JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Volatile Compounds Emitted by Sclerotia of *Sclerotinia minor*, *Sclerotinia sclerotiorum*, and *Sclerotium rolfsii*

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Volatile compounds emitted by sclerotia of *Sclerotinia minor*, *Sclerotinia sclerotiorum*, and *Sclerotium rolfsii* were identified by solid phase microextraction followed by gas chromatography and mass spectometry. Both *S. minor* and *S. sclerotiorum* emitted 2-methylenebornane and 2-methylisoborneol. In addition, *S. minor* emitted mesityl oxide, γ-butyrolactone, *cis*- and *trans*-linalool oxide, linalool, and *trans*-nerolidol. *S. sclerotiorum* emitted 2-methyl-2-bornene, 1-methylcamphene, and a diterpene with a molecular weight of 272. *Sclerotium rolfsii* did not emit any of these compounds but did emit δ-cadinene and *cis*-calamenene. Chemicals emitted by *S. minor* and *S. sclerotiorum* were tested to determine if they could stimulate germination of conidia of *Sporidesmium sclerotivorum*, a mycoparasite on sclerotia of *Sclerotinia* spp. Chemicals were tested at 1 part per billion to 100 parts per million, both in direct contact with conidia and near, but not in, physical contact. None of the chemicals alone nor a combination of all chemicals induced germination of conidia of *S. sclerotivorum*.

KEYWORDS: Biological control; *Sclerotinia minor*; *Sclerotinia sclerotiorum*; *Sclerotium rolfsii*; *Sporidesmium sclerotivorum*; volatiles; 2-methylenebornane; 2-methylisoborneol; mesityl oxide; γ-butyrolactone; *cis*-linalool oxide; *trans*-linalool oxide linalool; *trans*-nerolidol; 2-methyl-2-bornene; 1-methylcamphene; δ-cadinene; *cis*-calamenene

INTRODUCTION

The soil-borne plant pathogens *Sclerotinia minor* and *Sclerotinia sclerotiorum* cause severe economic losses of many vegetable and ornamental crops around the world, including plants in 148 genera in the United States. Lettuce drop, white mold on beans and onions, and sunflower head rot are particularly devastating. *Sporidesmium sclerotivorum* is a soil-inhabiting dematiaceous hyphomycete that has been investigated as a biocontrol agent against these plant pathogens (*1*). In nature, *S. sclerotivorum* behaves as an obligate parasite attacking sclerotia of *Sclerotinia* spp., *Sclerotium cepivorum*, and *Botrytis cinerea*. It does not attack *Sclerotium rolfsii* or *Macrophomina phaseolina*. Conidia of *S. sclerotivorum* germinate in response to the presence of host sclerotia and can detect host sclerotia up to 3 cm away in soil (*2*). Because they can be detected at this distance, these chemical signals may have sufficient volatility to be detected and analyzed by solid phase microextraction (SPME). SPME is a solventless technique to extract volatiles from the headspace above a sample. The volatiles are adsorbed onto a fiber coating and subsequently desorbed into the heated injection port of a gas chromatograph−mass spectrometer (GC-MS) for analysis. The SPME technique detects trace levels of organic compounds with minimal sample disturbance. Chemicals associated with living organisms that are detected by this technique are likely to be naturally occurring and not artifacts commonly seen when using other methods of sample extraction. The sensitivity of this method is in the nanogram range and is subject to change from very small environmental differences.

At least three compounds that stimulate germination of *Sp. sclerotivorum* were extracted from melanized sclerotia of *S. minor* but have not been identified (*3*). Volatile organic compounds are known to be germination stimulants for fungal spores (*4*). If bioactive volatiles from *S. minor* can be identified, it may be possible to use them in a control strategy to reduce crop disease caused by *Sclerotinia* spp.

