Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus

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***Muscodor albus* is a recently described endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* (cinnamon tree). This xylariaceae fungus effectively inhibits and kills certain other fungi, and bacteria, by virtue of a mixture of volatile compounds that it produces. The majority of these compounds were identified by gas chromatography/mass spectrometry and then made into an artificial mixture that mimicked the antibiotic effects of the mixture of volatile compounds given off by the fungus. Each of the five classes of volatile compounds produced by the fungus (alcohols, esters, ketones, acids and lipids) had some inhibitory effect against the test fungi and bacteria, but none was lethal. However, collectively they acted synergistically to kill a broad range of plant- and human-pathogenic fungi and bacteria. The most effective class of inhibitory compounds was the esters, of which 1-butanol, 3-methyl-, acetate was the most active biologically. This report describes the ecological implications and potential practical benefits of the 'mycofumigation' effects of *M. albus*.**

Keywords: endophyte, organic volatiles, pathogens, esters, aldehydes

INTRODUCTION

A conservative estimate of the number of fungi on our planet is 1.5 million (Hawksworth & Rossman, 1987). However, the number of species described is only in the range of 70 000–100 000 (Hawksworth & Rossman, 1987). One of the major niches to be explored for novel fungi and their products is the diminishing rainforests of the world. Besides expanding the world's mycological collections and learning more about microbial biodiversity, an alternative compelling reason for seeking out these unique microbes is the prospect that they may offer promising new biological systems for solving major problems in medicine, agriculture and commerce.

While exploring the rainforests of Central America we discovered a novel endophytic fungus, *Muscodor albus* (Woropong *et al.*, 2001) and here we report on the production of volatile compounds by this filamentous fungus that are both inhibitory and, for the most part, lethal to a broad range of pathogenic fungi and bacteria. Many fungal species are known to emit low concentrations of gaseous substances, especially ones that have distinctive obnoxious odours, and this has prompted appropriate chemical analyses of the fungal volatiles (Bjurman & Kristensson, 1992). Some of these volatile substances are common to many fungi, whereas others seem to be unique for one species (Schnurer *et al.*,

1999; Rapior *et al.*, 2000). Particularly relevant to this report are the observations of Dennis & Webster (1971), who noted that certain *Trichoderma* spp. produced volatile antibiotics. These compounds inhibited the growth of such test fungi as *Rhizoctonia solani*, *Pythium ultimum* and *Fusarium oxysporum*. No lethality to any of the test fungi was reported by these authors and comprehensive chemical analyses of the volatile components of the fungal cultures were not performed, although acetaldehyde was suggested as one of the volatiles. Thus, in spite of some attention having been given to the volatile compounds of fungal cultures over the years, to our knowledge no lethal mixtures of volatile antimicrobials produced by fungi have been reported. However, here we show the separation and identification of volatile compounds from *M. albus* and a successful mimicry of their inhibitory activity with compounds obtained from synthetic and commercial sources. We also present *in vivo* data illustrating the practical implications for the use of the volatiles of this fungus in biological control measures as a mycofumigant.

METHODS

Culturing and storing *M. albus*. The culture of *M. albus* used in this study was obtained as an endophyte from small limbs of *Cinnamomum zeylanicum* located in the Lancetilla Bot-

Fig. 1. A Petri plate (9 cm) illustrating the geometry of the bioassay system. An *M. albus* mycelial colony (2 weeks old) is on one side of the plate, a central agar strip has been removed, and the opposite side of the plate contains a small agar block supporting a test microbe.

anical Garden near La Ceiba, Honduras. Because plant materials being handled in the laboratory are infested with microscopic phytophagous mites, we placed all Petri plates containing water agar and the internal tissues of the ethanol-treated cinnamon tree stems into a plastic box in order to preclude invasion of the agar plates by these mites. After a few days, most plant tissues had sported fungal growth, but only one endophytic fungus was still alive. It seemed that this lone fungus had produced volatiles that had 'mycofumigated' the entire environment of the plastic container. The procedures used to isolate this organism have been described previously and include a thorough exposure of the external tissues to 70% ethanol prior to placement of the carefully excised internal tissues on water agar (Woropong *et al.*, 2001). *M. albus*, a sterile xylariaceous fungus, has been taxonomically treated primarily on the basis of its 18S rDNA sequences and their relatedness, but not identity, to other fungi in this group (Woropong *et al.*, 2001). The fungus could best be stored after growth on sterile filter paper strips placed on the surface of plates of potato dextrose agar (PDA), subsequently removed, dried in a laminar flow hood, and the strips placed at -70°C . The fungus, under these conditions, remained viable for over 1 year. The fungus was also stored by placing its mycelium, on plugs of PDA, in sterile distilled water at 4°C . It remained viable for over 4 years.

