Chem. Senses 33: 379–387, 2008
doi:10.1093/chemse/bjn005
Advance Access publication February 17, 2008

Antennal and Behavioral Responses of *Cis boleti* to Fungal Odor of *Trametes gibbosa*

Prodpran Thakeow, Sergio Angeli, Bernhard Weißbecker and Stefan Schütz

Institute of Forest Zoology and Forest Conservation, University of Göttingen, 37077 Göttingen, Germany

Correspondence to be sent to: Prodpran Thakeow, Institute of Forest Zoology and Forest Conservation, University of Göttingen, 37077 Göttingen, Germany. e-mail: pthakeo@gwdg.de

Abstract

Cis boleti (Coleoptera: Ciidae) preferentially colonizes fungi from the genus *Trametes* that are known as important wood decomposers. The aim of our research was to investigate if *C. boleti* uses the chemical volatile composition of its fungal host, *Trametes gibbosa*, as a key attraction factor. Therefore, the *T. gibbosa* fruiting body volatiles were analysed by using gas chromatography–mass spectrometry, with parallel electroantennographic detection (GC-MS/EAD) using adults of *C. boleti*. Furthermore, we examined the behavioral responses of *C. boleti* to the *T. gibbosa* volatile compounds. The dominant component of the *T. gibbosa* fruiting body bouquet was 1-octen-3-ol. Other volatiles, like the aldehydes hexanal, nonanal, and (*E*,*E*)-2,4-decadienal and the terpene α-bisabolol, were present in minor quantities. 1-Octen-3-ol was released with a ratio of the (*R*)- and (*S*)-enantiomers of 93:7, respectively. Electroantennography (EAG) employing *C. boleti* antennae yielded consistently dominant responses to 1-octen-3-ol. GC–EAD and EAG responses to pure standard compounds showed that *C. boleti* also perceived other host fungal volatiles. A highly significant attraction to 1-octen-3-ol was observed in behavioral tests. Female beetles were significantly attracted to the (*S*)-(+)- enantiomer at 10 times lower doses than male beetles. Our finding is the first direct proof that ciid beetles use 1-octen-3-ol as a key cue for host finding.

Key words: EAG, fungivorous insect, GC–MS/EAD, host selection, (*S*)-(+)-1-octen-3-ol, VOCs

Introduction

Wood-rotting fungi, like bracket fungi, are known to release a wide range of volatile organic compounds (VOCs) such as alcohols, aldehydes, esters, ketones, acids, and terpenoids (Gross et al. 1989; Fäldt et al. 1999; Rösecke and König 2000; Rösecke et al. 2000; Ewen et al. 2004; Thakeow et al. 2006; Ziegenbein et al. 2006). Two important features of these fungal volatile patterns are the group of 8-carbon aliphatic compounds (C8 compounds) and the group of terpenoids (Fäldt et al. 1999; Thakeow et al. 2007). The C8 compounds as octanols, octenols, and octanones are common and contribute to the typical fungal odor (Tressl et al. 1982; Wurzenberger and Grosch 1982; Gross et al. 1989). Especially, 1-octen-3-ol, known also as mushroom alcohol, is the most characteristic fungal volatile, being used as aroma in several food industries (Hadar and Dosoretz 1991). The pattern of VOCs released by fungi is often species specific (Wheatley 2002). However, recent analyses showed that VOC pattern may be affected considerably by the developmental stage and/or the type of fungal substrate (Wheatley et al. 1997; Zeppa et al. 2004; Thakeow et al. 2007). Fungi of the genus *Trametes* (Basidiomycetes) are growing on different substrates as the trunks of dead trees, stored wood logs, and construction wood causing considerable economic losses (Zabel and Morrell 1992). At the other side, these fungi are ecologically important as major organisms decomposing biomass in forests (Boddy 1991; Boddy and Watkinson 1995).

Ciid beetles belong to the family Ciidae (Coleoptera) and are a group of fungivorous insects that colonize bracket fungi (Basidiomycetes: Polyporaceae) (Lawrence 1973; Hanski 1989). Most ciid beetles are smaller than 3 mm, and host specificity ranges from monophagous to polyphagous. For example, *Cis bilamellatus* has no host preference among bracket fungi, whereas *Cis nitidus* is specialized to *Ganoderma adspersum* (Guevara et al. 2000b). *Cis boleti* is oligophagous, its host range is restricted to the genus *Trametes* (Fossli and Andersen 1998; Guevara et al. 2000b), and it is often found associated to *Trametes gibbosa* and *Trametes versicolor* in the German beech forests. To the best of our knowledge, there are no direct evidences of which chemicals are perceived for host selection of ciid beetles, although

© The Author 2008. Published by Oxford University Press. All rights reserved.
For permissions, please e-mail: journals.permissions@oxfordjournals.org

Downloaded from http://chemse.oxfordjournals.org/ by guest on October 3, 2012

it has been hypothesized that the chemical composition of fungal volatiles plays an important role (Jonsell and Nordlander 1995, 2004; Guevara et al. 2000, 2000b; Komonen and Kouki 2005).

To find out whether fungal volatiles play a role in host selection of ciid beetles, we characterized the fungal volatiles of *T. gibbosa* using gas chromatography–mass spectrometry (GC–MS), verified the antennal responses by GC–electroantennographic detection (GC–EAD), and performed behavioral tests for host selection of *C. boleti*.

Materials and methods

Collection of fungi and insects

Fruiting bodies of *T. gibbosa* (Pers.) Fr., growing on decayed beech (*Fagus sylvatica*), were collected from a natural forest protection area close to Göttingen (Germany), called Königsbuche. The fruiting bodies were stored in a cool, dark room at 10 °C throughout the experiments. In order to prevent desiccation, the fruiting bodies were sprayed with tap water every week. Adults of *C. boleti* were taken directly from the *T. gibbosa* fruiting bodies, sexed, and used for all experiments.

Extraction of *T. gibbosa* volatiles

In order to obtain a sufficient sample of volatile compounds from the *T. gibbosa* fruiting bodies, we performed a Soxhlet extraction. Twelve grams of *T. gibbosa* fruiting bodies were cut into small pieces and were put in an extraction thimble. Soxhlet extraction was carried out using a conventional system consisting of 250-ml round bottom flask. The extraction was done with 150 ml dichloromethane (GC grade, Merck, Darmstadt, Germany) for 6 h at the solvent reflux temperature. The original extract was reduced to 2.0 ml on a rotary evaporator at 40 °C and standard atmosphere. The final extract was stored at −80 °C and used for *T. gibbosa* volatile identification as well as for the physiological and behavioral tests of *C. boleti*.

