Electroantennographic Responses of *Dryocosmus kuriphilus* to *Castanea sativa* Leaf Volatiles

G.S. Germinara, A. De Cristofaro and
G. Rotundo
Department of Animal, Plant and
Environmental Sciences
University of Molise
Via De Sanctis, I-86100 Campobasso
Italy

B. Paparatti, S. Speranza and
E. Stacchiotti
Department of Plant Protection
University of Tuscia
Via S. Camillo de Lellis, I-01100 Viterbo
Italy

Abstract
      Electroantennographic (EAG) responses from adults of the chestnut gall wasp, *Dryocosmus kuriphilus* were recorded on stimulation with a given dose of 22 volatile compounds previously identified from *Castanea sativa* leaves. All compounds elicited measurable EAGs. The largest EAG amplitudes were recorded in response to the oxygenated monoterpenes, α-terpineol and linalool, and the green leaf volatiles (GLVs), (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-3-hexen-1-ol and (*E*)-2-hexen-1-ol. EAGs to hydrocarbon monoterpenes and aromatics were generally weak. In preliminary Y-tube olfactometer bioassays the GLVs, hexyl acetate, (*E*)-2-hexen-1-ol and (*E*)-3-hexen-1-ol were attractive when individually tested compared to a solvent control suggesting their possible role in the *D. kuriphilus* host plant selection. Identification of host plant attractants is useful to develop semiochemically based pest control strategies of the chestnut gall wasp.

INTRODUCTION

      The chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), is an economically important pest of chestnut trees (*Castanea* spp.). Native of China, this species was accidentally introduced in Japan (1951), Korea (1958), the US (1974) and Nepal (1999) (Abe et al., 2007). In Europe, it was recorded for the first time in 2002 in north-western Italy (Brussino et al., 2002) and in 2005 in central Italy (Paparatti and Speranza, 2005). The species is univoltine and thelytokous. Adults lay their eggs in chestnut buds in early summer and larvae remain latent until buds expand the following spring. Larvae induce the formation of leaf and twig galls that disrupt shoot elongation, reduce fruit yield by 50-75% and even determine the death of young trees as a result of severe, consecutive attacks (Payne et al., 1983; Kato and Hijii, 1997).

      The effectiveness of chemical control is limited by the difficulty to accurately detect the emergence of adults and by concerns related to the natural distribution of chestnut trees, which often render it impracticable. In this context, the identification of attractant compounds could be useful both to detect the presence of adult wasps in new areas and to improve the timing of control measures.

      Gall-inducing insects are characterized by a high degree of host plant specificity (Stone et al., 2002) and plant volatiles are expected to play a significant role in host-plant selection by gall-inducing cynipids. However, the importance of this host-plant trait is yet little investigated (Tooker et al., 2002, 2005; Tooker and Hanks, 2004).

      Volatile compounds of chestnut (*Castanea sativa* Miller) leaves have been identified in a previous study (Rotundo et al., 1987). In the present research we used the electroantennographic technique (EAG) to test the hypothesis that chestnut leaf volatiles are perceived by *D. kuriphilus* adults. In addition, preliminary behavioural bioassays using some strong antennal stimulants were carried out.

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MATERIALS AND METHODS

Insects

Galls containing immature stages of the chestnut gall wasp were collected in an infested chestnut orchard near Viterbo (central Italy) during July and kept in glass containers at $25\pm2°C$, $55\pm5\%$ r.h., under natural light conditions. Newly emerged adults were collected daily and kept individually in glass vials (Ø 2×8 cm) covered with a fine mesh net (1 mm) until used.

Odour Stimuli

Olfactory stimuli were selected among compounds identified from *C. sativa* leaves (Rotundo et al., 1987) and classified among the green leaf volatiles (GLVs) [(*E*)-2-hexenal, (*E*)-2-hexen-1-ol, (*E*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol, hexyl acetate, (*E*)-2-hexenyl acetate, (*Z*)-3-hexenyl acetate], terpenoids [α-(−)-pinene, α-(+)-pinene, β-pinene, S-(−)-limonene, R-(+)-limonene, (+)-3-carene, caryophyllene, geraniol, linalool, α-terpineol] and aromatics (phenol, eugenol, toluene, styrene, benzothiazole). Test chemicals were purchased from Sigma-Aldrich (Milan, Italy) and their chemical purities ranged from 97 to 99%.

