Electrophysiological and behavioral responses of *Tomicus minor* (Coleoptera: Scolytidae) to host volatiles

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To screen for host volatiles that effectively attract the lesser pine shoot beetle, *Tomicus minor* (Coleoptera: Scolytidae), an important forest pest, water vapor distillation extraction and gas chromatography-mass spectrometry were combined to analyze volatiles emitted from uninfested and infested shoots of *Pinus yunnanensis* (Pinaceae). Electroantennography and Y-tube olfactometer behavioral tests were used to compare and analyze the responses of male and female beetles to each of selected eight compounds. The spectrometry results showed that monoterpenes are the primary chemicals emitted by *P. yunnanensis*. The electroantennogram responses of adult *T. minor* to six of the plant compounds peaked at 10 μg/μL; the exceptions were terpinolene and myrcene, which had maxima above 100 μg/μL. (+)-α-Pinene and β-phellandrene were strongly attractive to the male and female adults.

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1. Introduction

Volatile organic chemicals released by host plants can attract insects and stimulate their feeding and oviposition (Stanjek *et al.* 1997, Ruther *et al.* 2000, Du 2001, Inui *et al.* 2003, Inui & Itioka 2007, Hu *et al.* 2009). Such kairomones include monoterpenes, fatty acid derivatives, phenylpropanoids, and isoprenoids, among others (Visser 1986, Bernays & Graham 1988, Bruce *et al.* 2005, Zhuge *et al.* 2010).

Zhang *et al.* (2007) reported that *Ips subelongatus* Motschulsky was preferentially attracted to α-pinene, β-pinene, and p-isopropyltoluene released by its host *Larix gmelinii* (Rupr.) Kuzen. Likewise, Miao *et al.* (2004) showed that *Dendroctonus valens* LeConte was attracted to (+)-3-carene of its host in China. Furthermore, a mixture of α-pinene and ethanol was strongly attractive to *T. piniperda* (Byers 1992). These studies suggest that plant volatile chemicals can be important for attraction and host location by herbivorous insects and may be effectively used to monitor and control pest activities (Allison & Cardé 2008, Williams *et al.* 2008, Ngumbi *et al.* 2009). In many cases, electroantennography followed by Y-tube olfactometer behavioral tests is used to screen and select candidate volatile chemicals that elicit responses by insects (Han & Han 2007, David *et al.* 2009).

In this study, composition and amounts of volatile chemicals were compared between uninfested and infested *Pinus yunnanensis* Franchet shoots, and electrophysiological and behavioral responses of *Tomicus minor* Hartig, 1834 (Coleoptera: Scolytidae) to synthetic compounds were assessed. This work may lead to the identification of attractants that can be used to control and monitor *T. minor*.

2. Material and methods

2.1. Lesser pine shoot beetle

2.1.1. Basic biology

The lesser pine shoot beetle, *Tomicus minor*, is an oligophagous wood boring beetle that feeds on more than 20 host species of Pinaceae, including *Pinus yunnanensis*, *P. massoniana* Lamb, *P. tabuliformis* Carr, *P. densiflora* Sieb. *et* Zucc, *Larix* spp., and *Picea* spp. (Långström 1983, Ying & Huang 1984). *Tomicus minor* is widely distributed in China, Japan, Russia, and Europe (Lundgren 2004, Alonzo-Zarazaga 2013). The beetle undergoes one generation per year and overwinters as an adult in bark (Ye *et al.* 2004, Xue *et al.* 2007). Adults exit their holes and feed on fresh shoots from April to May (Kirkendall *et al.* 2008). In China, *P. yunnanensis* is the preferred host for both larvae and adults (Ye *et al.* 2004).

After eclosion, the adults of *T. minor* fly to the crowns of adjacent *P. yunnanensis* trees and feed on shoots. After sexual maturity, they move to the trunk where they mate and oviposit. The damage to stems may cause tree mortality by disrupting water and nutrient flow between the foliage and roots (Li *et al.* 2006) and can also result in withered and broken shoots (Ying *et al.* 1984, Ye & Ding 1999). Thus, controlling the virgin male and female adults during the shoot-feeding period is critical to prevent *T. minor* outbreaks in *P. yunnanensis* stands.