Trametes gibbosa headspace analysis and emission rate of 1-octen-3-ol

In order to investigate the headspace constitution of the *T. gibbosa* bouquet, we decided to collect the fungal headspace volatiles using prepacked Tenax—TA type adsorbent tubes (Gerstel, Mülheim an der Ruhr, Germany, article number 012260-005-00). The adsorbents (60/80 mesh) have a surface area of 35 m^2 g^{-1} and a density of 0.25 g ml^{-1}. The dimension of adsorbent bed is 60 mm in length and 4 mm in diameter. The sampling was carried out for 30 min using close-loop stripping technique with a flow rate of 160 ml min^{-1}. Each sample, ~1 cm^3 of fruiting body, was placed in a 250-ml polytetrafluoroethylene (PTFE) bottle. We tested 2 samples of minimally (<10%) and 3 samples of fully (~100%) colonized *T. gibbosa* fruiting bodies. The sampling was started after each sample was placed in the PTFE bottle for 10 min. The headspace volatiles were then analyzed by means of GC and MS. After the headspace sampling was done, we dried the fruiting bodies in an oven at 105 °C till a constant weight was obtained. The emission rate of 1-octen-3-ol was calculated by considering the quantity of released compound and expressed per mass of dried fruiting body and time (nanogram per gram per hour).

Chemicals

1-Pentanol (99%, Aldrich, Steinheim, Germany), 1-hexanol (99%, Aldrich), 3-octanol (>97%, VWR, Darmstadt, Germany), (*E*)-2-octen-1-ol (97%, Merck), 1-octanol (98%, Aldrich), 1-octen-3-ol (>98%, Merck), (*S*)-(+)-1-octen-3-ol (99.8%, ACROS, Geel, Belgium), (*R*)-(−)-1-octen-3-ol (99%, ACROS), 3-octanone (>96%, VWR), hexanal (98%, Aldrich), heptanal (95%, ACROS), (*E*)-2-heptenal (97%, Aldrich), nonanal (>98%, Merck), (*E*,*E*)-2,4-heptadienal (90%, Aldrich), (*E*,*E*)-2,4-decadienal (90%, ABCR, Karlsruhe, Germany), acetic acid (>99.8%, Fluka, Buchs, Switzerland), propanoic acid (>99.5%, Aldrich), butanoic acid (99%, ABCR), hexanoic acid (98%, Merck), octanoic acid (>99%, Merck), α-bisabolol (>85%, Fluka), 2-pentyl furan (98%, ABCR), and 2-methyl phenol (>99%, Aldrich).

GC–MS analysis

A GC (6890N, Agilent Technologies, Palo Alto) coupled to an MS (5973, Agilent Technologies) was used for analyzing the constituents in the extract and the headspace VOCs of *T. gibbosa*. Two column types, HP-5MS (nonpolar column, Agilent Technologies), 30 m × 0.25 mm internal diameter (i.d.) and 0.25 μm film thickness, and INNOWAX (polar column, Agilent Technologies), 30 m × 0.25 mm i.d. and 0.25 μm film thickness, were used in order to validate the composition of the samples. The extract was injected in a quantity of 1 μl into the injector in the pulsed splitless mode at a temperature of 250 °C. Helium was used as a carrier gas at a flow rate of 1.0 ml min^{-1}. The oven temperature for the extract sample was programmed for an initial temperature of 50 °C, held for 1.5 min, heated at a rate of 6.5 °C min^{-1} to 250 °C, and held for 10.0 min at 250 °C. The operating conditions of GC–MS for analyzing the Tenax headspace samples were as follows. A thermodesorption system with a cold injection system (Gerstel) was used. The tubes were heated up to 280 °C in order to desorb the VOCs. The temperature program started at 40 °C, held for 3 min, heated at a rate of 7.5 °C min^{-1} to 200 °C, and held for 5 min at 200 °C. Helium was used as a carrier gas at a flow rate of 1.0 ml min^{-1}. The MS was operated in the scan mode in a range of 20–400 amu, a source temperature of 230 °C, and electron ionization mode at 70 eV.

Identification and quantification of *T. gibbosa* volatiles

The chromatograms were preliminarily interpreted with Enhanced ChemStation version D.02.00.275 (Agilent Technologies), the Mass Spectral Search library of the National

Downloaded from http://chemse.oxfordjournals.org/ by guest on October 3, 2012

Institute of Standards and Technology (Gaithersburg, MD), and the Massfinder version 3.0 software together with the library "Terpenoids and Related Constituents of Essential Oils" (Hochmuth, König, Joulain, Hamburg, Germany). The interpretation was confirmed by matching the mass spectra and retention times with those of authentic standards on the 2 different columns used. Linear retention indices were calculated for each identified volatile using the retention times of *n*-alkane series (from C_{10} till C_{23}) as reference compounds, applying the linear equation developed by Van den Dool and Kratz (1963). The quantification of 1-octen-3-ol was done by 5-point calibration of the standard compound and evaluation of peak areas in the selected ion-monitoring mode. Moreover, quantification of the volatile compounds was performed by passing the *T. gibbosa* extract on a GC and a flame ionization detector (GC–FID; Agilent Technologies) using 1-octen-3-ol as an internal standard.

The enantiomer identification of 1-octen-3-ol of the *T. gibbosa* extract was done in cooperation with Dr Holm Frauendorf (Institute of Organic and Molecular Chemistry, Faculty of Organic Chemistry, Göttingen University) by performing enantioselective GC on a Carlo Erba Instrument, HRGC 5300 Mega Series (Milan, Italy). The instrument was equipped with a 25 m × 0.25 mm i.d. column coated with heptakis (2,3-di-*O*-acetyl-6-*O*-TBDMS)-β-cyclodextrin (50% in OV1701) (Prof König, Hamburg, Germany). The injector (split/splitless) and FID were operated at 220 °C and 240 °C, respectively. The experiment was run isothermally at 80 °C. Two standards of *rac*-1-octen-3-ol and (*S*)-(+)-1-octen-3-ol were used.

Electrophysiological measurements

The electrophysiological analyses were carried out with GC–MS/EAD, as described by Weissbecker et al. (2004). The insect antenna was dissected and placed in an antenna holder (Professor Koch, Kaiserslautern, Germany) (Färbert et al. 1997), where both ends of the antenna were immersed in an adapted insect hemolymph electrolyte solution (Kaissling and Thorson 1980). The antennal activity of 10 males and 10 females of *C. boleti* adults was recorded. As the antennae of *C. boleti* adults are quite small (0.7–1 mm long), each excised antenna was mounted on antenna holder using a piece of filter paper to make sure that the contact with the electrolyte solutions was effective. This experiment was done by injecting 1 μl of *T. gibbosa* extract on an HP 6890N GC equipped with INNOWAX column, with the same parameters as for GC–MS analyses. The EAD was carried out under humidified air at room temperature with a flow rate at 20 l h^{-1}.

