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Electrophysiological and Behavioral Responses of Vine Weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae), Adults to Host Plant Odors

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Abstract

Vine weevil, *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae), is an economically important pest species in many soft-fruit and ornamental crops. Economic losses arise from damage to the roots, caused by larvae, and to the leaves, caused by adults. As adults are nocturnal and larvae feed below ground, infestations can initially be missed, and controls may, as a result, be applied too late. In the absence of a vine weevil sex or aggregation pheromone being identified, the development of an effective semiochemical lure for better management of this pest is likely to focus on host-plant volatiles. Here, we investigate the electrophysiological and behavioral responses of adult vine weevils to volatile organic compounds (VOCs) originating from their preferred host plant *Euonymus fortunei*, and synthetic VOCs associated with this host when presented individually or as blends. Consistent electroantennographic responses were observed to a range of generalist VOCs. Behavioral responses to VOCs, when presented individually, were found to be influenced by the concentration of the compound to which the weevils were exposed. Vine weevil adults showed directional movement towards a mixture of seven plant volatiles (methyl salicylate, 1-octen-3-ol, (*E*)-2-hexenol, (*Z*)-3-hexenol, 1-hexanol, (*E*)-2-pentenol, and linalool) even though either no response or negative responses were recorded to each of these compounds when presented individually. Similarly, vine weevils showed directional movement towards a 1:1 ratio mixture of (*Z*)-2-pentenol and methyl eugenol. Results presented here point to the importance of blends of generalist compounds and concentrations of VOCs in the optimization of a lure.

Key Words – Vine weevil, *Euonymus fortunei*, monitoring, electroantennography, air entrainment, olfactometry.

Introduction

Vine weevil (also known as black vine weevil), *Otiorhynchus sulcatus* F. (Coleoptera: Curculionidae), is one of the most economically important pest species of soft-fruit and ornamental crops globally (van Tol et al. 2012). Vine weevils are female and reproduce via thelytokous parthenogenesis, resulting in very little genetic diversity within the species (Lundmark 2010). The flightless adults lay their eggs at night into cracks in the soil or growing medium (Smith 1932). Upon hatching, the larvae complete four to nine molts before pupating in earthen cells (Masaki and Ohto 1995). Typically, vine weevils are a univoltine species, but as a winter diapause is not required and their development rate is temperature-dependent (Son and Lewis 2005), overlapping generations may occur in protected environments. Crop damage and economic losses are the result of feeding on plant roots, corms and rhizomes by larvae and on leaves by adults (Moorhouse et al. 1992).

The conventional method of controlling vine weevil has been with synthetic chemical insecticides incorporated into plant growing media for larval control and foliar applications at dusk to target the adults (van Tol et al. 2012). Despite the widespread use of these insecticides, vine weevil has remained an important pest. Furthermore, repeated applications of insecticides in fruit crops can have negative impacts on beneficial arthropods (Solomon et al. 2001), resulting in outbreaks of secondary pests (van Tol et al. 2012). Over recent years there is has been a move to use entomopathogenic nematodes and fungi to control vine weevil larvae (e.g. Ansari et al. 2008; Georgis et al. 2006; Shah et al. 2006; Willmott et al. 2002), but management of adult weevils still typically relies on the use of broad-spectrum insecticides (Moorhouse et al. 1992; van Tol et al. 2012).

A key component of an integrated pest management (IPM) program is to base the use of control methods on careful monitoring of pest numbers in relation to action thresholds (e.g. Kogan 1998). For vine weevil, this is made difficult by the nocturnal activity of the adults

and the fact that the larvae feed below ground. Consequently, growers often do not observe vine weevils within crops through direct monitoring before significant damage has already occurred (van Tol et al. 2012). During the day adult weevils can be found in leaf litter, under pots or other suitable refuges. This behavior has been exploited using grooved boards placed on the ground (Gordon et al. 2003; Li et al. 1995), corrugated card wrapped around stems of larger bushes (Phillips 1989), and plastic crawling insect 'traps' (Pope et al. 2018) that provide adult weevils with artificial refuges and so help to focus monitoring efforts. Indirect monitoring of adult weevils is based on the presence of damage in the form of characteristic 'leaf-notching' from adult feeding to determine whether a crop is infested (Moorhouse et al. 1992), but this approach also leads to a delay in detecting the presence of adult weevils within crops, allowing oviposition to occur before control measures can be applied (van Tol et al. 2012).

Development of an effective semiochemical lure would improve vine weevil monitoring reliability and sensitivity, which may contribute to development of novel control methods such as autodissemination of entomopathogenic fungi (Pope et al. 2018). Identification of semiochemicals suitable for use as a vine weevil lure has proved challenging. As vine weevil reproduce asexually they do not produce a sex pheromone (van Tol et al. 2012). However, adults display strong aggregation behavior (Kakizaki 2001; Pickett et al. 1996) and attraction to conspecifics in a laboratory bioassay has been reported by van Tol et al. (2004b) and Nakamuta et al. (2005). Although male-produced aggregation pheromones have been identified from several weevil species (e.g. Blight and Wadhams 1987; Faustini et al. 1982; Gunawardena et al. 1998; Jayaraman et al. 1997; Rochat et al. 1991; Tumlinson et al. 1969), an aggregation pheromone for the vine weevil has not yet been identified.

Attraction of vine weevil to kairomones from host plants has also been investigated. Attraction to weevil frass has been reported by Pickett et al. (1996) and van Tol et al. (2004b), and it is possible this may explain the attraction of vine weevils to conspecific weevils. Although considered to be polyphagous, vine weevil adults show preferences for plants such as yew, *Taxus baccata* (L.) and spindle tree, *Euonymus fortunei* (Turcz.) Hand.-Maz. (van Tol and Visser 2002; van Tol et al. 2012), and two compounds identified in volatiles from *E. fortunei*, (*Z*)-2-pentenol and methyl eugenol, have been found to increase numbers of weevils in the area around traps (van Tol et al. 2012). These studies suggest that vine weevil adults use olfactory cues to locate host plants and for aggregation. Here we report on the electrophysiological and behavioral responses of adult vine weevils to volatile chemical stimuli originating from host plants and synthetic chemical compounds, presented both individually and as blends, with the aim of informing development of an effective lure for improved monitoring and potentially control of this pest.

Methods and Materials

Insect Cultures Adult vine weevils, *Otiohynchus sulcatus* F., were collected from commercial strawberry crops grown in Shropshire and Staffordshire UK during the summer in 2012, 2017 and 2018. Recovered vine weevils were maintained on branches of yew, *Taxus baccata* (L.), and moist paper towels inside insect cages (47.5 x 47.5 x 47.5 cm) (Bugdorm, MegaView, Taiwan) placed in a controlled environment room (20 °C; 60% RH; L:D 16:8 h) (Fitotron, Weiss Technik, Ebbw Vale, Wales).

Collection of Volatiles For collection of volatiles from intact plants during 2012, potted *E. fortunei* plants (Homebase, Buckinghamshire, UK) carrying approximately 50 g foliage were enclosed individually in a polyethyleneterephthalate oven bag (37 x 25 cm x 12 µ thick; J

Sainsbury plc, London, UK) (Stewart-Jones and Poppy 2006). Charcoal-filtered air (600 ml/min) was pumped into the bag to maintain positive pressure while air was drawn out (500 ml/min) through a collection filter containing Porapak Q (200 mg, 50-80 mesh; Supelco, Gillingham, Dorset, UK) held between two silanized glass wool plugs in a disposable glass pipette (4 mm i.d.). Six collections were carried out over a period of 24 h in a controlled environment room at 25 °C, 12:12 h L:D and 50% relative humidity.

During 2018, volatiles were collected from samples of cut branches of *E. fortunei* as used in the bioassay experiments. Cut stems (approx. 60 g) from one of the authors' garden in Kent, UK, were contained in round-bottomed flasks (3 liter) maintained in a controlled environment room as above. Air was drawn into the flask through an activated charcoal filter (20 cm x 2 cm; 8-10 mesh) and out through a filter containing Porapak Q as above. Collections were made for 6 h and then a further 18 h from four samples.

Volatiles were eluted from the Porapak Q filters with dichloromethane (2 x 0.5 ml; Pesticide Residue Grade; Fisher Scientific, Loughborough, UK), concentrated approximately five times by evaporation under a gentle stream of purified nitrogen, and stored at -20 °C before analysis. All collections were analyzed by GC/MS before and after concentration to confirm that no impurities had been introduced and no significant changes in composition had occurred during concentration. In other work, this concentration step caused a 15% loss of limonene but no detectable loss of less volatile compounds such as decyl acetate.

For GC/EAG analyses, the collections estimated to contain most material were used. For collections from intact plants in 2012, this was one made from 50 g plant material over 18 h, estimated to contain 4.4 ng/g plant material/h of the major component, (*E*,*E*)-α-farnesene, by comparison with an external standard. For collections from cut plants in 2018, the collection was made from 54 g plant material for 21 h, estimated to contain 5.9 ng/g/h (*E*,*E*)-α-farnesene.