2.1.2. Beetle collection and rearing

Lesser pine shoot beetles were collected from Yunnan pine plants in fields near Luding, Sichuan Province, China in May, 2013. We cut five *P. yunnanensis* trees (age: 30 years, height: 18–20 m, diameter at breast height: 20–25 cm) into 1–2 metre long pieces, and put them into several mesh cages (2.5 × 2.5 × 2.0 m) at room temperature (25 °C ± 2). The emerging female and male beetles were collected and placed in individual cages (5 cm × 5 cm × 5 cm, stainless steel mesh) at the room temperature. Female and male beetles were distinguished by the fact that male adults chirp but female adults do not (Ye 1996).

2.2. Extraction and identification of volatile chemicals emitted from shoots

Branches from both infested and uninfested 2-year-old *P. yunnanensis* were collected from Yunnan pine plants in Luding County, Sichuan Province. Branches were then cut into 1 cm pieces, and 600 g were placed into a water vapor distiller filled with 1200 mL deionized water for 4 hours as described in Wang *et al.* (2002). The distilled liquid was extracted with anhydrous ether (analytically pure; Chengdu Kelong Chemical, Chengdu, China) and dewatered with anhydrous sodium sulfate (analytically pure; Chengdu Kelong Chemical). Then, the diethyl ether was removed with concentrated N_2. Finally, 2 mL of a pale yellow transparent grease with a strong aromatic odor was obtained and stored in an ultracold freezer (–80 °C) (Sanyo, Osaka, Japan) and used for chemical identification.

The extracts were analyzed by gas chromatography (GC) (Shimadzu, model 17A; Kyoto, Japan) coupled with a Shimadzu QP5050A electron ionization mass detector (MS). The GC-MS was operated in the splitless mode, and was equipped with a DB-5 (30 m × 0.25 mm × 25 µm) capillary column (Agilent Technologies, Santa Clara, CA, USA). The column oven temperature was programmed to rise from the initial temperature of 70 °C (1 min) to 100 °C at 10 °C min^{-1}, then to 250 °C at 5 °C min^{-1}, at which the column was maintained for 2 minutes.

For the mass spectrometer, the voltage of the electron impact ion source was 70 eV, the temperature of the GC-MS connector was 250 °C, the temperature of the ion source was 200 °C, the scanning speed was 0.4 s, the scanning range was mass-to-charge ratio (m/z) 40–450, and the electric current in the filament was 150 μA. The mass spectrogram was directly checked against the standard mass spectrogram from the NIST (the national institute of standards and technology) database of this device to identify the chemical component.

2.3. Synthetic compounds

Based on the GC-MS analysis, we selected eight compounds of the highest relative abundance to be tested for EAG dose-response. Only the eight compounds were selected for the EAG dose-response tests in order to match to the limited laboratory conditions, expenditure etc. The synthetic compounds of these eight chemicals were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan): (+)-α-pinene (95% pure) and β-caryophyllene (90% pure), and from Aladdin Chemistry Co. (Shanghai, China): (β-pinene (98% pure),(+)-3-carene (90% pure), (+)-limonene (97% pure), myrcene (90% pure), terpinolene (85% pure), and β-phellandrene (99% pure). They were each dissolved in distilled hexane at concentrations of 0.01, 0.1, 1, 10, and 100 μg/μL and used for the electroantennogram analysis below.

2.4. Electroantennograms

Antennal recordings of the responses of female and male *T. minor* to the eight individual compounds were made using Syntech (Hilversum, The Netherlands) equipment comprising micromanipulators, a CS-05 stimulus air controller, and an IDAC signal connection box for data acquisition. Electroantennogram (EAG) signals and data were analyzed using a customized software package (EAG for Windows XP, Syntech). The antennae of *T. minor* were excised and mounted between Ag and AgCl glass electrodes filled with Ringer solution.

