



# Muscodor species- Endophytes with Biological Promise

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***Muscodor* species- Endophytes with Biological Promise**

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**Key words:** Mycofumigation, Endophytes, Volatile antibiotics, Biological Control

## **Abstract**

A novel fungal genus is described that produces extremely bioactive volatile organic compounds (VOC's). The initial fungal isolate was discovered as an endophyte in *Cinnamomum zeylanicum* in a botanical garden in Honduras. This endophytic fungus was named *Muscodor albus* because of its odor and its white color. This fungus produces a mixture of VOC's that are lethal to a wide variety of plant and human pathogenic fungi and bacteria. It also is effective against nematodes and certain insects. The mixture of VOC's has been analyzed using GC/MS and consists primarily of various alcohols, acids, esters, ketones, and lipids. Final verification of the identity of the VOC's was carried out by using artificial mixtures of the putatively identified compounds and showing that the artificial mixture possessed the identical retention times and mass spectral qualities as those of the fungal derived substances. Artificial mixtures of the available VOC's mimicked some but not all of the biological effects of the fungal VOC's when tested against a wide range of fungal and bacterial pathogens. Other species and isolates of this genus have been found in various tropical forests in Australia, Bolivia, Ecuador, and Thailand. The most recent discovery is *Muscodor crispans* whose VOCs are active against many plant and human pathogens. Potential applications for "mycofumigation" by members of the *Muscodor* genus are currently being investigated and include uses for treating plant diseases, buildings, soils, agricultural produce and many more. This report will describe how the fungus was discovered, identified, and found potentially useful to agriculture, medicine and industry.

Key words; Endophytes, Mycofumigation, Volatile organic compounds, DNA,GC/MS

## **Microbes for humankind**

Microorganisms have long served mankind by virtue of the myriad of the enzymes and secondary products that they make [1]. Interestingly, however, is the fact that only a relatively small number of microbes are used directly in human applications i.e. bread, cheese, and alcoholic beverage making. It seems that a more comprehensive search of the earth's niches may reveal novel microbes having other direct utilities to human societies. Of course, the advantages of direct use are numerous including reduced economic and regulatory factors while at the same time successfully completing a useful task. The diversity of microbial life is enormous and the niches in which microbes live is truly amazing ranging from deep ocean sediments to the earth's thermal pools [2]. A relatively untapped source of microbial diversity is the world's rainforests. Each plant supports a suite of microorganisms known as endophytes [3]. These organisms cause no overt symptoms on the plants in which they live [4]. Furthermore, since so little work on these endophytes has been done it is suspected that untold numbers of novel fungal and bacterial genera exist as plant-associated microbes [5]. The rationale for sampling rainforest species is the fact that high plant biodiversity existing in the world's rainforest areas may also be accompanied by high microbial diversity [3, 6]. Thus, we have begun a concerted search for novel endophytic microbes and the prospects that they may produce novel bioactive products as well as processes that may prove useful at the organismal level. This report concentrates mostly on the discovery of one novel endophytic fungal genus – *Muscodor* and the systematic study of its VOC production and its future for human use, biological discovery, and biological control.