To prevent early evaporation, test compounds were dissolved in mineral oil (Sigma-Aldrich). For EAG tests, compound solutions in molar equivalents (1 mol L^{-1}) were prepared. To obtain dose-response curves, mineral oil solutions of (*Z*)-3-hexen-1-ol (from 0.05 mmol L^{-1} to 2.0 mol L^{-1}) were also prepared. Immediately before the experiment, each test solution (20 µl) was pipetted onto a filter paper strip (1 cm^2, Whatman No. 1), placed in a Pasteur pipette (15 cm long) and used as an odour cartridge. For behavioural experiments, 0.1 mol L^{-1} solutions of individual compounds were prepared; solutions were stored at -20°C until needed.

Electroantennogram Recording

The EAG technique was similar to that used in previous studies (Den Otter et al., 1996; Germinara et al., 2007). Antennae were excised from 1-day-old insects. The base of the antenna was placed into a glass pipette filled with 0.1 M KCl solution which served as indifferent electrode. The tip of the antenna was put in contact with the end of a similar pipette (Ø 0.2-0.3 mm) serving as different electrode.

AgCl-coated silver wires were used to maintain electrical continuity between the antennal preparation and an AC/DC UN-6 amplifier in DC mode (Syntech Laboratories, Hilversum, The Netherlands). Stimuli were puffed into a constant stream of charcoal-filtered humidified air (500 ml min^{-1}) flowing in a stainless steel delivery tube (Ø 1 cm) with the outlet positioned at approximately 1 cm from the antenna. During 1 s, 2.5 cm^3 of vapour from an odour cartridge were added. Stimuli were applied in random order at 30 s intervals. In dose-response experiments, stimuli were applied in ascending dose whereas in the other experiments they were randomly selected. Control (20 µl of mineral oil) and reference (20 µl of a 1 mol L^{-1} (*Z*)-3-hexen-1-ol solution) stimuli were applied at the beginning of the experiment and after each group of 3 test odours. For each compound tested, EAG responses were recorded from 8 antennae of different insects.

Y-Tube Olfactometer Bioassay

The preference of chestnut gall wasps to individual compounds was investigated in a glass Y-tube olfactometer (length per arm, 23 cm at a 75° angle; stem length, 30 cm; internal diameter, 3 cm). Both arms of the Y-tube were connected to a glass cylinder (length, 9 cm; internal diameter, 3 cm) as an odour source container. The apparatus was placed into an observation chamber (90×75×40 cm) and illuminated from above by two 36-W cool, white fluorescent tubes providing uniform lighting (2500 lux) inside the tube. Purified (activated charcoal) and humidified airflow maintained at 60 ml min^{-1} by a flowmeter was pumped through each of the olfactometer arms. For the bioassay, the odour chamber contained a filter paper disk (0.5 cm^2) loaded with 5 µl of a 0.1 mol L^{-1} oil

solution of a test compound, whereas the control chamber contained a filter paper disk loaded with 5 µl of mineral oil. One-day-old chestnut wasps were released individually at the open end of the stem. Each experiment lasted 10 min. A choice was recorded when the insect moved 3 cm up an arm of the Y-tube, crossing the decision line (marked on both arms), and remained beyond that line for more then 30 s. The time spent by test insects in each arm was also recorded.

After each group of 5 insects had been tested, the olfactometer was cleaned with acetone and dried (200°C for 30 min); treatments between arms were switched to avoid position bias. The bioassays were repeated until there where 25 wasps responding to each test compound.

