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Authors: Gary A. Strobel, Amy Ericksen, Joe Sears, Jie Xie, Brad Geary, and Bryan Blatt

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Urnula sp., an Endophyte of *Dicksonia antarctica*, Making a Fragrant Mixture of Biologically Active Volatile Organic Compounds

Gary Strobel¹ · Amy Ericksen² · Joe Sears³ · Jie Xie⁴ · Brad Geary⁵ · Bryan Blatt²

Abstract *Urnula* sp. was isolated as an endophyte of *Dicksonia antarctica* and identified primarily on the basis of its ITS sequence and morphological features. The anamorphic state of the fungus appeared as a hyphomyceteous-like fungus as based on its features in culture and scanning electron microscopy examination of its spores. On potato dextrose agar (PDA), the organism makes a characteristic fragrance resembling peach pie with vanilla overtones. A GC/MS analysis done on the volatile organic compounds (VOCs) of this organism, trapped by carbotrap methodology, revealed over 150 compounds with high MS matching quality being noted for 44 of these. Some of the most abundantly produced compounds included 4-decene, tridecane, 2-decene (E), 2-dodecene, (Z,E)-alpha-farnesene, butanoic acid, pentyl ester, and 1-hexanol,2-ethyl. In addition, vanillin, methyl vanillin, and many other fragrant substances were noted including isomenthol, pyrazine derivatives, and 3-decanone. In split plate bioassay tests on potato dextrose agar (PDA), *Botrytis cinerea*, *Ceratocystis ulmi*, *Pythium ultimum*, *Fusarium solani*, and *Rhizoctonia solani* were inhibited at levels of 24 to 50% of their normal growth on this medium. Bioreactors supporting fungal growth on 50 g of beet pulp

waste, using stainless steel carbotraps, yielded over 180 mg of hydrocarbon-based products collected over 6 weeks of incubation. Similarly, because this organism is making one of the largest sets of VOCs as any fungus examined to date, producing many compounds of commercial interest, it has enormous biotechnical potential. The role of the VOCs in the biology and ecology of this endophyte may be related to the antimicrobial activities that they possess.

Keywords Endophyte · 4-Decene · Hydrocarbons · Fragrance · Pathogenic fungi · Biofuel

Introduction

It appears that all plants have the potential of hosting endophytic microorganisms. In fact, these microbes may be considered the core of the plant microbiome [1]. While they do not cause any overt signs or symptoms of their appearance in the plant, endophytes may exist as symbionts providing the host with some form of protection from various environmental insults such as insect or pathogen attack or potential environmental stresses [2]. Most of the evidence for the involvement of secondary products of the endophyte in the microbe/plant interaction is circumstantial. Nevertheless, it appears that these microbes, as a biological group, continually seem to be proven as exciting novel sources of natural products [3]. Thus, unlike freely living microbes which may produce principles that are toxic to eukaryotes, this prospect seems less likely with endophytes. This may be the case since the microbe lives in association with a higher organism, and the presence of toxic factors from the microbe would certainly limit the prospects of an endophytic association.

While a plethora of natural products with varying activities and biological promise have been isolated from endophytes in the recent past, most of these compounds have been chemically characterized as solids obtained after extraction of the culture

✉ Gary Strobel
uplgs@montana.edu

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

² Endophytics, 920 Technology Blvd, Bozeman, MT 59718, USA

³ Center for Lab Services/RJ Lee Group, 2710 North 20th Ave., Pasco, WA 99301, USA

⁴ State Key Laboratory of Silkworm Genome Biology, College of Biotechnology, Southwest University, Chongqing 400715, People's Republic of China

⁵ Department of Plant and Wildlife Sciences, Brigham Young University, Provo, UT 84602, USA

fluid [3–5]. The discovery of *Muscodor albus*, an endophyte of cinnamon, revealed yet another potentially interesting trait of endophytic microbes. This fungus produces a potent mixture of volatile organic compounds (VOCs) that either kill or strongly inhibit a wide range of pathogenic microorganisms [6]. Since its initial discovery, at least 15 other species of this organism have been discovered and all have in common the ability to produce biologically active (VOCs) [7, 8]. Interestingly, many of the other strains of *Muscodor* were obtained using the original *M. albus* isolate as an agent to screen or eliminate unwanted microbes in the isolation process [9, 10]. On occasion, however, some fungi are able to withstand the lethality of the *M. albus* VOCs and have been recovered from culture using *M. albus* as a selection source. Some of the fungi recovered from such sources have proven both interesting and worthy of investigation since many of them also make VOCs with biological activity and fuel potential. Among these have been *Ascocoryne sarcoides*, *Hypoxyton* sp. (*Nodulisporium* sp.), and others [11–13].

