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ORIGINAL ARTICLE

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Muscodor albus and its biological promise

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Abstract We have found a novel fungal genus that produces extremely bioactive volatile organic compounds (VOCs). This fungal isolate was initially discovered as an endophyte in *Cinnamomum zeylanicum* in a botanical garden in Honduras. This endophytic fungus, *Muscodor albus*, produces a mixture of VOCs that are lethal to a wide variety of plant and human pathogenic fungi and bacteria. It is also effective against nematodes and certain insects. The mixture of VOCs has been analyzed using GC/MS and consists primarily of various alcohols, acids, esters, ketones, and lipids. Final verification of the identity of the VOCs 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 VOCs nicely mimicked the biological effects of the fungal VOCs when tested against a wide range of fungal and bacterial pathogens. Potential applications for "mycofumigation" by *M. albus* are currently being investigated and include uses for treating various plant parts, and human wastes. Another promising option includes its use to replace methyl bromide fumigation as a means to control soil-borne plant diseases.

Keywords Volatile antibiotics · Mycofumigation · Endophyte · Plant pathogen

Introduction

As a field, microbiology has witnessed an evolution of its own. It was initially spawned out of interest in brewing and fermentation activities on which mankind had relied for millennia. The discipline was formalized when it was realized that microbes have the potential to cause infection and death. Ultimately, in the earlier part of the last century the discovery of antibiotic producing microbes triggered a search for useful microbes in every conceivable niche in the world. Today, as a result of this search, microbes produce a vast array of products ranging from antibiotics, enzymes, vitamins, to important secondary products [6]. Eventually, microbes served as models for the development of new fields including genetics, molecular biology, and industrial microbiology. The discovery processes in microbiology seem endless especially with the advent of biotechnology as it inspires new uses for microbial products and systems [3].

However, in spite of all of the knowledge gained about myriads of microbes and their products that have been put to use, only a few dozen have ever been domesticated, that is used directly, to carry out specific biological functions for mankind. Some of these include the rhizobia that nodulate legumes, cheese and wine/beer making bacteria and fungi, as well as the yeasts that are a vital part in bread making. More recently, other microbes have been directly utilized in cleaning up environmental contamination and some have been found and developed for agricultural applications. The advantages of direct use of an organism to do a specific task are great considering the reduced costs in development and production. Now, the question, given the relatively small number of microbes that have been domesticated, is it possible that there are still other previously undiscovered microbes that may have potential usefulness to mankind by direct application?

The most important and critical aspect of embarking on the endeavor of prospecting for new microbes should directly hinge upon having some ideas concerning the specific needs of society (industry/medicine/agriculture) that may drive the process. Ultimately, it seems as if the mind must be prepared to recognize uniqueness in a world filled with things that appear common. Thus, as we set out to find microbial novelty we are driven by a whole set of massive problems faced by humankind

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including rampant and deadly infectious diseases in humans, plants and domestic animals, environmental degradation, starvation, lack of drinkable water, waste build-up, and a myriad of others. These are some very strong and apparent drivers that can spark innovation in microbe discovery.

So where is it possible to find one or more unique microbes that may be useful in helping solve one or more societal problems? Outwardly, it appears that the search for novel microorganisms having potentially useful functions should center on higher organisms (biotopes) that they may inhabit. That is, to focus a search for microbes that may have developed a symbiotic or near symbiotic relationship with another life form. This approach has the potential of an immediate selection of microbes that are less toxic and thus more biologically compatible with higher life forms because of their constant and perhaps specific affiliation such an organism. Thus, it behooves the investigator to carefully study and select the potential biological sources before proceeding, rather than to have a totally random approach in selecting the biotope to be studied. Careful study also indicates that biotopes that are subjected to constant metabolic and environmental interactions as well as ones existing in areas of enormous biodiversity should have even a greater likelihood of hosting greater genetic diversity in the microbial populations that they may support [20].

While microbes seem to be in almost every niche on our planet, it appears that some niches may be more productive than others in yielding novel and useful microflora.

