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

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Muscodor crispans is a recently described novel endophytic fungus of *Ananas ananassoides* (wild pineapple) growing in the Bolivian Amazon Basin. The fungus produces a mixture of volatile organic compounds (VOCs); some of the major components of this mixture, as determined by GC/MS, are propanoic acid, 2-methyl-, methyl ester; propanoic acid, 2-methyl-, 1-butanol, 3-methyl-, 1-butanol, 3-methyl-, acetate; propanoic acid, 2-methyl-, 2-methylbutyl ester; and ethanol. The fungus does not, however, produce naphthalene or azulene derivatives as has been observed with many other members of the genus *Muscodor*. The mixture of VOCs produced by *M. crispans* cultures possesses antibiotic properties, as does an artificial mixture of a majority of the components. The VOCs of the fungus are effective against a wide range of plant pathogens, including the fungi *Pythium ultimum*, *Phytophthora cinnamomi*, *Sclerotinia sclerotiorum* and *Mycosphaerella fijiensis* (the black sigatoka pathogen of bananas), and the serious bacterial pathogen of citrus, *Xanthomonas axonopodis* pv. *citri*. In addition, the VOCs of *M. crispans* killed several human pathogens, including *Yersinia pestis*, *Mycobacterium tuberculosis* and *Staphylococcus aureus*. Artificial mixtures of the fungal VOCs were both inhibitory and lethal to a number of human and plant pathogens, including three drug-resistant strains of *Mycobacterium tuberculosis*. The gaseous products of *Muscodor crispans* potentially could prove to be beneficial in the fields of medicine, agriculture, and industry.

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INTRODUCTION

Micro-organisms have long served mankind by virtue of the myriad of enzymes and secondary products, including pharmaceuticals, that they make (Demain, 1981). However, only a relatively small number of microbes are used directly in human applications such as the production of bread, cheese and alcoholic beverages, and environmental clean-up activities. A more comprehensive search of the earth's niches may reveal novel microbes of practical value to human societies.

The diversity of microbial life and the niches in which microbes live is enormous and truly amazing, ranging from deep ocean sediments to the earth's thermal pools (Bull, 2004). A relatively untapped source of microbial diversity is the world's rainforests. Each plant supports a suite of micro-organisms known as endophytes (Strobel & Daisy, 2003). These organisms cause no overt damage to the

plants in which they live (Bacon & White, 2000). Furthermore, since so little work on these endophytes has been done it is suspected that untold numbers of novel fungal genera exist as plant-associated microbes (Smith *et al.*, 2008). The rationale for sampling rainforest species is that high plant biodiversity in the world's rainforest areas may be accompanied by high microbial diversity (Strobel & Daisy, 2003; Mittermeier *et al.*, 1999). Thus, we have begun a concerted search for novel endophytic microbes that may produce novel bioactive products or carry out processes that may prove useful at the organismal level. This report concentrates on the biological activity of the novel endophytic fungal isolate *Muscodor crispans* (Mitchell *et al.*, 2008).

While fungi producing volatile organic compounds (VOCs) have been isolated and studied chemically in the past 30–40 years, none have been found that have such a comprehensive spectrum of antimicrobial activity as that of *Muscodor albus* (Strobel *et al.*, 2001; McAfee & Taylor, 1999). *M. albus* was the first endophytic fungus demonstrated to produce VOCs with broad-spectrum killing

Abbreviation: VOC, volatile organic compound.

The GenBank/EMBL/DDBJ accession number for the partial rDNA sequence of *M. crispans* is EU195297.

properties against other microbes, including plant and human pathogens (Strobel *et al.*, 2001). This fungus produces no spores in culture, has a white mycelium, and releases a mixture of more than 25 VOCs (Strobel *et al.*, 2001). Other isolates of this fungus have been obtained in rainforests ranging from the upper Amazon countries to Indonesia, Thailand and Australia (Strobel, 2006). Thus far, each species of this genus examined has been found to produce one or more biologically active VOCs (Strobel, 2006). Likewise, each species produces no spores, and generally possesses a rope-like mycelium. The most recently isolated novel species of this genus is *M. crispans*. It differs markedly from all of the other species; among other differences, its hyphae have associated with them small and quite unique cauliflower-like protuberances (Mitchell *et al.*, 2008).