A 2-μL odor source sample (one of the eight monoterpenoid compounds) was taken with a micro-sampler. It was uniformly dripped onto a folded filter paper (1.5 × 1.5 cm) that was put into a 10-cm sample tube. The end of the sample tube was connected to an odor-stimulating control device. When the baseline was stable, the antenna was stimulated. The stimulation time was 0.5 s, and the interval between stimuli was 30 s, which permitted recovery of the antennal receptors. For each compound, twelve antennae (from six different males and females) were tested, and each antenna was stimulated five times. Distilled hexane was the standard, and the mean of the observed five values for each antenna was divided by the mean of the two standard values to give the relative antennal response (Yang *et al.* 2013).

2.5. Bioassay of behavioral responses

To test the behavioral responses of adult beetles, a glass Y-tube olfactometer with the inside diameter of 0.5 cm was used. The main arm of the device was 6 cm long, as was each side arm. The angle between the two side arms was 75 degrees, and the ends of the arms had ground-glass edges. The two side arms were connected to two 250-mL volumetric flasks by Teflon tubes. A micro-sampler was used to extract 10 μL of a compound and solution (distilled hexane) for each comparison, and the liquid was dropped onto a filter paper (1 × 1 cm).

Filter papers were separately placed into the two volumetric flasks, which were connected by Teflon tubes to a distilled water humidification bottle and a charcoal filter. The air flow speed was controlled at 0.5–0.6 L/min. The tests were run at 08:00–12:00, when the temperature of the laboratory was 25 ± 2 °C.

Adult *T. minor* was introduced into the inlet of the main arm of the Y-tube, and timing began after they had moved forward 2 cm from the inlet. Adults had to make a choice at the junction of the Y-tube. The tests were conducted for 5 min for each adult. If a beetle went forward 2 cm into a side arm and stayed for at least 1 min, it was recorded as having made a choice of that odor; otherwise, it was recorded to have made no choice. After every five adults, the two arms of the Y-tube were interchanged to eliminate the possible influence of individual arms on the insect behavior. After each treatment, the Y-tube olfactometer, Teflon tubes, and volumetric flasks were washed with ethanol and allowed to air dry. For each treatment, three replicates of 30 females and 30

Table 1. Gas chromatography-mass spectrometry results as relative abundances (%, mean ± SE, *N*=3) of 42 compounds of uninfested and infested shoots of *Pinus yunnanensis*. Retention times are shown below relative abundances.

Compound	Uninfested	Infested	*t*
Tricyclene	0.28±0.01	0.06±0.01	10.02
	3.72	3.72	*P*=0.001
(+)-α-pinene	40.8±1.28	8.29±0.80	16.23
	3.84	3.84	*P* 0.001
Camphene	1.10±0.10	0.34±0.05	7.01
	4.04	4.04	*P*=0.002
Sabinene	0.92±0.08	0.10±0.01	9.76
	4.32	4.32	*P*=0.001
β-pinene	10.48±0.70	12.39±0.94	–1.64
	4.39	4.39	*P*=0.177
α-phellandrene	0.12±0.02	0.11±0.01	0.08
	4.76	4.76	*P*=0.448
(+)-3-carene	4.72±0.05	4.12±0.11	4.90
	4.86	4.86	*P*=0.008
2,6-dimethyl benzene methyl ether	0.16±0.01	–	–
	4.96	–	–
(+)-limonene	6.05±0.11	5.09±0.21	4.09
	5.15	5.15	*P*=0.015
β-phellandrene	11.02±0.15	12.48±0.38	–3.61
	5.17	5.17	*P*=0.023
Ocimene	0.22±0.01	0.14±0.01	9.80
	5.40	5.40	*P*=0.001
γ-terpinene	0.28±0.02	0.16±0.03	3.52
	5.63	5.63	*P*=0.024
Terpinolene	3.52±0.14	3.92±0.14	–2.03
	6.15	6.15	*P*=0.112
Fenchol	–	0.10±0.02	–
	–	6.59	–
Camphor	0.22±0.02	0.33±0.04	–2.61
	7.15	7.15	*P*=0.059
Camphol	0.24±0.04	0.21±0.01	0.78
	7.49	7.49	*P*=0.478
4-terpenol	0.33±0.04	0.26±0.03	1.30
	7.67	7.67	*P*=0.263
α-terpilenol	0.60±0.04	2.42±0.18	–10.00
	7.89	7.89	*P*=0.001
Verbenone	0.06±0.01	–	–
	8.26	–	–
2-isopropyl-5-methyl anisole	0.13±0.01	0.17±0.03	–1.12
	8.63	8.63	*P*=0.325
Bornyl acetate	1.78±0.19	1.02±0.09	3.59
	9.65	9.65	*P*=0.023
(-)-α-cubebene	–	0.04±0.01	–
	–	10.93	–
α-pinene	0.10±0.01	0.36±0.05	–5.34
	11.48	11.48	*P*=0.006
β-elemene	0.29±0.07	0.52±0.05	–3.63
	11.76	11.76	*P*=0.022
β-caryophyllene	7.42±0.34	14.75±1.21	–5.85
	12.32	12.32	*P*=0.004
Aromadendrene	–	0.05±0.01	–
	–	12.65	–
(E)-β-farnesene	0.19±0.02	–	–
	12.82	–	–