Analysis by Gas Chromatography coupled to Mass Spectrometry (GC/MS) Analyses were carried out on a CP3500 GC coupled to a CP2200 Ion Trap Detector (Varian, now Agilent Technologies, Cheadle, UK). The GC was fitted with a fused silica capillary column (30 mm x 0.25 mm x 0.25 µm film thickness) coated with DBWax (Supelco). Manual injections (1 µl) were made in splitless mode (220 °C), with oven temperature programmed from 40 °C for 2 min then at 10 °C/min to 240 °C. Compounds were identified according to their mass spectrum, retention index relative to retention times of *n*-alkanes, and co-chromatography with authentic compounds.

The enantiomeric composition of linalool present in collections of volatiles from *E. fortunei* was determined by GC with flame ionization detection (FID) using a fused silica capillary column (25 mm x 0.32 mm i.d. x 0.25 µm film thickness) coated with a cyclodextrin stationary phase (Chirasil-DEX CB; Varian). Injection was splitless (220 °C), detection was by FID (250 °C), and carrier gas was helium (2.4 ml/min). The oven temperature was programmed at 60 °C for 2 min then at 5 °C/min to 200 °C. Retention times of (*R*)-(-)- and (*S*)-(+)-linalool were 11.39 min and 11.48 min, respectively.

Analysis by Gas Chromatography coupled to Electroantennography (GC/EAG) Analyses were carried out with a HP6890 GC (Agilent Technologies) fitted with flame ionization detector (FID) and fused silica capillary columns (30 m x 0.25 mm x 0.32 µm film thickness) coated with DBWax and DB1 (Supelco). Injections onto the DBWax column were in splitless mode (220 °C), with the oven temperature programmed from 50 °C for 2 min and then at 10 °C/min. to 250 °C for analyses in 2012 and from 50 °C for 2 min and then at 20 °C/min. to 250 °C for analyses in 2018. The effluents of the two columns were combined with a glass push-fit Y-tube connector (Agilent Technologies) connected to a second Y-tube connector

with deactivated fused silica tubing (10 cm x 0.32 mm i.d.). One arm of this connector was connected with fused silica tubing (50 cm x 0.32 mm i.d.) to the FID (250 ˚C) and the other to an equal length of deactivated silica tubing passing through a heated transfer line (250°C; Syntech, Hilversum, The Netherlands, now Kirchzarten, Germany) into a glass tube (4 mm i.d.) through which air passed (500 ml/min) over the EAG preparation.

Electroantennogram recordings were made with a portable INR-02 device (Syntech) connected as a second detector of the GC for A/D conversion. Glass electrodes containing electrolyte solution (0.1 M potassium chloride with 1 % polyvinylpyrrolidone) were attached to silver wires held in micromanipulators. Vine weevils were anaesthetized using carbon dioxide before excising the head using a scalpel. The reference electrode was inserted into the back of the head and the circuit was completed by bringing the recording electrode into contact with the tip of one antenna. Both the FID and EAG signals were collected and analyzed with EZChrom software (Elite v3.0; Agilent Technologies).

This system was used both for analyses of collections of volatiles from *E. fortunei* plants (2 µl injected) and for measurement of EAG responses to synthetic compounds. For the latter, the test compound (10 ng) was injected with 1-hexanol (10 ng) as internal standard eliciting a strong EAG response from vine weevil antennae in our system.

Chemicals Unless stated otherwise, chemicals were purchased from Sigma Aldrich (Gillingham, Dorset, UK) and were > 95% pure by GC analysis. 1-Octen-3-ol was from International Flavors and Fragrances (Haverhill, Suffolk, UK). (*E*)-4,8-Dimethyl-1,3,7-nonatriene (DMNT) was synthesized by Wittig reaction of methylphosphonium bromide with geranial prepared by oxidation of geraniol with pyridinium dichromate in dichloromethane. (*E,E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene (TMTT) was synthesized analogously from (*E,E*)-farnesal prepared by oxidation of (*E,E*)-farnesol. Pure (*E,E*)-α-farnesene was obtained

by washing apples in hexane followed by chromatography on silica gel eluted with hexane. Germacrene-D was a major component (40%) of ylang-ylang essential oil (Holland and Barrett, Nuneaton, Warwks, UK) with (*E,E*)-α-farnesene (45%) and β-caryophyllene (15%). Cadinols were isolated from Hinoki essential oil from *Chamaecyparis obtusa* (Shieh et al. 1981) by liquid chromatography on silica gel eluted with a gradient of diethyl ether in hexane. The fraction eluted with 50% diethyl ether in hexane contained 16% tau-cadinol and 32% alpha-cadinol according to their mass spectra and GC retention times in GC/MS analyses (El-Sayed 2019).

Y-tube Olfactometer Bioassays The behavioral responses of adult vine weevils to chemical stimuli were tested using a Y-tube olfactometer (Sci-Glass Consultancy, Bere Alston, UK) (Supplementary Information Fig. S1) based upon the design previously used by van Tol et al. (2002). The stem length was 120 mm, arm length 190 mm and internal diameter 18 mm. Airflow in each arm was 600 ml/min and odor sources were held in Drechsel bottles (500 ml). *Euonymous fortunei* plant material (20 g) was cut from the plant and the cut stem wrapped in moist cotton wool and aluminum foil. Synthetic compounds were diluted in paraffin oil (Fisher Scientific) to give the required amount in 10 µl which was applied to Whatman glass microfibre filters (934-AH grade; 47 mm diameter; Sigma Aldrich). Test samples equilibrated in the Drechsel bottles for 1 h prior to tests.

All bioassays were undertaken in complete darkness in a controlled environment room (20 °C; 60 % RH; 16:8 h L:D). Prior to their use in a bioassay, vine weevils were starved for a minimum of 24 h unless stated otherwise. Groups of 40 vine weevils were introduced into the olfactometer via a release chamber (100 mm diameter). Each pair of odor sources was tested six times with fresh individuals for 20 min, and the numbers of weevils reaching the end of each arm during this time were recorded. The positions of the odor

sources were alternated between replicates to eliminate directional bias. After each pair of odor sources had been tested six times, all glassware was thoroughly cleaned by rinsing with warm water followed by HPLC-grade acetone (Sigma Aldrich) before baking in a glassware oven at 120 °C for 15 min.

Nine compounds were tested individually at source loadings of 0.1 mg, 1 mg and 5 mg to provide a wide range of doses that caused significant behavioral effects in previous work by Karley et al. (2012). These were (*Z*)-2-pentenol and methyl eugenol, compounds reported to be attractive to vine weevil by van Tol et al. (2012), and methyl salicylate, 1-hexanol, (*Z*)-3-hexenol, (*E*)-2-hexenol, (*E*)-2-pentenol, (±)-linalool, and 1-octen-3-ol, compounds found to elicit strong EAG responses from vine weevil in our own work. Two blends were tested at loadings of 1 mg total: a binary blend of equal amounts of (*Z*)-2-pentenol and methyl eugenol (van Tol et al. 2012) and a blend of equal amounts of the other seven electrophysiologically active compounds: methyl salicylate, 1-hexanol, (*Z*)-3-hexenol, (*E*)-2-hexenol, (*E*)-2-pentenol, linalool and 1-octen-3-ol.

Statistical Analyses All statistical analyses were performed using R (Version 3.5-3) (R Core Team, 2019). Y-Tube olfactometer bioassay data were analyzed using an exact binomial test against the null hypothesis that the number of vine weevils reaching the end of either olfactometer arm had a 50:50 distribution. Prior to performing statistical analyses, the replicated results from each of the odor pairs tested were pooled with non-responding individuals being excluded from statistical analyses.

Results

Y-Tube Bioassay with Host Plant Material Vine weevils starved for at least 24 h exhibited a preference for the Y-tube olfactometer arm containing air blown over *E. fortunei* plant

material with 83 % of responding individuals choosing this arm over the clean-air control arm ($P < 0.001$).

GC/EAG and GC/MS Analyses of Volatiles from *Euonymus fortunei* In analyses of volatiles from *E. fortunei* by GC coupled to EAG recording from a vine weevil antenna, 22 reproducible responses were observed in analyses of volatiles from intact plants (Fig. 1) and 20 responses in analyses of volatiles from cut branches (Fig. 2). The identities of the compounds responsible are summarized in Table 1 along with their relative proportions present in the collections used for GC/EAG analyses. There were responses to 13 compounds present in both sets of samples: aldehydes hexanal, (*Z*)-3-hexenal, heptanal, (*E*)-2-hexenal, octanal, nonanal, and decanal; alcohols 1-hexanol, (*Z*)-3-hexenol, (*R*)-(-) linalool; aromatic 1,2-dimethoxybenzene (veratrole), sesquiterpene (*E,E*)-α-farnesene; and the oxylipin *cis*-jasmone. (*E,E*)-α-Farnesene was the most abundant compound in both sets of samples, although the EAG response to this was always relatively weak (Figs. 2 and 3).

In analyses of volatiles from intact plants, responses were also observed to ketones 6-methyl-5-hepten-2-one and geranyl acetone; alcohols 1-octen-3-ol and 2-ethyl-1-hexanol; acetic acid; sesquiterpene α-cadinol; and three unidentified compounds present at very low levels. Acetic acid and 2-ethyl-1-hexanol were also present in collections of volatiles from cut plants, although no EAG response was observed, while the other compounds were not detected in volatiles from cut plants.

