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## ***Muscodor albus* strain GBA, an endophytic fungus of *Ginkgo biloba* from United States of America, produces volatile antimicrobials**

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*Muscodor albus* strain GBA is a newly isolated endophytic fungus from *Ginkgo biloba* (family Ginkgoaceae) collected in Newport, RI, USA. The cultural characteristics (color, growth pattern) and mycelial/hyphal characteristics resemble many isolates of *Muscodor albus*. The ITS rDNA sequence of the strain has at least 98% similarity with other isolates of *M. albus* and *M. crispans*. This xyleriaceous species effectively inhibits and kills certain test microbes via a mixture of volatile organic compounds (VOCs) that it produces. Some of the target test microbes were totally inhibited by *M. albus* strain GBA and not by other *M. albus* isolates, making this isolate unique in its biological activity. The VOCs of this fungus were identified by gas chromatography/mass spectrometry as esters, lipids, alcohols, acids and ketones, including proportionally large quantities of 1-butanol, 3-methyl-, acetate. A terpenoid, not observed in other strains of this fungus, vitrene was tentatively identified in the VOCs of this organism. This is the first record of *M. albus* in *Ginkgo biloba* and is the first report of any *M. albus* strain from the United States. The organism is normally found in tropical latitudes (16° north/ south) but the plant host *M. albus* strain GBA is located at 41° north latitude. Most importantly, however, the discovery of *M. albus* in the USA has enormous implications vis-a-vis governmental regulation of *M. albus* for use as a biological control agent in agriculture and industry, as this organism naturally occurs in the USA. A discussion on the relationship of this taxon with its host is also included.

Q1

**Keywords:** antibiotics; biological control; endophyte; organic volatiles; pathogens

### **Introduction**

*Muscodor albus*, Woropong, Strobel & Hess strain CZ620, a volatile organic compound (VOC)-producing fungus, was first isolated from a cinnamon tree in a Central American rainforest in the late 1990s (Strobel et al., 2001). The organism is usually whitish, sterile, with rosy, strongly intertwined hyphae forming rosy strands. Subsequently, our laboratory reported several strains and species of this fungus, always present as an endophyte of higher plants from different parts of the world (Strobel et al., 2001, 2007; Ezra et al., 2004; Atmosukarto et al., 2005; Strobel, 2006; Mitchell et al., 2010). These fungi produce many volatile compounds which, as a mixture, are both inhibitory and, for the most part, lethal to a broad range of pathogenic fungi and bacteria (Strobel, 2006; Mitchell et al., 2010). In addition, each isolate of *Muscodor* sp. seems to produce its own unique set of volatile compounds (Strobel, 2006). While there are many reports on VOC production by microorganisms, many of these volatiles are common to many microorganisms, whereas others seem to be unique for one species (Schnurer et al., 1999; Rapior

et al., 2000; Schulz and Dickschat, 2007; Korpi et al., 2009). Furthermore, it appears that only a limited number of VOC-producing fungi possess any biological activity via the gas phase. As isolates of *M. albus* can inhibit and kill a broad range of both bacterial and fungal pathogens in practical test situations, they are being considered for use in agriculture, medical and industrial applications (Strobel, 2006).

Since *Muscodor* species are found in mostly tropical locations (16° north/ south), it seems reasonable to assume that other isolates of this organism may exist in other locales and that the biological activities of these isolates may prove to have previously unrealized potential applications. In this communication, we report a new strain of *M. albus*, designated as GBA, having genetic, biological and morphological similarities with the first isolate (*M. albus* strain CZ620), which inhibits and kills many plant pathogens (Strobel et al., 2001). *Muscodor albus* strain GBA also has a unique set of VOCs and biological activities and, for the first time, this organism has been isolated from a plant existing in the USA (at 41° north latitude)

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from a novel host and host family for this organism, i.e. *Ginkgo biloba* (Ginkgoaceae). The demonstration that *M. albus* exists in the natural environment of the USA has enormous implications for governmental regulation of this organism for practical biological uses in agriculture and industry.