Data Analysis

The amplitudes (mV) of EAG responses to test compounds were adjusted to compensate for solvent and/or mechanosensory artefacts by subtracting the mean EAG response of nearest controls. To compensate for the decrease in antennal responsiveness, the corrected EAG amplitudes were normalized by expressing them as percentages relative to those of adjacent reference stimuli. One-way analysis of variance (ANOVA), followed by Tukey's HSD test (P=0.05), was used to rank mean relative EAG responses to all compounds. A χ^2 test was employed to determine the significance of differences between the number of wasps choosing the treatment or control arm of the olfactometer. A paired-sample t-test was used to analyse the differences between the percentage time spent by wasps in each arm.

RESULTS

EAG Responses

On stimulation with various doses of (Z)-3-hexen-1-ol a typical sigmoid-shaped dose-dependent response, with the activation dose of 0.1 µmol and the saturation one of 20 µmol, was found. Based on these data the other compounds were tested at the 20 µmol dose.

The corrected absolute EAG response to the standard (Z)-3-hexen-1-ol was 0.62±0.21 mV. Normalized EAG responses to the other 21 chemicals varied from 25.9±9.3% (phenol) to 125.1±16.8% (α-terpineol) (Fig. 1).

One-way ANOVA indicated significant differences among the EAG responses to different compounds (F=42.9, d.f.=21, P<0.0001). The rank order of mean EAG responses to the stimuli tested is shown in Figure 1. The largest EAG responses were elicited by α-terpineol and (E)-2-hexenal. High EAGs were also induced by (Z)-3-hexen-1-ol, (E)-3-hexen-1-ol, linalool and (E)-2-hexen-1-ol. These compounds elicited peak amplitudes significantly lower (P=0.05) than α-terpineol but similar to (E)-2-hexenal (Fig. 1). The least effective chemicals were phenol, styrene, S-(−)-limonene, toluene, α-(+)-pinene, R-(+)-limonene, β-pinene and (+)-3-carene.

Among GLVs, the aldehyde and alcohols were significantly more EAG-active than acetates. EAG response to α-terpineol was significantly higher compared to other terpenoids. Among aromatics, benzothiazole elicited the significantly highest EAG amplitude (Fig. 1).

Behavioural Bioassays

Olfactometer bioassays were designed to assess the attraction of chestnut gall wasps to α-terpineol, linalool and GLVs which were the most EAG-active compounds. α-Terpineol and linalool did not elicit a significant attraction response from adult wasps either as first choice or time spent in the treatment arm (Table 1). Among GLVs, hexyl acetate, (E)-2-hexen-1-ol and (E)-3-hexen-1-ol were significantly attractive. As a first choice, the highest level of attraction was recorded in response to hexyl acetate (χ^2=17.64, d.f.=1, P=2.67×10^{-5}) followed by (E)-3-hexen-1-ol and (E)-2-hexen-1-ol (χ^2=4.84, d.f.=1, P=0.028) (Table 1). Similarly, a significantly longer time in the treatment than in the

control arm was spent by insects when presented with hexyl acetate (t=17.19, d.f.=24, P=5.0×10^{-15}), (*E*)-2-hexen-1-ol (t=4.85, d.f.=24, P=7.0×10^{-5}) and (*E*)-3-hexen-1-ol (t=2.61, d.f.=24, P=0.015) (Table 1).

DISCUSSION

Antennae of *D. kuriphilus* adults can detect a wide range of volatiles emitted by chestnut leaves and that some general green leaf odours are attractive to adult wasps. EAG screening with plant volatiles appears to have been performed for the first time in Cynipidae. High EAG amplitudes were evoked by the oxygenated monoterpenes, α-terpineol and linalool, and the GLVs, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*E*)-3-hexen-1-ol and (*E*)-2-hexen-1-ol. Moderate-to-low EAG responses were elicited by the aromatics, benzothiazole and eugenol, the acetate GLVs, and the hydrocarbon terpenoids. The lowest responses were obtained on stimulation with the aromatics, phenol and styrene.