In analyses of volatiles from cut plants, responses were also observed to alcohols (*E*)-2-hexenol, benzyl alcohol and 2-phenylethanol; acids hexanoic acid and 2-ethylhexanoic acid; methyl salicylate; and indole. Of these compounds, only methyl salicylate was detected in samples of volatiles from intact plants.

EAG responses were not observed to other relatively ubiquitous plant volatiles present in one or both sets of collections, such as (*E*)-ocimene, (*Z*)-3-hexenyl acetate, copaene, α- and β-caryophyllene, and (*E*)-nerolidol, or to the stress-related volatiles DMNT and TMTT.

(*E*)-2-Pentenol, (*Z*)-2-pentenol and methyl eugenol, compounds reported from *E. fortunei* by van Tol et al. (2012), could not be detected (<0.1%) in any of the collections from intact plants (6 samples) or cut plants (8 samples) by comparison of GC retention times and mass spectra with those of authentic standards (Table 2).

GC-EAG Analysis of Synthetic Chemicals In experiments during 2012, consistent EAG responses were observed to (*E*)-2-pentenol, (*Z*)-2-pentenol, (*E*)-2-hexenol, (*Z*)-3-hexenol, 1-hexanol, 1-octen-3-ol, (±)-linalool, and methyl salicylate (Table 2 and Supplementary Material Figs. S2, S3), while (*E,E*)-α-farnesene and methyl eugenol elicited responses approximately 50 % of the time they were tested (Table 2). No response was observed to DMNT or β-caryophyllene, consistent with the absence of responses to these compounds in volatiles from *E. fortunei* (Table 2 and Supplementary Material Figs. S2, S3).

Subsequent experiments during 2018 confirmed the consistent response to 1-hexanol and showed a similarly consistent response to *cis*-jasmone (Table 2 and Supplementary Material Fig. S4). Analysis of the cadinol fraction from Hinoki essential oil showed a consistent response to tau-cadinol but not to α-cadinol (Table 2 and Supplementary Material Fig. S5), in contrast to what was observed previously in GC-EAG analyses of volatiles from intact *E. fortunei* plants. No EAG response was observed to indole (Table 2) even though a response was sometimes observed to this compound in volatiles from cut *E. fortunei* plants (Fig. 1 and Table 1).

Y-Tube Bioassays When tested at 0.1 mg at source, significantly more vine weevils moved to the control arm relative to the treatment arm for five of the nine chemical compounds tested : 1-hexanol ($P < 0.001$), (*Z*)-3-hexenol ($P = 0.02$), (*E*)-2-hexenol ($P < 0.001$), 1-octen-3-ol ($P < 0.001$), and (*Z*)-2-pentenol ($P = 0.02$) (Fig. 3). For the other four compounds, methyl salicylate, (*E*)-2-pentenol, linalool and methyl eugenol, there was no significant difference between the numbers in control and treatment arms ($P > 0.05$) (Fig. 3).

When tested at 1 mg at source, significantly more weevils were recorded in the control arm relative to the number in the treatment arm for three chemicals: 1-octen-3-ol ($P < 0.001$), linalool ($P < 0.001$), and 1-hexanol ($P = 0.02$) (Fig. 4). Significantly more weevils were recorded in the treatment arm for two of the compounds: (*Z*)-2-pentenol ($P = 0.03$) and methyl eugenol ($P = 0.001$), while there was no significant difference between the numbers in control and treatment arms for (*E*)-2-pentenol, (*Z)*-3-hexenol, (*E*)-2-hexenol, and methyl salicylate ($P > 0.05$) (Fig. 4).

When tested at 5 mg at source, seven of the nine chemical compounds tested evoked a behavioral response in the vine weevils. Significantly more weevils were recorded in the control arm relative to the treatment arm for five compounds: methyl salicylate ($P < 0.001$), (*E*)-2-hexenol ($P < 0.001$), linalool ($P < 0.001$), 1-octen-3-ol ($P < 0.01$), and methyl eugenol ($P < 0.001$) (Fig. 5). Significantly more weevils were observed in the treatment arm relative to the number in the control arm for two compounds: 1-hexanol ($P = 0.01$) and (*Z*)-2-pentenol ($P < 0.001$) (Fig. 5). There was no significant difference between the number of weevils in the control and treatment arms of (*Z*)-3-hexenol and (*E*)-2-pentenol ($P > 0.05$).

Two blends of chemicals were tested at 1 mg total at source (Fig. 6). With a binary blend of equal amounts of (*Z*)-2-pentenol and methyl eugenol, a significantly greater number of the responding vine weevils demonstrated a preference for the olfactometer arm containing the treatment over the control arm ($P = 0.01$). Similarly, with a seven-component blend

containing equal amounts of methyl salicylate, 1-hexanol, (*Z*)-3-hexenol, (*E*)-2-hexenol, (*E*)-2-pentenol, linalool and 1-octen-3-ol, significantly more of the weevils responding were recorded in the treatment arm ($P < 0.001$) (Fig. 6).

Discussion

Vine weevil larvae and adults are polyphagous (Masaki et al. 1984; Smith 1932), although adults show a strong preference for certain plant species in the families Rosaceae, Ericaceae and Taxaceae (van Tol et al., 2004a). Olfactory cues play an important role in these behavioral responses, with vine weevil adults preferring the odor of *E. fortunei* over blank controls and other potential hosts such as *Fragaria* or *Rhododendron* (van Tol et al., 2004a). The results presented here confirm that adult vine weevils move toward air streams containing VOCs originating from *E. fortunei*. Furthermore, it is apparent that it is the blend of generalist compounds and the concentrations of VOCs that are important in determining whether a positive behavioral response is elicited.

In GC-EAG analyses of volatiles from intact and cut *E. fortunei* plants, consistent EAG responses from vine weevil antennae were observed to 13 common plant volatiles present in both sets of collections. These were seven aliphatic aldehydes, three alcohols, one sesquiterpenes, one aromatic and the oxylipin, *cis*-jasmone. In GC-EAG analyses of volatiles from intact plants, 9 additional EAG responses were observed. These additional EAG responses included those to 6-methyl-5-heptenone and geranyl acetone, which are, paradoxically, characteristically emitted by damaged plants (War et al. 2011). In analyses of volatiles from cut plants, as used in the laboratory bioassays, additional EAG responses were observed to three alcohols, two acids, methyl salicylate, and indole. Several of the volatiles eliciting EAG responses in this study, such as (*Z*)-3-hexenol, (*E*)-2-hexenol, 1-octen-3-ol, and (*E*,*E*)-α-farnesene, have previously been detected from GC-EAG analyses of volatiles from

342 crushed and variously damaged *E. fortunei* plants (van Tol et al. 2012). Other relatively
343 ubiquitous plant volatiles detected in one or both sets of collections, such as (*E*)-ocimene, β-
344 caryophyllene, germacrene D and the stress-related volatiles DMNT and TMTT, did not elicit
345 EAG responses despite being previously reported to do so (van Tol et al. 2012). van Tol et al.
346 (2012) also reported EAG responses to (*Z*)-2-pentenol, methyl benzoate, estragole, 4-*tert*-
347 butylcyclohexanol, 2-phenoxyethanol and methyl eugenol presented in *E. fortunei* air
348 entrainments. These volatiles were never detected in our analyses even though they were
349 specifically looked for by comparison with mass spectra and GC retention times of the
350 authentic compounds. This may in part be due to the poor resolution of the GC peaks in the
351 analyses of van Tol et al. (2012), and questionable structural assignments in this earlier study
352 – e.g. (*E*)-ocimene does not elute after DMNT on the column used.
353 Results of GC-EAG analyses of volatiles from *E. fortunei* plants were supported by
354 similar analyses of a selection of synthetic compounds using vine weevil antennae. In
355 particular, strong and consistent EAG responses were observed to alcohols 1-hexanol, (*E*)-2-
356 hexenol, (*Z*)-3-hexenol, 1-octen-3-ol and linalool. These results are similar to those reported
357 by van Tol and Visser (2002). Strong and consistent EAG responses were also observed in
358 this study to *cis*-jasmone, which has not previously been reported. DMNT and β-
359 caryophyllene did not elicit EAG responses, as observed in GC-EAG analyses of the plant
360 volatiles, but (*Z*)-2-pentenol and methyl eugenol, although not detected in the GC-EAG
361 analysis of the plant volatiles, did elicit EAG responses.
362 Despite their absence in our study samples, both (*Z*)-2-pentenol and methyl eugenol
363 were tested in Y-tube olfactometer bioassays as van Tol et al. (2012) reported that release of
364 (*Z*)-2-pentenol on its own or as 1:1 ratio mixture with methyl eugenol from boll weevil traps
365 in strawberry crops increased numbers of vine weevil adults in the area surrounding the traps.
366 This response could result from orientated movement of vine weevil adults from distance