Table 1 continued

Compound	Uninfested	Infested	*t*
β-sesquiphellandrene	–	0.34±0.08	–
	–	12.83	–
α-caryophyllene	1.21±0.17	2.68±0.12	–6.94
	12.91	12.91	*P*=0.002
Myrcene	5.56±0.30	20.52±1.52	–9.67
	13.38	13.38	*P*=0.001
α-selinene	–	0.32±0.02	–
	–	13.48	–
Germacrene	0.12±0.02	0.46±0.06	–5.50
	13.59	13.59	*P*=0.005
α-muurolene	–	2.35±0.27	–
	–	13.66	–
δ-cadinene	–	0.22±0.01	–
	–	13.81	–
2, 6-di-tert-butyl-4-methylphenol	–	0.62±0.14	–
	–	13.85	–
γ-cadinene	0.32±0.02	1.18±0.10	–8.27
	13.94	13.94	*P*=0.001
δ- cadinene	1.24±0.11	–	–
	14.09	–	–
α-elemene	–	0.15±0.02	–
	–	14.14	–
(-)-isocaryophyllene	–	0.30±0.05	–
	–	15.29	–
t-cadinol	–	1.48±0.19	–
	–	16.43	–
t-muurolol	–	1.95±0.18	–
	–	16.69	–
a-cadinol	0.52±0.05	–	–
	16.70	–	–

– = Compound not detected.

males of the same eclosion time were performed, and each adult was used only once.

2.6. Statistical analyses

Statistical analyses were performed using the SPSS 18.0 statistical package (IBM, Chicago, IL, USA). Independent-sample t-tests and Bonferroni correction were used to compare the relative abundances of individual compounds in uninfested and infested plants as well as EAG responses of female and male adults to the same dose. Chi-squared tests were used to test whether female and male adults were attracted to the selected eight compounds in the olfactometer trials (Yang *et al.* 2013). A one-factor randomized complete block analysis of variance (ANOVA) was conducted on the olfactometer data of behavioral responses to different compounds, separately for females and males. After ANOVAs, Duncan's multiple range tests were used to test the differences in the attraction among the compounds from *P. yunnanensis* (Dong *et al.* 2000, Liu *et al.* 2005). The rates of attraction, repellence and indifference (no choice) were calculated as follows (Ding *et al.* 1996, Yan *et al.* 2006):

$$A = 100 \times N_t / N \quad (1)$$

where A is attraction rate (%), N_t is the total number of *T. minor* in the treatment arm of the olfactometer, and N is the total number of *T. minor* tested.

