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Antennal response of *Prinobius myardi* to synthetic tree volatiles

Highlights

- *Prinobius myardi* is a wood borer considered a major threat for Mediterranean oaks, especially *Quercus suber* and *Q. ilex*.
- We performed electroantennographic bioassays to assess olfactory sensitivity of *P. myardi* to synthetic plant volatiles.
- *P. myardi* exhibits a broad sensitivity to common tree volatiles, including those emitted by oaks (α-pinene and β-pinene) or non-host volatiles (1,8-cineole).

Abstract

Prinobius myardi Mulsant is a wood borer implicated in the decline of Mediterranean oaks, especially *Quercus suber* L. and *Quercus ilex* L. Plant volatiles play an important role in plant-insect interactions, and electroantennography (EAG) is an effective tool for exploring the electrophysiological activity of host plant volatiles on insects. To improve our understanding of the olfactory sensitivity of *P. myardi*, we recorded EAG responses to 20 tree volatiles, and analyzed the dose-dependent response to five doses (10^{-4}:1 to 1:1 v/v) of the three most EAG-active compounds. Antennae of *P. myardi* responded to 13 chemicals, mainly monoterpenes and green leaf volatiles, with the strongest EAG responses being observed with β-pinene, (+)-α-pinene and 1,8-cineole. Dose–response profiles showed positive dose-dependent responses for all three compounds. Our results suggest a broad sensitivity of *P. myardi* to common tree volatiles, particularly some host-related compounds and volatiles associated with wounded trees; the olfactory recognition of ratios of these compounds could play a role in host selection by *P. myardi*.

1 Introduction

Wood-boring insects play a significant role in oak decline in the Mediterranean area (Sallé et al. 2014). As they colonize weakened trees, they act as an aggravating factor, hindering tree recovery (López-Pantoja et al. 2011). Mediterranean *Quercus ilex* L. and *Quercus suber* L. dehesa open woodlands of the Andalucía region (Southern Spain) have high ecological, economic and cultural importance. Hence, problems affecting stands of these species, including invasion by wood-boring beetles such as *Prinobius myardi* Mulsant (Coleoptera, Cerambycidae), deserve particular attention especially in a context of climate change (López-Pantoja et al. 2011; Sallé et al. 2014).

The importance of volatile semiochemicals in host selection by Cerambycidae species has mostly been studied for species attacking conifers (see Allison et al. 2004); and relatively little is known about the role of such compounds in host location by wood borers infesting deciduous trees – especially *Quercus* species – though notable results have been obtained for *Cerambyx welensii* Küster (Torres-Vila et al. 2012 and 2013; Sánchez-Osorio et al. 2015). Electroantennography (EAG) is an effective tool for measuring the total response of insect antennal receptor cells to olfactory stimuli (Bruce et al. 2005; Gullan and Cranston 2014), and has already been applied to several cerambycid wood borers, including *C. welensii* and *P. myardi* (Sánchez-Osorio et al. 2007 and 2009), *Saperda populnea* L. (Chi et al. 2011) and *Batocera lineolata* Chevrolat (Yang et al. 2013).

Prinobius myardi is a large (up to 60 mm long), holomediterranean species with nocturnal habits, whose larvae bore into wood of several deciduous tree species, especially weakened cork and holm oaks*,* causing tree branches and trunks to break (López-Pantoja et al. 2011). In addition, these insects facilitate infection by both plant pathogens and wood-decaying fungi (Martin et al. 2005). Despite these effects, several aspects of the behavior of *P. myardi* are not well known, in particular, regarding its role in oak decline, host selection cues and the development of control methods (López-Pantoja et al. 2011; Torres-Vila et al. 2013).

Quercus suber and *Q. ilex* are strong emitters of foliar monoterpenes, mainly α-pinene, β-pinene, limonene, sabinene and myrcene (Staudt et al. 2008; Loreto et al. 2014). Several green leaf volatiles (GLVs), in particular (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate and (*E*)-2-hexenol, are also emitted by freshly cut branches and leaf extracts of *Q. suber* (Fürstenau et al. 2012). Recently, it has been suggested that colonization of *Q. suber* trees by *C. welensii* is positively associated with foliar emission of limonene (Sánchez-Osorio et al. 2008).