367 towards the release point of these VOCs or be due to the VOCs arresting the weevils after a
368 chance arrival in the area close to the traps (Kennedy 1977). The olfactometry results
369 presented here confirm that vine weevil adults do indeed show orientated movement towards
370 both compounds when 1 mg was presented individually. For (*Z*)-2-pentenol, when 5 mg was
371 presented a similar response was recorded but at 0.1 mg weevils were found to move away
372 from this odor source. In addition, when 1 mg of a 1:1 ratio mixture of (*Z*)-2-pentenol and
373 methyl eugenol was presented, vine weevils were again found to orientate towards this odor.
374 The apparent sensitivity to concentration seen for (*Z*)-2-pentenol was also seen for the
375 other individual compounds tested, which elicited either no or negative behavioral responses
376 depending on the concentration presented. The only exceptions were (*E*)-2-pentenol, which
377 did not elicit a behavioral response at any of the loadings tested, and 1-hexanol, to which
378 weevils responded positively only at the 5 mg loading. Few studies have investigated the
379 importance of plant VOC concentration in determining the behavioral responses of
380 phytophagous insects during host plant location or selection (Dicke and Baldwin 2010). For
381 example, a meta-analysis of using plant VOCs to manipulate phytophagous insect pest
382 behavior does not list concentration as a factor influencing insect behavior in any of the 34
383 published studies analyzed (Szendrei and Rodfriguz-Saona 2009). Despite this, it has been
384 argued that VOC concentration is important (e.g. Bruce et al. 2005). Often in the case of
385 individual compounds where an insect encounters higher concentrations than would be found
386 in nature a misleading response may be recorded, as demonstrated in electrophysiological
387 experiments (e.g. Anderson et al. 1995; Blight et al. 1995; Hansson et al. 1999; Larsson et al.
388 2005). Nonetheless, in this study negative behavioral responses were recorded to many plant
389 VOCs considered common throughout the plant kingdom at either low or high concentrations
390 (War et al. 2011). The results presented in this study suggest that the concentration of
391 individual VOCs can influence the behavioral response of vine weevil adults under controlled

392 laboratory conditions. Webster et al. (2010) reported similar results for the black bean aphids
393 (*Aphis fabae*) responding to different concentrations of individual VOCs identified from their
394 host plants.
395 With green leaf volatiles (GLVs) dominating the list of VOCs detected by vine weevil
396 adults, the EAG results presented here appear to support the hypothesis that this group of
397 plant volatiles play a role in host-plant location by phytophagous arthropods (Bruce et al.
398 2005). With either no or negative behavioral responses to compounds when presented
399 individually, vine weevil adults responded positively to a mixture of equal amounts of seven
400 plant volatiles (methyl salicylate, 1-octen-3-ol, (*E*)-2-hexenol, (*Z*)-3-hexenol, 1-hexanol, (*E*)-
401 2-pentenol, and linalool) when 1 mg of the blend was presented. As such, these olfactometry
402 results with individual and simple blends of VOCs demonstrate that components of a host-
403 plant blend may not be recognized when perceived outside the context of that blend as
404 suggested by Bruce and Pickett (2011). This is again comparable to the study by Webster et
405 al. (2010) who found black bean aphids respond positively to a nine-component blend of host
406 plant VOCs that evoked a negative behavioral response when presented individually.
407 In the absence of a vine weevil sex or aggregation pheromone, the development of an
408 effective semiochemical lure for vine weevil adults is likely to focus on host-plant volatiles.
409 As the VOC's known to be detected by vine weevil adults are found in many plant families,
410 rather than those that are taxonomically characteristic, it seems likely that optimization of a
411 lure must focus on the blend and the concentration of each component in it. The fact that both
412 blends tested here elicited positive behavioral responses suggests that there is redundancy in
413 the composition of the blends used by vine weevil adults to recognize host plants with the
414 potential to substitute some compounds with others (Bruce and Pickett 2011). Further work is
415 required to identify a blend of VOCs that is effective enough to be deployed within crops as a
416 lure to attract adult vine weevils into traps for monitoring or control purposes.

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548 **Table 1** Compounds identified in GC-EAG analyses of volatiles from intact and cut plants of
549 *Euonymous fortunei* and relative amounts (EAG responses numbered as in Figs. 1 and 2; RI
550 is retention index relative to retention times of *n*-alkanes on polar GC column; DMNT is 4,8-
551 dimethyl-1,3,7-nonatriene; TMTT is 4,8,12-trimethyl-1,3,7,11-tridecatetraene)

Intact plants				Cut plants				
EAG response	RT (min)	RI	Area (%)	EAG response	RT (min)	RI	Area (%)	Compound
1	4.79	1074	1.2	1	3.97	1086	0.48	hexanal
2	5.55	1136	2.0	2	4.47	1147	0.54	(*Z*)-3-hexenal
3	6.15	1185	1.4	3	4.85	1193	0.31	heptanal
4	6.60	1220	0.7	4	5.11	1226	1.58	(*E*)-2-hexenal
	7.06	1255	2.2					(*E*)-ocimene
5	7.57	1293	1.0	5	5.67	1301	0.32	octanal
	7.82	1312	3.7		5.79	1317	3.98	DMNT
	7.97	1323	1.3					(*Z*)-3-hexenyl acetate
6	8.23	1343	2.1					6-methyl-5-hepten-2-one
7	8.41	1356	0.0	6	6.10	1358	1.07	1-hexanol
8	8.84	1389	4.7	7	6.33	1388	3.63	(*Z*)-3-hexenol
9	8.98	1399	4.2	8	6.43	1402	1.98	nonanal
				9	6.48	1409	0.54	(*E*)-2-hexenol
10	9.50	1441	0.0		6.70	1443	0.35	acetic acid?
11	9.66	1454	0.0					1-octen-3-ol
12	10.18	1496	1.6		7.03	1495	1.07	2-ethyl-1-hexanol
13	10.31	1507	4.4	10	7.13	1510	2.43	decanal
					7.17	1516	1.99	copaene
14	10.86	1551	0.8	11	7.39	1550	2.32	(*R*)-(-)-linalool
	11.46	1600	1.7					hexadecane
	11.58	1610	1.3		7.84	1623	1.79	β-caryophyllene

					8.28	1700	1.25	α-caryophyllene
	12.80	1720	2.8					ethyl-benzaldehyde
					8.40	1721		germacrene D
15	12.88	1727	0.8	12	8.45	1730	1.28	1,2-dimethoxy-benzene
	13.00	1738	0.5		8.50	1739	1.14	α-muurolene
	13.14	1750	2.3		8.55	1747	1.14	farnesene isomer
16	13.19	1754	15.6	13	8.61	1758	25.99	(*E*,*E*)-α-farnesene
	13.54	1786	2.7	14	8.85	1800	0.65	methyl salicylate
	13.82	1811	0.6					TMTT
				15	9.12	1852	0.45	hexanoic acid
17	14.34	1863	2.1					(*E*)-geranyl acetone
				16	9.31	1889	0.92	benzyl alcohol
				17	9.51	1928	0.70	2-phenylethanol
					9.61	1948	1.94	benzyl cyanide
					9.70	1965	0.20	*trans*-jasmone
18	15.28	1956	0.0	18	9.73	1971	1.99	*cis*-jasmone
				19	9.89	2002	0.20	2-ethylhexanoic acid
	16.14	2044	0.8		10.09	2044	6.06	(*E*)-nerolidol
	17.44	2183	1.8					tau-cadinol
19	17.99	2245	1.4					α-cadinol
20	18.92	2353	0.0					unknown
				20	12.01	2468	3.31	indole
21/22	20.94	2588	0.0					unknown

552

553

554 **Table 2** Frequency of EAG responses of vine weevil to 10 ng of synthetic compounds
555 delivered by GC-EAG (number of times a response was observed out of the total number of
556 runs); RI is retention index relative to retention times of *n*-alkanes on polar GC column;
557 DMNT is 4,8-dimethyl-1,3,7-nonatriene; TMTT is 4,8,12-trimethyl-1,3,7,11-tridecatetraene)