$$R = 100 \times N_c / N \quad (2)$$

where R is repellence rate (%), N_c is the total num-

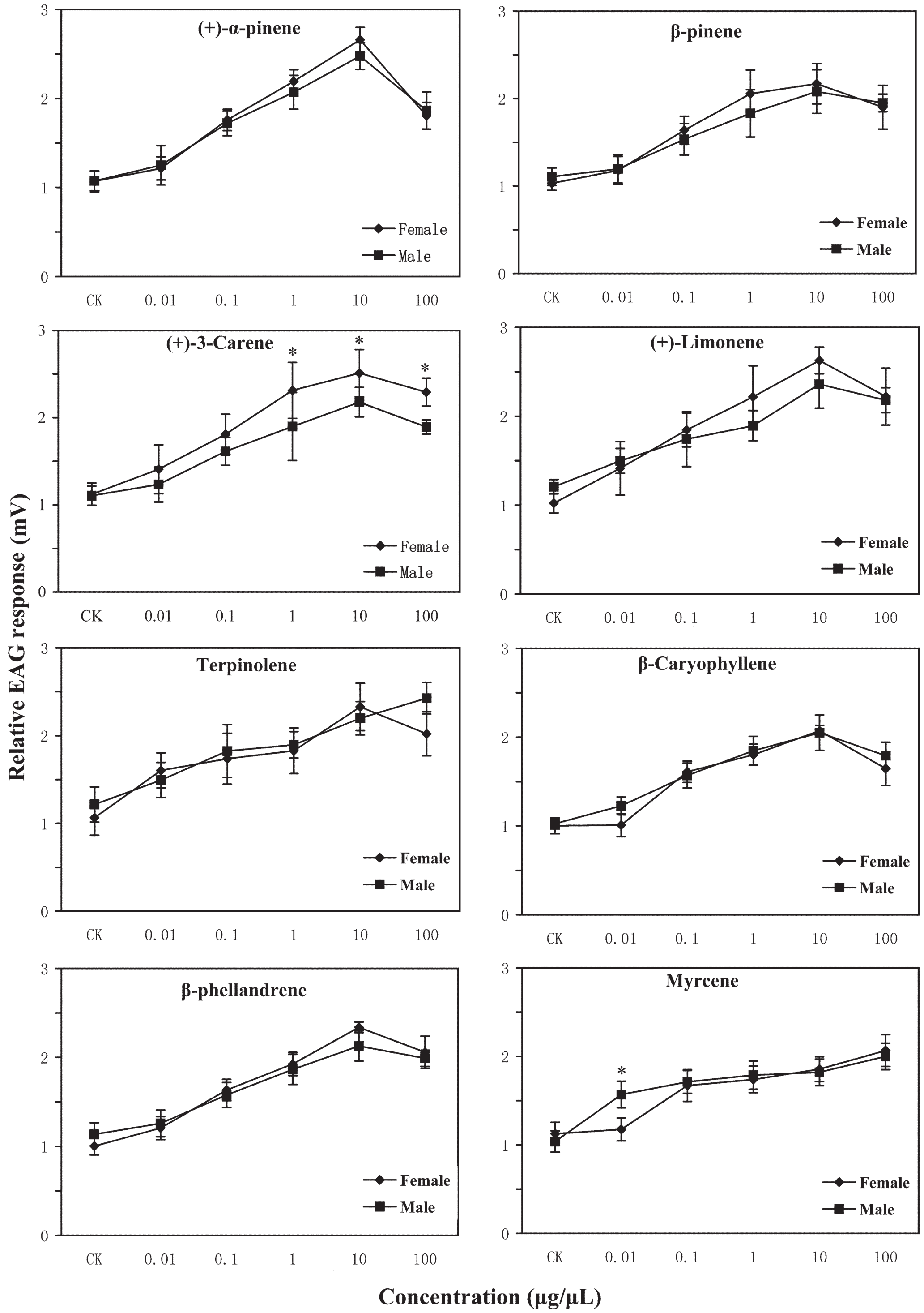

Fig. 1. Electrophysiological dose–response curves of *Tomicus minor* antennae to eight selected combounds (CK = distilled hexane, as a standard reference). Asterisks indicate significant differences between female and male beetles at the specified dose (independent-samples t-tests with Bonferroni correction, $P < 0.05$, $N = 6$ males and 6 females).

ber of *T. minor* in the control arm of the olfactometer, and N is as in equation (1) above.

$$I = 100 - (A + R) \tag{3}$$

where I is rate of indifference (%), A and R as in equations (1) and (2).

3. Results

3.1. Comparison of chemical compositions of tissues from uninfested and infested *P. yunnanensis*

From uninfested and infested *P. yunnanensis*, 30 and 37 compounds were identified by GC-MS, respectively. Monoterpenes are the primary chemicals emitted by *P. yunnanensis*. Thereinto, eight of those compounds had the highest relative abundances in both types of plants: (+)-α-pinene, β-pinene, (+)-3-carene, (+)-limonene, terpinolene, β-carophyllene, β-phellandrene, and myrcene. The retention times and relative proportion of total peak areas of all identified compounds are listed in Table 1.