The objectives of this study were to identify volatile organic compounds emitted by sclerotia of *S. minor*, *S. sclerotiorum*, and *Sclerotium rolfsii* and to determine if the compounds from *S. minor* and *S. sclerotiorum* would induce germination of conidia of *Sp. sclerotivorum*. Volatiles emitted by *S. minor* were the main focus of this study because this species demonstrates

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10.1021/jf0117098
Published on Web 05/11/2002

Table 1. Volatile Compounds Emitted by Sclerotia of *S. minor*, *S. sclerotiorum*, and *Sclerotium rolfsii* in Their Order of Elution

compound[a]	RT[b]	*S. minor*	*S. sclerotiorum*	*S. rolfsii*	MW[c]	MS fragments
4-methyl-3-penten-2-one (mesityl oxide)	7.50	A	–	–	98	98 (5), 83 (11), 55 (12, 43 (100)
dihydro-2(3*H*)-furanone (δ-butyrolactone)	10.42	C	–	–	86	86 (35), 56 (33), 42 (100)
2-methyl-2-bornene	13.10	–	B	–	150	150 (34), 135 (54), 122 (29), 107 (100)
1-methylcamphene	13.71	–	B	–	150	150 (15), 135 (10), 121 (18), 107 (100)
2-methylenebornane	14.55	B	B	–	150	150 (22), 135 (41), 121 (21), 107 (100)
trans-linalool oxide	15.87	A	–	–	170	170 (2), 111 (19), 94 (48), 59 (100)
cis-linalool oxide	15.53	A	–	–	170	170 (1), 111 (35), 94 (41), 59 (100)
linalool	16.01	A	–	–	154	154 (1), 136 (8), 93 (63), 71 (100)
2-methylisoborneol	18.08	A	A	–	168	168 (4), 135 (20), 108 (42), 95 (100)
trans-nerolidol	22.80	A	–	–	222	222 (1), 204 (5), 93 (64), 69 (100)
δ-cadinene	22.57	–	–	C	204	204 (42), 189 (18), 161 (100), 134 (50)
cis-calamenene	22.64	–	–	C	202	202 (6), 159 (100), 144 (7), 129 (8)
diterpene, MW 272	26.10	–	C	–	272	272 (27), 257 (46), 137 (100)

[a] Compound identification was based on (A) mass spectrum and GC retention time consistent with authentic sample, (B) mass spectrum consistent with spectrum reported in Schumann and Pendleton (*7*), or (C) mass spectrum closely matches entry in Wiley mass spectral library. A dash indicates that the compound was not found or was present in a concentration that was too low for a reliable identification. [b] Retention time. [c] Molecular weight.

a greater ability to germinate *Sp. sclerotivorum* than does *S. sclerotiorum* (*1*).

MATERIALS AND METHODS

Culture of Fungi. *S. minor* isolate135L, *S. sclerotiorum* isolate 88, both originally isolated from lettuce in New Jersey by P. B. Adams, and *Sclerotium rolfsii* isolate 111 originally isolated from bean in Maryland by G. C. Papavizas were maintained on potato dextrose agar (PDA; Sigma, St. Louis, MO) in 9-cm-diameter Petri dishes under ambient conditions. Sclerotia of *Sclerotium rolfsii* were included in this study as a negative control to more clearly indicate which volatiles found exclusively in *S. minor* and *S. sclerotiorum* would have the greatest potential for activity and, therefore, be the best candidates for inclusion in germination bioassays. Cultures were produced in Beltsville, MD, and shipped to New Orleans, LA, for analysis. Sclerotia of *S. minor* were harvested from 6−41-day-old cultures, those of *S. sclerotiorum* from 9−41-day-old cultures, and those of *Sclerotium rolfsii* from 14−41-day-old cultures. Noninoculated PDA plates incubated under the same conditions along with the cultures served as controls. Alhtough PDA does not emit the same volaties as soil, *S. sclerotivorum* behaves the same toward sclerotia produced on PDA as it does to those produced in soil. Volatiles emitted by the PDA were analyzed the same day as were the volatiles from the sclerotia. Sclerotia of *S. minor* used for spore germination studies were removed from plates with forceps and rinsed briefly in sterile distilled water before they were placed in drops of spore suspensions.

Sp. sclerotivorum strain CS-5 was originally isolated in Maryland by P. B. Adams. It was grown in deep-dish Petri dishes (9-cm-diameter, 7.5 cm tall) on sand−vermiculite moistened with SM-4 medium (*5*) under ambient conditions. Cultures were 4−6 months old when used.