As is generally the case with each new isolate of *Muscodor*, there is a corresponding novel list of associated VOCs that it produces (Strobel, 2006). *M. crispans* follows this general observation. This report describes the identity of these compounds and their wide range of biological activities, with an emphasis on effects on pathogenic microbes that are responsible for serious health problems to both humans and plants.

METHODS

Fungal isolation and storage. The culture of *M. crispans* used in this study was obtained as an endophyte from a wild pineapple plant, *Ananas ananassoides*, growing in the Bolivian Amazon Basin (Mitchell *et al.*, 2008). Several small portions of *A. ananassoides* plant were recovered from the Heath river region and immediately brought back to Montana State University for analysis. At least five other plant species, including *Drosera montana*, *Roupala montana*, *Virola sebifera* and *Platino gigantica*, were also collected from the same area, but *M. crispans* was not recovered from any of these plants. Small pieces of tissue from the limbs were plated onto Petri plates, which were specially prepared as part of a selection system designed to isolate *Muscodor* spp. and/or fungi which produce gaseous antibiotic products (Ezra *et al.*, 2004). In this manner, *M. crispans* was isolated; it has been classified based on the similarity of its partial 18S rDNA sequence to other members in this group (Mitchell *et al.*, 2008). The fungus was stored by placing potato dextrose agar (PDA) plugs containing the *M. crispans* mycelium into 15% glycerol and storing the solution at -70°C , or by growing the fungus on sterile barley seed, drying the seed, and storing it at -70°C . *M. crispans* is deposited in the living Montana State University mycological collection as acquisition number 2347 (2/29/2008) and rDNA sequence information in GenBank as EU195297.

Bioassays for *M. crispans* VOCs against plant pathogens. The VOCs produced by *M. crispans* were tested for their microbial inhibitory activities using a relatively simple test, as previously described (Strobel *et al.*, 2001). A strip of agar (2 cm wide) in a standard PDA Petri dish was removed and *M. crispans* was inoculated and allowed to grow on one side of the plate for about a week. The test fungus or bacterium was then inoculated on the other side of the Petri dish, using small plugs of agar for the fungi. The bacteria and yeasts were streaked onto the agar (1.5 cm long). The plate was then wrapped with one piece of Parafilm and incubated at 23°C for 48 h. The effect of *M. crispans* on the growth of the test organisms was

determined first by verifying the presence or absence of growth where the inoculations had taken place. If growth was observed, measurements of the diameter in two locations of the fungal hyphae were taken. The biological activity of the VOCs on bacteria and yeasts was assessed by estimating the degree to which their growth was affected as percentage of growth on a control plate (Strobel *et al.*, 2001). If no growth was observed, the test organism was aseptically removed from the test plate and inoculated onto a fresh PDA plate after exposure to the VOCs in order to ascertain viability. All test fungi were sourced from the Montana State University mycological collection (Strobel *et al.*, 2001).

The age of *M. crispans* cultures that was most effective against test organisms was assessed by inoculating the fungus on PDA plates and growing it for specific time periods (0–28 days) before adding the test organism onto the plate. The test organism chosen for these tests was *Pythium ultimum*, due to its rapid growth and sensitivity to the fungal VOCs. The viability of *P. ultimum* was assessed after 48 h exposure to the VOCs. All tests were repeated at least three times with essentially identical results.

Bioassays with *M. crispans* against human-pathogenic bacteria. The fungus was grown for 3–5 days at room temperature on PDA in one quadrant of X-plates and incubated prior to inoculation with one or more test organisms (Fig. 1). Control plates were made at the same time of inoculation, using the same medium as used for the test plates (see below). *Staphylococcus aureus* ATCC 6538, *Salmonella choleraesuis* ATCC 10708, *Escherichia coli* ATCC 11229, *Staph. aureus* ATCC 43300 (MRSA) and *Vibrio cholerae* ATCC 14035 were grown on Trypticase Soy Agar (TSA) in the three remaining quadrants of the X-plate. Three plates of each organism, with appropriate controls, were exposed to the VOCs of the fungus for approximately 2, 4 and 6 days at room temperature. In order to check the viability of the test microbe, the fungus was then removed, and the control and test plates were placed in an incubator at $35 \pm 1^{\circ}\text{C}$ for a minimum of 3–4 days (the *Mycobacterium* spp. – see below – were incubated for approximately one additional month). This was done in order to ascertain if the VOCs had inhibited or killed the test organism. This same protocol was followed for *Yersinia pestis* and *Bacillus anthracis*, except that the exposure times were 3 and 5 days, and *Y. pestis* was incubated at $28 \pm 1^{\circ}\text{C}$ and in 5% CO_2 after exposure to the fungus. *Mycobacterium marinum* ATCC 927 was grown on Mycobacteria