To understand the olfactory sensitivity of *P. myardi* to plant volatiles, we examined: (a) the EAG responses of *P. myardi* to individual synthetic tree volatiles; and (b) the dose–response relationships for the three most EAG-active compounds.

2 Material and methods

2.1 Test substances

The odorant test panel comprised 20 synthetic chemicals. The main group included 16 analogues of volatiles identified in foliar emissions from *Q. suber* and *Q. ilex*: the monoterpenes, β-pinene, (+)-α-pinene, (+)-limonene, β-myrcene, 1,8-cineole, (*E*)-β-ocimene, α-terpinene, γ-terpinene, α-phellandrene, ρ-cymene and α-terpineol (Sánchez-Osorio et al. 2008; Llusià et al. 2012); the GLVs, (*E*)-2-hexenal, (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate (Fürstenau et al. 2012); and the triterpenes, lupenone and erithrodiol (Monaco and Previtera 1984). We also tested several analogues of compounds found in *Q. suber* cork, namely gallic and ellagic acids, the triterpene friedelin and 3-methyl-butanol (Rocha et al. 1996). Turpentine oil was used as the standard stimulus (Sánchez-

Osorio et al. 2007). All chemicals were purchased from Sigma-Aldrich (Madrid, Spain). Purity was ≥94%, except in the cases of ρ-cymene and turpentine, which had >90% purity.

2.2 Electroantennography

Prinobius myardi adults were collected by hand picking during the flight period (July, 2004) in a *Q. suber* dehesa (Huelva, SW Spain). Insects (3.8 ± 0.4 cm long, 1.2 ± 0.3 g weight; on average) were kept in individual containers with access to a 4% sugar solution, in semi-darkness (at 23–32 °C). Notably, *P. myardi* females were difficult to capture (the average female:male capture ratio being 0.28 over eight years (López-Pantoja et al. 2011)), and most of those collected died within 24 hours.

EAG responses were recorded 1 to 7 days (mean of 4 days) after insect collection, as in Sánchez-Osorio et al. (2007), using glass capillary electrodes (0.5-mm internal diameter) filled with saline solution (KCL 0.1 N, with 3% polyvinylpyrrolidone) and slipped over silver wire. The distal end of the antenna was inserted into the tip of the recording electrode; and the indifferent electrode (same glass capillary, custom-pulled to an approximately 100-μm tip diameter) was inserted through the membrane between scape and pedicel. Each chemical compound tested was dissolved in hexane (Sigma-Aldrich, 99% purity) to prepare odor sources, and 20-μl aliquots of these solutions were applied to filter paper strips (Whatman No 1). The solvent was allowed to evaporate for 5 min before each paper strip was inserted into a glass Pasteur pipette. For stimulation, 10-ml puffs of air were blown through the Pasteur pipettes into a purified air stream continuously flowing over the antenna ($4 \, \mathrm{l} \, \mathrm{min}^{-1}$), using a Windjet 727-RY-15 diffuser (Spraying Systems GmbH, Hamburg, Germany) to ensure covering the entire antenna length. EAG responses were recorded (in mV) using Syntech electrode holders, IDAC-4 acquisition controller and Autospike-32 software (Syntech, Hilversum, The Netherlands).

In Experiment 1, EAG responses were recorded in five stimulation series of four randomly-puffed individual odor sources (1:1 v/v dilution of each compound in hexane). Each series was preceded by a stimulus with turpentine standard as a reference for EAG response over the session; a last turpentine stimulus was also applied 10 min after the last series. There was a 10-min interval between series, and 1-min interval between stimulations within a series. The same seven insects (five males and two females) were tested with all the compounds (one replication per individual and per compound).

In Experiment 2, the three compounds eliciting the strongest EAG responses in Experiment 1 – β-pinene, (+)-α-pinene and 1,8-cineole – were presented at five doses increasing by orders of magnitude from 10^{-4}:1 (corresponding to 858–923 ηg depending on the chemical) to 1:1 v/v in hexane. For each chemical, dose–response was tested in five stimulation series with three replicates for each dose, preceded by a stimulus with turpentine standard. In this case, there were 1-min intervals between series, and between replicates within series; a last turpentine stimulus was applied 1 min after the last series, and 10-min intervals separated tests with different compounds. We calculated the mean response from the three replicates of a specific dose to minimize variation associated with manual injections. The dose–response was tested for each of the three chemicals using the same five insects (three males and two females, different individuals from those used for experiment 1).