## Materials and methods

### Culturing and storing *M. albus* GBA

The culture of *Muscodor albus* strain GBA used in this study was obtained as an endophyte from terminal stem segments of *Ginkgo biloba* (Maidenhair Tree) located in Newport, Rhode Island, USA at N 41° 28'499", W 071° 18' 736". To find close relatives of the originally isolated *M. albus* strain CZ620, a potato dextrose agar (PDA) plate selection technique was used (Mitchell et al., 2010). Surface-sterilized plant segments were placed in a Petri plate (PDA) already supporting the growth of *M. albus* strain CZ620. The production of its VOCs facilitates selection pressure, allowing the growth and development of only those fungal species that tolerate the VOCs of *M. albus*. Thus, other isolates of *M. albus* and usually other members of this family Xylariaceae will grow on plates with this organism (Mitchell et al., 2010).

This fungus has been taxonomically treated primarily on the basis of its partial ITS- rDNA sequences and compared to others in GenBank (Woropong et al., 2001). The fungus was grown on PDA plates and stored on infested barley grain at -70 °C for future use. It is deposited in the Montana State University Culture Collection as culture *Muscodor albus* strain GBA 2375.

### Scanning electron microscopy (SEM and ESEM)

The fungus was grown on PDA or gamma-irradiated carnation leaves for several weeks and then was processed for SEM. The samples were slowly dehydrated in ethanol, as previously described, and then critically point-dried, coated with gold and examined with an FEI XL30 scanning electron microscope (SEM) FEG (Stinson et al., 2003).

### Fungal DNA isolation and acquiring ITS-5.8S rDNA sequence information

A pure GBA culture, growing on PDA, was used as a source of DNA after incubation at 25 °C using the GebE-lute™ Plant Genomic DNA Miniprep kit (Sigma, Rehovot, Israel). Some of the techniques used were similar to those used to genetically characterize *M. albus* isolate E-6 from Ecuador (Strobel et al., 2007). Squares of the cultured mycelia (0.5 cm<sup>2</sup>) were cut from 1-week-old cultures. The agar was scraped from the bottom of the pieces to exclude as much agar as possible. The pieces were

ground in the presence of liquid nitrogen using a mortar and pestle. The DNA was then extracted according to the instructions of the kit manufacturer. Extracted DNA was diluted (1:9) in sterile, double-distilled water and 1 µl samples of this solution were used for PCR amplification. The ITS1-5.8-ITS2 rDNA sequence was amplified by PCR using the primers ITS1 (TCCGTAGGTGAACCT-GCGG) and ITS4 (TCCTCCGCTTATTGATATGC).

The PCR procedure was carried out in a 25-µl reaction mix containing 1 µl DNA extracted from the fungal culture (1:9 dilution), 1 µl primer ITS1 and 1 µl primer ITS4, 0.125 µl DreamTaq™ DNA polymerase (Fermentas, Vilnius, Lithuania), supplemented with its buffer (2.5 µl per reaction) and dNTPs (2.5 mM each). The final volume (25 µl) was adjusted using PCR-grade ddH<sub>2</sub>O (Fisher Scientific, Wembley, Western Australia). The PCR amplification was performed in a Biometra personal cycler (Goettingen, Germany): 96 °C for 5 min. The PCR products were examined using gel electrophoresis, on a 1.0% agarose gel for 25 min at 100 V with 0.5× TAE buffer in the GelXLUltra V-2 (Labnet International Inc., Woodbridge, NJ, USA) or Wealtec GES cell system (Wealtec Inc., Kennesaw, GA, USA). Gels were soaked in a 0.5 µg ml<sup>-1</sup> ethidium bromide solution for 3 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, Kiryat Anavim, Israel). A ~500-bp PCR product was purified using the GFX™ PCR DNA and Gel Band purification kit (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions. Purified products were sent to Hy Laboratories (Rehovot, Israel) for direct PCR sequencing. Automated sequencing was carried out on both strands of the PCR products using a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems Inc.) on ABI PRISM 3730xl DNA analyzer sequencing analysis software v. 5.2 with ITS1 and ITS4 primers. 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 BLAST software on the NCBI website: <http://ncbi.nlm.nih.gov/BLAST/>.