Electroantennography (EAG) was employed to confirm the responsiveness of the antennae to 10^{-3} dilution in paraffin oil (Uvasol, Merck) of the following chemicals: 1-pentanol, 1-hexanol, 3-octanol, (*E*)-2-octen-1-ol, 1-octanol, 1-octen-3-ol, 3-octanone, hexanal, heptanal, (*E*)-2-heptenal, octanal, (*E*)-2-octenal, nonanal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-decadienal, acetic acid, propanoic acid, butanoic acid, hexanoic acid, octanoic acid, α-bisabolol, 2-pentyl furan, and 2-methyl phenol. For the 2 enantiomers of 1-octen-3-ol, we performed a dilution series from 10^{-6} to 10^{-3} in order to obtain a dose–response curve. Small pieces of filter paper (2 cm^2; Schleicher & Schuell, Dassel, Germany) were soaked with 100 μl of the standard dilution or paraffin oil only (control). The filter paper was inserted into a 10-ml glass syringe (Poulten & Graf GmbH, Wertheim, Germany). A reproducible stimulus was supplied by puffing 5 ml of air over the antenna (Schütz et al. 1999). The EAG response of the antennae was recorded for each standard dilution from at least 5 male and 5 female beetles. The response to paraffin oil was considered as negative control and was subtracted from all the reported EAG measurements.

Behavioral tests

Behavioral investigations were done by using a dual-choice bioassay. The setup consisted of a closed Petri dish (13 cm diameter) used as an arena. A filter paper (1.5 cm^2) treated with the tested sample (as *T. gibbosa* fruiting body extract or 1-octen-3-ol) was placed in a side part of the Petri dish (1 cm from the border), and a control filter paper treated with paraffin oil as control was placed in the opposite side. In a typical experiment, 10 animals of the same sex were placed in the middle of the Petri dish and were allowed to walk for 15 min in the darkness. The number of beetles was then counted, regarding the test compound area, the neutral area (a central segment of 2 cm), and in the control area. Two indices were calculated: the activity index (A_cI) as the number of active animals (=total number of beetles placed in the arena minus the beetles in the neutral area) divided by the total number of beetles placed in the arena and the attraction index (A_tI) as the number of beetles found in the test compound area divided by the number of active animals. The significance of the results was statistically evaluated by an analysis of variance using Statistica 7 (2004) (Statsoft Inc., Tulsa, OK).

We performed different bioassays with this setup. Experiment 1: This experiment was done in order to ensure the similarity of the *T. gibbosa* extract to the fruiting body volatiles. *Trametes gibbosa* extract was diluted with GC grade dichloromethane (Merck) to obtain a concentration of 1-octen-3-ol, which showed to have a similar release rate of 1-octen-3-ol as the fresh *T. gibbosa* fruiting body by performing headspace analysis of both samples. In one site of the Petri dish, 50 μl of the diluted *T. gibbosa* extract was dropped on the filter paper, and on the opposite side, a cube (1 × 1 × 0.5 cm^3) of fresh fruiting body was placed. Experiment 2: This experiment was done in order to measure the attraction of *C. boleti* to different doses of (*R*)-(−)- and (*S*)-(+)-1-octen-3-ol. Dilution series of 10^{-5}–10^{-3} were prepared by mixing the pure enantiomers with paraffin oil. In one site of the Petri dish, 20 μl of paraffin oil solution was

Downloaded from http://chemse.oxfordjournals.org/ by guest on October 3, 2012

dropped on the filter paper, whereas on the opposite side, only paraffin oil (20 μl) was dropped on the filter paper as a control.

Results

Volatile compounds of *T. gibbosa* extract

A total of 23 volatile chemical compounds were characterized with GC–MS and GC–FID analyses of *T. gibbosa* extract. The identified compounds are listed in Table 1, where they are shown in an elution order according to the nonpolar column and they are classified with regard to their chemical functions. The volatile compounds were categorized into 6 broad groups: alcohols, ketones, acids, aldehydes, terpenoids, and aromatics. The most abundant compounds were 5 alcohols (49%) comprising 1-octen-3-ol, 1-hexanol, 1-octanol, 3-octanol, and (*E*)-2-octen-1-ol; 8 aldehydes (45%) comprising hexanal, heptanal, octanal, nonanal, (*E*)-2-heptenal, (*E*)-2-octenal, (*E*,*E*)-2,4-heptadienal, and (*E*,*E*)-2,4-decadienal; 1 ketone (5%) 3-octanone; and 1 terpene α-bisabolol. Two aromatics comprising 2-pentyl furan and 2-methyl phenol and 2 acids comprising hexanoic and octanoic acids were present in trace quantities. The most dominant compound was 1-octen-3-ol (37%), followed by hexanal (25%), nonanal (6%), (*E*)-2-octenal (5%), and 3-octanone (5%). More detailed information is given in Table 1.

Table 1 Volatile compounds of *Trametes gibbosa* identified by GC–MS in fruiting body headspace and extract

No.	Compounds[a]	Linear retention indices		Quantity in extract[b] (ppm)	Proportion in extract[c]	Proportion in headspace[c]	Chemical classes
		HP-5MS	INNOWAX				
1	Acetic acid	n.d.	1460	tr.	tr.	—	Acid
2	Propanoic acid	n.d.	1540	tr.	tr.	—	Acid
3	Butanoic acid	n.d.	1629	tr.	tr.	—	Acid
4	1-Pentanol	779	1237	tr.	tr.	0.01	Alcohol
5	Hexanal	803	1082	55	0.25	0.15	Aldehyde
6	1-Hexanol	879	1341	10	0.04	0.02	Alcohol
7	Heptanal	907	1183	3	0.01	tr.	Aldehyde
8	(*E*)-2-heptenal	962	1324	7	0.03	0.01	Aldehyde
9	1-Octen-3-ol	980	1438	80	0.37	0.69	Alcohol
10	3-Octanone	990	1251	10	0.05	0.03	Ketone
11	2-Pentyl furan	993	1228	tr.	tr.	tr.	Aromatic
12	3-Octanol	995	1380	5	0.02	0.03	Alcohol
13	Hexanoic acid	998	1843	tr.	tr.	—	Acid
14	Octanal	1001	1287	1	0.01	0.01	Aldehyde
15	(*E*,*E*)-2,4-heptadienal	1020	1494	tr.	tr.	—	Aldehyde
16	2-Methyl phenol	1060	1993	tr.	tr.	0.01	Aromatic
17	(*E*)-2-octenal	1062	1428	11	0.05	—	Aldehyde
18	(*E*)-2-octen-1-ol	1067	1603	7	0.03	0.01	Alcohol
19	1-Octanol	1074	1542	5	0.02	0.03	Alcohol
20	Nonanal	1106	1390	12	0.06	0.01	Aldehyde
21	Octanoic acid	1182	2050	tr.	tr.	—	Acid
22	(*E*,*E*)-2,4-decadienal	1320	1806	8	0.04	—	Aldehyde
23	β-Bisabolene	1516	—	—	—	tr.	Terpene
24	α-Bisabolol	1693	2228	4	0.02	—	Terpene

n.d., not detected.
[a]The compounds were verified by comparing the mass spectra and retention indices of authentic standards, except β-bisabolene which was identified by matching the mass spectra with library and internal databases.
[b]tr.: trace, <1 ppm.
[c]tr.: trace, less than 0.01.