Since the original discovery of *M. albus*, we have constantly been aware of potential VOC components in endophytic cultures because of their biological activities and the intrinsic value of the volatile compounds. It is surmised that tropical and semi-tropical areas of the world possess still other interesting endophytes because of the high moisture and the biological diversity that exists there. For this reason, a plant collection was made in south coastal Australia especially in the limited areas in which tree ferns, i.e., *Dicksonia antarctica*, and other endemic plant species abound. The search yielded an endophyte that appears to be an *Urmula* sp. that makes a plethora of VOCs, and the mixture possesses biological activity. While certain *Urmula* spp. make secondary products that are antagonistic to fungi, there are no reports of this fungal genus making bioactive VOCs [14, 15]. The imperfect stage of the fungus is hyphomycetous like; in that, it makes large conidia in culture on sterile carnation leaves. Thus, this report describes the fungal endophyte, the composition of its VOC mixture, and the biological activities associated with the VOCs. The report also points out the economic potential of some of the *Urmula* sp. VOCs and the importance of these individual compounds to a number of industries including foods, flavoring, fragrance, bioenergy, and synthetic chemistry. The role of this fungus in the environment can only be surmised at this point, but the availing circumstantial evidence strongly points to a definite role of fungal VOCs in the host/plant relationship leading to a potential protection device to the host plant.

Methods

Isolating the Endophyte

A sample of the host plant (*D. antarctica*) (38° 45' 245" S, 143° 33' 329" E) was obtained by clipping terminal stem pieces (ca. 1 × 20 cm) from readily accessible portions of

the plants. The harvested specimens were surface treated, and internal tissue pieces were set out on plates of water agar (WA) and glycerol-arginine medium (GAM) [12]. The tissue pieces were incubated at room temperature for several days. Visible fungal growth from the tissue samples were picked as hyphal tips and sub-cultured and eventually transferred to PDA plates. The endophyte of interest was designated as En-22. It possessed an extremely pleasant fragrance of a freshly baked “peach pie” with a vanilla overtone. The organism was not found in any other plant specimen collected in this area including such species as *Banksia marginata*, *Phebalim squameum*, *Leptospermum juniperium*, *Solanum aviculae*, and *Pimelea axiflora*. The organism En-22 was stored at –70 °C in the living Mycological Collection of the Department of Plant Sciences at Montana State University as entry 2409. Long-term storage of these rain forest endophytes is best done on doubly autoclaved, thoroughly wetted, and leached (sterile) barley seeds at –70 °C.

Scanning Electron and Light Microscopy

The fungus, En-22, was viewed and photographed using scanning electron microscopy (SEM). Scanning electron microscopy was done in order to determine the morphological features of the endophyte. The fungus was grown on γ -irradiated carnation leaves on water agar plates that were incubated for 1 month at 23 °C. The En-22 samples (mycelium and spores) were placed in ethanol with further drying to a critical point and coated with gold for SEM. The instruments used were an FEI-XL 30 SEM-FEG on high-vacuum mode and an Everhart-Thornley detector [12].

ITS-Based Phylogenetic Analysis

Ribosomal gene sequence of ITS-5.8 S ribosomal gene sequence was used for En-22. Two samples of En-22 were used for analysis. Samples of the fungus were taken at both 3 and 5 weeks after growing on PDA [16]. Prepman Ultra Sample Preparation Reagent was used for DNA template preparation using instructions from the manufacturer (Applied Biosystems, USA). Regions of ITS that were amplified in the polymerase chain reaction (PCR) process were with the universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3'). The program for ITS region amplification had the following conditions: lid temperature of 103 °C; initial denaturation done for 3 min at 94 °C; annealing and Taq operation cycle done for 35 times with 94 °C for 15 s, 50 °C for 30 s, and 72 °C for 45 s; followed by the final lengthening process at 72 °C for 5 min. The reaction mixture that was used in the PCR amplification was 20 μ l with 11.8 μ l of autoclaved, distilled water, 2.1 μ l of $\times 10$ PCR buffer with 1.5 mM MgCl₂, 1.1 μ l of 2 mM dNTP mix, 1.7 μ l of REDTaq DNA polymerase (1 U), 1.1 μ l of the