GC-MS Analysis. Sclerotia (0.2−0.3 g) were removed from PDA plates with forceps, inserted into 1.5-mL autosampler vials, and sealed with septum caps. A small amount of PDA adhered to the sclerotia. The vials and caps had been baked at 120° C overnight to remove extraneous volatiles before the sclerotia samples were added. The PDA controls weighed 0.1−0.2 g, which was more PDA than that which adhered to the sclerotia. Peaks appearing both in the PDA control and in sclerotial samples were excluded from further consideration.

Analyses were performed using an HP 5973 GC-MS (Palo Alto, CA) fitted with a Leap Technologies Combi Pal autosampler (Carrboro, NC). The headspace from the vials was analyzed 4−8 h after capping. Longer times could not be used because fungal depletion of oxygen from respiration could alter the physiology of the fungus and therefore the volatiles produced. Adsorption of headspace volatiles by SPME with a 100 μm poly(dimethylsiloxane) or a divinylbenzene/carboxen in poly(dimethylsiloxane) fiber occurred under agitation (10 s on and 1 s off) at 250 rpm for 1 h at 25° C. Volatiles were desorbed from the SPME fiber under pulsed splitless conditions at 25 psi for 1 min at 250° C. The helium carrier flow was set at a constant linear velocity at 40 cm/s. The GC column was a Phenomenex, 95% dimethyl/5% diphenyl siloxane, 30 m × 0.25 mm i.d., with a 0.3-μm film. The GC oven was held initially at 50 °C for 1 min, then ramped at 5 °C/min to 100 °C, at 10 °C/min to 200 °C, and at 25 °C/min to 275 °C, and held for 3 min for a total run time of 27 min.

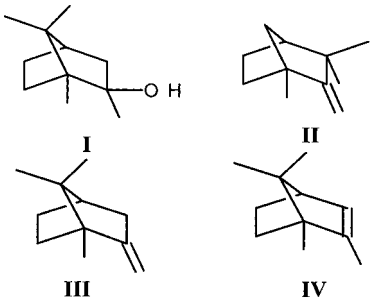

Figure 1. Structures of the dehydration or biodegradation products of 2-methylisoborneol: (**I**) 2-methylisoborneol; (**II**) 1-methylcamphene; (**III**) 2-methylenebornane; and (**IV**) 2-methyl-2-bornene.

The HP 5973 MS was operated in scan mode from *m*/*z* 33 to 300 under EI conditions at 70 eV. The ion source was held at 230 °C. Data were acquired and analyzed using the HP Chemstation software, which contained the Wiley mass spectral library (version 7.0).

A tentative identification of sclerotial volatiles was made using a computerized search of this library. Compounds were considered to be positively identified if their retention times and mass spectra closely matched those obtained for authentic compounds. Authentic samples of these chemicals were obtained from Sigma except for the mixture of 2-methylenebornane, 1-methylcamphene, and 2-methyl-2-bornene, which was obtained by dehydration of 2-methylisoborneol according to the method of Lapalme et al. (*6*). Structures of 2-methylisoborneol and its dehydration products are shown in **Figure 1**.

***Sp. sclerotivorum* Germination Bioassays.** Chemicals tested for stimulation of germination of conidia of *Sp. sclerotivorum* are listed in **Table 1**. Chemicals were tested mixed with conidial suspensions, as well as near, but not in contact with, conidial suspensions (**Figure 2**). In both cases, 6-cm-diameter plastic Petri dishes were lined with 6-cm-diameter filter paper (Whatman No. 1) moistened with 1 mL of sterile distilled water. A plastic paper clip was placed in each dish, and a 22-mm-square glass cover slip was placed on the plastic paper clip.

Except for 2-methylisoborneol, chemicals were diluted in ethanol (95.0%, ACS spectrophotometric grade; Aldrich, Milwaukee, WI) to result in 1 part per thousand. These were then diluted with sterile distilled water to give 100, 10, and 1 ppm and 100, 10,, and 1 ppb. Except for 2-methylisoborneol, these six concentrations were tested.