Downloaded from http://chemse.oxfordjournals.org/ by guest on October 3, 2012

In the headspace analysis, there were totally 15 volatiles identified. The volatile pattern was different from the extract, but both displayed similar volatile profile with 1-octen-3-ol being the dominant volatile and followed by hexanal. In addition to 1-octen-3-ol, and the other 4 alcohols previously identified, here we detected 1-pentanol. However, this compound was also present in the fruiting body extract, but it was coeluted with the solvents. Among the other chemicals, we detected 4 aldehydes comprising heptanal, (*E*)-2-heptenal, octanal, and nonanal; 1 ketone 3-octanone; 2 aromatic compounds 2-pentyl furan and 2-methyl phenol; and 1 terpene β-bisabolene.

The emission rate of 1-octen-3-ol in the headspace was calculated by comparing minimally and fully colonized fruiting bodies. 1-Octen-3-ol was found to be released at almost 20 times higher dose in minimally colonized fruiting bodies (960 ± 250 ng g^{-1} h^{-1}) than in fully colonized fruiting bodies (50 ± 10 ng g^{-1} h^{-1}). This result was highly significant [$F(1,7) = 277.97$, $P < 0.001$].

The enantioselective GC of 1-octen-3-ol showed an average α-value of 1.04 ± 0.02 for the 2 enantiomers (*R*)-(−)- and (*S*)-(+)-1-octen-3-ol. In the fruiting body extract, the enantiomer ratios of (*R*)-(−)- and (*S*)-(+)-1-octen-3-ol were 92.9% and 7.1%, respectively.

Electrophysiological response of *C. boleti* antennae to *T. gibbosa* volatiles

Two types of experiments, GC–EAD and EAG, were carried out to study the electrophysiological response of *C. boleti* to its host volatiles as shown in Table 2. The GC–EAD analysis of *T. gibbosa* extract yielded consistent responses to 1-octen-3-ol. The antennal activity of 10 males and 10 females of *C. boleti* adults was recorded, and only 1 male and 2 female antennae did not show antennal response in correspondence with the elution of 1-octen-3-ol at the retention time of 12.93 min (Figure 1). We also recorded few responses to 3-octanone, in both male and female antennae (2 males and 1 female). Afterward, all the volatiles (23) present in the fruiting volatile body extract were tested in a 10-ng quantity by performing GC–EAD to *C. boleti* antennae. It was found that 6 out of the 23 compounds were able to elicit antennal activity at different intensity. Those compounds were (*E*)-2-heptenal, 1-octen-3-ol, 3-octanone, octanal, (*E*,*E*)-heptadienal, and nonanal. Among them, 1-octen-3-ol induced the strongest response (0.46 mV), whereas the other compounds elicited weaker responses in a range of 0.1–0.2 mV.

The EAG experiments of the same 23 standard compounds (10^{-3} dilution in paraffin oil) showed that acetic acid elicited similar responses as the paraffin oil; therefore, it was not considered as a stimulating compound. Propanoic and butanoic acids as well as (*E*,*E*)-2,4-decadienal and α-bisabolol elicited very weak responses. 1-Hexanol as well as the 4 C8 alcohols (1-octanol, 3-octanol, 1-octen-3-ol, and (*E*)-2-octen-1-ol) elicited strong EAG responses. A lower response was observed for 1-pentanol. In addition, there were 4 aldehydes (heptanal, (*E*)-2-heptenal, (*E*,*E*)-2,4-heptadienal, and nonanal) that excited the antennae in the same range as the alcohol compounds. Dose–response curves of both enantiomers of the most sensitively detected compound, (*R*)-(−)- and (*S*)-(+)-1-octen-3-ol (10^{-6}–10^{-3} in paraffin oil) for *C. boleti* males and females were recorded (Figure 2). No significant differences were observed in the EAG response to either enantiomer between the sexes [(*R*)-form, $F(1,36) = 3.72$, $P = 0.06$; (*S*)-form, $F(1,36) = 2.55$, $P = 0.12$]. There were, however, differences with respect to the sexes and doses [(*R*)-form, $F(3,36) = 10.41$, $P < 0.001$; (*S*)-form, $F(3,36) = 8.32$, $P < 0.001$]. Among the ranges of the tested doses,

Table 2 Electroantennographic responses of *Cis boleti* antennae to *Trametes gibbosa* volatiles and standard compounds

No.	Compounds	Quantity in extract (ppm)	EAG	GC–EAD	
			10^{-3} Dilution in paraffin oil	Standard compound 10 ng	*T. gibbosa* extract
1	Acetic acid	tr.			
2	Propanoic acid	tr.	+		
3	Butanoic acid	tr.	+		
4	1-Pentanol	tr.	++		
5	Hexanal	55	++		
6	1-Hexanol	10	++++		
7	Heptanal	3	++++		
8	(*E*)-2-heptenal	7	++++	*	
9	1-Octen-3-ol	80	+++++	*****	**
10	3-Octanone	10	+++	**	*
11	2-Pentyl furan	tr.	++		
12	3-Octanol	5	++++		
13	Hexanoic acid	tr.	+		
14	Octanal	1	+++	**	
15	(*E*,*E*)-2,4-heptadienal	tr.	+++++	**	
16	2-Methyl phenol	tr.	+++		
17	(*E*)-2-octenal	11	+++		
18	(*E*)-2-octen-1-ol	7	+++		
19	1-Octanol	5	++++		
20	Nonanal	12	+++	**	
21	Octanoic acid	tr.	++		
22	(*E*,*E*)-2,4-decadienal	8	+		
23	α-Bisabolol	4	+		

+, 0.0–0.3 mV; ++, 0.3–0.6 mV; +++, 0.6–0.9; ++++, 0.9–1.2; +++++, 1.2–1.5 mV; *, 0.0–0.1 mV; **, 0.1–0.2 mV; ***, 0.2–0.3 mV; ****, 0.3–0.4 mV; *****, 0.4–0.5 mV.