The evolutionary history of *M. albus* strain GBA was inferred using the neighbor-joining method (Saitou and Nei, 1987). An optimal phylogenetic tree with the sum of branch length = 0.82135348 was constructed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is illustrated next to the branches (Felsenstein, 1985). The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 379 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

### Test fungi and bacteria

All plant pathogenic fungi used in the bioassay test system were obtained from D. Mathre of the MSU Department of Plant Sciences. *Candida albicans* and all bacterial cultures were supplied by M. Franklin of the MSU Department of Microbiology. All fungi and bacteria were grown on PDA at 23 °C.

### Bioassay test for volatile antimicrobials

A relatively simple bioassay test system was devised which allows for volatiles only being the agents for any microbial inhibition being assessed (Strobel et al., 2001). Initially, an agar strip 2.5 cm wide was completely removed from the mid-portion of a PDA Petri plate. Then, *Muscodor albus* strain GBA was then inoculated and grown on one side of the plate for about 6–7 days prior to testing. On the half moon strip of agar on the opposite side of the plate was placed the test fungus or bacterium (Strobel et al., 2001). Individual fungi were inoculated on the test side of the plate on a 3-mm<sup>3</sup> plug of agar. Bacteria, *Saccharomyces cerevicae* and *Candida albicans* were simply streaked (1.5 cm long) on to the PDA on the test side of the plate. The act of removing a strip of agar from the mid-portion of the plate effectively precluded the diffusion of any inhibitory soluble compounds emanating from *Muscodor albus* strain GBA or *Muscodor albus* strain CZ620 to the fungi or bacteria being tested (Figure 1). The plate was wrapped with two individual pieces of parafilm and incubated at 23 °C. The growth of these latter 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 for each test microorganism by either removing the agar plug containing the

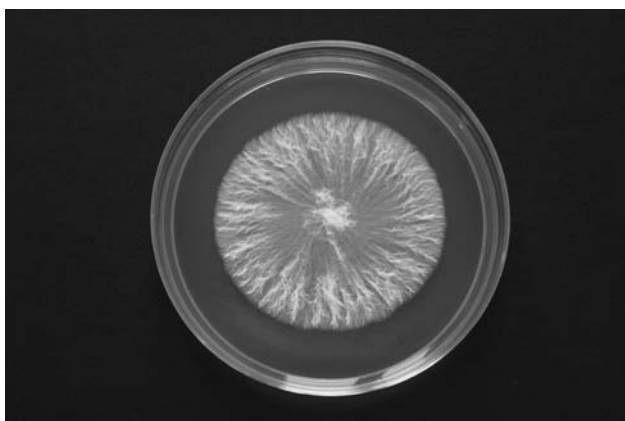


Figure 1. Petri plate supporting the growth of an *M. albus* strain GBA mycelial colony (10 days old).

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. Each bacterium and fungus for testing was used when producing fresh growth. In addition, appropriate control experiments were conducted in which the test fungus or bacterium was subjected to the same procedures minus *M. albus* strain GBA, *M. albus* strain CZ620 or *M. albus* strain E-6 on the test side of the Petri plate. The others isolates, including the original *M. albus* strain CZ620, were used in the tests for comparative purposes, especially isolate *M. albus* strain E-6, since its mycelia characteristics (ropy and closely interwoven) closely resembled that of *M. albus* strain GBA. In each case, the growth and viability of each test organism was noted in the experimental set-up. It should be noted that, while PDA is not the ideal medium for the bacteria or human pathogenic fungi used in this study, it satisfactorily supported the growth of these organisms. Its use also precluded the need to pour additional 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* strain GBA volatiles

