Naphthalene, an insect repellent, is produced by *Muscodor vitigenus*, a novel endophytic fungus

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Muscodor vitigenus is a recently described endophytic fungus of Paullinia paullinioides, a liana growing in the understorey of the rainforests of the Peruvian Amazon. This fungus produces naphthalene under certain cultural conditions. Naphthalene produced by *M. vitigenus* was identified by gas chromatography/mass spectrometry. Its chromatographic and mass spectral properties were identical to authentic naphthalene. Agar plugs supporting growth of the fungus and producing known amounts of naphthalene effectively repelled the adult stage of the wheat stem sawfly, *Cephus cinctus*, in Y-tube bioassay tests. Authentic naphthalene, at comparable concentrations to those in tests involving the fungus itself, mimicked the insect repellency of the fungus. Although other *Muscodor* spp. produce volatile antimicrobials, *M. vitigenus* is unique in its ability to produce naphthalene almost exclusively. This report also describes the potential practical implications of *M. vitigenus*.

Keywords: endophyte, organic volatiles, insects

INTRODUCTION

Plants appear to be a reservoir of untold numbers of endophytic organisms (Bacon & White, 2000). By definition, these micro-organisms (mostly fungi and bacteria) reside in the tissues beneath the epidermal cell layers and cause no apparent harm to the host (Stone *et al.*, 2000). The exact physical relationship of the endophyte to the plant has, in most cases, remained obscure because it is extremely difficult to find an endophyte within plant tissues. Conceivably, the microbes live within the interstitial spaces of the tissues, and it also seems likely that penetration of living cells may occur, but this possibility remains difficult to observe directly.

Some of these endophytes produce bioactive substances that are involved in the host-microbe relationship. While there are many epiphytic micro-organisms associated with plants, the endophytic associations may be more complex since living host tissues are involved. Thus, closer biological associations may have developed between these endophytes in their respective hosts than is the case with epiphytes or soil-related organisms. The result of these associations may be the production, by the endophyte, of bioactive chemicals that are involved in the relationship. This assumption has prompted our search for novel endophytes and an examination of their secondary bioactive products. One remote and extremely biodiverse area of the world in which we focused our search for unique microbe–plant relationships is the Manu region of the upper Amazon.

Recently, we described two novel endophytic fungi, *Muscodor albus* from *Cinnamomum zeylanicum* from Honduras (Worapong *et al.*, 2001), and *Muscodor roseus* from two monsoonal rainforest trees in Northern Australia (Worapong *et al.*, 2002). These endophytes produce a mixture of volatile antimicrobials that effectively inhibit and kill a wide spectrum of plantassociated fungi and bacteria (Strobel *et al.*, 2001). On the other hand, the gases of *M. albus* did not kill fungi that were related to it, some of which were producers of other lethal gas mixtures (Worapong *et al.*, 2001, 2002). Thus, using *M. albus* as a selection tool, it was possible to isolate still other endophytic fungi that produce volatile compounds of biological interest and importance.

In this report we describe some of the biology and biochemistry of another endophytic *Muscodor* sp. that was obtained by using the volatiles of *M. albus* as a selection tool. Representive forest plants, collected in the southern Peruvian Amazon, were examined for the

Department of Plant Sciences¹, Department of Chemistry and Biochemistry² and Department of Entomology³, Montana State University, Bozeman, MT 59717, USA presence of novel *Muscodor* spp. From this effort we isolated *Muscodor vitigenus*, which is endophytic on a liana identified as *Paullina paullinioides*. This unique endophytic fungus produces naphthalene in quantities sufficient to cause modifications in insect behaviour, and this is the subject of this report.