Test fungi and bacteria. All plant-pathogenic fungi used in the bioassay system were obtained from Dr Don Mathre of the Montana State University Department of Plant Sciences. *Candida albicans*, and all bacterial cultures, were supplied by Dr Jim Cutler and Dr Mike Franklin, respectively, both of the Montana State University Department of Microbiology. All fungi and bacteria were grown on PDA at 23°C .

Bioassay for volatile antimicrobials. A relatively simple bioassay system was devised that allows only for volatiles being the caustive agents for any microbial inhibition being examined. An agar strip 2.5 cm wide was completely removed from the mid-portion of a Petri plate of PDA. 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 inoculated on the test side of the plate on a $3\text{ mm} \times 3\text{ mm} \times 3\text{ mm}$ plug of agar (Fig. 1). Bacteria and *Candida albicans* were simply streaked (1.5 cm long streak) on to the PDA on the test side of the plate. Each bacterium and fungus was tested when it was producing fresh growth. The removal of a strip of agar from the mid-portion of the plate precluded the diffusion of any inhibitory soluble compounds emanating from *M. albus* to the fungi or bacteria being tested. The plate was wrapped with two layers of Parafilm and incubated at 23°C . The growth of the 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) and the viability of each test fungus and bacterium were evaluated. The latter was done by either removing the agar plug containing the test fungus and placing it on to a PDA Petri plate, or restreaking the test bacterium or yeast from the original test streak made on the test side of the plate. Appropriate control experiments were conducted in which the test fungus or bacterium was subjected to the same procedures minus *M. albus* on the test side of the Petri plate; in each case, normal growth and viability of each organism was noted. It should be noted that while PDA is not an ideal medium for the bacteria and human-pathogenic fungi used in this study, it did satisfactorily support the growth of these organisms. Its use, while adequately supporting the growth of *M. albus*, also obviated the need to pour other agar into the other half of the Petri plate to support the growth of the test fungus or bacterium.

Quantitative and qualitative analyses of *M. albus* volatiles.

A method was devised to analyse the gases in the air space above the *M. albus* mycelium growing in Petri plates. First, the use of a solid-phase micro-extraction syringe (Supelco) was shown to be a convenient method for trapping the fungal volatiles. The fibre material 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 $30\text{ m} \times 0.25\text{ mm}$ ID 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 $5^{\circ}\text{C min}^{-1}$. 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. A 30 s injection time was used to introduce the sample fibre into the gas chromatograph. The gas 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 a.m.u. Data acquisition and data processing were performed on the VG SIOS/OPUS interface and software package. Initial identification of the unknown compounds produced by *M. albus* was made through library comparison using the NIST database on the mass spectrometer.

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 20 of 28 compounds was done by comparison with authentic standards using the GC/MS methods described above. However, the eight remaining compounds have only been tentatively identified on the basis of the NIST database information; these were not included in any of the bioassays that employed artificial mixtures of *M. albus* compounds.

As a first approximation, the quantitative analysis of each compound found in the *M. albus* culture headspace was based on its relative peak area obtained after GC/MS analysis. These values were used to prepare artificial atmospheres of the *M. albus* gases in the relative proportions that they occur in culture.

Sourcing of fungal volatile compounds. The majority of the commercially available compounds produced by *M. albus* were obtained from Aldrich; however, valencene was obtained from Fluka. Synthetic bulnesene was a generous gift of Dr Clayton Heathcock of the Department of Chemistry, University of California Berkeley (Heathcock & Ratcliffe, 1971). However, most of the major compounds (esters) found in the *M. albus* volatiles that were unavailable commercially or otherwise were synthesized by standard organic synthetic methods using the acylation methods of Hoefle *et al.* (1978) as follows.