A method was devised to analyze the gases in the air space above the *M. albus* strain GBA mycelium growing in Petri plates. First, a solid-phase micro-extraction syringe was shown to be a convenient method for trapping the fungal volatiles. The fiber material (Supelco) was 50/30 divinylbenzene/carburen on polydimethylsiloxane on a stable flex fiber. The syringe was placed through a small hole drilled in the side of the Petri plate and exposed to the vapor 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 μm was used for the separation of the volatiles. The column was temperature programmed as follows: 25 °C for 2 min followed to 220 °C at 5 °C min<sup>-1</sup>. The carrier gas was helium (He) of ultra high purity (local distributor) and the initial column head pressure was 50 kPa. The He 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 fiber 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 fiber into the GC. The gas chromatograph was interfaced to a VG 70E-HF double focusing magnetic mass spectrometer operating at a mass resolution of 1500. The MS was scanned at a rate of 0.50 s per mass decade over a mass range of 35–360 amu. Data acquisition and data processing was performed on the VG SIOS/OPUS interface and software package. Initial identification of the unknowns produced by *M. albus* strain GBA

was made through library comparison using the WILEY  
270 and NIST databases. Thus, all chemical nomenclature in  
this report is based on that used by these databases.

Comparable analyses were conducted on Petri plates  
containing only PDA and the compounds obtained, mostly  
styrene, were subtracted from the analyses done on plates  
275 containing the fungus. Final identification of 10 com-  
pounds was done on a comparative basis to authentic stan-  
dards using the GC/MS methods described above.  
However, other compounds of the volatile mixture have  
only been tentatively identified on the basis of the compar-  
280 ative information in the databases.

## Results and discussion

### Identification of *Muscodor albus* strain GBA

This isolate was obtained by using the *M. albus* selection  
technique on small pieces of limb tissue of *G. biloba*  
285 placed on split PDA plates. The organism appeared to  
have a whitish mycelium with heavily intertwining  
hyphae (Figure 1). When trying to transfer it to other  
plates, the entire mycelial mat appeared to lift off the  
surface of the agar, similar to that of *M. albus* strain E-6  
290 (Strobel et al., 2007). For this reason, comparative bio-  
logical and other experiments were done with strain E-6  
as well as the original *M. albus* strain CZ620. The SEMs  
showed hyphae as strongly intertwined and appearing in  
rope-like and coiled strands, which is similar to other *M.*  
295 *albus* strains (Figure 2A,B) (Woropong et al., 2001).  
Under no circumstances was it ever possible to observe  
any fruiting bodies or spores being produced by this  
fungal isolate.

The ITS-5.8S rDNA-ITS sequence data of *Muscodor*  
*albus* strain GBA were obtained and deposited as entry 300  
GU797134 in GenBank. A BLAST search of the database  
indicated at least 98% sequence identity to the isolates  
*M. albus* strain CZ620 (original isolate) and *M. albus*  
strain E-6, and an equally close genetic relationship to  
other isolates of this fungus, including the original *M.* 305  
*crispans* isolate as per the phylogenetic tree (Figure 3).

### Chemical composition of the fungal volatiles

The compounds produced by *M. albus* strain GBA were  
tentatively identified by the initial GC/MS separation of  
the fungal VOCs. These compounds ultimately fell into 310  
several classes of chemical substances. Present in the mix-  
ture of a 2-week-old culture were esters, alcohols, acids,  
lipids and ketones (Table 1). Comparable analyses were  
done on the gas phase above a regular PDA Petri plate and  
several compounds, including such major components as 315  
styrene, propanone, acetaldehyde and ethyl benzene, were  
identified and subsequently eliminated from the analysis  
done on the Petri plate containing *M. albus* strain GBA.  
Final identification of 10 compounds was done on a  
comparative basis to authentic standards obtained from 320  
Sigma/Aldrich or Fluka. The standards yielded relatively  
the same retention times and mass spectra as the fungal  
products. However, other compounds have only been  
tentatively identified on the basis of the database informa-  
325 tion (Table 1). The most abundant compound, based on the  
total area of the GC analysis, was 1-butanol, 3-methyl-,  
acetate followed by vitrene (a terpenoid) and 1-butanol,  
3-methyl (Table 1). Interestingly, vitrene (tentatively