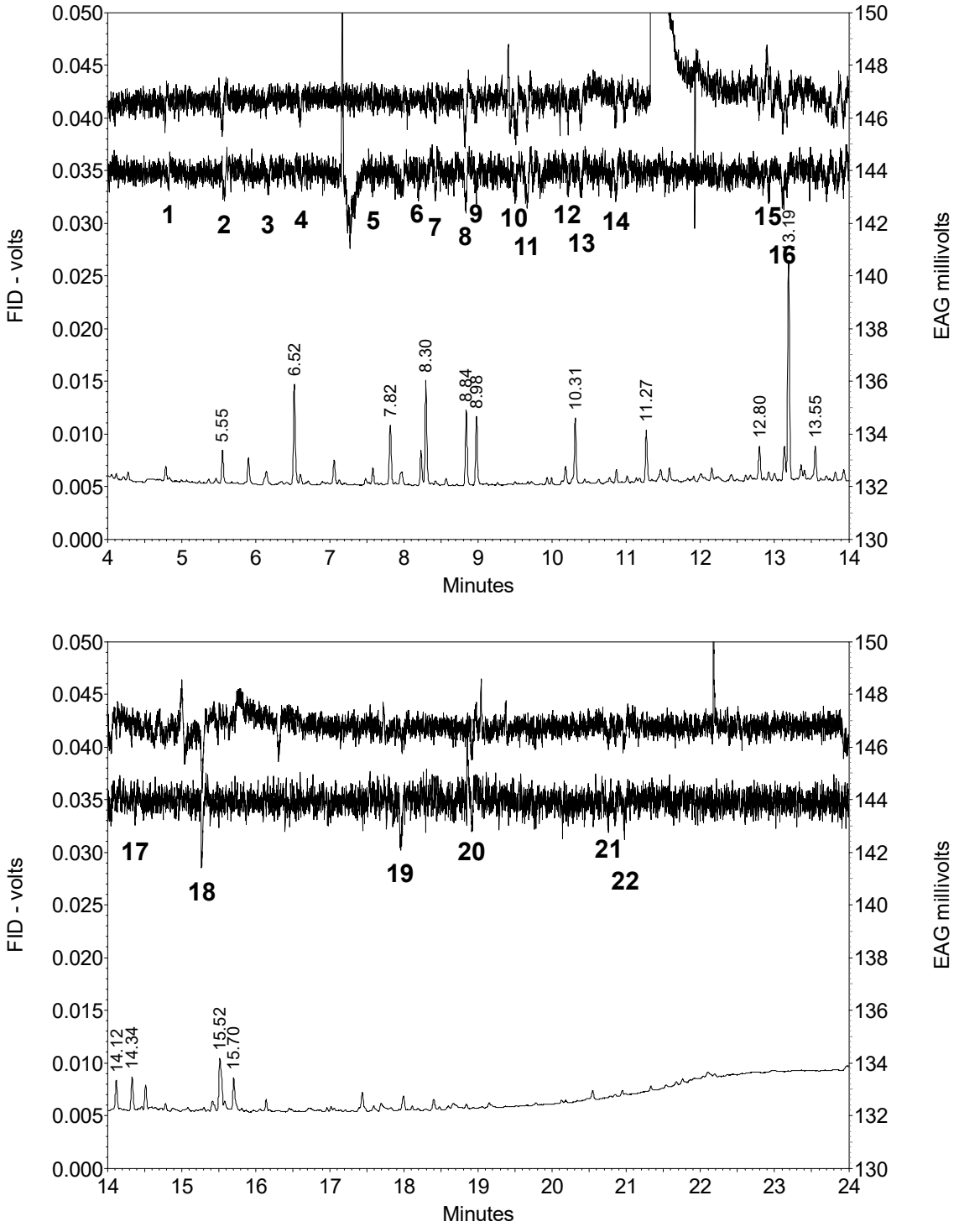
Compound	RT (mins)	RI	Frequency of EAG response
2012 Experiments			
DMNT	6.82	1310	0/2
(*E*)-2-pentenol	6.88	1315	3/4
(*Z*)-2-pentenol	6.98	1323	4/4
1-hexanol	7.41	1356	16/16
(*Z*)-3-hexenol	7.85	1389	4/4
(*E*)-2-hexenol	8.11	1410	18/18
1-octen-3-ol	8.67	1455	2/2
linalool [a]	9.87	1552	4/4
β-caryophyllene	10.59	1612	0/16
germacrene-D	11.88	1727	0/16
(*E*,*E*)-α-farnesene	12.22	1757	5/16
methyl salicylate	12.56	1788	4/4
methyl eugenol	14.91	2018	1/2
2018 Experiments			
1-hexanol	6.09	1358	15/15
cis-jasmone	9.72	1973	14/14
tau-cadinol	10.79	2196	5/5
α-cadinol	11.08	2261	0/5
indole	12.00	2468	0/2

558

559 [a] Present as a minor component in ylang ylang essential oil

560

561 **Figures**

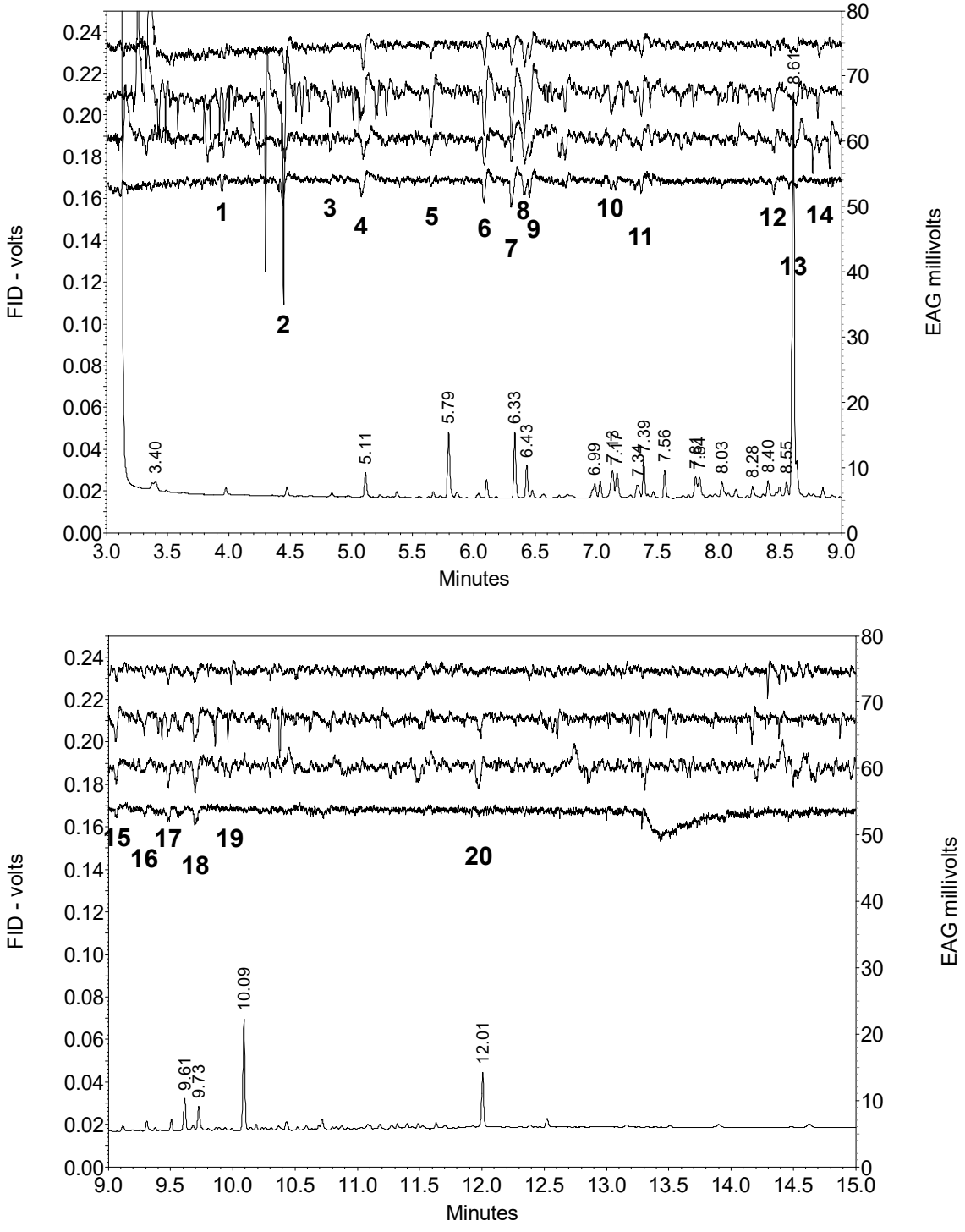


562

563

564 **Fig. 1** GC-EAG analysis of volatiles from intact *Euonymus fortunei* plants on polar GC