### **The discovery of *Muscodor albus***

In the late 90's I was on a collecting trip in the jungles near to the Caribbean coast of Honduras. I had selected this area to visit because Central America is one of the world's "hot spots of biodiversity" [6]. One modestly sized tree, not native to the new world, was introduced to me as *Cinnamomum zeylanicum*. Small limb specimens were taken and placed in a plastic bag and brought back to Montana and the sample was processed according to standard isolation procedures for endophytes [3]. However, we had been plagued with microscopic phytophagous mites in the lab for many months. This is not uncommon for labs in which plant materials are being brought on a regular basis. Mites infest the bench tops and invade parafilm sealed Petri plates containing agar in which they take up residence. Thus, in order to eliminate this persistent mite problem we decided to place the Petri plates, with plant tissues, in a large plastic box having a firmly fitting lid. This maneuver would make it difficult for the tiny animals to find their way from the bench surfaces to the inside of the box. After a few days most plant specimens had sported endophytic fungal growth. Eventually the plates were removed and the individual hyphae transferred to fresh plates of potato dextrose agar. After a day or two of incubation we noted that no transferred endophyte grew except one. Had the placement of the endophytes in the large plastic box killed the endophytes by limiting oxygen availability? Soon it became obvious that the one endophytic fungus (designated isolate 620) remaining alive was producing volatile organic compounds (VOCs). The hypothesis that an endophyte can make VOCs having antibiotic activities was born. It was quickly learned that although many wood inhabiting fungi make volatile substances, none of these possessed the biological activity of isolate 620 [7]. However some early data

supported the observations that *Trichoderma* sp. produced some VOCs, however, with only modest biological activity and no attempt was made to identify the VOCs of this organism [8]. Later, another report on *Trichoderma* sp. showed the identity of the VOCs and pointed out that inhibitory activity was associated with these compounds [9].

### ***Muscodor albus* and its VOCs**

Isolate 620 is a sterile (not producing spores) endophytic fungus possessing some interesting hyphal characteristics including coiling, ropyness, and some right angle branching. The mycelia of the fungus on most media are whitish and suppressed. In fact, the mycelia commonly make undulations on the agar surface. However, it has a tendency to produce aerial discrete organized mycelia yielding what appear to be synemma, however closer inspection reveals that they too are sterile. All attempts to initiate spore formation have failed. Isolate 620 grows well on a number of agar media including potato dextrose agar and other media containing plant extracts or various media made with wood shavings supplemented with starch since it appears that a rich carbohydrate source is critical to VOC production by this organism [10].

Therefore, in order to taxonomically characterize this organism, the partial regions of the internal transcribed spacer region and the 5.8S of the nuclear ribosomal DNA operon (ITS- 5.8 rDNA) were isolated, sequenced, and deposited in GenBank as **AF 324336** and **AF 324337** [10]. It turns out that isolate 620 is unique but it has a 82-92% sequence similarity to several *Xylaria* spp. [10]. The GC/MS analysis of the fungal VOCs showed the presence of at least 28 VOCs (Table 1) [11]. These compounds represented at least

five general classes of organic substance (acids, alcohols, esters, and lipids). All chemical compounds, in this review, are described on the basis of their appropriate NIST data base names since this base was used to provide preliminary identification information. Final identification of the volatile compounds was done by GC/MS of authentic compounds obtained from commercial sources or synthesized by us or others and compared directly by GC/MS to the VOCs of the fungus (Table 1) [11]. With this chemical information in hand, along with the DNA sequence data, we felt secure in proposing a binomial for this fungus derived from the Latin –*Muscodor* (stinky) *albus* (white) [10].

Ultimately, artificial mixtures of the compounds were used in a biological assay system to demonstrate the relative activity of individual compounds [11]. Although over 80% of the volatiles could be identified, this seemed to be adequate to achieve an excellent reproduction of the lethal- antibiotic effects of the VOCs that were being produced by the fungus [11]. The bioassay test was used to examine the five general classes of VOCs. Each class possessed some inhibitory activity with the esters being the most active (Table 2) [11]. Of these, the most active individual compound was 1-butanol, 3 methyl-, acetate. However, it is to be strongly stressed that no individual compound or class of compounds was lethal to any of the test microbes which consisted of representative plant pathogenic fungi, Gram positive and Gram negative bacteria, and others [11]. Obviously, the antibiotic effect of the VOCs of *M. albus* is strictly related to the synergistic activity of the compounds in the gas phase (Table 2).