3.2. EAG activity of synthetic compounds

There was a clear electrophysiological dose–response relationship for all eight compounds. The EAG responses of *T. minor* adults peaked when the compound concentrations were 10 µg/µL, except for terpinolene and myrcene, for which the responses were still rising at 100 µg/µL (Fig. 1).

In most cases, female and male beetles showed no differences in their EAG responses to the compounds (Fig. 1). The EAG response of female adults was significantly higher than those of male adults for (+)-3-carene at 1 µg/µL (t = 3.844, df = 5, P = 0.018), 10 µg/µL (t = 3.733, df = 5, P = 0.020), and 100 µg/µL (t = 2.761, df = 5, P = 0.044), while the EAG response of male adults to myrcene was significantly higher than those of female adults at 0.01 µg/µL (t = -2.739, df = 5, P = 0.045).

3.3. Laboratory bioassay of *T. minor* adult responses to synthetic compounds

Except for (+)-limonene, β-carophyllene and myrcene, the remaining compounds tested had significant attraction rates for female *T. minor* (Fig. 2a). Of those five compounds, (+)-α-pinene, β-pinene and β-phellandrene had significant attraction also to male adults (Fig. 2b).

Among the five compounds that attracted females, there were significant differences in the rate of attraction (P = 0.030, df_1 = 4, df_2 = 10, F = 2.85). (+)-α-pinene had a higher attraction rate for females than β-pinene and terpinolene, while (+)-3-carene and β-phellandrene did not differ from any of the other compounds (Fig. 2a). However, among the three compounds that attracted males, there were no significant differences in the rate of attraction (P = 0.127, df_1 = 2, df_2 = 6, F = 1.71).

4. Discussion

Host volatiles identified in infested and uninfested shoots of *P. yunnanensis* were different. Feeding by *T. minor* induced new chemical compounds in the tissues, such as fenchol, (-)-α-cubebene, aromadendrene, β-sesquiphellandrene, α-selinene, α-muurolene, δ-cadinene, α-elemene, (-)-isocaryophyllene, t-cadinol, t-muurolol, and 2,6-di-tert-butyl-4-methylphenol. Production of novel compounds due to herbivory feeding is in parallel to the findings of earlier studies that the types and quantities of chemicals released by plants chewed by phytophagous insects are different from "constitutive" chemicals (Mamiya & Enda 1972) and are affected by the feeding activities of insects (Lou & Cheng 2000, Degenhardt & Lincoln 2006). Paré and Tumlinson (1997) used CO_2-impulse labeling to prove that only insect feeding induced production of new volatile organic compounds that are not released by undamaged plants. Likewise, Loughrin *et al.* (1995) showed that crabapple leaves can be induced by the Japanese beetle to release a great deal of terpenoids.

Different host plant volatile compounds can have varying impacts on insect activities, because the physiological activity of a compound can vary