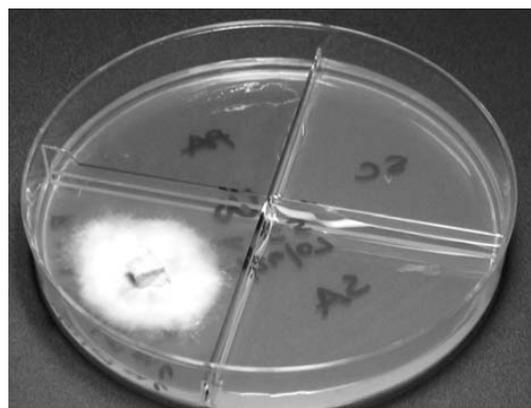


Fig. 1. An X-plate illustrating how assays of the VOCs of *M. crispans* were conducted with human-pathogenic bacteria. The *M. crispans* culture is in the left quadrant and streaks of the various test organisms (indicated by initials) were each placed in the other quadrants.

7H11 agar (Difco) in the remaining three quadrants, using the previously stated protocol, and incubated at 33 ± 1 °C. All three replicates in the tests with each organism behaved identically.

For all four *Mycobacterium tuberculosis* strains, also grown on Mycobacteria 7H11 agar, a section of agar was removed from the plate and a plug of PDA carrying *Muscodor crispans* mycelium was inserted. The plates were then inoculated from a broth culture. Control plates, where no fungus was present, were also inoculated. At each appointed time interval, a section of agar was removed from the plates and transferred to a separate and empty plate and placed in an incubator at 35 ± 1 °C in order to determine the viability of the microbe. The plates were placed in a plastic bag with moistened paper towels to prevent desiccation.

Pseudomonas aeruginosa ATCC 15442 and *Burkholderia thailandensis* ATCC 70038 were both grown on TSA. They were left at room temperature for the optimal growth time for the organism and then moved to an incubator at 35 ± 1 °C and observed.

All tests using human pathogens were conducted under strict and federally approved biosafety conditions. All tests on human pathogens were repeated at least twice.

Quantitative and qualitative analyses of *M. crispans* VOCs. The method used to analyse the gases in the air space above the mycelium of *M. crispans* involved trapping the gases using a solid-phase micro-extraction syringe and then injecting them into a gas chromatograph interfaced to a mass spectrometer, as described by Strobel *et al.* (2001). First, a baked solid-phase micro-extraction syringe (Supelco) consisting of 50/30 divinylbenzene/carburen on polydimethylsiloxane on a stable flex fibre was placed through a small hole drilled in the side of the Petri plate and exposed to the vapour phase for 45 min. The syringe was then inserted into the splitless injection port of a Hewlett Packard 6890 gas chromatograph containing a 30 m × 0.25 mm i.d. ZB Wax capillary column with a film thickness of 0.50 mm. The column was temperature programmed as follows: 30 °C for 2 min increasing to 220 °C at 5 °C min⁻¹. The carrier gas was ultra-high-purity helium, and the initial column head pressure was 50 kPa. Prior to trapping the VOCs, the fibre was conditioned at 240 °C for 20 min under a flow of helium gas. A 30 s injection time was used to introduce the sample fibre into the gas chromatograph. The chromatograph was interfaced to a Hewlett Packard 5973 mass-selective detector (mass spectrometer) operating at unit resolution. The MS was operated 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. Initial identification of the unknowns produced by *M. crispans* was made via library comparison using the NIST database, and all chemical compounds described in this report use the NIST database chemical terminology.