Measured EAG responses were corrected by subtracting the mean response to two hexane control injections (20 μl) recorded at the beginning and end of each series in both experiments, to compensate for solvent and/or mechanoreceptive artifacts. Corrected measures were then normalized to percentages relative to the EAG response obtained to the standard stimuli, namely, the mean of the EAG responses to the two turpentine injections between which the response to each chemical or specific dose was recorded (Yang et al. 2013).

2.3 Data analysis

Grubbs's tests showed outliers in male EAG responses, probably due to the heterogeneous vitality of field-caught insects, and hence, normality and homoscedasticity assumptions were not met. In such cases, robust statistical methods have been recommended (Wilcox 2012). EAG responses were compared using functions implemented in the WRS package (Wilcox 2012), based on 20% trimmed means (Mean$_{0.2}$: mean calculated after discarding the top and bottom 20% of values). Two-sided confidence intervals for the difference in Mean$_{0.2}$ EAG responses revealed no differences between sexes for any of the tested compounds ($P > 0.1$), and hence, EAG data from males and females were pooled.

Data from both experiments were analyzed using heteroscedastic ANOVA for related samples (two-way analysis for Experiment 2), followed by Rom's modified Bonferroni tests for multiple comparisons. One-sided confidence intervals based on the Mean$_{0.2}$ for related samples were used to assess whether normalized (uncorrected) EAG responses to individual compounds were larger than the response to the hexane control. All statistical analyses were performed using the R framework (R Development Core Team 2014, version 3.1.1), with $\alpha = 0.05$, employing corrected (except when comparing to hexane controls) and then normalized EAG values.

3 Results

EAG signals showed the typical rapid depolarization followed by a slower recovery phase. The EAG response to standard turpentine was weak (0.38 ± 0.17 mV, Mean$_{0.2} \pm$ Error$_{0.2}$, $N = 7$) and to the hexane control was negligible (Mean$_{0.2} < 0.01$ mV, $N = 7$). Compared to the hexane control, EAG responses were significantly higher for 13 compounds: all tested monoterpenes except ρ-cymene and α-terpineol, the three GLVs and 3-methyl-butanol.

In Experiment 1 (Fig. 1), there were significant differences in EAG responses to individual compounds (ANOVA: $F_{6\ 24.1} = 14.6$, $P < 0.001$). Three compounds, β-pinene, 1,8-cineole and (+)-α-pinene, clearly elicited the highest responses, with no significant differences between them. Stimulation with the other 10 active compounds produced moderate-intensity EAG signals (normalized response range: 35–48%), with six compounds being significantly stronger stimulants than ρ-cymene: α-terpinene, (E)-β-ocimene, β-myrcene, (E)-2-hexenal, α-phellandrene and (Z)-3-hexenol.