Propanoic acid, 2-methyl, 3-methylbutyl ester. Isobutyryl chloride (2 ml, 19.1 mmol) was slowly added to a 0 °C solution of isoamyl alcohol (1 ml, 9.5 mmol), 4-dimethylaminopyridine (583 mg, 4.8 mmol) and pyridine (0.85 ml, 10.5 mmol) in dichloromethane. A precipitate was evident 5 min after addition was complete. After stirring for 12 h under argon, the reaction was poured into 20 ml 0.1 M HCl. The layers were separated and the aqueous layer was extracted with 20 ml methylene chloride. The organic layers were combined and washed with 10 ml saturated aqueous ammonium chloride then 10 ml saturated aqueous sodium bicarbonate. The organic layers were dried over magnesium sulfate, filtered, concentrated *in vacuo*, and purified by distillation through a 14 mm Vigreux column (b.p. 60–62 °C, 25 mm). The resulting clear, colourless oil was stirred over Amberlyst 15 to remove any remaining isobutyryl chloride. ¹H NMR (250 MHz, CDCl₃) 4.09 (t, 2H, *J* 6.7), 2.53 (m, 1H), 1.68 (m, 1H), 1.52 (q, 2H, *J* 6.5), 1.16 (d, 6H, *J* 7.0), 0.92 (d, 6H, *J* 6.5).

Propanoic acid, 2-methylethyl ester. Isobutyryl chloride (2 ml, 19.1 mmol) was slowly added to a 0 °C solution of ethyl alcohol (0.55 ml, 9.5 mmol), 4-dimethylaminopyridine (583 mg, 4.8 mmol) and pyridine (0.85 ml, 10.5 mmol) in dichloromethane. A precipitate was evident 5 min after addition was complete. After stirring for 12 h under argon, the reaction was poured into 20 ml 0.1 M HCl. The layers were separated and the aqueous layer was extracted with 20 ml methylene chloride. The organic layers were combined and washed with 10 ml saturated aqueous ammonium chloride then 10 ml saturated aqueous sodium bicarbonate. The organic layers were dried over magnesium sulfate, filtered, concentrated *in vacuo*, and purified by distillation through a 14 mm Vigreux column (b.p. 102 °C). ¹H NMR (300 MHz, CDCl₃) 4.12 (q, 2H, *J* 7.2), 2.52 (m, 1H), 1.25 (t, 3H, *J* 6.9), 1.16 (d, 6H, *J* 7.2).

1-Butanol, 3 methyl-, acetate. Under an atmosphere of argon, acetyl chloride (6.5 ml, 91.8 mmol) was added dropwise to a 0 °C solution of isoamyl alcohol (5 ml, 45.9 mmol), *N,N*-dimethylpyridine (2.8 g, 23 mmol) and anhydrous pyridine (4.1 ml, 50.5 mol) in dichloromethane (92 ml). The reaction mixture was poured into 100 ml 0.1 M HCl, and the resulting

layers were separated. The organic layer was washed with 50 ml saturated aqueous ammonium chloride then dried over magnesium sulfate. The organic layer was filtered and concentrated *in vacuo* to a clear oil, which was purified by distillation (bp 134–136 °C) to give isoamyl acetate. ¹H NMR (300 MHz, CDCl₃) 4.08 (t, 2H, *J* 6.9), 2.03 (s, 3H), 1.68 (m, 1H), 1.51 (q, 2H, *J* 6.9), 0.92 (d, 6H, *J* 6.6).