DNA template, and then 1.1 μ l the primer ITS4 (10 pmol) with primer ITS5 (10 pmol) or with the primer ITS1 (10 pmol). After amplification, 5 μ l of the product was used to confirm PCR in 1% (w/v) agarose gel. Single bands of amplified products were visible under ultraviolet fluorescent lighting. PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions and sequenced by the University of California–Berkeley [16]. After sequences had been run, GenBank by BLASTN program was used to determine relatedness in reference to other organisms [17]. Relevant sequences were downloaded and the evolutionary position of the organism was ascertained by constructing a phylogenetic tree [18, 19]. Both samples revealed identical sequences. Phylogenetic tree of En-22 based on ITS-5.8S ribosomal DNA (rDNA) was constructed by MEGA6 using the neighbor-joining (NJ) method with the Kimura two-parameter substitution model [18]. The resulting tree was evaluated with 1000 bootstrap replications.

GC/MS Analyses of Fungal VOCs

Gas (VOC) analyses was done on a 13-day-old culture of the fungus grown on PDA. First, a baked “solid phase microextraction” SPME fiber (Supelco) consisting of 50/30 divinylbenzene/carboxen on polydimethylsiloxane on a StableFlex fiber was inserted into a small hole made in the side of the Petri plate containing the fungus and exposed to the fungal VOCs for 45 min [6]. The fiber was then inserted into the splitless injection port of a Hewlett Packard 6890 gas chromatograph containing a 30 m $\times$ 0.25 mm I.D. A 30-s injection time was used to introduce the sample fiber into the GC, ZB Wax capillary column with a film thickness of 0.50 μ m. The column was temperature programmed as follows: 30 $^{\circ}$ C for 2 min increased to 220 $^{\circ}$ C at 5 $^{\circ}$ C min⁻¹. The carrier gas was ultrahigh-purity helium, and the initial column head pressure was 50 kPa. Prior to trapping the volatiles, the fiber was conditioned at 240 $^{\circ}$ C for 20 min under a flow of helium gas. The gas chromatograph was interfaced to a Hewlett Packard 5973 mass selective detector (mass spectrometer) operating at unit resolution. The MS was scanned at a rate of 2.5 scans per second over a mass range of 35–360 amu. Data acquisition and data processing were performed on the Hewlett Packard ChemStation software system. Tentative identification of the compounds produced by the fungus was made via library comparison using the NIST database, and all chemical compounds described in this report use the NIST database chemical terminology. Compounds that are listed have at least an 70% quality score indicating a high likelihood of product identity, but most are in the 85–95% range. The control experiment was also conducted in which VOCs were trapped in the PDA plate not supporting fungal growth. In all such experiments, the individual compounds obtained in the control were subtracted from the set of

compounds found in the treatment. Authentic standard compounds (Sigma-Aldrich, USA), when available, were also subjected to the same GC/MS analysis, and their relative retention times and mass analyses were compared to those of the fungal samples and these indicated as such on the tables.

Now, in the case of the analysis of VOCs having been trapped in the carbotrap experiment, the SPME fiber was inserted, through the septum in the top of the vial directly into the air space in the 30-ml trapping vial. The fiber had a 45-min exposure to the VOCs in the vial [20]. The control consisted of VOCs trapped in a bioreactor treated in the exact same manner but not having been inoculated with the fungus. Then, any compound appearing the control GC/MS analysis was subtracted from the carbotrap analysis of the bioreactor possessing the fungus.