The mechanism of action of the VOCs of *M. albus*. on target fungi and bacteria is unknown. However, a microarray study to analyze the transcriptional response of

*Bacillus subtilis* cells exposed to *M. albus* VOCs showed that genes involved in DNA repair and replication were increased in expression (RA Britton, Michigan State University, unpublished observations). These preliminary results would indicate that *M. albus* VOCs are inducing some type of DNA damage in cells, possibly through the effects of one of its naphthalene derivatives, since such related compounds had previously shown to cause chromosome decondensation in bacteria [12]. Likewise, similar microarray studies on other microorganisms susceptible to *M. albus* gases would be helpful in elucidating the mechanism of antimicrobial action.

Using the bioassay test system it was possible to calculate the 50% inhibitory concentration (IC<sub>50</sub>) for each test microbe and compare the killing ability of the artificial mixture with that of the *M. albus* VOCs [11]. One of the most sensitive fungi was *Rhizoctonia solani* and one of the least sensitive was *Fusarium solani*. In the later case, *F. solani* is only inhibited by both the artificial VOC mixture as well as the VOCs of the fungus. Finally, the viability of all test organisms was also determined after exposure to either the fungus or to its artificial mixture of VOCs (Tables 1& 2) [11]. The artificial mixture generally mimicked the effects of the fungus itself (Table 2) [11]. A minimum of three VOCs (naphthalene, propanoic acid and butanol, 3-methyl) can quite effectively mimic the killing activity of the fungal VOCs (13). Not all compounds in the fungal VOCs are necessary for biological activity, but there may still be others that have gone undetected that are critical to bioactivity [11].

### **Other isolates and species of *Muscodor***

Recently, seven new *M. albus* isolates were retrieved from the Northern Territory of Australia [13]. These organisms were obtained by using *M. albus* (isolate 620) as a selection tool in Petri plates containing agar in the presence of the plant tissues containing endophytic fungi [13]. The host plants of these isolates were *Terminalia prostrata* (Combretaceae), *Kennedia nigricans* (Leguminosae) and *Grevillea pterifolia* (Proteaceae). Each isolate was biologically active, produced some but not all of the VOCs made by *M. albus* 620, and had between 96 % and 99% ITS-5.8S rDNA sequence similarity to *M. albus* 620 [13].

Another isolate of *M. albus* has been recovered as an endophyte from nutmeg (*Myristica nutans*- family Myristaceae) in Thailand [14]. This isolate has 98% sequence similarity to the ITS-5.8 rDNA of *M. albus* and produces many of the same volatiles as isolate 620 and it also possesses antibiotic properties. Furthermore, again using the selection technique, *M. albus* isolate 1-41-3s was obtained from an unidentified vine in the Sumatran jungle of Tesso Nilo in Indonesia, and it possessed 98% ITS-5.8SrDNA sequence similarity to *M. albus* 620 [15]. This isolate possesses unusual hyphae, a slime layer, and some VOCs not observed before in other *M. albus* isolates including aciphyllene, 2-butanone, 2-methyl furan, tetrahydrofuran and large amounts of an unusual azulene derivative [15]. Discovery of these new isolates of *M. albus* confirms that this organism may be a *bona fide* novel endophytic fungal genus rather than the

original 620 isolate occurring as a localized phenomenon in nature. The discovery also shows that several plant families other than Lauraceae (*Cinnamomum* sp.) can serve as a host for *M. albus*.

Two other species of *Muscodor* have been reported including *M. roseus* (endophytic on *Grevillea pteridifolia*, Australia) and *M. vitigenus* (endophytic on *Paullinia paullinoides*, Peru) [16,17]. These isolates are also closely genetically related (ITS-5.8S rDNA sequences) to the Australian isolates of *M. albus* at the 96%-99% level [13]. The later organism makes only naphthalene as a VOC and its repellency towards a plant associated insect has been demonstrated [18].