It turns out that plants represent some of the most diverse and numerous biotopes on earth. It is estimated that there are between 300,000 and 500,000 different species. The vast majority of these organisms are capable of making their own food and structural forms by the virtue of photosynthesis. They also have a myriad of niches on, and in between their tissues which presents perfect protection for microbes. Those that cause disease are in the minority of plant-associated microbes. On the other hand, we are learning that virtually all plants are hosts to microbes and these are known as endophytes [2]. Bacon and White [2] give an inclusive and widely accepted definition of endophytes—‘‘Microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects’’. Endophytes are currently considered a wellspring of novel microorganisms as well as secondary metabolites offering the potential for medical, agricultural and/or industrial exploitation. Currently, endophytes are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of unique biological niches (higher plants) growing in so many unusual environments. Thus, it would appear that these biotypical factors could be important in plant selection since they may govern the novelty and biological activity of the products associated with endophytic microbes.

While the symptomless nature of endophyte occupation in plant tissue has prompted focus on symbiotic or mutualistic relationships between endophytes and their hosts, the observed biodiversity of endophytes suggests they can also be aggressive saprophytes or opportunistic pathogens. Both fungi and bacteria are the most common microbes existing as endophytes. It would seem that other microbial forms most certainly exist in plants as endophytes, but no evidence for them has yet been presented, e.g., mycoplasmas, and archaebacteria. The most frequently isolated endophytes are the fungi. It turns out that the vast majority of plants have not been studied for their endophytes. Thus, enormous opportunities exist for the recovery of novel fungal forms, taxa, and biotypes. Hawksworth and Rossman [11] estimated there might be as many as 1 million different fungal species, yet only about 100,000 have been described. As more evidence accumulates, estimates keep rising as to the actual number of fungal species. It seems obvious that endophytes are a rich and reliable source of genetic diversity and novel, undescribed species. Finally, in our experience, novel microbes usually have associated with them, novel natural products and processes. This fact alone helps eliminate the problems of dereplication in discovery.

Rationale for plant selection

It is important to understand the methods and rationale used to provide the best opportunities to isolate novel endophytic microorganisms. Since the number of plant species in the world is so great, creative and imaginative strategies must be used to quickly narrow the search for endophytes displaying bioactivity [20].

A specific rationale for the collection of each plant for endophyte isolation and natural product discovery is used. Several reasonable hypotheses govern this plant selection strategy and these are as follows:

1. Plants from unique environmental settings, especially those with an unusual biology, and possessing novel strategies for survival.
2. Plants that have an ethnobotanical history (use by indigenous peoples) that are related to the specific uses or applications of interest are selected for study. These plants are chosen either by direct contact with local peoples or via local literature. Ultimately, it may be learned that the healing powers of the botanical source, in fact, may have nothing to do with the natural products of the plant, but of the endophyte (inhabiting the plant).
3. Plants that are endemic, having an unusual longevity, or that have occupied a certain ancient land mass, such as Gondwanaland, are also more likely to have had millions of years to form symbiotic relationships with microbes.
4. Plants growing in areas of great biodiversity also have the prospect of hosting endophytes with great biodiversity.

Anatomy of a discovery

In the late 1990s, I was on a collecting trip in the jungles near to the Caribbean coast of Honduras one of the world's "hot spots of biodiversity" [16]. I was eventually led to the Lancetilla Botanical Garden near La Ceiba. It is not often that I ever make any plant collections in or around botanical gardens since they are usually located in metropolitan areas. The smog and pollution in these areas generally limits the frequency of the exploitation of plants by endophytes. However, to my amazement this garden was well removed from any major city. It was a great place to visit since many tropical plants from around the world had been planted there nearly a century before in order to learn which ones may be eventually used for agricultural purposes in that area. One modestly sized tree, not native to the new world, was introduced to me as *Cinnamomum zeylanicum*. I immediately seized the opportunity to sample and taste the bark of this plant since I had only known of it from botany books, yet had feasted on its delicious and wonderful flavor for my entire life. Small limb specimens were taken and placed in a plastic bag and brought back to Montana.

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 processed on a regular basis. These nasty creatures infest the bench tops and find Petri plates containing agar in which to take up residence. We have even found that they can easily navigate their way through a parafilm seal on a Petri plate and eventually crawl down to or drop to the agar surface. If they make their move on an agar plate sporting fungi one can never know that they are present unless the hyphae are carefully examined under a binocular microscope. However, if an infested culture is transferred to a liquid broth medium it will turn cloudy with bacterial growth overnight.