METHODS

Fungal isolation and storage. The culture of M. vitigenus used in this study was isolated as an endophyte from a liana growing in the upper Amazon (Daisy et al., 2002). Several small limbs of P. paullinioides Radlk. were removed from a plant growing near Lake Sandoval in the Bahuaja Sonene National Park in the extreme south of Peru. Attempts to find this same organism from P. paullinioides growing in the Manu River area of the upper Amazon were not successful. Furthermore, the fungus was not isolated from at least 30 other plants in the Lake Sandoval area that were sampled. Small pieces of the inner tissues of the plant were placed in specially prepared Petri plates that were part of a selection system designed to specifically isolate endophytes related to the Muscodor spp. and/or other fungi that produce volatile antibiotics (Strobel et al., 2001). This selection system has been described previously and includes the use of the known Muscodor spp. isolates as selection tools (Worapong et al., 2002). M. vitigenus, a sterile, gas-producing fungus, has been classified as a member of the genus Muscodor primarily due to the relatedness of its 18S rDNA sequences to previously known members of this group (Daisy et al., 2002). The fungus could best be stored after growth on sterile filter paper that had been placed on natural potato dextrose [glucose] agar (PDAN). This agar was made with 10 g potato starch pellets (Basic American, Rexburg, ID, USA), 15 g glucose, 15 g agar, and distilled water up to 1 litre. The paper with mycelial growth was removed from the plates and dried under a laminar-flow hood, cut into pieces, and stored at -70 °C. The fungus remained viable for at least 8 months in this manner. M. vitigenus also remained viable, but to a lesser degree, when its mycelium was stored on PDAN plugs in glycerol/distilled water and stored at -70 °C. M. vitigenus is stored in the Montana State University Mycological Culture Collection (MONT) as no. 2116.

Quantitative and qualitative analyses of *M. vitigenus* volatiles. The gases in the air space above the mycelium of *M. vitigenus* growing in Petri plates were analysed by trapping the fungal volatiles with a solid-phase micro-extraction syringe and injecting them into a gas chromatograph interfaced to a mass spectrometer, as described by Strobel *et al.* (2001). Initial identification of the unknown compounds produced by *M. vitigenus* was made through library comparison using the NIST database. Control analyses were conducted using Petri plates containing only PDAN; the compounds obtained from these controls were deleted from the analyses done on Petri plates containing PDAN and *M. vitigenus*.

Synthetic naphthalene (Willert Home Products, St Louis, MO, USA) was used to create an artificial atmosphere mimicking that produced by *M. vitigenus* for use in preliminary testing of bioactivity. It too was subjected to the GC/MS assay as described above.

Source and handling of insects. The wheat stem sawfly (*Cephus cinctus*) was selected as a model for its behavioural responses to naphthalene because of its availability, its economic importance, and the experience that we have in dealing with this insect. It is a major pest in the winter-wheat-

growing areas of the Great Plains of North America that cannot be controlled sufficiently with current agricultural management practices (Morrill *et al.*, 2001). Overwintering pre-pupae were collected from a wheat field in September after the 2001 harvest in Broadwater County, MT. Stubble was removed from this heavily infested field with a shovel and taken to the laboratory. The uninfested residue was separated from wheat stems containing sawfly larvae. These sawfly-cut stems were stored at 4 °C for at least 100 days to complete diapause. The sawfly-cut stems were then removed from the refrigerator and placed in individual shell vials for 4–5 weeks to complete metamorphosis. Vials were sealed with a cotton plug that was wetted twice weekly with distilled water. Upon adult emergence, insects were held in darkness, at room temperature, in the shell vial until used.

Collection of volatiles and analytical methods for insect repellency bioassay tests. To determine the rate of naphthalene release from M. vitigenus cultures, collections of volatile compounds were made from circular (0.785 cm²) plugs of the fungus growing on PDAN. The volatile collection apparatus used was that described by Heath & Manukian (1992). Specifically, agar plugs containing the 14-day-old fungus culture were placed in Pyrex glass volatile collection chambers (1.5 cm diameter \times 8 cm long) fitted at one end with an air diffuser inlet cap and sealed at both ends with no. 7 ChemThread inlets [0.25 inch (0.64 cm) i.d.] using rubber Orings (Analytical Research Systems). A volatile collector trap $[0.25 \text{ inch o.d.} \times 3 \text{ inch long } (0.64 \text{ cm} \times 7.62 \text{ cm}) \text{ glass tube}]$ containing 30 mg SuperQ adsorbent (Alltech Associates) was inserted into one end of the volatile collection chamber. Purified air was delivered at a rate of 0.3 l min⁻¹ over the fungal plugs, and the flow and pressure were maintained by both a compressed air source and a vacuum pump. Volatiles were collected for 3 h. Fungal plugs from four colonies on individual Petri plates containing PDAN were used and the volatile aerations were replicated three times for each colony.