VOC Analyses in Carbotrap Experiments

In an attempt to measure VOC production of the fungus and identify as many as possible of the VOCs being produced, a bioreactor with En-22 was set up in a bioreactor with a solid state configuration. Initially, 50 g of dry shredded beet pulp (Standlee, USA) was hydrated with tap water and left to stand for 0.5 h until expansion of the pulp occurred. Beet pulp was selected since it is heavily enriched source of simple as well as complex carbohydrates, minerals, and proteins, and it is easily compatible with a solid state bioreactor. It is also an agricultural waste that is in plentiful supply and quite inexpensive. The beet pulp was thoroughly drained and then transferred into a 2-l bioreactor having wire mesh inserted between layers of the pulp (2–4 cm deep) for air and VOC transfer (Fig. 1). The bioreactor was autoclaved on a slow cycle for 20 min along with other equipment needed for the experiment. A flask containing the fungus in 25 ml of PD broth (agitated at 130 rpm at 22 $^{\circ}$ C for 8 days) was used as the inoculum. The inoculum was evenly poured over the beet pulp in the bioreactor. Metal tubing was inserted to the bottom of the bioreactor, and through it, filtered air (50 vent, 0.2 μ m PTFE) was passed through autoclaved, distilled, water at the rate of 25 psi, for optimal aeration and humidification. The VOCs were trapped via an air outlet for the system which was passed through a stainless steel column containing carbon black material that had been previously sized and conditioned (Fig. 1) [20]. A control bioreactor was also set up but it did not contain fungal inoculum, and its gas content was trapped and subtracted from the bioreactor containing the fungus.

Desorption of the stainless steel columns occurred weekly. Each time, appropriate water desorption steps were done at 60 $^{\circ}$ C for 10 min until a constant weight of the column was obtained [20]. Then, the column temperature was ramped rapidly to 300 $^{\circ}$ C for 1 h and the final weight of the cooled

Fig. 1 The bioreactor setup used to do solid state fermentation, with 50 g of sugar beet pulp on which *Urnula* sp. was grown. The VOCs of the fungus were trapped in the stainless steel gas trap on the left. The mycelium is at the 3-week growth stage. Details for all conditions used are described in the “Methods” section

column was recorded [20]. Trapping of the effluent was done in a glass vial trap cooled with dry ice in acetone. The effluent was subjected to GC/MS using the SPME technique.

Bioactivity of En-22

Bioactivity testing of volatiles from En-22 fungus was done on 13-day-old cultures against various fungi. The fungus (En-22) was grown on one half-moon side of a PDA plate with a 2-cm section removed from the center leaving the other half moon for the test organism [6]. Fungal inocula (1-mm³ sections on an agar plug) were placed on the test plate and hyphal measurements made after 30 h of incubation at 22 °C. At least six measurements of hyphal growth (from the edge of the agar

plug) were made in three separate trials, and the mean growth and standard deviation of the mean were calculated. Fungal test organisms, representing the controls, were grown on PDA without the En-22. A number of well-selected, plant-associated test organisms (mostly plant pathogenic fungi) were used in the bioassays, and they included *Pythium ultimum*, *Cercospora beticola*, *Sclerotinia sclerotiorum*, *Fusarium solani*, *Trichoderma viridae*, *Botrytis cinerea*, *Aspergillus niger*, *Ceratocystis ulmi*, and *Rhizoctonia solani*.

Results and Discussion

Classification of Endophytic Isolate En-22 as an *Urnula* sp.

When grown on PDA, the fungus produced a whitish, creamy aerial mycelium. No fruiting structures were ever observed when this fungus was grown on a range of substrates, i.e., pycnidia, ascervuli, or perithecia. However, macroconidia and microconidia were noticed when the organism was grown on γ -irradiated carnation leaves placed on water agar, but distinctive conidiophores were not obvious. The macroconidia were 10–11 $\times$ 22–27 μ m in size, and single-celled, smooth, straight ellipsoid with an obtuse apex and a base abruptly tapered to a truncated scar (Fig. 2). The microconidia ranged from 3 to 8 $\times$ 1–1.5 μ m. Both spore types are nearly identical in all respects to those formed by *Cryptosporiopsis* spp. (the imperfect stage (anamorph) of *Pezicula* spp. (Pezizales), but in this case, the conidiomata were never observed [21].

The ITS-5.8S rDNA sequence of En-22 was obtained by using universal primers ITS1 and ITS4 according to the methods described, followed by submission to GenBank with an assigned deposit number of KT358892. BLAST analysis of the ITS-5.8 S revealed that En-22, twice repeated, was most phylogenetically similar (96% level and 77% coverage) to the perfect stage fungus *Urnula campylospora* (accession numbers JX669831 and JX669830) with an *E* value of 0 (Fig. 3). Based on the BLAST results, several fungal strains were used in a comparative phylogenetic analysis of En-22 strain, and

Fig. 2 Scanning electron micrographs of the imperfect stage fungus En-22. The microspores are pictured on the left and a macrospore is shown on the right. The bar (left) is equal to 3 μ m and the bar (right) equals 4 μ m