Thus, in order to eliminate this nagging mite problem, we treated all plant tissues with 95% ethanol and then flamed them and this treatment effectively killed all of the mites as well as surface contaminating microbes. The plates were handled in a biosafety hood that was free of any mites. Then, we decided to place the plates (free of mites) in a large plastic box making it difficult for the tiny animals to find their way from the untreated bench surfaces to the inside of the box and ultimately to the inside of the plates. After a few days, most plant specimens had sported endophytic fungal growth. The plates were incubated for another week until the colonies grew a bit larger for culture isolation and purification. When the lid of the box was opened a strange odor emerged. Plates were removed and the individual hyphae transferred to fresh plates of potato dextrose agar. After a day or two of incubation, no transferred endophyte grew except one. Had the placement of the endophytes in the large plastic box killed the endophytes by limiting oxygen availability? Should we continue to use the boxes as barriers to mites? Then, it became obvious that the one endophytic fungus (designated isolate 620) remaining alive was producing volatile antibiotics or volatile organic compounds (VOCs). When the original culture of this lone living endophyte was "sniffed" it seemed to be producing the same odor as that in the box when it was originally opened. The hypothesis that an endophyte can make volatile antibiotic substances with a wide range of biological activity was born. We had serendipitously discovered, that in the processes of trying to free our cultures of mites, an extremely useful VOC producing fungus with enormous biological potential.

A literature search revealed that although many wood inhabiting fungi make volatile substances, none of these possessed the biological activity of isolate 620 [13]. 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 [7]. Later, another report on Trichoderma showed the identity of the VOCs and pointed out that the inhibitory activity was associated with these compounds [22]. Thus, although some fungal species have been known to produce low concentrations of volatile substances, to date, none have been demonstrated to have wide ranging and potentially useful lethality to other microbes when compared to isolate 620.

Muscodor albus and its VOCs

Isolate 620 is a sterile (not producing spores) endophytic fungus possessing some interesting hyphal characteristics including coiling, ropyness, and right angle branching. The mycelia of the fungus on most media are whitish and suppressed (Fig. 1).

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 get initiate spore formation have failed. These include placement on various media including pieces of stems of the original host and tissues

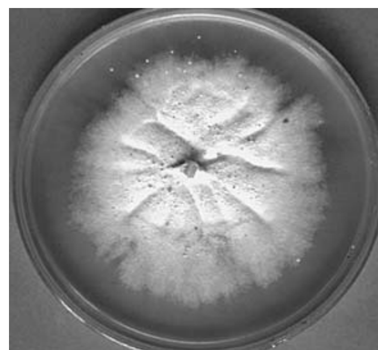

Fig. 1 A culture of isolate 620 or *Mucodor albus*

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

RT	Total area (%)	*M/z*	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	[a]	Ethanol	46
8:03	2.07	116	Propanoic acid, 2-methyl-ethyl ester	116
11:45	0.58	[a]	Propanoic acid, 2-methyl 2-methylpropyl ester	144
12:05	2.06	74	Isobutyl alcohol	74
12:50	22.24	[a]	1-Butanol, 3-methyl, acetate	130
14:57	1.53	[a]	Propanoic acid, 2-methyl, 3-methylbutyl ester	158
15:28	22.99	[a]	1-Butanol, 3-methyl-	88
16:08	0.29	138	[b]Furan, 2-pentyl-	138
18:53	0.29	142	[b]4-Nonanone	142
20:38	0.41	142	2-Nonanone	142
21:07	0.30	204	[b]Naphthalene, decahydro-4a-methyl-1-methylene-7—(1-methylethylidene)-, (4aR-trans)-	204
22:54	1.51	204	[b]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	[b]Cyclohexene, 4-(1,5-dimethyl-1,4-hexadienyl)-1-methyl-	204
25:20	3.63	204	[b]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	[b]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	[b]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.)] 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)-,[1R-(1.alpha.,7.beta.,8a.alpha.)] Common Name: Valencene	204
31:12	1.74	[a]	Acetic acid,2-phenylethyl ester	164
33:17	1.06	122	Phenylethyl alcohol	122
39:00	9.76	204	[b]Unknown	204

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

[a]No molecular-ion peak was observed in the spectrum of either the standard compound or the compound undergoing the analysis