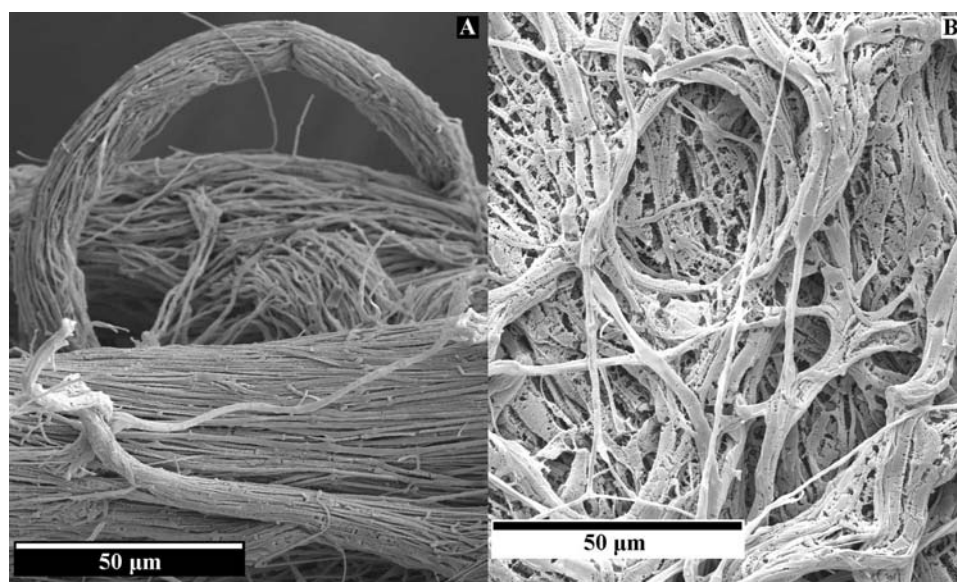


Figure 2. SEMs of a 10-day-old culture of *M. albus* strain GBA. Note the rope-like quality of the mycelium (A). Characteristic circular coils of the mycelium quite like that of other *M. albus* cultures also appear (B) (Woropong et al., 2001).

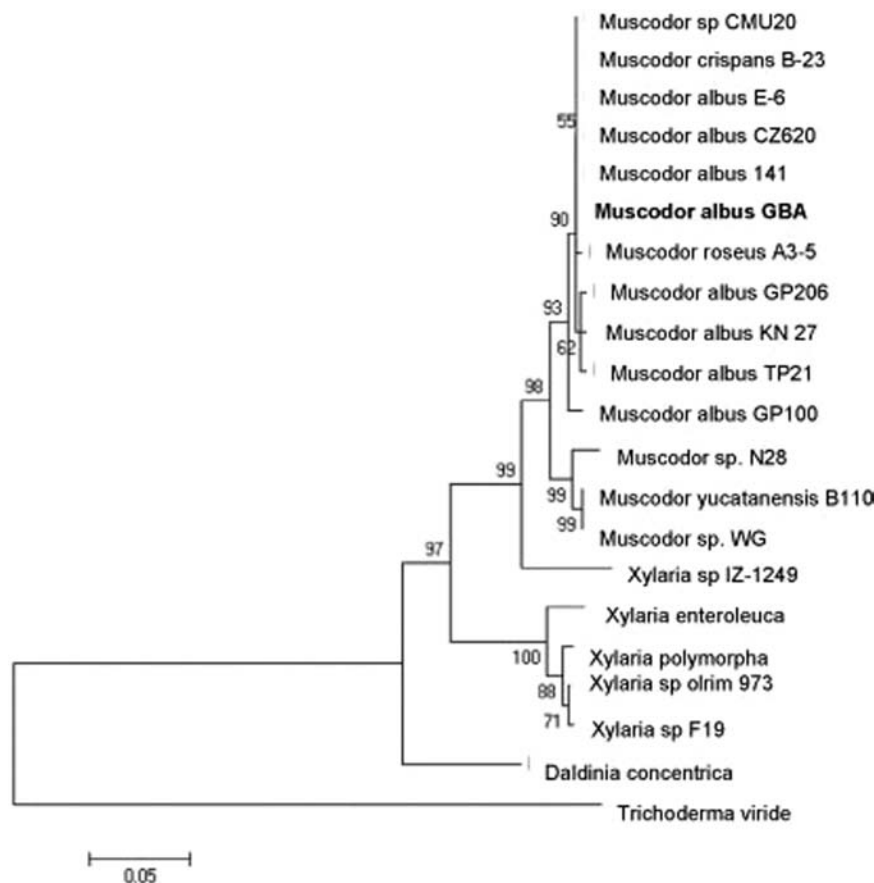


Figure 3. Evolutionary relationships of *Muscodor* spp. and some related fungi. The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