Another isolate of *M. albus* (E-6) with unusual biochemical and biological properties, has been obtained from the branches of a mature *Guazuma ulmifolia* (Sterculiaceae) tree growing in a dry tropical forest in SW Ecuador [19]. This unique organism produces many VOC's not previously observed in other *M. albus* isolates including: butanoic acid, 2-methyl-; butanoic acid, 3-methyl-; 2-butenal, 2-methyl-; butanoic acid, 3-methylbutyl ester; 3-buten-1-ol, 3-methyl; guaiol; 1-octene, 3-ethyl-, formamide, N-(1-methylpropyl), and along with certain azulene and naphthalene derivatives. On the other hand, some compounds usually seen in *M. albus* isolates also appeared in the VOCs of isolate E-6 including: caryophyllene; phenylethyl alcohol; acetic acid, 2-phenylethyl ester; bulnesene; and various propanoic acid, 2-methyl- derivatives. Scanning electron micrographs of the mycelium showed substantial intertwining of the hyphal strands. These strands seemed to be held together by an extracellular matrix accounting for the strong mat-like nature of the mycelium which easily lifts off the agar surface upon

transfer unlike any other isolate of this fungus. The ITS -5.8S rDNA partial sequence data showed 99% similarity to the original *M. albus* strain- 620. Now, for the first time, a successful establishment of *M. albus* into its natural host, followed by recovery of the fungus, was accomplished in seedlings of *G. ulmifolia*. The biological activity of the VOCs of E-6 appears different from the original isolate of this fungus- 620 since a Gram positive bacterium was killed and *Sclerotinia sclerotiorum* along with *Rhizoctonia solani* were not.

### **Physiological aspects of VOC production**

The composition of the medium greatly influences the quality and effectiveness of the VOCs emitted by *M. albus*-620 [20]. For instance, a sucrose enriched medium primarily yields methyl isobutylketone and acetic acid, butyl ester as the primary volatiles, yet neither of these compounds appeared in any other medium. Furthermore, the VOCs under these conditions, were limited in their bioactivity. More enriched media were more effective in inhibiting a suite of plant pathogens used as test microbes [20].

Although only qualitative methodology was initially used to gather information on the fungal VOCs there was a need to obtain quantitative data on VOC production. A relatively new technology called proton transfer reaction-mass spectrometry (PTR-MS) was used to monitor the concentration of VOCs emitted by *M. albus* [21]. PTR-MS is a particularly useful technique because it can be run on line while continuously yielding data on the concentrations of specific ions of interest. Data gathered from a long term *M. albus* culture in a carboy by PTR-MS indicated that the production of VOCs is

temperature-dependent with diurnal fluctuations in gas production occurring as the temperature varied [21]. Furthermore, continuous monitoring after 3 weeks revealed a slow, but steady decline in VOC production which is probably a reflection of the depletion of the carbohydrate sources in the potato dextrose agar. This is consistent with the observations showing VOC production is related to the presence of a carbohydrate source [20].

The PTR-MS technique was also applied to soils containing *M. albus* along with the plant pathogen *Pythium ultimum* and it was possible to show the production of VOCs from *M. albus in situ*. An estimation of the range of concentrations of total VOCs being produced by *M. albus* is in the order of 100-300 ppb based on the determination of the concentration of propanoic acid, 2-methyl, methyl ester.

Interestingly, since *M. albus* has not been artificially established in any host or non-host plant, no data are available on the *in planta* production of VOCs by endophytically established *M. albus*. This is an important phenomenon awaiting observation and the PTR-MS technique seems like the most useful method to make such observations. Establishing a successful *M. albus*/ plant relationship is critical in doing these *in planta* mass spectral studies.