[b]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

of other tropical plants. Therefore, in order to taxonomically characterize this organism, the partial regions of the ITS-5.8 rDNA were isolated and sequenced. The sequences were entered into GenBank as AF 324336 and AF 324337, respectively. Based on comparative sequence data the organism (isolate 620) was not identical, in this respect, to any other fungus whose DNA had been previously entered into GenBank, but it did show some relatedness to several *Xylaria* spp. including *X. arbuscula*, *X. longipes* and *X. mali*, at the 82–92% level [24]. Initially, unsuccessful attempts were made to identify the VOCs of isolate 620 using a gas tight syringe. Then, with the advent of the micro-extraction syringe (Supelco) technology we trapped and did GC/MS of the VOCs of this fungus [21]. The GC/MS analysis of the fungal VOCs showed the presence of at least 28 VOCs (Table 1). A control PDA plate also contained VOCs and these were represented by such compounds as ethylbenzene, bicyclo[4.2.0]octa-1,3,5-triene, benzene,1,3-dimethyl-, *p*-xylene, and styrene and they were subtracted from the analysis of the plates containing *M albus* [21]. The fungal VOCs represented at least five general classes of organic substance (lipids, esters, alcohol, ketones, and acids). Final identification of the VOCs was done by GC/MS of authentic compounds obtained from commercial sources or synthesized by us or others and compared directly to the VOCs of the fungus [21]. This is a critical step in the identification of VOCs since at times MS data can be misleading and result in inaccurate structural assignments. 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) [24] (Fig. 1).

Ultimately, artificial mixtures of the compounds were used in a biological assay system to demonstrate the relative activity of individual compounds. 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 identified being produced by the fungus [21]. We then examined each of the five general classes of VOCs in the bioassay test and each possessed some inhibitory activity with the esters being the most active [21]. Of these, the most active individual compound was 1-butanol, 3 methyl-, acetate. However, 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 [21]. Obviously, the antibiotic effect

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

Test microbe	Percent growth over control after a 2 days exposure to *M.albus*	Viability after 3 days exposure to *M. albus* culture	IC_{50} in artificial atmosphere for 2 days (μl/cc)	Percent growth (mm) over control in artifical atmosphere	Viability after 3 days exposure artificial atmosphere
Pythium ultimum	0	Dead	0.48 ± 0.01	0	Dead
Phytophthora cinnamoni	0	Dead	0.29 ± 0.06	0	Dead
Rhizoctonia solani	0	Dead	0.08 ± 0.02	0	Dead
Ustilago hordei	0	Dead	0.31 ± 0.09	0	Dead
Stagnospora nodorum	0	Dead	0.15 ± 0	0	Dead
Sclerotinia sclerotiorum	0	Dead	0.17 ± 0.05	0	Alive
Aspergillus fumigatus	0	Dead	0.41 ± 0.05	0	Alive
Fusarium solani	19.4 ± 0.28	Alive	1.13 ± 0.07	42.0 ± 2	Alive
Verticillum dahliae	0	Dead	0.3 ± 0	0	Dead
Cercospora beticola	17.5 ± 3.5	Alive	0.12 ± 0.15	8 ± 2	Alive
Tapesia yallundae	0	Dead	0.64 ± 0	0	Dead
Xylaria sp.	25 ± 0	Alive	0.41 ± 0.03	0	Alive
Muscodor albus	100 ± 0	Alive	0.6 ± 0	17.5 ± 7.5	Alive
Escherichia coli	0	Dead	[a]	0	Dead
Staphylococcus aureus	0	Dead	[a]	0	Dead
Micrococcus luteus	0	Dead	[a]	0	Dead
Candida albicans	0	Dead	[a]	trace	Alive
Bacillus subtilus	0	Alive	[a]	0	Alive

After exposure to *M. albus* gases, 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 (in this table). The amount of each positively identified compound used in the artificial mixture was obtained by applying the electron ionization cross-section (percent 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 (4×6 mm^2) 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 two 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 . 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/50 cc in order to obtain IC_{50}s. The percent growth over the control and viability were measured after exposure to 60 μl/50 cc. Viability was determined after the removal of the compounds at 3 days

[a]Not measured in this experimental design

of the VOCs of *M. albus* is strictly related to the synergistic activity of the compounds in the gas phase. We know very little about the mode of action of these compounds on the test microbes thus, this represents an interesting academic avenue to pursue in the future.

Using the bioassay test system, employing small plastic microcups and varying amounts of the artificial mixture of VOCs, it was possible to calculate the IC_{50} for each test microbe. We also compared the killing ability of the artificial mixture with that of the *M. albus* VOCs. 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 and 2) [21]. The artificial mixture nicely mimicked the effects of the fungus itself (Table 2)[21]. We have also learned that a minimum of three of the fungal compounds (naphthalene, propanoic acid and butanol, 3-methyl) can quite effectively mimic the killing activity of the fungal VOCs, thus certainly not all compounds in the fungal VOCs are necessary for biological activity [9].