Volatiles were eluted from the SuperQ in each volatile collection trap with 225 μ l hexane, and 7 ng decane was added as an internal standard. Volatiles were analysed by coupled GC/MS. The GC was an Agilent Technologies 6890 instrument fitted with a 30 m DB-1MS capillary column (0.25 mm i.d. with a 0.25 μ m film thickness; J & W Scientific). The temperature programme increased from 50 °C to 280 °C at 10 °C min⁻¹. The MS instrument used was an Agilent Technologies 5973. Authentic naphthalene was used as a standard.

Insect repellency bioassay tests. Insect repellency bioassay tests were done with a Y-tube olfactometer (Fig. 1). The olfactometer design incorporated two 38 cm long glass tubes upwind of the Y-tube for the stimulus and control odour sources. Each tube was made of Corning 22 mm o.d. glass with a 24/25 inner ground-glass joint, which connected it to either of the upper arms of the Y-tube. A charcoal-purified and humidified airstream was connected to these tubes by a Teflon-lined, threaded 24-410 cap coupled to a 0.64 cm Swagelock union that delivered air through 0.64 cm o.d. Teflon tubing. The airflow was set at 0.8 l min⁻¹ using a flowmeter. The lower arm of the Y-tube was a 30 cm length of 28 mm o.d. Corning glass tubing that branched at 20 cm from the tip to form the upper arms. The interior angle of the upper arms of the Y was 120°, and the arms branched for 4 cm before becoming parallel for the final 10 cm of each arm.

An individual test insect was introduced into the Y-tube approximately 1-2 cm from the outlet. Positive phototaxis was used to enhance movement of the test insect by means of



Fig. 1. Schematic drawing of the olfactory Y-tube used to measure insect behaviour in the presence of various naphthalene sources. A 6.3 V light source (not shown) is placed at the base of the Y-tube, giving an intensity of 0.1384 W m⁻² inside the tube where the insect is placed.

a 6.3 V light source that was carefully positioned at the midpoint between the upper arms of the Y. Trials were terminated after 7 min if there was no response. A 'successful' trial was one in which the upwind-responding insect moved entirely past the midpoint of the bifurcation in the Y-tube and into one of the arms of the 'Y'. A trial was designated 'unsuccessful' when the insect did not make either choice. Two sets of 30 successful trials, each set using a different concentration of authentic naphthalene, and 60 successful trials using M. vitigenus fungal plugs, were performed. Statistical analyses were performed using a chi-squared test for small sample sizes (Sokal & Rohlf, 1981). It was initially established that the insects had no preference for or aversion to either side of the Y-olfactory tube used in the bioassay tests: 50 individual insect tests were done using only air in both arms, with the result being no statistical difference in insect behaviour between them. Also, in accordance with the standard method of doing Y-tube olfactory testing, the treatment side of the tube in each of the test situations was alternated with the control side on every fifth measurement, to eliminate any positional effect that may have favoured one side or the other of the Y-tube in measuring the insect response.

Y-tube olfactometry with authentic naphthalene as odour source. Naphthalene (in acetone) was delivered to a circular (6 mm diameter) piece of filter paper (created using a paper hole punch) and this, along with a wheat plant (natural host of the insect) served as odour sources in the Y-tube (cf. Fig. 1). Naphthalene (10 µl) in acetone was used in two insect behaviour tests: (A) 0.05 g naphthalene (ml acetone)⁻¹, and (B) a 10-fold dilution of (A). The release rate of naphthalene from test system (A) was approximately 12 ng h⁻¹. The rate of release of naphthalene on filter paper was determined using the volatile collection methodology described above. Thirty successful trials were performed for each of the two solutions. The control consisted of a wheat plant and 10 µl acetone placed on a piece of filter paper (also 6 mm diameter). Wheat (Triticum aestivum L., McNeal) was grown in an insect-free growth chamber (15 h light:9 h dark; 22 °C day/20 °C night). Plants at growth stage 30 (Zadoks et al., 1974) were carefully pulled from the soil and the roots were wrapped with wet cotton prior to use.