Table 2. Semiquantitative Relative Amounts of 2-Methylisoborneol and Its Dehydration or Biodegradation Products 2-Methylenebornane and 1-Methylcamphene Emitted by Eclerotia of *S. minor* and *S. sclerotiorum*[a]

	GC peak area ratio (range)[b]		
	2-methylenebornane /	1-methylcamphene /	1-methylcamphene /
fungus	2-methylisoborneol	2-methylisoborneol	2-methylenebornane
S. minor	0.7 (0.45–1.1)		
S. sclerotiorum	8.7 (4.1–14.3)	0.9 (0.42–1.73)	0.1 (0.09–0.122)

[a] Figures represent the proportion of 2-methylisoborneol and its dehydration or biodegradation products to each other. [b] Average of seven samples. Sclerotia were grown on potato dextrose agar for 9–23 days in the dark at 25 °C.

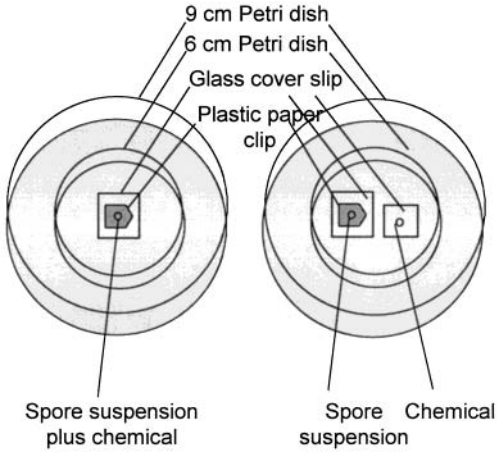

Figure 2. Germination of spores of *Sp. sclerotivorum* in response to chemicals tested both with chemicals mixed with spores (left) and with spores exposed to the vapors of the chemicals (right).

2-Methylisoborneol is supplied as 1% in methanol. This was diluted in sterile distilled water to obtain 100, 10, and 1 ppb, and these three concentrations were tested. When chemicals were tested in combination, they were mixed to result in 1 part per thousand of each in ethanol and then further diluted in sterile distilled water to achieve the same six concentrations as above.

Conidia of *Sp. sclerotivorum* were harvested with forceps and placed in sterile distilled water. Conidia were counted using an eosinophil counting slide (Thomas Scientific, Philadelphia, PA). Conidial suspensions were adjusted with sterile distilled water to achieve 10^3 spores/mL.

When chemicals were tested mixed with the conidial suspension, a 25-μL drop of conidial suspension was placed on the cover slip and then a 25-μL drop of the chemical was added to this drop (**Figure 2**). When the chemical was tested apart from the conidial suspension, an 18-mm-square cover slip was placed on the filter paper near the paper clip with the larger cover slip. A 25-μL drop of conidial suspension plus a 25-μL drop of sterile distilled water was placed on the 22-mm cover slip, and a 25-μL drop of the diluted chemical was placed on the 18-mm cover slip. The two different sizes of cover slips were used to help distinguish which held the chemical and which held the conidial suspension. Control treatments included conidial suspension alone, conidial suspension plus a single sclerotium of *S. minor*, conidial suspension plus 1:10 (v:v) ethanol/sterile distilled water, and condial suspension plus a single sclerotium plus ethanol. When 2-methylisoborneol was tested, 1 part per thousand of methanol was substituted for ethanol in the control treatment.

Lids were placed on each 60-mm Petri dish, and these were placed inside 9-cm-diameter Petri dishes lined with filter paper (Whatman No. 3) moistened with 5 mL of sterile distilled water. These were placed under glass cylinders (22.5-cm height, 11.5-cm diameter) closed at one end. Each glass cylinder contained only one concentration of the chemical. All work was conducted under a fume hood. Except for when combinations of chemicals were specifically tested, only one chemical was tested at a time. Treatments were replicated twice, with one Petri dish considered a replicate. All experiments were repeated once.

After 4−5 days, cover slips were inverted onto glass slides and observed microscopically to determine the percentage of conidial germination.