The second isolate of *M. albus* to be recovered from nature as an endophyte is from nutmeg (*Myristica nutans*-family Myristaceae) in Thailand [17]. This isolate bears 98% homology 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. It too, by virtue of its molecular biology, is related to other xylariaceous fungi to the same degree as isolate 620. The discovery of the second isolate 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 a plant family other than Lauraceae (*Cinnamomum* sp.) can serve as a host for *M. albus*. localized phenomenon in nature. The discovery also shows that a plant family other than Lauraceae (*Cinnamomum* sp.) can serve as a host for *M. albus*.

Recently, we have obtained seven new *M. albus* isolates from the Northern Territory of Australia [9]. 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 (Fig. 2) [9]. The host plants of these isolates were *Terminalia prostrata* (Combretaceae), *Kennedia nigriscans* (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 95 and 99% ITS-5.8S rDNA sequence similarity to *M. albus* 620, *M. roseus* and *M. vitigenus* [9]. 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 [1]. This isolate possesses unusual hyphae, a slime layer, and some VOCs not observed before in other *M. albus* isolates including tetrahydrofuran, 2-methyl furan; 2-butanone; aciphyllene, and large amounts of an unusual azulene derivative [1]. In addition, several new isolates of Muscodor-like organisms have been obtained in the jungles of central Venezuela and they are currently being more completely characterized using standard methods [9].

Two other species of Muscodor were found in Australia and Peru, respectively. These organisms were relatively easily obtained by the use of the VOCs of *M. albus* as the selection tool. Plant tissues were placed in the presence of the *M. albus* cultures and those microbes emerging were either close or more distant relatives of this fungus. *M. roseus* produces a reddish mycelium and has about the same biological activity as the original isolate−620 [23]. Then, *M. vitigeneus* was obtained from *Paullinia paullinoides*, (Sapindaceae) a small vine, growing in the upper Amazon region of Peru [5]. This organism makes only naphthalene as a VOC and its repellency towards a plant-associated insect was demonstrated [4].

Numerous other areas (representative) of the world have been examined for the presence of *Muscodor* spp. For instance, no volatile antibiotic producing endophytic fungi have been recovered from 20 plant species representing such families as Fagaceae, Podocarpaceae, Eucryphiaceae, and Myrtaceae in Western Australia and Tasmania. Likewise, several plant families including Burseraceae and Bombacaceae representing 21 different plant species on the exotic dry tropical island of Socotra, Yemen (Arabic sea) yielded no volatile antibiotic producing fungi. Furthermore, plants representing a wide range of families including Mimosaceae, and Rosaceae, growing in Israel, S. Utah, Hawaii (Kawai), Central Alaska, and Morocco (Atlas Mts), did not yield these fungi. Likewise, no *Muscodor* spp. were recovered from at least 25 different plant species growing in several locations in Patagonia (Chile).

There are several critically important facts about isolates of *M. albus* and *Muscodor* spp. in general.

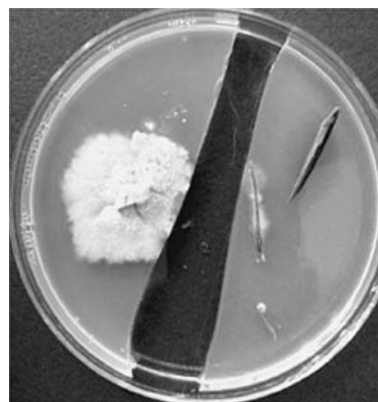

Fig. 2 The *M. albus* plate selection technique for obtaining new *Muscodor* spp. isolates. A culture of *M. albus* is on the left side of the plate and fungi are emerging from the plant materials on the right side of the plate

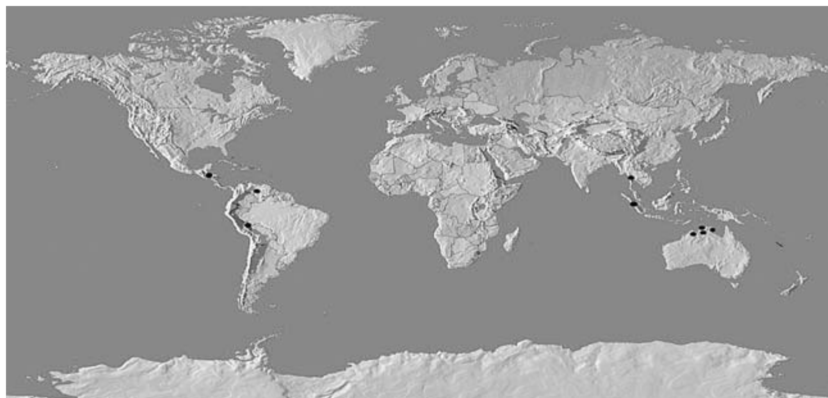

Fig. 3 Areas of the world in which *Muscodor* spp. have been recovered indicated as *small dots*