As far as possible, each compound identified by GC/MS was tested for its authenticity by doing independent GC/MS analysis on a set of authentic standards obtained from Sigma-Aldrich or by organic synthesis (Strobel *et al.*, 2001).

Testing of an artificial mixture of *M. crispans* VOCs. After obtaining the results of the VOC analysis of *M. crispans*, most of the identified compounds (those indicated with an asterisk in Table 1) were obtained from Sigma-Aldrich or synthesized and used to prepare a mixture containing the compounds in the same proportions as those determined by GC/MS analysis of the natural mixture, as described by Strobel *et al.* (2001). The propanoic acid, 2-methyl-, 2-methylbutyl ester in the natural mixture was substituted with propanoic acid, 2-methyl-, 3-methylbutyl ester in the artificial mixture. The mixture was placed in a tightly sealed container and stored at 0 °C. Several Petri plates, each with a sterile plastic well

(caps removed from microcentrifuge tubes) inserted into the agar in the centre of the plate, were inoculated with test plant-pathogenic micro-organisms around the periphery. The artificial mixture was added simultaneously to the well, and the plate was immediately sealed with two strips of Parafilm. The plates were incubated at 23 °C for 2 days and then assessed for growth (linear measurement of the fungal mycelium from the edge of the agar inoculum block) of the test organisms. Measurements were taken of the growth of the test organisms, and those with no growth were checked for viability by aseptically inoculating them onto fresh PDA plates and incubating at 23 °C for several days. The IC₅₀s were calculated for several target organisms as described by Strobel *et al.* (2001). The colony growth of non-mycelial organisms (yeasts and bacteria), was assessed visually relative to the control according to Strobel *et al.* (2001).

The artificial VOC mixture was also assessed for its activity against four *M. tuberculosis* isolates with different drug-resistance properties. To test each isolate, 10 µl of an active culture was placed in the middle of a Mycobacteria 7H11 agar plate and then evenly spread across the whole surface of the plate with a sterile plastic loop. Autoclaved plastic lids (micro-caps) served as wells for the VOCs. The control plates (one for each isolate) did not receive VOCs in the micro-caps. Three plates for each isolate were made, containing 5, 10 or 20 µl of the artificial VOC mixture. The plates were then placed into a zip-lock sealed plastic bag with a damp paper towel and incubated at 36 ± 1 °C. After 48 h of exposure, the micro-cap was removed, and the plates were returned to the incubator for 28 days. The paper towels were frequently checked and remoistened to prevent dehydration of the media. The viability of the bacterial cultures after incubation was assessed by virtue of colony development.

RESULTS AND DISCUSSION

Composition of VOCs produced by *M. crispans*

The VOCs produced by a 10-day-old culture of *M. crispans* were identified by GC/MS. Controls consisted of uninoculated PDA Petri plates; the compounds appearing in the control plate were subtracted from those acquired from the *M. crispans* plate. Comparison of the MS data with the NIST database was the basis for the identification of the compounds in both plates. Based upon the total integrated peak area of the GC elution profile, the most abundant compound appearing in the *M. crispans* culture was propanoic acid, 2-methyl-, with a retention time of 13:37 min (Table 1). Several other major compounds appearing as VOCs of *M. crispans* included propanoic acid, 2-methyl-; 1-butanol, 3-methyl-; 1-butanol, 3-methyl-, acetate; propanoic acid, 2-methyl-, 2-methylbutyl ester; and ethanol (Table 1). Unlike various other members of the genus *Muscodor* (Strobel *et al.*, 2001; Strobel, 2006; Ezra *et al.*, 2004; Atmosukarto *et al.*, 2005). *M. crispans* did not produce naphthalene or azulene derivatives, or a number of other compounds such as caryophyllene, phenylethyl alcohol, or 2- or 4-nonanone (Table 1).

Biological activities of the VOCs of *M. crispans*

When *M. crispans* was grown for 7–10 days at 23 °C on PDA, the VOCs of the fungus proved to be lethal to several fungi and bacteria in the bioassay Petri plate test system (Strobel *et al.*, 2001). Gram-negative and Gram-positive

Table 1. GC/MS analysis of the VOCs produced by *M. crispans*

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. AU, arbitrary units.