565 column (responses numbered as in Table 1



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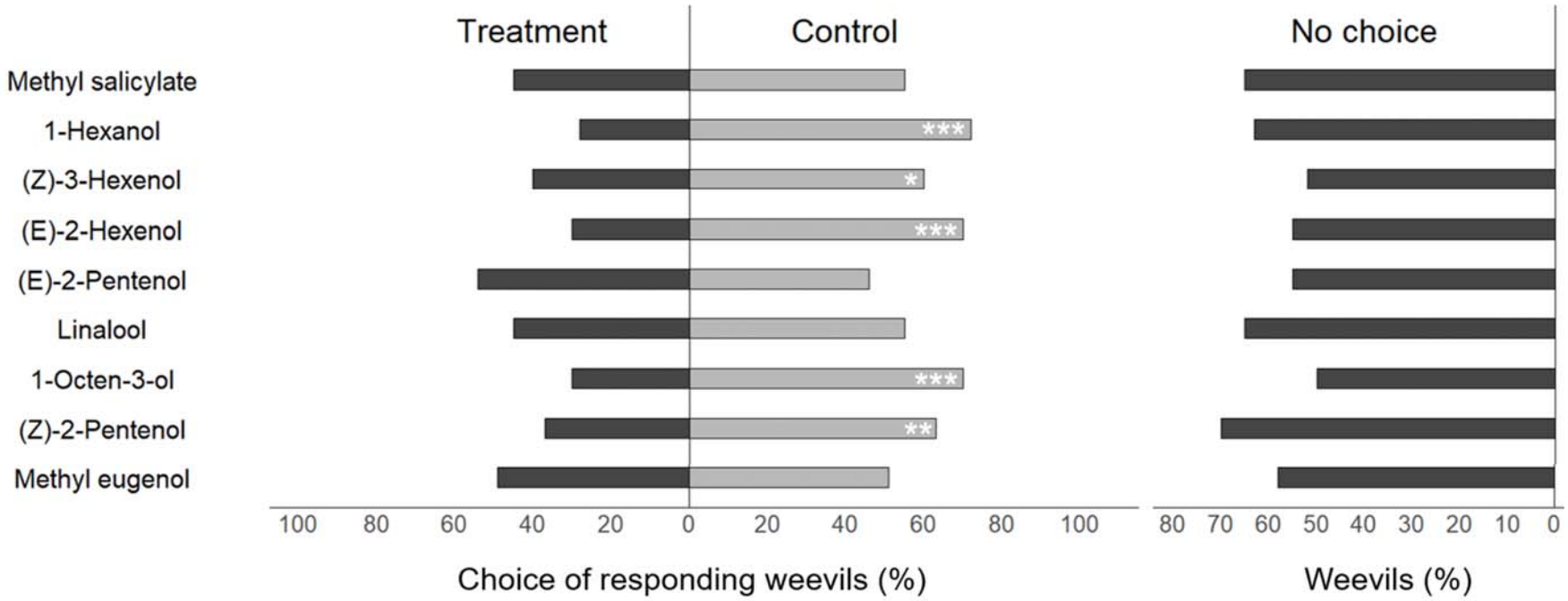
567

568 **Fig. 2** GC-EAG analysis of volatiles from cut *Euonymus fortunei* plants on polar GC column

569 (responses numbered as in Table 1)

570



571

572 **Fig. 3** Behavioral responses of adult vine weevils toward synthetic chemical compounds when offered against paraffin oil in a Y-tube

573 olfactometer at a concentration of 10 mg/ml (0.1 mg at source). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by binomial exact test

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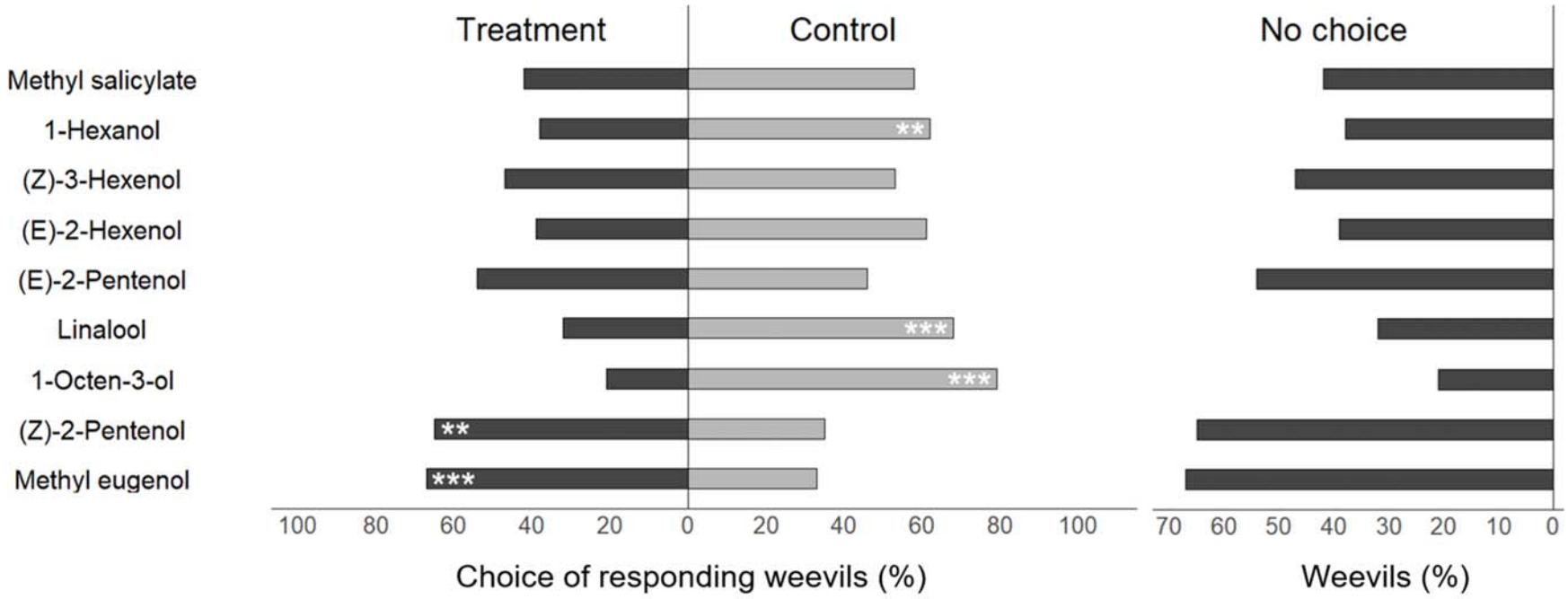
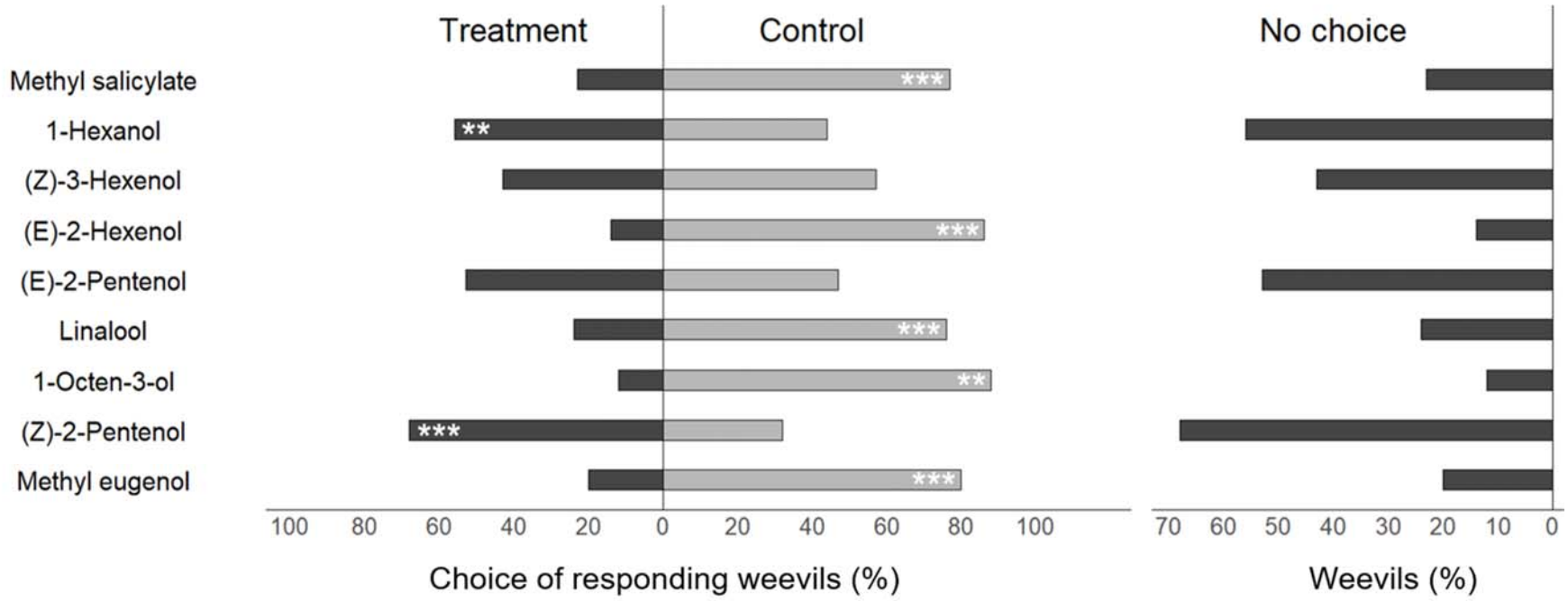


575

576 **Fig. 4** Behavioral responses of adult vine weevils toward synthetic chemical compounds when offered against paraffin oil in a Y-tube

577 olfactometer at a concentration of 100 mg/ml (1 mg at source). ** $P < 0.01$, *** $P < 0.001$ by binomial exact test