While each isolate obtained thus far shares features in common to each of the other isolates, it turns out that each one is biologically as well a biochemcially/chemically distinct. Each isolate has a ropy mycelium, is sterile, and is closely similar to all other isolates relative to its 18S r DNA sequence. The fungus, with limited observations, seems to inhabit wet tropical areas near equatorial regions of the earth (Fig. 3). Other VOC producing fungi pale to its biological activity by comparison [13].

An extremely important aspect on any of the *Muscodor* spp. relates to our total lack of information on the life cycle of the organism. Ultimately it will be important to understand how a sterile organism (producing no spores, having no fruiting structures, and thus having no apparent sexual stage) is able to pass from one generation of plants to the next and continue to exist in nature. Based on our past experience, *M. albus* definitely appears to have host preferences, i.e., several non-hosts (of non- related plant families) of *M. albus* 620 could not be successfully inoculated in order to establish a microbe–host interaction (Ezra and Strobel, unpublished). Outwardly, this suggests the existence of plant factors that control the outcome of any ingress, natural or artificial, the fungus may have to establish a relationship with the plant. The most likely hypothesis is that the fungus survives exclusively in a sterile state by establishing itself in vegetative tissues of the host including the structures associated with seeds including pods, enlarged hypanthiums, endocarps, stamens, styles, and other structures including seed coats. Then, the fungus grows into the newly developing plant tissues when the seed germinates and the newly developing plant is penetrated by the fungus as it becomes systemic in it. The life cycle is completed when the seed pod or seed coat is subsequently invaded by the fungus only to be perpetuated when the newly formed seed falls from the plant and is moved to new locations.

Physiological aspects of VOC production

The composition of the medium greatly influences the quality and effectiveness of the VOCs emitted by *M. albus* [8]. For instance, a sucrose enriched medium primarily yielded 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 [8]. A mixture of propanoic acid, 2-methyl-, and several related esters were associated with the elevated bioactivity of these fungal support media. An artificial mixture of the volatile compounds emitted by the *M. albus* on the various growth media mimicked the effects of the natural volatiles in the bioassay test system. Both IC_{50} and IC_{100} values, for each test organism, were generally the lowest for the artificial test mixture representing the atmosphere of the fungus grown on PDA [8].

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* [10]. This on-line technique is fast, accurate and provided data at the detection limits of ppb. The PTR-MS instrument ionizes organic molecules in the gas phase through their reaction with H_3O^+, forming mostly MH^+ molecules (where M is the neutral organic molecule), which can then be detected by a standard quadrupole/multiplier mass analyzer. This process can be run on real air samples without dilution, since the primary constituents of air (nitrogen, oxygen, argon, and carbon dioxide) have a proton affinity less than water and thus are not ionized. Most organic molecules (excepting alkanes) have a proton affinity greater than water and are therefore ionized and detected. A further advantage of PTR-MS is by experimentally determining the reaction time, the amount of H_3O^+ present, and the theoretical reaction rate constant for a proton transfer reaction and ultimately the absolute concentration of constituents in a sample can be quantified. This technique seems never to have been used to study VOCs of microbes [10]. An enormous advantage of PTR-MS is that it can be run on line while yielding data on the concentrations of specific ions of interest continuously.

Critical signature ions associated with only the masses at m/z 103, m/z 117 and m/z 131 are sufficiently unique and correlated directly to the size and biological activity of the *M. albus* culture. Thus, the emission profiles for m/z 103 (propanoic acid, 2-methyl, methyl ester), and m/z 131 (isoamyl acetate) were monitored as a function of drift tube temperature. 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 decreased gas production occurring at higher temperatures [10]. Temperature dependent enzyme mediated reactions in *M. albus* probably control the volatile emission rate from the fungus itself. 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 PDA. This is consistent with the observations showing VOC production and its relationship to a carbohydrate source [8]. The PTR-MS technique was also applied to soils containing *M. albus* along with the plant pathogen *Pythium ultimum*, and it was possible to successfully monitor VOC production in situ and 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.