Determination of IC₅₀ values and other data on microbe inhibition. The relative ability of the authenticated volatile *M. albus* compounds to inhibit and kill test organisms was measured. Test solutions were prepared by placing compounds in vials in the relative proportions that they occurred in the gas phase of *M. albus* cultures. The test mixture was placed in a presterilized microcup (4 × 6 mm) located in the centre of a Petri plate containing PDA. When not in use, the mixture was stored at 0 °C. The test organisms, freshly growing and excised on 3 mm × 3 mm × 3 mm agar blocks (at least three agar blocks per test fungus), were placed 2–3 cm from the microcup and the plate wrapped with two layers of Parafilm. Measurements of linear mycelial growth were made from the edge of the agar blocks to the edge of the colony after a given time period. However, in the case of bacteria and *Candida albicans* the organisms were streaked on the test side of the PDA plate and checked for new visible growth and viability by restreaking from the original area of the agar plate that had been inoculated. Appropriate controls were also set up in which no test solution was placed into the microcup. Tests on 3.2–90 µl of the artificial mixture per 50 ml of air space above the PDA plate were done on three replicates in order to obtain IC₅₀ data for each test organism. Individual classes of compounds were also tested in the relative amounts in which they occur at the optimum concentration of the entire mixture, which is 60 µl of test mixture per 50 ml of air space above the culture in a standard Petri plate. For instance, the esters represent 44 % of the mixture of the identified volatiles and were tested at 26.4 µl per 50 ml air space; the same procedure was used for each of the other classes of compounds that were identified. Finally, each individual compound, especially among the esters, was tested at the concentration or relative percentage in which it occurs in 60 µl. Viability of the test microbes was checked by aseptically removing the small agar block, placing it on a PDA plate and observing growth after 1–3 d.

Plant mycofumigation tests. Two 2-week-old cultures of *M. albus* were each used to expose, for 4 d, 25 seeds of barley that had been naturally contaminated with the smut fungus *Ustilago hordei*. During this time the seed germinated. Plates containing only PDA were used as controls in which 25 infested seeds per plate were placed for 4 d. Non-infested seeds were used as another control. The seeds of each treatment were placed in plastic greenhouse pots (15 cm diameter) with a standard soil mix and watered liberally every 2 d for 15 weeks. Eventually, the plants were harvested and the number of seedheads that were infested determined versus the number of uninfested seedheads.

RESULTS AND DISCUSSION

Effects of *M. albus* volatiles on various fungi and bacteria

Initially, we ascertained that *M. albus* growing on PDA has maximal lethality to fungi and bacteria when grown for 10–14 d at 23 °C. We then tested a wide range of freshly growing test fungi and bacteria in the standard

Table 1. Effects of the volatile compounds of *M. albus* and an artificial mixture of *M. albus* compounds on a group of test fungi and bacteria

After exposure to *M. albus* gases, each test organism was evaluated for its growth and viability after removal from the gases. The artificial atmosphere consisted of the compounds identified after analysis of the *M. albus* gases (see text and Table 2). The growth of the test organisms in the artificial atmosphere was measured after exposure to the artificial mixture of compounds at 3.2–90 µl per 50 ml in order to obtain IC₅₀ values (see text for details). The percentage growth relative to the control and viability were measured after exposure to 60 µl per 50 ml. Viability was determined after the removal of the compounds at 3 d. The results are means of three determinations ± SD.

Test microbe	Growth after 2 d exposure to <i>M. albus</i> (% vs control)	Viability after 3 d exposure to <i>M. albus</i> culture	IC ₅₀ in artificial atmosphere for 2 d (µl ml ⁻¹)	Growth (mm) in artificial atmosphere (% vs control)	Viability after 3 d exposure to artificial atmosphere
<i>Pythium ultimum</i>	0	Dead	0.48 ± 0.01	0	Dead
<i>Phytophthora cinnamomi</i>	0	Dead	0.29 ± 0.06	0	Dead
<i>Rhizoctonia solani</i>	0	Dead	0.08 ± 0.02	0	Dead
<i>Ustilago hordei</i>	0	Dead	0.31 ± 0.09	0	Dead
<i>Stagnospora nodorum</i>	0	Dead	0.15 ± 0	0	Dead
<i>Sclerotinia sclerotiorum</i>	0	Dead	0.17 ± 0.05	0	Alive
<i>Aspergillus fumigatus</i>	0	Dead	0.41 ± 0.05	0	Alive
<i>Fusarium solani</i>	19.4 ± 0.28	Alive	1.13 ± 0.07	42.0 ± 2	Alive
<i>Verticillium dahliae</i>	0	Dead	0.3 ± 0	0	Dead
<i>Cercospora beticola</i>	17.5 ± 3.5	Alive	0.12 ± 0.15	8 ± 2	Alive
<i>Tapesia yallundae</i>	0	Dead	0.64 ± 0	0	Dead
<i>Xylaria</i> sp.	25 ± 0	Alive	0.41 ± 0.03	0	Alive
<i>Muscodor albus</i>	100 ± 0	Alive	0.6 ± 0	17.5 ± 7.5	Alive
<i>Candida albicans</i>	0	Dead	ND	trace	Alive
<i>Escherichia coli</i>	0	Dead	ND	0	Dead
<i>Staphylococcus aureus</i>	0	Dead	ND	0	Dead
<i>Micrococcus luteus</i>	0	Dead	ND	0	Dead
<i>Bacillus subtilis</i>	0	Alive	ND	0	Alive