identified) has never before been observed in any *Muscodor* spp. isolates. Collectively, the esters comprised the greatest percentage of compounds present in the gas phase of the *M. albus* strain GBA culture followed by lipids, alcohols, acids and ketones (Table 1). The VOC data also follow a distinct pattern for this fungal isolate since no other *Muscodor* studied ever revealed a pattern identical to this (Table 1) (Strobel, 2006).

#### **Biological effects of *Muscodor albus* strain GBA volatiles on various fungi and bacteria**

A wide range of freshly growing fungi and bacteria were tested in the standard bioassay test. The test organisms were selected on the basis of a broad taxonomic representation of major plant and human fungal pathogens as well as representative Gram positive and Gram negative bacteria. Most test organisms were completely inhibited and, in fact, killed after a 2-day exposure to the *M. albus* GBA gases (Table 2). A time-frame of 4 days 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 4-day exposure to *M. albus* strain GBA; for more prolonged periods, these test organisms never seemed to expire (Table 2). Thus, it is important to note that the fungal volatiles are biologically selective (Table 2). The range of microorganisms affected by the volatiles of *M. albus* strain GBA is also impressive, given the fact that representative oomycetes, basidiomycetes, ascomycetes, deuteromycetes, and Gram negative and Gram positive bacteria were all inhibited after exposure to the gases of this fungus. Most importantly, some of the target test microbes were almost totally inhibited by this organism, including *B. subtilis* and *E. coli*, but not by other *M. albus* strains, making this isolate unique in its biological activity (Table 2). Finally, the fungal human pathogen *Candida albicans* was 100% inhibited (after 2 days) by the *M. albus* strain GBA volatiles in contrast to the other *Muscodor* strains which demonstrated only slight inhibition (Table 2).

Table 1. GC/MS analysis of the VOCs of *M. albus* strain GBA after 12 days incubation at 23 °C on PDA followed by gas collection with a SPME fiber.

Retention time (min)	Compound	Relative %	Molecular mass (Da)
1.98	Acetic acid, methyl ester <sup>a</sup>	1.5	74
2.68	2-Butanone <sup>a</sup>	0.5	72
4.52	Acetic acid, 2-methylpropyl ester <sup>a</sup>	7.0	116
6.58	1-Propanol, 2-methyl- <sup>a</sup>	1.0	74
6.96	1-Butanol, 3-methyl-, acetate <sup>a,b</sup>	30.0	130
8.99	Cyclohexane, 1-methyl-4-methylene-	0.7	110
9.01	2,3-Dimethyl-3-isopropyl-cyclopentene	1.0	138
9.49	1-Butanol, 3-methyl- <sup>a,b</sup>	15	88
11.89	Pyrrolidine	1.3	71
14.54	Germacrene B	0.8	204
16.09	2-Heptanoic acid, 4-cyclopropyl-	0.8	194
16.17	Unknown	4.1	175
16.57	A-Sinensal	2.4	218
19.08	Bicyclo[3.1.1]heptane, 6-methyl-2-	2.5	204
19.20	Propanoic acid, 2-methyl- <sup>a,b</sup>	5.3	88
22.51	<i>Trans</i> -caryophyllene <sup>a,b</sup>	4.5	204
24.51	4-Piperidinone, 1-methyl-	0.5	113
24.67	Acetic acid, 2-phenylethyl ester <sup>a,b</sup>	1.4	164
26.76	Benzeneethanol <sup>a</sup>	0.7	122
32.45	(+)-Vitrene	18.9	204

<sup>a</sup>Denotes that the spectrum and retention time of this component were identical to those of an authentic standard compound.

<sup>b</sup>Compounds found in original *M. albus* strain CZ620.

Compounds found on the control PDA plate or having less than 0.5% of total area are not included in the table.