Y-tube olfactometry with *M. vitigenus* **as odour source.** In all trials, a wheat plant and a circular (0.785 cm^2) fungal plug of *M. vitigenus* served as odour sources. The control consisted of a wheat plant and a circular (0.785 cm^2) plug of uninoculated PDAN. The stage and handling of wheat plants were as described above.

RESULTS AND DISCUSSION

Chemical composition of volatiles in *M. vitigenus* cultures

The compounds produced as volatile secondary metabolites of a 21-day-old culture of M. vitigenus were initially identified by GC/MS. Comparable analyses (controls) were done on the gas phase trapped above an uninoculated PDAN Petri plate. Compounds appearing in the control plate were subtracted from the analyses of those trapped on the plate supporting fungal growth. Some of the compounds in the control Petri plate were styrene, benzaldehyde, butylated hydroxytoluene, toluene, and a number of minor benzene derivatives including benzene, 1,3,5-trimethyl and benzene, 1methoxy-3-methyl. Initial identification of the volatile compounds in both analyses was based on a comparison of the MS data acquired after separation by GC/MS with the NIST database. The most abundant compound appearing in the M. vitigenus culture atmosphere, based on the total integrated peak area of the GC elution profile, was naphthalene, with a retention time of 28:03 (min:s) (Fig. 2). Final identification of the naphthalene was by done comparison with an authentic standard. The authentic standard had the same retention time and an identical mass spectrum to the compound trapped in the atmosphere of the fungal culture. Several other naphthalene derivatives were also tentatively identified from the gas phase of M. vitigenus, including traces of naphthalene, 2-methyl (retention time 30:24) and naphthalene, 1-methoxy (36:38). None of the other volatiles commonly associated with M. albus and M. roseus appeared in M. vitigenus except traces of acetone, caryophyllene and azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4dimethyl-7-(1-methylethenyl)-, [1S-(1.alpha.,7.alpha., 8a.beta.)] with retention times of 3:19 and 37:12, respectively (Strobel et al., 2001).

Release of volatiles from M. vitigenus

Six plugs of PDAN (0.785 cm^2) supporting growth of 14day-old *M. vitigenus* cultures were placed in a specialized volatile collection chamber (see Methods) to



Fig. 2. Elution profile of volatiles trapped in the air space above a 3-week-old colony of *M. vitigenus* and subjected to GC/MS. The major peak (28 min 3 s) in this trace has been identified as naphthalene (structure shown inset).

determine the rate of naphthalene released from each of them. Volatiles were collected for 3 h and were then analysed by GC/MS. Naphthalene identification was again confirmed by comparison with an authentic standard and quantified using decane as an internal standard. The rate of naphthalene production, on six agar plugs, ranged from 9.45 to 106.85 ng h⁻¹, with a mean production rate of 25.7 ± 11.8 ng h⁻¹. Although the range of naphthalene production is large, even the smallest yield of naphthalene proved to be sufficient to modify insect behaviour. Naphthalene production did not occur on media that were high in lipids as an energy source or on media that was not enriched in carbohydrates (starch or sucrose) (Daisy *et al.*, 2002). On PDAN, naphthalene production began to occur at 7 days and continued for several weeks.

M. vitigenus compounds as insect repellents

M. vitigenus, in limited insect bioassay tests, demonstrated insect repellency. In controlled, replicated experiments, both authentic naphthalene and M. vitigenus were used as odour sources in Y-tube olfactometry tests. The responses of C. cinctus, a major local crop pest, were measured with plugs of the naphthalene-producing fungus as compared to comparable amounts of authentic naphthalene. In 30 successful trials using naphthalene test solution A (naphthalene release rate 12 ng h^{-1}), 24 out of 30 insects responded by moving away from the naphthalene source (Table 1). In a second set of 30 successful trials using naphthalene test solution B, 21 out of 30 insects were repelled by the naphthalene source. Finally, 60 successful trials were performed using 0.785 cm² fungal plugs (2-3 weeks old) of M. vitigenus as the odour source. Out of the 60 insects, 49 moved away from the fungus and its volatiles. The results of all three sets of tests were significantly different from the respective controls (Table 1). Finally, many of the insects tested became more disoriented than usual in the presence of both commercial naphthalene and the M. vitigenus volatiles.