RESULTS

Volatile organic compounds emitted by sclerotia of *S. minor*, *S. sclerotiorum*, and *Sclerotium rolfsii* and identified by GC-MS are presented in **Table 1**. The mass spectrum of 2-methylisoborneol was somewhat distorted when the ion source temperature was 230 °C, and a better match with the spectral library resulted when the temperature was reduced to 150 °C.

S. minor also emitted 2-methylenebornane, whereas *S. sclerotiorum* emitted 2-methylenebornane plus 2-methyl-2-bornene and 1-methylcamphene. These MW 150 terpenoids are dehydration or biodegradation products of 2-methylisoborneol, and their relative concentrations in the headspace volatiles are given in **Table 2**. Identification of these compounds was made by comparison of the mass spectra with those reported in Schumann and Pendleton (*7*). 2-Methyl-2-bornene appeared only in trace quantities. These dehydration products of 2-methylisoborneol were not produced in significant amounts by thermal breakdown in the GC, nor did they result from microbiological contamination of the sclerotia.

In addition to the volatiles named in **Table 1**, *S. minor* occasionally emitted 2-ethyl-1-hexanol and *S. sclerotiorum* emitted small quantities of hydrocarbons, several unidentified MW 150 terpenoids, MW 204 sesquiterpenes, and an MW 272 diterpene, the spectrum of which most closely matched that of pimara-8(14),15-diene or sandaracopimaradiene in the spectral library. *Sclerotium rolfsii* emitted small quantities of δ-cadinene, *cis*-calamenene and several unidentified MW 204 sesquiterpenes. Approximate quantities of 2-methylisoborneol, 2-methylenebornane, and 2-methylcamphene are listed in **Table 2**.

The age of the fungal growth affected the production of volatiles. Sclerotia that grew for 6 days on PDA emitted few volatiles, whereas those growing for 9 days and longer emitted sufficient quantities of volatiles for routine detection.

Germination of *Sp. sclerotivorum* conidia in the presence of sclerotia of *S. minor* ranged from 30 to 90%. No conidia germinated in the absence of sclerotia. None of the chemicals tested, alone or as combinations of all chemicals, induced germination (data not shown).

DISCUSSION

Although the effects of volatile compounds emitted by higher plants on germination of fungal spores are well-known (*4*), less is known about the effects of volatiles from one fungus on other fungi. We were not able to identify the chemicals from sclerotia that trigger germination in *Sp. sclerotivorum*, but we think it is important to document chemicals emitted by these sclerotia. The chemicals that trigger germination may not have sufficient volatility to be detected by the SPME-based GC-MS method employed.

A surprising discovery was 2-methylisoborneol, which has an earthy/musty/camphorus odor and heretofore was associated only with actinomycetes, cyanobacteria, blue-green algae, and *Penicillium* spp. (*8*−*13*). 2-Methylisoborneol was emitted both by both *S. minor* and *S. sclerotiorum* but not *Sclerotium rolfsii*. 2-Methylisoborneol emitted by actinomycetes was correlated with spore germination of *Gigaspora margarita*, a vesicular-arbuscular mycorrhizal fungus (*14*). Degradation of 2-methylisoborneol to 2-methylenebornane has also been reported for a *Pseudomonas* sp. (*15*).

Basidiomycetes are known to produce many volatile compounds including 4-methyl-3-penten-2-one, δ-cadinene, nerolidol, linalool, and linalool oxides (*16*−*19*). An ascomycete, *Ceratocystis populina*, has been reported to produce trace amounts of linalool and δ-cadinene (*20*). Small changes in the composition of the culture medium for ascomycetes and basidiomycetes can cause changes in the production of volatiles (*19*). Linalool, nerolidol, and δ-cadinene are prominent green tea aroma and flavor volatiles and each has shown antimicrobial activity (*21*).

Dead fungal propagules in soil are quickly degraded. Live propagules, particularly if they are dormant, defend themselves from microbial attack. Because of the antimicrobial properties of some of the volatiles we identified, it is possible that these are involved in the defense of sclerotia from microbial degradation, and this possibility should be investigated.

ACKNOWLEDGMENT

We thank Rebecca Batista for assistance with mass spectommetric analysis.

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Received for review December 27, 2001. Revised manuscript received April 2, 2002. Accepted April 3, 2002. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

JF0117098