Fig. 3 Phylogenetic tree of EN-22 based on ITS-5.8S rDNA. The tree was constructed by MEGA6 using neighbor-joining (NJ) method with Kimura two-parameter substitution model. The resulting tree was evaluated with 1000 bootstrap replications

they were *Plectania* spp., *Pseudoplectania nigrella*, and *Urnula* spp. Meanwhile, *Geotrichum* sp. was used as outgroup. The results showed that En-22 was close to *Urnula campylospora* but was not in the same minimum clade (Fig. 3). *U. campylospora* is an ascomycete and in the order *Pezizales* [22]. Most commonly, *Urnula* spp. have only *Conoplea* (*Strumella*) spp. reported for them as the anamorphic state [22]. Generally, *Conoplea* spp. produce dark echinulated spores on distinctive conidiophores; however, one species *Colletotrichum mangenotii* produces single-celled, dark brown, smooth-walled conidia that are about 50% of the size of the conidia of En-22 [23]. Thus, no *Conoplea* spp. are known whose morphological characteristics are identical to the observations made on the anamorphic state of En-22 described above. Therefore, from this perspective, it appears that the imperfect stage of En-22 is best defined as a hypomyceteous-like fungus, which is not referable to any current anamorphic fungal genus with certainty.

In the teleomorphic state, both the *Pezicula* spp. and *Urnula* spp. make disk-shaped fruiting bodies as they usually exist on rotting matter on the forest floor. In the case of En-22, the teleomorphic state was never observed. It is also the case that many *Ascomycetes* have more than one anamorphic state including *Pleospora* spp. for which eight distinct anamorphic genera have been described [22]. Thus, it is conceivable that another anamorph exists for *Urnula* spp. now including the one described above, which has morphological relevance to the *Pezizales* as per the observed micronidia and macroconidia being nearly identical to those of *Cryptosporiopsis* spp. It is

also entirely possible that En-22 represents an entirely new fungal genus, but until more observations can be made on this fungus, it will be tentatively assigned as an *Urnula* sp., primarily based on the ITS-5.8 S sequence information (Fig. 3).

It should also be noted that at times when a fungus is isolated from its endophytic state, it is difficult, if not impossible, to find the conditions that will entice the organism to make the morphological structures normally associated with its taxonomic description. At times, specific plant host compounds do control the development of fungal fruiting structures [24]. However, in the case of *Gliocladium roseum*, a VOC-producing fungus, it was isolated as an endophyte from *Eucryphia cordifolia* in a Patagonian forest [11, 25]. However, ITS-5.8 S sequence information indicated that the organism best fit the teleomorphic fungus *A. sarcoides* [26]. The later fungus was found in the field and was shown to make all stages of the fungus in culture including *Gliocladium*-like conidiophores, synnema, and the *Coryne*-like synnema [26]. However, the original isolate only made isolated single individual conidiophores that were identical to *G. roseum*, and it could not be enticed to produce other fruiting structures. The final taxonomic judgment on the fungus being *A. sarcoides* was not only based on ITS rDNA but also on the complete genome sequence plus extensive annotation of the genetic information, making it the best annotated endophytic fungus to date [27].

VOC Production by *Urnula* sp.

An examination of the VOCs of a 13-day-old culture of *Urnula* sp. growing on PDA revealed the presence of at least

10 VOCs (Table 1). The most relative abundant of these was 2-octene (Z) followed by ethanol, squalene, and cis-4-decene (Table 1). However, the GC/MS analysis performed on VOCs being emitted from the fungus on a PDA plate did not account for the kinds of substances observed by olfactory detection. Namely, the organism produces a pleasing fragrance of peach pie with vanilla overtones, suggestive of a VOC mixture more complex than that detected on the PDA plate (Table 1). To this end, the fungus was scaled up by growing it under solid state conditions on 50 g of hydrated sugar beet pulp with the VOCs trapped on the stainless steel column trap [20] (Fig. 1). The VOCs on the column were quantified weekly with a total production of 184 mg of hydrocarbon-like materials over the course of 42 days of incubation. The maximum production occurred between 24 and 32 days. At this time, the effluent from the column was trapped in a vial bathed in dry ice/acetone as it exited the column. The contents of the vial were then subjected to SPME and GC/MS. Over 150 compounds were detected in this mixture, after subtraction of those also found in the control bioreactor supporting no fungal growth (Fig. 4). And only about one third of the total substances eluting from the GC column could be identified with some certainty, mostly at the 90% level or better. Nevertheless, all of the compounds detected in the fungal VOCs on the PDA plate were also detected in the VOCs from the bioreactor (Tables 1 and 2). However, in the bioreactor, the most abundantly produced compound was 4-decene at 5.54 min (Table 2 and Fig. 3, arrow). There were a number of other hydrocarbons produced by this fungus including 2-decene (E), tridecane, 2-dodecene (Z), squalene, and a plethora of benzene derivatives (Tables 1 and 3). All of the hydrocarbons are of interest as potential fuel-related substances. However, other compounds such as isomenthol, the pyrazine derivatives, and vanillin and