"Mycofumigation" with *Muscodor albus*

The VOCs of *M. albus* kill many of the fungi and bacteria that affect plants, people and even buildings [1, 21](Table 2). Recently, some nematode and arthropod species have been shown to be affected by the VOCs of *M. albus* (Jacobsen, MSU and Agraquest, unpublished). Furthermore, in limited lab test trials, rodents exposed to the VOCs of *M. albus* were unaffected and larger trials are underway (AgraQuest). Thus, there appears to be many practical applications of *M. albus* and its VOCs in agriculture, medicine and industry. The term "mycofumigation" has been applied to the practical aspects of this fungus [21]. 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 [21]. An additional extremely important use of this technology is for the treatment of fruits in storage and transit [14]. Soil treatments have also been effectively used in both field and green house situations [12, 15, 19]. In these cases, soils are pretreated with a *M. albus* formulation in order to preclude the development of infected seedlings.

AgraQuest, an agricultural biotech company, of Davis, Calif., is developing *M. albus* for numerous agricultural applications. Recently, conditional EPA approval was received to use *M. albus* as a product. The USDA has given unconditional approval for its use in agricultural applications. It is anticipated that the company will have a product available to the American public in 2006. In the meantime, medical and industrial applications of *M. albus* are also being investigated. The concept of mycofumigation, for a multitude of applications, has the potential to replace the use of otherwise 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 cease in 2007 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.

There are still other more unconventional uses for *M. albus*. For instance, Phillips Environmental Products of Belgrade Montana announced in June, 2005, that it will begin using *M. albus* in its product line. Since *M. albus* kills *E. coli* and other bacteria found in human wastes an appropriate technology has been developed to promote the growth of *M. albus* in this situation. The fungus is being produced in 100 lb lots using a fermentation broth medium containing the fungus as inoculum over a solid-state medium that utilizes pasterurized grain. This fungus will be placed in their biodegradable WAG bags that are sold to trap human wastes. *M. albus* also reduces the odor associated with human and animal wastes (full US Patent filed). Phillips makes a portable toilet containing a WAG bag that is being used by military organizations, those finding themselves in emergency situations and people in transit or on sporting outings. In addition, their product line is being used in many National Parks in the USA and abroad. Finally, In June, 2005, the US Patent Office allowed virtually all claims on *M. albus* (US patent 6,911,338) including all members of this novel fungal genus.

Other VOC producing fungi

Using *M. albus* as a selection tool at least one non-Muscodor VOC producing endophyte has been isolated by us (Fig. 2). This endophyte was identified as *Gliocladium* sp. and this organism has taxonomic similarity/identity to *Trichoderma* spp. [18]. As is generally true with *Trichoderma*-like organisms, its VOCs possess a wide range of inhibitory biological activity, but lethality to target organisms is not generally observed [7, 13]. This organism is a *Gliocladium* sp. whose major recoverable VOC is [9] annulene, a compound not previously seen as a natural product. The isolate was obtained from "Ulmo" (*Eucryphia cordifolia*-Eucryphiaceae—a Gondwanaland plant family). Annulene was unequivocally shown to be the major VOC in *Gliocladium* sp. [18]. *Trichoderma* spp. per se do not produce the proper VOCs to have wide ranging use of their VOC-produced-antimicrobial effects. Thus, *Trichoderma* sp. and its relatives, although possessing biological activity, are not active enough 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 *Verticillum dahliae* [18]. What, however, is most interesting about [9] annulene is that it is explosive and was used during WWII as rocket fuel [18]. By itself it is quite biologically active [18].

Pending questions about the VOC producing fungi

Obviously, because of the impressive biological activity of *M. albus* and its VOCs, as well as its potential for a myriad of practical applications and its novelty, the fungus should be more fully studied relative to its location and role in nature. Information relating to the distribution, ecology, chemistry, and biology of this organism is needed. Knowledge of its host preferences 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 and it could find still more applications and direct uses. This idea may be exemplified by the direct inoculation of plants including those used in agriculture and forestry for their protection against insects and diseases. In addition, it is extremely important to possess information on the distribution, life cycle and other aspects of the chemistry of this organism. It may be possible to learn what factors govern the biology and interactivity of *Muscodor* spp. with higher plants and other organisms and it may find use in many other ways. Such information will have broad implications for the discovery and development of other rainforest microbes.