ND, Not determined in this experiment.

bioassay. The test organisms were selected to include a broad taxonomic representation of major plant and human fungal pathogens as well as representative Gram-positive and Gram-negative bacteria (Table 1). Most test organisms were completely inhibited, and in fact killed, after 1 d exposure to the *M. albus* gases, but some, such as *Rhizoctonia solani*, required at least 2 d exposure to be killed (data not shown). Therefore, a more inclusive time frame of 3 d was used as the exposure period for all test fungi and bacteria. However, a few microbes, including *Fusarium solani* and *Cercospora beticola*, were only partially inhibited after a 3 d exposure to *M. albus* (Table 1), and some were not killed even by longer exposure (not shown). Also, the volatiles of *M. albus* did not kill *M. albus* itself or its close relative *Xylaria* sp., although they did inhibit the growth of *Xylaria* sp. (Table 1). Thus, the fungal volatiles are biologically selective. The range of microorganisms affected by the volatiles of *M. albus* is impressive: representative oomycetes, basidiomycetes, ascomycetes, deuteromycetes, and Gram-negative and Gram-positive bacteria were each inhibited after exposure to the gases of this fungus. Some, however, while being inhibited were not killed, as with *Bacillus subtilis*

(Table 1). The fungal human pathogens *Aspergillus fumigatus* and *Candida albicans* were sensitive to *M. albus* volatiles and killed by them (Table 1).

Chemical composition of the volatiles

In order to begin to understand the nature of the lethal and inhibitory effects of the volatile substances produced by *M. albus* on the suite of test microbes, it was necessary to chemically analyse the gas phase of this fungus in culture. The compounds were tentatively identified by initial GC/MS separation. They fell into several classes of chemical substances: esters, alcohols, acids, lipids and ketones were present in the mixture of a 2-week-old culture (Table 2). Comparable analyses were done on the gas phase above an uninoculated PDA Petri plate; several compounds, including such major components as styrene, methyl isobutyl ketone, naphthlene and butylated hydroxytoluene, were identified and subsequently subtracted from the analysis done on the Petri plate containing *M. albus*. Final identification of 20 out of 28 compounds was done by comparison with authentic standards that were synthesized, acquired from Aldrich or Fluka, or

Table 2. GC/MS analysis of the volatile compounds produced by *M. albus*

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 (%)	<i>m/z</i>	Possible compound	<i>M_r</i>
3:45	0.33	114	Octane	114
4:19	0.93	58	Acetone	58
4:37	0.68	74	Acetic acid, methyl ester	74
5:56	7.63	88	Acetic acid, ethyl ester	88
6:51	0.31	102	Propanoic acid, 2-methyl, methyl ester	102
7:16	6.24	*	Ethanol	46
8:03	2.07	116	Propanoic acid, 2-methylethyl ester	116
11:45	0.58	*	Propanoic acid, 2-methyl 2-methylpropyl ester	144
12:05	2.06	74	Isobutyl alcohol	74
12:50	22.24	*	1-Butanol, 3-methyl-, acetate	130
14:57	1.53	*	Propanoic acid, 2-methyl, 3-methylbutyl ester	158
15:28	22.99	*	1-Butanol, 3-methyl-	88
16:08	0.29	138	†Furan, 2-pentyl-	138
18:53	0.29	142	†4-Nonanone	142
20:38	0.41	142	2-Nonanone	142
21:07	0.30	204	†Naphthalene, decahydro-4a-methyl-1-methylene-7-(1-methylethylidene)-, (4a <i>R-trans</i>)-	204
22:54	1.51	204	†Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1 <i>S</i> -(1.alpha.,4.alpha.,7.alpha.)]	204
23:16	0.94	204	†Cyclohexene, 4-(1,5-dimethyl-1,4-hexadienyl)-1-methyl-	204
25:20	3.63	204	†1 <i>H</i> -3a,7-methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8-tetramethyl-, [3 <i>R</i> -(3.alpha.,3a.beta.,7.beta.,8a.alpha.)]	204
25:30	6.08	88	Propanoic acid, 2-methyl	88
26:04	0.48	204	Caryophyllene	204
27:55	0.34	204	†Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, [1 <i>R</i> -(1.alpha.,4a.alpha.,8a.alpha.)]	204
28:34	0.36	204	†Spiro[5.5]undec-2-ene, 3,7,7-trimethyl-11-methylene-	204
28:50	1.07	204	Azulene, 1,2,3,5,6,7,8, 8a-octahydro-1, 4-dimethyl-7-(1-methylethyenyl)-, [1 <i>S</i> -(1.alpha.,7.alpha.,8a.beta.)] (common name: bulnesene)	204
28:57	3.24	204	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1 <i>R</i> -(1.alpha.,7.beta.,8a.alpha.)] (common name: valencene)	204
31:12	1.74	*	Acetic acid, 2-phenylethyl ester	164
33:17	1.06	122	Phenylethyl alcohol	122
39:00	9.76	204	†Unknown	204