#### **“Mycofumigation” with *Muscodora* spp.**

The VOCs of *M. albus* kill many of the pathogens that affect plants, people and even buildings [11,14](Table 2). The term “mycofumigation” has been applied to the practical

aspects of this fungus [11]. The first practical demonstration of its effects against a pathogen was the mycofumigation of covered smut infected barley seeds for a few days. The seeds were eventually planted and the resulting plants, in contrast to the untreated control group, produced no infected heads [11]. Mycofumigation is also important for the treatment of fruits in storage and transit [22]. Soil treatments have also been effectively used in both field and greenhouse situations [23-25]. In these cases, soils are pretreated with a *M. albus* formulation in order to preclude the development of infected seedlings. *M. albus* is now being produced, by solid state fermentation, by the ton in order for its use in many practical applications.

AgraQuest, an agricultural biotech company, of Davis, Calif., is developing *M. albus* for numerous agricultural applications. The concept of mycofumigation, for a multitude of uses, has the potential to replace hazardous substances that are currently applied to humans, food, soil and buildings. The most notable of which is methyl bromide for soil sterilization which use will soon cease because of its toxicity and negative influences on the world's ozone layer. On the other hand, it turns out that the VOCs of *M. albus* appear safe, effective and environmentally friendly and may serve as a replacement soil treatment [25].

### **Other VOC producing fungi**

Using *M. albus* as a selection tool, *Gliocladium* sp. was isolated from *Eucryphia cordifolia* in Patagonia and this organism is phylogenetically related to *Trichoderma* spp.(Fig.2)[26]. It produces a series of VOC that have some bioactivity. Generally,

*Trichoderma* spp. although possessing VOCs with biological activity, are not sufficiently active for practical applications to be made of them. However, in the case of *Gliocladium* sp. at least two target organisms were killed by its VOCs including *Pythium ultimum* and *Verticillium dahliae* [26].

### **Concluding remarks and future perspectives**

Obviously, because of the impressive biological activity of *M. albus* and its introduction into practical agriculture/industry, it seems that the fungus should be more fully studied relative to its location and role in nature. Overall, the most pressing question regarding *M. albus* is the mode of action of a multitude of volatile compounds and how they act synergistically to cause the death of microbes.

Certainly, knowledge of its hosts and those factors controlling host preference may eventually allow for the use and development of this organism for hosts that it does not naturally frequent. Such information may result in still more applications and direct uses of *M. albus*. Further, we need to learn if *Muscodora* isolates can be directly inoculated into agricultural and forest species in order to provide protection against invading pests and pathogens. In this regard, it has been recently learned that *M. albus* as well as a mixture of its VOCs has activity against certain plant pathogenic nematodes [27]. Other studies are in progress to test its biological activity against plant insect pests.

It seems that there may be some factors that may limit the use of *M. albus* for biological control. One of the most important of which is the inability of *M. albus* to kill, and only

inhibit isolates of *Fusarium* spp. This seems to be a universal observation with all isolates of *Muscodor* sp. [11,13,15]. The fusarial species are some of the most important plant pathogens occurring on a wide range of agricultural and forest species. Perhaps, other *Muscodor* spp. will be found in places that have not yet been sampled including such locations as Australia, Brazil, Malaysia, USA, Venezuela, and tropical Africa that will be effective against *Fusarium* spp. Furthermore, unless adequate sources of carbohydrates are available, *M. albus* ceases producing its VOCs [20]. This may be present a problem in situations in which VOC production stops even though unwanted organisms persist.

In addition, it is extremely important to possess information on the distribution, life cycle and other aspects of the chemistry of this organism. This will aid in expanding its utility as a biological control agent. Hopefully, the development of information on *M. albus* will have broad implications for the discovery and development of other rainforest microbes. Work on this important fungal genus has just begun.

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Table 1. 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 [11].