In Experiment 2, responses to the three strongest stimulants was significantly dose dependent (Fig. 2), with no significant dose and compound interaction (ANOVA: $P < 0.001$, $P = 0.462$, and $P = 0.181$; respectively for dose, compound, dose × compound). No or negligible EAG responses were elicited by 10^{-2}:1 and lower doses. Dose–response profiles did not indicate that any saturation threshold had been reached; however, a 1:1 dose of 1,8-cineole did not elicit a significantly higher EAG response than a 10^{-1}:1 dose ($P = 0.160$).
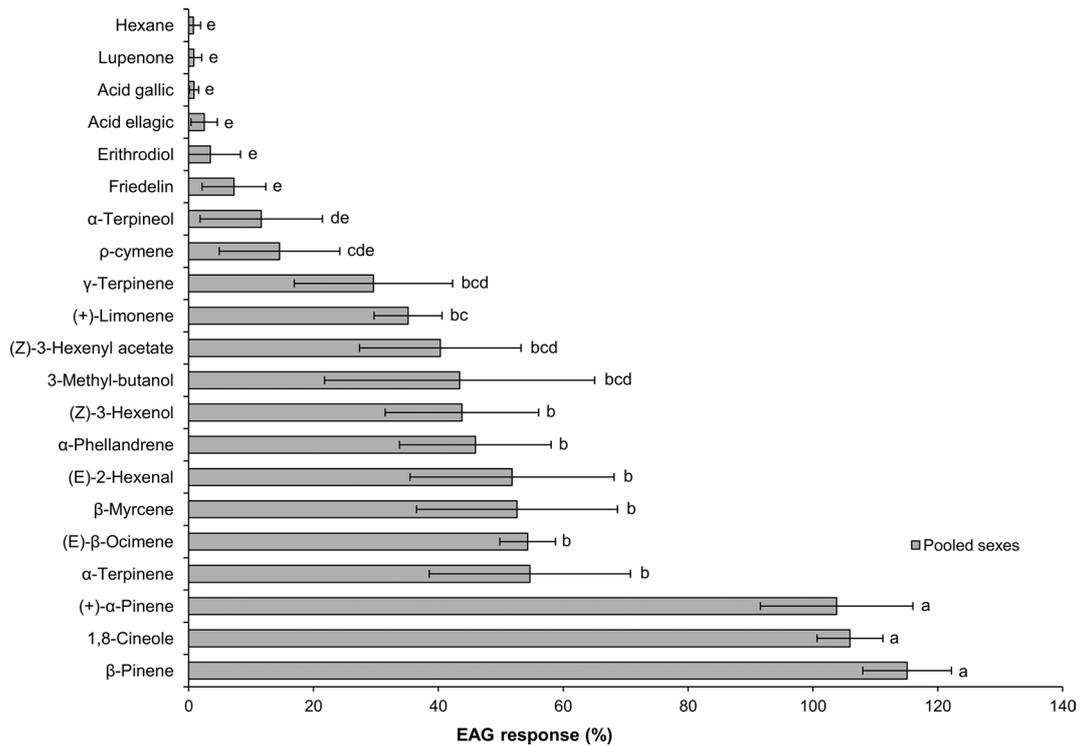
Fig. 1. $\text{Mean}_{0.2}$ ($\pm \text{Error}_{0.2}$) EAG responses of *Prinobius myardi* to tree volatiles. Responses are expressed as normalized percentages relative to the standard reference (turpentine oil), after subtracting the response to the hexane control. The hexane control response is included for comparison. Bars with the same letter are not significantly different (heteroscedastic ANOVA for related samples, $N = 7$).
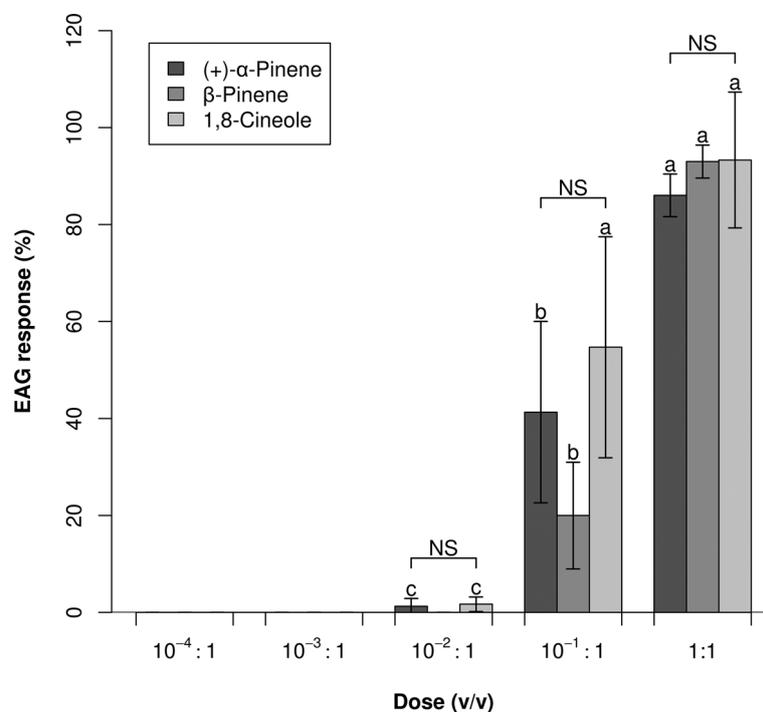

Fig. 2. Dose–response EAG profiles ($\text{Mean}_{0.2} \pm \text{Error}_{0.2}$) of *Prinobius myardi*. Responses are expressed as normalized percentages relative to the standard reference turpentine oil (not shown). For each compound, letters on the columns denote differences between doses; NS indicates no significant difference between compounds at each dose (heteroscedastic ANOVA for related samples, $N = 5$).