* No molecular-ion peak was observed in the spectrum of either the standard compound or the compound undergoing the analysis.

† Denotes that a spectrum and retention time of this component were observed and the substance matched to the most likely compound in the NIST database, but the data have not been confirmed by use of an appropriate identical standard compound by either retention time or MS. These compounds were not placed in the artificial mixture in the bioassay.

obtained from those who did the original synthesis of them. The standards yielded identical retention times and mass spectra to the fungal products. However, the remaining eight compounds have only been tentatively identified on the basis of the NIST database information; these were not included in any of the bioassays. The most abundant compound, based on the total area of the

GC analysis, was 1-butanol, 3-methyl- followed by 1-butanol, 3 methyl-, acetate and an unknown compound with a 39 min retention time (Table 2). Collectively, the esters constituted the greatest percentage of compounds present in the gas phase of the *M. albus* culture, followed by alcohols, acids and lipids and ketones, in that order (Table 2).

Artificial test mixtures of the *M. albus* volatiles

An artificial mixture containing each of the positively identified compounds in the *M. albus* volatiles was prepared and tested. The amount of each compound placed into the mixture was based on its relative proportional content to all other positively identified compounds in the gas phase of the *M. albus* culture (Table 2), as determined by the electron ionization cross-section (percentage of the total area) of the compound obtained in the GC/MS analysis. Agar plugs containing freshly growing test fungi (or streaked bacteria and *Candida albicans*) were positioned 2–3 cm from the centre microcup. The microcups contained various amounts of the artificial mixture of *M. albus* volatiles. In this manner, it was possible to calculate the IC_{50} in $\mu\text{l ml}^{-1}$ (the concentration in μl per ml of air space in the Petri plate required to produce a 50 % reduction in microbe growth relative to an untreated control) (Table 1) for the filamentous fungi. However, the bacteria and *C. albicans* were evaluated only for their inhibition and viability after exposure to the fungus or the artificial atmosphere (Table 2).

The test rate that was the most effective per plate was 60 μl per 50 ml air space ($1.2 \mu\text{l ml}^{-1}$). The general response of the test microbes to the artificial mixture of volatiles, in many respects, mimicked the effects of the *M. albus* atmosphere (Table 1). For instance, the two plant-pathogenic oomycetes, *Pythium ultimum* and *Phytophthora cinnamomi*, responded identically to the artificial mixture as they did to the *M. albus* atmosphere (Table 1), being both inhibited and killed. The same was noted for the pathogenic basidiomycetes *Ustilago hordei* and *Rhizoctonia solani*. Likewise, the ascomycetes and fungi imperfecti, *Stagnospora nodorum*, *Tapesia yallundae* and *Verticillium dahliae*, also followed this pattern (Table 1). Just as important, however, was the response of certain test microbes such as *Fusarium solani* and *Cercospora beticola*. These two fungi were only inhibited, not killed, by *M. albus* gases, and in the artificial atmosphere they behaved in the same manner (Table 2). On the other hand, *Xylaria* sp. and *M. albus* are relatives, and while neither was killed by the artificial atmosphere, *M. albus* was about 83 % inhibited in its growth and *Xylaria* sp. was completely inhibited (Table 1). This suggests that the antimicrobial volatiles of the fungus are not general biocides, but selective in the microbes they affect. The same results with the artificial test mixture as with the fungal volatiles were also noted with each of the bacteria tested (Table 2). However, notable exceptions to an ideal correlation were the human pathogens *Candida albicans* and *Aspergillus fumigatus*, and the plant pathogen *Sclerotinia sclerotiorum* (Table 2), in that they were only inhibited and not killed in the artificial atmosphere.