RT	Total Area (%)	<i>m/z</i>	Possible compound	MW
3:45	0.33	114	Octane	114
4:19	0.93	58	Acetone	58
4:37	0.68	74	Methyl acetate	74
5:56	7.63	88	Ethyl acetate	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-methyl-ethyl ester	116
11:45	0.58	*	Propanoic acid, 2-methyl 2-methylpropyl ester	144
12:05	2.06	74	1-Propanol, 2-methyl-	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)-, (4aR-trans)-	204
22:54	1.51	204	# Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(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	# 1H-3a,7-methanoazulene, 2,3,4,7,8,8a-hexahydro-3,6,8,8 tetramethyl-, [3R-(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)-, [1R-(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)-, [1S-(1.alpha.,7.alpha.,8a.beta.)]	204
28:57	3.24	204	Common Name: Bulnesene Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-	204

methylethenyl)-,[1R-  
(1.alpha.,7.beta.,8a.alpha.)]  
Common Name: Valencene

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 was observed and the substance matched to the most likely compound in the NIST data base, 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 test.

Table 2. The 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* gasses, the 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 (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 $\mu$ l/50CC in order to obtain IC<sub>50</sub>'s. The % growth over the control and viability were measured after exposure to 60 $\mu$ l/50CC. Viability was determined after the removal of the compounds at 3 days [11].

Test Microbe	% Growth over control after a 2 day exposure to <i>M.albus</i>	Viability after 3 days exposure to <i>M.albus</i> culture	IC <sub>50</sub> in artificial atmosphere for 2days ( $\mu$ l/CC)	% Growth (mm) over control in artificial atmosphere	Viability after 3 days exposure artificial atmosphere
<i>Pythium ultimum</i>	0	Dead	0.48 $\pm$ 0.01	0	Dead
<i>Phytophthora cinnamoni</i>	0	Dead	0.29 $\pm$ 0.06	0	Dead
<i>Rhizoctonia solani</i>	0	Dead	0.08 $\pm$ 0.02	0	Dead
<i>Ustilago hordei</i>	0	Dead	0.31 $\pm$ 0.09	0	Dead
	0	Dead	0.15 $\pm$ 0	0	Dead
<i>Stagnospora nodorum</i>					
<i>Sclerotinia sclerotiorum</i>	0	Dead	0.17 $\pm$ 0.05	0	Alive
<i>Aspergillus fumigatus</i>	0	Dead	0.41 $\pm$ 0.05	0	Alive
<i>Fusarium solani</i>	19.4 $\pm$ 0.28	Alive	1.13 $\pm$ 0.07	42.0 $\pm$ 2	Alive
<i>Verticillium dahliae</i>	0	Dead	0.3 $\pm$ 0	0	Dead
	17.5 $\pm$ 3.5	Alive	0.12 $\pm$ 0.15	8 $\pm$ 2	Alive
<i>Cercospora beticola</i>					
<i>Tapesia yallundae</i>	0	Dead	0.64 $\pm$ 0	0	Dead
<i>Xylaria</i> sp.	25 $\pm$ 0	Alive	0.41 $\pm$ 0.03	0	Alive
<i>Muscodor albus</i>	100 $\pm$ 0	Alive	0.6 $\pm$ 0	17.5 $\pm$ 7.5	Alive
<i>Escherichia coli</i>	0	Dead	#	0	Dead
<i>Staphylococcus aureus</i>	0	Dead	#	0	Dead
<i>Micrococcus luteus</i>	0	Dead	#	0	Dead
<i>Candida albicans</i>	0	Dead	#	trace	Alive
<i>Bacillus subtilis</i>	0	Alive	#	0	Alive

\* The amount of each positively identified compound used in the artificial mixture was obtained by applying the electron ionization cross section (% of the total area) of the compound obtained in the GC/MS analysis (Table 1). The artificial mixtures were subsequently tested by placing them in a presterilized microcup (4x6 mm) located in the center of a test Petri plate containing PDA. Agar plugs containing freshly growing test microbes (or streaked microbes) were positioned about 2-3 cm from the center microcup. Then the plate was wrapped with 2 layers of parafilm and incubated for two or more days at 23°C. Measurements of linear mycelial growth were made from the edge of the inoculum agar plug to the edge of the mycelial colony.

#Not measured in this experimental design.