578

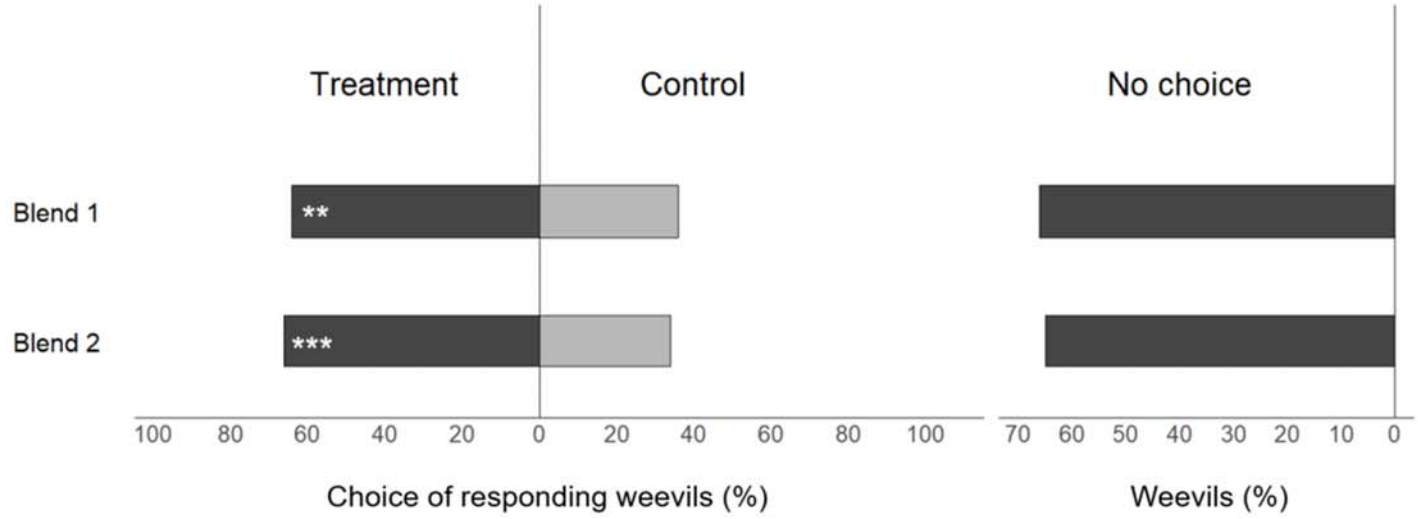


579

580 **Fig. 5** Behavioral responses of adult vine weevils toward synthetic chemical compounds when offered against paraffin oil in a Y-tube

581 olfactometer at a concentration of 500 mg/ml (5 mg at source). ** $P < 0.01$, *** $P < 0.001$ by binomial exact test

582



583

584 **Fig. 6** Behavioral responses of adult vine weevils toward synthetic chemical blends when offered against paraffin oil in a Y-tube olfactometer at
585 a concentration of 100 mg/ml (10 mg at source). Blend 1 contains equal amounts of (*Z*)-2-pentenol + methyl eugenol; Blend 2 contains equal
586 amounts of methyl salicylate + 1-octen-3-ol + (*E*)-2-hexenol + (*Z*)-3-hexenol + 1-hexanol + (*E*)-2-pentenol + (±)-linalool. ** $P < 0.01$, *** $P <$
587 0.001 by binomial exact test

588

SUPPLEMENTARY INFORMATION

Electrophysiological and Behavioral Responses of Vine Weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae), Adults to Host Plant Odors

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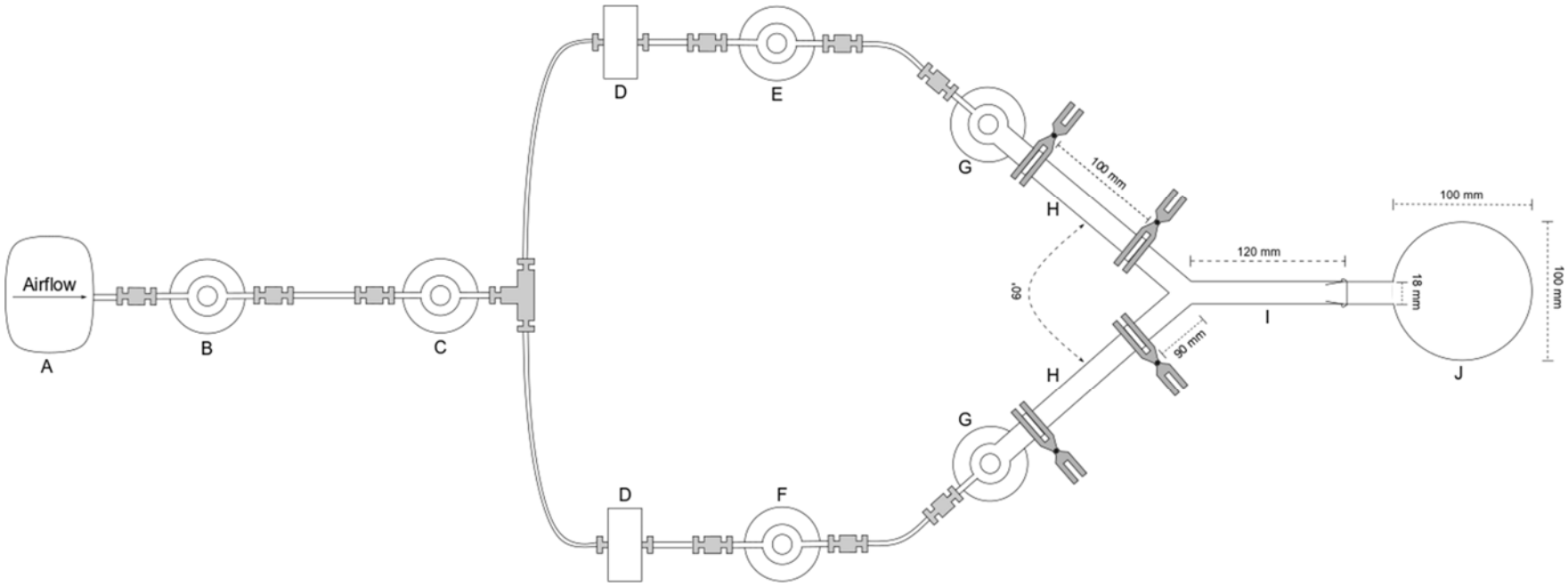

Fig. S1 Y-tube olfactometer: (A) pump, (B) 500 ml Drechsel bottle containing 500 g of activated charcoal, (C) 500 ml Drechsel bottle containing 450 ml distilled water, (D) air flow regulators set at 600 ml/min, (E) 500 ml Drechsel bottle containing the odor source, (F) 500 ml Drechsel bottle containing odor source, (G) 500 ml Drechsel bottles acting as vine weevil collection points, (H) conex tubes connected to parts G and I using stainless steel clips, (I) Y-tube, and (J) vine weevil release point. All glassware and air pumps were connected using PTFE tubing with an outside diameter of 3 mm, 6 mm brass tube fitting unions, 6 mm to 3 mm brass tube reducing unions, and 3 mm brass 'T' unions (Swagelok, Manchester UK).

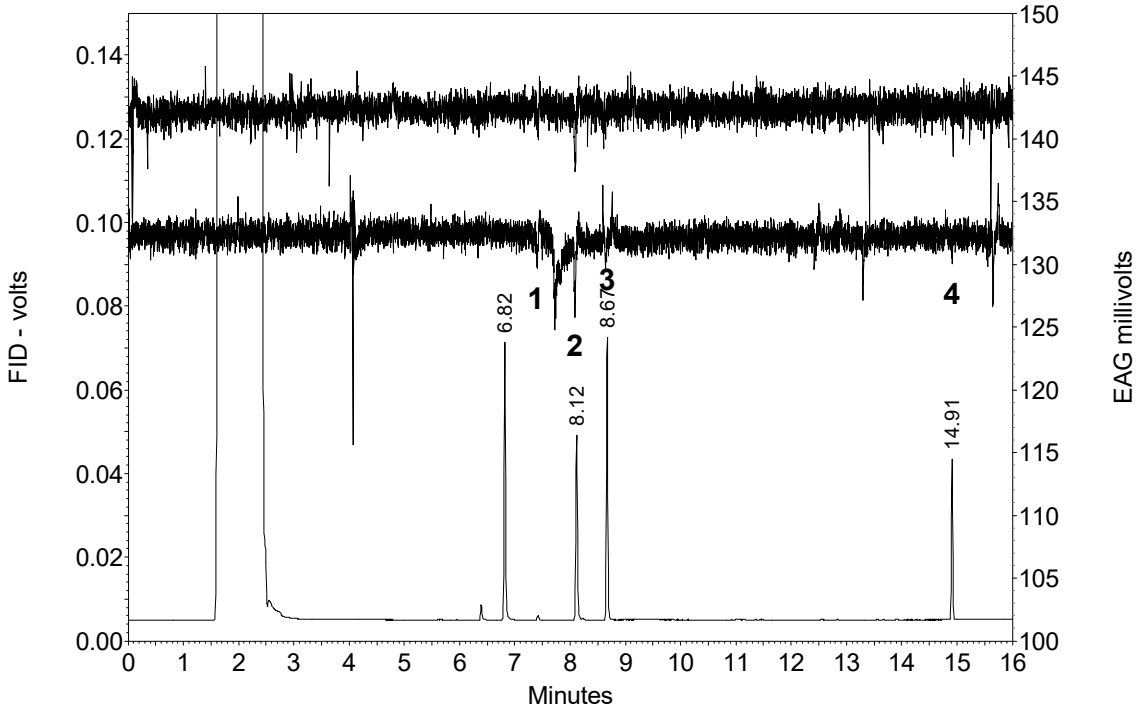

Fig. S2 GC-EAG Analyses of DMNT (8.82 min), (*E*)-2-hexenol (8.12 min), 1-octen-3-ol (8.67 min) and methyl eugenol (14.91 min), showing EAG responses to the latter three compounds and no response to DMNT; EAG response (1) is due to the trace of 1-hexanol (7.40 min) in the (*E*)-2-hexenol