There appeared to be no relationship of the IC_{50} values of the artificial mixture for the test organisms to their resistance to being killed or inhibited by this atmosphere (Table 1). However, since some test organisms (e.g. *C. albicans*, *S. sclerotiorum* and *A. fumigatus*) were not

killed in the artificial atmosphere but were killed by the *M. albus* atmosphere (Table 2), one might suspect that a key ingredient that kills cells in these cases is missing in the artificial test atmosphere. Only the chemically confirmed volatiles were placed into the artificial mixture and one or more of the non-confirmed compounds may be critical to making a perfect mimic of the results with the *M. albus* atmosphere. Furthermore, the method of administering the artificial atmosphere probably does not perfectly mimic that of the *M. albus* culture itself by providing a constant flow of volatiles to the test microbe, and this may be critical to the response of some microbes.

Since the artificial atmosphere did closely mimic the inhibitory and lethal activities of the *M. albus* atmosphere, we further evaluated the individual classes of compounds in the natural volatiles of *M. albus* in order to determine the relative biological activity of each. Each class of compounds, in the relative proportions that they occur, was tested at the level of the percentages that they occur in the total 60 μl per 50 ml air space ($1.2 \mu\text{l ml}^{-1}$) (Table 3). This was done with a selected group of seven test fungi. Each group of compounds possessed some inhibitory activity against the test organisms, but on a comparative basis the esters had more inhibitory activity than any other group of compounds (Table 3). Quite obviously, some microbes were more sensitive within and between chemical classes than others; for instance *Pythium ultimum* was highly sensitive to the alcohols while it showed little or no response to the ketones and lipids (Table 3). Indeed, the lipids and ketones were the least biologically active group of substances (Table 3). It should be noted that the majority of compounds omitted from the assay were tentatively identified as lipids and as such may not be substantial contributors to the bioactivity of the gas atmosphere of *M. albus* (Tables 1 and 3). Overall, these observations on the biological effectiveness of the individual classes of compounds warranted further study.

Testing of individual volatiles

Each compound in the class of esters was individually evaluated. When a comparable test on each ester was conducted according to the conditions in Table 3, 1-butanol, 3-methyl-, acetate almost completely mimicked the results of all esters as in Table 3. It represented 62 % of all of the identified combined esters and was therefore tested at the level of $0.32 \mu\text{l ml}^{-1}$. Only slight inhibitory bioactivity was displayed by propionic acid, 2-methyl, 3-methylbutyl ester and little or no activity was noted on the part of the other esters. Although the esters (including 1-butanol, 3 methyl-, acetate) had inhibitory activity in the bioassays, under no conditions in any test was death of any test fungus observed after the standard 3 d exposure period. This is a significant observation, since the test organisms died both in the complete artificial atmosphere and in the natural Petri plate atmosphere of *M. albus*. The result strongly suggests

Table 3. Inhibitory influence of each class of volatile compounds

The compounds were tested for a 2 d exposure at the relative concentrations that they occur in *M. albus* at the optimum test concentration of 60 µl per 50 ml air space (1.2 µl ml⁻¹). Inhibition is expressed as the percentage of test fungus growth compared to a control not exposed to the test compounds. All measurements of mycelial growth compared to the untreated control were made as described in Table 1. None of the microbes was killed after a 3 d exposure to any of the artificial test mixtures given in this table. The results are means of three determinations ± SD.