Table 2. Response of several test fungi and bacteria to the volatiles of *M. albus* strain GBA, *M. albus* strain E6 and *M. albus* CZ620. The test organism was exposed to the VOCs of these Muscodor strains for the times given. Percentage inhibition was measured relative to the growth of the control culture. Viability was tested after the original inoculum was replaced on a regular PDA plate and checked for growth. The experiment was repeated twice with comparable results.

Group of microbes	Pathogen tested	<i>M. albus</i> GBA						<i>M. albus</i> E6		<i>M. albus</i> CZ 620	
		% Inhibition (condition)									
		After 2 day	After 4 day	After 2 day	After 4 day	After 2 day	After 4 day	After 2 day	After 4 day	After 2 day	After 4 day
Phycomycetes	<i>Pythium ultimum</i>	100 (dead)	100 (dead)	100 (living)	90 (living)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)
	<i>Phytophthora cinnamomi</i>	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)
Ascomycetes	<i>Geotrichium candidum</i>	100 (dead)	100 (dead)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)
	<i>Aspergillus fumigatus</i>	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)
	<i>Trichoderma</i> sp.	60 (living)	30 (living)	70 (living)	20 (living)	0 (living)	0 (living)	0 (living)	0 (living)	0 (living)	0 (living)
	<i>Verticillium dahliae</i>	100 (living)	100 (living)	100 (living)	95 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)
	<i>Cercospora beticola</i>	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)	100 (living)
	<i>Sclerotinia sclerotiorum</i>	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)
	<i>Botrytis cinerea</i>	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)
Basidiomycetes	<i>Fusarium solani</i>	85 (living)	75 (living)	90 (living)	70 (living)	60 (living)	40 (living)	60 (living)	40 (living)	60 (living)	40 (living)
	<i>Rhizoctonia solani</i>	100 (living)	95 (living)	95 (living)	80 (living)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)	100 (dead)
Yeasts	<i>Candida albicans</i>	100 (living)	20 (living)	60 (living)	40 (living)	20 (living)	0 (living)	20 (living)	0 (living)	20 (living)	0 (living)
	<i>Saccharomyces cerevisiae</i>	100 (dead)	100 (dead)	80 (living)	50 (living)	10 (living)	0 (living)	10 (living)	0 (living)	10 (living)	0 (living)
Bacteria	<i>Bacillus subtilis</i>	100 (living)	70 (living)	0 (living)	0 (living)	10 (living)	0 (living)	10 (living)	0 (living)	10 (living)	0 (living)
	<i>Escherichia coli</i>	100 (living)	100 (living)	20 (living)	10 (living)	10 (living)	0 (living)	10 (living)	0 (living)	10 (living)	0 (living)

### *Muscodor albus* strain GBA and *M. albus* strain CZ620 comparisons

The original isolate of *M. albus* strain CZ620 was obtained as an endophyte from *Cinnamomum zeylanicum* in Central America. Subsequently, many other isolates and species of this fungal genus were obtained from tropical locations

(at 16° north/south) all over the world (Strobel, 2006). Now, for the first time and uniquely, *M. albus* strain GBA was isolated from a *Ginkgo biloba* tree in Newport (Rhode Island, USA) located at 41° North. The tree, according to the owner James Coombs, may have been brought to the USA many years ago by a local man who traded in the far



Eastern. He apparently brought several ginkos to the USA  
 380 and planted them in his yard in the late 1800s. This loca-  
 tion is the maximum north or south latitude from which  
 this organism has ever been found. Conceivably, the  
 imported trees may have carried the fungus with them in  
 an endophytic form and been retained therein for over  
 385 100 years. Alternatively, the trees could have made an  
 association with this fungus from local biological sources  
 when they were planted in their present location.