Downloaded from http://chemse.oxfordjournals.org/ by guest on October 3, 2012

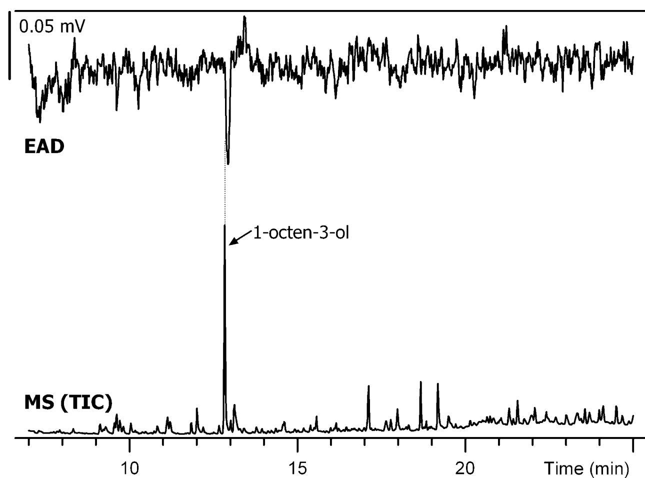

Figure 1 GC–EAD and MS chromatograms of *Cis boleti* response to 1-octen-3-ol in *Trametes gibbosa* fruiting body extract. TIC, total ion current.

the female beetles showed a small amount of scatter in their responses yielding 3 stages of dose discrimination at 10^{-6}, overlap of 10^{-5}–10^{-4}, and 10^{-3}. In comparison, the male beetles showed a higher degree of scatter in their responses yielding only 2 stages of discrimination at 10^{-6}–10^{-4} and 10^{-3}.

Behavioral test

To ensure the similarity between the fruiting body and the extract, a behavioral test was carried out. The attraction index of the fruiting body against the extract was 0.53. The activity index, which measures the induction of insect movement and searching behavior, was equal to 0.65. The attraction index revealed no statistical difference, showing that the extract has a similar attraction as the fruiting body.

A second experiment was carried out to test the preference of *C. boleti* males and females to the 2 enantiomers of 1-octen-3-ol. The results are illustrated in Figure 3A,B, showing the attraction index and the activity index, respectively. No statistical difference was found between the sexes in the attraction to the 2 enantiomers [$F(1,52) = 0.49$, $P = 0.48$]. In each sex, significant differences were observed in the attraction to each of the enantiomers [$F(1,52) = 6.50$, $P < 0.059$] and among the different dilutions [$F(2,52) = 34.67$, $P < 0.001$]. The male beetles were significantly more attracted to both enantiomers at a dilution of 10^{-3} compared with a dilution of 10^{-4} and 10^{-5}. The female beetles were significantly more attracted to (*R*)-(−)-1-octen-3-ol at a dilution of 10^{-3}, but for the (*S*)-(+)-1-octen-3-ol, they were already significantly more attracted to a dilution of 10^{-4}. In Figure 3B, the activity index induced by (*R*)-(−)-1-octen-3-ol was high for both male and female beetles throughout the range of dilutions examined. The activity index of the male beetles induced by (*S*)-(+)-1-octen-3-ol was increased to the level of the (*R*)-(−)-enantiomer at a dilution of 10^{-3}, whereas the female activity reached this level already at dilutions of 10^{-4}.

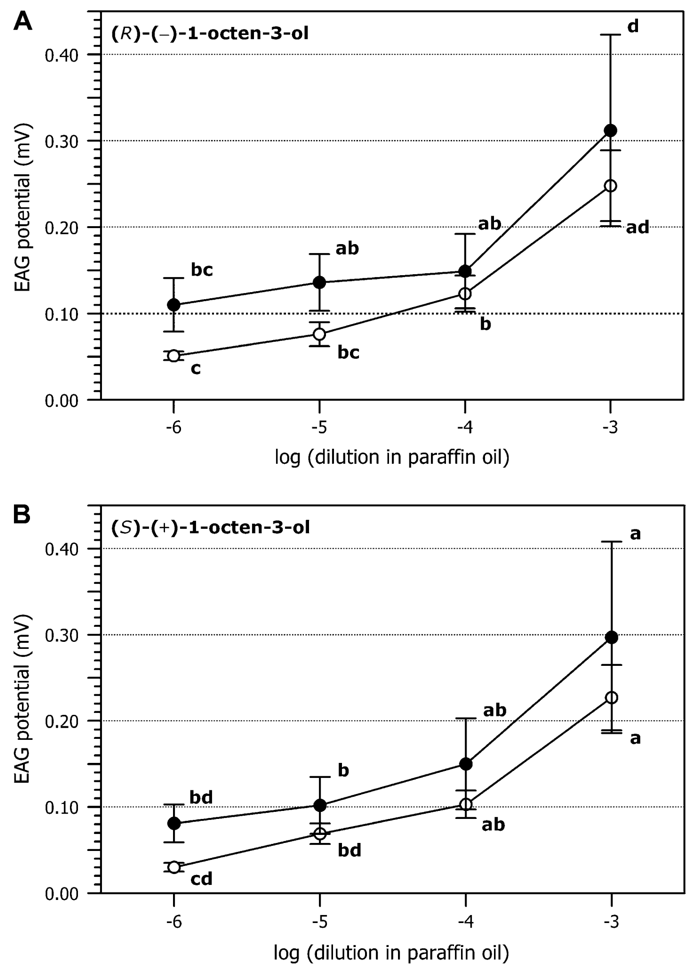

Figure 2 EAG responses (mean ± standard error) of *Cis boleti* males (filled circle) and females (open circle) to 1-octen-3-ol. **(A)** (*R*)-(−)-1-octen-3-ol and **(B)** (*S*)-(+)-1-octen-3-ol. Different letters indicate significant differences between samples.