Test fungus	Growth (% of control) in presence of:				
	Alcohols (0.48 µl ml ⁻¹)	Esters (0.53 µl ml ⁻¹)	Ketones (0.02 µl ml ⁻¹)	Acids (0.09 µl ml ⁻¹)	Lipids (0.08 µl ml ⁻¹)
<i>Pythium ultimum</i>	11.2 ± 4	0	67.5 ± 7	40.9 ± 3	75 ± 0
<i>Rhizoctonia solani</i>	55 ± 5	0	67.5 ± 7.5	67.5 ± 7.5	40 ± 0
<i>Tapesia yallundae</i>	35 ± 15	0	75 ± 25	100 ± 0	100 ± 0
<i>Xylaria</i> sp.	75 ± 25	0	100 ± 0	100 ± 0	100 ± 0
<i>Sclerotinia sclerotiorum</i>	29 ± 3	8.1 ± 1.5	20.6 ± 12	40 ± 0	78 ± 2
<i>Cercospora beticola</i>	58 ± 8	5 ± 5	100 ± 0	83 ± 17	100 ± 0
<i>Fusarium solani</i>	70 ± 10	55 ± 5	90 ± 10	80 ± 20	80 ± 10

that an additive or synergistic mechanism is operational in the case of the *M. albus* volatiles. Thus, while each class of compounds possesses more or less inhibitory activity, a complete mixture of the ingredients is needed to bring about death of the test fungi and bacteria (Table 1).

M. albus as a mycofumigant

M. albus, in limited greenhouse experiments, was demonstrated to have potential practical uses. In controlled, replicated experiments, 25 barley seeds infested with *Ustilago hordei* (covered smut) were placed in each of two agar plates with the gases of *M. albus* for 4 d and then planted in test pots in the greenhouse. After 15 weeks the plants were harvested and evaluated for smut in the seed heads. There was 100% control of this disease in two groups of plants that had been exposed to *M. albus* gases and no sign of any inhibition or damage to the plants caused by the gas treatment. An identical number of control plants (untreated and *U. hordei* infested seed) had 50% and 41%, respectively of infected seed heads. Also, as expected, uninfected seed yielded plants having no diseased grains. Similar experiments have been conducted with *Muscodor albus* in soil infested with soil pathogens such as *Rhizoctonia solani*, *Pythium ultimum* and *Verticillium dahliae*, with significant effective biological control of the soil-borne plant diseases incited by these pathogens (A. Stinson, B. Jacobsen, N. Zidack & G. A. Strobel, unpublished results). These initial results are encouraging, given that in a few years the use of methyl bromide as a fumigant, widely used in agricultural soil treatment applications, will be banned for environmental and health reasons (Nelson, 1998). Conceivably, a mycofumigant may supplant the need for methyl bromide.

M. albus and other gas producers as endophytes

Generally, it is accepted that endophytic fungi may exist in their host plants in a range of biological associations from near-pathogenic to symbiotic (Bacon & White, 2000). In the latter case, a number of endophytes have been discovered that make products that are extremely biologically active and selective against certain microbes that may be a potential threat to the host plant (Yang *et al.*, 1994). In this manner the endophyte seems to have the potential to contribute to the benefit of the host by protecting it from a major biological threat – a plant pathogen. Some protective compounds recently isolated from endophytes are taxol, oocydin A, cryptocin, ambuic acid and jesterone (Stierle *et al.*, 1993; Strobel *et al.*, 1999; Li *et al.*, 2000, 2001; Li & Strobel, 2001). Each is active against a select group of pathogens and each is soluble in organic solvents. While this mechanism for the protection of the host plant may exist, with endophytes producing such compounds, it seems that no comparable situation involving a mixture of volatile lethal and inhibitory antimicrobials has been previously demonstrated. Given the current status of our understanding on the phenomenon of volatile antimicrobials, it appears that we have at least one operational hypothesis as to how *M. albus* may function in nature. That is, it may protect plant tissues against invading pathogens upon the release of a suite of inhibitory molecules.

M. albus is not the only lethal-gas-producing endophyte that we have been able to recover from plants. Recently, we have isolated two rainforest endophytes that are lethal to other microbes, by producing an entirely different suite of volatile compounds than *M. albus*. We have used *M. albus* as a selection tool to acquire these other lethal-gas producers. This can be done since

organisms related to *M. albus* seem to be immune to the effects of these lethal volatiles (Table 1). Thus, it appears that the emission of gases from endophytic fungi that are lethal to other microbes is not a case strictly confined to *M. albus* in *C. zeylanicum* in Central America, but it may be a common phenomenon in the life of all rainforests. This work again illustrates the importance of conserving the biodiversity in all of the world's ecosystems.

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