4   Discussion

Prinobius myardi shows olfactory sensitivity to common tree volatiles, particularly some volatiles emitted by host species. Host location by insects involves recognition of specific semiochemicals or, more often, specific mixtures of common plant volatiles (Allison et al. 2004; Bruce et al. 2005; Smart et al. 2013). *Quercus suber* and *Q. ilex*, the main hosts of *P. myardi*, are considered strong monoterpene emitters, with four leaf chemotypes which are genetically based: a pinene type, a limonene type, and either an intermediate limonene/pinene type (in *Q. suber*) or a myrcene type (in *Q. ilex*) (Staudt et al. 2008; Loreto et al. 2014). Two monoterpenes widely emitted by trees (especially from resinous species), β-pinene and (+)-α-pinene, together with 1,8-cineole, elicited the highest EAG responses in *P. myardi*, and these were dose-dependent. Limonene and myrcene elicited lower EAG responses than the two pinene isomers, suggesting *P. myardi* is able to discriminate between leaf chemotypes, particularly pinene from both limonene and myrcene types.

The mediation of plant volatiles in host location has been reported within Cerambycidae (Allison et al. 2004; Hanks et al. 2012). Sánchez-Osorio et al. (2008) found limonene emission from *Q. suber* leaves to be higher in trees heavily colonized by *C. welensii* than neighboring non-colonized trees. Further, (+)-Limonene and two related compounds (α-phellandrene and α-terpinene) elicited moderate EAG responses in *P. myardi*, high concentrations of α-phellandrene having been found in leaf emissions from *Q. ilex* (Llusià et al. 2012). Plant volatiles are also probably involved in the recognition of weakened, damaged hosts (Allison et al. 2004; Hanks et al. 2012). Moderate EAG responses were elicited in *P. myardi* by three compounds associated with emissions from wounded leaves of *Quercus* species: the known ubiquitous GLV (*E*)-2-hexenal, two acyclic monoterpenes, β-myrcene and (*E*)-β-ocimene (Paris et al. 2011; Pearse et al. 2013), and 3-methyl-butanol (found in microbially colonized cork from *Q. suber* (Rocha et al. 1996)).

Using olfactory receptor neurons for common plant compounds allows insects to evaluate greater ranges of potential hosts (Bruce et al. 2005). It is not possible to directly match EAG responses to a behavioral activity (e.g., attraction or repulsion); and moreover, the heterogeneous vitality of the insects (survival after EAG test: 3–18 days in males; 1–2 days for females) and the small sample could have biased EAG responses. However, our results allow us to formulate a tentative two-stage hypothesis concerning reliance on common tree volatiles in host location by *P. myardi*, which should be further explored. At a first stage, the specific recognition of strong EAG-active compounds – β-pinene, (+)-α-pinene and 1,8-cineole – could help distinguish suitable from unsuitable hosts at patch scale (e.g., avoiding non-host *Pinus* and *Eucalyptus* genera, which include 1,8-cineole as a major terpenic component. Barata et al. 2000; Blanch et al. 2007) and possibly also within patches, enabling *P. myardi* to recognize trees with pinene chemotype (and, in turn, this could influence the probability of conspecific encounters, as suggested for *C. welensii* based on *Q. suber* leaf chemotypes). At a second stage, *P. myardi* could use specific ratios of several compounds eliciting moderate EAG responses to identify blends of volatiles characterizing host species, even at the intraspecific level (e.g., relative fractions of limonene and myrcene in terpenic emission from *Q. ilex* and *Q. suber*), or wounded trees (e.g., some GLVs, (*E*)-β-ocimene or 3-methyl-butanol).

Behaviorally-active semiochemicals, such as host plant volatiles, have potential applications in integrated pest management including for monitoring and mass-trapping programs (Allison et al. 2004; Smart et al. 2013). Our results are promising as a basis for such applications in *P. myardi*, especially in the context of declining oak populations, as is the case for *Q. suber* and *Q. ilex* dehesas. However, there is a need for further studies focused on improving electrophysiological testing (especially with females) for tree volatiles, as well as performing behavioral bioassays (field trials with baited traps, and flight tunnels) to thoroughly investigate activity induced by EAG-active compounds.

Acknowledgements

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