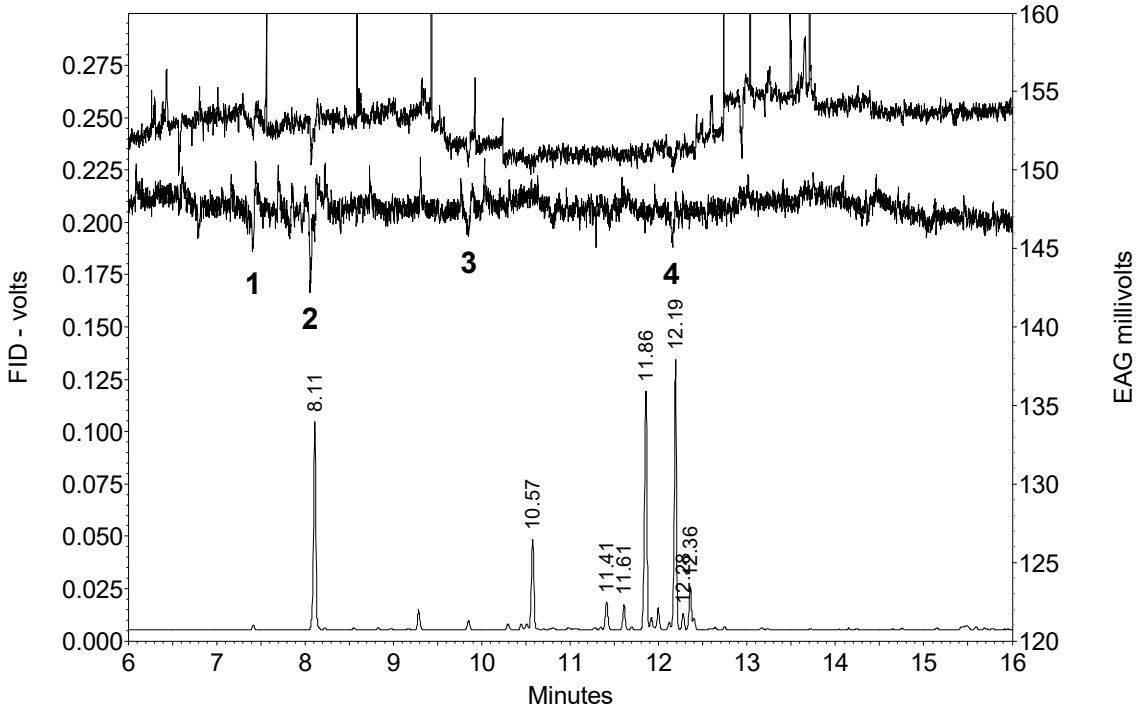

Fig. S3 GC-EAG Analyses of (*E*)-2-hexenol (8.11 min; response 2) and ylang-ylang essential oil showing EAG responses to linalool (9.85 min; 3) and (E,E)-α-farnesene (12.19 min; 4), but no EAG response to β-caryophyllene (10.57 min) and germacrene-D (11.88 min); EAG response (1) is due to the trace of 1-hexanol (7.40 min) in the (*E*)-2-hexenol

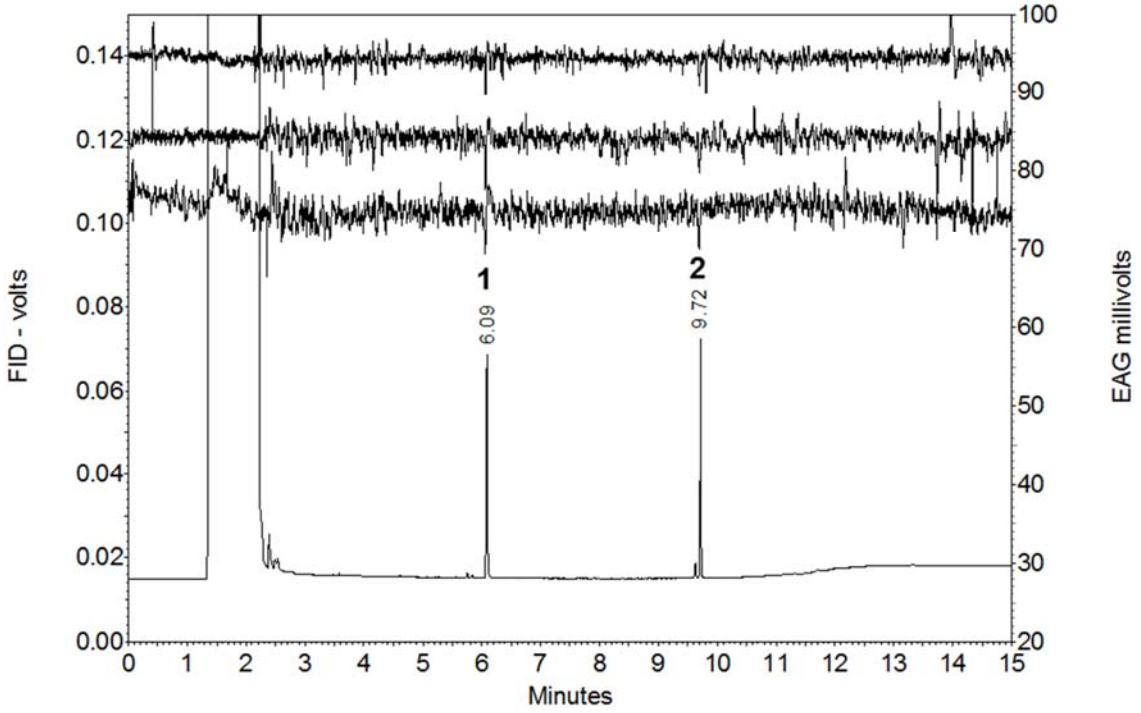

Fig. S4 GC-EAG Analyses of 1-hexanol (6.09 min) and *cis*-jasmone (9.72 min) showing EAG responses (1) and (2) respectively

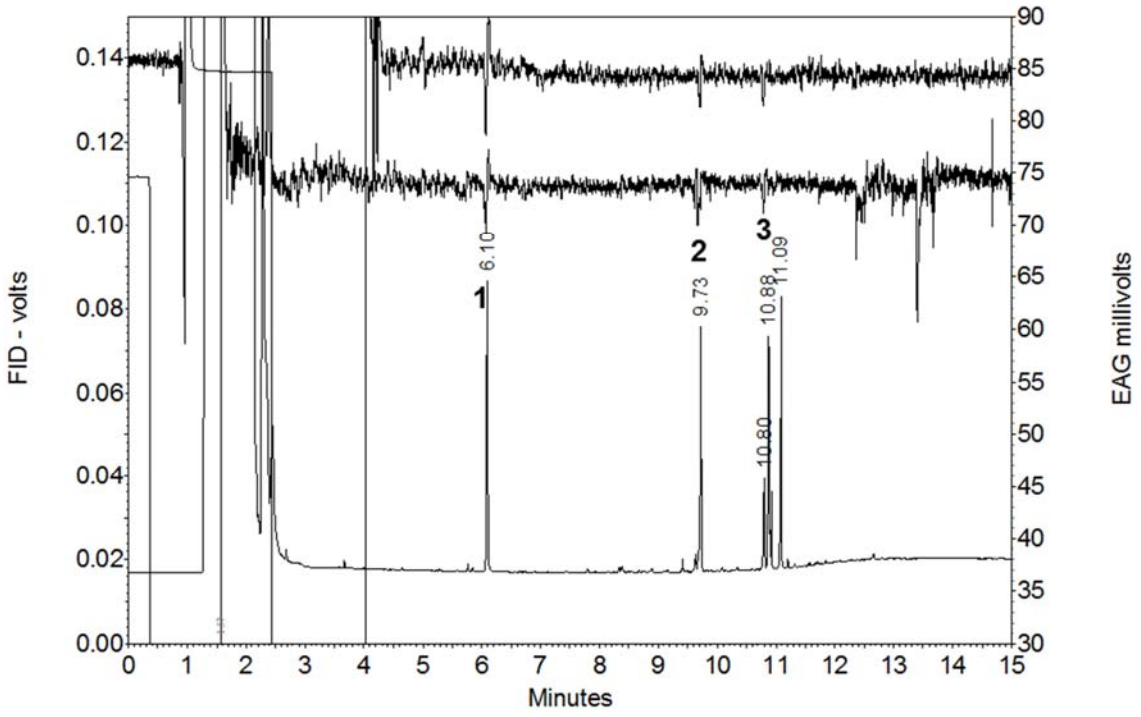

Fig. S5 GC-EAG Analyses of 1-hexanol (6.10 min; response 1), *cis*-jasmone (9.73 min; 2) and the cadinol fraction from Hinoki essential oil showing EAG response to tau-cadinol (10.80 min; 3) but not α-cadinol (11.09 min)