EAG studies with compounds of different volatility showed that insect sensitivity mainly accounts for differences in EAG amplitudes (Van Der Pers, 1981; Visser et al., 1996) despite the different number of molecules reaching the antennal preparation (Dickens et al., 1991). Nevertheless, in this study olfactory stimuli were compared on an equimolar rather than on an equal-weight basis to limit the effect of differences in volatility over the range of volatiles tested. Therefore, the large differences found among relative EAG responses indicate the ability of the peripheral receptor system of *D. kuriphilus* to differentiate among host-plant chemical cues.

Oxygenated terpenoids produced higher EAG responses than did hydrocarbon terpenoids also in other unrelated species including *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Visser, 1979), *Psila rosae* (F.) (Diptera: Psilidae) (Guerin and Visser, 1980), *Yponomeuta* sp. (Lepidoptera: Yponomeutidae) (Van Der Pers, 1981) and *Megastigmus spermotrophus* Wachtl (Hymenoptera: Torymidae) (Thiery and Marion-Poll, 1998). Among chestnut leaf volatiles, α-terpineol was also found to be the most antennal stimulant in three adult chestnut moths (Rotundo and Tremblay, 1993). This may suggest that coevolution of these phylogenetically distant species with the same host plant has resulted in a high antennal capability to perceive a common compound.

In preliminary behavioural bioassays, α-terpineol and linalool did not show any significant attraction; therefore, their potential biological activity in relation to the chestnut gall wasp remains unknown. The three GLVs, hexyl acetate, (*E*)-2-hexen-1-ol, and (*E*)-3-hexen-1-ol, significantly attracted adult wasps when individually compared to a control in the Y-tube olfactometer. GLVs occur in the foliage of numerous plant species but in highly varying proportions and therefore may play an important role in host plant location by phytophagous insects (Visser, 1986; Reinecke et al., 2002; Ruther and Mayer, 2005). A species-specific ratio of the behaviourally-active GLVs may provide a signal for host plant selection by *D. kuriphilus*.

Previous studies have shown the biological activity of some hydrocarbon monoterpenes to the cynipid *Antistrophus rufus* Gillette. Residues of α-pinene, camphene, and β-pinene in dead stems of host plants are used by males as cues for mate location (Tooker et al., 2002; Tooker and Hanks, 2004), whereas a blend of α-pinene, (−)-camphene, β-pinene, and (+)-limonene acts as a behavioural signal for adult females (Tooker et al., 2005). In this study, hydrocarbon monoterpenes evoked low antennal responses from *D. kuriphilus* adults; however, their behavioural activity remains to be investigated.

In conclusion, the study shows that various chestnut leaf volatiles are perceived by *D. kuriphilus*, and some GLVs act as attractants. Further behavioural and field studies are needed to evaluate the potential of attractant compounds in control strategies of the chestnut gall wasp.

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Tables

Table 1. Response of adult *D. kuriphilus* in a Y-tube olfactometer to individual compounds versus a solvent control (mineral oil).

Compound	n^a	First choice			Time spent in arm	
		Treated[b]	χ^2	P-value	Treated[c]	P-value[d]
α-terpineol	28 (25)	36	1.96	0.162	55±9	0.619
linalool	30 (25)	44	0.36	0.549	41±6	0.166
hexyl acetate	25 (25)	92	17.64	<0.001	95±3	<0.001
(Z)-3-hexenyl acetate	28 (25)	40	1.00	0.317	46±8	0.608
(E)-2-hexenyl acetate	29 (25)	60	1.00	0.317	60±9	0.275
(E)-2-hexenal	28 (25)	56	0.04	0.841	48±9	0.780
(Z)-3-hexen-1-ol	28 (25)	40	1.00	0.317	48±7	0.814
(E)-3-hexen-1-ol	26 (25)	72	4.84	0.028	67±7	0.015
(E)-2-hexen-1-ol	26 (25)	72	4.84	0.028	71±4	<0.001