Discussion

In ciid beetles, fungal VOCs have been proposed as a mechanism in host finding (Jonsell and Nordlander 1995, 2004; Fäldt et al. 1999; Guevara et al. 2000a). It has been hypothesized that 2 main VOC fractions may play an important role in host finding by ciid beetles: C8 compounds and terpenoids (Fäldt et al. 1999; Guevara et al. 2000a). In *T. gibbosa* fruiting body, we found a range of volatile compounds, including alcohols, terpenoids, aldehydes, and aromatic compounds. Dichloromethane extract of *T. gibbosa* fruiting body showed a composition dominated by 1-octen-3-ol (37%) and other 7 C8 compounds. The terpenoid fraction was characterized only by 1 sesquiterpenic alcohol, α-bisabolol. This compound was found in high proportion also in other wood-rotting fungi as *Schizophyllum commune* (Ziegenbein et al. 2006) and *Phlebia radiate* (Gross et al. 1989). Interestingly, in the headspace analysis, we did not find this compound but β-bisabolene in trace quantity. Bisabolene and bisabolol

Downloaded from http://chemse.oxfordjournals.org/ by guest on October 3, 2012

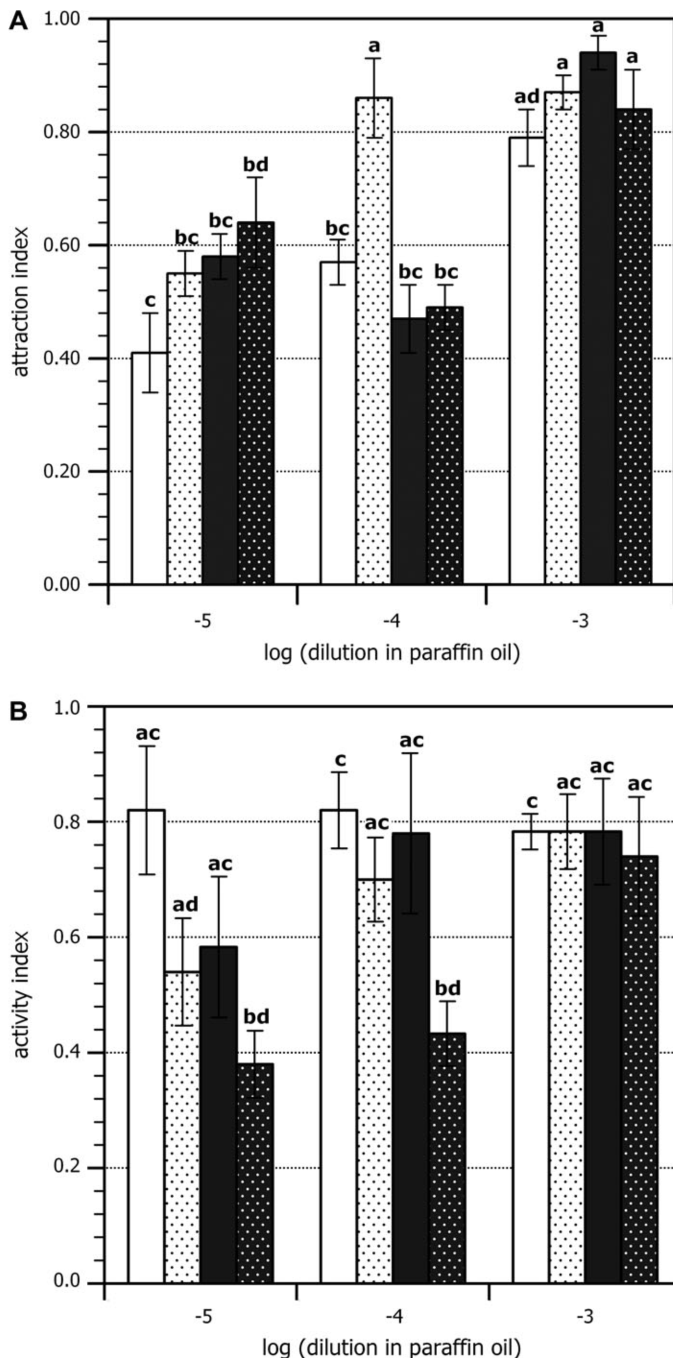

Figure 3 Behavioral responses to 1-octen-3-ol enantiomers. **(A)** Attraction index and **(B)** activity index; white bar: female, dark bar: male, bar without dots: (*R*)-form, and bar with dots: (*S*)-form. Values = (mean ± standard error). Different letters indicate significant differences between samples.

share the same precursor, farnesyl diphosphate (Jones et al. 2006). Bisabolene is released by enzymatic reaction from fungal tissues, and bisabolol is proposed to be a product of hydrolysis (Benedict et al. 2001; Köllner et al. 2004). Fruiting body extract and headspace share a similar volatile profile, although acidic compounds were not detected in the headspace, being possibly by-products of acid hydrolysis of Soxhlet extraction. Extraction method allowed the identification of 3 aldehydes, which were not identified by headspace analysis, comprising (*E*,*E*)-2,4-heptadienal, (*E*)-2-octenal, and (*E*,*E*)-2,4-decadienal.

We analyzed the release rate of 1-octen-3-ol in 2 different colonization stages, and we found that *T. gibbosa* releases from 50 to 1000 ng g^{-1} $h^{-1}$of 1-octen-3-ol, with a higher release rate in case of minimally colonized fruiting body. The higher release rate of 1-octen-3-ol in the minimally colonized fruiting body was most probably due to the fact that it was in a growing stage and had been additionally damaged by the insects. Whereas, the likely reason why there was a lower release rate in the fully colonized fruiting body was that tissues had been already consumed. Comparable release rates of 1-octen-3-ol have been found in other species of bracket fungi, also with a variation depending on the fungal age (factor of 100) and on the season (factor of 10) (Fäldt et al. 1999; Wu et al. 2005).

The enantiomeric composition of 1-octen-3-ol released by *T. gibbosa* displayed a ratio of 93:7 of the (*R*)- and (*S*)-enantiomers, respectively. The R:S of other bracket fungi species range in a species-specific manner, from a minimum of 89% to a maximum 98% of the (*R*)-enantiomer (Ziegenbein et al. 2006). For example, these authors found ratios of the (*R*)- and (*S*)-1-octen-3-ol of 90:10 in the wood-rotting fungi *S. commune* and 98:2 in *Datronia mollis* hydrodistillates.