methyl vanillin are important flavorings and fragrances (Table 2).

Biological Activity of the Fungal VOCs

Since the organism was making VOCs (by virtue of the odor of the culture), it was deemed important to determine if any biological activity was associated with these products. Using the standard VOC assay that was previously established for *M. albus*, this fungus was also assayed using 13-day-old cultures in the half-moon agar assay [6]. The majority of the plant pathogenic fungi that were tested were inhibited in mycelial growth between 24 and 50% with *P. ultimum*, *F. solani*, and *C. ulmi* being the most sensitive test organisms (Table 1). On the other hand, the organism with the least sensitivity was *T. viridae*, which is also one of the least sensitive organisms to the VOCs of *M. albus* [6]. The production of VOCs by the endophyte may add complexity to its interaction of the host which ultimately may affect the outcome of the relationship or the potential involvement of other microorganisms in the interaction [28]. Thus, since *Urnula* sp. possessed VOCs with antifungal activities, it can be strongly suggested that these compounds may have some role in the ability of the organism to compete with other microorganisms in its environment and in nature.

The Biologically Active Components of the VOC Mixture

There are over 150 VOCs being produced by *Urnula* sp. Unlike the VOCs of *Muscodor*, none of the target organisms are either 100% inhibited or even killed by the gas mixture (Table 2 and Fig. 4) [6]. An important consideration is which of the substances in the VOC mixture does possess biological

Table 1 GC/MS air-space analysis of the volatile compounds produced by *Urnula* sp. after 14 days of incubation at 23 °C on PDA

RT (min)	Relative response+	Possible compounds	Molecular weight	Quality
3.28	24.33	2-Octene (Z)	112	96
4.66	12.74	Ethyl alcohol ^a	46	86
5.67	3.26	2,4-Octadiene	110	95
7.17	5.26	Cis-4-decene	140	96
8.55	3.38	1-Propanol, 2-methyl ^a	74	90
29	1.01	Butylated hydroxytoluene	220	94
29.2	1.31	Phenylethyl alcohol ^a	122	97
29.85	5.08	2-Decene,(E)-	140	92
40.44	13.71	Squalene	410	94
41.83	4.19	6-Octadecenoic acid	282	97

Compounds present in a control PDA Petri plate have been subtracted from the data set, and compounds not having at least a 70% matching quality have not been shown

^a The retention time and MS spectrum closely matched or were identical to an authentic standard compound. Those compounds without a designated footnote have a MS spectrum that most closely matched the appropriate compound in the NIST database at the percent matching given in the last column

⁺ Relative response is indicative of compound abundance

Fig. 4 A SPME fiber GC/MS analysis of the VOCs resulting from the solid state fermentation of *Urnula* sp. Peaks at 2.51, 10.08, 10.77, 12.77, 13.07, and 17.90 also appeared in the control flask, whereas the remainder appears to be from fungal sources. *Y axis* = relative abundance. *X axis* = time. The most abundant compound detected in the mixture was 4-decene = arrow at 5.54 min. The peaks of high-percent match are specifically identified in Table 2

activity? It turns out that 1-butanol,3-methyl-acetate and a number of other esters, when tested alone or in combination, do possess moderate to weak inhibitory properties against many of the same test organisms used in this study [6]. This ester is present in the VOC mixture of En-22 along with others (Table 2). In addition, it has been noted that 1-butanol,3-methyl-acetate is a synergist [29]. It can interact with other organic substances such as small molecular weight acids and more complex compounds such as 6-pentyl-2H-pyran-2-one to reveal an enhanced biological activity over that with either test compound alone [29]. In another study, an *Oidium* sp. possessing lesser biological activity to that shown in Table 3, and producing only a mixture of esters, was completely enhanced in its VOC biological activity with the addition of propanoic acid—2 methyl [30]. Given the complex mixture of the VOCs of *Urnula* sp., until more is known about the chemistry of the mixture, it seems likely that the esters that are present are interacting with one or more other

components in the mixture to be exhibiting synergistic microbial inhibitory effects.