M. vitigenus and other gas producers as endophytes

Naphthalene has very rarely been described as a product from biological sources, and never before, to our knowledge, from an endophytic fungus. Its role in *M. vitigenus* may be related to the ability of naphthalene to inhibit some types of fungal proliferation (Chen *et al.*, 1998), thus providing the endophyte with the capability to ward off competitors in its natural environment,

Table 1. Influence of authentic naphthalene and *M. vitigenus* volatiles, containing mostly naphthalene, on the behaviour of adult wheat stem sawflies (*C. cinctus*)

Bioassay tests were conducted in Y-olfactory tubes as described in Methods.

Test material*	No. of insects†	Insect ratio (control/naphthalene source)‡	Statistical evaluation (chi-squared test)§
Authentic naphthalene, concn A	15F, 15M	24/6	$\chi^2 = 9.63, \alpha = 0.005$
Authentic naphthalene, concn B	15F, 15M	21/9	$\chi^2 = 4.03, \ \alpha = 0.05$
PDAN plugs of a 2-week-old	26F, 34M	49/11	$\chi^2 = 22.81, \alpha = 0.001$
M. vitigenus culture			

* The control side of the Y-tubes used in these tests consisted of agar plugs (PDAN supporting no fungal growth), or in the case of authentic naphthalene a paper disk with an application of acetone containing no naphthalene. The naphthalene concentrations were: A, 0.05 g (ml acetone)⁻¹; B, 0.005 g (ml acetone)⁻¹.

†F, number of females; M, number of males. No differences were noted between male and female behaviour in any of the tests.

[‡]The numerator is the number of insects moving toward the control side of the Y-tube; the denominator is the number of insects moving toward the naphthalene source.

Statistical evaluation was performed on the differences in the behaviour of insects in each test group between those moving toward the control side of the Y-tube and those moving toward the side containing naphthalene or *M. vitigenus*.

assisting not only the host, but its own survival. In this study, however, we focused on the insect-repellent activity of this compound (Table 1). Generally, in commerce, naphthalene is used as an antimicrobial, an insecticide, an insect repellent, an anthelmintic and a vermicide (Bolton & Eaton, 1968). Naturally, naphthalene is found in fossil fuels and produced by the burning of coal, oil and wood (see http:// www.atsdr.cdc.gov/tfacts67.html). Only recently has naphthalene been found as a constituent of other natural biological systems, having been reported from Magnolia flowers and in the nest material of Formosan subterranean termites; it has been suggested that the presence of the chemical in both of these places may function as protection from insects (Azuma et al., 1996; Chen et al., 1998).

It remains unknown whether M. vitigenus can grow successfully in nature in plants other than P. *paullinioides*. However, the fungus has proven to be uneven in its production of naphthalene, with a strong dependency upon the presence of starch as an energy source and precursor to naphthalene. On occasion, individual mycelial transfers to a new Petri plate containing PDAN will fail to produce naphthalene (B. H. Daisy & G. A. Strobel, unpublished). It is a common observation that plant-associated fungi will sometimes halt production of interesting active products when cultured in artificial media (Pinkerton & Strobel, 1976). This could result from the absence of critical activator compounds that are present in the host (Pinkerton & Strobel, 1976). We have not yet been able to determine the optimum conditions for the maximum production of naphthalene by this fungus.

The presence of naphthalene in M. vitigenus is important in understanding the biology of the fungus and P. paullinioides as well as their evolutionary relationship. A number of endophytes have been isolated that make biologically active products that have selective activity against potential threats to the host plant, especially in the Muscodor group (Strobel et al., 2001). *M. vitigenus* is quite different from the other *Muscodor* isolates in that it produces a distinct set of volatile compounds that are less lethal to micro-organisms but much more inhibitory of arthropods. It appears that these gas-producing fungi are not restricted to just one area of the world: they have been recovered from Australia, Honduras, and now Peru. It remains a mystery why these nearly genetically identical fungi with similar functions can exert such biologically diverse phenomena in such a diversity of places. The discovery of this fungus again adds support to the growing necessity to conserve these areas of the world harbouring yet untold numbers of endophytic micro-organisms.

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