By coupling GC with MS and EAD (GC–MS/EAD) of fruiting body extract, we proved for the first time that 1-octen-3-ol elicits consistent responses to *C. boleti* antennae. Furthermore, 3-octanone was found to elicit occasionally antennal responses in this concentration range. To prove if other *T. gibbosa* volatiles are perceived by *C. boleti* beetles, we performed GC–EAD analyses of all identified compounds in the extract by running 10 ng of each authentic standard through the GC–EAD. In these experiments, we found that the highest antennal response was elicited by 1-octen-3-ol, followed by 3-octanone, octanal, (*E*,*E*)-2,4-heptadienal, nonanal, and (*E*)-2-heptenal (Table 2). A further investigation was done by performing EAG experiments with all volatiles identified in *T. gibbosa* at higher odor concentration (10^{-3} dilution in paraffin oil). Here we found that the acid compounds elicited very weak antennal responses, in agreement with the observation that they are not constituents of fruiting body volatiles but present in the extract as by-products. The alcohol fraction together with the aldehyde fraction elicited strong EAG responses. (*E*,*E*)-2,4-heptadienal showed similar response as 1-octen-3-ol, although in GC–EAD experiment we recorded a lower response than the one of 1-octen-3-ol. The only terpenoid with more than trace contribution to the fungal volatile pattern, α-bisabolol, elicited very weak EAG response. Moreover, in behavioral experiments, fresh fruiting body compared with fruiting body extract showed similar attraction to the beetles. We may therefore suppose that terpenoids do not play a major role in host finding. Therefore, we focus our attention on

Downloaded from http://chemse.oxfordjournals.org/ by guest on October 3, 2012

1-octen-3-ol, which is the major component of the fungal volatile bouquet and elicits the strongest antennal response. In behavioral test, the female beetles showed a statistically higher attraction to a dose of the (*S*)-enantiomer equal to 10^{-4}, whereas a dose equal to 10^{-3} was necessary to show a statistically higher attraction for the (*R*)-enantiomer. The males were strongly attracted only to the high dose (10^{-3}) and did not show a discriminated behavior between the 2 enantiomers. Moreover, the searching activity of female beetles was increased selectively by even the lower release rates of (*S*)-(+)-1-octen-3-ol. Such an enantiomeric discrimination has been observed in several other insect species, both at the electrophysiological and at the behavioral level (Ulland et al. 2006), and convincing examples have been found in pheromone perception (Kozlov et al. 1996). For instance, the cabbage moth, *Mamestra brassicae*, detected enantiomers of linalool with different intensity, showing 10 times higher sensitivity to (*R*)-(−)-linalool than to (*S*)-(+)-linalool (Ulland et al. 2006). However, in this study, it is the first time that an enantiomeric discrimination for ciid beetles has been demonstrated. The results of the behavioral assays suggested that female beetles play a major role in the fruiting body colonization of bracket fungi. This assumption is in agreement with the results reported by Fäldt et al. (1999), where a higher number of wood-decomposing female beetles were found in 1-octen-3-ol baited traps. Moreover, also in the deathwatch beetle, *Xestobium rufovillosum* (Coleoptera, Anobiidae), females perform the first colonization of fungal decayed wood (Fisher 1940; Belmain et al. 1998). The task of colonizing pioneers is not only to locate and to identify a suitable fungus species but also to assess the developmental stage and age of the fruiting body. *Cis boleti* is reported to be frequently a second colonizer following a first colonization made by the ciid beetle *Octotemnus glabriculus* (Guevara et al. 2000a). The activity of the first insects colonizing a fungal host causes damage to the fruiting body and consequently alters the pattern of VOCs. This variation in the volatile profile may be an important hint for host suitability to second-colonizer ciid beetles and allows a successive colonization of the fungal fruiting bodies.

In this study, it was shown that minimally colonized fruiting bodies release 1-octen-3-ol at high emission rates. Moreover, it was demonstrated in the electrophysiological and behavioral tests that *C. boleti* beetles are able to perceive and to behaviorally respond to 1-octen-3-ol. The 2 enantiomers of 1-octen-3-ol affect the behavior of female and male beetles in a different manner. Thus, 1-octen-3-ol emission rate and enantiomeric ratio are important information for host fungus suitability. These results do not exclude that other volatiles detected at higher concentrations may contribute to host discrimination, whereas 1-octen-3-ol is important in host finding. Whether the differences in the enantiomeric ratios of 1-octen-3-ol in various fungal species are sufficient to allow ciid beetles to differentiate between fungal species needs further confirmation. In order to understand better the mechanisms of host selection in ciid beetles, more investigations of the volatile composition during fungal development are needed. Moreover, the distribution of the antennal sensilla as well as analysis with single sensillum recording could allow characterizing the olfactory structures responsible for enantiomeric discrimination and fungal volatile perception in these insects.

Funding

Royal Thai Government, Chiang Mai University (P.T.).

Acknowledgements

The authors would like to thank Dr Norbert Menke, Forestry Experimental Station, Lower Saxony, Germany, for insect classification and Dr Holm Frauendorf, Institute of Organic and Molecular Chemistry, Faculty of Chemistry, Göttingen University, for 1-octen-3-ol enantiomer identification.

References

Belmain SR, Blaney WM, Simmonds MSJ. 1998. Host selection behaviour of deathwatch beetle, *Xestobium rufovillosum*: oviposition preference choice assays testing old *vs* new oak timber, *Quercus* sp. Entomol Exp Appl. 89:193–199.

Benedict CR, Lu JL, Pettigrew DW, Liu J, Stipanovic RD, Williams HJ. 2001. The cyclization of farnesyl diphosphate and nerolidyl diphosphate by a purified recombinant δ-cadinene synthase. Plant Physiol. 125:1754–1765.

Boddy L. 1991. Importance of wood decay fungi in forest ecosystems. In: Arora DK, Rai B, Mujerji KG, Knudsen GR, editors. Handbook of applied mycology. Soils and plants. Vol. 1. New York: Marcel Dekker, Inc. p. 507–540.

Boddy L, Watkinson SC. 1995. Wood decomposition, higher fungi, and their role in nutrient redistribution. Can J Bot. 73(Suppl):S1377–S1383.

Ewen RJ, Jones PRH, Ratcliffe NM, Spencer-Phillips PTN. 2004. Identification by gas chromatography-mass spectrometry of the volatile organic compounds emitted from the wood-rotting fungi *Serpula lacrymans* and *Coniophora puteana*, and from *Pinus sylvestris* timber. Mycol Res. 108:806–814.

Fäldt J, Jonsell M, Nordlander G, Borg-Karlson AK. 1999. Volatiles of bracket fungi *Fomitopsis pinicola* and *Fomes fomentarius* and their functions as insect attractants. J Chem Ecol. 25:567–590.

Färbert P, Koch UT, Färbert A, Staten RS, Cardé RT. 1997. Pheromone concentration measured with electroantennogram in cotton fields treated for mating disruption of *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). Environ Entomol. 26:1105–1116.

Fisher RC. 1940. Studies of the biology of the deathwatch beetle, *Xestobium rufovillosum* de Geer: part III, fungal decay in timber in relation to the occurrence and rate of development of the insect. Ann Appl Biol. 27:545–557.

Fossli TE, Andersen J. 1998. Host preference of Cisidae (Coleoptera) on tree-inhabiting fungi in northern Norway. Entomol Fenn. 9:65–78.

Gross B, Gallois A, Spinnler HE, Langlois D. 1989. Volatile compounds produced by the ligninolytic fungus *Phlebia radiata* Fr. (Basidiomycetes) and influence of the strain specificity on the odorous profile. J Biotechnol. 10:303–308.