[a]Total sample size (number of individuals that made a choice).
[b]Proportion of responding individuals that chose the treated arm first.
[c]Proportion of time spent (mean±SE) in the treated arm by responding individuals.
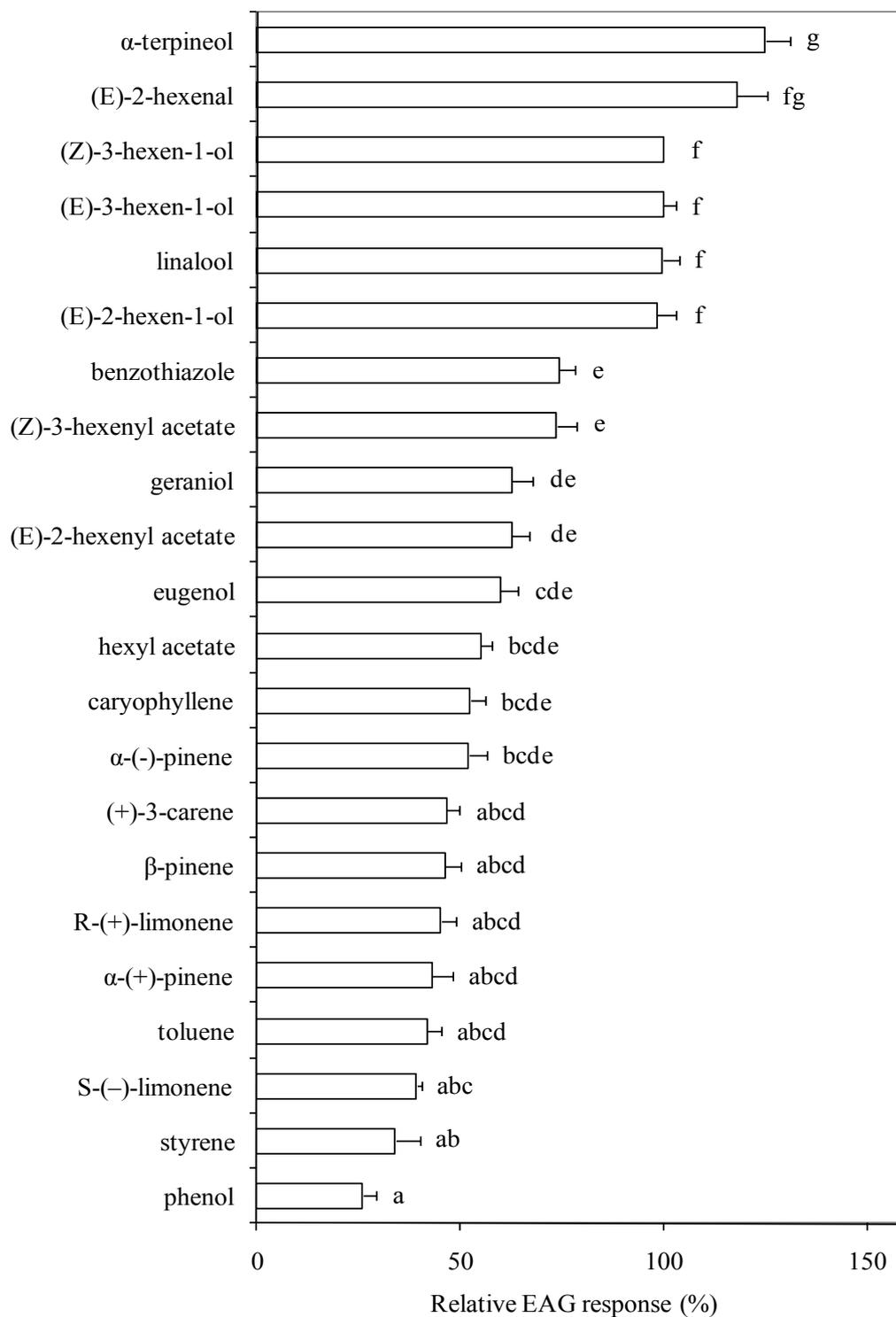[d]Paired-sample *t*-test.

Figures

Fig. 1. Relative EAG responses of adult *D. kuriphilus* to plant volatiles ranked according to amplitude increase. EAG responses are expressed relative to the reference (*Z*)-3-hexen-1-ol. Lines indicate standard errors. Means with the same letter are not significantly different by P=0.05 (Tukey's HSD test).