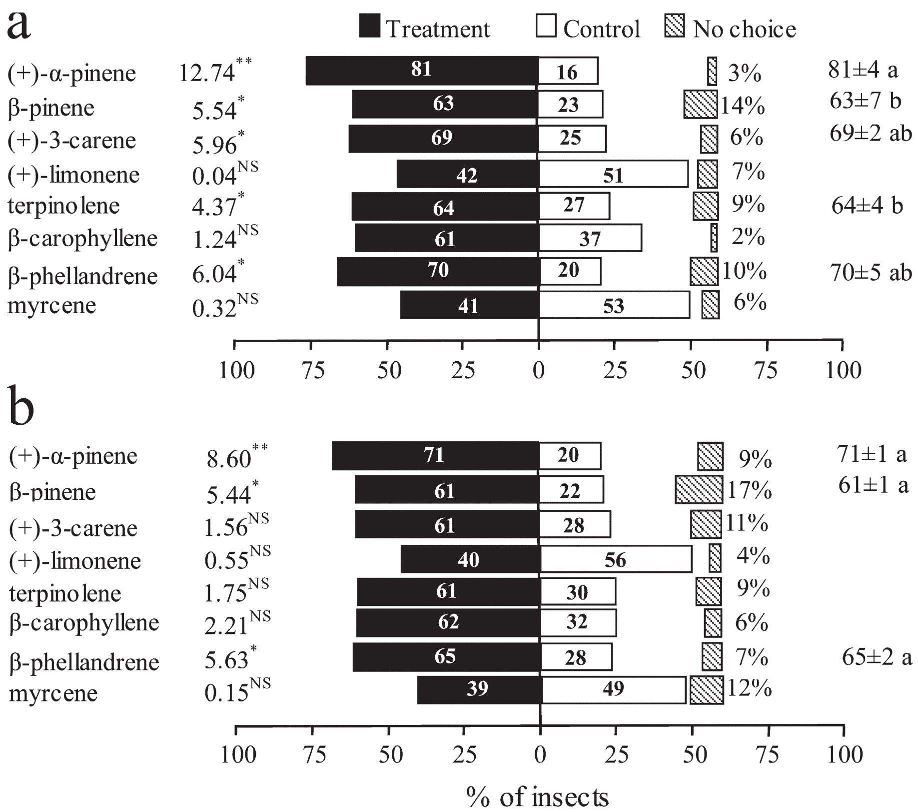

Fig. 2. Y-tube olfactometer responses of *Tomicus minor* adults to 10 μg/μL of different odors. – a. Females. – b. Males. Mean percentages (± SE) of adults choosing the test compound, control, or with no choice are reported ($N = 90$). To the left of the bars, significant differences between treatment and control are denoted; Chi-squared tests: ** ($P < 0.01$), * ($P < 0.05$), NS ($P \geq 0.05$). For the compounds showing significant attraction, differences in the rate of attraction among the compounds are shown to the right of the bars; compounds with different letters differ significantly ($P < 0.05$, Duncan's multiple comparison test). Beetles were considered to make no choice when they spent the whole test period in main arm of the olfactometer or visited either test arm for less than 1 min.

with concentration (Fan *et al.* 2004). There is an optimum dose range for the insect response to each volatile compound. Within this range, the response rate rises as the dose increases. Beyond this range, insect attraction declines as the dose changes (Fan *et al.* 2003). We found that male and female *T. minor* responses reached maximum at 10 μg/μL for (+)-α-pinene, β-pinene, (+)-3-carene, (+)-limonene, β-carophyllene, and β-phellandrene, while for myrcene they peaked at 100 μg/μL. Interestingly, in some cases, the EAG responses of male and female antennae were different, e.g. for terpinolene, the EAG responses of females were maximum at 10 μg/μL, while those of males were highest at 100 μg/μL. This strongly suggests that the antennae of female and male *T. minor* adults might differ in the type and number of receptors or in physiological functioning (Lu 2008).

We studied the antennal response for only the eight compounds with the highest abundances in the GC-MS analyses. However, there were considerable differences between infested and uninfested plants also in some less abundant compounds (Table 1). Insects can be responsive to very low concentrations of some compounds, meaning that it is worth to include low-concentration combounds in future studies of antennal responses and attraction to insects.

Plant chemicals are of vital importance in host-plant selection by bark beetles. In this study, both sexes showed basically similar responses to the tested compounds in the selection tests in a Y tube, in spite of the fact that the EAG responses of female and male antennae were different for some compounds. (+)-α-pinene, β-pinene, and β-phellandrene were attractive to both male and female beetles, while (+)-3-carene and terpinolene were attractive only to female beetles. Some bark beetle species have been previously shown to be attracted to host plant volatiles, including (+)-α-pinene, (+)-3-carene, and β-pinene (Hobson *et al.* 1993). Likewise, Miao *et al.* (2004) reported that (+)-α-pinene, (+)-3-carene, and β-pinene were repellent to *Dendroctonus valens* when released at 150 mg/day. These results may imply no major differences in olfaction between male and female beetles, but further studies are warranted. However, our electrophysiological and behavioral data indicate that future field studies may yield results that are useful for the development of traps to monitore lesser pine shoot beetle populations.

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