Biotechnical Aspects

It has not escaped our attention that the VOC mixture being produced by *Urnula* sp. on more complex substrates has economic potential. For instance, most abundant set of molecules were 4-decene, 2-decene (E), tridecane, and (Z,E)-alpha-farnesene. Each of these molecules, along with octane, 4-methyl, heptane, 2,3 dimethyl-, 2-octene, 3-octene, 2,4-octadiene, tetradecane, octane, 2–6-dimethyl, and tridecane plus all of the methylated benzene derivatives are hydrocarbons, and each (or combinations thereof) has potential as a fuel or fuel additive. In addition, some of the hydrocarbons have uses for synthetic chemistry (Table 2). The compound in the VOC mixture seems to be one of the most complex of any fungus studied to date in terms of both the kinds and numbers

Table 2 GC/MS air-space analysis of the volatile compounds produced by *Urmla* sp. after 14 days of incubation at 23 °C on PDA

RT (min)	Relative response+	Possible compound	Molecular weight	Quality (%)
1.32	4	Acetaldehyde ^a	44	86
1.52	1	Hexane, 2,4-dimethyl-	114	90
1.67	6	Octane ^a	114	95
1.72	3	Heptane, 2,4-dimethyl-	128	72
2.09	1	Octane, 4-methyl	128	87
2.16	150	3-Octene,(E)-	94	94
2.79	6	Octane, 2-6-dimethyl-	112	78
3.28	24	2-Octene (Z)	112	96
4.65	52	2-Pentanone, 3-methyl-	100	83
4.66	12	Ethyl alcohol ^a	46	86
5.26	51	2-Butenal ^a	70	91
5.54	1062	4-Decene	140	94
5.67	3	2,4-Octadiene	110	95
7.16	271	1-Butanol, 3-methyl-acetate ^a	130	78
7.57	119	Benzene, 1,3-dimethyl-	106	95
8.55	3	1-Propanol, 2-methyl ^a	74	90
9.86	24	Furan, 2-methyl-	82	91
9.91	91	Benzene, 1-ethyl-3-methyl-	120	92
10.41	128	Benzene, 1,3,5-trimethyl-	120	94
10.88	161	2-Dodecene,(Z)-	168	74
11.04	413	Butanoic acid, pentyl ester	158	83
12.06	754	Tridecane ^a	184	86
12.06	188	Pyrazine, 2,5-dimethyl-	108	93
14.26	182	Pyrazine, 2-ethyl-5-methyl-	122	81
14.66	177	Tetradecane ^a	198	96
16.40	161	3-Decanone	156	72
17.06	487	1-Hexanol, 2-ethyl-	130	90
19.42	550	(Z,E)-alpha-farnesene ^a	204	78
20.61	100	Isomenthol	156	96
22.73	173	Benzene, 1,2-dimethoxy-	138	95
25.67	27	Phenol, 2-methoxy-	124	95
26.00	167	4,7,7-Trimethylbicyclo-[3.3.0]-octan-2-one	166	90
26.66	111	Phenylethyl alcohol ^a	122	94
27.05	507	2-Decene,(E)-	140	91
27.76	223	Phenol, 2-methoxy-acetate	166	91
28.61	111	Phenol	94	94
29.06	223	Phenol, 4-ethyl-2-methoxy-	152	91
29.19	2	Benzene, 4-ethyl-1,2-dimethoxy	164	96
30.97	16	Phenol, 4-ethyl-2-methoxy- ^a	152	87
35.69	2	Benzaldehyde, 3,4-dimethoxy-(methyl vanillin)	166	95
37.01	5	Methanone, diphenyl	182	94
37.51	4	Benzaldehyde, 4-hydroxy-3-methoxy-(vanillin)	152	90
40.44	13	Squalene	410	94
41.83	4	6-Octadecenoic acid	282	97

Compounds present in a control PDA Petri plate have been subtracted from the data set, and compounds not having at least a 70% matching quality have not been shown

^a The retention time and MS spectrum closely matched or were identical to an authentic standard compound. Those compounds without a designated footnote have a MS spectrum that most closely matched the appropriate compound in the NIST database at the percent matching given in the last column