Retention time (min : s)	Total area (AU)	Possible compound	m/z
2:05	1.39	Acetaldehyde*	44.03
3:40	6.23	Ethyl acetate*	88.05
3:51	2.83	2-Butanone*	72.06
4:08	30.56	Propanoic acid, 2-methyl-, methyl ester*	102.07
4:18	12.41	Ethanol*	46.04
5:29	2.29	Acetic acid, 2-methylpropyl ester*	116.08
6:39	1.09	Propanoic acid, 2-methyl-, 2-methylpropyl ester*	144.12
6:46	1.78	1-Propanol, 2-methyl-*	74.07
6:52	1.51	2-Butenal, 2-methyl-, (E)-*	84.06
7:12	4.79	1-Butanol, 3-methyl-, acetate*	130.10
8:18	3.01	Hexane, 2,3-dimethyl-†	114.14
8:21	4.78	Propanoic acid, 2-methyl-, 2-methylbutyl ester*	158.13
8:31	5.38	1-Butanol, 3-methyl-*	88.09
13:37	351.18	Propanoic acid, 2-methyl-*	88.05
14:41	3.94	Formamide, N-(1-methylpropyl)-	101.08
16:44	1.31	Acetic acid, 2-phenylethyl ester*	164.08
20:44	7.20	Cyclohexane, 1,2-dimethyl-3,5-bis(1-methylethenyl)-	192.19

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*Indicates that the compound had the same retention time and mass spectrum as an authentic standard. Also indicates that the compound was used in the artificial mixture of VOCs. All other compounds in the list are matched to the most likely compound in the NIST database, but the data have not been confirmed by use of an appropriate identical standard compound by either retention time or MS.

†Indicates that the authentic standard did not have the same retention time as the compound indicated.

bacteria, as well as yeasts and each of the major classes of fungi, were utilized as test organisms. Most of the test organisms were 100 % inhibited and died after a 2 day exposure to the VOCs of *M. crispans* (Table 2). Some of the test organisms (e.g. *Penicillium roquefortii*, *Bipolaris sorokiniana*, *Stagonospora* sp. and *Fusarium oxysporum*) did not succumb to the VOCs of *M. crispans* after a 2 day exposure, but their growth was significantly inhibited by the VOCs, and they were killed after a 4 day exposure. Organisms relatively or completely unaffected by exposure to *M. crispans* included *Trichoderma viride* and *Fusarium solani*.

The susceptibility of *Pythium ultimum* to the VOCs of *M. crispans* was dependent upon the age of the *M. crispans* culture. For instance, when a 0-day-old culture (simultaneous inoculation with the test organism) of *M. crispans* was tested, 67.7 % inhibition of growth was observed, compared with 100 % inhibition of growth for cultures between 2 and 15 days old, in which *P. ultimum* was killed. A test of a 1-day-old culture showed 49.2 % inhibition, but the organism did not grow after exposure to *M. crispans*. Once the age of the *M. crispans* culture reached 16 days, the inhibition of *P. ultimum* was less than 100 % (Table 3). Therefore, the peak of effective VOC production by *M. crispans* was between 2 and 15 days after inoculation of the test plate (Table 3). It is suspected that the gradual loss of biological activity beginning at 16–17 days is related to the depletion of the carbohydrate source, as shown by Ezra &

Strobel (2003) with strains of *Muscodor albus*. The delay in the build-up of activity of the *M. crispans* culture against *P. ultimum* is thus presumably related to the time needed for VOC production to occur.

Biological activities of the artificial mixture of *M. crispans* VOCs

The IC₅₀ of the artificial gas mixture experiments was calculated for some of the test organisms (Table 4). All of the test organisms were 100 % inhibited when 15 µl of the artificial mixture was used, and several of them were killed with as little as 10 µl. *Verticillium dahliae*, *Botrytis cinerea* and *Aspergillus fumigatus* were not killed by even the largest volume of the mixture (30 µl), but all three were 100 % inhibited with 10 or 15 µl of the test mixture. The most sensitive organism was *Pythium ultimum*, which was killed with 10 µl and 100 % inhibited with 2 µl. Thus the IC₅₀ values do not necessarily reflect the killing ability of the VOCs since both *P. ultimum* and *B. cinerea* showed virtually the same IC₅₀ but one was killed and the other was not (Table 4).