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References

1. Atmosukarto I, Castillo U, Hess WM, Sears J, Strobel G (2005) Isolation and characterization of *Muscodor albus* I-41.3s, a volatile antibiotic producing fungus. Plant Sci 169:854–861
2. Bacon CW, White JF (2000) Microbial endophytes. Marcel Dekker , NY
3. Bull AT (2004) Microbial diversity and bioprospecting. ASM press, Washington, DC
4. Daisy B, Strobel G, Ezra D, Castillo U, Baird G, Hess WM (2002) *Muscodor vitigenus* sp.nov. an endophyte from *Paullinia paullinoides*. Mycotaxon 84:39–50
5. Daisy B, Strobel G, Ezra D, Castillo U, Ezra D, Sears J, Weaver D, Runyon J (2002) Naphthalene, an insect repellent, is produced by *Muscodor vitigenus*, a novel endophytic fungus. Microbiology 148:3737–3741
6. Demain A (1981) Industrial microbiology. Science 214:987–995
7. Dennis C, Webster J (1971) Antagonistic properties of species-groups of *Trichoderma*. 11. Production of volatile antibiotics. Trans Br Mycol Soc 57:41–48
8. Ezra D, Strobel GA (2003) Effect of substrate on the bioactivity of volatile antimicrobials emitted by *Muscodor albus*. Plant Sci 165:1229–1238
9. Ezra D, Hess WH, Strobel GA (2004) New endophytic isolates of *M. albus*, a volatile antibiotic-producing fungus. Microbiology 150:4023–4031
10. Ezra D, Jasper J, Rogers T, Knighton B, Grimsrud E, Strobel GA (2004) Proton-transfer reaction—mass spectroscopy as a technique to measure volatile emissions of *Muscodor albus*. Plant Sci 166:1471–1477
11. Hawksworth DC, Rossman AY (1987) Where are the undescribed fungi? Phytopathology 87:888–891
12. Jacobsen BJ, Zidack NK, Strobel GA, Ezra D, Grimme E, Stinson AM (2004) Mycofumigation with *Muscoder albus* for control of soilborne microorganisms. IOBC WPRS Bull 27:103–113
13. McFee BJ, Taylor A (1999) A review on the volatile metabolites of fungi found on wood substrates. Nat Toxins 7:283–303
14. Mercer J, Jimenez JI (2004) Control of fungal decay of apples and peaches by the biofumigant fungus *Muscodor albus*. Post Harvest Biol Tech 31:1–8
15. Mercier J, Manker D (2005) Biocontrol of soil-borne disease and plant growth enhancement in green house soilless mix by the volatile-producing fungus. *Muscodor albus*. Crop Protect 24:355–362
16. Mittermeier RA, Meyers N, Gil PR, Mittermeier CG (1999) Hotspots: Earth's biologically richest and most endangered ecoregions. Toppan Printing Co, Japan
17. Sopalun K, Strobel GA, Hess WM, Worapong J (2003) A record of *Muscodor albus*, an endophyte from *Myristica fragrans*, in Thailand. Mycotaxon 88:239–247
18. Stinson AM, Ezra D, Hess WM, Sears J, Strobel GA (2003) An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. Plant Sci 165:913–922
19. Stinson M, Zidack NK, Strobel GA, Jacobsen B. (2003) Mycofumigation with *Muscodor albus* and *Muscodor roseus* for control of seedling diseases of sugar beet and verticillium wilt of eggplant. Plant Dis 87:1349–1354
20. Strobel GA, Daisy B (2003) Bioprospecting for microbial endophytes and their natural products. Microbiol Mol Biol Rev 67:491–502
21. Strobel GA, Dirksie E, Sears J, Markworth C (2001) Volatile antimicrobials from a novel endophytic fungus. Microbiology 147:2943–2950
22. Wheatly R, Hackett C, Bruce A, Kundzewicz A (1997) Effect of substrate composition on production of volatile organic compounds from *Trichoderma* spp. inhibitory to wood decay fungi. Int Biodeter Biodeg 39:199–205
23. Worapong J, Strobel GA, Daisy B, Castillo UF, Baird G, Hess WM (2002) *Muscodor roseus* anam. sp. nov., an endophyte from *Grevillea pteridifolia*. Mycotaxon 81: 463–475
24. Woropong J, Strobel GA, Ford EJ, Li JY, Baird G, Hess WM (2001) *Muscodor albus* anam. nov. an endophyte from *Cinnamomum zeylanicum*. Mycotaxon 79:67–79