⁺ Relative response is indicative of compound abundance

of chemicals being produced, and this complexity is related to the complex substrate on which the fungus was grown (Table 1 vs Table 2). All of the esters on the list contribute to the fruity nature of the VOC mixture, but the overall culture odor is enhanced by virtue of all of the other VOC components; many of which are food and fragrance-related substances. Some interesting examples of valuable fragrance compounds include the pyrazines, which have intrinsic

woody/chocolate/coffee qualities. Squalene and 6-octadecenoic acid are used for cosmetics along with 2-phenylethyl alcohol. Vanillin and methyl vanillin are important flavoring substances imparting the vanillin fragrance to the fungal VOCs, while related compounds such as phenol,4-ethyl-2-methoxy have a clove-spicy aroma and phenylethyl alcohol has a floral fragrance. In addition, isomenthol has a woody smell and 1-hexanol,2-ethyl has a faint pleasant odor.

Table 3 Percent inhibition (means) and standard deviation for plant pathogenic fungi grown in the presence of 13-day-old culture of *Urnulla* sp. using the gas test assay as described in the “Methods” section

Test organism	Inhibition after a 30-h exposure plus standard deviation
<i>Cercospora beticola</i>	38.1 ± 18.7
<i>Sclerotinia sclerotiorum</i>	33.8 ± 4.6
<i>Fusarium solani</i>	42.1 ± 10
<i>Trichoderma viridae</i>	7.0 ± 2.5
<i>Botrytis cinerea</i>	30.3 ± 5
<i>Aspergillus niger</i>	28 ± 2.9
<i>Ceratocystis ulmi</i>	47 ± 10
<i>Rhizoctonia solani</i>	24 ± 12
<i>Pythium ultimum</i>	50 ± 5

Other compounds have use in industry such as methanone, diphenyl, which is used as a photo-initiator, and phenol, 2-methoxy, which is a precursor to vanillin- and eugenol-flavoring substances. Finally, the chemical nature of the remainder of the VOCs of *Urnulla* sp. is not known but surely will be eventually be shown to contain other important and valuable compounds, and this probably includes γ -decalactone, which is the peach lactone (Fig. 4). This seems likely since the VOC mixture contains a family of decane (decene) derivatives including the most prevalent compound in the VOC mixture which is 4-decene.

Interestingly, while microbes are known to make food, flavoring, and fragrance-related substances, it is usually the case that any selected microbe may carry out one step in a long engineering process to yield the final product [31]. And in order to acquire the final product, an appropriate precursor must be added to the medium on which the microbe is growing [31]. This is exemplified by the well-studied and industrial example of the conversion of ricinoleic acid to the peach lactone aroma γ -decalactone by yeasts such as *Sporidiobolus salmonicolor* [31]. Otherwise, there are some examples of microbes making an array of esters plus low molecular weight acids, alcohols, and terpenoids. Some of these are *Ceratocystis moniliformis* making a wide range of esters and the yeast *Kluyveromyces lactis* making de novo such flavor terpenes as citronellol, linalool, and geraniol [31]. *M. albus* and *Muscodor crispans* also make a wide range of esters, acids, ketones, and terpenes [6, 9, 10]. However, there seems to be no examples, to date, of a fungus that makes such a wide array of important flavoring, fragrance, and other volatile substances as does *Urnulla* sp. (Fig. 4 and Table 2). The production of this broad array of VOCs is undoubtedly related to the complex substrate on which it was grown.

The role of endophytic fungal VOCs in the host/microbe relationship has only ever been a point of conjecture. That is, because these bioactive VOCs mixtures are produced in

culture, does not necessarily mean that the same or nearly the same mixture is produced in vivo (within a host). No direct studies have ever been reported on the presence of fungal VOCs being detectable in the host/endophyte relationship, although there are a number of studies showing the importance of fungal VOCs in modulating the physiology of higher plants [32, 33]. It is certainly interesting to propose that these compounds provide some protection to a host plant via their production, dispersal, and bioactivity within the host. As such, they could then be claimed as playing a role in the natural microbial environment. Surprisingly, in this and a few other cases, the fungal VOCs have aroused interest and excitement in the biotechnology community since some of the molecules have economic potential such as those with fuel properties or food and fragrance substances. Furthermore, the endophytes themselves have the potential to be used directly in soil or other plant growth substratum as a source of volatile antimicrobials as with the case of the *Muscodors* [8].

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