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Activity of *M. crispans* VOCs against human-pathogenic bacteria

All four *Mycobacterium tuberculosis* strains were killed after 2, 4, 7 and 14 days exposure to actively growing *Muscodor*

Table 2. Effects of the *M. crispans* VOCs on many fungal pathogens of plants and some selected bacteria

The inhibition values were calculated as percentage growth inhibition as compared to an untreated control test organism. The tests were repeated at least three times with comparable results. Inhibition of the test organisms was recorded 48 h after exposure to the VOCs (see Methods for details).

Test organism	Inhibition (%) after 48 h exposure	Alive after 48 h exposure	Alive after 96 h exposure
<i>Alternaria helianthi</i>	100	N	N
<i>Aspergillus fumigatus</i>	100	Y	N
<i>Bacillus subtilis</i> *	100	N	N
<i>Bipolaris sorokiniana</i>	100	Y	N
<i>Botrytis cinerea</i>	100	N	N
<i>Candida albicans</i> *	100	N	N
<i>Cephalosporium gramineum</i>	100	N	N
<i>Ceratocystis ulmi</i>	100	Y	N
<i>Cochliobolus carbonum</i>	100	N	N
<i>Colletotrichum lagenarium</i>	100	N	N
<i>Curvularia lunata</i>	100	Y	N
<i>Drechslera portulacae</i>	100	N	N
<i>Drechslera teres</i>	100	N	N
<i>Drechslera tritici-repentis</i>	100	N	N
<i>Escherichia coli</i> *	100	N	N
<i>Fusarium avenaceum</i>	100	N	N
<i>Fusarium culmorum</i>	100	N	N
<i>Fusarium oxysporum</i>	100	Y	N
<i>Fusarium solani</i>	50	Y	Y
<i>Ganoderma</i> sp.	100	Y	N
<i>Geotrichum candidum</i>	100	Y	N
<i>Mycosphaerella fijiensis</i>	100	N	N
<i>Penicillium roquefortii</i>	100	Y	N
<i>Phytophthora cinnamomi</i>	100	N	N
<i>Phytophthora palmivora</i>	100	N	N
<i>Pythium ultimum</i>	100	N	N
<i>Rhizoctonia solani</i>	100	N	N
<i>Saccharomyces cerevisiae</i> *	90–95	N	N
<i>Sclerotinia sclerotiorum</i>	100	N	N
<i>Stagonospora</i> sp.	100	Y	N
<i>Tapesia yallundae</i>	100	N	N
<i>Trichoderma viride</i>	10	Y	Y
<i>Verticillium dahliae</i>	100	Y	N
<i>Xanthomonas axonopodis</i> pv. <i>citri</i> *	100	N	N

*These organisms were streaked onto the test plate, and growth was scored as positive if colony development eventually occurred. After appropriate exposure to the VOCs of *M. crispans*, the streaked area was compared to the growth on the control plate and the percentage inhibition estimated. Each organism was also restreaked onto a PDA plate to test for viability.

crispans (6–10-day-old cultures) (Table 5). Other bacteria which were killed after at least 2 days of exposure to *M. crispans* were *Staphylococcus aureus* ATCC 6538, *Mycobacterium marinum*, *Yersinia pestis* and *Salmonella choleraesuis*. The following bacteria were affected only slightly or were unaffected by exposure to *M. crispans*: *Pseudomonas aeruginosa*, *Burkholderia thailandensis*, *Staph. aureus* (MRSA), *Escherichia coli*, *Vibrio cholerae* and *Bacillus anthracis*. However, the growth of *Staph. aureus* (MRSA) was only as a slimy film rather than distinct

colonies, indicating an effect of the VOCs (Table 5). In addition, the *Bac. anthracis* plate had only a few colonies left on the exposure plate, but more colonies grew after removal of *M. crispans* and subsequent incubation. Therefore, it is suspected that *M. crispans* is only effective against the vegetative cells of *Bac. anthracis*, but not against the spores. One month after the last observation time (14 days), no growth was observed on any of the plates exposed to the fungus, and growth was observed on all of the control plates.

