do, it becomes reasonable to hypothesize a new biological role of *M. albus*. That is, as an endophyte in plants, it may deter insects that would otherwise inhabit and destroy plant tissues. Certainly this concept is worthy of further testing.

Using the approach of finding and isolating fungi that are tolerant of the VOCs of *M. albus* as a selection tool, it has been possible to find not only these new isolates of this organism, but others as well, including *M. vitigenus* and *M. roseus* (Daisy *et al.*, 2002b; Worapong *et al.*, 2001). Most recently, at least eleven new isolates of VOC-producing fungi with a white sterile mycelium have appeared from a number of plants obtained in the tropical zones of Venezuela. These each possess volatile antibiotic properties, but their morphological and molecular biological properties have not been defined. A pattern is beginning to emerge that indicates the preferred habitat of the *Muscodor* spp. They have only been isolated as endophytes in tropical or monsoonal rainforests, e.g. Thailand, Hondouras, Peru, Venezuela and Australia. Attempts to find them in temperate rainforests, using the original *M. albus* isolate as a selection tool, such as those in Tasmania or southwestern Australia, and the temperate zones of Chile have failed. Also, attempts to find them in tropical, but more seasonally dry climates such as the island of Socotra, Yemen, also have failed. However, an antibiotic VOC-producing *Gliocladium* sp. was isolated from a temperate rainforest in central Chile using *M. albus* VOCs as the selection technique (Stinson *et al.*, 2003a). Initially it was thought that *M. albus* was a unique organism confined to only one locale, but as more studies are done, it appears that it and related organisms are common inhabitants of the world's tropical rainforests. Their life cycle remains a mystery.

Potential applications for *M. albus* and its VOCs are currently being investigated. These include uses for treating various seeds, fruits and cut flowers, to reduce or eliminate harmful or disease-causing micro-organisms (Mercier & Jimenez, 2004). Another promising option is as an alternative to methyl bromide fumigation of soil to control soil-borne plant pathogens (Stinson *et al.*, 2003b).

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