Guevara R, Hutcheson KA, Mee AC, Rayner ADM, Reynolds SE. 2000. Resource partitioning of the host fungus *Coriolus versicolor* by two ciid

Downloaded from http://chemse.oxfordjournals.org/ by guest on October 3, 2012

beetles: the role of odour compounds and host ageing. Oikos. 91: 184–194.

Guevara R, Rayner ADM, Reynolds S. 2000a. Effects of fungivory by two specialist ciid beetles (*Octotemnus glabriculus* and *Cis boleti*) on the reproductive fitness of their host fungus, *Coriolus versicolor.* New Phytol. 145:137–144.

Guevara R, Rayner ADM, Reynolds S. 2000b. Orientation of specialist and generalist fungivorous ciid beetles to host and non-host odours. Physiol Entomol. 25:288–295.

Hadar Y, Dosoretz CG. 1991. Mushroom mycelium as a potential source of food flavour. Trends Food Sci Technol. 2:214–218.

Hanski I. 1989. Fungivory: fungi, insects and ecology. In: Wilding N, Collins NM, Hammond PM, Webber JF, editors. Insect-fungus interactions. London: Academic Press. p. 25–68.

Jones CG, Ghisalberti EL, Plummer JA, Barbour EL. 2006. Quantitative co-occurrence of sesquiterpenes; a tool for elucidating their biosynthesis in Indian sandalwood, *Santalum album.* Phytochemistry. 67:2463–2468.

Jonsell M, Nordlander G. 1995. Field attraction of Coleoptera to odours of the wood-decaying polypores *Fomitopsis pinicola* and *Fomes fomentarius.* Ann Zool Fenn. 32:391–402.

Jonsell M, Nordlander G. 2004. Host selection patterns in insects breeding in bracket fungi. Ecol Entomol. 29:697–705.

Kaissling KE, Thorson J. 1980. Insect olfactory sensilla: structural, chemical and electrical aspects of the functional organisation. In: Satelle DB, Hall LM, Hildebrand JG, editors. Receptors for neurotransmitters, hormones and pheromone in insects. Amsterdam (The Netherlands): Elsevier/North-Holland Biomedical Press. p. 261–282.

Köllner TG, Schnee C, Gershenzon J, Degenhardt J. 2004. The variability of sesquiterpenes emitted from two *Zea mays* cultivars is controlled by allelic variation of two terpene synthase genes encoding stereoselective multiple product enzymes. Plant cell. 16:1115–1131.

Komonen A, Kouki J. 2005. Occurrence and abundance of fungus-dwelling beetles (Ciidae) in boreal forests and clearcuts: habitat associations at two spatial scales. Anim Biodivers Conserv. 28:137–147.

Kozlov MV, Zhu J, Philipp P, Francke W, Zvereva EL, Hansson BS, Löfstedt C. 1996. Pheromone specificity in *Eriocrania semipurpurella* (Stephens) and *E. sangii* (Wood) (Lepidoptera: Eriocraniidae) based on chirality of semiochemicals. J Chem Ecol. 22:431–454.

Lawrence JF. 1973. Host preference in ciid beetles (Coleoptera: Ciidae) inhabiting the fruiting body of Basidiomycetes in North America. Bull Mus Comp Zool Harv. 145:163–212.

Rösecke J, König WA. 2000. Constituents of various wood-rotting basidiomycetes. Phytochemistry. 54:603–610.

Rösecke J, Pietsch M, König WA. 2000. Volatile constituents of wood-rotting basidiomycetes. Phytochemistry. 54:747–750.

Schütz S, Weißbecker B, Koch UT, Hummel HE. 1999. Detection of volatiles released by diseased potato tubers using a biosensor on the basis of intact insect antennae. Biosens Bioelectron. 14:221–228.

Thakeow P, Holighaus G, Schütz S. 2007. Volatile organic compounds for wood assessment. In: Kües U, editor. Wood production, wood technology and biotechnological impacts. Göttingen: Universitätsverlag Göttingen. p. 197–228.

Thakeow P, Weißbecker B, Schütz S. 2006. Volatile organic compounds emitted from fungal-rotting beech (*Fagus sylvatica*). Mitt Dtsch Ges Allg Angew Entomol. 15:157–160.

Tressl R, Bahri D, Engel KH. 1982. Formation of eight-carbon and ten-carbon components in mushrooms (*Agaricus campestris*). J Agric Food Chem. 30:89–93.

Ulland S, Ian E, Borg-Karlson A-K, Mustaparta H. 2006. Discrimination between enantiomers of linalool by olfactory receptor neurons in the cabbage moth *Mamestra brassicae* (L.). Chem Senses. 31:325–334.

Van den Dool H, Kratz PD. 1963. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. J Chromatogr. 11:463–471.

Weissbecker B, Holighaus G, Schütz S. 2004. Gas chromatography with mass spectrometric and electroantennographic detection: analysis of wood odorants by direct coupling of insect olfaction and mass spectrometry. J Chromatogr A. 1056:209–216.

Wheatley RE. 2002. The consequences of volatile organic compound mediated bacterial and fungal interactions. Antonie van Leeuwenhoek. 81:257–264.

Wheatley RE, Hackett C, Bruce A, Kundzewicz A. 1997. Effect of substrate composition on production and inhibitory activity against wood decay fungi of volatile organic compounds from *Trichoderma* spp. Int Biodeterior Biodegradation. 39:199–205.

Wu S, Zorn H, Krings U, Berger RG. 2005. Characteristic volatiles from young and aged fruiting bodies of wild *Polyporus sulfureus* (Bull.:Fr.) Fr. J Agric Food Chem. 53:4525–4528.

Wurzenberger M, Grosch W. 1982. The enzymic oxidative breakdown of linoleic acid in mushrooms (*Psalliota bispora*). Z Lebensm Unters Forsch. 175:186–190.

Zabel RA, Morrell JJ. 1992. Wood microbiology: decay and its prevention. New York: Academic Press, Inc.

Zeppa S, Gioacchini AM, Guidi C, Guescini M, Pierleoni R, Zambonelli A, Stocchi V. 2004. Determination of specific volatile organic compounds synthesised during *Tuber borchii* fruit body development by solid-phase microextraction and gas chromatography/mass spectrometry. Rapid Commun Mass Spectrom. 18:199–205.

Ziegenbein RC, Hanssen HP, König WA. 2006. Chemical constituents of the essential oils of 3 wood-rotting fungi. Flavour Frag J. 21:813–816.

Accepted January 7, 2008

Downloaded from http://chemse.oxfordjournals.org/ by guest on October 3, 2012