Table 3. Susceptibility of *Pythium ultimum* to the VOCs of *M. crispans* as a function of the age of the *M. crispans* culture

Age of <i>M. crispans</i> culture (days)	Inhibition of <i>P. ultimum</i> growth (%)	Growth after isolation
0	67.7	Y
1	49.2	N
2	100	N
3	100	N
4	100	N
5	100	N
6	100	N
7	100	N
8	100	N
9	100	N
10	100	N
11	100	N
12	100	N
13	100	N
14	100	N
15	100	N
16	20.2	Y
17	2.8	Y
18	2.6	Y
21	0	Y
28	0	Y

Effect of the artificial mixture of VOCs on *M. tuberculosis* strains

Of the human bacteria used as test organisms in this work, the most important overall threat to human health is *M. tuberculosis*. For this reason, this organism was selected for testing with the artificial VOC mixture in the assay described above. The artificial mixture of the *M. crispans* VOCs was placed in varying amounts on plates that had

been inoculated with *M. tuberculosis* strains. These strains had either no drug resistance or resistance to one or more specific drugs. Interestingly, each strain succumbed to at least a 2 day exposure to the VOCs at the 20 µl level, with the exception of strain 50001106, which is resistant to streptomycin (Table 6). There was no killing effect against any strain at lower levels of the artificial VOC mixture (Table 6). In general, the artificial VOC mixture mimicked the effects of the fungal VOCs against the *M. tuberculosis* strains. The lack of effect on strain 50001106 is presumably due to the absence from the artificial mixture of some component(s) present in the natural mixture.

Bioactive fungal VOCs

Each individual isolate of *Muscodor* that has been examined makes its own unique set of VOCs, and it appears that *M. crispans* is no exception to this (Table 1; Strobel, 2006). Interestingly, however, the majority of the VOCs produced by *M. crispans*, in contrast to *Muscodor albus*, are on the US Food and Drug Administration’s GRAS list of harmless substances (see the FDA website for details: <http://www.fda.gov/>). For instance, no azulene or naphthalene derivatives were detected in the VOCs of this organism (Table 1; Strobel *et al.*, 2001). When a mixture of the GRAS compounds produced by *M. crispans* was tested, the results were virtually identical to those of the natural mixture (Table 2; A. M. Mitchell, unpublished). Thus this mixture may have potential utility in applications ranging from food preservation to agricultural, household and industrial uses. The mixture was also active against *M. tuberculosis*, including at least two drug-resistant strains, making this mixture a candidate for testing as an inhalant in the treatment of tuberculosis (Table 6).

The mechanism of action of the VOCs of *Muscodor* spp. on target fungi and bacteria is unknown. However, a

Table 4. IC₅₀s of the artificial mixture of VOCs of *M. crispans* on various plant pathogens

Amounts of the mixture, ranging from 1 to 30 µl, were added to a sterile plastic well in the centre of the test plate, and the pathogenic organisms were placed around the edge of the plate. Viability was assessed after 48 h and compared to a control plate with no mixture added but with the sterile well in place. Any organisms which showed no growth after that period were determined to be 100 % inhibited, while those which showed no growth after 48 h and no growth after inoculation onto PDA immediately following the 48 h assessment were considered dead. The IC₅₀ calculation was determined by dividing the amount of the artificial mixture required to cause 50 % inhibition (in µl) by the total air space in the Petri dish (50 ml). NA, Not applicable.

Test organism	Minimum vol. to cause 100 % inhibition (µl)	Vol. to cause death (µl)	IC ₅₀ (µl ml ⁻¹)
<i>Aspergillus fumigatus</i>	2.0	20	0.031 ± 0.003
<i>Botrytis cinerea</i>	10.0	>30	0.035 ± 0.004
<i>Phytophthora cinnamomi</i>	5.0	30.0	0.056 ± 0.009
<i>Phytophthora palmivora</i>	1.0	5.0	<0.02
<i>Pythium ultimum</i>	2.0	10.0	0.030 ± 0.004
<i>Rhizoctonia solani</i>	20.0	15.0	0.039 ± 0.006
<i>Sclerotinia sclerotiorum</i>	NA	>30	0.15 ± 0.016
<i>Verticillium dahliae</i>	5.0	>30	0.062 ± 0.004

Table 5. Effects of the VOCs of *M. crispans* on various Gram-positive and Gram-negative bacteria on X-plates

See Methods and Fig. 1 for the set-up of X-plates. The exposure times were varied according to the organism under test, and the viability of the organism was determined after that period (listed as growth or no growth).