This isolate has some properties in common with other  
 isolates of *Muscodor* spp. The most interesting is tough  
 390 intertwined mycelia, allowing it to be easily lifted up from  
 the agar surface in a manner comparable to *M. albus* strain  
 E-6 (Strobel et al., 2007). On the other hand, it kills *Geot-*  
*richium candidum* and *Saccharomyces cerevicae*, though  
*M. albus* strains CZ620 and E-6 do not (Table 2) ( Strobel  
 395 et al., 2001, 2007). It also strongly inhibits *E. coli* and *B.*  
*subtilis*, while *M. albus* strains CZ620 and E-6 do not  
 (Table 2). Furthermore, the major volatile component in  
*M. albus* strain GBA is an ester, 1-butanol, 3-methyl-, ace-  
 400 tate, whereas, in *M. albus* strain CZ620, the major compo-  
 nent is an alcohol, 1-butanol, 3-methyl. Napthalene and  
 azulene derivatives were not detected in *M. albus* strain  
 GBA although they are some of the major volatile compo-  
 nents in *M. albus* strains CZ620 and E-6 mixtures (Strobel  
 et al., 2001, 2007). However, vitrene, a terpenoid, was  
 405 detected in the VOCs of *M. albus* strain GBA, which is the  
 first time it has been recorded in the VOC mixtures of any  
*Muscodor* spp. This suggests that it has a terpenoid  
 synthetase critical to the production of this compound  
 (Table 1).

#### 410 **Taxonomic position and significance of *Muscodor albus* strain GBA**

This is the first report showing that an *M. albus* isolate  
 exists as a naturally occurring endophyte in the United  
 States and also from *G. biloba* (Ginkgoaceae). However, its  
 415 phenotypic characteristics may not be substantial enough to  
 qualify it for new species status. This was also the conclu-  
 sion drawn for other isolates of this organism found in Indo-  
 nesia, Australia and Thailand (Atmosukarto et al., 2005;  
 Ezra et al., 2004; Sopalun et al., 2003). Certainly, *M. albus*  
 420 GBA possesses some chemically (VOC) distinct pheno-  
 typic characteristics (Table 2); however, other *M. albus*  
 isolates obtained from other plant species also seem to  
 produce some unique VOCs. Furthermore, this isolate  
 does not have any hyphal or mycelial characteristics that  
 425 are distinctive, such as the hyphal cauliflower projections  
 and undulating hyphae of *Muscodor crispans* (Mitchell et  
 al., 2009). Thus, it is reasonable to simply designate this  
 organism as a new isolate of *M. albus*.

Most importantly is the fact that a *Muscodor* sp. has  
 430 appeared under natural circumstances as an endophyte in the  
 United States. This has extremely important implications for

federal/state and local regulatory authorities controlling  
 the use of this organism in agriculture and industry. Since  
 it occurs naturally, the restrictions on its use to control  
 unwanted bacteria and fungi will be subsequently ameliorated,  
 435 making the observations in this report of critical  
 significance.

#### **Conclusions**

In addition to the practical implications for the use of  
*Muscodor* spp. for the biological control of unwanted  
 440 microbes in agricultural production, food storage, food  
 transportation and other agri/industrial uses, other interesting  
 questions arise concerning the natural role of this organism  
 in its environment. Generally, it is accepted that endo-  
 phytic fungi may exist in their host plants in a range of  
 445 biological associations from near pathogenic to symbiotic  
 (Bacon and White, 2000). In the latter case, a number of  
 endophytes produce compounds which are extremely bio-  
 logically active and selective against certain microbes that  
 may be potential threats to the host plant (Yang et al.,  
 450 1994). Thus, the endophyte seems to have the potential to  
 contribute to the benefit of the host by providing protection  
 from a major biological threat – a plant pathogen. Some  
 protective compounds recently isolated from endophytes  
 are exemplified by taxol, oocydin A, cryptocin, ambuic  
 455 acid and jesterone (Strobel et al., 2004). Each is active  
 against a select group of pathogens and each is soluble in  
 organic solvents. While this host plant protection mecha-  
 nism may involve endophytes producing such compounds,  
 no comparable situation involving inhibitory and lethal  
 460 volatiles has been demonstrated previously, except for the  
 various isolates of *Muscodor* spp. In addition, there is no  
 conclusive evidence that the endophytic microbial prod-  
 ucts are actually produced in the plant (in vivo) and at  
 levels that affect potential pathogens. Direct host/endo-  
 465 phyte relationship studies are, therefore, warranted for  
 various isolates of *Muscodor*. This group of endophytes is  
 important as they are associated with numerous plant  
 families worldwide (Strobel, 2006).

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