Organism	Type of cell wall	Exposure time (days)	Growth in presence of <i>M. crispans</i>	Comments
<i>Staph. aureus</i> ATCC 6538	Gram +	2, 4 and 6	N	
<i>Staph. aureus</i> ATCC 43300 (MRSA)	Gram +	2, 4 and 6	Y	No actual colonies formed, just a slightly filmy growth
<i>Sal. choleraesuis</i> ATCC 10708	Gram –	2, 4 and 6	N	
<i>P. aeruginosa</i> ATCC 15442	Gram –	2	Y	No visible difference between exposed and control plates
<i>M. marinum</i> ATCC 927	Acid-fast	2, 4 and 6	N	
<i>Burk. thailandensis</i> ATCC 70038	Gram –	2	Y	No visible difference between exposed and control plates
<i>E. coli</i> 11229	Gram –	2, 4 and 6	Y	No visible difference between exposed and control plates
<i>V. cholerae</i> ATCC 14035	Gram –	2, 4 and 6	Y	Growth at 4 and 6 day exposures slightly inhibited in comparison to control plates
<i>Y. pestis</i> 91-3365	Gram –	3 and 5	N	
<i>Bac. anthracis</i> A2084	Gram +	3 and 5	Y	Only a few colonies left after exposure; when incubated, more grew
<i>M. tuberculosis</i> 3081 (resistant to isoniazid)	Acid-fast	2, 4, 7 and 14	N	
<i>M. tuberculosis</i> 50001106 (resistant to streptomycin)	Acid-fast	2, 4, 7 and 14	N	
<i>M. tuberculosis</i> 59501228 (resistant to streptomycin and ethambutol)	Acid-fast	2, 4, 7 and 14	N	
<i>M. tuberculosis</i> 59501867 (susceptible)	Acid-fast	2, 4, 7 and 14	N	

microarray study to analyse the transcriptional response of *Bacillus subtilis* cells exposed to *M. albus* VOCs showed that genes involved in DNA repair and replication increased in expression (R. A. Britton, Michigan State University, unpublished observations). These preliminary results imply that *M. albus* VOCs are inducing some type of DNA damage in cells, possibly through the effects of one of the naphthalene derivatives; such compounds have been shown to cause chromosome decondensation in bacteria (Trun & Gottesman, 1990). This mechanism of action is unlikely to be similar to the mechanism of killing found in

M. crispans since this organism does not produce naphthalene, but it seems to provide some guidance on how to proceed with such a system. Similar microarray studies on organisms susceptible to *M. crispans* VOCs would be helpful in elucidating the mechanism of antimicrobial action.

The VOCs of *M. crispans* are active against a wide spectrum of fungal pathogens of plants, including the banana pathogen *Mycosphaerella fijiensis*, arguably the most serious plantation disease in the world. They also showed activity

Table 6. Lethal effects of an artificial mixture of *Muscodor crispans* VOCs on growth of various strains of *Mycobacterium tuberculosis*

Three different volumes of the artificial mixture were utilized to test its effects on *M. tuberculosis*. The mixture of VOCs was prepared according to the composition of substances in Table 1. The plates were exposed to 5, 10 µl or 20 µl of the synthetic mixture of VOCs in the presence of the *M. tuberculosis* strains. The organisms were exposed to the VOCs for 2 days and then incubated for 28 days without the VOCs, prior to assessment of their viability (see Methods). +, Isolate grew in the presence of the VOCs; –, no growth.

<i>M. tuberculosis</i> isolate (drug resistance in parentheses)	5 µl	10 µl	20 µl
3081 (isoniazid)	+	+	–
50001106 (streptomycin)	+	+	+
59501228 (streptomycin and ethambutol)	+	+	–
59501867 (susceptible)	+	+	–

against *Xanthomonas axonopodis* pv. *citri*, the causal agent of citrus canker (Table 2), raising the possibility of developing either the fungal VOCs or artificial VOC mixtures for the treatment of plants, plant parts or equipment associated with the canker disease. Other pathogenic organisms can also be considered as potential targets for the *M. crispans* VOCs.

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